Menno van Lummel,[1] Gaby Duinkerken,[1] Peter A. van Veelen,[1] Arnoud de Ru,[1] Robert Cordfunke,[1] Arnaud Zaldumbide,[2] Iria Gomez-Touriño,[3] Sefina Arif,[3] Mark Peakman,[3] Jan W. Drijfhout,[1] and Bart O. Roep[1]

Posttranslational Modification of HLA-DQ Binding Islet Autoantigens in Type 1 Diabetes

Posttranslational modification (PTM) of islet autoantigens can cause lack of central tolerance in type 1 diabetes (T1D). Tissue transglutaminase (tTG), involved in PTM of gluten antigens in celiac disease, creates negatively charged peptides favored by T1D-predisposing HLA-DQ molecules, offering an attractive candidate modifying islet autoantigens in T1D. The highly predisposing HLA-DQ8*cis*/*trans* molecules share preferences for negatively charged peptides, as well as distinct peptide-binding characteristics that distinguish their peptide-binding repertoire. We screened islet autoantigens with the tTG substrate motif for candidate-modified epitopes binding to HLA-DQ8*cis*/*trans* and identified 31 candidate islet epitopes. Deamidation was confirmed for 28 peptides (90%). Two of these epitopes preferentially bound to HLA-DQ8*cis* and six to HLA-DQ8*trans* upon deamidation, whereas all other peptides bound equally to HLA-DQ8*cis*/*trans*. HLA-DQ8*cis*–restricted T cells from a new-onset T1D patient could only be generated against a deamidated proinsulin peptide, but cross-reacted with native proinsulin peptide upon restimulation. The rate of T-cell autoreactivity in recent-onset T1D patients extended from 42% to native insulin to 68% adding responses to modified proinsulin, versus 20% and 37% respectively, in healthy donors. Most patients responded by interferon-γ, whereas most healthy donors produced interleukin-10 only. Thus, T-cell autoreactivity exists to modified islet epitopes that differs in quality and quantity between patients and healthy donors.

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Type 1 diabetes (T1D) is an autoimmune disease characterized by T cell–mediated destruction of the insulin-producing islet β-cells (1). The disease process involves autoreactive T cells to various islet antigens, such as insulin, insulin secretory granules, islet T cells, islet antigen-2 (IA-2), and the 65 kDa isoform of GAD (GAD65) (2). Posttranslational modification (PTM) of islet autoantigens provides a mechanism by which pathogenic T cells can escape thymic deletion and become activated when encountering a modified islet autoantigen in the periphery (3). Several lines of evidence implicate preproinsulin (PPI) as a target autoantigen in T cell–mediated β-cell destruction in humans and in the NOD mouse model of T1D (4–11), rendering this islet protein a likely candidate for PTM. Notably, CD4 T cells from a T1D patient reacted against the human insulin A-chain after PTM (12).

T1D is genetically strongly associated with HLA-DQ2 and HLA-DQ8 (13,14). HLA-DQ2/8 heterozygous individuals carry the highest odds ratio (OR) for T1D, exceeding that of HLA-DQ2 or HLA-DQ8 homozygous individuals (13,15). Besides the two HLA-DQ*cis* molecules, two additional molecules in the *trans*-configuration

[1]Department of Immunohematology and Blood Transfusion, Leiden University Medical Center, Leiden, the Netherlands
[2]Department of Molecular Cell Biology, Leiden University Medical Center, Leiden, the Netherlands
[3]Department of Immunobiology, School of Medicine, King's College London, London, U.K.

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

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(*DQA1*05:01-DQB1*03:02*; HLA-DQ8*trans*) and *DQA1*03:01-DQB1*02:01*; HLA-DQ2*trans*) can be formed in DQ2/8 heterozygote subjects. HLA-DQ8*trans* appears to be responsible for the increased OR in heterozygous individuals (13). HLA-DQ molecules are believed to exert their dominant effect on islet autoimmunity via thymic selection and/or peripheral activation of autoreactive T cells. Furthermore, certain HLA-DQ molecules may delete autoreactive thymocytes poorly, thus permitting potentially pathogenic T cells to reach the periphery (16,17). Therefore, self-peptides that undergo PTM may form neoautoantigens binding to HLA-DQ that are recognized by the immune system and form the target of autoimmunity.

The HLA-DQ8*trans* peptide-binding motif (18) reveals a strong preference for negatively charged residues (E or D) in the peptides capable of binding HLA-DQ8*trans*. HLA-DQ8*trans* and HLA-DQ8*cis* share the HLA-DQ8 β-chain (encoded by *DQB1*0302*) and have overlapping but distinct peptide-binding characteristics. Therefore, they display overlapping as well as distinct peptide repertoires. Although both HLA-DQ8 molecules prefer negatively charged residues at p9, HLA-DQ8*trans* prefers aliphatic residues at p1, and HLA-DQ8*cis* prefers negatively charged residues at p1.

Tissue transglutaminase (tTG), an enzyme able of converting Q residues into negatively charged E residues required for binding to HLA-DQ molecules, is an attractive candidate to create PTM associated with T1D. tTG is known to modify gluten antigens in celiac disease (CD), making these more potent HLA-DQ binders able to activate CD4 T cells, causing lesions in CD pathogenesis (19). In addition, CD4 T cells from patients with CD can cross-react between HLA-DQ8*cis* and HLA-DQ8*trans* or can even be HLA-DQ8*trans*–dependent (20). On the basis of the comorbidity of CD and T1D and shared HLA-DQ susceptibility, we hypothesized that tTG is able to modify islet autoantigens, converting these into more potent HLA-DQ binders and amplifying the immune response. Here, we took an unbiased approach to screen candidate islet autoantigens (e.g., PPI, islet tyrosine phosphatase IA-2, and GAD65), using the peptide-binding motifs of HLA-DQ8*cis/trans* for candidate epitopes that can be modified by tTG, using the tTG algorithms (21). Binding of the predicted epitopes (nondeamidated vs. deamidated) was validated to all four *cis*- and the *trans*- molecules of HLA-DQ2 and HLA-DQ8. As proof-of-concept that this approach identifies functionally relevant autoimmune responses, we demonstrate the existence of CD4 T cells specific for a PPI epitope modified by tTG and isolated from peripheral blood of a new-onset HLA-DQ8*cis* homozygous T1D patient. T-cell responses in new-onset T1D patients and in healthy donors matched for age and HLA underscored the potential relevance of PTM in disease.

