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DISCOVERY OF A SELECTIVE ISLET PEPTIDOME PRESENTED BY THE HIGHEST-RISK HLA-DQ8*TRANS* MOLECULE

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ABSTRACT

HLA-DQ2/8 heterozygous individuals are at far greater risk for type 1 diabetes (T1D) development by expressing HLA-DQ8*trans* on antigen-presenting cells compared to HLA-DQ2 or DQ8 homozygous individuals. Dendritic cells (DC) initiate and shape adaptive immune responses by presenting HLA-epitope complexes to naive T-cells. To dissect the role of HLA-DQ8*trans* in presenting natural islet epitopes, we analyzed the islet peptidome of HLA-DQ2, DQ8 and DQ2/8 by pulsing DC with preproinsulin (PPI), IA-2 and GAD65. Quality and quantity of islet-epitopes presented by HLA-DQ2/8 differed from DQ2 or DQ8. We identified two PPI epitopes solely processed and presented by HLA-DQ2/8 DC; an HLA-DQ8*trans*-binding signal-sequence epitope previously identified as CD8 T-cell epitope and a second epitope that we previously identified as CD4 T-cell epitope with increased binding to HLA-DQ8*trans* upon posttranslational modification. IA-2 epitopes retrieved from HLA-DQ2/8 and DQ8 DC bound to HLA-DQ8*cis/trans*. No GAD65 epitopes were eluted from HLA-DQ. T-cell responses were detected against the novel islet-epitopes in blood from T1D patients but hardly in healthy donors. We report the first PPI and IA-2 natural epitopes presented by highest-risk HLA-DQ8*trans*. The selective processing and presentation of HLA-DQ8*trans*-binding islet-epitopes provides insight in the mechanism of excessive genetic risk imposed by HLA-DQ2/8 heterozygosity and may assist immune monitoring of disease progression and therapeutic intervention, as well as provide therapeutic targets for immunotherapy in subjects at risk for T1D.

INTRODUCTION

Type 1 diabetes (T1D) is an autoimmune disease characterized by autoreactive T-cell mediated destruction of the insulin-producing pancreatic beta-cells (1-4). The search for naturally processed and presented epitopes (NPPEs) for high-risk HLA class II as target for autoreactive CD4 T cells in T1D has been focus of attention over the years. Most attention was given to HLA-DR binding epitopes (5, 6), whereas HLA-DQ binding epitopes deserve investigation, too. Indeed, subject heterozygous for HLA-DQ2 and -DQ8 are endorsed with by far the highest risk for development T1D, but it remains unclear what functional consequences explain this synergistically increased risk compared to a double dose of HLA-DQ2 or -DQ8. We previously revealed the unique peptide binding properties of HLA-DQ molecules composed of the products of DQA1*0201 (coding for the alpha chain of DQ2) and DQB1*0302 (coding for the beta-chain of DQ8), the so-called HLA-DQ8*trans* molecule (7-9). The islet epitopes presented by HLA-DQ8*trans* are largely unknown. Importantly, DQ8*cis*/*trans*-restricted CD4 T cell clones have been isolated from human insulitis lesions and T cell autoreactivity was confirmed for several pro-insulin peptides, underscoring the potential relevance of PPI peptides presented by HLA-DQ in diabetogenesis (10-13). We contend that knowledge of the HLA-DQ8trans islet peptidome provides insight in the mechanism by which HLA-DQ2/8 heterozygosity imposes excessive risk for T1D. In turn, HLA-DQ-restricted islet epitopes could yield key reagents for biomarker assays (ELISPOT, DQ tetramers) and feed the pipeline of islet tissue specific reagents being developed for peptide immunotherapy (8, 14-16).

Islet-epitopes for T1D predisposing HLA class II molecules have been identified by *in silico* prediction of T cell epitopes (17-19), using overlapping islet-peptides (14, 15, 20-22) or by pulsing B-cells with islet-antigens (23, 24). Although these approaches can identify CD4 T cell epitopes, epitopes derived from islet-autoantigens are generally presented by HLA class

II molecules after a sequence of events termed naturally processing and presentation by professional antigen-presenting cells APC (25). Dendritic cells (DC) are the master regulators of the immune orchestra and initiate and shape both the innate and adaptive immune responses. Here, we investigated presentation of islet-epitopes by DC expressing T1D highest-risk HLA-DQ. For this purpose, HLA-DQ2 and/or DQ8 expressing DC were pulsed with three islet-autoantigens, namely preproinsulin (PPI), islet tyrosine phosphatase IA-2 (insulinoma-associated antigen-2) and glutamic acid decarboxylase (65 kDa isoform; GAD65). After HLA-DQ isolation and peptide elution, using an HLA-elution method that we optimized for low cell numbers, eluted NPPE were analyzed using high-resolution tandem mass spectrometry. To verify the relevance of these peptides derived from the islet-autoantigens in the context of T1D immunopathology, we analyzed peripheral blood of HLA- and age-matched T1D patients and non-diabetic patients for the presence of autoreactive T cells. We report the first islet-epitopes that are uniquely processed and presented by the T1D highest-risk HLA-DQ2/8 molecules expressed on DC. These islet-epitopes are preferentially presented by HLA-DQ8*trans*. Such novel HLA-DQ restricted epitopes can be used as biomarkers for disease progression and/or contribute to the development of novel immunotherapeutic strategies (e.g. tolerogenic dendritic cell therapy).

RESEARCH DESIGN AND METHODS

Blood donors. After informed consent, peripheral blood was drawn from patients diagnosed with T1D (n=30, age: 17±6; disease duration ranging from 0 to 43 years) and from age and HLA-DQ matched healthy controls (n=36, age: 27±6). Peripheral blood mononuclear cells (PBMC) were freshly isolated by Ficoll centrifugation and resuspended in culture medium (Iscove's modified Dulbecco medium (Gibco BRL, Paisely, UK) containing 10% pooled human, heat inactivated serum. PBMC were subsequently tested for the presence of autoreactive CD4 T cells using ELISPOT.

Proteins and peptides. Recombinant proteins were produced as previously described (26). Briefly, PPI, IA-2 and GAD65 were amplified by PCR from human islet cDNA. PCR products were cloned by Gateway technology (Invitrogen, Carlsbad, CA) in a bacterial expression vector containing a histidine tag at the N-terminus. Proteins were overexpressed in Escherichia coli BL21(DE3) and affinity purified using anti-His antibody (Invitrogen, Carlsbad, CA). Size and purity of recombinant proteins were analyzed by gel electrophoresis and Western blotting using anti-His antibody. Endotoxin contents were below 50 IU/mg recombinant protein which is below the detection threshold, as tested using a Limulus Amebocyte Lysate (LAL) assay (Cambrex, East Rutherford, NJ). All proteins were tested in lymphocyte stimulation assays in order to exclude antigen-nonspecific T-cell stimulation. Peptides were synthesized according to standard fluorenylmethoxycarbonyl (Fmoc) chemistry using a SyroII peptide synthesizer (MultiSynTech, Witten, Germany). The integrity of the peptides was checked using UPLC-MS and Maldi-Tof MS. The following biotinylated indicator peptides were used in the cell-free HLA-DQ peptide binding studies: CLIP: KMRMATPLLMQAL (DQ2*cis*); AAEAALEAEEWAA (DQ2*trans*); AAPHTTQPAVEAA (DQ8*trans*); HSV-2: EEVDMTPADALDDFD (DQ8*cis*).

