Menno van Lummel,[1] Peter A. van Veelen,[1] Arnoud H. de Ru,[1] Jos Pool,[1] Tatjana Nikolic,[1] Sandra Laban,[1] Antoinette Joosten,[1] Jan W. Drijfhout,[1] Iria Gómez-Touriño,[2] Sefina Arif,[2] Henk J. Aanstoot,[3] Mark Peakman,[2] and Bart O. Roep[1,4]

Discovery of a Selective Islet Peptidome Presented by the Highest-Risk HLA-DQ8*trans* Molecule

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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 with HLA-DQ2 or -DQ8 homozygous individuals. Dendritic cells (DC) initiate and shape adaptive immune responses by presenting HLA-epitope complexes to naïve 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 patients with T1D but scantly detected in healthy donor subjects. 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.

Type 1 diabetes (T1D) is an autoimmune disease characterized by autoreactive T-cell–mediated destruction of the insulin-producing pancreatic β-cells (1–4). The search for naturally processed and presented epitopes (NPPE) for high-risk HLA class II as a 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, subjects heterozygous for HLA-DQ2 and -DQ8 are endorsed with by far the highest risk for development of T1D, but what functional consequences explain this synergistically increased risk compared with a double dose of HLA-DQ2 or -DQ8 remain unclear. We previously revealed the unique peptide binding properties of HLA-DQ molecules composed of the products of DQA1*0201 (coding for the α-chain of DQ2) and DQB1*0302 (coding for the β-chain of DQ8), the so-called HLA-DQ8*trans* molecule (7–9). The islet epitopes presented by HLA-DQ8*trans* are largely unknown. Importantly, HLA-DQ8*cis/trans*–restricted CD4 T-cell clones have been isolated from human insulitis lesions, and T-cell autoreactivity was confirmed for several proinsulin peptides, underscoring the potential relevance of preproinsulin (PPI) peptides presented by HLA-DQ in diabetogenesis (10–13). We contend that knowledge of the HLA-DQ8*trans* 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 (enzyme-linked immunospot [EPISPOT], DQ tetramers)

[1]Department of Immunohematology and Blood Transfusion, Leiden University Medical Center, Leiden, the Netherlands
[2]Department of Immunobiology, School of Medicine, King's College London, London, U.K.
[3]Diabeter, Center for Pediatric and Adolescent Diabetes Care and Research, Rotterdam, the Netherlands
[4]Department of Diabetes Immunology, Diabetes & Metabolism Research Institute at the Beckman Research Institute of City of Hope, Duarte, CA

Corresponding author: Bart O. Roep, boroep@lumc.nl.

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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 the innate and adaptive immune responses. Here, we investigated presentation of islet epitopes by DC expressing T1D highest-risk HLA-DQ. For this purpose, DC expressing HLA-DQ2 and/or -DQ8 were pulsed with three islet-autoantigens, namely PPI, islet tyrosine phosphatase IA-2 (insulinoma-associated antigen-2), and GAD (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 (MS/MS). 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 patients with T1D and patients without diabetes 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 DC therapy).

RESEARCH DESIGN AND METHODS

Blood Donors

After informed consent, peripheral blood was drawn from 30 patients diagnosed with T1D (age: 17 ± 6; disease duration: 0–43 years) and from 36 healthy control subjects matched for age and HLA-DQ (age: 27 ± 6). Peripheral blood mononuclear cells (PBMC) were freshly isolated by Ficoll centrifugation and resuspended in culture medium (Iscove's Modified Dulbecco's Medium; Gibco BRL, Paisely, U.K.) 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). 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 Assay (Cambrex, East Rutherford, NJ). All proteins were tested in lymphocyte stimulation assays to exclude antigen-nonspecific T-cell stimulation. Peptides were synthesized according to standard fluorenylmethoxycarbonyl chemistry using a Syro II peptide synthesizer (MultiSynTech, Witten, Germany). The integrity of the peptides was checked using ultra-performance liquid chromatography-MS and matrix-assisted laser desorption/ionization time-of-flight MS. The following biotinylated indicator peptides were used in the cell-free HLA-DQ peptide binding studies: CLIP: KMRMATPLLMQAL (DQ2*cis*), AAEAALEAEEWAA (DQ2*trans*), and AAPHTTQPAVEAA (DQ8*trans*) and HSV-2: EEVDMTPADALDDFD (DQ8*cis*).