RESEARCH DESIGN AND METHODS

Peptide Synthesis

Peptides were synthesized according to standard fluorenylmethoxycarbonyl chemistry using a SyroII peptide synthesizer (MultiSynTech, Witten, Germany). The integrity of the peptides was checked using ultra-performance liquid chromatography–mass spectrometry (MS) and matrix-assisted laser desorption/ionization–time-of-flight (TOF) MS. The following peptides have been used in the cell-free peptide-binding studies as biotinylated indicator peptides: CLIP: KMRMATPLLMQAL (HLA-DQ2*cis*); AAEAALEAEEWAA (HLA-DQ2*trans*); AAPHTTQPAVEAA (HLA-DQ8*trans*); and HSV-2: EEVDMTPADALDDFD (HLA-DQ8*cis*). HLA-DQ8*cis/trans* candidate epitopes used in the peptide-binding studies, predicted as 9-mers, were synthesized as 13-mers containing two overhanging amino acids (called overhanging peptide segments) according to the native protein sequences of their respective islet antigens both N- and C-terminally for proper binding in the HLA-DQ binding groove. Predicted HLA-DQ8*cis/trans* candidate epitopes selected for deamidation studies with tTG were synthesized as 11-mers with the Q in the peptide center and 5 additional native residues, both N- and C-terminally, to form a proper substrate for tTG.

Prediction of HLA-DQ8*cis/trans* Candidate Epitopes With the Potential To Be Deamidated

The peptide-binding motifs of HLA-DQ8*trans* (18) and HLA-DQ8*cis* (22,23) were used to predict candidate epitopes from known islet autoantigens (e.g., PPI, IA-2, and GAD65). Because HLA-DQ8*trans* and HLA-DQ8*cis* prefer peptides having negatively charged residues (E and/or D) and tTG generates such acid residues through deamidation (Q to E), a Q was added to the HLA-DQ8*cis/trans* peptide-binding motifs at the anchor positions p1/p7/p9 where E is preferred. For predictions, we used our in-house algorithm software program MOTIFS. After the predictions were performed, the candidate epitopes were screened with the tTG algorithms (21) to identify epitopes with the potential to be deamidated by tTG. Selected epitopes were subsequently tested for deamidation.

Deamidation of HLA-DQ8*cis/trans* Predicted Candidate Epitopes by tTG

HLA-DQ8*cis*– and HLA-DQ8*trans*–predicted candidate epitopes were tested for deamidation by tTG in a purified system. Peptides and tTG (4.1 units/mg protein; Sigma-Aldrich) were dissolved in deamidation buffer (containing 200 mmol/L HEPES and 8 mmol/L $CaCl_2$, pH 6.5) at a concentration of 1 mg/mL. For the reaction mixture, 15 μL tTG solution was added to 50 μL peptide solution, followed by an overnight incubation at 37°C. Deamidation of peptides was measured by MS.

Mass Spectrometry

Electrospray ionization MS was performed on a hybrid quadrupole TOF MS, the Q-TOF Ultima (Waters,

Manchester, U.K.). Peptides were analyzed by online nano–high-performance liquid chromatography (HPLC) tandem MS (MS/MS) using an 1100 HPLC system (Agilent Technologies). Peptides were trapped at 10 μL/min on a 15-mm column (100 μm ID; ReproSil-Pur C18-AQ, 3 μm, Dr. Maisch GmbH) and block-eluted with 50% acetonitrile in 0.1% formic acid. MS/MS was performed on selected precursors before and after deamidation. The collision gas applied was argon (pressure 4×10^{-5} mbar), and the collision voltage was ~30 V. From the mass shift in the MS1 spectra and altered fragmentation pattern after deamidation, the degree and position of deamidation was established.

Cell-Free HLA-DQ Peptide-Binding Assays

Binding of predicted HLA-DQ8*cis*/*trans* candidate epitopes was studied in cell-free peptide-binding assays. These assays are based on competition between a biotinylated reporter peptide and the peptide of interest. For HLA-DQ2*cis* and HLA-DQ8*cis*, Epstein-Barr virus–transformed B lymphoblastoid cell lines were used as a source of HLA-DQ. As a source of HLA-DQ2*trans* and HLA-DQ8*trans*, human embryonic kidney 293 cells were lentiviral-transduced to express a single HLA-DQ molecule (18). These binding assays have been described earlier (9,18). The half-maximal effective concentration (EC_{50}) values were calculated from the observed binding of the tested peptides against the fixed concentration indicator peptide; the concentration of tested peptide required for half-maximal inhibition of binding of the reporter peptide indicate the EC_{50} value. Although binding of the same peptide to different HLA-DQ molecules is difficult to compare because indicator peptides differ, a difference in EC_{50} value $\geq$10 times was considered substantial.

Patient, Isolation, and Culture of InsB30-C13–Specific T-Cell Lines

After informed consent, peripheral blood was drawn from an 11-year-old Caucasian girl diagnosed with new-onset T1D. Seropositivity for islet autoantibodies was confirmed. Peripheral blood mononuclear cells (PBMC) were isolated by Ficoll centrifugation and resuspended in Iscove's modified Dulbecco culture medium (Gibco BRL, Paisely, U.K.) containing 10% pooled human, heat-inactivated serum. PBMC were tested in a lymphocyte stimulation assay using culture medium, interleukin (IL)-10, or 10 μg/mL InsB30-C13, as described previously (4). In all stimulation assays, InsB30-C13 was used synthesized as peptides with Q or E ("deamidated"). For the generation of T-cell lines, 1.5×10^5 frozen PBMC (HLA-DQ8 homozygous) were incubated in culture medium containing 10 μg/mL InsB30-C13 and 0.5% leuco A (Sigma-Aldrich) in round-bottomed 96-well plates (Costar, Cambridge, MA). The cells were incubated for 6 days and then expanded in culture medium containing 5% T-cell growth factor (Promega). At day 14, 2×10^5 T cells were restimulated with 1% phytoagglutinin-A (GE Healthcare, Uppsala, Sweden) and 1×10^6 feeder cells (mix of six different donor PBMC irradiated with 3000 Rad) and, after an additional 3 days, were expanded by adding 10% IL-2 to the culture medium. T-cell cultures were repeatedly split, and after a further 10 days of incubation, were frozen.

T-Cell Proliferation Assay

A T-cell proliferation assay was performed on T-cell lines derived from the T1D patient as described before (4). T-cell lines (1.0×10^4 cells) and partly HLA-DQ–matched irradiated (3,000 Rad) PBMC, isolated from healthy donors (5×10^4 cells) in Iscove's modified Dulbecco's culture medium [Gibco BRL] with 10% heat-inactivated human serum, were seeded per well in flat-bottomed 96-well microculture plates (Greiner, Nürtingen, Germany) and cultured for 3 days at 37°C in 5% CO_2, in a humidified atmosphere. Cells were cultured in triplicates in medium alone, with 10 μg/mL InsB30-C13 (with Q or E), or recombinant IL-2 10% (25 units/mL; Genzyme, Cambridge, MA) as positive control. In the final 16 h of culture, 50 μL RPMI 1640 (Dutch modification; Gibco) containing 0.5 μCi ^{3}H-thymidine (DuPont NEN, Boston, MA) was added per well. After the cells were harvested on glass filters with an automated harvester, proliferation was determined by the measurement of ^{3}H-thymidine incorporation in an automatic liquid scintillation counter. All results are calculated as mean counts per minute in the presence of antigen and compared with medium alone.