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Generation of dendritic cells. Isolation and generation of DC from homozygous HLA-DQ2, homozygous HLA-DQ8 or heterozygous HLA-DQ2/8 healthy blood donors was performed as described previously (27). PBMC isolated from each buffy coat were separately cultured and pulsed with islet antigens. For each elution a total of 40x106 pulsed mDCs were obtained from three donors per HLA-DQ genotype that were pooled after pulsing with islet autoantigen prior to the HLA-peptide elutions. PBMC were isolated by Ficoll gradient from three HLA-typed buffy coats per HLA-DQ typing and subsequently CD14+ monocytes were isolated and cultivated with GM-CSF (800 U/ml) and IL-4 (500 U/ml) (Invitrogen, Breda, The Netherlands) for 6 days to obtain immature DC (iDC). The iDCs were pulsed with PPI, IA-2 and GAD65 for 6 hrs after which iDC were matured by incubating 0.5×10^{6} DCs/well in a 24 well plate with LPS (100ng/mL) for 24 hrs in the continuous presence of the three islet-autoantigens. After 30 hrs pulsed mDC were harvested, washed three times with PBS to remove excess of islet-autoantigens and lysed in 1mL lysis buffer (50mM Tris, 150mM NaCl, 5mM EDTA, 0.5% zwitterion, 10mM iodoacetamide and protease inhibitors (complete inhibitor mix, Roche)) and subsequently high-speed centrifuged for 60min at 10,000 x g to remove nuclei and insoluble material.

Proteome analysis of dendritic cells pulsed with islet-autoantigens. Islet autoantigens pulsed mDC were lysed and proteins were digested using the filter aided sample preparation (FASP) method (28). Briefly, 100μg of protein was loaded on a 30kDa filter. SDS was removed in three washes by 8M urea. The proteins were alkylated using iodoacetamide, and the excess reagent was washed through the filters by three additional washes with 8M urea. Proteins were overnight digested using endoproteinase LysC (endoLysC) followed by a four hour digestion using trypsin at room temperature. Tryptic peptides were desalted on C18 SepPak. Peptides were subsequently fractionated by SCX on an Agilent 1100 system

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equipped with an in-house packed SCX-column (320μm ID, 15cm, polysulfoethyl A 3μm, Poly LC), run at 4μl/min. The gradient started with 10min at 100% solvent A 70/30/0.1 (water/acetonitrile/formic acid), after which a linear gradient was started to reach 100% solvent B (250mM KCl, 35% acetonitrile. 0.1% formic acid) in 15min, followed by 100% solvent C (500mM KCl, 35% acetonitrile 0.1% formic acid) in the following 15min. The eluent was held at 100% solvent C for 5 minutes to clean the column, then switched back to 100% solvent A. 15 fractions were collected in 1min intervals, lyophilized and reconstituted in 30μl 95/3/0.1 (water/acetonitrile/formic acid). Dissolved fractions were analyzed by on-line nano-HPLC MS with a system, consisting of an Agilent 1100 gradient HPLC system (Agilent, Waldbronn, Germany) and a LTQ-FT Ultra mass spectrometer (Thermo, Bremen, Germany). Fractions (5μl) were injected onto a home-made precolumn (100μm×15mm; Reprosil-Pur C18-AQ 3μm, Dr. Maisch, Ammerbuch, Germany) and eluted via a home-made analytical nano-HPLC column (15cm×50μm; Reprosil-Pur C18-AQ 3μm). The gradient was run from 0% to 30% solvent B (10/90/0.1 water/acetonitrile/formic acid) in 10-155min. A tip of approximately 5μm was drawn from the tip of the nano-HPLC column to act as electrospray needle. Full scan mass spectra were acquired in the FT-MS with a resolution of 25,000 at a target value of $5*10^6$. The five most intense ions were selected and fragmented in the linear ion trap using collision-induced dissociation at a target value of 10,000. For MS2 spectral matching Mascot 2.2.04 (Matrix Science) was used, with 2 ppm precursor and 0.5 Da fragment accuracy. Variable modifications included N-terminal protein acetylation and methionine oxidation. Carbamidomethylation of cysteine was selected as a fixed modification. False discovery rate was set to 1%.

Peptide elution and isolation from affinity-purified HLA-DQ. Affinity-purification of HLA-DQ molecules from mDC and subsequent peptide elutions were performed as follows.

We optimized our existing peptide elution protocol (8) for low DC numbers. All HLA-DQ isolation and washing steps were performed using a 100μL pipet tip. Lysate was precleared by running it through a 100μL pipet tip containing a small filter and packed with 100μL Sepharose beads. The precleared lysate was collected and HLA-DQ molecules were subsequently isolated using a pan-DQ (SPV-L3) antibody coupled to Sepharose beads and packed in a 100μL pipet tip; the lysate was passed through the SPV-L3 microcolumn to isolate HLA-DQ molecules by gravity force. Columns were washed with four bed volumes lysis buffer, subsequently followed by four bed volumes low salt buffer (120mM NaCl, 20mM Tris-HCl, pH 8.0), high salt buffer (1M NaCl, 20mM Tris-HCl, pH 8.0), no salt buffer (20mM Tris-HCl, pH 8.0) and low Tris buffer (10mM Tris-HCl, pH 8.0). The HLA-peptide complexes were eluted with two bed volumes 10% acetic acid. HLA-DQ eluates (containing both peptides and HLA) were fractionated with an HPLC system. The material was eluted using a gradient of 0-50% acetonitrile supplemented with 0.1% trifluoroacetic acid.

