American Diabetes Association. diabetes

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Journal:	*Diabetes*
Manuscript ID	DB18-0295.R1
Manuscript Type:	Original Article: Immunology and Transplantation
Date Submitted by the Author:	04-Jul-2018
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Original Article

Inflammation-induced citrullinated glucose-regulated protein 78 elicits immune responses in human type 1 diabetes.

Running title: Immunogenicity of CitGRP78 in human type 1 diabetes

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Word count: 4647
Figure count: 6 figures, one table

Abstract

The beta-cell has become recognized as a central player in the pathogenesis of type 1 diabetes, with the generation of neo-antigens as potential triggers for breaking immune tolerance. Here, we report that post-translationally modified (PTM) glucose-regulated protein 78 (GRP78) is a novel autoantigen in human type 1 diabetes. When human islets were exposed to inflammatory stress, induced by IL-1β, TNF-α and IFN-γ, arginine residue R510 within GRP78 was converted into citrulline, as evidenced by liquid chromatography-tandem mass spectrometry (LC-MS/MS). This conversion, known as citrullination, led to the generation of neo-epitopes, which could effectively be presented by HLA-DRB1*04:01 molecules. Using HLA-DRB1*04:01 tetramers and ELISA techniques, we demonstrated enhanced antigenicity of citrullinated GRP78, with significantly increased $CD4^+$ T-cell responses and autoantibody titers in type 1 diabetes patients compared to healthy control subjects. Interestingly, patients with type 1 diabetes had a predominantly higher percentage of central memory cells and a lower percentage of effector memory cells directed against citrullinated GRP78, as compared to the native epitope. These results strongly suggest that citrullination of beta-cell proteins, exemplified here by the citrullination of GRP78, contributes to loss of self-tolerance towards beta-cells in human type 1 diabetes, indicating that beta-cells actively participate in their own demise.

Introduction

Type 1 diabetes is an autoimmune disease characterized by T-cell mediated destruction of insulin-producing pancreatic beta-cells, leading to insulin deficiency [1]. Although it is well-established that loss of central and peripheral tolerance towards native self-proteins leads to the escape and accumulation of autoreactive T-cells, the exact mechanisms that trigger this break in self-tolerance are not entirely understood [2].

Growing evidence now suggests that the beta-cell is not a passive target of autoimmunity, but actively contributes to its own destruction by triggering the immune system [2]. Many of the triggers associated with type 1 diabetes, such as inflammatory cytokines, free radicals and viral infections, result in endoplasmic reticulum (ER) or oxidative stress [3–11]. These stresses can lead to modifications of beta-cell proteins, including post-translational modifications (PTMs) [10,12–17], mRNA alternative splicing, hybrid peptide formation [18,19], and non-conventional translation, potentially generating neo-epitopes against which no tolerance exists in the immune system [20]. In several autoimmune diseases, such as rheumatoid arthritis, multiple sclerosis, systemic lupus erythematosus and celiac disease, PTM proteins are established autoantigens [21,22], but their relevance in type 1 diabetes is only starting to be explored.

One PTM that received recent attention in type 1 diabetes is citrullination, which involves the deimination of arginine to citrulline by peptidyl arginine deiminase (PAD) enzymes [15,17,23]. Our group previously demonstrated that the ER chaperone glucose-regulated protein 78 (GRP78), classically known as an important regulator of the unfolded protein response [21], is citrullinated in beta-cell lines when exposed to inflammatory stress [17]. Here, we provide the first direct evidence by 2-dimensional difference gel electrophoresis (2D-DIGE) and liquid chromatography-tandem mass spectrometry (LC-MS/MS) that GRP78 is citrullinated in human islets after cytokine exposure. Together with the identification of higher $CD4^+$ T-cell frequencies directed against citrullinated GRP78 (citGRP78) epitopes and significantly elevated autoantibody titers in type 1 diabetes patients, these results suggest that citrullination of beta-cell proteins contribute to loss of tolerance towards beta-cells in human type 1 diabetes and identify citrullinated GRP78 as a novel islet autoantigen.

Research Design and Methods

Islet culture and treatment. Human islets were obtained from Alberta Diabetes Institute Islet Core and Pisa University, with the agreement of the local Ethical Committee from the University of Alberta (Pro00013094; Pro00001754) and Pisa University (#2615) [24,25](Supplementary Table 1), were cultured in DMEM, supplemented with 1% l-glutamine (Gibco), 10% FBS and 100 U/ml penicillin–streptomycin. Islets were exposed to human IL-1β (50 U/ml; R&D systems), murine TNF-α (1000 U/ml; R&D systems) and human IFN-γ (1000 U/ml; Preprotech) for 24 or 72 h [26], as indicated in the figure legends.

Human subjects. Blood samples for plasma and peripheral blood mononuclear cells (PBMCs) collection were obtained from individuals with type 1 diabetes and healthy controls (Supplementary Table 2, 3, and 4). All procedures were approved by the ethical committee UZ-Leuven (S52697) and conducted in accordance with the principles of the Declaration of Helsinki, with written informed consent obtained from all participants prior to inclusion.

Quantitative RT-PCR (qRT-PCR). qRT-PCR was performed as described [17]. Primers used: CHOP-Forward: GAACGGCTCAAGCAGGAAATC; CHOP-Reverse: TTCACCATTCGGTCAATCAGAG. GRP78: Hs.PT.58.22715160 (IDT). ATF3: Hs.PT.56a.41052403.g (IDT).

Cell death assays. Islets were incubated with propidium iodide (Invitrogen) and Hoechst 33342 (Invitrogen) for 15 min at 37°C. Imaging was performed using a Zeiss COLIBRI fast LED microscope. At least 15 islets were analyzed by two independent researchers (one blinded for sample identity).

2D-DIGE. Two-dimensional gel electrophoresis analysis was performed with protein lysates from 1000 islets/condition, as described [5].

2D-Western blotting. 2D-gels were blotted onto a PVDF membrane (GE Healthcare), and probed with anti-GRP78 antibody (Santa Cruz) and goat anti-rabbit HRP (Dako) as described [17].

Orbitrap LC-MS/MS analysis. Human islets were lysed in lysis buffer (7 M urea, 2 M thiourea, 4% (w/v) CHAPS, 40 mM Tris base, 1% (w/v) dithiothreitol (DTT), and a mixture of protease inhibitors (Complete protease inhibitor, Roche Diagnostics)). Cell lysates (10 µg) were reduced in 5 mM DTT for 30 min at 37ºC, followed by alkylation with 25 mM iodoacetamide (IAA) and quenching of excess IAA with 25 mM DTT, both by incubating for 30 min at 37ºC in the dark. Reduced and alkylated cell lysates were protein precipitated using the Wessel-Fluegge method [27] and were digested with 1 µg modified trypsin (Pierce) or 1.5 µg Glu-C (Promega) at pH 8 in the presence of 5% acetonitrile and 0.01% ProteaseMAX (Promega), subjected to desalting with C18 ZipTip pipette tips (Millipore) [28] and analyzed by unbiased LC-MS/MS or targeted data dependent acquisition (DDA) with inclusion list on a hybrid quadrupole-Orbitrap mass spectrometer (Q Exactive, Thermo Fisher). Peptides were identified by MASCOT (Matrix Science) using SwissProt (Homo sapiens, 169779 entries) as a database via Proteome Discoverer 2.2, incorporating Percolator for peptide validation. "Oxidation (M)", "deamidation (N/Q)", and "deamidation (R)" (referring to citrullination), were included as variable modifications, "carbamidomethylation (C)" as a fixed modification. Two missed cleavages were allowed for both trypsin and Glu-C digestion. Peptide tolerance was set at 15 ppm and MS/MS tolerance at 20 mmu. Only peptides displaying high and medium confidence (PEP < or equal to 0.01 and 0.05) were retained.

As citrullination and deamidation result in a mass increment of 0.98402 Da, whereas a C^{13} isotope of a native peptide results in a mass increment of 1.003355 Da, we manually checked differences in MH^+ of native versus modified peptide. Moreover, we checked differences in retention time between modified and native peptides, as described [29]. In order to discriminate between citrullination and deamidation we manually checked the MS/MS spectra of the putative citrullinated peptides, inspecting the isotopic distribution of peptide fragments containing N, Q and R.

Synthetic peptides (Synpeptide) IDVNGILRVTAE, IDVDGILRVTAE and IDVNGILXVTAE were used to distinguish between citrullinated and deamidated peptides, using LC-MS/MS fragmentation data and retention time as discriminating criteria.

Peptide-HLA binding prediction and affinity assays. Native and citGRP78 epitope candidates with predicted binding affinity for HLA-DRB1*04:01 were identified using the *in silico* approach outlined in previous work [30] and synthesized by Synpeptide. Binding affinity to HLA-DRB1*04:01 was measured by incubating increasing concentrations of

peptides in competition with 0.02 μmol/l biotinylated Influenza hemagglutinin (HA) 306–318 in wells coated with DRB1*04:01 protein. After washing, residual biotin-HA306–318 was detected using europium-conjugated streptavidin and quantified using a Victor2 D time-resolved fluorometer (PerkinElmer). IC50 values were calculated as the concentration needed to displace 50% of the biotin-HA306–318 peptide.

HLA-DRB1*04:01 protein and tetramer (Tmr) reagents. Recombinant HLA-DRB1*04:01 was purified from insect cell culture supernatants by affinity chromatography and dialyzed against phosphate buffer, pH 6.0. To prepare Tmrs, DRB1*04:01 was biotinylated *in vitro* and incubated with 0.2 mg/ml peptide at 37°C in 0.2% n-octyl-β-D-glucopyranoside and 1 mmol/l Pefabloc SC. Monomers were conjugated into Tmrs using RPE streptavidin at a molar ratio of 8:1.

Isolation of PBMCs. PBMCs from HLA-DRB1*04:01^{+} donors (Supplementary Table 2 and 3) were isolated from heparinized blood using Lymphoprep^{TM} and frozen in AIM-V medium (Gibco) containing 10% DMSO.

***In vitro* Tmr assays.** PBMCs were expanded *in vitro* as described [31]. Briefly, 5 × 10^6 PBMCs were stimulated with 6 μM of pooled citGRP78 peptides. After 14 d of *in vitro* stimulation, PE labeled Tmrs were used to stain T-cells specific for the GRP78 peptides (75 min at 37°C). Analysis was performed by FACSCalibur (BD Biosciences) using FlowJo software (TreeStar). Positivity was defined as the presence of a distinct population of CD4 bright cells at a percentage more than twofold above background (staining of cells from the unstimulated well, set to 0.1% for most experiments).

***Ex vivo* Tmr assays.** Cryopreserved PBMCs (20-30 x 10^6) were treated with 50 nM of Dasatinib (Cell Signaling) for 7 min at 37°C, and stained with Tmrs for 2 h at room temperature [32]. Cells were labeled with anti-PE and anti-APC magnetic beads for 20 min at 4°C. Next, 1/40th of the cells (pre-enriched fraction) was reserved to determine the total cell number used in the assay. The remaining fraction was enriched using Miltenyi Biotec MS magnetic columns (enriched fraction) according to the manufacturer's instructions. Both fractions were stained for 15 min at 4°C with CD4-BV421 (Biolegend), CCR7-APC-Cy7 (Biolegend), CD45RA-AF700 (BD Bioscience), Sytox Green (Thermo Fisher) and CD20/CD14-FITC (eBioscience). Samples were fully collected on a LSRII flow cytometer

(BD Bioscience) and analyzed using FlowJo software v.10.1. Data was expressed as the frequency of Tmr^+ cells per 10^6 living $CD4^+$ T-cells, and was considered positive when > 5 [33].