Detection of InsB30-C13–Specific CD4 T Cells Secreting Interferon-γ and IL-10

Detection of interferon (IFN)-γ and IL-10 production by CD4 T cells in response to the native (Q) and modified (E) InsB30-C13 epitopes and InsB6-22 was performed using an enzyme-linked immunospot (ELISPOT), as described previously (6,24). After informed consent, blood was collected from 22 new-onset T1D patients (mean age 26 ± 11 years) and 19 HLA-DQ–matched healthy control subjects (mean age 31 ± 7 years). PBMCs were freshly isolated and incubated in the presence of 10 μg/mL peptide or diluent alone for 48 h. The cells were then transferred onto microtiter precoated with monoclonal anti–IFN-γ or anti–IL-10 capture antibody. Detection of cells producing IFN-γ and IL-10 was performed using biotinylated anti–IFN-γ or anti–IL-10 detector antibody. Data are expressed as the mean number of spots per triplicate divided by the mean number of spots in triplicate in the presence of diluent alone. Stimulation index values of ≥3 are considered as positive.

RESULTS

Prediction of HLA-DQ8*cis*/*trans* Candidate Epitopes With the Potential To Be Deamidated

HLA-DQ8*cis* has a major preference for negatively charged amino acid residues E (and D) at the anchor residues at positions p1 and p9 and a minor preference at p7. The HLA-DQ8*trans* peptide-binding motif, identifying

novel candidate T-cell epitopes (18), revealed a major preference for such residues at position p9 and minor preferences at p1 and p7. For the screening studies, we used the peptide-binding motifs of HLA-DQ8*cis* and HLA-DQ8*trans* and added a Q in the motifs at the anchor positions p1/p7/p9. We also included in the search double mismatches at the anchor positions because peptide-binding to HLA-DQ molecules does not necessarily need to have all anchor positions occupied by amino acid residues within the binding groove (18).

Next, we screened these candidate epitopes for potential deamidation sites at the anchor positions p1/p7/p9 by making use of the tTG algorithms (21). This tTG algorithm defines which amino acid residues are preferred C-terminally after a Q residue for proper modification by tTG. Supplementary Table 1 summarizes the predicted candidate epitopes containing Q residues in bold at the anchor residues having the potential to be deamidated by tTG into E. In this way, 63 candidate islet epitopes were identified having one or more Q residues, 17 epitopes (27%) with p1Q, 11 epitopes (17%) with p7Q, and 37 epitopes (59%) with p9Q. Two peptides, the PPI peptide EDLQVGQVE and the IA-2 peptide QRLQGVLRQ, had a p4Q with the potential to be deamidated. However, both HLA-DQ8 molecules do not prefer p4E, and these p4Q residues were initially not tested for deamidation. Because the anchor position p9 has the highest preference for negatively charged residues for both HLA-DQ8*cis* and HLA-DQ8*trans*, from the 63 we selected 31 candidate epitopes with the p9Q for deamidation studies, including the PPI epitope with p7Q, because this was the only predicted PPI epitope (Supplementary Table 2).

For deamidation studies, peptides were synthesized with the Q residue in the peptide center flanked by five amino acid residues at both the N- and C-terminus. These native residues were derived from the sequences of the islet autoantigen the epitopes were predicted from. From the 31 selected candidate epitopes, 28 (90%) were deamidated by tTG at the predicted Q residues, illustrating the predictive power of the tTG algorithms. For one peptide, ERLLYPDYQIQAT (Znt-8) p7Q was deamidated, although this position did not fulfill the tTG algorithm. That the p9Q residues of the candidate epitopes MEYGTTMVSY**Q**PL (GAD65) and PSLSYEPALL**Q**PY (IA-2) (predicted core nonamers underlined) were not deamidated is explained by the presence of the P residue positioned directly after the Q residue. The p9Q in PYEFTTLKSL**Q**DP (ICA69), despite fulfilling the tTG algorithms, was not deamidated by tTG.

Binding of Deamidated Versus Nondeamidated Predicted HLA-DQ8*cis*/*trans* Candidate Epitopes to all Four HLA-DQ *cis*- and *trans*-Dimers

To examine whether deamidation of the predicted HLA-DQ8*cis* and HLA-DQ8*trans* candidate epitopes induces better peptide binding to HLA-DQ, we tested the candidate epitopes as 13-mers containing two overhanging amino acid residues, both N- and C-terminally, according to the native protein sequences of their respective islet antigens necessary for proper HLA-DQ binding. The tested candidate epitopes were synthesized with Q (nondeamidated) or E (deamidated) residues. Peptide binding to the four HLA-DQ molecules was divided into four groups by the calculated EC_{50} values: no binding (EC_{50} >100 μmol/L), weak binding (EC_{50} 10–100 μmol/L), intermediate binding (EC_{50} 1–10 μmol/L), and high binding (EC_{50} <1 μmol/L). Figure 1 shows binding of the 28 tested candidate epitopes to HLA-DQ8*cis* and HLA-DQ8*trans*. Table 1 summarizes the EC_{50} values of the 28 tested peptides to all four HLA-DQ molecules.

Of the 28 nondeamidated epitopes, 7 (25%) showed binding to HLA-DQ8*cis,* and 2 of these 7 epitopes, EAEDLEVGEVELG (PPI) and NQQESTDAAVQEP (ICA-69), showed increased binding to HLA-DQ8cis upon deamidation. A total of 18 of 28 epitopes (64%) showed increased binding to HLA-DQ8*cis* upon deamidation, whereas no binding was observed with the native encoded epitopes. Of 28 epitopes, 2 (7%) did not bind HLA-DQ8*cis* after deamidation and were thus considered as false positives from the prediction studies with the combined HLA-DQ8*cis* binding motif and the tTG algorithm.

For HLA-DQ8*trans*, 3 of 28 (11) nondeamidated epitopes showed binding, for which only the PPI epitope EAEDLEVGEVELG binding was improved after deamidation. Upon deamidation, 22 of 28 epitopes (79%) bound to HLA-DQ8*trans*, whereas the same nondeamidated epitopes did not bind. Of the 28 the islet peptides, 6 (22%) bound preferentially to HLA-DQ8*tran*. Binding to the other three tested HLA-DQ molecules was absent or reduced 10-fold or more compared with HLA-DQ8*trans*. Similar percentages of false positives (7%) were observed as for HLA-DQ8*cis*.

