Epitope Stealing as a Mechanism of Dominant Protection by HLA-DQ6 in Type 1 Diabetes

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The heterozygous DQ2/8 (DQA1*05:01-DQB1*02:01/DQA1*03:01-DQB1*03:02) genotype confers the highest risk in type 1 diabetes (T1D), whereas the DQ6/8 (DQA1*02:01-DQB1*06:02/DQA1*03:01-DQB1*03:02) genotype is protective. The mechanism of dominant protection by DQ6 (DQB1*06:02) is unknown. We tested the hypothesis that DQ6 interferes with peptide binding to DQ8 by competition for islet epitope ("epitope stealing") by analysis of the islet ligandome presented by HLA-DQ6/8 and -DQ8/8 on dendritic cells pulsed with islet autoantigens preproinsulin (PPI), GAD65, and IA-2, followed by competition assays using a newly established "epitope-stealing" HLA/peptide-binding assay. HLA-DQ ligandome analysis revealed a distinct DQ6 peptide-binding motif compared with the susceptible DQ2/8 molecules. PPI and IA-2 peptides were identified from DQ6, of DQ6/8 heterozygous dendritic cells, but no DQ8 islet peptides were retrieved. Insulin B6-23, a highly immunogenic CD4 T-cell epitope in patients with T1D, bound to both DQ6 and DQ8. Yet, binding of InsB6-23 to DQ8 was prevented by DQ6. We obtained first functional evidence of a mechanism of dominant protection from disease, in which HLA molecules associated with protection bind islet epitopes in a different, competing, HLA-binding register, leading to "epitope stealing" and conceivably diverting the immune response from islet epitopes presented by disease-susceptible HLA molecules in the absence of protective HLA.

The strongest single genetic determinant of type 1 diabetes (T1D) risk resides in the HLA class II region. Specifically, DQA1*05:01-DQB1*02:01 (hereafter denoted as DQ2) and DQA1*03:01-DQB1*03:02 (DQ8) have been found to be the major susceptible haplotypes in most populations, whereas DQA1*02:01-DQB1*06:02 (DQ6) has been described to be protective in a dominant manner. Most notably, the risk of the DQ2/8 heterozygote genotype is markedly increased (approximately fivefold) compared with individual DQ2 and DQ8 homozygous genotypes, indicating an epistatic or synergistic effect. The other T1D-critical HLA-DQ molecule is DQ6 that is strongly associated with dominant protection from T1D.

The dominant protective role of HLA-DQ6 in T1D has been well documented, but its mechanism remains unclear until today. Several plausible mechanistic explanations may apply; those that rely upon αβ pairings between DQ8 or DQ2 and DQ6 are most likely not operative because these pairings do not form (1). Rather, we favored epitope stealing as the most conceivable mechanism. Epitope stealing may occur when protective HLAs bind critical autoantigenic peptides, thereby preventing binding by susceptible HLA molecules in such a manner (e.g., higher affinity) that the pathway to autoantigen presentation and disease is inhibited (2). The peptide-binding characteristics of DQ6 have been studied based upon three-dimensional models of DQ6 molecules, by molecular modeling of DQ6-eluted peptides, and by eluting natural processed and presented insulin peptides (3–6). These studies suggest that T1D high-risk DQ molecules bind a distinct peptide repertoire as compared with the protective DQ6 molecule

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(7,8). Recently, we defined the peptide-binding motifs of high-risk HLA-DQ molecules (9); the high-risk DQ8*cis* molecule harbors peptide-binding motifs preferring binding peptides with negatively charged amino acids in the p1 and p9 pocket and mainly aliphatic residues at the other anchor positions in the HLA peptide-binding grooves. However, we already have proof-of-concept that a prototype proinsulin epitope (proinsB6-23) can bind both DQ8 and DQ6 with overlapping amino acids in proinsB6-23 (10). In the same study, HLA-DQ8–restricted CD4 T cells reactive against proinsB6-23 cells were isolated from an HLA-DQ8 homozygous patient with T1D. The phenotype of these CD4 T cells changed from proinflammatory (interferon-γ) to anti-inflammatory (interleukin-10) in the presence of antigen-presenting cells (APCs) expressing the high-risk HLA-DQ8cis as well as the protective HLA-DQ6.

In this study, we deciphered the DQ6 peptidome of the major islet antigens preproinsulin (PPI), IA-2, and GAD65 and the protective mechanism of DQ6 in T1D. First, we studied in detail the peptide-binding characteristics of DQ6 and compared its binding motif with the established binding motifs of the high-risk HLA-DQ molecules. Next, we examined the capacity of DQ6 to prevent binding of islet epitopes to high-risk HLA-DQ8. Finally, we explored the effect of DQ6 on the islet ligandome and on specific antigen presentation by DQ8 coexpressed by the same APCs (dendritic cells [DCs]), including an examination of "epitope stealing." This new knowledge reinforces the concept of using immunomodulatory APCs carrying the protective HLA-DQ6 molecule as targets of T1D therapy.

