

# Recognition of HLA Class I–Restricted $\beta$ -Cell Epitopes in Type 1 Diabetes

Qin Ouyang,<sup>1</sup> Nathan E. Standifer,<sup>2</sup> Huilian Qin,<sup>1</sup> Peter Gottlieb,<sup>3</sup> C. Bruce Verchere,<sup>1</sup> Gerald T. Nepom,<sup>2</sup> Rusung Tan,<sup>1</sup> and Constadina Panagiotopoulos<sup>4</sup>

**Type 1 diabetes results from the autoimmune destruction of insulin-producing pancreatic  $\beta$ -cells by cytotoxic T-lymphocytes (CTLs). In humans, few  $\beta$ -cell epitopes have been reported, thereby limiting the study of  $\beta$ -cell-specific CTLs in type 1 diabetes. To identify additional epitopes, HLA class I peptide affinity algorithms were used to identify a panel of peptides derived from the  $\beta$ -cell proteins islet amyloid polypeptide (IAPP), islet-specific glucose-6-phosphatase catalytic subunit-related protein (IGRP), insulin, insulinoma-associated antigen 2 (IA-2), and phogrin that were predicted to bind HLA-A\*0201. Peripheral blood mononuclear cells from 24 HLA-A\*0201 recent-onset type 1 diabetic patients and 11 nondiabetic control subjects were evaluated for  $\gamma$ -interferon secretion in response to peptide stimulation in enzyme-linked immunospot assays. We identified peptides IAPP9-17, IGRP215-223, IGRP152-160, islet IA-2(172-180), and IA-2(482-490) as novel HLA-A\*0201-restricted T-cell epitopes in type 1 diabetic patients. Interestingly, we observed a strong inverse correlation between the binding affinity of  $\beta$ -cell peptides to HLA-A\*0201 and CTL responses against those peptides in recent-onset type 1 diabetic patients. In addition, we found that self-reactive CTLs with specificity for an insulin peptide are frequently present in healthy individuals. These data suggest that many  $\beta$ -cell epitopes are recognized by CTLs in recent-onset type 1 diabetic patients. These epitopes may be important in the pathogenesis of type 1 diabetes. *Diabetes* 55:3068–3074, 2006**

From the <sup>1</sup>Department of Pathology and Laboratory Medicine, Child and Family Research Institute, University of British Columbia and British Columbia Children's Hospital, Vancouver, Canada; the <sup>2</sup>Benaroya Research Institute at Virginia Mason, Seattle, Washington; the <sup>3</sup>Barbara Davis Center for Childhood Diabetes, University of Colorado Health Sciences Center, Denver, Colorado; and the <sup>4</sup>Department of Pediatrics, Child and Family Research Institute, University of British Columbia and British Columbia Children's Hospital, Vancouver, Canada.

Address correspondence and reprint requests to Dr. Rusung Tan, Department of Pathology and Laboratory Medicine, Child and Family Research Institute, University of British Columbia and British Columbia Children's Hospital, 4480 Oak St., Vancouver, British Columbia V6H 3V4, Canada. E-mail: roo@interchange.ubc.ca.

Received for publication 13 January 2006 and accepted in revised form 25 July 2006.

R.T. and C.P. contributed equally to this study.

BIMAS, BioInformatics and Molecular Analysis Section; CMV, cytomegalovirus; CTL, cytotoxic T-lymphocyte; EBV, Epstein-Barr virus; ELISpot, enzyme-linked immunospot; FITC, fluorescein isothiocyanate; HCV, hepatitis C virus; IA-2, insulinoma-associated antigen 2; IAPP, islet amyloid polypeptide; IFN- $\gamma$ ,  $\gamma$ -interferon; IGRP, islet-specific glucose-6-phosphatase catalytic subunit-related protein; MHC, major histocompatibility complex; PBMC, peripheral blood mononuclear cell.

DOI: 10.2337/db06-0065

© 2006 by the American Diabetes Association.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

**T**ype 1 diabetes arises from the selective destruction of pancreatic islet  $\beta$ -cells through a coordinated effort by T-cells of the immune system (1). Although both CD4<sup>+</sup> and CD8<sup>+</sup> T-cells are necessary for diabetes onset, cytotoxic T-lymphocytes (CTLs) are considered to be the major contributors to  $\beta$ -cell destruction (2,3). Self-reactive CTLs recognize short peptides displayed on the  $\beta$ -cell surface in the context of HLA class I molecules, and upon T-cell receptor activation, they induce  $\beta$ -cell apoptosis.

Despite the essential role of T-cells during diabetes progression, prediction of disease in humans has been based primarily on the presence of circulating autoantibodies to  $\beta$ -cell antigens, including insulin, GAD, and the protein tyrosine phosphatase insulinoma-associated antigen 2 (IA-2) (4,5). Because  $\beta$ -cell damage, precipitated by CTL attack, is likely to precede the development of a humoral response, we hypothesize that  $\beta$ -cell-specific CTLs may appear before autoantibodies are detectable and thus may provide an earlier and more accurate means of predicting disease development. Indeed, strategies to predict or prevent diabetes in NOD mice have been successfully predicated on identifying and modifying the  $\beta$ -cell-specific CTL response (6,7). The identification of HLA class I-restricted  $\beta$ -cell epitopes would enable the testing of similar strategies in humans.

To date, a limited number of HLA class I epitopes have been reported. The first two, GAD114-123 (8) and insulin B chain 22-30 (9), were chosen for study based on homology to known NOD CD4<sup>+</sup> T-cell epitopes and then confirmed by culturing and expanding CD8<sup>+</sup> T-cells in vitro from the peripheral blood of HLA-A\*0201 and HLA-A\*2402 type 1 diabetic patients, respectively. A limitation of in vitro stimulation and expansion is that the true in vivo frequency of peptide-specific CTLs cannot be determined. Using computer-based binding algorithms, we identified preproIAPP5-13 as an HLA-A\*0201 epitope recognized by CTLs in individuals with recent-onset type 1 diabetes (10). In addition, Toma et al. (11) recently reported three peptide epitopes located in the proinsulin region that were predicted by proteasomal cleavage databases. In both of the latter studies, peripheral blood mononuclear cells (PBMCs) were tested directly ex vivo for their ability to recognize peptide, using  $\gamma$ -interferon (IFN- $\gamma$ ) enzyme-linked immunospot (ELISpot) assays.