Peptide identification by mass spectrometry. Mass spectrometry analysis of HLA eluted peptides was performed as described previously (8) with some modifications. After immune precipitation, proteins and HLA-peptides in the unfiltered eluate were separated by selective elution from a small C18-column in two fractions with 20% and 30% acetonitrile, respectively (29). Subsequently the HLA-peptides were analysed via on-line C18-nano-HPLC-MS with a system consisting of an Easy nLC 1000 gradient HPLC system (Thermo, Bremen, Germany), and a Q-Exactive mass spectrometer (Thermo). Fractions were injected onto a homemade precolumn (100μm×15mm; Reprosil-Pur C18-AQ 3μm, Dr. Maisch, Ammerbuch, Germany) and eluted via a homemade analytical nano-HPLC column (15cm×50μm; Reprosil-Pur C18-AQ 3μm). The gradient was run from 0% to 30% solvent B (10/90/0.1 water/ACN/FA) in 120 min. The nano-HPLC column was drawn to a tip of ~5μm

and acted as the electrospray needle of the MS source. The Q-Exactive mass spectrometer was operated in top10-mode. Parameters were resolution 70,000 at an automatic gain control (AGC) target value of 3 million maximum fill time of 100ms (full scan), and resolution 35,000 at an AGC target value of 1 million/maximum fill time of 128ms for MS/MS at an intensity threshold of 78,500. Apex trigger was set to 1 to 5 seconds, and allowed charges were 1-3. In a post-analysis process, raw data were converted to peak lists using Proteome Discoverer 1.4. For peptide identification, MS/MS spectra were submitted to the human IPI 3.87 database using Mascot Version 2.2.04 (Matrix Science) with the following settings: 10ppm and 20 millimass units (mmu) deviation for precursor and fragment masses, respectively; no enzyme was specified. All reported hits were assessed manually.

Cell free HLA-DQ/peptide binding assays. Binding of identified islet-peptides to all four HLA-DQ molecules was studied in cell-free HLA-DQ peptide binding assays. As source of HLA-DQ, EBV-BLCL cells lentiviral transduced to express a single HLA-DQ molecule were used (8). These assays are based upon competition between a fixed concentration biotinylated reporter peptide (0.6μM) and an unbiotinylated islet-peptide (0-300μM); once the islet peptide competes with the indicator peptide a drop in signal (counts per minute; CPM) is observed representing binding of the islet-peptide. Affinities are subsequently calculated using GraphPad software (version 5); the concentration of islet-peptide required for half-maximal inhibition of binding of the reporter peptide indicate the EC_{50} value. Although binding of the islet-peptides to different HLA-DQ molecules cannot be compare accurately due to amino acid sequence of indicator peptides differing between the HLA-DQ assays, different HLA-DQ molecules with different properties), a difference in EC_{50} value ≥ 10 times was considered substantial.

Detection of DQ naturally processed and presented epitopes specific IFN-γ and IL-10 secreting CD4 T-cells. Detection of IFN-γ and IL-10 production by CD4 T-cells in response to the identified NPPE (10μg/mL) was performed using an enzyme-linked immunospot (ELISPOT) as described previously (30). Data are expressed as the total number of spots per triplicate divided by the total number of spots in triplicate in the presence of diluent alone (=SI). Stimulation index (SI) ≥ 3 are considered as positive.

RESULTS

Uptake of islet antigens by pulsed dendritic cells. Since our islet-antigens were not labeled with a fluorescent dye and antigen uptake and processing cannot be measured by flowcytometry or fluorescent microscopy, we performed proteome analysis of DC pulsed with PPI, IA-2 and GAD65 to confer intracellular uptake of the islet antigens. Lysates of islet-antigen pulsed mDC ($\sim$40x10^6) were digested with trypsin and tryptic peptides were analyzed by MS. Identified peptides were screened against a human protein database containing the amino acid sequences of PPI, IA-2 and GAD65. We independently analyzed the proteome of two lysates pulsed with PPI/IA-2/GAD65. Of all three islet-antigens we retrieved peptides derived from the antigens in the two lysates. Peptides of both the N- and C-terminus were identified in two independent proteome analysis covering 32.0±0% of PPI, 23.0±1.0% of GAD65 and 26.5±0.5% of IA-2 (*Supplemental Figure 1*). In addition, nested sets of peptides were retrieved, indicating accurate MS analysis. These data show that islet-antigen pulsed mDC efficiently take up whole antigens.

Identification of peptides eluted from highest-risk HLA-DQ2/8 and HLA-DQ8. To decipher the HLA-DQ islet-peptidome, immature DC homozygous for HLA-DQ2 or HLA-DQ8 or heterozygous for HLA-DQ2/8 were pulsed with PPI, IA-2 and GAD65 and subsequently matured with LPS and IFN-γ. Complete maturation of DC was confirmed by phenotype (*not shown*). A total of 353 unique peptides (derived from 56 proteins) were eluted from HLA-DQ2/8. In total, more islet-peptides were retrieved from heterozygous HLA-DQ2/8 (19/353; 5.4%) compared to homozygous HLA-DQ8 DC (6/459; 1.3%). From DQ2 no islet peptides were eluted. PPI and IA-2 peptides were retrieved from HLA-DQ2/8 and HLA-DQ8. GAD65 islet-peptides were not identified. From PPI, peptides with a mean length of 13 amino acids (range 8-16) were retrieved solely from DC expressing the highest-risk

HLA-DQ2/8 molecules (*Figure 1* and *Table 1*). In total, three PPI peptides were eluted from HLA-DQ2/8 encompassing two distinct core-regions: PPI_{17-24} (part of the PPI signal-sequence) and PPI_{54-69}. Intriguingly, PPI_{17-24} has been identified as a CD8 T cell epitope that is naturally processed and presented by HLA-A2 (31). Now, for the first time PPI_{17-24} is identified as a naturally processed and HLA-DQ presented peptide. Recently, we identified PPI_{54-69} (TRREAEDLQVGQVELG) as a CD4 T cell epitope that preferentially is presented by HLA-DQ8*trans* and becoming highly immunogenic after posttranslational modification (30). Here, this same PPI_{54-69} epitope is naturally processed and presented by heterozygous HLA-DQ2/8 expressing DC. Peptides from the C-terminus of PPI were not retrieved.

From HLA-DQ2/8 a total of 16 IA-2 peptides (*Figure 1 and Table 1*) were eluted with a mean peptide-length of 14 amino acids (range 11-19). All peptides are members of nested peptide sets covering three distinct core-regions of the N-terminus of IA-2. Similar nested peptide sets were retrieved from HLA-DQ8. Peptides from the C-terminus of IA-2 were not retrieved. To validate MS analysis of the identified peptides, we synthesized PPI and IA-2 eluted peptides (PPI_{17-24}, $IA-2_{296-311}$ and $IA-2_{319-333}$); MS spectra of the eluted peptides and the synthesized peptides fully overlapped (*Supplemental Figure 2*).

Binding of identified eluted PPI and IA-2 peptides to HLA-DQ. HLA-DQ2/8 heterozygous cells can express four types of HLA-DQ molecules: HLA-DQ2*cis*, HLA-DQ2*trans*, HLA-DQ8*trans* and HLA-DQ8*cis* (11). Therefore, HLA-DQ binding of the PPI and IA-2 eluted peptides was validated in competitive HLA-DQ/peptide binding assays. Binding of PPI_{54-69} to HLA-DQ was already validated in our previous study (30). PPI_{17-24} only bound to HLA-DQ8*trans* (*Figure 2 and Table 1*). IA-2 peptides, encompassing the same core-regions, were retrieved from HLA-DQ2/8 and HLA-DQ8. Binding of $IA-2_{142-159}$, $IA-2_{293-311}$ and $IA-2_{318-333}$ was observed for HLA-DQ8*trans* and -DQ8*cis* (*Figure 3* and *Table 1*).