Generation of DC

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, 40×10^6 pulsed mature DC (mDC) were obtained from three donors per HLA-DQ genotype that were pooled after pulsing with islet autoantigen before the HLA-peptide elutions. PBMCs were isolated by Ficoll gradient from three HLA-typed buffy coats per HLA-DQ typing, and $CD14^+$ monocytes were isolated and cultivated with granulocyte macrophage colony-stimulating factor (800 units/mL) and interleukin (IL)-4 (500 units/mL) (Invitrogen, Breda, the Netherlands) for 6 days to obtain immature DC (iDC). The iDC were pulsed with PPI, IA-2, and GAD65 for 6 h, after which iDC were matured by incubating 0.5×10^6 DC/well in a 24-well plate with lipopolysaccharide (100 ng/mL) for 24 h in the continuous presence of the three islet autoantigens. After 30 h, pulsed mDC were harvested, washed three times with PBS to remove excess of islet autoantigens, and lysed in 1 mL lysis buffer (50 mmol/L Tris, 150 mmol/L NaCl, 5 mmol/L EDTA, 0.5% zwitterion, 10 mmol/L iodoacetamide, and protease inhibitors [complete inhibitor mix; Roche]); they were centrifuged at high speed for 60 min at 10,000*g* to remove nuclei and insoluble material.

Proteome Analysis of DC Pulsed With Islet Autoantigens

Islet autoantigens pulsed mDC were lysed, and proteins were digested using the filter-aided sample preparation method (28). Briefly, 100 μg of protein was loaded on a 30-kDa filter. SDS was removed in three washes by 8 mol/L urea. The proteins were alkylated using iodoacetamide, and the excess reagent was washed through the filters by three additional washes with 8 mol/L urea. Proteins were digested overnight using endoproteinase LysC, followed by a 4-h digestion using trypsin at room temperature. Tryptic peptides were desalted on C18 SepPak. Peptides were subsequently

fractionated by strong cation exchange on an Agilent 1100 gradient high-performance liquid chromotography (HPLC) system (Agilent, Waldbronn, Germany) equipped with an in-house packed strong cation exchange-column (320 μm ID, 15 cm, polysulfoethyl A 3 μm; Poly LC), run at 4 μL/min. The gradient started with 10 min at 100% solvent A 70/30/0.1 (water/acetonitrile/formic acid), after which a linear gradient was started to reach 100% solvent B (250 mmol/L KCl, 35% acetonitrile, and 0.1% formic acid) in 15 min, followed by 100% solvent C (500 mmol/L KCl, 35% acetonitrile, and 0.1% formic acid) in the following 15 min. The eluent was held at 100% solvent C for 5 min to clean the column, then switched back to 100% solvent A. Fifteen fractions were collected in 1-min intervals, lyophilized, and reconstituted in 30 μL 95/3/0.1 (water/acetonitrile/formic acid). Dissolved fractions were analyzed by online nano–HPLC MS with a system consisting of an Agilent 1100 gradient HPLC system and a LTQ FT Ultra MS (Thermo Fisher Scientific, Bremen, Germany). Fractions (5 μL) were injected onto a homemade precolumn (100 μm × 15 mm; Reprosil-Pur C18-AQ 3μm, Dr. Maisch, Ammerbuch, Germany) and eluted via a homemade analytical nano-HPLC column (15 cm × 50 μm; Reprosil-Pur C18-AQ 3 μm). The gradient was run from 0 to 30% solvent B (10/90/0.1 water/acetonitrile/formic acid) in 10–155 min. A tip of ~5 μm was drawn from the tip of the nano-HPLC column to act as electrospray needle. Full-scan MS spectra were acquired in the Fourier transform-ion cyclotron resonance-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 MS/MS 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. The 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 100-μL pipet tip. Lysate was precleared by running it through a 100-μL pipet tip containing a small filter and packed with 100 μL 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 100-μL 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 of lysis buffer, followed by four bed volumes of low-salt buffer (120 mmol/L NaCl and 20 mmol/L Tris-HCl, pH 8.0), high-salt buffer (1 mol/L NaCl and 20 mmol/LTris-HCl, pH 8.0), no-salt buffer (20 mmol/L Tris-HCl, pH 8.0), and low-Tris buffer (10 mmol/L Tris-HCl, pH 8.0). The HLA-peptide complexes were eluted with two bed volumes of 10% acetic acid. HLA-DQ eluates (containing 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 MS