ELISA. Immunoreactivity of human plasma to native and citrullinated GRP78 was performed by ELISA (Supplementary Table 4). Briefly, 0.125 µg of recombinant human native or citGRP78 protein (constructed and produced as described, [17]) in 0.05 M carbonate-bicarbonate buffer, pH 9.6 (Sigma) was coated onto Immulon 2 HB plates (Thermo Fisher) overnight at 4°C. The plate was blocked with 1% BSA in PBS containing 0.05% Tween-20 (PBS-T) for 2 h at room temperature. Plasma was diluted 1:100 in PBS-T containing 0.3% BSA and incubated for 1 h. Mouse anti-GRP78 monoclonal antibody (Santa Cruz) served as a positive control. Species-specific goat anti-human IgG (Southern Biotech) or rat anti-mouse IgM alkaline phosphatase (Santa Cruz) was added as secondary reagent. Color was developed with para-nitrophenylphosphate substrate (Sigma). All readings were normalized to non-specific plasma binding from blank wells (no antigen added). In parallel assays, wells coated with PAD enzymes only confirmed specific binding to citGRP78.

Statistical analyses. Data are shown as mean ± SD. Significant differences between experimental conditions were assessed by paired Student's t-test, Wilcoxon ranked-sum test, Kruskal-Wallis test with Mann-Whitney U comparison, or multivariate analysis of variance (MANOVA), as indicated (GraphPad Prism v.7.02 and SPSS version 22). P-values < 0.05 were considered statistically significant.

Results

GRP78 is citrullinated in human islets of Langerhans upon inflammatory stress.

To study the effect of inflammation on the modification of GRP78, islets were exposed *in vitro* to IL-1β, IFN-γ and TNF-α for 24 or 72 h, followed by 2D-DIGE analyses. Cytokine exposure significantly induced cell death after 72 h (11.39% vs. 4.41% in control islets; $p < 0.05$, $n = 5$) (Figure 1A). Whereas after 24 h no increased cell death was observed (Figure 1A), upregulation of *CHOP*, *GRP78* and *ATF3* mRNA ($p < 0.05$, $n = 3$) suggested that increased ER stress preceded the induction of cell death (Figure 1B-D).

Analysis of the islet 2D-DIGE pattern in control islets revealed the presence of five GRP78 spots (I1-I5), differing in isoelectric point but not in molecular weight (Figure 1E). This pointed to the presence of at least five GRP78 isoforms, of which the identity was confirmed by 2D-Western blotting (Figure 1F). Quantitative 2D-DIGE analysis of cytokine-exposed vs. control islets ($n = 5$; D1-5, Supplementary Table 1) revealed a significant upregulation of the two most abundant GRP78 isoforms (isoform I2: 1.5-fold upregulated, $p < 0.05$; isoform I3: 1.64-fold upregulated, $p < 0.05$). In three out of five islet donors (D1-3), this upregulation was paralleled by a shift in relative abundance towards the isoform with the lower isoelectric point (isoform I3), indicative of an increased acidic nature consistent with citrullination or other PTMs, such as phosphorylation, deamidation and ribosylation amongst others (Figure 1G-H).

To identify which GRP78 amino acid residues are modified, we investigated the human islet proteome by unbiased LC-MS/MS ($n = 5$; D6-10, Supplementary Table 1). Based on our earlier observation that GRP78 is citrullinated in INS-1E cells [17], we specifically focused on the identification of citrullinated arginine (R) residues. GRP78 contains 28 arginines, all potential sites for citrullination. Combined LC-MS/MS analysis of trypsin and Glu-C digested islets from 5 islet donors, control or cytokine-exposed, resulted in 67.58% and 71.71% coverage of GRP78, respectively, including 22 of the 28 arginines (Figure 2A). However for none of these covered R-residues a conversion to citrulline could be unambiguously identified.

We hypothesized that the inability to detect citrullination of GRP78 could either indicate that citrullination does not occur, that the citrullinated R-residues are not covered in the current approach, or that they are not abundant enough to be detected by unbiased LC-MS/MS. We therefore applied a targeted MS/MS strategy (data dependent acquisition), specifically focusing on R510, the arginine residue we have previously identified as being citrullinated in

INS-1E upon cytokine-exposure and against which diabetic NOD mice have elevated levels of circulating autoantibodies and autoreactive T-cells [17]. As citrullination is often difficult to distinguish from deamidation, we first analyzed a native, citrullinated and deamidated version of a synthetic Glu-C peptide harboring R510 by LC-MS/MS. We measured a clear difference in retention time of the different peptides: 182.53 ± 1.87 min, 209.92 ± 1.91 min and 189.18 ± 0.66 min, respectively (Figure 2B). Unbiased LC-MS/MS analysis of 5 different human islet samples, control or cytokine-exposed, revealed the presence of precursor ions with a mass, charge and retention time corresponding to those of the synthetic native and citrullinated peptides. By subsequent targeted LC-MS/MS, fragmentation data were obtained which enabled us to unambiguously assign these precursor ions to the native and citrullinated R510 harboring peptides and to distinguish them from the deamidated peptide, the latter based on the diagnostic b+-ion of m/z 612.34 (Figure 2C). A combination of correct m/z, retention times and diagnostic fragmentation data led us to conclude that R510 is citrullinated in human islets.

Citrullinated GRP78 peptides can be presented in the context of HLA-DRB1*04:01.

Based on the finding that GRP78 is citrullinated in cytokine-exposed human islets, we investigated if GRP78 citrullination could result in the generation of neo-epitopes recognized in patients with type 1 diabetes. To this end, we first identified GRP78 peptide sequences containing possible HLA-binding motifs. Since we could not cover the full length GRP78 protein sequence by mass spectrometry analysis, thus possibly missing other citrullinated R-residues, we screened the entire GRP78 sequence for potential HLA-DRB1*04:01 binders. We specifically focused on HLA-DRB1*04:01, as binding to this allotype has been shown to be enhanced by citrullination at certain amino acid positions [34,35]. A previously developed algorithm was used to *in silico* predict peptide sequences with a high affinity for HLA-DRB1*04:01 in their native or citrullinated forms. Thirteen peptides were identified as potential binders (Table 1). Interestingly, among these peptides were 498-512X and 500-514X, citrullinated at R510, the residue identified here by LC-MS/MS as citrullinated in cytokine-exposed islets and previously identified as autoantigenic epitope in NOD mice [17]. *In vitro* binding affinity assays demonstrated that 3 of the 13 citrullinated peptides bound to DRB1*04:01, namely 195-209X (citrullinated on R197, covered by LC-MS/MS, but not modified), 498-512X (citrullinated on R510) and 500-514X (citrullinated on R510) (Table 1). Two other peptides, i.e. 58-72R and 65-79R, bound DRB1*04:01 only in their native form, but with much lower affinity.

Circulating CD4$^+$ T-cells directed against citrullinated GRP78 498-512X are present at higher frequencies in patients with type 1 diabetes compared to healthy subjects.

We assessed the ability of the citrullinated peptides 195-209X, 498-512X and 500-514X to elicit CD4$^+$ T-cell responses in PBMCs from type 1 diabetes patients and healthy subjects (Supplementary Table 2) stimulated *in vitro* for 14 d, followed by detection with DRB1*04:01 tetramers (Tmrs). Despite the low binding affinity of 498-512X and 500-514X (Table 1), stable Tmrs could be generated. All peptides elicited positive CD4$^+$ T-cell responses in multiple subjects (Figure 3). One epitope, 498-512X was exclusively recognized by CD4$^+$ T-cells from patients with type 1 diabetes. Peptide 195-209X elicited T-cell responses in one out of six healthy subjects and in one patient with type 1 diabetes. In two out of six healthy subjects, a response against peptide 500-514X was observed, whereas only one patient with type 1 diabetes was responsive (Figure 3).

Based on this first evidence that citGRP78 epitopes are able to elicit T-cell responses, we assessed the frequencies of CD4$^+$ T-cells that recognize citGRP78 epitopes in PBMCs from patients with new-onset type 1 diabetes (< 1 year, n = 4), patients with longstanding type 1 diabetes (≥ 1 year, n = 11), and healthy subjects (n = 8) by direct *ex vivo* Tmr assays (Figure 4A-H) (donor characteristics listed in Supplementary Table 3). We focused on peptides that elicited a similar or higher T-cell response in subjects with type 1 diabetes when compared to healthy controls based on the *in vitro* Tmr assay results (Figure 3). 498-512X reactive CD4$^+$ T-cells were more frequent in patients with type 1 diabetes than in healthy controls, with a positive response in 7 out of 15 patients (Figure 4J). Although the native variant 498-512R also elicited a positive response in 5 out of 15 patients, the frequency of 498-512R reactive CD4$^+$ T-cells was not significantly higher in patients with type 1 diabetes than in healthy controls (Figure 4I). For peptide 195-209X (Figure 4L) and its corresponding unmodified sequence (Figure 4K), a positive response was observed in a subset of patients, but the frequency of Tmr$^+$ cells was not significantly higher than in healthy subjects. Although no significant difference was observed when comparing T-cell frequencies recognizing the native and citrullinated form of each peptide in each individual (Figure 4M-P), a trend toward higher frequencies of Tmr$^+$ T-cells directed against 498-512X was observed ($p = 0.058$) (Figure 4N).

In samples from type 1 diabetes patients with more than 10 Tmr$^+$ cells, we further examined the activation phenotype of 498-512R and 498-512X experienced Tmr$^+$ T-cells based on CCR7 and CD45RA (Figure 5A-C). In patients with longstanding type 1 diabetes, the 498-512X Tmr$^+$ fraction displayed a higher percentage of central memory and a lower percentage

of effector memory cells, as compared to the 498-512R Tmr^+ fraction (Figure 5D-E). No differences were observed in the frequency of naive T-cells specific for 498-512R and 498-512X (Figure 5F).

Native and citrullinated GRP78 are targets for autoantibodies in patients with type 1 diabetes

Next, we assessed autoantibody reactivity against citGRP78 and its native counterpart in plasma of patients with new-onset type 1 diabetes (n = 47), longstanding type 1 diabetes (n = 61) and of healthy subjects (n = 89) (Figure 6A) (Supplementary Table 4). With 33% of the longstanding type 1 diabetes patients being autoantibody-positive, these patients had significantly higher anti-citGRP78 autoantibody titers than healthy controls (Figure 6A-B). When comparing the reactivity against native and citGRP78 of the same subjects, patients with new-onset and longstanding type 1 diabetes had significantly higher titers of anti-citGRP78 autoantibodies. Interestingly, 4 out of 5 citGRP78 autoantibody-positive patients with new-onset type 1 diabetes and 15 out of 20 citGRP78 autoantibody-positive patients with longstanding type 1 diabetes also showed reactivity against the native epitope (Figure 6C-E).
Autoantibodies against native and citrullinated GRP78 were primarily present in patients with multiple autoantibodies against insulin, GADA and/or IA2-A (Figure 6F-G). When comparing autoantibody positivity for native and/or citrullinated GRP78 in relation to diabetes duration, autoantibodies against native and/or citrullinated GRP78 were most often detected in patients diagnosed within 1-5 years (Figure 6H).