Strong binding of these IA-2 peptides was observed for HLA-DQ8*trans* and weak binding for HLA-DQ8*cis*. Binding of the IA-2 eluted peptides to HLA-DQ2*cis/trans* was not observed.

Identification of the minimal HLA-DQ-binding registers. The minimal binder registers (MBR) in the eluted peptides responsible for HLA-DQ binding, were *in silico* predicted by employing the predictions algorithm software MOTIFS and the peptide-binding motifs of all four HLA-DQ molecules (8). Predicted MBR in the IA-2 peptides for HLA-DQ8*cis/trans* (*Table 1*) were consistent with the observed binding of the eluted IA-2 peptides to HLA-DQ8*cis/trans*. MBR in the eluted IA-2 peptides for HLA-DQ2*cis/trans* were not predicted. The majority of eluted IA-2 peptides contained three to four anchor residues (crucial for HLA-peptide binding; in bold) in the predicted MBR for HLA-DQ8*trans* (black) and two anchor residues for HLA-DQ8*cis* (gray). Indeed, stronger binding of the IA-2 peptides to HLA-DQ8*trans* was observed compared to HLA-DQ8*cis*. To validate HLA-DQ binding of the putative registers in the IA-2 peptides, synthetic peptides spanning the MBR (underlined in *Table 1*) were synthesized with two extra alanines both N- and C-terminally necessary for proper HLA-DQ binding. Strong binding to DQ8*trans* was observed for five predicted DQ8*trans* registers and two predicted DQ8*cis* registers bound with low affinity to DQ8*cis* (*data not shown*). These *in silico* predictions support the results from the DQ elution studies; number of eluted IA-2 peptides DQ8*trans* > DQ8*cis*.

Cytokine responses of T1D patients and healthy controls with naturally processed and presented islet peptides eluted from high-risk HLA-DQ. We examined proliferative responses in fresh peripheral blood of T1D patients and HLA-DQ and age matched non-T1D control subjects against the DQ NPPEs derived from PPI and IA-2 using ELISPOT. ELISPOT studies with T1D patients and the identified PPI$_{54-69}$ natural epitope was already

performed previously (30). Collectively, T-cell responses in T1D patients could be observed against three DQ naturally epitopes *ex vivo* in 12/30 (40%) for PPI_{17-24}, 16/30 T1D patients (53%) for $IA-2_{293-311}$ and 16/30 (53%) for $IA-2_{318-333}$ (*Figure 3* and *Supplemental Tables 1 and 2*). Only few patients (2/21; 9.5%) responded to $IA-2_{142-159}$ by producing IL-10 (*not shown*). Healthy individuals showed less frequent responses to the tested DQ natural epitopes (15-28%) with the majority of healthy individuals responding with IFN-γ to PPI_{17-24} and with IL-10 to the IA-2 epitopes. Patients responding to PPI_{17-24} produced IFN-γ (31%), IFN-γ+IL-10 (8%) or IL-10 (61%). As we recently reported that the PPI_{17-24} epitope is also presented by HLA-A2 to autoreactive CD8 T cells (31) we checked the HLA class I typing when available, but we did not see a trend in that direction; all five cases in which PBMC from patients responded to PPI_{17-24} by production of IFN-γ were HLA-A2 negative. This excludes the possibility that this responsiveness can be attributed to HLA-A2 restricted CD8 T cells.

Patients showed predominantly IFN-γ to the two IA-2 epitopes (75% and 88%, respectively); patients responding to $IA-2_{293-311}$ produced IFN-γ (62.5%) or IFN-γ+IL-10 (12.5%) and 25% patients responded solely with IL-10. Patient responses against $IA-2_{318-333}$ showed even a more proinflammatory phenotype as 75% produced IFN-γ and 12.5% produced IFN-γ+IL-10. A decrease in patients responding to $IA-2_{318-333}$ exclusively with IL-10 (12.5%) was observed as compared to $IA-2_{293-311}$. Our data demonstrate that autoreactive CD4 T cell responses can be detected against these novel PPI and IA-2 naturally epitopes that preferentially bind the T1D highest-risk HLA-DQ8*trans* molecule. Furthermore, both the quality and prevalence of these T-cell responses differ between T1D patients and non-T1D case controls. While our cohort remains insufficiently sized to draw firm conclusions regarding the influence of HLA status on the presence of CD4 T-cell responses, these data demonstrate the presence of autoreactive T cell responses against the identified novel DQ epitopes.

DISCUSSION

We provide evidence on the functional consequences of presentation of islet-proteins by the highest-risk HLA-DQ8trans that may contribute to the association of this molecule with the highest genetic predisposition to T1D. Both qualitative and quantitative differences are observed between the islet peptidomes of HLA-DQ2/8 heterozygosity versus homozygosity. First, the total number of peptides derived from PPI and IA-2 presented by highest-risk HLA-DQ2/8 is greater compared to homozygous HLA-DQ8, whereas no islet peptides were retrieved from homozygous HLA-DQ2. Second, HLA-DQ2/8 DC generate an exclusive islet peptidome uniquely presented by the highest-risk HLA-DQ8*trans* molecule. Finally, we provide evidence that DC selectively present islet autoantigens PPI and IA-2 epitopes but not GAD65 epitopes by high-risk HLA-DQ.

The identified PPI naturally processed peptides were retrieved solely from highest-risk HLA-DQ2/8 were confirmed to bind HLA-DQ8*cis*/*trans* selectively. The unusually short DQ binding PPI_{17-24} peptide is located in the signal-sequence of PPI and has been described as a naturally processed and HLA-A2 restricted epitope of diabetogenic CD8 T cells (31). We now report islet autoreactive CD4 T cell responses against the same PPI epitope in T1D patients, but not healthy age and HLA matched subjects. The PPI_{17-24} epitope as an 8-mer does not fulfill the high-risk HLA-DQ peptide-binding motif (8). Yet, binding of PPI_{17-24} was confirmed for HLA-DQ8*trans*. Unconventional length and binding of peptides to HLA class II molecules has been reported (32, 33). Our finding that PPI_{17-24} as an 8-mer is naturally presented by T1D highest-risk HLA-DQ8trans extends the notion that CD4 T cells can respond to unusually presented self-peptides. This signal peptide was generated by DC pulsed with whole PPI. The question that emerged is how peptides from the signal-sequence of PPI can be presented by HLA-DQ on DC. In the NOD mouse model of autoimmune diabetes, insulin-secretory granules from beta-cells and even whole beta-cells are ingested by APC and

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transported to the pancreatic lymph node (34, 35) and T cells from T1D patients respond to beta-cell derived insulin secretory granules (36). Subsequently, intact or partially synthesized PPI that is embedded in the ER can become a natural source of signal peptides presented by HLA-DQ8trans on the surface of DC. Pancreatic beta-cells do not secrete whole PPI as extracellular source for uptake by DC. However, a small proportion of whole PPI (including the signal sequence) is present in the cytosol of human islets that may become accessible to DC under pathological conditions in T1D such as beta-cell stress, thus ending up in HLA-DQ for presentation to the immune system (37, 38). We previously reported PPI_{54-69} as preferentially presented by the highest-risk HLA-DQ8trans molecule (30). The proportion of patients responding against this epitope doubled after posttranslational modification, with the majority of patients showing a proinflammatory immune response. This PPI_{54-69} epitope is now confirmed as processing product presented solely by HLA-DQ2/8 heterozygous DC.