RESEARCH DESIGN AND METHODS

Culture of B-Cell Lines

Epstein-Barr virus–transformed B-lymphoblastoid cell lines (EBV-BLCLs) homozygous for DQ6 (LD2B, MGAR) or DQ8 (DUCAF, BSM) or heterozygous for DQ6/8 (CST) were generated from B cells of healthy individuals (11). For HLA-DQ peptide-binding assays, EBV-BLCLs were harvested by centrifugation, washed three times with PBS, and lysed with Nonidet P-40 lysis buffer; for HLA-DQ peptidome analysis, BLCLs were lysed using zwitter detergent. To remove nuclei and insoluble material, lysates were centrifuged at 3,500 revolutions/min for 20 min at 4°C. Lysates were stored at −80°C. Lysates were used for HLA-DQ peptide-binding assays and HLA-DQ peptide elution studies.

Islet Autoantigens

Recombinant islet autoantigens were produced as described previously (12,13).

HLA-DQ Affinity Purification, Peptide Elutions, and Peptidome Analysis

To decipher the peptide-binding characteristics of DQ6, affinity purifications and peptidome analysis of HLA-DQ6–expressing myeloid DCs (mDCs) were performed as described previously (9). Affinity-purification of HLA-DQ6 or DQ8 molecules from islet autoantigen–pulsed DQ6/8 heterozygous mDCs and subsequent peptide elutions were performed as follows. The HLA-peptide elution protocol was optimized for low mDC numbers according to our existing protocol for EBV-BLCLs. All HLA-DQ isolation and washing steps were performed using a 100-μL pipette tip. Lysate was precleared using 100 μL Sepharose beads. HLA-DQ8 was isolated using Sepharose beads coupled with the DQ8-capturing antibody IVD12 (Department of Immunohematology and Blood Transfusion, Leiden University Medical Center). Subsequently, from the same lysate, HLA-DQ6 molecules were isolated using SPV-L3 (Department of Immunohematology and Blood Transfusion, Leiden University Medical Center) coupled to Sepharose beads. mDC lysates were passed sequentially over the IVD12 and SPV-L3 Sepharose columns. Columns were washed as previously described (9). DQ–peptide complexes were eluted with two bed volumes 10% acetic acid. The HLA-DQ eluates (containing both peptides and HLA) were fractionated with a high-performance liquid chromatography system. The material was eluted using a gradient of 0–50% acetonitrile supplemented with 0.1% trifluoroacetic acid.

Peptide Identification by Mass Spectrometry

Natural processed and presented peptides (NPPPs) eluted from HLA-DQ molecules were analyzed as described previously (13).

Molecular Modeling

HLA-DQ homology modeling of the complexes between eluted peptides and the DQ6 molecule were performed as described previously (10).

Hybridomas and Affinity Purification of HLA-DQ–Specific Antibodies

Hybridomas producing the pan-DQ monoclonal antibody (Moab) SPV-L3 or the HLA-DQ8–specific Moab IVD12 were first cultured in Iscove's modified Dulbecco's medium supplemented with 8% FCS, penicillin/streptomycin, and L-glutamine. Gradually, FCS was depleted from the Iscove's modified Dulbecco's medium by diluting the cultures with protein-free hybridoma medium (PFHM) until cells were cultured in 100% PFHM. The IVD12 hybridoma did not survive in the absence of FCS, and therefore, PFHM was supplemented with 1% FCS. For antibody production, hybridomas were cultured in PFHM in CELLine Bioreactor Flasks (CL1000; Sigma-Aldrich) according to the manufacturer's instructions. SPV-L3 was affinity purified from PFHM using protein A Sepharose and Moab IVD12 using protein G Sepharose. Protein concentration was determined by A280 spectrophotometry.

HLA-DQ Peptide-Binding Assays

To obtain an indicator peptide for the HLA-DQ6 peptide-binding studies, core nonamers of a selection of peptides eluted from HLA-DQ6 EBV-BLCLs were predicted by

molecular modeling. The core nonamers, containing the anchor residues crucial for HLA class II binding, were synthesized with two flanking alanines both at the N- and C-terminus including a biotin label at the N-terminus. The flanking alanine residues (forming 14-mer peptides) are essential for peptide binding outside the HLA class II binding grooves. Binding studies were further performed as described previously (8). HLA-DQ6 and -DQ8 competitive peptide-binding assays were performed as described previously (13). We took these as a starting point to set up the epitope-stealing assay in which DQ6 interference on binding a peptide to DQ8 could be examined. In this assay, peptide binding to HLA-DQ8, HLA-DQ6, or DQ6/8 is measured using a specific DQ8 or DQ6 indicator peptide. The 96-well plates are coated with the pan-DQ antibody SPV-L3, and lysates from homozygous DQ6/6 and DQ8/8 or heterozygous DQ6/8 BLCL are incubated overnight at 4°C. After washing and blocking the plates, fixed concentrations of DQ8 or DQ6 indicator peptides (0.6 μmol/L) are incubated in the presence of increasing concentrations of test peptide (InsB6-23) that can bind both DQ8 and DQ6 (0–900 μmol/L). For the DQ8/8 or DQ6/6 plate, once the test peptide binds to HLA, the indicator peptide is outcompeted, and a drop in signal (counts per minute) is measured.