Discussion

Here we report on the citrullinated ER chaperone GRP78 as a novel autoantigen in human type 1 diabetes. We demonstrate that citrullination of GRP78 occurs in human islets exposed to inflammatory stress. With some citGRP78 peptides displaying strong binding to HLA DRB1*04:01, we identified the presence of $CD4^+$ T-cells and circulating autoantibodies reactive against citGRP78 in a subset of type 1 diabetes patients. We thus provide the first direct evidence that beta-cell proteins can be citrullinated and that islet cell-derived GRP78 epitopes may be involved in the pathogenesis of human type 1 diabetes through the formation of citrullinated neo-antigens that evoke $CD4^+$ T-cell and B-cell responses.

In recent years, PTMs of beta-cell proteins have emerged as a potential source for neo-antigens in type 1 diabetes, with many studies focusing on their role in inducing T-cell responses [12–16,18,19,36]. Here, we provide a more comprehensive insight by covering not only the interaction between the modified proteins and the immune system, but also the role of inflammation in inducing these modifications. Given that the PTM citrullination is closely associated with inflammatory conditions and inflammation plays a pivotal role in the induction, amplification and maintenance of type 1 diabetes [2], this modification may also be important in this disease [37]. The observation that T-cell responses are detected against citrullinated peptides derived from GAD65 [15], IAPP [14], and now also GRP78, is an indication that citrullination indeed plays a role in human type 1 diabetes. Importantly, we show here for the first time that beta-cell proteins can be citrullinated when human islets are exposed to inflammatory cytokines (IL-1β, IFN-γ and TNF-α), as exemplified by the citrullination of GRP78. This finding reinforces the concept that inflammation modulates the cross-talk between the beta-cell and the immune system, not only by mediating beta-cell dysfunction and death [3], but also by inducing beta-cell neo-antigen formation. Recent data on islet-reactive $CD8^+$ T cells suggests that the autoimmune repertoire between T1D and healthy controls is largely overlapping [38], and that the critical ingredient leading to the priming of this repertoire may be the availability of its target islet antigens in the inflammatory milieu of insulitis. In light of the evidence that GRP78 citrullination is induced by pro-inflammatory cytokines, we hypothesize that this paradigm may also apply to citrullinated GRP78.

We have used a combination of IL-1β, IFN-γ and TNF-α, where TNF-α may be crucial to evoke cytokine-induced PTMs in human islets, as the combination of IL-1β and IFN-γ alone does not induce GRP78 modifications in human islets [39]. Of importance, PTMs of GRP78

were not induced in all islet donors investigated; in two islet preparations, the levels of modified GRP78 were already elevated in control islets and could not be further enhanced by cytokine exposure. This may be explained by higher basal levels of stress which are intrinsically associated with the islet isolation procedure, such as a more harsh enzymatic digestion [40] or a longer cold ischemia time [41]. On the other hand, it may also reflect the inherent heterogeneity in humans, with not every individual being prone to citrullination, even when exposed to inflammation. Whether this is due to variations in genetic background, possibly leading to abnormal expression levels of PAD enzymes, or to differences in susceptibility to environmental triggers, which may result in increased Ca^{2+} fluxes necessary for the activation of PAD enzymes, remains to be elucidated.

Using mass spectrometry, we identified R510 as a citrullination site in human GRP78. The 2D-DIGE profile shows however at least 5 different isoforms, suggesting modifications on several residues. With a sequence coverage of 68-72%, identifying 22 of the 28 arginines within GRP78, we potentially missed other citrullination sites. The use of alternative digestion enzymes, such as Lys-C or elastase, or the use of a more sensitive targeted approach as performed here for the R510 region, may result in a more complete picture of GRP78 citrullination. Next to this, it may also be that other PTMs, such as deamidation, result in the multiple isoform spots observed in the 2D-DIGE [42].

Alterations in peptide epitopes are known to affect immune recognition by modulating HLA binding or influencing T-cell recognition [34,35,43]. Out of the thirteen citGRP78 peptides predicted to bind HLA-DRB1*04:01, two peptides with a R510 citrullination site (498-512X and 500-514X) were confirmed to be preferential binders compared with their native counterparts. This observation supports the notion that some HLA allotypes, such as HLA-DRB1*04:01, prefer citrulline over arginine at key peptide binding positions [34,35]. This may reflect either a direct interaction of citrulline with HLA anchor positions or an indirect effect mediated by a change in peptide orientation within the binding groove. The binding motif of HLA-DRB1*04:01 displays a strong preference for aliphatic and aromatic residues in the first binding pocket. According to this motif, the canonical nonamer motif of epitope 498-512X most likely to bind to HLA-DRB1*04:01 would be FEIDVNGIL, because it contains an optimal F residue at P1, a highly favorable D at P4, and a preferred N at P6 [44–46]. This places citrulline in position 10, which, according to Yassai et al. [47], can modulate peptide binding affinity and induce an alternative downward configuration of the peptide in the binding groove, thus shifting the TCR contact positions.

Although the binding affinity of peptide 195-209 was improved upon citrullination, the frequency of Tmr^+ T-cells directed against this epitope was not significantly increased in patients with type 1 diabetes compared to healthy subjects. Together with our observation that R197 is not citrullinated in cytokine-treated human islets (Fig. 2A), these results may indicate that citrullination of R197 does not readily occur in human type 1 diabetes.

R510 is exactly the same arginine residue as previously found to be citrullinated in INS-1E cells upon cytokine exposure and against which diabetic NOD mice have elevated levels of circulating autoantibodies and autoreactive T-cells [17]. Using *ex vivo* Tmr and ELISA assays, we now demonstrate elevated T-cell and autoantibody responses against 498-512X in type 1 diabetes patients, which strongly indicates that the same epitope as in NOD mice is recognized in human type 1 diabetes. The observation that there are patients with $CD4^+$ T-cells and autoantibody responses to the native, the citrullinated, or to both epitopes, may suggests that epitope spreading occurs [48]. There is great interest in knowing to which epitope the immune system initially responds, following the hypothesis that this epitope may behave as a disease driver [49] and may be a potential target for antigen-specific treatments [48].

The observation that B- and T-cell responses were more readily observed in patients with long-standing ($\geq$ 1 year) type 1 diabetes than in new-onset patients, underscores our hypothesis that citrullinated GRP78, rather than being a primary autoantigen, may be involved in the amplification of islet autoimmunity, once inflammation is already ongoing. The activation of PAD enzymes requires high levels of cytosolic Ca^{2+} [11], which is favored by ER dysfunction in combination with excessive insulin demand. A similar process has been described for the Ca^{2+}-dependent PTM enzyme tissue transglutaminase 2, which activity is increased by ER stress, leading to beta-cell stress-dependent immunogenicity [10]. In this scenario, our observation that GRP78 is translocated to the plasma membrane upon cytokine exposure in rodent beta cells [17] and human islets (unpublished data) is noteworthy. Although the mechanisms have not yet been elucidated, the combination of membrane translocation and increased cytosolic Ca^{2+} concentrations may be a prerequisite for GRP78 citrullination to occur.

Similar to other autoantigens [12,15], we show that both B- and T-cell responses to citGRP78 are not homogeneous across the patient population, which may reflect the heterogeneity of the disease or the sequestration of autoimmune T-cells in diseased organs [38]. This heterogeneity suggests that it is unlikely that a single marker can predict disease onset or progression, emphasizing the importance to identify other autoantigens and to map T-cell

responses and autoantibody generation in at-risk individuals and patients with type 1 diabetes during disease initiation and progression.

In conclusion, this study reinforces the notion that beta-cells are actively involved in their own destruction, by generating citrullinated beta-cell protein epitopes when exposed to inflammatory stress, as shown here for GRP78. We demonstrate that one of these citGRP78 epitopes is able to elicit $CD4^+$ T-cell and autoantibody responses, primarily in patients with longstanding type 1 diabetes. These results may suggest that immune responses against citGRP78 contribute to accelerate disease rather than to precipitate its development. On the other hand, it may also be possible that GRP78-specific immune cells were mainly sequestered in the pancreas and pancreatic lymph nodes until after disease onset, after which they emerged in the periphery. Our findings further suggest that epitope spreading occurs, with detectable T- and B-cell responses directed against both the native and citrullinated epitope. Given the heterogeneity of type 1 diabetes, the identification of PTM beta-cell proteins as potential neoantigens is critical for a better understanding of disease pathology, the discovery of new markers for earlier diagnosis and patient stratification, and the development of novel disease interventions, especially in the view of more personalized therapies. Determining the stage of disease during which T- and B-cell reactivity towards modified and native self-antigens occurs will shed more light on the question if responses to modified epitopes are primary responses and thus drivers of the disease process. This will be of crucial importance for disease prediction, prevention and development of antigen-specific therapies.

Author's Contributions

MB, AC, FMCS and IC contributed to the design, conduction, analysis and interpretation of the data and wrote and edited the manuscript. AC, EW and RD conducted, analyzed and interpreted the proteomics data. GB-F, DA-L, DPC and EAJ contributed to the design, analysis and/or interpretation of the peptide binding affinity and Tmr data. M-LY and MaM designed and performed the ELISA experiments, as well as contributed to the analysis and interpretation. MaB and PM provided human islets. MeM, JP and RM provided intellectual input. CM and LO designed research, interpreted data and wrote and edited the paper. All authors revised the article and gave their final approval of the version to be published. CM and LO are the guarantors of this work and, as such, had full access to all the data in the study and take responsibility for the integrity of the data and the accuracy of the data analysis.

Acknowledgements

We gratefully acknowledge Frea Coun (KU Leuven, Leuven, Belgium) and Dries Swinnen (KU Leuven, Leuven, Belgium) for technical assistance. We wish to thank Prof. Patrick MacDonald (Alberta Diabetes Institute, University of Alberta, Edmonton, Canada) for the supply of human islets. We thank Dr. Sebastien Carpentier, head of SyBioMA Mass Spectrometry facility (KU Leuven) for helpful advice. We acknowledge Hilde Morobé (UZ Leuven, Leuven, Belgium) for patient recruitment and collecting the blood samples. We thank all patients and healthy volunteers for participating in this study. This work was supported by the IMI2-JU under grant agreement No 115797 (INNODIA). This Joint Undertaking receives support from the Union's Horizon 2020 research and innovation program and "EFPIA", 'JDRF" and "The Leona M. and Harry B. Helmsley Charitable Trust"; the KU Leuven (GOA14/010); the JDRF (2-SRA-2015-52-Q-R, 1-INO-2018-638-A-N and a JDRF-PD fellowship for MB (3-PDF-2018-593-A-N)); and FWO (for a PhD fellowship for AC (1189518N) and for DPC (11Y6716N). The authors have declared that no conflict of interest exists.