IA-2 peptides were presented both by HLA-DQ2/8 and to a lesser extent HLA-DQ8 expressing DC and encompassed identical core-regions that were derived solely from the IA-2 extracellular domain. Binding of the IA-2 peptides was validated for HLA-DQ8*cis/trans*. As humoral responses have been detected against the IA-2 intracellular domain, several immune studies hitherto focused on CD4 T cell epitopes derived from the IA-2 intracellular domain. Uptake of IA-2 by pulsed DC was confirmed but peptides from the IA-2 intracellular domain were not retrieved, which may imply a limited relevance of these peptides as target for CD4 T cells in the context of high-risk HLA-DQ. Preliminary data show that, peptides from the IA-2 extracellular domain were retrieved derived solely from DQ2/8 heterozygous DC that were deamidated at one Q residue, suggesting posttranslational modification of islet-peptides presented by highest-risk DQ2/8 expressed on DC.

The naturally processed PPI and IA-2 epitopes identified approved to be targets of CD4 T cells in approximately half of the T1D patients. Patient T cells responding to the IA-2

epitopes showed a proinflammatory phenotype (IFN-γ), whereas the IA-2 specific T cells from few responding non-diabetic donors showed an anti-inflammatory response (IL-10). Intriguingly, immune responses to PPI$_{17-24}$ showed an inverse pattern of cytokine production that we speculate to reflect differential regulatory mechanisms against different islet epitopes in T1D patients versus healthy donors. T-cell responses to peptides eluded from HLA-DQ2/8 DCs were not exclusively recognized by HLA-DQ2/8 heterozygous T1D patients. While it is certainly conceivable that other HLA molecules than those tested here may act as alternative restriction element in such cases (notably HLA-A2) (31), this discrepancy between the HLA-DQ ligandome generated by processing of the whole PPI protein versus exogenous pulsing with an excess of synthetic PPI peptide only in the T-cell assay may also relate to the notion that the amount of PPI peptides in the latter case may overcome the threshold required for T-cell activation in T1D donors expressing either DQ2 or DQ8. This peptide was repeatedly undetectable in elution studies from DQ2 or DQ8 homozygous donors, but exclusively retrieved from HLA-DQ2/8 heterozygous DCs upon processing of PPI protein. Yet, our HLA-DQ binding studied demonstrate that once a peptide is generated, it may also bind to other HLA-DQ molecules, be it to a lesser extent.

GAD65 peptides were not retrieved from HLA-DQ, although uptake of the protein was confirmed by proteome analysis of pulsed DC. Peptide loading pathways in APC are diverse; peptide loading into HLA class II molecules in the MHC class II compartment requires CLIP derived from the invariant chain and HLA-DM for presentation in DC, but in B-cells a modifier of HLA-DM is expressed (HLA-DO) that associates with HLA-DM and restricts HLA-DM activity to more acidic compartments in B-cells. Although the exact role of HLA-DO in different APCs remains largely unknown, DO can function as a competitive and irreversible inhibitor of HLA-DM in subsets of APCs (39, 40); when present, DO subtly alters the repertoire of HLA class II-bound peptides displayed at the surface of APCs. DO is

mainly expressed in B-cells and its effect on HLA class II presentation would therefore be mostly observed in B-cells. Epitopes recognized in the context of DQ display a DM-sensitive phenotype, whereas for DR molecules a tendency towards DM-resistant epitopes is observed (41); presentation of DM-sensitive (DQ) antigens benefited more from maturation of DCs compared with DM-resistant (DR) antigens. Thus peptide binding to HLA class II molecules is modulated differently in B-cells compared to DC providing a plausible explanation why DC do not present GAD65 peptides in HLA-DQ. Since GAD65 epitopes have been identified to be naturally processed and presented by B-cells as target of CD4 T cells, this implies that DC, uniquely involved in priming of naïve CD4 T cells, present a relative small set of islet-epitopes in HLA-DQ to autoreactive T cells derived from a limited number of islet autoantigens.

The recombinant islet proteins used in this study are not necessarily in their native conformation. For dendritic cells (DCs) the 3D-structure is not crucial for protein uptake. This may differ for B-cells, for which the 3D-structure of an antigen is important for uptake via the B-cell receptor and subsequent processing. For identification of B-cell epitopes, proper folding of the recombinant proteins will therefore be more important. Nonetheless, the actual processing occurs in lysozymes, in which the acidic milieu will affect the natural confirmation of proteins. Considering the time of antigen processing that we elected, the possibility remains that additional peptides may generated and presented by DCs. Studies in mice show that DCs are able to retain and present antigens long after uptake (42) and that the same peptides are presented by DC at early and later time points. Rapidly generated peptides from proximal parts of antigens may be over-represented in our approach. It is conceivable that additional peptides presented by (subsets of) DCs at later time points after uptake in vivo may arise.

It is conceivable that our identified PPI and IA-2 DQ-NPPEs represent the tip of the iceberg of the total islet peptide ligandome. The identified peptides in this study might impose a bias regarding most favorable binding characteristics and peptides having unfavorable properties are more challenging to identify due to technical limitations. Nevertheless, our observations that the particular islets eluted from HLA-DQ proved immunogenic in T1D patients in particular compared to non-diabetic subjects, even if their binding affinity to HLA was relatively weak, implies that our strategy of epitope discovery with potential relevance to the disease is suitable and rewarding.

Our identification of naturally processed and HLA-DQ presented epitopes by DC may bear relevance to the selection of islet peptides for prevention of T1D to avoid loss of tolerance to the triggering epitopes, impacting the choice of potential therapeutic agents for the prevention of T1D, as well as immune monitoring of islet autoreactive CD4 T-cell responses in prediction, disease progression and possibly as surrogate end-points in immunotherapeutic trials and as part of the developing area of HLA-DQ tetramer technology in T1D.