In the current study, we used computer-based algorithms to predict candidate peptide epitopes from islet amyloid polypeptide (IAPP), islet-specific glucose-6-phosphatase catalytic subunit-related protein (IGRP), insulin,

IA-2, and IA-2 $\beta$  (phogrin). After determining HLA binding, we assessed IFN- $\gamma$  secretion by PBMCs to a panel of 11 peptides in patients with recent-onset type 1 diabetes. Here, we report the identification of five additional HLA-A\*0201-restricted  $\beta$ -cell epitopes derived from IAPP, IGRP, and IA-2.

## RESEARCH DESIGN AND METHODS

Peripheral blood samples were collected from patients with recent-onset type 1 diabetes (disease duration 1–182 days) as well as from healthy HLA-A\*0201 control subjects with no family history of type 1 diabetes. PBMCs were isolated by density gradient centrifugation, using Ficoll-Paque PLUS (Amersham Bioscience, Sweden), and cryopreserved in 10% dimethylsulphoxide, 40% FCS, and 50% RPMI. HLA-A\*0201-positive patients and control subjects were identified by flow cytometry (FACSCalibur; Becton Dickinson, San Diego, CA) using an aliquot of the cells stained with fluorescein isothiocyanate (FITC)-conjugated anti-HLA-A2 mAb (BB7.2; Pharmingen, San Diego, CA). HLA typing was performed using PEL-FREEZ HLA A/B/C kits (Clinical Systems, Milwaukee). A total of 24 HLA-A\*0201-positive (age [mean  $\pm$  SD] 9.9  $\pm$  5.1 years, range 1.4–17.7 years, 54% female) and 5 HLA-A\*0201-negative (10.2  $\pm$  5.7, 4.4–16.4, 60%) type 1 diabetic patients as well as 11 HLA-A\*0201 nondiabetic control subjects (24.3  $\pm$  14.6, 7.3–44, 54.5%) were enrolled in the study. The study protocol was approved by the clinical research ethics board of the University of British Columbia. Parents of all participants provided written informed consent, and patients provided written assent.

**Peptides.** Putative HLA-A\*0201 CD8<sup>+</sup> T-cell epitopes were predicted using the online programs SYFPEITHI (available at <http://syfpeithi.bmi-heidelberg.com>) (12,13) and National Institutes of Health, Center for Information Technology, Bioinformatics and Molecular Analysis Section (BIMAS) HLA Peptide Binding Predictions (available at [http://thr.cit.nih.gov/molbio/hla\\_bind/](http://thr.cit.nih.gov/molbio/hla_bind/)) (14). HLA-A\*0201 control peptides used were the nucleocapsid epitope of hepatitis C virus (HCV; negative control, DLMGYPLV) and a mix of immunodominant positive control viral peptides from influenza A matrix protein (GILGFVFTL), Epstein-Barr virus (EBV), EBV BMLF1 lytic cycle antigen (GLCTLVAML), and cytomegalovirus (CMV; NLVPMVATV). A peptide known to bind to HLA-B\*0801 but not HLA-A\*0201 (EBV BZLF1 antigen, RAKFKQLL) was used as a negative control. Predicted  $\beta$ -cell peptides and control peptides were synthesized by NAPS (the Nucleic Acid Protein Service) at the University of British Columbia, using automated fluorenylmethoxycarbonyl chemistry (432A; Applied Biosystems), and purified by reverse-phase high-performance liquid chromatography. Analytical high-performance liquid chromatography tracing and mass spectrometry (MALDI-TOF) were performed for quality control.

**Peptide binding assays.** The ability of peptides to bind HLA-A\*0201 was confirmed by cell membrane stabilization of the HLA-A2 molecule in TAP (transporter associated with antigen processing)-deficient 174xCEM.T2 cells (15). Briefly, T2 cells were loaded with 50  $\mu$ g/ml peptide during an overnight incubation at room temperature in the presence of 3  $\mu$ g/ml  $\beta_2$ m (Sigma-Aldrich, Oakville, ON, Canada) in serum-free medium (X-VIVO 10; BioWhittaker, Walkersville, MD) and then washed and stained with FITC-conjugated anti-HLA-A2 monoclonal antibody (BB7.2; Pharmingen). The surface HLA-A2 expression was measured by flow cytometry (FACSCalibur), and the mean fluorescence intensity was recorded. The high-affinity immunodominant HLA-A2 CMV peptide (14,16) was used as a positive control. Results of  $\beta$ -cell peptide binding to HLA-A\*0201 are expressed as: percentage relative binding of the CMV peptide to HLA-A\*0201 =  $100 \times [(MFI \text{ with given peptide} - MFI \text{ without peptide}) / (MFI \text{ with CMV/A2 peptide} - MFI \text{ without peptide})]$ , where MFI is the mean fluorescence intensity.

The temporal stability of peptide/HLA-A\*0201 complexes was assessed as previously described (15). Briefly, T2 cells were cultured with synthetic peptides overnight at room temperature as performed for peptide binding assay. The following day, after removing peptide and adding emetine (10<sup>-4</sup> mol/l; Sigma-Aldrich) to block protein synthesis, cells were incubated at 37°C for the indicated time periods. At each time point, an aliquot of cells was washed and stained with FITC-conjugated anti-HLA-A2 monoclonal antibody (BB7.2). Surface HLA-A2 expression was assessed by flow cytometry (FACSCalibur), and mean fluorescence intensity was recorded. The CMV peptide forms highly stable complexes with HLA-A\*0201 and was used as a positive control. Results are expressed as: relative complex stability =  $100 \times [(MFI \text{ with given peptide} - MFI \text{ without peptide}) / (MFI \text{ with CMV peptide} - MFI \text{ without peptide})]$ , where MFI is the mean fluorescence intensity.

**ELISpot assays.** IFN- $\gamma$  ELISpot assays were performed as previously described (17). We precoated 96-well MAHA S4510 plates (Millipore, Bedford, MA) with anti-IFN- $\gamma$  monoclonal antibody 1-DIK (Mabtech, Mariemont, OH) and blocked with complete medium containing FCS. Thawed PBMCs were

added to wells in triplicate ( $2 \times 10^5$  cells per well) and incubated for 24 h in the absence or presence of peptide or phytohemagglutinin (Invitrogen). IFN- $\gamma$  secretion was detected with a second biotin-conjugated anti-IFN- $\gamma$  monoclonal antibody (7-B6-1; Mabtech) followed by the addition of streptavidin-alkaline phosphatase (Mabtech). Spots were developed using an alkaline phosphatase-conjugated substrate kit (Bio-Rad Laboratories, Hercules, CA), counted by an automated ELISpot reader (Bio-Sys, Karben, Germany) and expressed as number of spots per  $2 \times 10^5$  PBMCs after subtraction of the background values without peptide. The intra- and interassay coefficients of variation (CVs) were calculated as: SD/(mean  $\times$  100). For intra-assay CVs, we used the differences between triplicates of the number of IFN- $\gamma$ -producing spots to calculate the CV. The intra-assay CVs calculated by analysis of 15 triplicates of two samples in one ELISpot plate performed in one assay were 10.7 and 11.3%, respectively. The interassay CVs determined from the mean of triplicate IFN- $\gamma$ -producing spots of three samples in three independent ELISpot assays were 10.8, 11.7, and 15.15%, respectively.