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Tables

Table 1

Motif analysis for native and citrullinated DRB1*0401 GRP78 sequences

Native GRP78 peptides			Citrullinated GRP78 peptides			
Peptide	**Sequence**	**IC_{50} (µmol/L)[a]***	**Peptide**	**Cit. site**	**Sequence[b]**	**IC_{50} (µmol/L)[a]***
7-20R	AAMLLLLSAARAEE	ND	7-20X	R17	AAMLLLLSAA**X**AEE	ND
39-53R	YSCVGVFKNGRVEII	ND	39-53X	R49	YSCVGVFKNG**X**VEII	ND
58-72R	GNRITPSYVAFTPEG	63	58-72X	R60	GN**X**ITPSYVAFTPEG	ND
65-79R	YVAFTPEGERLIGDA	97	65-79X	R74	YVAFTPEGE**X**LIGDA	ND
97-110R	RLIGRTWNDPSVQQ	ND	97-110X	R97;R101	RLIG**X**TWNDPSVQQ	ND
172-186R	VPAYFNDAQRQATKD	ND	172-186X	R181	VPAYFNDAQ**X**QATKD	ND
195-209R	VMRIINEPTAAAIAY	0.04	195-209X	R197	VM**X**IINEPTAAAIAY	0.20
292-305R	VEKAKRALSSQHQA	ND	292-305X	R297	VEKAK**X**ALSSQHQA	ND
434-448R	TKLIPRNTVVPTKKS	ND	434-448X	R439	TKLIP**X**NTVVPTKKS	ND
498-512R	EVTFEIDVNGILRVT	ND	498-512X	R510	EVTFEIDVNGIL**X**VT	2.71
500-514R	TFEIDVNGILRVTAE	ND	500-514X	R510	TFEIDVNGIL**X**VTAE	4.89
523-536R	KITITNDQNRLTPE	ND	523-536X	R532	KITITNDQN**X**LTPE	ND
554-566R	KLKERIDTRNELE	ND	554-566X	R558;R562	KLKERIDT**X**NELE	ND

[a]IC_{50}: the peptide concentration that displaces half of the reference peptide (biotinylated HA306–318 peptide). A lower IC50 indicates better binding. [b] The residue that is modulated is bolded in each sequence. X indicates citrulline. *ND: not detectable.

Figure legends

Figure 1

GRP78 is post-translationally modified in human islets of Langerhans upon cytokine exposure. (**A**) Apoptosis in human control islets and after exposure to IL-1β (50 U/ml), IFN-γ (1000 U/ml) and TNF-α (1000 U/ml) for 24 or 72 h (n = 5). (**B-D**) Changes in mRNA levels of ER stress markers *CHOP* (**B**), *GRP78* (**C**), and *ATF3* (**D**) in human control islets and after exposure to IL-1β (50 U/ml), IFN-γ (1000 U/ml) and TNF-α (1000 U/ml) for 24 h (n = 3). (**E-F**) Human islets express 5 isoforms (I1-I5) of GRP78 that differ in isoelectric point but not in molecular weight, indicated by 2D-DIGE (pH 4-7) (**E**) and 2D-WB (**F**). (**G**) Three-dimensional graph view of the DeCyder analysis of the 2D-DIGE of human control islets and cytokine exposed islets (n = 5). I1-I5 represent the 5 different isoforms of GRP78. (**H**) The ratio of GRP78 isoforms I3/I2 (shown in %) is increased in cytokine exposed islets from donor D1, D2 and D3, while decreased in islets from D4 and D5. Data in A-D are expressed as mean ± SD and analyzed by a paired student's t-test. * $p < 0.05$.

Figure 2

Identification of citrullinated GRP78 residues in control and cytokine-exposed human islets using LC-MS/MS. (**A**) Protein sequence coverage of GRP78 in control and cytokine-exposed (IL-1β (50 U/ml), IFN-γ (1000 U/ml) and TNF-α (1000 U/ml) for 72 h) human islets. Coverage is indicated in red, arginine residues are indicated in bold, the signal peptide in italic, and the synthetic peptide used for LC-MS/MS is underlined. (B) Fragmentation data, m/z values and retention times (RT) of synthetic IDVNGILRVTAE (native), IDVNGILXVTAE (citrullinated) and IDVDGILRVTAE (deamidated) peptides using LC-MS/MS. (C) Fragmentation data, m/z values and RT of the native and citrullinated peptides found in human islets using targeted LC-MS/MS (data dependent acquisition). Detected ions are indicated in bold, the diagnostic ion to distinguish citrullination from deamidation is underlined.

Figure 3

Analysis of $CD4^+$ T-cell responses to citrullinated GRP78 peptides using *in vitro* Tmr assays. To identify immunogenic citrullinated peptides derived from GRP78, PBMCs from 6 subjects with type 1 diabetes and 6 healthy controls were expanded for two weeks by stimulating with peptide and then stained with the corresponding DRB1*04:01 Tmr. The

representative FACS plots shown depict Tmr^+ $CD4^+$ T-cells (percentages shown in each upper right quadrant) directed against the citrullinated GRP78 peptide 195-209X (**A-B**), 498-512X (**C-D**), or 500-514X(**E-F**) in healthy controls (**A,C,E**) and in subjects with type 1 diabetes (**B,D,F**). Using a previously established cutoff, Tmr staining of twice the mean value of the negative control (Tmr staining of unstimulated T-cells) (**G**), were considered to be positive responses (**H**).

Figure 4

Identification of $CD4^+$ T-cell frequencies directed against native and citrullinated GRP78 peptides in healthy controls and type 1 diabetes patients, analyzed by *ex vivo* Tmr assays. (**A-H**) Representative FACS plots of $CD4^+$ T-cells in the enriched fraction directed against the native GRP78 peptide 498-512R in (**A**) healthy controls and (**B**) type 1 diabetes patients; against the citrullinated GRP78 peptide 498-512X in (**C**) healthy controls and (**D**) type 1 diabetes patients; against the native GRP78 peptide 195-209R in (**E**) healthy controls and (**F**) type 1 diabetes patients; and against the citrullinated GRP78 peptide 195-209X in (**G**) healthy controls and (**H**) type 1 diabetes patients. (**I-L**) Total $CD4^+$ T-cell frequencies in the enriched fraction directed against (**I**) 498-512R, (**J**) 498-512X, (**K**) 195-209R, and (**L**) 195-209X in healthy controls ($n = 8$, ■) and in type 1 diabetes patients ($n = 15$), subdivided in patients with new-onset type 1 diabetes (< 1 year, $n = 4$, Δ) and patients with longstanding type 1 diabetes (≥ 1 year, $n = 11$, •). $CD4^+$ T-cell frequencies > 5 Tmr^+ cells/10^6 (dashed line) are considered positive (indicated in red). (**M-P**) Total $CD4^+$ T-cell frequencies in the enriched fraction comparing the native and citrullinated forms of peptide 498-512 in (**M**) healthy controls and in (**N**) patients with type 1 diabetes, and of peptide 195-209 in (**O**) healthy controls and in (**P**) patients with type 1 diabetes. Data in I-L are expressed as mean ± SD and are analyzed by Mann-Whitney U test. * $p < 0.05$. M-P are analyzed using the Wilcoxon rank-sum test.

Figure 5

Phenotypic characterization of 498-512R and 498-512X experienced $CD4^+$ T-cells in patients with type 1 diabetes. (**A-C**) CCR7 and CD45RA were used to discriminate between antigen experienced central memory $CD4^+$ T-cells (TCM) ($CD45RA^-CCR7^+$), effector memory $CD4^+$ T-cells (TEM) ($CD45RA^-CCR7^-$), and naive $CD4^+$ T-cells ($CD45RA^+CCR7^+$). Gating was performed on the $CD4^+$ T-cells of the pre-enriched fraction (**A**) and imposed on the $CD4^+$ Tmr^+ cells against 498-512R (**B**) and 498-512X (**C**). Frequency of antigen

experienced central memory (TCM) (**D**), effector memory (TEM) (**E**) and naive (**F**) $CD4^+$ T-cells in patients with longstanding type 1 diabetes (n = 6). Data in D-F are analyzed using the Wilcoxon rank-sum test. * $p < 0.05$.

Figure 6

Autoantibody responses directed against native and citrullinated GRP78 in healthy controls and patients with type 1 diabetes. (A-B) Titers of anti-GRP78 and anti-citGRP78 autoantibodies **(A)** and frequencies of anti-GRP78 and anti-citGRP78 autoantibody positivity **(B)** in plasma of healthy controls (n = 89, ■), patients with new-onset type 1 diabetes (nT1D, < 1 year, n = 47, Δ), and patients with longstanding type 1 diabetes (T1D, ≥ 1 year, n = 61, •). Titers are considered positive (indicated in red) when the optical density (OD) is higher than the mean + 2 SDs of the control group. **(C-E)** Comparison of anti-GRP78 and anti-citGRP78 autoantibody titers in **(C)** healthy controls, **(D)** patients with new-onset type 1 diabetes and **(E)** patients with type 1 diabetes. **(F-G)** Anti-GRP78 and anti-citGRP78 autoantibody positivity in patients with new-onset **(F)** and longstanding **(G)** type 1 diabetes who are positive for none, one or multiple IAA, GADA and/or IA2-A autoantibodies. **(H)** Anti-GRP78 and anti-citGRP78 autoantibody positivity plotted against diabetes duration. Data are analyzed using MANOVA with Bonferroni-corrected post-hoc comparisons within (native vs. citrullinated) and between (HC vs. nT1D vs. T1D) subjects. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

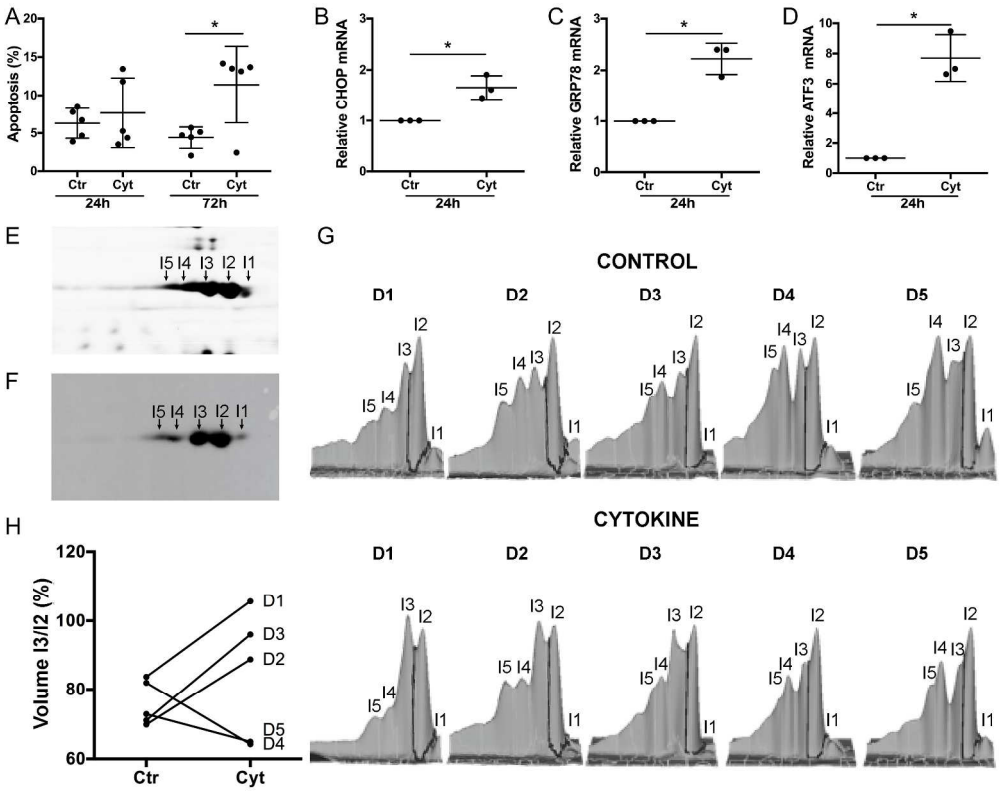

Figure 1┼ GRP78 is post-translationally modified in human islets of Langerhans upon cytokine exposure. (A) Apoptosis in human control islets and after exposure to IL-1β (50 U/ml), IFN-γ (1000 U/ml) and TNF-α (1000 U/ml) for 24 or 72 h (n=5). (B-D) Changes in mRNA levels of ER stress markers CHOP (B), GRP78 (C), and ATF3 (D) in human control islets and after exposure to IL-1β (50 U/ml), IFN-γ (1000 U/ml) and TNF-α (1000 U/ml) for 24 h (n=3). (E-F) Human islets express 5 isoforms (I1-I5) of GRP78 that differ in isoelectric point but not in molecular weight, indicated by 2D-DIGE (pH 4-7) (E) and 2D-WB (F). (G) Three-dimensional graph view of the DeCyder analysis of the 2D-DIGE of human control islets and cytokine exposed islets (n=5). I1-I5 represent the 5 different isoforms of GRP78. (H) The ratio of GRP78 isoforms I3/I2 (shown in %) is increased in cytokine exposed islets from donor D1, D2 and D3, while decreased in islets from D4 and D5. Data in A-D are expressed as mean ± SD and analyzed by a paired student's t-test. *p<0.05.