To measure InsB6-23 binding to DQ8 in the presence of DQ6, binding of the test peptide to the DQ6/8-coated plate is measured in the presence of the DQ6- or DQ8-specific biotinylated (Z) indicator peptide (DQ6 indicator: ZAAVKGSSLHVGAA; DQ8 indicator: ZEEPRAPWIEQEGPEYWDQE [EPRAP]). Once InsB6-23 binds to DQ6, the DQ8-specific indicator peptide will not be outcompeted by InsB6-23, and a drop in signal is not measured, resulting in a stable signal. However, in the presence of the DQ6-specific indicator peptide, a drop in signal will be observed once InsB6-23 binds, preferentially, DQ6 and not DQ8 and thus outcompetes the DQ6 indicator peptide. DQ6 competition is defined as InsB6-23 binding to the DQ6/8-coated plate in the presence of the DQ8-specific indicator peptide; DQ8 competition is defined as InsB6-23 binding to DQ6/8 in the presence of the DQ6-specific indicator peptide.

Generation of DCs

For elution of natural processed and presented islet peptides from HLA-DQ–expressing professional APCs, generation of DCs from heterozygous HLA-DQ6/8 healthy blood donors was performed as described previously (13,14). Immature DCs were pulsed with the islet autoantigens PPI, IA-2, and GAD65 for 6 h, and immature DCs were matured with LPS (100 ng/mL) for 24 h in the continuous presence of the three islet autoantigens. After 30 h, pulsed mDCs were harvested, washed three times with PBS to remove excess islet autoantigens, lysed in 1 mL zwitter lysis buffer, and subsequently high-speed centrifuged for 60 min at 10,000*g* to remove nuclei and insoluble material. For each HLA-DQ type, a total of $\sim 40 \times 10^6$ pulsed mDCs were obtained from three donors.

RESULTS

Ligandome of HLA-DQ6

The nine-mer putative peptide-binding cores of protective HLA-DQ6 were deduced from the unique peptides eluted from homozygous DQ6-expressing EBV-BLCLs as we described previously for the T1D high-risk HLA-DQ molecules (9). In total, 7.5×10^9 EBV-BLCLs were used for the HLA/peptide elution studies. In total, 1,156 unique peptides from DQ6 were eluted (Supplementary Table 1). This number of unique peptides eluted from DQ6 EBV-BLCLs is consistent with the number of eluted peptides eluted from EBV-BLCLs expressing the susceptible HLA-DQ molecules, as we described previously (9). A unique peptide is defined as a peptide sequence that is unique to a protein; peptides with the same amino acid sequence can be detected by mass spectrometry multiple times (called hits), but such a particular peptide is then denoted as one unique peptide detected. Table 1 shows examples of the alignment and core predictions (with anchor and nonanchor residues) of length variants of eluted peptides from DQ6. Anchor and nonanchor residues in the minimal binding registers of the unique DQ6 peptide sequences were predicted. Molecular modeling studies with DQ6 were performed to verify the predictions of anchor and nonanchor residues (data not shown). Based on the prediction of the minimal binding cores and the molecular simulation studies, the frequency of the amino acids at both the anchor and nonanchor residues in the eluted peptides was calculated for DQ6. Amino acids with a frequency of 1.5 times higher than the same amino acids in the human proteome were considered as preferred residues (Supplementary Fig. 1). To visualize all amino acids in the peptide-binding groove (positions p1–p9) of DQ6 (referred to as binding motif), heat maps were generated

Table 1—Alignments and core predictions of length variants of DQ6-eluted peptides

HLA-DQ6
12345**6**78**9**
AGH**T**SG**A**A**MW**P**G**TDVK
YPFD**F**QG**A**R**II**T**G**QEEG
GPGAA**V**VA**G**A**VV**G**T**LVG
A**V**PV**M**V**PA**Q**S**QAGSLV
DNVL**M**SG**V**K**NN**V**G**RGIN
SQL**I**MQ**A**E**AE**A**A**SVR
EL**Q**EP**A**EL**V**E**S**DG
TD**Q**VQ**A**E**AK**E**S**GPT
TGET**Y**TC**V**V**AH**E**A**LPNR
GAL**T**SG**V**H**TF**P**A**VLQ
TD**T**DQ**A**C**SI**R**D**PNSG
KSGN**T**AS**L**T**IS**G**L**Q
VLH**V**WG**V**T**TE**K**S**KE
EPA**E**VT**A**T**VL**A**S**RDD
DS**N**EF**S**V**IA**D**P**RG

Overview of peptide alignments and binding core identifications of length variants eluted from DQ6-expressing EBV-BLCLs. Anchors are in boldface type.