REFERENCES

1. Coppieters KT, Dotta F, Amirian N, Campbell PD, Kay TW, Atkinson MA, et al. Demonstration of islet-autoreactive CD8 T cells in insulitic lesions from recent onset and long-term type 1 diabetes patients. JExpMed. 2012;209(1):51-60.
2. Roep BO. beta-Cells, autoimmunity, and the innate immune system: "un menage a trois"? Diabetes. 2013;62(6):1821-2.
3. Knight RR, Kronenberg D, Zhao M, Huang GC, Eichmann M, Bulek A, et al. Human beta-cell killing by autoreactive preproinsulin-specific CD8 T cells is predominantly granule-mediated with the potency dependent upon T-cell receptor avidity. Diabetes. 2013;62(1):205-13.
4. Peakman M. Immunological pathways to beta-cell damage in Type 1 diabetes. Diabetic medicine : a journal of the British Diabetic Association. 2013;30(2):147-54.
5. Mannering SI, Harrison LC, Williamson NA, Morris JS, Thearle DJ, Jensen KP, et al. The insulin A-chain epitope recognized by human T cells is posttranslationally modified. The Journal of experimental medicine. 2005;202(9):1191-7.
6. Mannering SI, Pang SH, Williamson NA, Naselli G, Reynolds EC, O'Brien-Simpson NM, et al. The A-chain of insulin is a hot-spot for CD4+ T cell epitopes in human type 1 diabetes. Clinical and experimental immunology. 2009;156(2):226-31.
7. Koeleman BP, Lie BA, Undlien DE, Dudbridge F, Thorsby E, De Vries RR, et al. Genotype effects and epistasis in type 1 diabetes and HLA-DQ trans dimer associations with disease. Genes Immun. 2004;5(5):381-8.
8. van Lummel M, van Veelen PA, Zaldumbide A, de Ru A, Janssen GM, Moustakas AK, et al. The type 1 diabetes associated HLA-DQ8-trans dimer accomodates a unique peptide repertoire. JBiolChem. 2011.
9. Erlich H, Valdes AM, Noble J, Carlson JA, Varney M, Concannon P, et al. HLA DR-DQ haplotypes and genotypes and type 1 diabetes risk: analysis of the type 1 diabetes genetics consortium families. Diabetes. 2008;57(4):1084-92.
10. Durinovic-Bello I, Steinle A, Ziegler AG, Schendel DJ. HLA-DQ-restricted, islet-specific T-cell clones of a type I diabetic patient. T-cell receptor sequence similarities to insulitis-inducing T-cells of nonobese diabetic mice. Diabetes. 1994;43(11):1318-25.
11. Eerligh P, van Lummel M, Zaldumbide A, Moustakas AK, Duinkerken G, Bondinas G, et al. Functional consequences of HLA-DQ8 homozygosity versus heterozygosity for islet autoimmunity in type 1 diabetes. Genes Immun. 2011.
12. Pathiraja V, Kuehlich JP, Campbell PD, Krishnamurthy B, Loudovaris T, Coates PT, et al. Proinsulin-specific, HLA-DQ8, and HLA-DQ8-transdimer-restricted CD4+ T cells infiltrate islets in type 1 diabetes. Diabetes. 2015;64(1):172-82.
13. Yang J, Chow IT, Sosinowski T, Torres-Chinn N, Greenbaum CJ, James EA, et al. Autoreactive T cells specific for insulin B:11-23 recognize a low-affinity peptide register in human subjects with autoimmune diabetes. Proceedings of the National Academy of Sciences of the United States of America. 2014;111(41):14840-5.
14. Yang J, James EA, Sanda S, Greenbaum C, Kwok WW. CD4+ T cells recognize diverse epitopes within GAD65: implications for repertoire development and diabetes monitoring. Immunology. 2013;138(3):269-79.
15. Roep BO, Peakman M. Antigen targets of type 1 diabetes autoimmunity. Cold Spring Harbor perspectives in medicine. 2012;2(4):a007781.
16. Di Lorenzo TP, Peakman M, Roep BO. Translational mini-review series on type 1 diabetes: Systematic analysis of T cell epitopes in autoimmune diabetes. Clinical and experimental immunology. 2007;148(1):1-16.
17. Chang KY, Unanue ER. Prediction of HLA-DQ8beta cell peptidome using a computational program and its relationship to autoreactive T cells. International immunology. 2009;21(6):705-13.
18. Cai R, Liu Z, Ren J, Ma C, Gao T, Zhou Y, et al. GPS-MBA: computational analysis of MHC class II epitopes in type 1 diabetes. PloS one. 2012;7(3):e33884.

19. Chen G, Han G, Feng J, Wang J, Wang R, Xu R, et al. Glutamic acid decarboxylase-derived epitopes with specific domains expand CD4(+)CD25(+) regulatory T cells. PloS one. 2009;4(9):e7034.

20. Endl J, Otto H, Jung G, Dreisbusch B, Donie F, Stahl P, et al. Identification of naturally processed T cell epitopes from glutamic acid decarboxylase presented in the context of HLA-DR alleles by T lymphocytes of recent onset IDDM patients. The Journal of clinical investigation. 1997;99(10):2405-15.

21. Lohmann T, Leslie RD, Hawa M, Geysen M, Rodda S, Londei M. Immunodominant epitopes of glutamic acid decarboxylase 65 and 67 in insulin-dependent diabetes mellitus. Lancet. 1994;343(8913):1607-8.

22. Chujo D, Foucat E, Nguyen TS, Chaussabel D, Banchereau J, Ueno H. ZnT8-Specific CD4+ T cells display distinct cytokine expression profiles between type 1 diabetes patients and healthy adults. PloS one. 2013;8(2):e55595.

23. Nepom GT, Lippolis JD, White FM, Masewicz S, Marto JA, Herman A, et al. Identification and modulation of a naturally processed T cell epitope from the diabetes-associated autoantigen human glutamic acid decarboxylase 65 (hGAD65). Proceedings of the National Academy of Sciences of the United States of America. 2001;98(4):1763-8.

24. Peakman M, Stevens EJ, Lohmann T, Narendran P, Dromey J, Alexander A, et al. Naturally processed and presented epitopes of the islet cell autoantigen IA-2 eluted from HLA-DR4. The Journal of clinical investigation. 1999;104(10):1449-57.

25. Blum JS, Wearsch PA, Cresswell P. Pathways of antigen processing. Annual review of immunology. 2013;31:443-73.

26. Franken KL, Hiemstra HS, van Meijgaarden KE, Subronto Y, den Hartigh J, Ottenhoff TH, et al. Purification of his-tagged proteins by immobilized chelate affinity chromatography: the benefits from the use of organic solvent. Protein expression and purification. 2000;18(1):95-9.

27. Unger WW, Laban S, Kleijwegt FS, van der Slik AR, Roep BO. Induction of Treg by monocyte-derived DC modulated by vitamin D3 or dexamethasone: differential role for PD-L1. EurJImmunol. 2009;39(11):3147-59.

28. Wisniewski JR, Zougman A, Nagaraj N, Mann M. Universal sample preparation method for proteome analysis. Nature methods. 2009;6(5):359-62.

29. Milner E, Gutter-Kapon L, Bassani-Strenberg M, Barnea E, Beer I, Admon A. The effect of proteasome inhibition on the generation of the human leukocyte antigen (HLA) peptidome. Molecular & cellular proteomics : MCP. 2013;12(7):1853-64.