MS 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). The HLA-peptides were analyzed via online C18-nano-HPLC-MS with a system consisting of an Easy-nLC 1000 gradient HPLC system (Thermo Fisher Scientific) and a Q Exactive MS (Thermo Fisher Scientific). Fractions were injected onto a homemade precolumn (100 μm × 15 mm; Reprosil-Pur C18-AQ 3 μm, Dr. Maisch, Ammerbuch, Germany) and eluted via a homemade analytical nano-HPLC column (15 cm × 50 μm; Reprosil-Pur C18-AQ 3μm). The gradient was run from 0 to 30% solvent B (10/90/0.1 water/acetonitrile/formic acid) in 120 min. The nano-HPLC column was drawn to a tip of ~5 μm and acted as the electrospray needle of the MS source. The Q Exactive MS was operated in top 10 mode. Parameters were resolution 70,000 at an automatic gain control target value of 3 million maximum fill time of 100 ms (full scan) and resolution 35,000 at an automatic gain control target value of 1 million/maximum fill time of 128 ms for MS/MS at an intensity threshold of 78,500. Apex trigger was set to 1 to 5 s, and allowed charges were 1–3. In a postanalysis 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 2.2.04 software with the following settings: 10 ppm and 20 millimass units 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 the source of HLA-DQ, Epstein-Barr virus–transformed B lymphocyte 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 (0.6 μmol/L) and an unbiotinylated islet peptide (0–300 μmol/L); once the islet peptide competes with the indicator peptide a drop in signal (counts/min) is observed representing binding of the islet-peptide. Affinities are subsequently calculated using GraphPad 5 software; the concentration of islet peptide required for half-maximal inhibition of binding of the reporter peptide indicate the half-maximal effective concentration (EC_{50}) value. Although binding of the islet peptides to different HLA-DQ molecules cannot be compared accurately due to

the 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 $\geq$10 times was considered substantial.

Detection of DQ NPPE-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 (10 μg/mL) was performed using ELISPOT, as described previously (30). Data are expressed as the simulation index (SI) = total number of spots per triplicate/total number of spots in triplicate in the presence of diluent alone. An SI $\geq$3 is considered as positive.

RESULTS

Uptake of Islet Antigens by Pulsed DC

Because our islet antigens were not labeled with a fluorescent dye and antigen uptake and processing cannot be measured by flow cytometry 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 ($\sim 40 \times 10^6$) 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 (Supplementary Fig. 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, iDC 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 lipopolysaccharide and IFN-γ. Complete maturation of DC was confirmed by phenotype (data not shown). A total of 353 unique peptides (derived from 56 proteins) were eluted from HLA-DQ2/8. More islet peptides were retrieved from heterozygous HLA-DQ2/8 (19 of 353 [5.4%]) than from homozygous HLA-DQ8 DC (6 of 459 [1.3%]). No islet peptides were eluted from HLA-DQ2. 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 (Fig. 1 and Table 1). 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. We recently identified $PPI_{54–69}$ (TRREAEDLQVGQVELG) as a CD4 T-cell epitope that preferentially is presented by HLA-DQ8*trans* and becoming highly immunogenic after post-translational 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 (Fig. 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. Similarly 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 (Supplementary Fig. 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* (Fig. 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* (Fig. 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 (MBRs) in the eluted peptides responsible for HLA-DQ binding were in silico predicted by using the predictions algorithm software MOTIFS and the peptide-binding motifs of all four HLA-DQ molecules (8). Predicted MBRs 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*. MBRs in the eluted IA-2 peptides for HLA-DQ2*cis*/*trans* were not predicted. Most of the eluted IA-2 peptides contained three to four anchor residues (crucial for HLA-peptide binding) in the predicted MBRs for HLA-DQ8*trans* and two anchor residues for HLA-DQ8*cis*. Indeed, stronger binding of the IA-2 peptides to HLA-DQ8*trans* was observed compared with HLA-DQ8*cis*. To validate HLA-DQ binding of the putative registers in the IA-2 peptides, synthetic peptides spanning the MBRs