To confirm correctness of the HLA-DQ8*cis*/*trans* epitope predictions for being specific HLA-DQ8*cis*/*trans* binders, we tested all 28 epitopes for binding to HLA-DQ2*cis* and HLA-DQ2*trans* (Table 1). Binding of the native PPI epitope was observed for HLA-DQ2*cis*, and improved binding was observed for 3 of 28 epitopes (11%) after deamidation, including the PPI epitope. For HLA-DQ2*trans*, 3 of 28 (11%) bound as native epitopes, whereas binding improved after deamidation for 4 of 28 epitopes (14%).

HLA-DQ8*cis* and HLA-DQ8*trans* only differ in their DQα-chain. We recently showed a dominant role of the DQβ-chain in peptide binding to HLA-DQ8*cis*/*trans* (20). Of the 28 tested peptide epitopes, 6 (22%) derived from PPI, phogrin, IA-2, and IGRP bound preferentially to HLA-DQ8*trans* after deamidation; binding to the other three HLA-DQ molecules was absent or reduced 10-fold or more.

In addition, 3 of 28 epitopes (11%) preferentially bound to HLA-DQ8*cis* upon deamidation. None of these

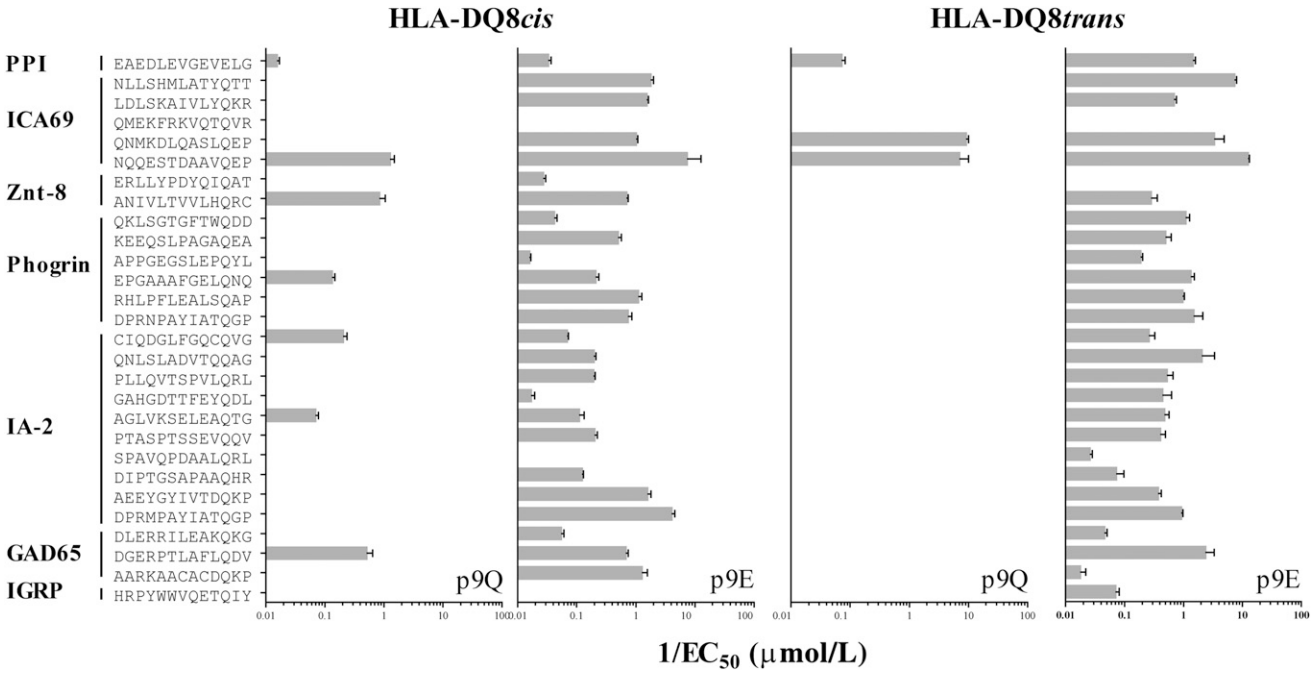

Figure 1—Binding of native and deamidated predicted HLA-DQ8*cis*/*trans* candidate epitopes to HLA-DQ8*cis* and HLA-DQ8*trans*. Prediction of HLA-DQ8*cis*/*trans* candidate epitopes within the top known diabetogenic proteins was performed with double mismatches at the anchor positions of the HLA-DQ8*cis*/*trans* binding motifs using our in-house algorithm program MOTIFS. Nondeamidated (Q) vs. deamidated (E) candidate epitopes were tested for binding HLA-DQ8*cis* and HLA-DQ8*trans* in competitive peptide-binding assays. EC_{50} values (μmol/L) were calculated on the basis of competition between the biotinylated indicator peptide and the test peptides. Data represent mean ± SEM (n = 3). Shown on the x-axis is $1/EC_{50}$, thereby illustrating that large bars represent better binding.

preferential binders showed binding to HLA-DQ2*cis*/*trans*. These results demonstrate improved binding to HLA-DQ8*cis* and HL-DQ8*trans* of predicted candidate epitopes after deamidation, and moreover, a subset of these predicted epitopes preferentially bind to HLA-DQ8*cis* or HLA-DQ8*trans*.

A Predicted Candidate Proinsulin Epitope Is Deamidated and Only Binds HLA-DQ8*cis* and HLA-DQ8*trans*

The islet autoantigen mostly investigated in T1D is PPI. Human CD4 and CD8 T cells have both been found to be reactive against PPI (5,7,10,25). During the screening studies, HLA-DQ8*cis*/*trans* candidate epitopes from PPI were not predicted to have a Q at position p9 (Supplementary Table 1). However, potential deamidation sites were observed at the anchor positions p4 and p7 in EDL**Q**VG**Q**VE (potential deamidation sites in bold). After examination of the PPI protein sequence, this candidate epitope was located in the InsB30-C13 sequence TRREAEPL**Q**VG**Q**VELG (potential p4 and p7 deamidation sites in bold) of proinsulin. Deamidation by tTG of this peptide was tested, and p4Q and p7Q residues in the predicted epitope (Table 1) were both deamidated (data not shown). However, we observed that the p4Q residue was not deamidated without having the p7Q residue deamidated initially.

Next, we tested binding of the InsB30-C13 peptides in the native form (QQ), the single-deamidated form (p7; QE), and the double-deamidated form (p4/p7; EE) to HLA-DQ2*cis*, HLA-DQ2*trans*, HLA-DQ8*trans*, and HLA-DQ8*cis* (Fig. 2). Binding of the InsB30-C13 peptide to all four HLA-DQ molecules was only observed after deamidation. Intriguingly, the highest binding of the InsB30-C13 peptide was observed for HLA-DQ8*trans* only after double deamidation (EE) (2.3 ± 0.3 μmol/L), although this was not significantly higher compared with the QE peptide (2.9 ± 1.0 μmol/L). The EE peptide bound to HLA-DQ2*cis* (61.1 ± 7.9 μmol/L), HLA-DQ2*trans* (57.1 ± 13.5 μmol/L), and HLA-DQ8*cis* (44.8 ± 1.9 μmol/L). The QE also bound to HLA-DQ8*cis* (43.9 ± 3.1 μmol/L). These data demonstrate that deamidation of the T1D prototype islet autoantigen PPI induces more potent HLA-DQ–specific binding.