**Statistical analysis.** Prism 4 for Macintosh (Graphpad Software, San Diego, CA) was used for statistical analysis. Relationships between the binding ability of  $\beta$ -cell peptides to HLA-A\*0201 and CTL responses to  $\beta$ -cell peptides in ELISpot assays were determined using paired Student's *t*-tests for the correlation coefficient. *P* values <0.05 were considered significant.

## RESULTS

**$\beta$ -Cell peptide binding to HLA-A\*0201.** Five  $\beta$ -cell proteins and candidate autoantigens in type 1 diabetes, including IAPP, IGRP, insulin, IA-2, and IA-2 $\beta$  (phogrin), were analyzed using SYFPEITHI and BIMAS algorithms to identify 21 HLA-A\*0201 candidate peptide epitopes for screening (Table 1). A threshold of 60 (BIMAS) or 20 (SYFPEITHI) resulted in a short list of the top 2% of peptides generated by both algorithms. The top 2% of peptides produced by the SYFPEITHI algorithm is thought to generate an 80% probability of identifying a naturally presented epitope (<http://www.syfpeithi.de/Scripts/MHC-Server.dll/Info.htm#scores>). To determine whether the candidate peptides were able to bind HLA-A\*0201 in vitro, the level of HLA-A\*0201 surface expression on T2 cells after the addition of exogenous peptides was measured (15). Given that the greatest stability of HLA-A2, correlating with the highest affinity peptide binding, was observed using the CMV peptide, this binding level was set at 100% with all other peptides expressed relative to this level. As shown in Fig. 1C, insulin2, insulinB10, IA-2(172), IA-2(180), IA-2(482), phogrin331, and the control peptide HCV stabilized HLA-A2 expression at levels >80%. Peptides IAPP5, IAPP9, IGRP152, IGRP215, IGRP293, IA-2(277), IA-2(341), IA-2(359), IA-2(577), phogrin11, phogrin335, phogrin387, and phogrin893 resulted in intermediate (40–80%) expression of HLA-A2, whereas insulin C6, phogrin7, and the negative control peptide EBV/B8 bound poorly to HLA-A\*0201 ( $\leq$ 40%). The relative binding affinities of  $\beta$ -cell peptides to HLA-A\*0201 did not correlate with the binding affinities predicted by SYFPEITHI ( $r = 0.227$ ) and BIMAS ( $r = 0.034$ ) (data not shown).

**Dissociation rate of  $\beta$ -cell peptides from HLA-A\*0201.** Although at nonphysiological temperatures ( $\leq$ 23°C), high-affinity peptides stabilize HLA-A2 levels on the surface of T2 cells, previous literature suggests that these peptide/HLA complexes may dissociate at physiological temperatures (37°C) (18,19). Therefore, we assessed the stability of complexes formed with the candidate peptide epitopes and HLA-A2 on T2 cells over a 4-h period at 37°C. After peptide removal and addition of emetine to inhibit protein synthesis, T2 cells were cultured at 37°C, and HLA-A2 expression was determined at different incubation times using anti-HLA-A2 antibody. The stability of the various peptide/HLA-A2 complexes were then normalized relative to that observed for CMV/HLA-A2 complexes

TABLE 1  
β-Cell peptides predicted to bind HLA-A\*0201

Peptide	Protein	Position	Sequence	SYFPEITHI	BIMAS
IAPP5	IAPP	5–13	KLQVFLIVL	26	268
IAPP9	IAPP	9–17	FLIVLSVAL	27	98
IGRP152	IGRP	152–160	FLWSVFMLI	20	5676
IGRP215	IGRP	215–223	FLFAVGFYL	22	11598
IGRP293	IGRP	293–301	RLLCALTSL	28	182
Insulin2	Insulin	2–10	ALWMRLPL	28	408
InsulinB10	Insulin	B10–B18	HLVEALYLV	27	22.3
InsulinC6	Insulin	C6–C14	DLQVGQVEL	25	1.6
IA-2(172)	IA-2	172–180	SLSPLQAEI	29	21
IA-2(180)	IA-2	180–188	LLPPLLEHL	29	41
IA-2(277)	IA-2	277–285	GLLYLAQEL	25	79
IA-2(341)	IA-2	341–349	VLGYGVEL	30	36.3
IA-2(359)	IA-2	359–367	TLTLLQLL	27	182
IA-2(482)	IA-2	482–490	SLAAGVKLL	29	49
IA-2(577)	IA-2	577–585	VLLTLVALA	24	72
Phogrin7	Phogrin	7–15	LLLLLLLLL	30	309
Phogrin11	Phogrin	11–19	LLLLLPPRV	26	437
Phogrin331	Phogrin	331–339	GMAELMAGL	27	146
Phogrin335	Phogrin	335–343	LMAGLMQGV	26	196
Phogrin387	Phogrin	387–395	RLYQEVHRL	26	157
Phogrin893	Phogrin	893–901	SLLDFRRKV	28	802