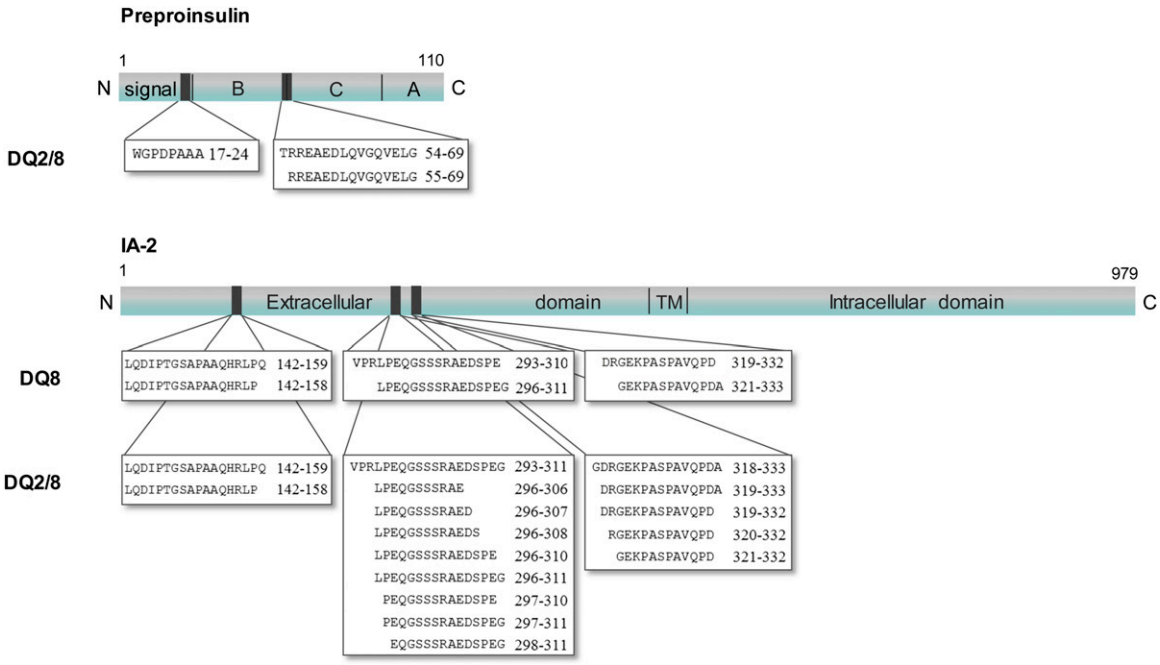

Figure 1—PPI and IA-2 peptides eluted from high-risk HLA-DQ2/8 and HLA-DQ8. HLA-DQ2 or HLA-DQ8 homozygous and HLA-DQ2/8 heterozygous DCs were pulsed for 6 h with islet autoantigens PPI, GAD65, and IA-2, after which the cells were matured for 24 h with lipopolysaccharide and IFN-γ in the continuous presence 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 MS. Nested sets of peptides, covering distinct core regions of PPI and IA-2, were eluted. TM, IA-2 transmembrane region.

(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* MBRs, and two predicted DQ8*cis* MBRs bound with low affinity to DQ8*cis* (data not shown). These in silico predictions support the results from the DQ elution studies; the number of eluted IA-2 peptides DQ8*trans* > DQ8*cis*.

Cytokine Responses of Patients With T1D and Healthy Control Subjects With Islet NPPEs Eluted From High-Risk HLA-DQ

We examined proliferative responses in fresh peripheral blood of patients with T1D and HLA-DQ– and age-matched control subjects without T1D against the DQ NPPEs derived from PPI and IA-2 using ELISPOT. ELISPOT studies with patients with T1D and the identified $PPI_{54–69}$ natural epitope were performed previously (30). Collectively, T-cell responses in 30 patients with T1D could be observed against three DQ naturally processed epitopes ex vivo in 12 (40%) for $PPI_{17–24}$, 16 (53%) for IA-$2_{293–311}$, and 16 (53%) for IA-$2_{318–333}$ (Fig. 3 and Supplementary Tables 1 and 2). Only a few patients (2 of 21 [9.5%]) responded to IA-$2_{142–159}$ by producing IL-10 (data not shown). Healthy individuals showed less frequent responses to the tested DQ natural epitopes (15–28%), with most of the 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%). Because 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 did not see a trend in that direction; all five cases in which PBMCs 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% of patients responded solely with IL-10. Patient responses against IA-$2_{318–333}$ showed even a more proinflammatory phenotype, because 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 compared with IA-$2_{293–311}$. Our data demonstrate that autoreactive CD4 T-cell responses can be detected against these novel PPI and IA-2 naturally processed epitopes that preferentially bind the T1D highest-risk HLA-DQ8*trans* molecule. Furthermore, the quality and prevalence of these T-cell responses both differ between patients with T1D and case control subjects without T1D. Although 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.

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

				EC_{50} (μmol/L)	
Observed m/z	Calculated m/z	Residues	Corresponding protein sequence	DQ8*trans*	DQ8*cis*
			PPI		
		Core 1			
784.36	783.35	17–24	WGPDPAAA	0.3 ± 0.01	nb
		Core 2			
600.64	1,798.92	54–69	TRREA**E**DLQVGQV**E**LG	16 ± 2 (26)	64 ± 8 (26)
566.96	1,697.87	55–69	RREAEDLQVGQVELG		
			IA-2		
		Core 1			
634.00	1,899.00	142–159	LQDI**P**TG**S**A**PA**AQHRLPQ	17.7 ± 3.3	29.2 ± 5.9
591.31	1,770.94	142–158	LQDIPTGS**A**P**AA**QHRLP		
		Core 2			
647.64	1,939.92	293–311	VP**R**LPEQ**G**SSSRAEDSPEG	13.0 ± 0.1	68.6 ± 44.5
580.78	1,159.55	296–306	LP**E**QG**S**SSRA**E**		
638.29	1,274.57	296–307	LPEQGS**S**SR**A**E**D**		
681.81	1,361.61	296–308	LPEQGSS**S**R**AE**DS		
794.85	1,587.70	296–310	LPEQGSSSRAEDSPE		
823.36	1,644.72	297–311	LPEQGSSSRAEDSPEG		
738.31	1,474.62	297–310	PEQGSSSRAEDSPE		
766.83	1,531.65	297–311	PEQGSSSRAEDSPEG		
718.29	1,434.59	298–311	EQGSSSRAEDSPEG		
		Core 3			
508.58	1,522.74	318–333	GDRG**E**KP**AS**P**A**VQPDA	5.2 ± 0.6	57.6 ± 7.0
733.86	1,465.71	319–333	DRGEK**P**ASPA**V**QP**D**A		
489.57	1,465.72	319–332	D**R**GEKP**A**SPAVQPD		
451.23	1,350.69	320–332	RGE**K**PASP**A**VQPD		
598.30	1,194.59	321–332	GEKPASPAVQPD		