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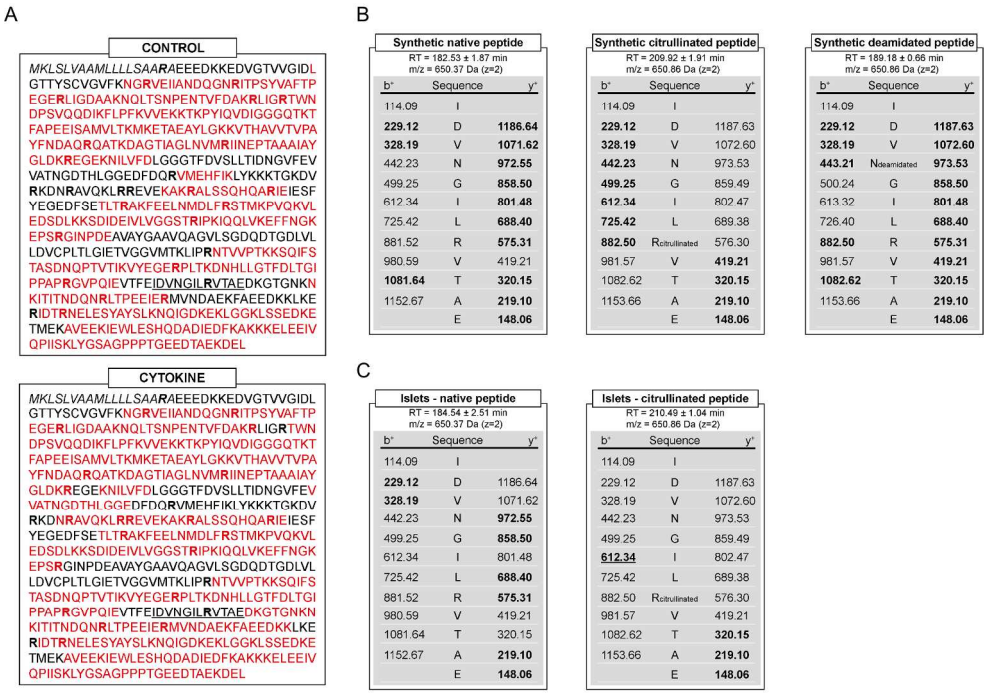

Figure 2 Identification of citrullinated GRP78 residues in control and cytokine-exposed human islets using LC-MS/MS. (A) Protein sequence coverage of GRP78 in control and cytokine-exposed (IL-1β (50 U/ml), IFN-γ (1000 U/ml) and TNF-α (1000 U/ml) for 72 h) human islets. Coverage is indicated in red, arginine residues are indicated in bold, the signal peptide in italic, and the synthetic peptide used for LC-MS/MS is underlined. (B) Fragmentation data, m/z values and retention times (RT) of synthetic IDVNGILRVTAE (native), IDVNGILXVTAE (citrullinated) and IDVDGILRVTAE (deamidated) peptides using LC-MS/MS. (C) Fragmentation data, m/z values and RT of the native and citrullinated peptides found in human islets using targeted LC-MS/MS (data dependent acquisition). Detected ions are indicated in bold, the diagnostic ion to distinguish citrullination from deamidation is underlined.

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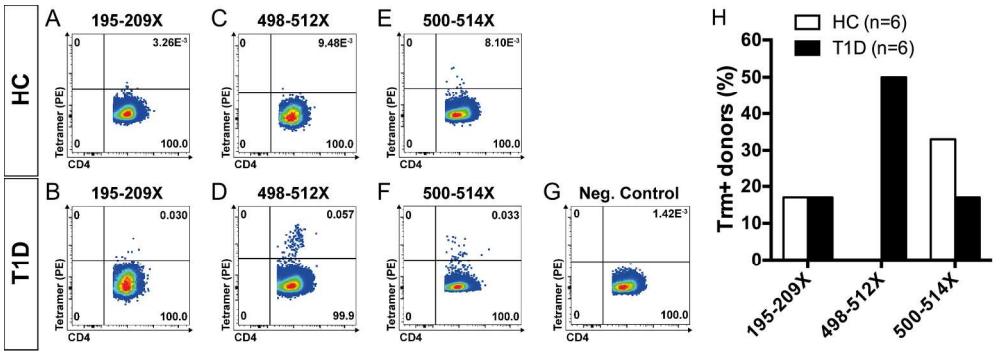

Figure 3

Analysis of CD4+ T-cell responses to citrullinated GRP78 peptides using in vitro Tmr assays. To identify immunogenic citrullinated peptides derived from GRP78, PBMCs from 6 subjects with type 1 diabetes and 6 healthy controls were expanded for two weeks by stimulating with peptide and then stained with the corresponding DRB1*04:01 Tmr. The representative FACS plots shown depict Tmr+ CD4+ T-cells (percentages shown in each upper right quadrant) directed against the citrullinated GRP78 peptide 195-209X (A-B), 498-512X (C-D), or 500-514X(E-F) in healthy controls (A,C,E) and in subjects with type 1 diabetes (B,D,F). Using a previously established cutoff, Tmr staining of twice the mean value of the negative control (Tmr staining of unstimulated T-cells) (G), were considered to be positive responses (H).

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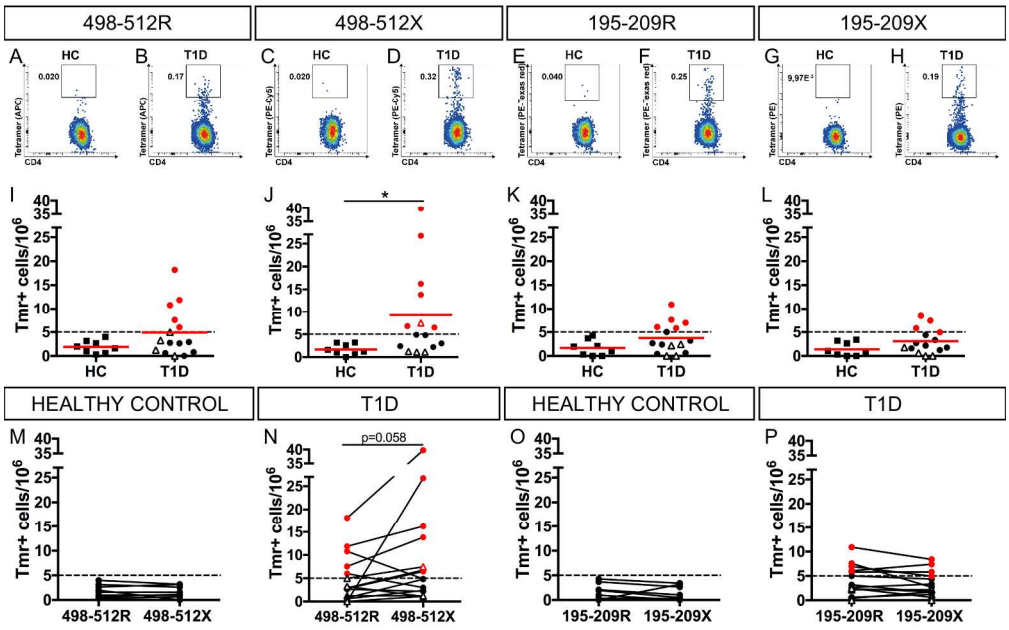

Figure 4┼ Identification of CD4+ T-cell frequencies directed against native and citrullinated GRP78 peptides in healthy controls and type 1 diabetes patients, analyzed by ex vivo Tmr assays. (A-H) Representative FACS plots of CD4+ T-cells in the enriched fraction directed against the native GRP78 peptide 498-512R in (A) healthy controls and (B) type 1 diabetes patients; against the citrullinated GRP78 peptide 498-512X in (C) healthy controls and (D) type 1 diabetes patients; against the native GRP78 peptide 195-209R in (E) healthy controls and (F) type 1 diabetes patients; and against the citrullinated GRP78 peptide 195-209X in (G) healthy controls and (H) type 1 diabetes patients. (I-L) Total CD4+ T-cell frequencies in the enriched fraction directed against (I) 498-512R, (J) 498-512X, (K) 195-209R, and (L) 195-209X in healthy controls (n=8, ■) and in type 1 diabetes patients (n=15), subdivided in patients with new-onset type 1 diabetes (<1 year, n=4, Δ) and patients with longstanding type 1 diabetes (≥1 year, n=11, ●). CD4+ T-cell frequencies >5 Tmr+ cells/10^6 (dashed line) are considered positive (indicated in red). (M-P) Total CD4+ T-cell frequencies in the enriched fraction comparing the native and citrullinated forms of peptide 498-512 in (M) healthy controls and in (N) patients with type 1 diabetes, and of peptide 195-209 in (O) healthy controls and in (P) patients with type 1 diabetes. Data in I-L are expressed as mean ± SD and are analyzed by Mann-Whitney U test. *p<0.05. M-P are analyzed using the Wilcoxon rank-sum test.