Identification of the Minimal HLA-DQ–Binding Register for InsB30-C13

After examination of the InsB30-C13 peptide TRREAEDL**Q**VG**Q**VELG (potential deamidation sites in bold), two minimal binding registers for HLA-DQ8*cis*/*trans* were observed, namely, binding register 1: EAEDL**Q**VG**Q** and binding register 2: EDL**Q**VG**Q**VE (p1 anchor positions underlined). Binding of both registers was tested for all four HLA-DQ *cis*- and *trans*-dimers (Fig. 2). Whereas the nondeamidated register 1 peptide (QQ) did not bind any of the HLA-DQ dimers (EC_{50} >100 μmol/L), and only the EE peptide bound with only weak affinity to HLA-DQ2*trans* (31.6 ± 2.8 μmol/L), the

Table 1—Binding of native and deamidated predicted HLA-DQ8*cis*/*trans* candidate epitopes to all four HLA-DQ*cis*/*trans* molecules

		DQ2*cis*		DQ2*trans*		DQ8*trans*		DQ8*cis*	
Predicted epitope	Antigen	Q	E	Q	E	Q	E	Q	E
EAEDLQVG**Q**VELG	PPI	48.7 ± 9.8	30.6 ± 1.2	44.2 ± 4.8	36.0 ± 5.2	13.8 ± 2.3	**0.7 ± 0.1**	62.8 ± 5.6	29.7 ± 3.7
NLLSHMLATY**Q**TT	ICA69	—	—	—	—	—	0.7 ± 0.01	—	0.5 ± 0.1
LDLSKAIVLY**Q**KR	ICA69	—	—	—	—	—	1.4 ± 0.2	—	0.6 ± 0.1
QMEKFRKVQT**Q**VR	ICA69	—	8.2 ± 1.3	—	—	—	—	—	—
QNMKDLQASL**Q**EP	ICA69	—	—	—	—	0.2 ± 0.1	0.4 ± 0.2	—	1.0 ± 0.1
NQQESTDAAV**Q**EP	ICA69	—	—	4.0 ± 0.3	1.0 ± 0.3	0.2 ± 0.1	0.1 ± 0.01	0.8 ± 0.2	0.2 ± 0.1
ERLLYPDYQI**Q**AT	Znt-8	—	6.8 ± 1.2	—	—	—	—	—	**36.0 ± 3.8**
ANIVLTVVLH**Q**RC	Znt-8	—	—	12.0 ± 1.6	1.1 ± 0.7	—	3.6 ± 1.2	1.2 ± 0.4	1.4 ± 0.1
QKLSGTGFTW**Q**DD	Phogrin	—	—	—	—	—	**0.9 ± 0.2**	—	23.6 ± 2.9
KEEQSLPAGA**Q**EA	Phogrin	—	—	—	—	—	2.0 ± 0.6	—	2.0 ± 0.3
APPGEGSLEP**Q**YL	Phogrin	—	—	—	9.3 ± 1.9	—	5.2 ± 0.5	—	61.6 ± 2.1
EPGAAAFGEL**Q**NQ	Phogrin	—	—	—	—	—	**0.7 ± 0.1**	7.4 ± 0.8	4.7 ± 0.7
RHLPFLEALS**Q**AP	Phogrin	—	—	—	—	—	0.9 ± 0.1	—	1.9 ± 0.2
DPRNPAYIAT**Q**GP	Phogrin	—	—	—	—	—	0.8 ± 0.4	—	1.3 ± 0.3
CIQDGLFGQC**Q**VG	IA-2	—	—	—	—	—	4.0 ± 1.3	4.9 ± 0.9	14.2 ± 0.5
QNLSLADVTQ**Q**AG	IA-2	—	—	—	—	—	0.7 ± 0.5	—	5.0 ± 0.4
PLLQVTSPVL**Q**RL	IA-2	—	—	—	—	—	2.0 ± 0.7	—	5.1 ± 0.4
GAHGDTTFEY**Q**DL	IA-2	—	—	—	—	—	**2.7 ± 1.6**	—	58.5 ± 10.3
AGLVKSELEA**Q**TG	IA-2	—	—	—	—	—	2.1 ± 0.4	14.1 ± 1.8	9.2 ± 2.5
PTASPTSSEV**Q**QV	IA-2	—	—	—	—	—	2.5 ± 0.7	—	4.9 ± 0.5
SPAVQPDAAL**Q**RL	IA-2	—	—	—	—	—	**38.0 ± 4.2**	—	—
DIPTGSAPAA**Q**HR	IA-2	—	—	—	—	—	15.0 ± 6.8	—	7.9 ± 0.3
AEEYGYIVTD**Q**KP	IA-2	—	—	—	—	—	2.7 ± 0.4	—	0.6 ± 0.1
DPRMPAYIAT**Q**GP	IA-2	—	—	—	—	—	1.1 ± 0.1	—	0.2 ± 0.1
DLERRILEAK**Q**KG	GAD65	—	—	—	—	—	21.7 ± 2.9	—	17.9 ± 1.8
DGERPTLAFL**Q**DV	GAD65	—	—	—	—	—	0.5 ± 0.2	2.0 ± 0.7	1.4 ± 0.1
AARKAACACD**Q**KP	GAD65	—	—	—	—	—	58.1 ± 18.2	—	**0.8 ± 0.2**
HRPYWWVQET**Q**IY	IGRP	—	—	—	—	—	**14.1 ± 2.5**	—	—

Binding of the predicted HLA-DQ8*cis*/*trans* candidate epitopes was tested for four HLA-DQ molecules in the competitive peptide-binding assays. Anchor positions in bold are the positions tested for binding as nondeamidated and deamidated by synthesis of the peptides with Q or E. EC_{50} values were calculated based on the observed binding of the tested peptides against the fixed concentration indicator peptide; and values are shown for the EC_{50} required for binding of the reporter peptide. Preferential HLA-DQ8*cis* or HLA-DQ8*trans* binders are shown with EC_{50} values in bold.

single-deamidated (QE) and the double-deamidated (EE) register 2 peptides showed improved binding to all four HLA-DQ dimers. High-affinity binding of the deamidated register 2 peptide (EE) was observed for HLA-DQ8*trans* (0.7 ± 0.1 μmol/L). In our previous experiments with InsB30-C13, we could not observe binding of the native InsB30-C13 peptide, whereas the native register 2 peptide present in InsB30-C13 bound to all four HLA-DQ molecules. We propose that the InsB30-C13 peptide is not presented properly by HLA-DQ due to its length, whereas the tested register 2 peptide EDLQVGQVE already has the appropriate length for binding HLA-DQ molecules. These results demonstrate that the minimal binding register 2, EDL**Q**VG**Q**VE, is responsible for HLA-DQ–specific binding of deamidated proinsulin peptide InsB30-C13.