(Fig. 1). To provide a proper comparison of both protective and high-risk HLA-DQ molecules, the heat maps of the HLA-DQ2/8 *cis*/*trans* molecules are included. Our previous studies showed that the high-risk HLA-DQ8*cis* has a strong preference for negatively charged amino acids (D/E) at the anchor positions p1 and p9. The binding motif of the protective DQ6 shows several similarities with the high-risk HLA-DQ molecules. Small aliphatic residues such as G, A, and V are dominantly present, similar as for the DQ2/8 molecules. The negatively charged amino acids D and E are virtually absent at the anchor positions p4, p6, and p9 of DQ6, where HLA-DQ2/8 molecules prefer such negatively charged residues. Importantly, similarities are observed between the peptide-binding preferences of protective DQ6 and susceptible DQ8; a p8E can be present in a DQ6-binding peptide, and an E at this p8 position within a DQ6-binding peptide is also important for peptide binding to DQ8*cis* (Fig. 1 and Supplementary Fig. 1). The basic amino acids K, R, and H are virtually absent at the anchor positions of the protective HLA-DQ6 similar to the high-risk HLA-DQ2/8 molecules.

In conclusion, the peptide-binding requirements for DQ6 show that peptides can bind both protective DQ6 as well as susceptible DQ8*cis*, which is a requirement for the hypothesized mechanism of epitope stealing.

Epitope Stealing by Protective HLA-DQ6

CD4 T cells from a homozygous HLA-DQ8 patient with T1D responded against the prototype InsB6-23 epitope in the presence of DQ8 homozygous APCs with a proinflammatory phenotype (10). Yet, CD4 T cells reactive against InsB6-23 in the presence of HLA-DQ6/8 heterozygous APCs elicited a cytokine production profile shifted from a proinflammatory to an anti-inflammatory profile. In this study, we hypothesize that protective DQ6 binds autoantigens in a different (competing) binding register, leading to epitope stealing, thereby inducing a regulatory rather than a pathogenic immune response. To examine the possibility that DQ6 steals the InsB6-23 epitope from DQ8, we set up an HLA-DQ/epitope-stealing assay based on our existing HLA peptide-binding assays (9) (graphic representation of this assay with extended explanation in Supplementary Fig. 2). A DQ6-specific indicator peptide was chosen from a selection of DQ6-eluted peptides that bound with an intermediate affinity to DQ6 and not to DQ8 (data not shown). Figure 2 shows that InsB6-23 can bind DQ6/6 or DQ8/8 with a half-maximal effective concentration (EC_{50}) of 0.11 ± 0.01 and 3.6 ± 0.6 μmol/L, respectively. When both DQ6 and DQ8 are present (DQ6/8), binding of InsB6-23 to DQ8 was not observed (DQ6 competition). DQ8 did not interfere with binding of InsB6-23 to DQ6 (DQ8 competition). In this study, we show that protective DQ6 interferes with binding of the diabetogenic InsB6-23 epitope to high-risk DQ8 in T1D.

DQ6 Interferes With Binding of Natural Processed Epitopes Presented by DQ8 Expressed on DCs

Epitopes derived from islet autoantigens are generally presented by HLA class II molecules after naturally processing by professional APCs (15). DCs are essential for initiation of diabetogenic T-cell responses and T1D development. Recently, we showed that DQ2/8 heterozygous APCs naturally process and present PPI and IA-2 peptides