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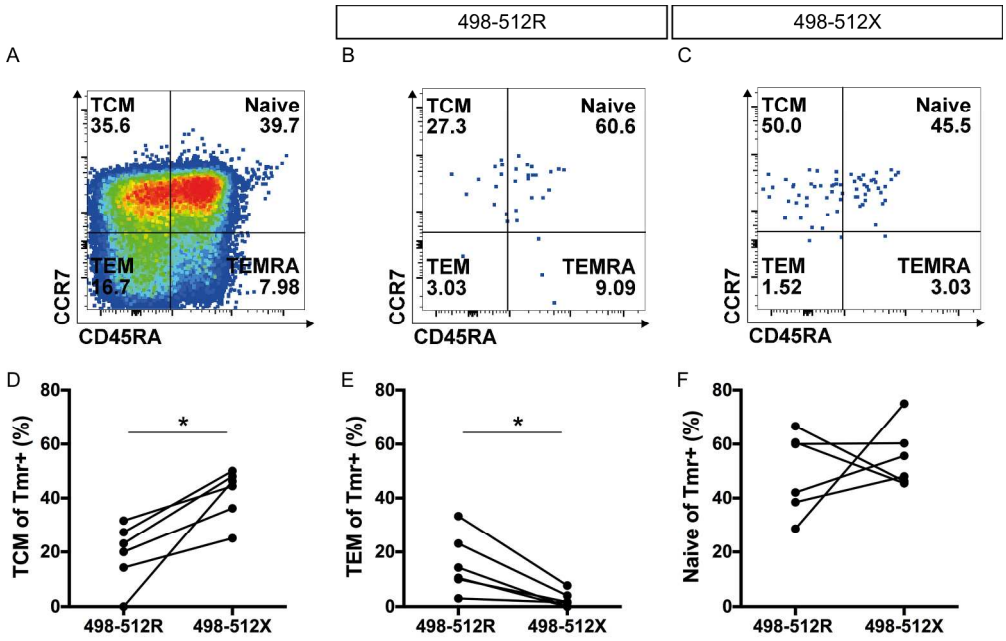

Figure 5
Phenotypic characterization of 498-512R and 498-512X experienced CD4+ T-cells in patients with type 1 diabetes. (A-C) CCR7 and CD45RA were used to discriminate between antigen experienced central memory CD4+ T-cells (TCM) (CD45RA-CCR7+), effector memory CD4+ T-cells (TEM) (CD45RA-CCR7-), and naive CD4+ T-cells (CD45RA+CCR7+). Gating was performed on the CD4+ T-cells of the pre-enriched fraction (A) and imposed on the CD4+ Tmr+ cells against 498-512R (B) and 498-512X (C). Frequency of antigen experienced central memory (TCM) (D), effector memory (TEM) (E) and naive (F) CD4+ T-cells in patients with longstanding type 1 diabetes (n=6). Data in D-F are analyzed using the Wilcoxon rank-sum test. *p<0.05.

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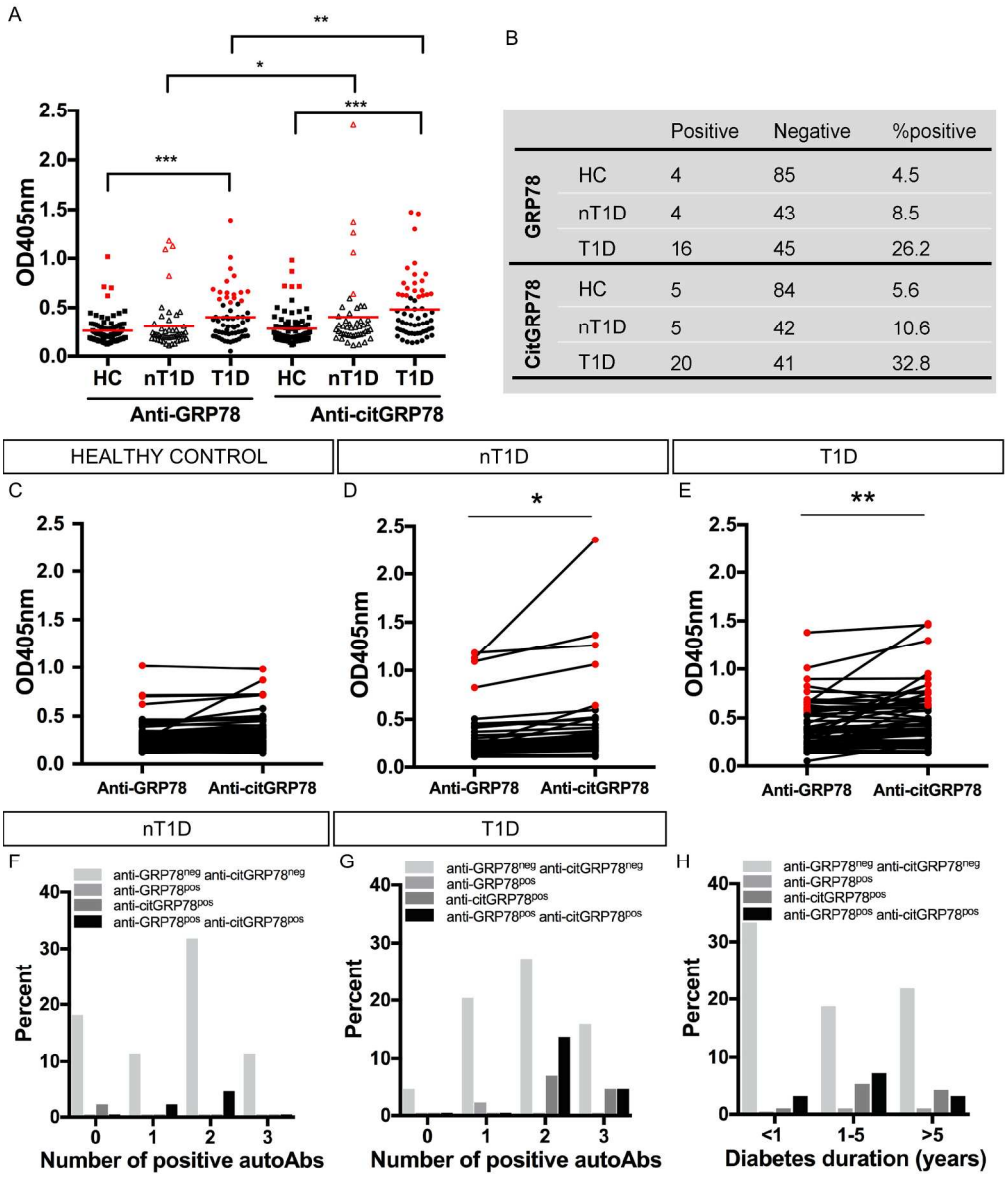

		Positive	Negative	%positive
GRP78	HC	4	85	4.5
	nT1D	4	43	8.5
	T1D	16	45	26.2
CitGRP78	HC	5	84	5.6
	nT1D	5	42	10.6
	T1D	20	41	32.8

Figure 6 Autoantibody responses directed against native and citrullinated GRP78 in healthy controls and patients with type 1 diabetes. (A-B) Titers of anti-GRP78 and anti-citGRP78 autoantibodies (A) and frequencies of anti-GRP78 and anti-citGRP78 autoantibody positivity (B) in plasma of healthy controls (n = 89, □), patients with new-onset type 1 diabetes (nT1D, < 1 year, n = 47, Δ), and patients with longstanding type 1 diabetes (T1D, ≥ 1 year, n = 61, □). Titers are considered positive (indicated in red) when the optical density (OD) is higher than the mean + 2 SDs of the control group. (C-E) Comparison of anti-GRP78 and anti-citGRP78 autoantibody titers in (C) healthy controls, (D) patients with new-onset type 1 diabetes and (E) patients with type 1 diabetes. (F-G) Anti-GRP78 and anti-citGRP78 autoantibody positivity in patients with new-onset (F) and longstanding (G) type 1 diabetes who are positive for none, one or multiple IAA, GADA and/or IA2-A autoantibodies. (H) Anti-GRP78 and anti-citGRP78 autoantibody positivity plotted against diabetes duration. Data are analyzed using MANOVA with Bonferroni-corrected post-hoc comparisons within (native vs. citrullinated) and between (HC vs. nT1D vs. T1D) subjects. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

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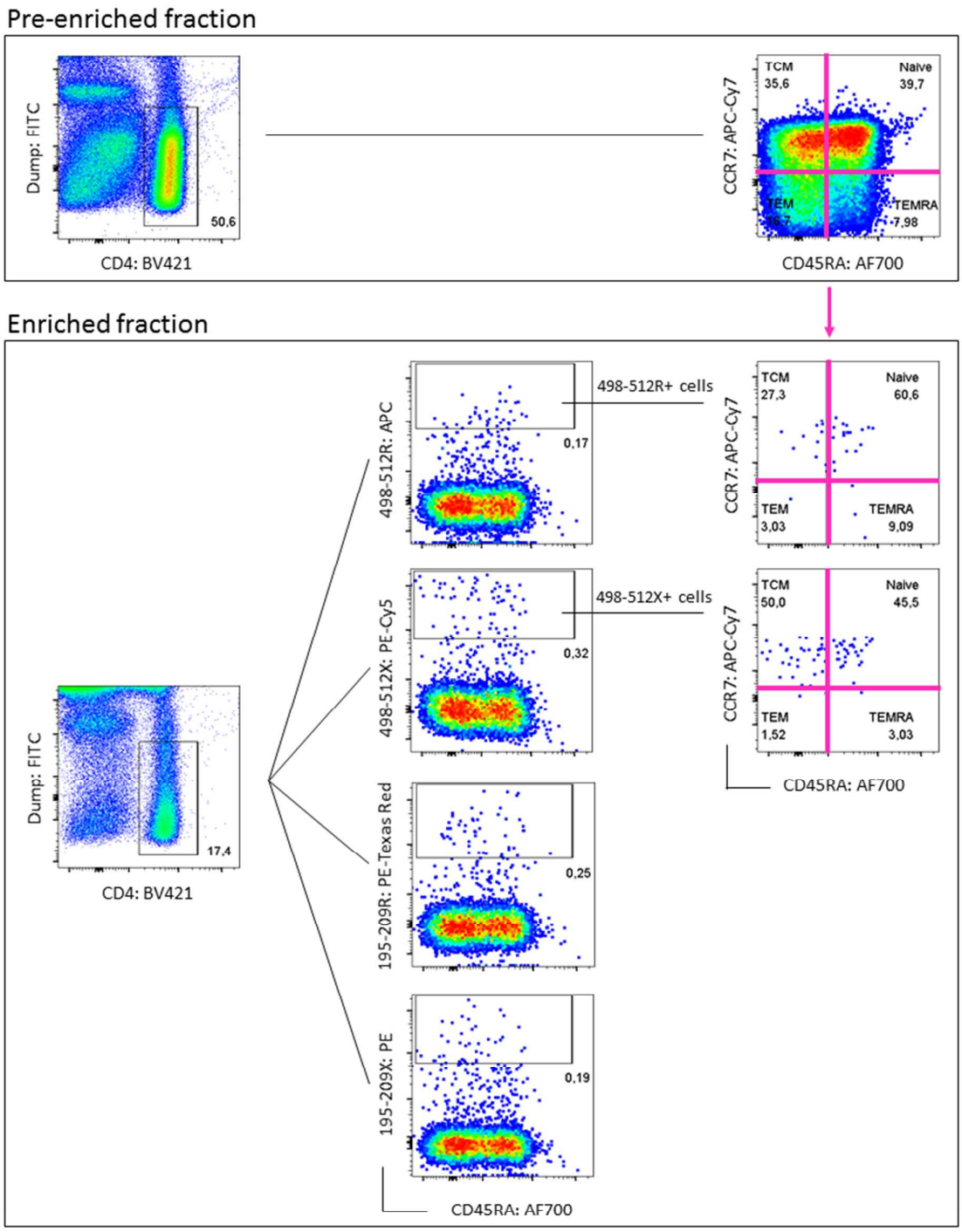

Supplementary Figure 1

Gating strategy for *ex vivo* Tmr assay. Initially, living CD4+ T cells were gated in the pre-enriched and enriched fractions, excluding dead cells, B cells and monocytes (gated out using Sytox green, CD20 and CD14-FITC dump channel). The living CD4+ population from the pre-enriched fraction was used to estimate the total number of cells used in the assay

(described in the equation below) and to set the gate for the phenotypic characterization (CD45RA vs. CCR7). Subjects in which there were less than 23 000 living CD4+ T cells in the pre-enriched fraction were excluded from the results. CD4+ T-cell population in the enriched fraction was sub-gated in the 4 Tmr+ populations: 498-512R+, 498-512X+, 195-209R+ and 195-209X+. The gated Tmr populations 498-512R+ and 498-512X+ were further sub-gated in central memory (TCM) (CD45RA-CCR7+), effector memory cells (TEM) (CD45RA-CCR7-), and naive cells (CD45RA+CCR7+) using the gate set for phenotypic characterization in the pre-enriched fraction. Frequencies of cells in each gate are expressed as the percentage of the parental population. The frequency of Tmr+ cells per 10^6 living CD4+ T cells (F) was calculated according to the following equation:

$$F = \left[\frac{Tmr^{+}\ events\ in\ enriched\ fraction}{(number\ of\ living\ CD4^{+}\ T\ cells\ in\ pre\text{-}enriched\ fraction)\ \times\ 40} \right] \times\ 10^{6}$$

Positive reactivity was predefined as more than 5 Tmr positive cells per million living CD4+ T cells, to exclude antigenic baseline.