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 (MBR) of the naturally processed epitopes for HLA-DQ8*trans* and HLA-DQ8*cis* (MBR starting with R or K). Minimal 9 MBRs for HLA-DQ8*cis/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. m/z, mass-to-charge ratio; nb, no binding.

DISCUSSION

We provide evidence on the functional consequences of presentation of islet proteins by the highest-risk HLA-DQ8*trans* that may contribute to the association of this molecule with the highest genetic predisposition to T1D. Qualitative and quantitative differences are both 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 with 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 that were retrieved solely from the 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 patients with T1D but not in 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-DQ8*trans* extends the notion that CD4 T cells can respond to unusually presented self-peptides. This signal peptide was

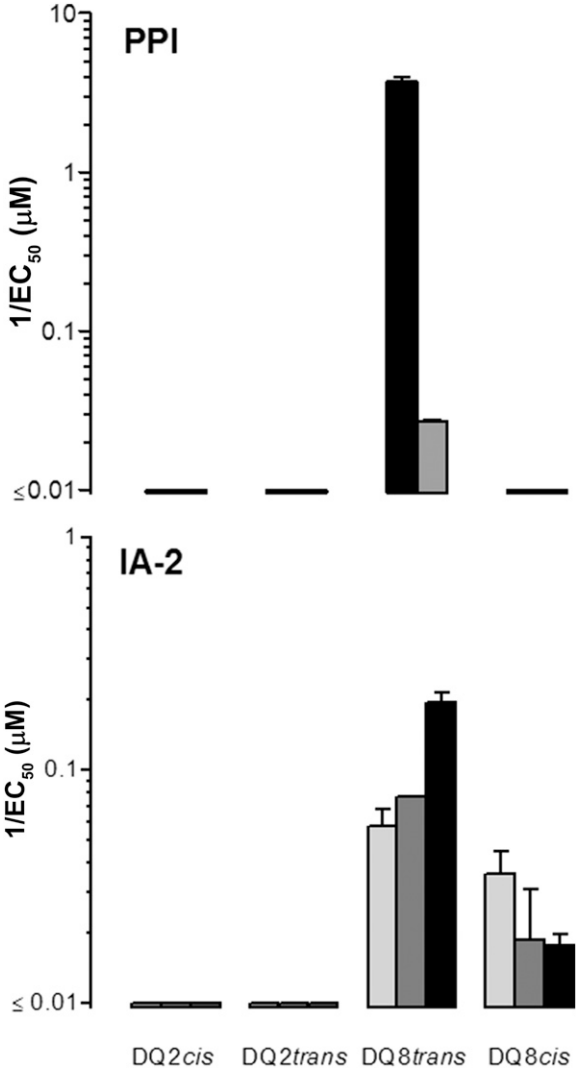

Figure 2—Binding validation of identified HLA-DQ–eluted peptides from PPI and IA-2. Binding of eluted $PPI_{17–24}$ (black) and $PPI_{54–69}$ (gray) peptides and the three longest peptides representing the three distinct core regions of IA-2 ($IA\text{-}2_{142–159}$: light gray; $IA\text{-}2_{293–311}$: medium gray; $IA\text{-}2_{318–333}$: 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-axis is $1/EC_{50}$, thereby illustrating that the large bars represent better binding.

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 β-cells and even whole β-cells are ingested by APC and transported to the pancreatic lymph node (34,35), and T cells from patients with T1D respond to insulin secretory granules derived from β-cells (36). Subsequently, intact or partially synthesized PPI that is embedded in the endoplasmic reticulum can become a natural source of signal peptides presented by HLA-DQ8*trans* on the surface of DC. Pancreatic β-cells do not secrete whole PPI as an 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 β-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-DQ8*trans* molecule (30). The proportion of patients responding against this epitope doubled after posttranslational modification, with most patients showing a proinflammatory immune response. This $PPI_{54–69}$ epitope is now confirmed as a 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*. Because 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 a target for CD4 T cells in the context of high-risk HLA-DQ. Preliminary data show that peptides retrieved from the IA-2 extracellular domain were derived solely from DQ2/8 heterozygous DC that were deamidated at one Q residue, suggesting posttranslational modification of islet-peptides presented by the highest-risk DQ2/8 expressed on DC.