CD4 T Cells Reactive Against Deamidated InsB30-C13 Occur in a New-Onset T1D Patient

InsB30-C13–specific T-cell lines were isolated from an HLA-DQ8 homozygous new-onset T1D patient upon restimulation of isolated PBMCs that proved HLA-DQ8 was restricted (Fig. 3). Although we were not able to generate T-cell lines reactive against the nondeamidated QQ line (not shown) or the single-deamidated QE line, a T-cell line against the double-deamidated EE line was

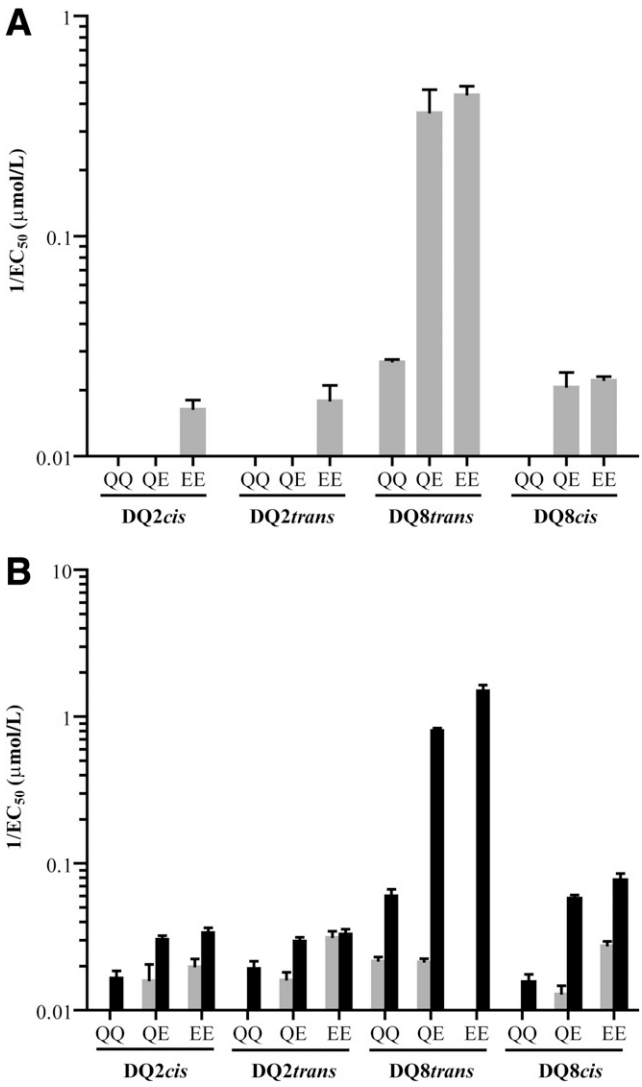

Figure 2—Binding of native and deamidated InsB30-C13 to all four HLA-DQ*cis* and HLA-DQ*trans* molecules. Deamidation of InsB30-C13 was confirmed by deamidation studies with tTG. InsB30-C13 peptides were synthesized as nondeamidated (QQ), single-deamidated (QE), and deamidated (EE). *A*: Binding of the QQ, QE, and EE peptides was tested for HLA-DQ2*cis*, HLA-DQ2*trans*, HLA-DQ8*trans*, and HLA-DQ8*cis*. *B*: Binding of the two InsB30-C13 minimal binding registers, EAEDL**Q**VG**Q** (gray bars) and EDL**Q**VG**Q**VE (black bars), was tested for binding HLA-DQ2*cis*, HLA-DQ2*trans*, HLA-DQ8*trans*, and HLA-DQ8*cis*. Both minimal binding registers were tested as native (QQ) or single- and double-deamidated (QE and EE) peptides. Data represent mean ± SEM (n = 3). Shown on the y-axes is $1/EC_{50}$, thereby illustrating that large bars represent better binding.

generated. The proliferation of the EE line was HLA-DQ–specific, because a blocking anti–HLA-DQ antibody could fully abrogate T-cell proliferation. Intriguingly, this EE line proved also to react against native (nondeamidated) and single-deamidated InsB30-C13. These results show that CD4 T cells against modified PPI exist in a T1D patient. Such T cells can be cross-reactive against non-modified islet autoantigens upon activation by a deamidated peptide.

T Cells to Native and Modified Proinsulin in T1D Patients and Healthy Control Subjects

We examined proliferative responses in fresh peripheral blood of newly diagnosed T1D patients and HLA-DQ– and age-matched healthy donors against native (Q) and modified (E) proinsulin epitopes (InsB30-C13; Fig. 4, Table 2, and Supplementary Table 3). Collectively, T-cell responses could be observed against proinsulin epitopes ex vivo in 15 of 22 T1D patients (68%) versus 7 of 19 healthy donors (37%). Most of the responding patients (11 of 15 [73%]) produced IFN-γ, whereas responses in healthy donors were dominated by IL-10 (5 of 7 [71%]). Two patients responded to the native epitope only (by IFN-γ) versus none of the healthy donors. The response rate of T cells from recent-onset T1D patients extended from 41% (9 of 22) to native proinsulin epitope to 68% (15 of 22) adding responses to modified epitopes versus 21% (4 of 19) and 37% (7 of 19), respectively, in healthy donors. Thirty-two percent (7 of 22) of patients and 21% (4 of 19) of healthy donors responded to both native and modified proinsulin epitopes.