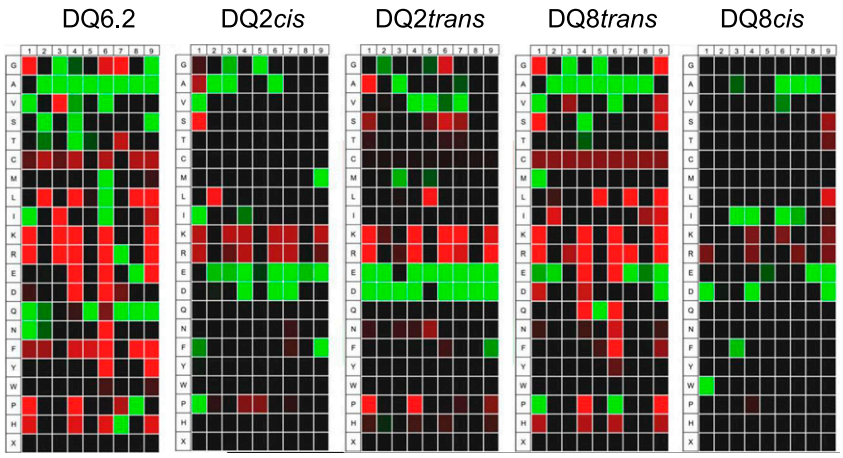

Figure 1—Frequency of the amino acids at anchor and nonanchor positions within the binding core of protective and susceptible HLA-DQ. After performing core analysis of the eluted unique peptides from HLA-DQ6–expressing EBV-BLCLs, the distribution of amino acids at both anchor (p1, p4, p6, p7, and p9) and nonanchor positions (p2, p3, p5, and p8) for protective DQ6 and susceptible DQ2 and DQ8 was calculated and compared with the amino acid frequency of the human proteome. The figure shows the heat map results of an iceLogo analysis for each HLA-DQ molecule. Heat maps for the susceptible HLA-DQ2 and -DQ8 molecules, already reported by us and others, have been added to the DQ6 heat map to demonstrate the differences between protective and susceptible T1D HLA-DQ molecules. Increased or decreased amino acid frequencies are shown in a gradient of, respectively, green or red shades.

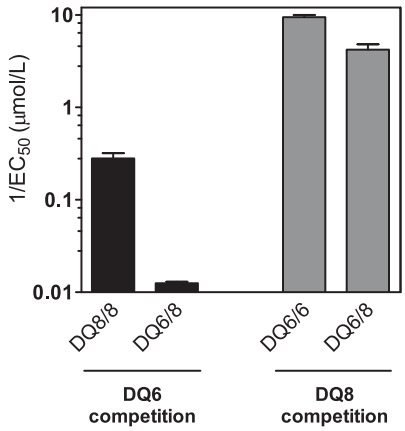

Figure 2—Binding of InsB6-23 to HLA-DQ6, -DQ8, and -DQ6/8. Binding of InsB6-23, harboring two distinct but partially overlapping binding InsB6-23 cores for HLA-DQ6 and -DQ8, was tested to HLA-DQ6, -DQ8, or -DQ6/8 in cell-free competitive HLA-DQ/peptide-binding assays. Indicator peptides were used at a fixed concentration (0.6 μmol/L) and were specific for either DQ6 (ZAAVKGSSLHVGAA) or DQ8 (ZEEPRAPWIEQEGPEYWDQE; i.e., EPRAP). InsB6-23 was tested in a concentration range (0–300 μmol/L). For DQ6/8-binding assays, binding of InsB6-23 was tested in the presence of the DQ8- or DQ6-specific reporter peptide. EC_{50} values (in μmol/L) were calculated based on competition between a biotinylated indicator peptide and the tested NPPP. Data represent mean ± SEM (n = 3). Shown on the x-axes is $1/EC_{50}$, thereby illustrating that large bars represent better binding.

recognized by proinflammatory autoreactive CD4 T cells in patients with T1D (13). To investigate if DQ6 interferes with binding of PPI and IA-2 peptides to DQ8 in this study, we analyzed the HLA-DQ6/8 islet peptidome by pulsing immature DCs heterozygous for HLA-DQ6/8 with PPI, IA-2, and GAD65. From these heterozygous HLA-DQ6/8 and pulsed mDCs, HLA-DQ8 was first immune precipitated using the HLA-DQ–specific antibody IVD12 subsequently followed by immune precipitation of HLA-DQ6 using the pan-DQ antibody SPV-L3. HLA-eluted peptides were identified using mass spectrometry. To confirm HLA-DQ specificity of IVD12 and SPV-L3, stainings of homozygous HLA-DQ6 (MGAR) and HLA-DQ8 (BSM) EBV-BLCLs were performed. As shown in Supplementary Fig. 3, IVD12 solely recognizes HLA-DQ8 expressed on BSM, whereas SPV-L3 recognizes both HLA-DQ6 and HLA-DQ8 expressed on MGAR and BSM, respectively.

From DQ8, PPI, IA-2, or GAD65 peptides were not identified. From DQ6, peptides derived from PPI and IA-2 and not from GAD65 were identified (Fig. 3). From PPI, several peptides of the signal sequence were identified with a mean length of 12 amino acids (range 9–16). Interestingly, a similar PPI signal peptide was identified by us naturally presented by DQ2/8 heterozygous DCs (13); this peptide was recognized by proinflammatory CD4 T cells isolated from both DQ8 homozygous and DQ2/8 heterozygous patients with T1D. Now, for the first time, PPI signal peptides are identified as naturally processed DQ6-presented peptides. Naturally processed and presented peptides by DQ6 were identified encompassing four core regions of IA-2, including its transmembrane region. No peptides were identified from the C-terminus of IA-2. These IA-2 core regions were distinct from the core regions of which peptides were identified presented by DQ8 homozygous and DQ2/8 heterozygous DCs. In this study, we show that protective DQ6 interferes with peptide binding to high-risk DQ8 in T1D.