The naturally processed PPI and IA-2 epitopes identified proved to be targets of CD4 T cells in approximately half of the patients with T1D. Patient T cells responding to the IA-2 epitopes showed a proinflammatory phenotype (IFN-γ), whereas the IA-2–specific T cells from a few responding donors without diabetes showed an anti-inflammatory response (IL-10). Intriguingly, immune responses to $PPI_{17–24}$ showed an inverse pattern of cytokine production that we speculate reflects differential regulatory mechanisms against different islet epitopes in T1D patients versus healthy donors. T-cell responses to peptides eluted from HLA-DQ2/8 DC were not exclusively recognized by HLA-DQ2/8 heterozygous patients with T1D. Although it is certainly conceivable that other HLA molecules than those tested here may act as an 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 donors with T1D expressing HLA-DQ2 or -DQ8. This peptide was repeatedly undetectable in elution studies from HLA-DQ2 or -DQ8 homozygous donors, but exclusively retrieved from HLA-DQ2/8 heterozygous DC upon processing of PPI protein. Yet, our HLA-DQ–binding study demonstrates that once a peptide is generated, it may also bind to other HLA-DQ molecules, be it to a lesser extent.

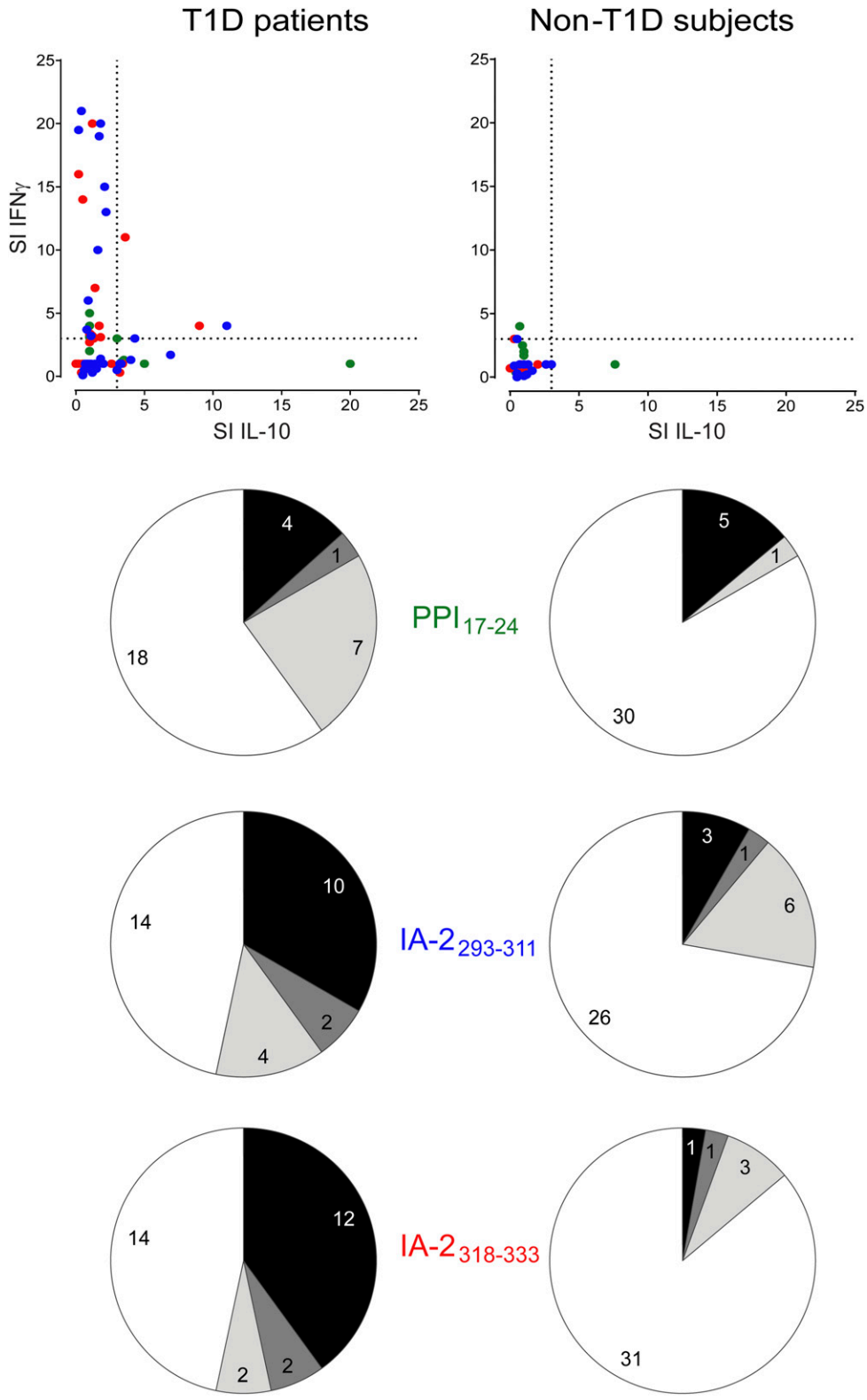

Figure 3—Cytokine responses of T1D patients and healthy control subjects against PPI and IA-2 naturally processed epitopes eluted from high-risk HLA-DQ. $PPI_{17–24}$ and IA-2 naturally processed peptides (core 1: IA-$2_{142–159}$; core 2: IA-$2_{293–311}$; and core 3: IA-$2_{318–333}$) eluted from high-risk HLA-DQ were tested for immune responses in 30 patients with T1D and in 36 healthy control subjects matched for age and high-risk HLA-DQ. Responses against IA-$2_{142–159}$ were observed in 2 of 21 patients with T1D (data not shown). Patient and control cytokine responses (IFN-γ vs. 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 dot plots. An overview of the quality of the immune responses against the three natural epitopes is viewed in separate pie charts and are indicated as proinflammatory (IFN-γ; black), regulatory (IL-10; light gray), or a combination of both (medium gray).

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 toward DM-resistant epitopes is observed (41); presentation of DM-sensitive (DQ) antigens benefited more from maturation of DC compared with DM-resistant (DR) antigens. Thus, peptide binding to HLA class II molecules is modulated differently in B cells compared with DC, providing a plausible explanation why DC do not present GAD65 peptides