30. van Lummel M, Duinkerken G, van Veelen PA, de Ru A, Cordfunke R, Zaldumbide A, et al. Post-Translational Modification Of Hla-Dq Binding Islet-Autoantigens In Type 1 Diabetes. Diabetes. 2013.

31. Skowera A, Ellis RJ, Varela-Calvino R, Arif S, Huang GC, Van-Krinks C, et al. CTLs are targeted to kill beta cells in patients with type 1 diabetes through recognition of a glucose-regulated preproinsulin epitope. The Journal of clinical investigation. 2008;118(10):3390-402.

32. Matsuzaki J, Tsuji T, Luescher I, Old LJ, Shrikant P, Gnjatic S, et al. Nonclassical antigen-processing pathways are required for MHC class II-restricted direct tumor recognition by NY-ESO-1-specific CD4(+) T cells. Cancer immunology research. 2014;2(4):341-50.

33. van de Wal Y, Kooy YM, van Veelen P, Vader W, August SA, Drijfhout JW, et al. Glutenin is involved in the gluten-driven mucosal T cell response. European journal of immunology. 1999;29(10):3133-9.

34. Tang Q, Adams JY, Tooley AJ, Bi M, Fife BT, Serra P, et al. Visualizing regulatory T cell control of autoimmune responses in nonobese diabetic mice. Nature immunology. 2006;7(1):83-92.

35. Unanue ER. Antigen presentation in the autoimmune diabetes of the NOD mouse. Annual review of immunology. 2014;32:579-608.

36. Roep BO, Arden SD, de Vries RR, Hutton JC. T-cell clones from a type-1 diabetes patient respond to insulin secretory granule proteins. Nature. 1990;345(6276):632-4.

37. Patzelt C, Labrecque AD, Duguid JR, Carroll RJ, Keim PS, Heinrikson RL, et al. Detection and kinetic behavior of preproinsulin in pancreatic islets. Proceedings of the National Academy of Sciences of the United States of America. 1978;75(3):1260-4.

38.   Guo H, Xiong Y, Witkowski P, Cui J, Wang LJ, Sun J, et al. Inefficient translocation of preproinsulin contributes to pancreatic beta cell failure and late-onset diabetes. The Journal of biological chemistry. 2014;289(23):16290-302.

39.   Guce AI, Mortimer SE, Yoon T, Painter CA, Jiang W, Mellins ED, et al. HLA-DO acts as a substrate mimic to inhibit HLA-DM by a competitive mechanism. Nature structural & molecular biology. 2013;20(1):90-8.

40.   Pezeshki AM, Azar GA, Mourad W, Routy JP, Boulassel MR, Denzin LK, et al. HLA-DO increases bacterial superantigen binding to human MHC molecules by inhibiting dissociation of class II-associated invariant chain peptides. Human immunology. 2013;74(10):1280-7.

41.   Kremer AN, van der Meijden ED, Honders MW, Goeman JJ, Wiertz EJ, Falkenburg JH, et al. Endogenous HLA class II epitopes that are immunogenic in vivo show distinct behavior toward HLA-DM and its natural inhibitor HLA-DO. Blood. 2012;120(16):3246-55.

42.   van Montfoort N, Camps MG, Khan S, Filippov DV, Weterings JJ, Griffith JM, et al. Antigen storage compartments in mature dendritic cells facilitate prolonged cytotoxic T lymphocyte cross-priming capacity. Proceedings of the National Academy of Sciences of the United States of America. 2009;106(16):6730-5.

LEGENDS

FIGURE 1. PPI and IA-2 peptides eluted from high-risk HLA-DQ2/8 and HLA-DQ8.
HLA-DQ2 or HLA-DQ8 homozygous and HLA-DQ2/8 heterozygous DC were pulsed for 6 hours with islet-autoantigens PPI, GAD65 and IA-2 after which the cells were matured for 24hrs with LPS and IFN-γ in the continuous presence of the islet-autoantigens. After cell lysis, HLA-DQ was purified using SPV-L3 (pan-DQ antibody) and peptides were acid eluted and analyzed by mass spectrometry. Nested sets of peptides, covering distinct core-regions of PPI and IA-2 were eluted.

FIGURE 2. Binding validation of identified HLA-DQ eluted peptides from PPI and IA-2. Binding of eluted PPI_{17-24} (black) and PPI_{54-69} (gray) peptides and the three longest peptides representing the three distinct core regions of IA-2 ($IA-2_{142-159}$: soft gray; $IA-2_{293-311}$: light gray; $IA-2_{318-333}$: dark gray), were tested in competitive peptide-binding assays for binding to HLA-DQ2*cis*, -DQ2*trans*, -DQ8*trans* and -DQ8*cis*. Data represent mean ± SEM (n=3). Shown on the x-axes is $1/EC_{50}$, thereby illustrating that large bars represent better binding.

FIGURE 3. Cytokine responses of T1D patients and healthy controls against PPI and IA-2 naturally epitopes eluted from high-risk HLA-DQ. PPI_{17-24} and IA-2 naturally peptides (core 1: $IA-2_{142-159}$; core 2: $IA-2_{293-311}$ and core 3: $IA-2_{318-333}$) eluted from high-risk HLA-DQ were tested for immune responses in T1D patients (n=30) and healthy controls matched for age and high-risk HLA-DQ (n=36). Responses against $IA-2_{142-159}$ were observed in 2/21 T1D patients (*not shown*). Patient and control cytokine responses (IFN-γ versus IL-10) against PPI_{17-24} (green circles), $IA-2_{293-311}$ (blue circles) and $IA-2_{318-333}$ (red circles) are viewed in two separate dotplots. An overview of the quality of the immune responses against

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the three natural epitopes is viewed in separate pie charts and are indicated as proinflammatory (IFN-γ; black), regulatory (IL-10; soft gray) or a combination of both (light gray).

TABLE 1. Experimentally observed masses of PPI and IA-2 peptides eluted from highest-risk HLA-DQ2/8. Alignments of naturally processed peptides derived from islet-autoantigens presented by T1D high-risk HLA-DQ2/8**.** Residues in bold represent the anchor residues in the predicted minimal binding cores of the naturally epitopes for HLA-DQ8*trans* (black) and HLA-DQ8*cis* (gray). Minimal 9-mer binding registers (MBR) for HLA-DQ8cis*/trans* are underlined. HLA-DQ2*cis*/*trans* MBR were not predicted. Shown are the EC$_{50}$ values of the longest eluted peptides from HLA-DQ8*trans* and -DQ8*cis* as validated by HLA-peptide binding studies.

TABLE 1. Experimentally observed masses of PPI and IA-2 peptides eluted from highest-risk HLA-DQ2/8.