Supplementary Table 1
Characteristics of islet donors

Donor	Gender (M/F)	Age (years)	BMI (kg/m^2)	HbA1c (% (mmol/mol))	Cold ischemia time (hours)
D1	M	52	23.8	5.3 (37)	NA
D2	M	53	34.1	5.6 (38)	7.9
D3	F	71	37.0	5.9 (41)	7.3
D4	F	62	28.3	6.1 (43)	13.8
D5	F	60	36.4	6.2 (44)	18.0
D6	M	59	19.8	NA	14
D7	F	84	28.2	NA	15
D8	F	75	NA	NA	17
D9	F	74	33.1	NA	16
D10	F	60	23.4	NA	12

NA: not available

Supplementary Table 2
DRB1*04:01^{+} type 1 diabetic patients (T1D) and healthy controls (HC) analyzed with *in vitro* tetramer assays.

Subject	Gender (M/F)	Age (yrs)	Time since diagnosis (yrs)	HLA-DQ	HLA-DR	Autoantibodies*
HC1	M	26	-	DQ8, DQ8	DR4, DR4	NA
HC2	F	23	-	DQ7, DQ8	DR4, DR4	NA
HC3	M	33	-	DQ2, DQ8	DR17, DR4	NA
HC4	F	42	-	DQ8, DQ5	DR4, DR10	NA
HC5	F	48	-	DQ8, DQ8	DR4, DR4	NA
HC6	F	24	-	DQ8, **	DR4, DR15	NA
T1D1	M	22	1	DQ5, DQ2	DR4, DR13	GADA
T1D2	F	26	5	DQ2, DQ8	DR17, DR4	GADA
T1D3	M	24	2	DQ8, DQ8	DR4, DR4	IA2-A
T1D4	F	39	2	DQ8, DQ2	DR17, DR4	Negative
T1D5	M	39	3	DQ7, DQ8	DR4, DR4	GADA
T1D6	F	24	1	DQ5, DQ8	DR4, DR13	GADA, IA2-A

*GADA: Glutamic acid decarboxylase autoantibodies
IA2-A: protein tyrosine phosphatase autoantibodies
** Allele DQB1*0619, not assigned by WHO
NA: not available

Supplementary Table 3
DRB1*04:01^{+} healthy control subjects (HC), patients with new-onset type 1 diabetes (nT1D), and patients with type 1 diabetes (T1D), analyzed with *ex vivo* tetramer assay

Subject	Gender (M/F)	Age (yrs)	Time since diagnosis (yrs)	HLA-DQ	HLA-DR	Autoantibodies *
HC1	M	36	-	DQ2, DQ8	DR17, DR4	NA
HC2	F	44	-	DQ8, DQ5	DR4, DR10	NA
HC3	F	27	-	DQ7, DQ6	DR4, DR13	NA
HC4	F	48	-	DQ8, DQ8	DR4, DR4	NA
HC5	F	24	-	DQ8, **	DR4, DR15	NA
HC6	F	39	-	DQ8, DQ4	DR4, DR8	NA
HC7	F	53	-	DQ7, DQ7	DR4, DR11	NA
HC8	F	32	-	DQ2, DQ8	DR17, DR4	NA
nT1D1	M	38	0	DQ7, DQ2	DR17, DR4	GADA
nT1D2	F	23	0	DQ8, DQ2	DR17, DR4	GADA, IA2-A
nT1D3	M	36	0	DQ8, DQ2	DR4, DR13	GADA
nT1D4	F	22	0	DQ5, DQ8	DR4, DR13	NA
T1D1	M	24	2	DQ5, DQ2	DR4, DR13	GADA
T1D2	F	24	5	DQ2, DQ8	DR4, DR7	IAA, GADA, IA2-A
T1D3	F	19	5	DQ7, DQ8	DR4, DR4	IAA, GADA
T1D4	F	29	7	DQ2, DQ8	DR17, DR4	NA
T1D5	F	40	3	DQ8, DQ2	DR17, DR4	Negative
T1D6	F	22	1	DQ8, DQ4	DR4, DR4	IAA, GADA
T1D7	M	41	3	DQ7, DQ8	DR4, DR4	GADA
T1D8	F	26	2	DQ5, DQ8	DR4, DR13	GADA, IA2-A
T1D9	F	22	4	DQ8, DQ9	DR4, DR4	IAA, GADA, IA2-A
T1D10	M	19	3	DQ8, DQ4	DR4, DR8	IAA, IA2-A
T1D11	F	24	2	DQ5, DQ8	DR4, DR13	NA

*GADA: Glutamic acid decarboxylase autoantibodies
IAA: insulin autoantibodies
ICA: islet cell cytoplasmic autoantibodies
IA2A: protein tyrosine phosphatase autoantibodies
** Allele DQB1*0619, not assigned by WHO
NA: not available

Supplementary Table 4
Characteristics of healthy control subject (HC), patients with new-onset type 1 diabetes (nT1D) and patients with type 1 diabetes (T1D) analyzed for autoantibody responses

Subject	Gender (M/F)	Age (yrs)	Time since diagnosis (yrs)	HLA-DQ	HLA-DR	AutoAb (IAA, GADA IA2-A)*	AutoAb (anti-GRP78 / anti-citGRP78)
HC1	F	46	-	NA	DR7, DR13	NA	-
HC2	F	39	-	NA	DR4, DR10	NA	-
HC3	F	40	-	NA	DR13, DR14	NA	-
HC4	F	23	-	NA	DR8, DR11	NA	-
HC5	F	22	-	NA	DR14, DR15	NA	-
HC6	F	23	-	NA	DR4, DR13	NA	-
HC7	F	24	-	NA	DR17, DR16	NA	-
HC8	F	44	-	NA	DR4, DR4	NA	-
HC9	F	24	-	NA	DR4, DR13	NA	-
HC10	M	55	-	NA	DR1, DR8	NA	-
HC11	M	28	-	NA	DR4, DR13	NA	-
HC12	F	44	-	NA	DR11, DR13	NA	-
HC13	M	49	-	NA	DR17, DR17	NA	-
HC14	F	28	-	NA	DR1, DR7	NA	-
HC15	F	48	-	NA	DR4, DR11	NA	-
HC16	F	22	-	NA	DR4, DR13	NA	anti-GRP78, anti-citGRP78
HC17	M	36	-	NA	DR4, DR7	NA	-
HC18	F	25	-	NA	DR17, DR12	NA	-
HC19	M	23	-	NA	DR4, DR15	NA	-
HC20	F	22	-	NA	DR11, DR15	NA	-
HC21	F	28	-	NA	DR17, DR7	NA	-
HC22	M	28	-	NA	DR1, D3	NA	-
HC23	M	38	-	NA	DR4, DR7	NA	-
HC24	F	34	-	NA	DR11, DR16	NA	-
HC25	F	29	-	NA	DR7, DR11	NA	-
HC26	F	31	-	NA	DR7, DR13	NA	-
HC27	M	32	-	NA	DR17, DR4	NA	-
HC28	F	24	-	NA	DR17, DR17	NA	-
HC29	F	29	-	NA	DR17, DR4	NA	-
HC30	M	25	-	NA	DR11, DR13	NA	-
HC31	F	22	-	NA	DR4, DR15	NA	-
HC32	F	27	-	NA	DR4, DR13	NA	-
HC33	F	50	-	NA	DR17, DR13	NA	-
HC34	M	25	-	NA	DR7, DR10	NA	-
HC35	F	42	-	NA	DR17, DR15	NA	-
HC36	F	24	-	NA	DR11, DR13	NA	-
HC37	F	33	-	NA	DR8, DR13	NA	-
HC38	F	36	-	NA	DR4, DR8	NA	-
HC39	F		-	NA	DR4, DR13	NA	-
HC40	F	22	-	NA	DR7, DR15	NA	-
HC41	F	23	-	NA	DR17, DR4	NA	-
HC42	M		-	NA	DR4, DR4	NA	-
HC43	F	21	-	NA	DR17, DR7	NA	-
HC44	M	22	-	NA	DR7, DR11	NA	anti-GRP78, anti-citGRP78
HC45	M	23	-	NA	DR1, DR16	NA	-
HC46	M		-	NA	DR1, DR13	NA	-
HC47	F	22	-	NA	DR4, DR15	NA	-
HC48	M	21	-	NA	DR7, DR7	NA	-
HC49	F	21	-	NA	DR17, DR15	NA	-
HC50	M	21	-	NA	DR11, DR16	NA	-
HC51	F	21	-	NA	DR17, DR8	NA	anti-citGRP78
HC52	F	22	-	NA	DR4, DR4	NA	-