in HLA-DQ. Because 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 relatively 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 DC, the three-dimensional structure is not crucial for protein uptake. This may differ for B cells, for which the three-dimensional 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 chose, the possibility remains that additional peptides may generated and presented by DC. Studies in mice show that DC 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 overrepresented in our approach. Additional peptides presented by (subsets of) DC at later time points after uptake in vivo may conceivably 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 the most favorable binding characteristics, and peptides with 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 patients with T1D, in particular, compared with subjects without diabetes, 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, affecting 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.

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Author Contributions. M.v.L. conceived experiments, researched data, and wrote and reviewed the manuscript. A.H.d.R., J.P., S.L., A.J., and I.G.-T. researched data. P.A.v.V., T.N., J.W.D., S.A., H.J.A., and M.P. contributed to discussion and reviewed and edited the manuscript. B.O.R. supervised this study and contributed to discussion and writing the manuscript. B.O.R. is the guarantor of this work and, as such, had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

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References

1. Coppieters KT, Dotta F, Amirian N, et al. Demonstration of islet-autoreactive CD8 T cells in insulitic lesions from recent onset and long-term type 1 diabetes patients. J Exp Med 2012;209:51–60

2. Roep BO. β-Cells, autoimmunity, and the innate immune system: "un ménage á trois"? Diabetes 2013;62:1821–1822

3. Knight RR, Kronenberg D, Zhao M, et al. Human β-cell killing by autoreactive preproinsulin-specific CD8 T cells is predominantly granule-mediated with the potency dependent upon T-cell receptor avidity. Diabetes 2013;62:205–213

4. Peakman M. Immunological pathways to beta-cell damage in Type 1 diabetes. Diabet Med 2013;30:147–154

5. Mannering SI, Harrison LC, Williamson NA, et al. The insulin A-chain epitope recognized by human T cells is posttranslationally modified. J Exp Med 2005; 202:1191–1197

6. Mannering SI, Pang SH, Williamson NA, et al. The A-chain of insulin is a hot-spot for CD4+ T cell epitopes in human type 1 diabetes. Clin Exp Immunol 2009;156:226–231