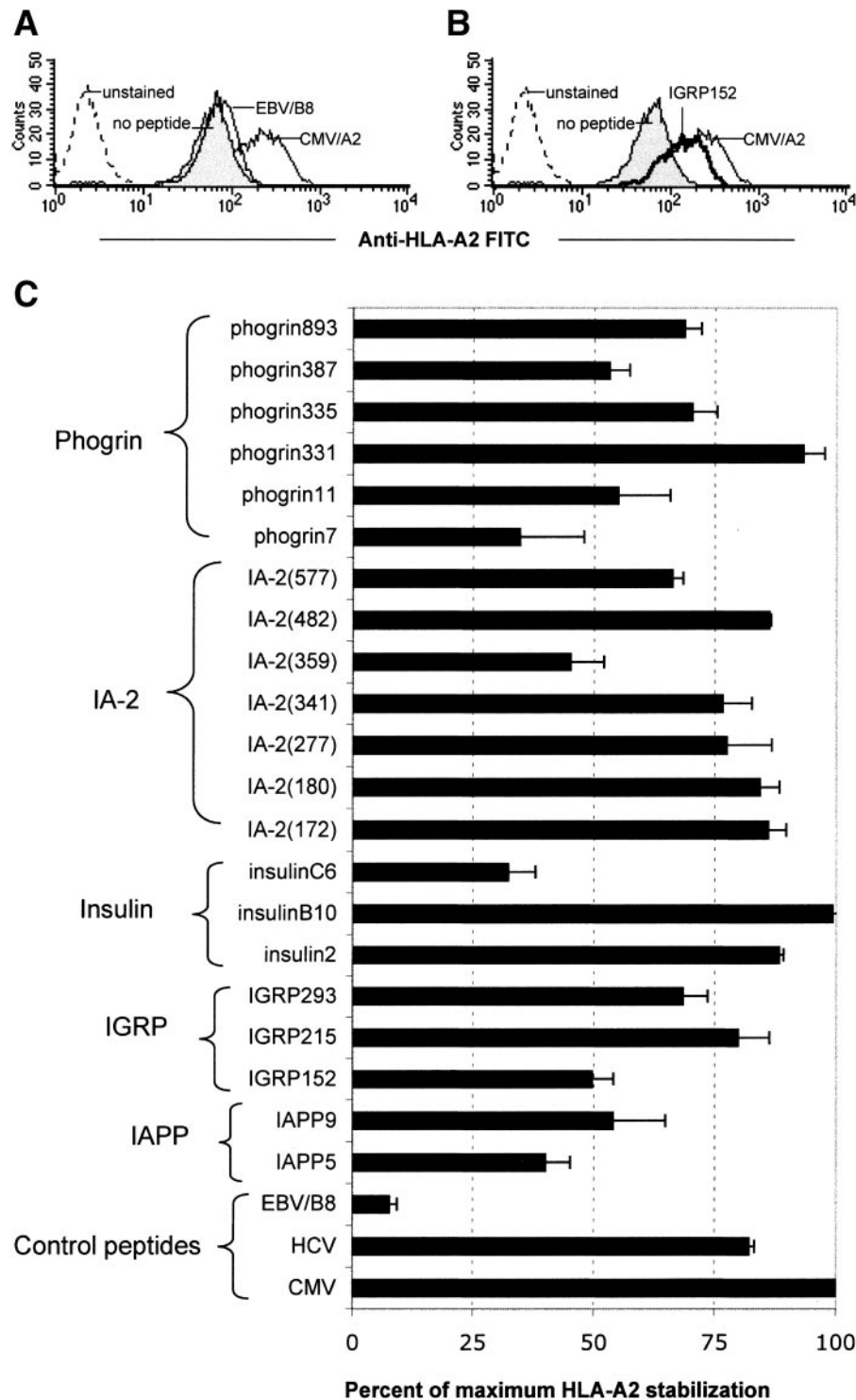
set as 100%. As shown in Fig. 3, peptide/HLA-A2 complexes formed with IA-2(182) and IA-2(482) were unstable, completely dissociating within 4 h of incubation at 37°C. Complexes of relatively low stability were observed for IGRP293, insulin2, and phogrin331 (~20% dissociated in 4 h), whereas all of the remaining peptides tested—IAPP5, IAPP9, IGRP152, IGRP215, insulinB10, and IA-2(172)—produced HLA complexes that were stable over a 4-h period (<5% dissociation) (data not shown).

**Recognition of β-cell peptides by PBMCs from patients with recent-onset type 1 diabetes.** The ability of the β-cell peptides to stimulate functional responses was tested next. PBMCs obtained from 24 HLA-A\*0201 and 5 non-HLA-A\*0201 patients with recent-onset type 1 diabetes and 11 HLA-A\*0201 nondiabetic control subjects were assayed for peptide recognition by IFN-γ ELISpot assays. Based on our previous findings, we chose for study all peptide epitopes identified by our prediction algorithms for IAPP (IAPP9) (10) and IGRP (IGRP152, IGRP215, IGRP293) (7,20). With respect to the other predicted peptides, we limited our analysis to those that stabilized HLA-A\*0201 expression at >80% of maximum levels, including insulin2, IA-2(172), IA-2(180), IA-2(482), and phogrin331. The previously reported epitopes IAPP5 (10) and insulin B chain 10-18 (11) were also studied. Because of the limited volume of blood available from our pediatric subjects, ELISpots were performed in separate studies using two sets of samples. We first assessed the recognition of IAPP and IGRP peptides by PBMCs of 19 HLA-A\*0201 and 5 non-HLA-A\*0201 recent-onset type 1 diabetic patients and 11 HLA-A\*0201 nondiabetic control subjects. Next, we determined the PBMC responses against peptides IGRP293, insulin2, insulinB10, IA-2(172), IA-2(180), IA-2(482), and phogrin331 in 11 HLA-A\*0201 and 5 non-HLA-A\*0201 recent-onset type 1 diabetic patients and 10 HLA-A\*0201 nondiabetic control subjects. Of the 11 patient samples analyzed in the second study, 6 used blood samples that were obtained and also analyzed in the first study because these samples had adequate numbers of frozen PBMCs for additional analysis.

In the first set of samples, PBMCs from ~50% of the HLA-A\*0201 recent-onset type 1 diabetic subjects but none of the control subjects, including non-HLA-A\*0201 recent-onset type 1 diabetic subjects and HLA-A\*0201 nondiabetic subjects, secreted IFN-γ in response to peptides IAPP5, IAPP9, IGRP152, and IGRP215 (Fig. 2B). None of the subjects responded to the control HCV peptide. The proportion of responsive type 1 diabetic subjects varied for each peptide (IAPP5: 7 of 19, 37%; IAPP9: 8 of 19, 37%; IGRP152: 8 of 19, 42%; and IGRP215: 13 of 19, 68%). In approximately one-third of the patients, the proportion of IFN-γ-secreting cells in response to the putative epitopes was comparable (20–50 spots per 2 × 10<sup>5</sup> PBMCs) to that observed with the positive control viral peptide mix. In the second panel of subjects, PBMCs from recent-onset type 1 diabetic patients demonstrated responses against insulinB10 (2 of 11, 18%), IA-2(172) (2 of 11, 18%), and IA-2(482) (3 of 11, 27%) (Fig. 2C). T-cell responses to IGRP293, IA-2(180), and phogrin331 could not be detected in diabetic patients nor in nondiabetic and non-HLA-matched control subjects. Interestingly, three of the five nondiabetic control subjects responded to the insulin2 peptide, but they did not respond to the other peptides.