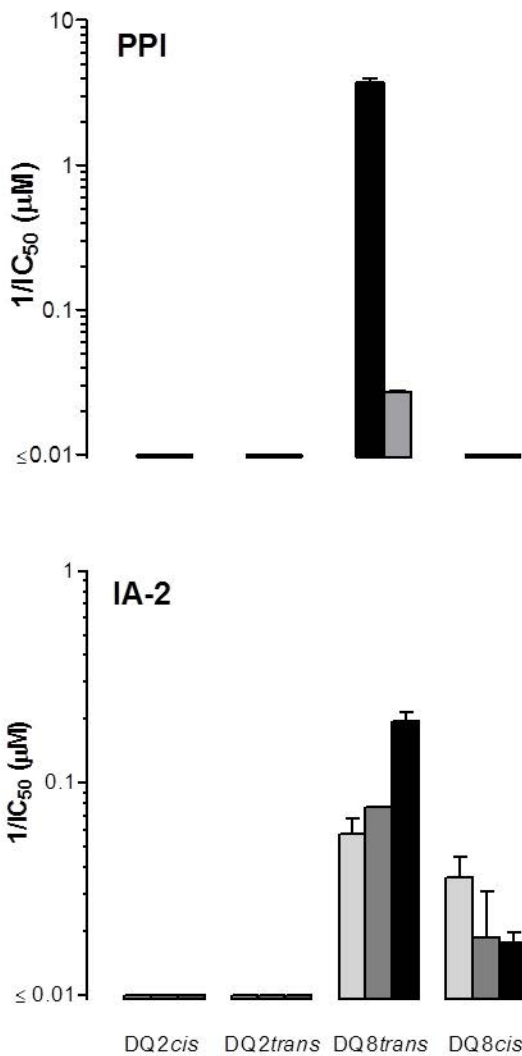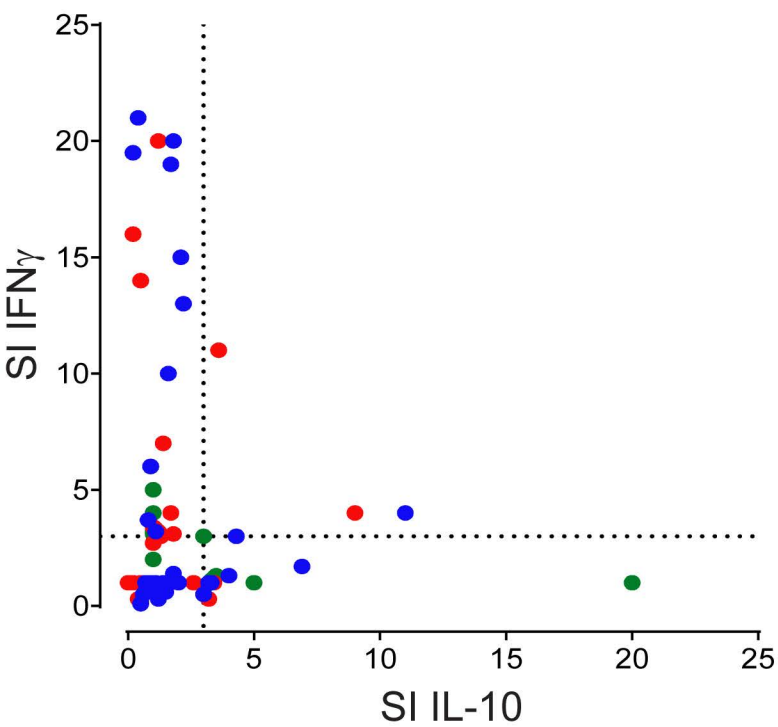| Observed m/z	Calculated m/z	Residues	Corresponding protein sequence	IC$_{50}$ (µM) DQ8*trans*	DQ8*cis*
			PPI		
		core 1			
784.36	783.35	17-24	WGPDPAAA	0.3±0.01	nb
		core 2			
600.64	1798.92	54-69	TRREA**E**DLQVGQV**E**LG	16±2 (26)	64±8 ([ref] 26)
566.96	1697.87	55-69	RREAEDLQVGQVELG		
			IA-2		
		core 1			
634.00	1899.00	142-159	LQDI**P**TG**S**A**PA**AQHRLPQ	17.7±3.3	29.2±5.9
591.31	1770.94	142-158	LQDIPTGS**A**P**AA**QHRLP		
		core 2			
647.64	1939.92	293-311	VP**R**LPEQ**G**SSSRAEDSPEG	13.0±0.1	68.6±44.5
580.78	1159.55	296-306	LP**E**QG**S**SSRA**E**		
638.29	1274.57	296-307	LPEQGS**S**SR**AE**D		
681.81	1361.61	296-308	LPEQGSS**S**R**AE**DS		
794.85	1587.70	296-310	LPEQGSSSRAEDSPE		
823.36	1644.72	297-311	LPEQGSSSRAEDSPEG		
738.31	1474.62	297-310	PEQGSSSRAEDSPE		
766.83	1531.65	297-311	PEQGSSSRAEDSPEG		
718.29	1434.59	298-311	EQGSSSRAEDSPEG		
		core 3			
508.58	1522.74	318-333	GDRG**E**KP**AS**PA**V**QPDA	5.2±0.6	57.6±7.0
733.86	1465.71	319-333	DRGEK**P**ASPA**V**QP**D**A		
489.57	1465.72	319-332	D**R**GEKP**A**SPAVQPD		
451.23	1350.69	320-332	RGE**K**PASP**A**VQPD		
598.30	1194.59	321-332	GEKPASPAVQPD		

FIGURE 1

Preproinsulin

1   110

N | signal | B | C | A | C

DQ2/8

WGPDPAAA 17-24

TRREAEDLQVGQVELG 54-69
RREAEDLQVGQVELG 55-69

IA-2

1   979

N | Extracellular | domain | TM | Intracellular domain | C

DQ8

LQDIPTGSAPAAQHRLPQ 142-159
LQDIPTGSAPAAQHRLP 142-158

VPRLPEQGSSSRAEDSPE 293-310
LPEQGSSSRAEDSPEG 296-311

DRGEKPASPAVQPD 319-332
GEKPASPAVQPDA 321-333

DQ2/8

LQDIPTGSAPAAQHRLPQ 142-159
LQDIPTGSAPAAQHRLP 142-158

VPRLPEQGSSSRAEDSPEG 293-311
LPEQGSSSRAE 296-306
LPEQGSSSRAED 296-307
LPEQGSSSRAEDS 296-308
LPEQGSSSRAEDSPE 296-310
LPEQGSSSRAEDSPEG 296-311
PEQGSSSRAEDSPE 297-310
PEQGSSSRAEDSPEG 297-311
EQGSSSRAEDSPEG 298-311

GDRGEKPASPAVQPDA 318-333
DRGEKPASPAVQPDA 319-333
DRGEKPASPAVQPD 319-332
RGEKPASPAVQPD 320-332
GEKPASPAVQPD 321-332

FIGURE 2