7. Koeleman BP, Lie BA, Undlien DE, et al. Genotype effects and epistasis in type 1 diabetes and HLA-DQ trans dimer associations with disease. Genes Immun 2004;5:381–388
8. van Lummel M, van Veelen PA, Zaldumbide A, et al. Type 1 diabetes associated HLA-DQ8-trans dimer accommodates a unique peptide repertoire. J Biol Chem 2012;287:9514–9524
9. Erlich H, Valdes AM, Noble J, et al.; Type 1 Diabetes Genetics Consortium. HLA DR-DQ haplotypes and genotypes and type 1 diabetes risk: analysis of the Type 1 Diabetes Genetics Consortium families. Diabetes 2008;57:1084–1092
10. Durinovic-Bellò 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;43:1318–1325
11. Eerligh P, van Lummel M, Zaldumbide A, et al. Functional consequences of HLA-DQ8 homozygosity versus heterozygosity for islet autoimmunity in type 1 diabetes. Genes Immun 2011;12:415–427
12. Pathiraja V, Kuehlich JP, Campbell PD, et al. Proinsulin-specific, HLA-DQ8, and HLA-DQ8-transdimer-restricted CD4+ T cells infiltrate islets in type 1 diabetes. Diabetes 2015;64:172–182
13. Yang J, Chow IT, Sosinowski T, et al. Autoreactive T cells specific for insulin B:11-23 recognize a low-affinity peptide register in human subjects with autoimmune diabetes. Proc Natl Acad Sci U S A 2014;111:14840–14845
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;138:269–279
15. Roep BO, Peakman M. Antigen targets of type 1 diabetes autoimmunity. Cold Spring Harb Perspect Med 2012;2: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. Clin Exp Immunol 2007;148:1–16
17. Chang KY, Unanue ER. Prediction of HLA-DQ8beta cell peptidome using a computational program and its relationship to autoreactive T cells. Int Immunol 2009;21:705–713
18. Cai R, Liu Z, Ren J, et al. GPS-MBA: computational analysis of MHC class II epitopes in type 1 diabetes. PLoS One 2012;7:e33884
19. Chen G, Han G, Feng J, et al. Glutamic acid decarboxylase-derived epitopes with specific domains expand CD4(+)CD25(+) regulatory T cells. PLoS One 2009;4:e7034
20. Endl J, Otto H, Jung G, 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. J Clin Invest 1997;99:2405–2415
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;343:1607–1608
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:e55595
23. Nepom GT, Lippolis JD, White FM, et al. Identification and modulation of a naturally processed T cell epitope from the diabetes-associated autoantigen human glutamic acid decarboxylase 65 (hGAD65). Proc Natl Acad Sci U S A 2001;98:1763–1768
24. Peakman M, Stevens EJ, Lohmann T, et al. Naturally processed and presented epitopes of the islet cell autoantigen IA-2 eluted from HLA-DR4. J Clin Invest 1999;104:1449–1457
25. Blum JS, Wearsch PA, Cresswell P. Pathways of antigen processing. Annu Rev Immunol 2013;31:443–473
26. Franken KL, Hiemstra HS, van Meijgaarden KE, et al. Purification of his-tagged proteins by immobilized chelate affinity chromatography: the benefits from the use of organic solvent. Protein Expr Purif 2000;18:95–99
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. Eur J Immunol 2009;39:3147–3159
28. Wiśniewski JR, Zougman A, Nagaraj N, Mann M. Universal sample preparation method for proteome analysis. Nat Methods 2009;6:359–362
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. Mol Cell Proteomics 2013;12:1853–1864
30. van Lummel M, Duinkerken G, van Veelen PA, et al. Posttranslational modification of HLA-DQ binding islet-autoantigens in type 1 diabetes. Diabetes 2014;63:237–247.
31. Skowera A, Ellis RJ, Varela-Calviño R, et al. CTLs are targeted to kill beta cells in patients with type 1 diabetes through recognition of a glucose-regulated preproinsulin epitope. J Clin Invest 2008;118:3390–3402
32. Matsuzaki J, Tsuji T, Luescher I, 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 Immunol Res 2014;2:341–350
33. van de Wal Y, Kooy YM, van Veelen P, et al. Glutenin is involved in the gluten-driven mucosal T cell response. Eur J Immunol 1999;29:3133–3139
34. Tang Q, Adams JY, Tooley AJ, et al. Visualizing regulatory T cell control of autoimmune responses in nonobese diabetic mice. Nat Immunol 2006;7:83–92
35. Unanue ER. Antigen presentation in the autoimmune diabetes of the NOD mouse. Annu Rev Immunol 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;345:632–634
37. Patzelt C, Labrecque AD, Duguid JR, et al. Detection and kinetic behavior of preproinsulin in pancreatic islets. Proc Natl Acad Sci U S A 1978;75:1260–1264
38. Guo H, Xiong Y, Witkowski P, et al. Inefficient translocation of preproinsulin contributes to pancreatic β cell failure and late-onset diabetes. J Biol Chem 2014;289:16290–16302
39. Guce AI, Mortimer SE, Yoon T, et al. HLA-DO acts as a substrate mimic to inhibit HLA-DM by a competitive mechanism. Nat Struct Mol Biol 2013;20:90–98
40. Pezeshki AM, Azar GA, Mourad W, et al. HLA-DO increases bacterial superantigen binding to human MHC molecules by inhibiting dissociation of class II-associated invariant chain peptides. Hum Immunol 2013;74:1280–1287
41. Kremer AN, van der Meijden ED, Honders MW, 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;120:3246–3255
42. van Montfoort N, Camps MG, Khan S, et al. Antigen storage compartments in mature dendritic cells facilitate prolonged cytotoxic T lymphocyte cross-priming capacity. Proc Natl Acad Sci U S A 2009;106:6730–6735