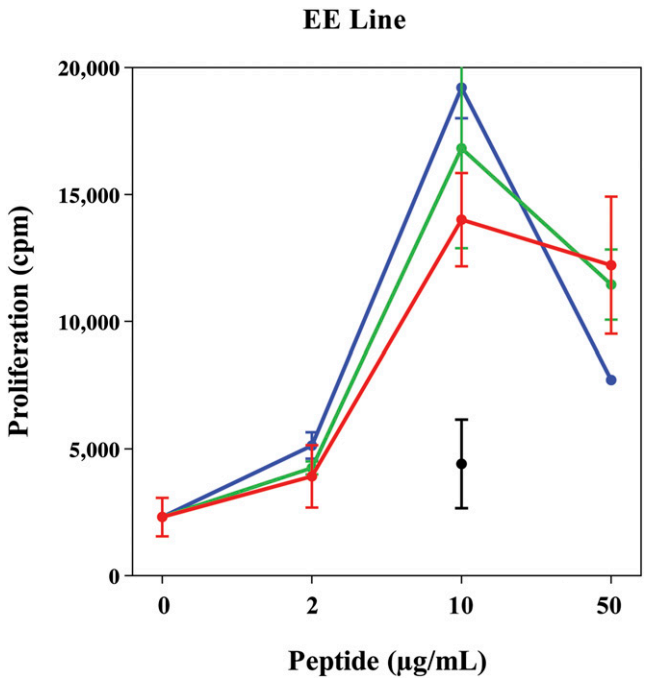

Figure 3—CD4 T cells reactive against deamidated InsB30-C13 are found in a new-onset T1D patient. InsB30-C13 reactive CD4 T cells are found in isolated PBMCs from a new-onset HLA-DQ8 homozygous T1D patient. T-cell lines reactive against native (QQ line; not shown) or single-deamidated InsB30-C13 (QE line; not shown) could not be generated. A T-cell line reactive against InsB30-C13 could only be generated with the double-deamidated InsB30-C13 peptide (EE line). The response was HLA-DQ restricted, because a blocking anti–HLA-DQ antibody at a concentration of 10 μg/mL (black dot) could fully abrogate proliferation of the EE line. The EE line is cross-reactive against nondeamidated (QQ) and single-deamidated (QE) InsB30-C13. Proliferation is shown as mean ± SEM counts per minute (cpm). QQ peptide, blue; QE peptide, green; EE peptide, red.

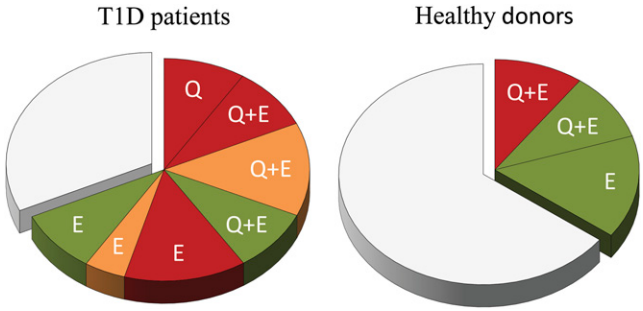

Figure 4—Distribution of immune responses to native and PTM proinsulin epitopes. Native (Q) and modified (E) proinsulin epitopes were tested for immune responses in 22 newly diagnosed patients and in 19 healthy donors matched for age and high-risk HLA. The quality of the response is indicated as proinflammatory (IFN-γ, red), regulatory (IL-10, green), or a combination of both (orange). Q, patients responding to native epitope only; Q+E, patients responding to both native and modified epitopes; E, patients responding to modified epitopes only. Responding case patients are calculated as percentages of the total case patients tested.

The polarized inverse relation between case subjects and control subjects was similar to that previously reported for other islet epitopes (6). Although our cohort remains insufficiently sized to draw firm conclusions regarding the influence of HLA status on the presence of CD4 T-cell responses, our data demonstrate that detecting T-cell autoreactivity to PTM proinsulin increases the response rate compared with responsiveness to native proinsulin alone. Furthermore, both the quality and prevalence of these T-cell responses differ between recent onset T1D patients and healthy donors.

DISCUSSION

Here, we demonstrate that PTM of islet autoantigens by tTG can improve their potency to bind to HLA-DQ, predisposing to T1D. Deamidation of islet antigens by tTG created potent HLA-DQ–specific binders. Indeed, CD4 T cells reactive against modified PPI could be identified in the blood of a T1D patient that cross-reacted with native PPI. Additional T-cell responses were observed upon deamidation of InsB30-C13 in new-onset patients that differ in quality and quantity from those in healthy donors. This demonstrates that deamidation of autoantigens might act as an initiating event for the induction of pathogenic T cells, which can subsequently be activated by nonmodified autoantigens. We thus provide a functional mechanism by which PTM of human islet autoantigens is involved in the pathogenesis of T1D through the generation of neoautoantigens and subsequent CD4 T-cell responses against native antigens, leading to epitope spreading. Our important observations rank T1D among autoimmune diseases in which PTMs occur that may lead to autoimmunity. In two other diseases, rheumatoid arthritis and CD, this novel insight

Table 2—Cytokine responses of T1D patients and healthy control subjects to InsB30-C13 epitopes

	Cytokine	QQ	QE	EE	HLA-DQ
TID patient #					
1	IFN-γ	4.3	<3	<3	DQ2/8
	IL-10	<3	<3	<3	
2	IFN-γ	<3	4	5	DQ2/2
	IL-10	<3	<3	<3	
3	IFN-γ	<3	4	<3	DQ5/8
	IL-10	<3	<3	<3	
4	IFN-γ	<3	<3	<3	DQ2/8
	IL-10	<3	<3	<3	
5	IFN-γ	<3	5	<3	DQ8
	IL-10	<3	<3	3	
6	IFN-γ	<3	<3	<3	DQ2/8
	IL-10	<3	<3	<3	
7	IFN-γ	<3	<3	<3	DQ2/5
	IL-10	<3	<3	3	
8	IFN-γ	4	<3	<3	DQ2/5
	IL-10	<3	<3	<3	
9	IFN-γ	<3	<3	<3	DQ2/2
	IL-10	3	<3	4	
10	IFN-γ	3	4	<3	DQ2/8
	IL-10	<3	<3	<3	
11	IFN-γ	<3	<3	<3	DQ2/2
	IL-10	<3	<3	<3	
12	IFN-γ	<3	4	<3	DQ5/8
	IL-10	<3	<3	<3	
13	IFN-γ	3	<3	6	DQ7/8
	IL-10	<3	4	<3	
14	IFN-γ	<3	3	<3	DQ2/8
	IL-10	8	5	4	
15	IFN-γ	<3	<3	<3	DQ2/8
	IL-10	<3	<3	<3	
16	IFN-γ	<3	<3	<3	DQ2/5
	IL-10	<3	<3	<3	
17	IFN-γ	<3	<3	<3	DQ2.2/7
	IL-10	<3	<3	<3	
18	IFN-γ	<3	<3	<3	DQ7/9
	IL-10	7	5	5	
19	IFN-γ	<3	<3	<3	DQ2/8
	IL-10	<3	4	<3	
20	IFN-γ	3	<3	4	DQ8/9
	IL-10	<3	<3	<3	
21	IFN-γ	5	<3	<3	DQ2
	IL-10	<3	4	<3	
22	IFN-γ	<3	<3	<3	N/A
	IL-10	<3	<3	<3	
HC #					
1	IFN-γ	<3	<3	<3	DQ5/8
	IL-10	<3	<3	4	
2	IFN-γ	<3	<3	<3	DQ2/7
	IL-10	4	5	8	
3	IFN-γ	<3	<3	<3	DQ7
	IL-10	<3	<3	<3	