Binding Confirmation of Naturally Processed and DQ6-Presented Peptides

The anchor residues and minimal binding registers of the DQ6-identified naturally processed and presented peptides were in silico predicted using the DQ6 and DQ8 peptide-binding motifs (Fig. 1) with our in-house MOTIFS software program. Anchor residues for DQ6 but not for DQ8 were predicted for all DQ6-eluted peptides (Table 2). First, an indicator peptide for DQ6 was selected to perform competitive peptide-binding assays. Core nonamers, predicted from a selection of nine peptides eluted from DQ6 EBV-BLCLs, were extended with alanine residues, labeled with biotin, and tested in direct DQ6-peptide binding assays (Supplementary Fig. 4). The peptide with sequence ZAAVKGSSLHVGAA was selected as indicator peptide for the competitive peptide-binding assays. Next, binding of the peptides eluted from DQ6/8 heterozygous DCs to DQ6 was measured in competitive HLA-DQ peptide-binding assays as described previously (13). The following binding affinities (EC_{50}) were calculated: $PPI_{14–22}$, 10.2 ± 1.2 μmol/L; $IA\text{-}2_{374–386}$, 4.6 ± 1.2 μmol/L; $IA\text{-}2_{498–511}$, 21.3 ± 2.4 μmol/L; $IA\text{-}2_{543–566}$, 23.2 ± 7.3 μmol/L; and $IA\text{-}2_{582–593}$, 7.6 ± 0.6 μmol/L (Supplementary Fig. 5). Binding of these tested PPI and IA-2 peptides to DQ8 was not observed. These data show exclusive binding of the eluted peptides by protective DQ6 expressed on DQ6/8 heterozygous DCs.

DISCUSSION

We provide a functional explanation for epitope stealing as a mechanism for genetic protection in T1D in which DQ6 (*DQB1*06:02*) prevents binding of diabetogenic epitopes by high-risk DQ8 as a target of autoreactive CD4 T cells in patients with T1D. First, DQ6 shows peptide-binding characteristics enabling this molecule to bind and present similar islet epitopes also presented by DQ8. Second, the prototype proinsulin epitope InsB6-23 binds both DQ6 and DQ8, yet has a strong preference for binding DQ6 in the presence of DQ8. Finally, we provide evidence that DQ6 interferes with binding of naturally processed and DQ8-presented islet epitopes on the surface of DQ6/8 heterozygous DCs. This DQ6 interference in peptide presentation by DQ8 on the same APC is due to sharing a similar PPI peptidome, but also by having a distinct IA-2 peptidome.

The identified PPI signal peptides naturally processed and presented by DQ6 showed profound similarities to the