**β-Cell peptide-HLA affinity inversely correlates with the self-reactive T-cell response in patients with type 1 diabetes.** Of 11 β-cell peptides screened by IFN-γ ELISpot assays with PBMCs from 24 patients with recent-onset type 1 diabetes, CD8<sup>+</sup> T-cell responses were detected against 7 peptides. As shown in Figs. 1 and 3, each of these peptides—IAPP5, IAPP9, IGRP152, IGRP215, insulinB10, IA-2(172), and IA-2(482)—had different relative binding affinities and dissociation rates. To determine whether a relationship exists between the affinity of peptide for HLA and the self-reactive CTL response, peptide binding affinities were plotted against the self-reactive ELISpot responses. This analysis revealed a strong inverse correlation between the relative binding affinity of β-cell peptides to HLA-A2 molecules and the average number of IFN-γ-producing spots per 2 × 10<sup>5</sup> PBMCs (*P* = 0.003, *r* = -0.958) (Fig. 4).

Downloaded from http://diabetesjournals.org/gdiabetes/article-pdf/55/11/3068/649897/zob01106003068.pdf by guest on 19 May 2022

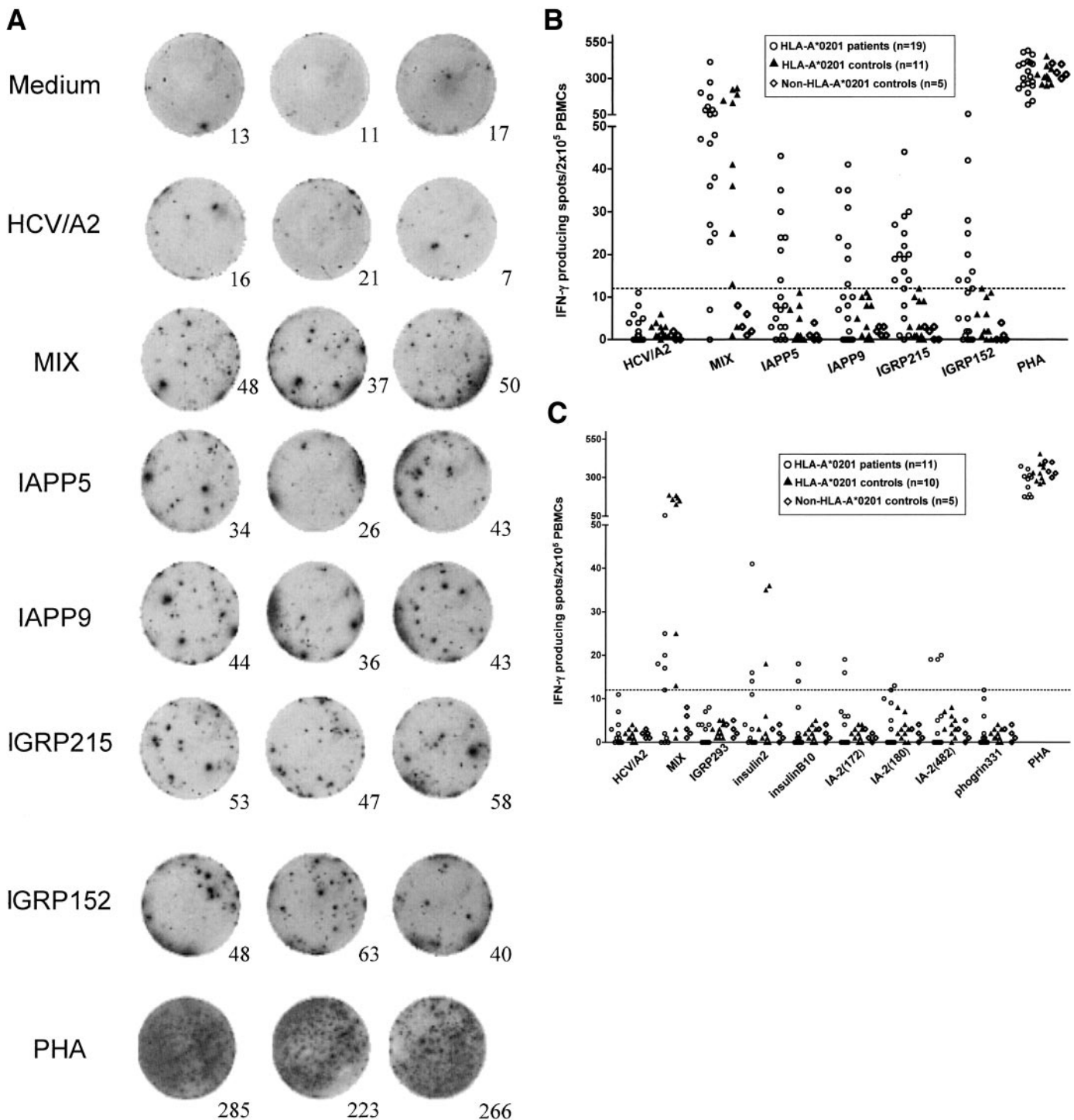


**FIG. 1.** Binding of  $\beta$ -cell peptides to HLA-A\*0201. T2 cells lacking stable HLA-A\*0201 surface expression were incubated with synthetic  $\beta$ -cell peptides, or equimolar amounts of control peptide known to bind to HLA-A\*0201 with high affinity (CMV, pp65 protein, NLVPMVATV), or peptide known to bind to HLA-B8 but not HLA-A\*0201 (EBV/B8, BZLF1 antigen, RAKFKQLL). **A:** Representative fluorescence-activated cell sorting histogram indicating the relative stability of HLA-A\*0201 on the surface of T2 cells when incubated in the absence of peptides, or with CMV/A2 or EBV/B8 peptides. **B:** Representative fluorescence-activated cell sorting histogram indicating T2 expression of HLA-A\*0201 in the presence of IGRP152 or CMV/A2. **C:** Summary of the relative affinity of  $\beta$ -cell peptides (derived from IAPP, IGRP, insulin, IA-2, and phogrin) for HLA-A\*0201. The error bars refer to the SE from three independent experiments. The relative binding affinity for each peptide is expressed as a percentage of maximal (CMV) binding as described in RESEARCH DESIGN AND METHODS. For details of peptide origin and amino acid sequence, refer to Table 1.