HC53	M	24	-	NA	DR1, DR4	NA	-
HC54	F	22	-	NA	DR11, DR13	NA	-
HC55	M	21	-	NA	DR4, DR15	NA	-
HC56	F	21	-	NA	DR17, DR11	NA	-
HC57	F	20	-	NA	DR1, DR17	NA	-
HC58	F	20	-	NA	DR4, DR8	NA	-
HC59	F	20	-	NA	DR4, DR13	NA	-
HC60	F	20	-	NA	DR13, DR15	NA	-
HC61	F	24	-	NA	DR13, DR13	NA	-
HC62	M	33	-	NA	DR13, DR15	NA	-
HC63	F	24	-	NA	DR8, DR13	NA	anti-GRP78, anti-citGRP78
HC64	F	28	-	NA	DR7, DR15	NA	-
HC65	F	24	-	NA	DR7, DR13	NA	-
HC66	M	26	-	NA	DR11, DR15	NA	-
HC67	F	18	-	NA	DR17/DR3, DR5/DR2	NA	-
HC68	F	28	-	NA	DR11, NA	NA	-
HC69	F	26	-	NA	DR7, DR8/DR11	NA	-
HC70	F	24	-	NA	DR1/DR101, DR13	NA	-
HC71	F	24	-	NA	DR8/DR11, DR12	NA	-
HC72	F	18	-	NA	DR7, DR11	NA	-
HC73	M	24	-	NA	DR4, DR7	NA	-
HC74	F	25	-	NA	DR9, DR13	NA	-
HC75	F	24	-	NA	DR4, DR7	NA	-
HC76	M	21	-	NA	DR7, DR11	NA	-
HC77	F	26	-	NA	DR4, DR7	NA	-
HC78	F	21	-	NA	DR17/DR3, DR5/DR2	NA	-
HC79	F	20	-	NA	DR4, DR7	NA	-
HC80	M	26	-	NA	DR10, DR5/DR2	NA	-
HC81	F	19	-	NA	DR8/DR11, DR13	NA	-
HC82	M	19	-	NA	DR11, DR5/DR2	NA	-
HC83	M	20	-	NA	DR11, DR5/DR2	NA	-
HC84	F	20	-	NA	DR13, DR5/DR2	NA	-
HC85	M	19	-	NA	DR4, DR12	NA	-
HC86	F	20	-	NA	DR1/DR101, DR11	NA	-
HC87	F	20	-	NA	DR17/DR3, DR11	NA	-
HC88	F	20	-	NA	DR7, DR11	NA	anti-GRP78, anti-citGRP78
HC89	F	32	-	NA	DR4, DR4/DR1403/DR1404/DR6/DR13	NA	-
nT1D1	M	20	0	DQ5, DQ2	DR1, DR17	GADA, IA2-A	-
nT1D2	M	47	0	DQ5, DQ2	DR4, DR13	-	-
nT1D3	M	43	0	DQ5, DQ2	DR1, DR17	-	-
nT1D4	V	25	0	DQ2, DQ2	DR17, DR17	GADA, IA2-A	-
nT1D5	NA	NA	0	NA	DR4, DR11	NA	-
nT1D6	M	20	0	DQ8, DQ2	DR7, DR11	IAA, GADA	-

nT1D7	M	39	0	DQ2, DQ2	DR1, DR4	IAA, GADA, IA2-A	-
nT1D8	M	36	0	DQ5, DQ7	DR4, DR7	-	-
nT1D9	V	36	0	DQ5, DQ8	DR1, DR4	GADA, IA2-A	-
nT1D10	M	21	0	DQ5, DQ2	DR4, DR13	GADA	-
nT1D11	M	36	0	DQ5, DQ7	DR1, DR15	-	-
nT1D12	V	20	0	DQ8, DQ4	DR4, DR4	IAA, GADA	-
nT1D13	V	26	0	DQ5, DQ8	DR4, DR13	GADA, IA2-A	-
nT1D14	M	65	0	NA	DR15, DR15	NA	-
nT1D15	V	22	0	DQ8, DQ8	DR9, DR14	GADA, IA2-A	-
nT1D16	M	25	0	DQ2, DQ2	DR7, DR13	IAA	anti-GRP78, anti-citGRP78
nT1D17	V	23	0	DQ5, DQ8	DR4, DR13	GADA, IA2-A	-
nT1D18	NA	NA	0	NA	NA	-	-
nT1D19	V	29	0	DQ7, DQ8	DR1, DR4	GADA, IA2-A	-
nT1D20	M	21	0	NA, DQ7	DR13, DR16	GADA	-
nT1D21	M	28	0	DQ2, DQ2	DR17, DR17	-	-
nT1D22	M	24	0	DQ5, DQ7	DR4, DR15	GADA, IA2-A	-
nT1D23	M	45	0	NA	DR17, DR16	GADA	-
nT1D24	V	47	0	NA	DR17, DR17	IAA, GADA, IA2-A	-
nT1D25	M	33	0	NA, DQ8	DR4, DR16	GADA	-
nT1D26	V	17	0	NA	DR1, DR11	GADA	-
nT1D27	M	18	0	DQ8, DQ2	DR17, DR4	GADA, IA2-A	-
nT1D28	M	18	0	DQ8, DQ2	DR17, DR4	IAA	-
nT1D29	M	17	0	DQ2, DQ2	DR17, DR17	GADA	-
nT1D30	M	20	0	DQ5, DQ8	NA	NA	-
nT1D31	M	27	0	DQ8, DQ2	DR17, DR4	-	-
nT1D32	M	18	0	DQ2, DQ8	DR4, DR4	IAA, GADA, IA2-A	-
nT1D33	M	35	0	DQ8, DQ2	DR4, DR13	GADA, IA2-A	-
nT1D34	M	52	0	DQ2, DQ2	NA	GADA	-
nT1D35	M	18	0	DQ5, DQ8	DR4, DR13	IAA, GADA, IA2-A	-
nT1D36	M	37	0	DQ7, DQ2	DR17, DR4	NA	-
nT1D37	V	27	0	DQ5, DQ5	DR13, DR13	NA	-
nT1D38	V	22	0	DQ8, DQ2	DR17, DR4	GADA	-
nT1D39	M	21	0	DQ8, DQ2	NA	-	-
nT1D40	M	26	0	NA	NA	NA	-
nT1D41	V	19	0	DQ8, DQ2	NA	NA	-
nT1D42	V	22	0	DQ8, DQ2	NA	NA	-
nT1D43	M	48	0	NA	NA	NA	-
nT1D44	V	20	0	NA	DR4, DR8	GADA, IA2-A	anti-GRP78, anti-citGRP78
nT1D45	V	25	0	DQ2, DQ4	DR7, DR8	IAA, GADA,	-
nT1D46	M	21	0	DQ5, DQ8	DR4, DR13	NA	-
nT1D47	M	30	0	DQ5, DQ5	DR1, DR13	-	-
T1D1	M	26	5	DQ8, DQ8	DR4, DR13	IA2-A	-
T1D2	V	25	16	DQ5, DQ2	DR8, DR11	GADA	anti-GRP78, anti-citGRP78
T1D3	V	23	8	NA	DR4, DR7	NA	anti-GRP78, anti-citGRP78
T1D4	M	21	7	DQ2, DQ2	DR17, DR17	NA	-
T1D5	M	23	4	NA	DR4, NA	GADA, IA2-A	anti-GRP78, anti-citGRP78
T1D6	M	29	5	DQ5, DQ8	DR1, DR4	GADA, IA2-A	-
T1D7	V	34	2	NA	DR17, DR13	NA	-
T1D8	M	21	7	DQ5, DQ2	DR1, DR17	GADA	-
T1D9	M	33	6	DQ8, DQ2	DR17, DR4	IAA, GADA, IA2-A	-

T1D10	V	39	6	DQ7, DQ7	DR11, DR12	IAA, GADA	anti-GRP78, anti-citGRP78
T1D11	V	20	6	DQ2, DQ4	DR17, DR8	IAA, GADA, IA2-A	-
T1D12	V	29	9	NA	DR17, DR13	NA	-
T1D13	V	28	5	DQ5, DQ2	DR17, DR13	-	-
T1D14	V	18	9	NA	DR17, DR17	NA	-
T1D15	V	22	6	NA	DR17, DR4	NA	anti-citGRP78
T1D16	V	21	14	DQ8, DQ2	DR17, DR4	GADA, IA2-A	-
T1D17	M	18	10	NA, DQ2	DR17, DR16	IA2-A	-
T1D18	M	25	6	DQ2, DQ2	DR17, DR7	IAA	-
T1D19	M	26	4	DQ5, DQ4	DR8, DR13	GADA, IA2-A	-
T1D20	M	28	4	DQ5, DQ2	DR17, DR13	GADA	-
T1D21	M	39	22	NA	DR17, DR4	NA	anti-GRP78, anti-citGRP78
T1D22	M	21	6	NA	DR4, DR4	NA	anti-GRP78
T1D23	V	24	5	DQ2, DQ8	DR4, DR7	IAA, GADA, IA2-A	anti-GRP78, anti-citGRP78
T1D24	M	27	7	DQ5, DQ8	DR1, DR4	IAA, GADA, IA2-A	-
T1D25	V	19	2	DQ8, DQ9	DR4, DR4	IAA, GADA, IA2-A	anti-GRP78, anti-citGRP78
T1D26	M	25	7	NA	NA	NA	-
T1D27	V	26	5	DQ8, DQ2	NA	GADA, IA2-A	-
T1D28	V	23	9	NA	NA	NA	-
T1D29	V	20	4	DQ2, DQ2	DR17, DR17	IAA, GADA	-
T1D30	M	33	3	DQ8, DQ2	NA	GADA	-
T1D31	M	39	22	NA	NA	NA	anti-GRP78, anti-citGRP78
T1D32	M	35	15	DQ5, DQ2	NA	GADA	-
T1D33	V	18	9	DQ8, DQ8	NA	IAA, GADA, IA2-A	-
T1D34	M	19	7	DQ5, DQ2	NA	IAA, GADA, IA2-A	-
T1D35	V	23	9	NA	NA	IAA, GADA, IA2-A	-
T1D36	M	30	4	NA	DR17, DR17	NA	anti-GRP78, anti-citGRP78
T1D37	M	22	6	NA	DR1, DR4	NA	anti-GRP78, anti-citGRP78
T1D38	M	24	8	DQ8, DQ2	DR17, DR4	GADA, IA2-A	anti-GRP78, anti-citGRP78
T1D39	M	20	6	DQ5, DQ2	DR17, DR13	GADA, IA2-A	-
T1D40	M	23	2	DQ5, DQ5	DR1, DR13	GADA, IA2-A	-
T1D41	V	18	8	DQ8, DQ2	DR17, DR4	IAA, IA2-A	-
T1D42	V	30	8	DQ8, DQ2	DR17, DR4	GADA, IA2-A	-
T1D43	V	26	5	NA	DR17, DR4	GADA	-
T1D44	M	20	4	DQ5, DQ5	DR1, DR13	GADA, IA2-A	anti-GRP78, anti-citGRP78
T1D45	V	18	4	DQ7, DQ8	DR4, DR4	IAA, GADA	anti-citGRP78
T1D46	M	24	15	NA	DR17, DR4	NA	-
T1D47	V	48	4	DQ8, DQ8	DR4, DR4	GADA	anti-GRP78
T1D48	V	27	5	NA	DR17, DR4	NA	-
T1D49	V	26	5	DQ2, DQ2	DR17, DR17	GADA, IA2-A	anti-citGRP78
T1D50	M	27	2	DQ8, DQ7	DR4, DR13	GADA, IA2-A	-
T1D51	M	19	4	DQ2, DQ2	DR17, DR7	GADA, IA2-A	anti-GRP78, anti-citGRP78
T1D52	M	22	3	DQ2, DQ2	DR17, DR7	IAA, GADA, IA2-A	anti-citGRP78

T1D53	M	23	4	DQ2, DQ8	DR4, DR7	GADA, IA2-A	-
T1D54	V	20	3	DQ2, DQ2	DR17, DR7	IAA, GADA, IA2-A	-
T1D55	M	18	1	DQ5, DQ8	DR4, DR13	IAA, GADA, IA2-A	anti-citGRP78
T1D56	V	19	4	DQ5, DQ2	NA	IAA, GADA	anti-citGRP78
T1D57	M	22	6	DQ2, DQ4	DR17, DR8	IAA	-
T1D58	M	18	3	DQ8, DQ4	DR4, DR8	IAA, IA2-A	-
T1D59	V	23	3	NA	DR1, DR17	NA	-
T1D60	V	23	3	NA	DR4, DR13	NA	-
T1D61	V	29	6	DQ5, DQ2	DR17, DR13	GADA	anti-GRP78, anti-citGRP78

*GADA: glutamic acid decarboxylase autoantibodies
IAA: insulin autoantibodies
IA2-A: protein tyrosine phosphatase autoantibodies
NA: not available