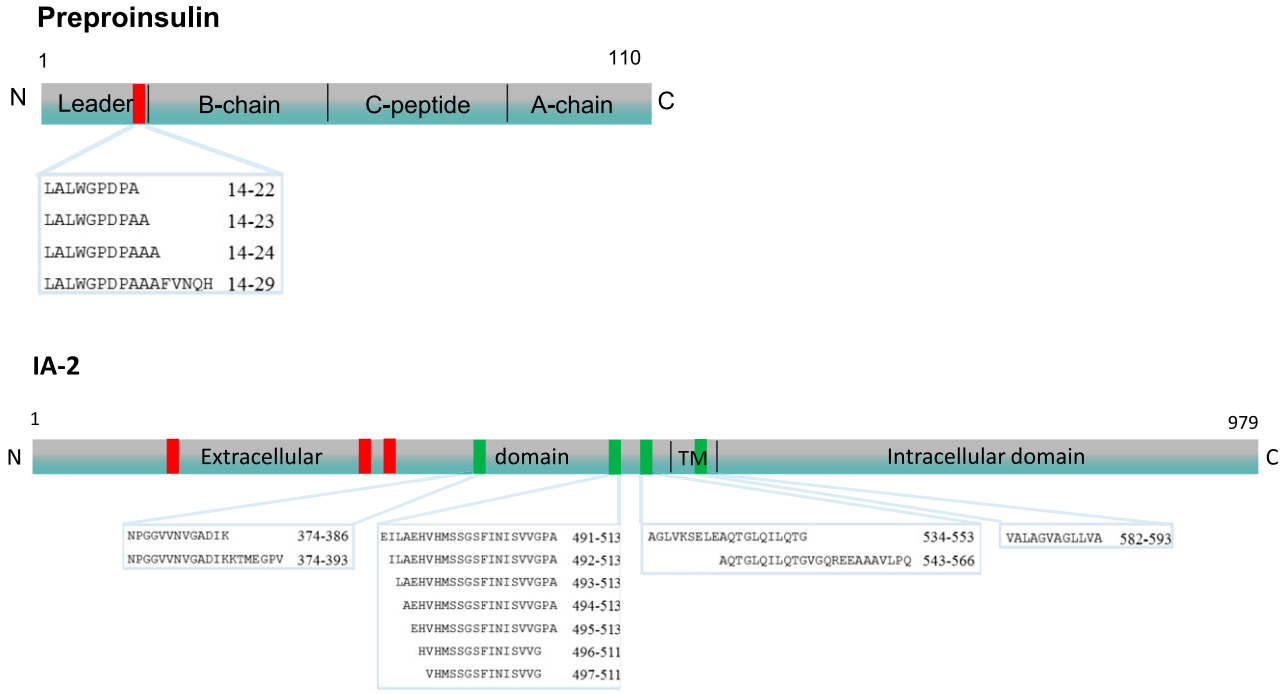

Figure 3—Elution of natural processed and presented islet peptides from DQ6/8 heterozygous DCs. HLA-DQ6/8 heterozygous DCs were pulsed for 6 h with the islet autoantigens PPI, IA-2, and GAD65, after which the DCs were matured for 24 h with LPS in the continuous presence of the three islet autoantigens. After cell lysis, HLA-DQ8 was purified using IVD12 (HLA-DQ8–capturing antibody) and HLA-DQ6 using SPV-L3 (pan-DQ), and peptides were acid eluted and analyzed by mass spectrometry. Nested sets of peptides, covering one or more core regions of PPI and IA-2, were identified.

PPI signal peptide ($PPI_{15–24}$) recently identified by us as a natural epitope presented by DQ2/8 heterozygous DCs. CD4 T cells isolated from patients with T1D either being DQ8 or DQ2/8 positive responded against this epitope (13). In this study, we show that the same signal peptide, although with a different binding register, can be naturally

Table 2—In silico binding predictions of natural islet peptides eluted from DQ6 expressed on DQ6/8 heterozygous DCs

Observed m/z	Calculated m/z	Residues	Corresponding protein sequence
PPI			
541.29	1,080.56	14–22	**L**ALWGPDP**A**
910.48	1,818.95	14–23	LALWGPDPAA
784.36	783.35	14–24	LA**L**WGPDPAA**A**
713.32	712.31	14–29	LALWGPDPAAAFVNQH
IA-2			
Core 1			
620.34	1,239.67	374–386	**N**PG**G**V**V**NV**G**ADIK
666.68	1,998.03	374–393	NPGGVVNVGADIKKTMEGPV
Core 2			
804.08	2,410.21	491–513	E**I**LAEH**V**HM**S**SGSFINISVVGPA
1,141.09	2,281.16	492–513	ILAEHVHMSSGSF**I**NI**S**V**VG**P**A**
723.37	2,168.08	493–513	LAEHVHMSSGSFINISVVGPA
685.67	2,055.00	494–513	AEHVHMSSGSFINISVVGPA
661.99	1,983.96	495–513	EHVHMSSGSFINISVVGPA
843.91	1,686.83	496–511	HVHMSSGSFINISVVG
775.39	1,549.77	497–511	VHMSSGSFINISVVG
Core 3			
1,028.57	2,056.13	534–553	AGLVKSELEAQTGLQILQTG
1,239.67	2,478.33	543–566	AQTGLQ**I**LQ**T**G**VG**QREEAAAVLPQ
Core 4			
1,053.67	1,053.67	582–593	**V**AL**A**G**VA**GLLVA

By using the peptide-binding motifs of protective DQ6 and susceptible DQ8, natural peptides derived from PPI and IA-2 eluted from autoantigen-pulsed DQ6/8-expressing DCs were in silico validated as DQ6-binding natural islet peptides (anchor residues in boldface type; minimal binding registers underlined). No prediction hits were observed for DQ8. m/z, charge/mass ratio.

presented by DQ6. Peptide-binding studies confirmed binding of the signal peptide to DQ6. Importantly, we show that in the presence of DQ6 on the surface of DQ6/8 heterozygous DCs, PPI signal peptides are not eluted from DQ8. This is the first time that a study provides evidence for epitope stealing in which DQ6 steals a diabetogenic naturally processed epitope normally presented by DQ8. InsB6-23 was not detected in the current study as an NPPP, which can be due to many technical restrictions making it currently impossible to elute and detect certain possibly low-quantitative peptide epitopes. However, a shorter variant, InsB9-23 (PPI 76–90), has been identified as a natural processed and eluted HLA class II peptide and as target for CD8 T cells (16–18). Also, T1D-derived T-cell clones reactive against InsB6-23 cross-react with naturally processed insulin. Recently, we reported that the T-cell receptor of these T cells cloned into T cells of humanized mice develop insulitis and cause selective β-cell destruction in these mice, mimicking T1D (19). This observation underscores that the InsB peptide is made in vivo and actually has biological and potentially pathological relevance.

The natural IA-2 peptides eluted from DQ6 encompassed four distinct core regions of the N-terminal part as well as the transmembrane region of IA-2. IA-2 peptides from DQ8, isolated from the same pulsed DQ6/8 DCs, were not identified. Binding of the IA-2 peptides was confirmed for DQ6, and no binding was observed for DQ8. This finding is supported by our previous findings that DQ8 presents a set of natural IA-2 peptides, although different from the DQ6-presented peptides, also derived from the N-terminus of IA-2 (13). In this study, instead of stealing peptides from DQ8 as for the PPI signal peptides, DQ6 completely abrogates the presentation of IA-2 peptides by DQ8 on the same DQ6/8 heterozygous APCs. Interestingly, the same epitope-stealing mechanism as described for InsB6-23 might in this case as well contribute to stealing of IA-2 peptides from susceptible DQ8 by protective DQ6.