## DISCUSSION

The affinity of peptide for major histocompatibility complex (MHC) class I is one critical factor determining peptide presentation and immune response by cognate

CTLs (21). Our data are consistent with previous literature in the mouse model (22), in that the predicted binding affinity of  $\beta$ -cell peptides to HLA-A\*0201 did not correlate well with the actual binding affinity. Thus, the actual

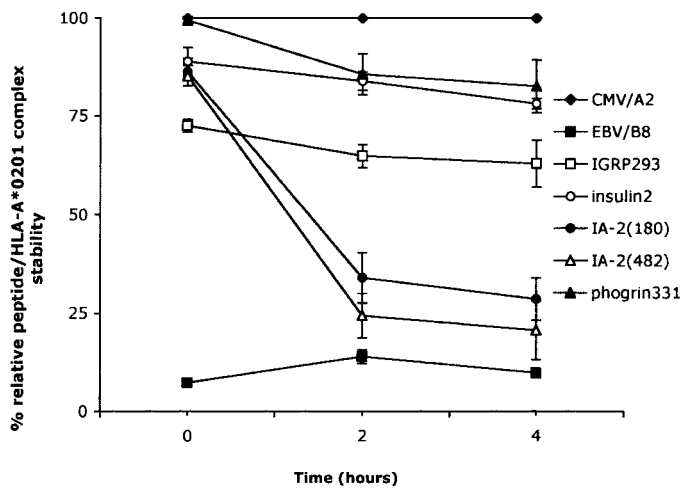


**FIG. 2.** Recognition of  $\beta$ -cell peptides by HLA-A2-restricted CD8<sup>+</sup> T-cells from recent-onset type 1 diabetic patients. **A:** Representative ELISpot assay (triplicate wells) demonstrates IFN- $\gamma$  responses to a peptide mix (CMV + EBV + Flu, positive control) and to individual  $\beta$ -cell peptides, including IAPP5, IAPP9, IGRP152, and PHA (positive control). Wells containing medium alone (Medium) and HCV peptide (HCV/A2) served as negative controls. **B** and **C:** CD8<sup>+</sup> T-cell responses to the indicated  $\beta$ -cell peptides are expressed as the absolute mean numbers of antigen-specific IFN- $\gamma$ -positive spots per  $2 \times 10^5$  PBMCs derived from HLA-A\*0201 recent-onset type 1 diabetic patients ( $\circ$ ), nondiabetic HLA-A\*0201 control subjects ( $\blacktriangle$ ), and non-HLA-A\*0201 control subjects ( $\diamond$ ). A threshold of 12 spots per  $2 \times 10^5$  cells (horizontal dotted line) was established as a cutoff for a positive result based on the ELISpot responses to the peptides that were at least 2 SDs above the mean of the nondiabetic control subjects. The peptide-specific responses are shown in two separate panels (**B** and **C**) for clarity. PHA, phytohemagglutinin.

affinity of peptides IA-2(172), IA-2(180), and phogrin331 was higher than that of IA-2(341), IA-2(359), phogrin7, and phogrin893, despite the higher predicted binding scores. The discrepancy between the predicted and actual binding affinities of candidate  $\beta$ -cell epitopes is likely attributable to the fact that the three-dimensional structure of the

peptide-HLA interaction cannot be accurately determined by existing algorithms. Nonetheless, computer-based prediction algorithms are helpful for identifying a pool of candidate epitopes in known target proteins that require confirmation of binding in vitro (23).

In this study, we identified 7 (of 11 tested) HLA-A\*0201-



**FIG. 3.** Temporal peptide dissociation of  $\beta$ -cell peptides from HLA-A\*0201. After overnight incubation with saturating amounts of peptide, T2 cells were treated with emetine (to inhibit protein synthesis) and incubated with peptides at 37°C. At the indicated time points, cells were washed and stained for HLA-A2 expression. Peptide/HLA-A\*0201 stability in the presence of EBV/B8 and the  $\beta$ -cell peptides IGRP293, insulin2, IA-2(180), IA-2(482), and phogrin331 has been normalized relative to that observed for the CMV/HLA-A\*0201 complex. Peptide/HLA complexes formed by IAPP5, IAPP9, IGRP215, IGRP152, insulinB10, and IA-2(172) were uniformly stable over the 4-h period (data not shown).

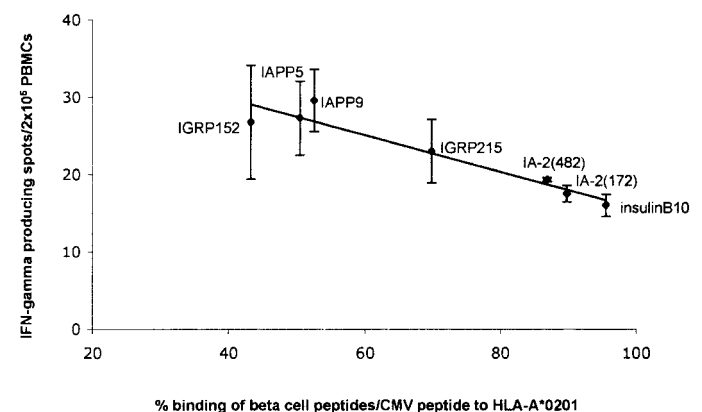
restricted  $\beta$ -cell peptides recognized by CD8<sup>+</sup> T-cells in recent-onset type 1 diabetic patients. Five of these peptides—IAPP9, IGRP152, IGRP215, IA-2(172), and IA-2(482)—have not been previously reported and represent novel type 1 diabetes autoepitopes. In addition to the IAPP5 epitope, which has previously been reported, we observed that more than one-third of the recent-onset type 1 diabetic patients responded to a second IAPP-derived peptide, IAPP9. MHC class I tetramer staining has previously identified IGRP-reactive T-cells as predominant in islets and peripheral blood of NOD mice (6,7,20). Our study indicates that human T-cells specific for IGRP epitopes are also found in recent-onset type 1 diabetic patients.

In addition to epitope identification, we observed a strong inverse relationship between  $\beta$ -cell peptide binding to HLA-A\*0201 and the corresponding self-reactive CD8<sup>+</sup> T-cell response, as measured by IFN- $\gamma$  spot-forming cells. Thus, CTL recognition of peptides with intermediate (lesser) HLA-binding affinity—IAPP5, IAPP9, IGRP152, and IGRP215—was significantly greater than that of peptides with higher HLA-binding affinity—insulin2, IA-2(172), and IA-2(482). This observed correlation is consistent with findings in the mouse model of experimental autoimmune encephalomyelitis, in which the capacity of peptides to induce experimental autoimmune encephalomyelitis correlates inversely with their affinity for MHC (24). This phenomenon may be explained by the incomplete deletion of autoreactive T-cells that recognize epitopes with lower binding affinity (25). Another factor that may play a role in CTL recognition of (self)  $\beta$ -cell peptides in the periphery is the HLA-peptide dissociation rate. Rapid peptide dissociation from HLA-A\*0201 may lead to a paucity of specific peptide/HLA-A\*0201 complexes at the surface of the cell, insufficient for recognition and triggering of circulating T-cells. For example, IA-2(180), which rapidly dissociates from HLA-A2, had a near complete lack of self-reactive CTL response in any patient studied.