Continued on p. 245

Table 2 — ***Continued***

	Cytokine	QQ	QE	EE	HLA-DQ
4	IFN-γ	<3	<3	<3	DQ2/5
	IL-10	<3	<3	3.3	
5	IFN-γ	<3	<3	<3	DQ5/8
	IL-10	<3	<3	<3	
6	IFN-γ	3	3	6	DQ2/8
	IL-10	<3	<3	<3	
7	IFN-γ	4	4	<3	DQ8
	IL-10	<3	<3	<3	
8	IFN-γ	<3	<3	<3	DQ2/7
	IL-10	<3	<3	<3	
9	IFN-γ	<3	<3	<3	DQ2/6
	IL-10	<3	<3	<3	
10	IFN-γ	<3	<3	<3	DQ2
	IL-10	<3	<3	<3	
11	IFN-γ	<3	<3	<3	DQ2/8
	IL-10	<3	<3	<3	
12	IFN-γ	<3	<3	<3	DQ2
	IL-10	<3	<3	<3	
13	IFN-γ	<3	<3	<3	DQ2
	IL-10	<3	<3	<3	
14	IFN-γ	<3	<3	<3	DQ8
	IL-10	<3	<3	<3	
15	IFN-γ	<3	<3	<3	DQ2/8
	IL-10	<3	<3	<3	
16	IFN-γ	<3	<3	<3	DQ8
	IL-10	5.1	3.1	<3	
17	IFN-γ	<3	<3	<3	DQ2
	IL-10	<3	<3	<3	
18	IFN-γ	<3	<3	<3	DQ8
	IL-10	<3	<3	<3	
19	IFN-γ	<3	<3	<3	DQ2
	IL-10	<3	3.1	<3	

HC, healthy control; N/A, not available. PBMCs were freshly isolated from new-onset T1D patients and healthy control subjects matched for HLA-DQ and age. PBMCs were incubated with peptide or diluent alone for 48 h, after which IFN-γ and IL-10 were measured using the ELISPOT. Stimulation index results >3 were considered positive.

has already been proven seminal to understanding mechanisms of disease, and in both diseases, this new insight has led to the design of new immune intervention strategies.

Using HLA-DQ8*cis* and HLA-DQ8*trans* peptide-binding motifs combined with the tTG algorithms (21), we identified HLA-DQ–specific candidate T-cell epitopes that are deamidated by tTG and become more potent HLA-DQ binders, with a subset of candidate epitopes preferentially binding to HLA-DQ8*trans*, the molecule most strongly associated with susceptibility to T1D. Only a few candidate epitopes bound to HLA-DQ2*cis* and HLA-DQ2*trans* after deamidation. Because PPI is a prototype islet autoantigen in T1D, we examined its protein sequence in detail and showed that the proinsulin peptide InsB30-C13 could deamidated, generating more potent HLA-DQ binders. The highest binding was observed for HLA-DQ8*trans*, which is suggested to be highly associated in HLA-DQ2/8 heterozygous individuals (13,15). These data underscore that modification of the protein by deamidation, creating negative charges that are prerequisite for HLA-DQ binding, creates genuine islet autoepitopes that are recognized by T1D patient–derived autoreactive T cells.

There is increasing evidence that pathogenic T cells in autoimmune disease recognize epitopes that are formed by PTM of self-antigens (26). For several inflammatory diseases in humans, such as rheumatoid arthritis and CD, PTM has been shown to induce potent HLA-DQ binders and induction of CD4 T-cell responses (21,27,28). Very recently, pathogenic CD4 T cells, reactive against modified chromogranin A, have been shown in NOD mice (29). Yet, there is only one example of PTM in human autoimmune diabetes, which was nonenzymatic (12). HLA-DQ8–restricted human autoreactive T-cell reagents are extremely rare and difficult to maintain. We are aware of only one other HLA-DQ8–restricted T-cell clone against insulin (9). Here, we provide the first proof that human islet autoantigens can undergo PTM by enzymatic modification, creating potent binders for the human disease–predisposing HLA-DQ8*cis* and HLA-DQ8*trans* molecules. Indeed, HLA-DQ8*cis*–restricted CD4 T-cell lines reactive against modified islet autoantigens (InsB30-C13) could be generated from the HLA-DQ8 homozygous T1D patient. Although the single-deamidated InsB30-C13 peptide binds with similar affinity to HLA-DQ8*cis* as the double-deamidated peptide, T-cell lines against the QE peptide could not be propagated from this particular HLA-DQ8 homozygous T1D patient. This phenomenon, although in one patient, can be caused by differences in avidity between the TCR and the peptide–HLA-DQ complex: low-avidity and high-avidity CD4 T cells. Low-avidity T cells can be undetectable in peripheral blood from T1D patients, despite the binding strength between the peptide and HLA-DQ. Of course, that we were unable to raise a T-cell line against a peptides does not exclude that T cells with such specificity exist, because this inability may result from the technical challenges associated with detecting and isolating peptide-specific autoreactive T cells.

One of the events inducing differences in PTM in β-cells is disruption of homeostasis of the endoplasmic reticulum (ER) in β-cells leads to cell death and contributes to T1D pathogenesis (30–32). Upon ER stress, proteins are misfolded or modified, changing the structure of the protein. In the context of T1D, this may generate new islet (neo)autoantigens (33). tTG is a transamidating acyltransferase that catalyzes Ca^{2+}-dependent protein modifications, is expressed in many somatic cells, and is generally considered to be a soluble cytoplasmic protein (34). There are reports

in favor of tTG being able to modify proinsulin in vivo and creating antigenic insulin peptides. Gene and protein expression of tTG has been found in human pancreatic cancer cell lines (35,36). Electron microscopy has shown that tTG is localized close to insulin and glucagon granules in human pancreatic islets (37,38), is expressed in antigen-presenting cells (39), that this tTG is active (40), and that various stimuli or insults that induce cell stress (e.g., inflammation) are able to activate tTG (41). Although deamidated proinsulin has not been detected in human islets by MS (42,43), it is conceivable that calcium levels change during ER stress in β-cells and T-cell death, creating the conditions required for tTG activation and resulting in the modification of islet autoantigens.

After the discovery of T cells to chemically modified proinsulin in 2005 (12), our findings are the first to demonstrate PTM in T1D after enzymatic modification of islet autoantigens associated with T-cell autoimmunity in T1D patients that differs in quality and quantity from those in healthy donors. Although antigen cross-reactivity and epitope-spreading is a generally appreciated inherent feature of T cells, and indeed, many responders reacted to both native and modified proinsulin epitope, 27% of patients and 16% of healthy donors responded to modified proinsulin exclusively. Such reactivity would seem particularly relevant in T1D patients, where stress and, subsequently, tTG activity are believed to be increased, thereby creating a substrate for these T cells.

In conclusion, PTM of islet autoantigens provides a mechanism by which pathogenic T cells can escape thymic deletion, and when encountering a modified islet (neo)autoantigen bound to HLA-DQ8*cis* or HLA-DQ8*trans* in the periphery, such T cells are activated, amplifying the immune response (33).

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