GAD65 peptides were retrieved from neither DQ6 nor DQ8, although uptake of all three islet antigens was confirmed by proteome analysis of the pulsed DCs (data not shown). Recently, we showed that DQ8 homozygous DCs did not present naturally processed and presented GAD65 peptides (13). This might imply that GAD65 peptides are not primary targets of autoreactive HLA-DQ–restricted CD4 T cells (pro- and anti-inflammatory) in patients with T1D. Intriguingly, as HLA-DQ8 is in linkage disequilibrium with HLA-DR4 (DRB1*04:01), it may be argued that GAD65 epitopes may be presented by HLA-DR4 rather than HLA-DQ8 molecules. In support of this notion, in our previous study (12) in which we eluted islet antigenic peptides from pulsed DCs, we sequentially purified HLA-DQ and HLA-DR, and we were able to elude GAD65 peptides from HLA-DR4, supporting the notion that GAD65 autoimmunity in T1D might be restricted by HLA-DR.

Additionally, although not investigated in the current study, DQ6 is in linkage disequilibrium with HLA-DR15 (*DRB1*15:01*), and as such, a role for DR15 in the protection from T1D cannot be excluded.

Because DCs were pulsed with islet autoantigens, one might expect an overrepresentation of eluted peptides from these antigens. However, this was not our observation; the majority of eluted peptides did not originate from the islet autoantigens. An explanation for this observation could be competition between endogenous processed peptides and peptides generated from the pulsed autoantigens. First, the pulsed autoantigens first have to be taken up by the DCs before being processed and presented by HLA class II molecules, creating a disadvantage versus endogenous processed peptides. Second, affinity differences likely play a role in HLA binding of endogenous processed and presented peptides and peptides generated from the pulsed autoantigens.

T1D is a multifactorial disease in which genetic factors inherited from the parents, such as protective HLA-DQ6 and environmental factors, reduce the risk of developing T1D (8,20,21). A new protective environmental factor with genetic implications could be the tolerizing effect of non-inherited maternal antigens (NIMAs) (22–24). The NIMA effect supports tolerance induction to specific non-inherited protective HLA from the mother in the offspring (25). The protective effect of HLA-DR13 as NIMA in rheumatoid arthritis has been found (26,27). Intriguingly, in our family cohort of children with T1D, the number of cases in which mothers carry protective HLA-DQ6 is much reduced, suggesting that HLA-DQ6 could protect offspring, even if the offspring does not inherit DQ6. As a mechanism, we propose the induction of mixed chimerism in which maternal leukocytes carrying DQ8 (shared with the fetus) and DQ6 (not inherited by the fetus) crossing the placenta could modulate the immune response in the fetus, possibly by mechanisms including epitope stealing. The disease-protective effect of HLA-DQ6 as NIMAs may be mimicked later in life by using immunomodulatory APCs carrying the protective DQ6 loaded with DQ6-specific islet epitopes in addition to high-risk HLA-DQ8 shared with the patient. To translate this concept clinically for patients with T1D, it is essential to understand the protective mechanism of these specific HLA subclasses as well as to delineate the natural islet peptidome of protective DQ6 in the context of DQ8.

Understanding the mechanism of dominant protection by DQ6 opens doors to novel immunotherapies that use epitope stealing as a protective mechanism for T1D. Candidate immunomodulatory APCs are mesenchymal stromal cells (MSCs), which have immunosuppressive and regenerative capacity. Beneficial effects of MSC transplantation in experimental models of T1D have been reported: MSCs dampen autoreactive T-cell responses (28), enhance development of regulatory T cells (29,30), and promote islet repair of endogenous or transplanted islets (31,32). MSCs that are hypoimmunogenic and

regulatory by nature expressing DQ6 and DQ8 could be used to install tolerance in DQ8-positive patients with T1D. As such, they resemble the way that mothers carrying DQ6 and DQ8 protect their DQ8-inherited children from T1D by microchimerism (NIMAs). In our view, this is an "experiment of nature" showing that the maternal protective haplotype during pregnancy indirectly protects a child from destructive autoimmunity later in life. The potential of MSCs to reduce proinflammatory autoimmune reactivity in an antigen-dependent fashion has not yet been explored. Therefore, it is attractive to mimic nature by exploring new therapeutic approaches using MSCs matched for DR4/DQ8 with the recipient but mismatched with the protective DR15/DQ6 haplotype and loaded with our identified DQ6 natural peptides. Natural peptides presented by DQ6 should be tested for their capacities to induce antigen-specific regulatory T cells. This will provide a novel antigen-specific immunomodulatory therapy for the treatment of patients with T1D to benefit from genetic protection endorsed by DQ6.

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