Insulin is clearly an important autoepitope in type 1 diabetes (26,27), and proinsulin-derived epitopes are targets of T-cells in both diabetic and prediabetic subjects (28,29). We observed that in addition to recent-onset patients, a significant number of nondiabetic control subjects also exhibited CD8<sup>+</sup> T-cell responses to the peptide insulin2. This finding is similar to other reports of CTL recognition of an IA-2-derived peptide (30) and CD4<sup>+</sup> T-cell recognition of a class II-restricted GAD peptide (31) present in healthy individuals. The importance of this observation is unclear. In our study, an insulin peptide, insulinB10, was recognized by 2 of 11 recent-onset type 1 diabetic patients, consistent with results of a recent study in which 4 of 10 recent-onset patients with type 1 diabetes demonstrated CD8<sup>+</sup> T-cell responses against the same peptide (11). Given the considerable interest in insulin as the primary inciting autoantigen in both the NOD mouse model of type 1 diabetes (26) and humans (32), further study of the CTL response to the proinsulin-derived peptides in humans is clearly needed.

Our findings correlate remarkably well with those of Standifer et al. (33) who found, using samples from a separate cohort and assayed by granzyme B ELISpots in a independent laboratory, that peptides IAPP9, IGRP152, and IGRP215 represent novel epitopes in recent-onset type 1 diabetic subjects. Given the difficulty encountered to date in standardizing and replicating ELISpot assays, the comparable findings obtained by two separate groups is reassuring and increases the likelihood that these identified epitopes are truly representative of recent-onset type 1 diabetic patients. Although definitive quantification of  $\beta$ -cell-specific CTLs awaits the availability of MHC class I tetramers with sufficient sensitivity to detect  $\beta$ -cell-reactive CTLs in humans, our findings suggest that standardization of ELISpots is possible and may be used for detection and enumeration of self-reactive T-cells in peripheral blood.

In summary, we have identified IAPP9-17, IGRP152-160, IGRP215-223, IA-2(172-180), and IA-2(482-490) as HLA-A\*0201-restricted CD8<sup>+</sup> T-cell epitopes in patients with recent-onset type 1 diabetes. In addition, we observed a strong inverse correlation between the affinity of  $\beta$ -cell peptides for HLA-A\*0201 and self-reactive CD8<sup>+</sup> T-cell responses. Finally, we confirmed the presence of CTLs in recent-onset type 1 diabetic patients that recognize the



**FIG. 4.** Inverse relationship between peptide/HLA-A\*0201 affinity and  $\beta$ -cell-reactive CD8<sup>+</sup> T-cell responses in patients with type 1 diabetes. Peptide/HLA affinity of the indicated peptides was plotted against the IFN- $\gamma$  ELISpot response for IAPP5, IAPP9, IGRP215, IGRP152, insulinB10, IA-2(172), and IA-2(482) (*y*-axis) ( $P = 0.003$ ,  $r = -0.958$ ).

previously reported β-cell epitopes IAPP5-13 and insulin B10-18, and we confirmed that insulin peptide-specific autoreactive CD8<sup>+</sup> T-cells are present in healthy individuals. The identification of these epitopes is an important first step toward understanding their potential role in the pathogenesis of type 1 diabetes and their possible value in the future prediction of this disease.

**ACKNOWLEDGMENTS**

This work was supported by the Juvenile Diabetes Research Foundation International (JDRF; 5-2004-538 to R.T. and C.P.) and the National Institutes of Health (FY04.062.020 to R.T. and C.P.). Q.O. is a recipient of a JDRF postdoctoral fellowship award, and R.T. is a Michael Smith Foundation Senior Scholar.

We are grateful to Lisa Hennan and Pamela Lutley for patient recruitment and to Dr. Lisa Xu (FACS Core), Xiaoxia Wang, Brian Chung, and I-Fang Lee for their comments and assistance.

**REFERENCES**

1. Atkinson MA, Eisenbarth GS: Type 1 diabetes: new perspectives on disease pathogenesis and treatment. *Lancet* 358:221–229, 2001
2. Delovitch TL, Singh B: The nonobese diabetic mouse as a model of autoimmune diabetes: immune dysregulation gets the NOD. *Immunity* 7:727–738, 1997
3. Liblau RS, Wong FS, Mars LT, Santamaria P: Autoreactive CD8 T cells in organ-specific autoimmunity: emerging targets for therapeutic intervention. *Immunity* 17:1–6, 2002
4. Verge CF, Stenger D, Bonifacio E, Colman PG, Pilcher C, Bingley PJ, Eisenbarth GS: Combined use of autoantibodies (IA-2 autoantibody, GAD autoantibody, insulin autoantibody, cytoplasmic islet cell antibodies) in type 1 diabetes: Combinatorial Islet Autoantibody Workshop. *Diabetes* 47:1857–1866, 1998
5. Eisenbarth GS: Prediction of type 1 diabetes: the natural history of the prediabetic period. *Adv Exp Med Biol* 552:268–290, 2004
6. Amrani A, Verdager J, Serra P, Tafuro S, Tan R, Santamaria P: Progression of autoimmune diabetes driven by avidity maturation of a T-cell population. *Nature* 406:739–742, 2000
7. Trudeau JD, Kelly-Smith C, Verchere CB, Elliott JF, Dutz JP, Finegood DT, Santamaria P, Tan R: Prediction of spontaneous autoimmune diabetes in NOD mice by quantification of autoreactive T cells in peripheral blood. *J Clin Invest* 111:217–223, 2003
8. Panina-Bordignon P, Lang R, van Endert PM, Benazzi E, Felix AM, Pastore RM, Spinaz GA, Sinigaglia F: Cytotoxic T cells specific for glutamic acid decarboxylase in autoimmune diabetes. *J Exp Med* 181:1923–1927, 1995
9. Kimura K, Kawamura T, Kadotani S, Inada H, Niihira S, Yamano T: Peptide-specific cytotoxicity of T lymphocytes against glutamic acid decarboxylase and insulin in type 1 diabetes mellitus. *Diabetes Res Clin Pract* 51:173–179, 2001
10. Panagiotopoulos C, Qin H, Tan R, Verchere CB: Identification of a β-cell-specific HLA class I restricted epitope in type 1 diabetes. *Diabetes* 52:2647–2651, 2003
11. Toma A, Haddouk S, Briand JP, Camoin L, Gahery H, Connan F, Dubois-Laforgue D, Caillat-Zucman S, Guillet JG, Carel JC, Muller S, Choppin J, Boitard C: Recognition of a subregion of human proinsulin by class I-restricted T cells in type 1 diabetic patients. *Proc Natl Acad Sci U S A* 102:10581–10586, 2005
12. Rammensee H, Bachmann J, Emmerich NP, Bachor OA, Stevanovic S: SYFPEITHI: database for MHC ligands and peptide motifs. *Immunogenetics* 50:213–219, 1999
13. Dick TP, Stevanovic S, Keilholz W, Ruppert T, Koszinowski U, Schild H, Rammensee HG: The making of the dominant MHC class I ligand SYF-PEITHI. *Eur J Immunol* 28:2478–2486, 1998
14. Parker KC, Bednarek MA, Coligan JE: Scheme for ranking potential

- HLA-A2 binding peptides based on independent binding of individual peptide side-chains. *J Immunol* 152:163–175, 1994
15. Valmori D, Gervois N, Rimoldi D, Fonteneau JF, Bonelo A, Lienard D, Rivoltini L, Jotereau F, Cerottini JC, Romero P: Diversity of the fine specificity displayed by HLA-A\*0201-restricted CTL specific for the immunodominant Melan-A/MART-1 antigenic peptide. *J Immunol* 161:6956–6962, 1998
16. Diamond DJ, York J, Sun JY, Wright CL, Forman SJ: Development of a candidate HLA A\*0201 restricted peptide-based vaccine against human cytomegalovirus infection. *Blood* 90:1751–1767, 1997
17. Lalvani A, Brookes R, Hambleton S, Britton WJ, Hill AV, McMichael AJ: Rapid effector function in CD8<sup>+</sup> memory T cells. *J Exp Med* 186:859–865, 1997
18. Neefjes JJ, Dierx J, Ploegh HL: The effect of anchor residue modifications on the stability of major histocompatibility complex class I-peptide interactions. *Eur J Immunol* 23:840–845, 1993
19. van der Burg SH, Visseren MJ, Brandt RM, Kast WM, Melief CJ: Immunogenicity of peptides bound to MHC class I molecules depends on the MHC-peptide complex stability. *J Immunol* 156:3308–3314, 1996
20. Lieberman SM, Evans AM, Han B, Takaki T, Vinnitskaya Y, Caldwell JA, Serreze DV, Shabanowitz J, Hunt DF, Nathenson SG, Santamaria P, DiLorenzo TP: Identification of the beta cell antigen targeted by a prevalent population of pathogenic CD8<sup>+</sup> T cells in autoimmune diabetes. *Proc Natl Acad Sci U S A* 100:8384–8388, 2003
21. Chen W, Khilko S, Fecondo J, Margulies DH, McCluskey J: Determinant selection of major histocompatibility complex class I-restricted antigenic peptides is explained by class I-peptide affinity and is strongly influenced by nondominant anchor residues. *J Exp Med* 180:1471–1483, 1994
22. Shao H, Peng Y, Liao T, Wang M, Song M, Kaplan HJ, Sun D: A shared epitope of the interphotoreceptor retinoid-binding protein recognized by the CD4<sup>+</sup> and CD8<sup>+</sup> autoreactive T cells. *J Immunol* 175:1851–1857, 2005
23. Pelte C, Cherepnev G, Wang Y, Schoenemann C, Volk HD, Kern F: Random screening of proteins for HLA-A\*0201-binding nine-amino acid peptides is not sufficient for identifying CD8 T cell epitopes recognized in the context of HLA-A\*0201. *J Immunol* 172:6783–6789, 2004
24. Anderson SM, Radu CG, Lowrey PA, Ward ES, Wraith DC: Negative selection during the peripheral immune response to antigen. *J Exp Med* 193:1–11, 2001
25. Chan S, Correia-Neves M, Benoist C, Mathis D: CD4/CD8 lineage commitment: matching fate with competence. *Immunol Rev* 165:195–207, 1998
26. Nakayama M, Abiru N, Moriyama H, Babaya N, Liu E, Miao D, Yu L, Wegmann DR, Hutton JC, Elliott JF, Eisenbarth GS: Prime role for an insulin epitope in the development of type 1 diabetes in NOD mice. *Nature* 435:220–223, 2005
27. Haffer DA, Kent SC, Chen Y, Bregoli L, Clemmings SM, Hering B, Kenyon NS, Ricordi C: Immunology: insulin auto-antigenicity in type 1 diabetes (Letter). *Nature* 438:E5–E6, 2005
28. Alleva DG, Crowe PD, Jin L, Kwok WW, Ling N, Gottschalk M, Conlon PJ, Gottlieb PA, Putnam AL, Gaur A: A disease-associated cellular immune response in type 1 diabetics to an immunodominant epitope of insulin. *J Clin Invest* 107:173–180, 2001
29. Durinovic-Bello I, Schlosser M, Riedl M, Maisel N, Rosinger S, Kalbacher H, Deeg M, Ziegler M, Elliott J, Roep BO, Karges W, Boehm BO: Pro- and anti-inflammatory cytokine production by autoimmune T cells against preproinsulin in HLA-DRB1\*04, DQ8 type 1 diabetes. *Diabetologia* 47:439–450, 2004
30. Takahashi K, Honeyman MC, Harrison LC: Cytotoxic T cells to an epitope in the islet autoantigen IA-2 are not disease-specific. *Clin Immunol* 99:360–364, 2001
31. Danke NA, Koelle DM, Yee C, Beheray S, Kwok WW: Autoreactive T cells in healthy individuals. *J Immunol* 172:5967–5972, 2004
32. Kent SC, Chen Y, Bregoli L, Clemmings SM, Kenyon NS, Ricordi C, Hering BJ, Haffer DA: Expanded T cells from pancreatic lymph nodes of type 1 diabetic subjects recognize an insulin epitope. *Nature* 435:224–228, 2005
33. Standifer NE, Ouyang Q, Panagiotopoulos C, Verchere CB, Tan R, Greenbaum CJ, Pihoker C, Nepom GT: Identification of novel HLA-A\*0201-restricted epitopes in recent-onset type 1 diabetic subjects and antibody-positive relatives. *Diabetes* 55:3061–3067, 2006

Downloaded from http://diabetesjournals.org/ by guest on 19 May 2022