In Vivo Cytotoxicity of Insulin-Specific CD8$^+$ T-Cells in HLA-A*0201 Transgenic NOD Mice

Irene Jarchum,[1] Jason C. Baker,[1,2] Tatsuya Yamada,[1] Toshiyuki Takaki,[1] Michele P. Marron,[3] David V. Serreze,[4] and Teresa P. DiLorenzo[1,2]

OBJECTIVE—CD8$^+$ T-cells specific for islet antigens are essential for the development of type 1 diabetes in the NOD mouse model of the disease. Such T-cells can also be detected in the blood of type 1 diabetic patients, suggesting their importance in the pathogenesis of the human disease as well. The development of peptide-based therapeutic reagents that target islet-reactive CD8$^+$ T-cells will require the identification of disease-relevant epitopes.

RESEARCH DESIGN AND METHODS—We used islet-infiltrating CD8$^+$ T-cells from HLA-A*0201 transgenic NOD mice in an interferon-γ enzyme-linked immunospot assay to identify autoantigenic peptides targeted during the spontaneous development of disease. We concentrated on insulin (Ins), which is a key target of the autoimmune response in NOD mice and patients alike.

RESULTS—We found that HLA-A*0201-restricted T-cells isolated from the islets of the transgenic mice were specific for Ins1 L3–11, Ins1 B5–14, and Ins1/2 A2–10. Insulin-reactive T-cells were present in the islets of mice as young as 5 weeks of age, suggesting an important function for these specificities early in the pathogenic process. Although there was individual variation in peptide reactivity, Ins1 B5–14 and Ins1/2 A2–10 were the immunodominant epitopes. Notably, in vivo cytotoxicity to cells bearing these peptides was observed, further confirming them as important targets of the pathogenic process.

CONCLUSIONS—The human versions of B5–14 and A2–10, differing from the murine peptides by only a single residue, represent excellent candidates to explore as CD8$^+$ T-cell targets in HLA-A*0201–positive type 1 diabetic patients. ***Diabetes* 56: 2551–2560, 2007**

Type 1 diabetes is an autoimmune disease characterized by T-cell–mediated elimination of insulin-producing pancreatic islet β-cells. Studies in the NOD mouse model established that both CD4$^+$ and CD8$^+$ T-cells are required for spontaneous diabetes development (1). Historically, the CD4$^+$ T-cell subset was the most intensively studied, due in part to the early association found between the expression of particular class II major histocompatibility complex (MHC) molecules and type 1 diabetes in both humans and NOD mice (2,3). However, multiple lines of investigation in NOD mice have led to a heightened appreciation for the importance of islet antigen-specific CD8$^+$ T-cells in the pathogenesis of type 1 diabetes. NOD mice depleted of CD8$^+$ T-cells by antibody treatment do not develop diabetes (4), nor do CD8α-deficient NOD mice (5). Similarly, diabetes does not occur in β2-microglobulin–deficient NOD mice, which lack class I MHC expression and therefore do not develop CD8$^+$ T-cells (6–9). Furthermore, splenocytes from young pre-diabetic NOD donors can transfer diabetes to class I MHC–positive, but not to class I–negative (i.e., β2-microglobulin–deficient), NOD-*scid* mice (10,11). Finally, CD8$^+$ T-cell clones can be isolated from the islets of NOD mice, and these are specifically cytotoxic to β-cells in vitro (10,12–15) and pathogenic in vivo (12,15–18).

In humans, islet-infiltrating CD8$^+$ T-cells are prevalent in pancreatic biopsies from graft-recurrent and new-onset type 1 diabetic patients (19–22). There have also been several recent reports documenting the direct ex vivo detection of islet antigen–specific CD8$^+$ T-cells in the peripheral blood of patients (23–28). These observations suggest the possibility that peptide-based predictive, diagnostic, monitoring, and therapeutic strategies targeting islet-reactive CD8$^+$ T-cells may be feasible. The development of these strategies will require knowledge of disease-relevant epitopes and, ideally, appropriate animal models for protocol optimization. The strength of binding of a peptide to an HLA molecule of interest, whether predicted or experimental, is a common criterion used to identify candidates to be examined for T-cell reactivity in patients. Here we instead use an unbiased approach, using islet-infiltrating CD8$^+$ T-cells from diabetes-susceptible HLA transgenic NOD mice to characterize the class I MHC-dependent responses that arise during the spontaneous development of disease. We recently identified peptides from the β-cell antigen islet-specific glucose-6-phosphatase catalytic subunit-related protein (IGRP) that are targeted by HLA-A*0201–restricted T-cells in HLA transgenic NOD mice (29). Two of these were subsequently shown to be recognized by peripheral CD8$^+$ T-cells in type 1 diabetic patients (23). This indicates that islet-infiltrating CD8$^+$ T-cells from class I HLA transgenic mice can indeed be used to identify peptides relevant to the pathogenic basis of type 1 diabetes in humans.

Insulin was the first β-cell protein to which an autoimmune response was documented in type 1 diabetic patients (30), and accumulating evidence suggests it is a key target of pathogenic T-cells in both NOD mice and humans

From the [1]Department of Microbiology and Immunology, Albert Einstein College of Medicine, Bronx, New York; the [2]Department of Medicine (Division of Endocrinology), Albert Einstein College of Medicine, Bronx, New York; the [3]Department of Pediatrics, Division of Genetic and Translational Medicine, University of Alabama at Birmingham, Birmingham, Alabama; and [4]The Jackson Laboratory, Bar Harbor, Maine.

Address correspondence and reprint requests to Teresa P. DiLorenzo, PhD, Albert Einstein College of Medicine, Microbiology and Immunology, 1300 Morris Park Ave., Bronx, NY 10461. E-mail: dilorenz@aecom.yu.edu.

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CFSE, carboxyfluorescein diacetate succinimidyl ester; ELISPOT, enzyme-linked immunospot; IFN, interferon; IGRP, islet-specific glucose-6-phosphatase catalytic subunit-related protein; MHC, major histocompatibility complex.

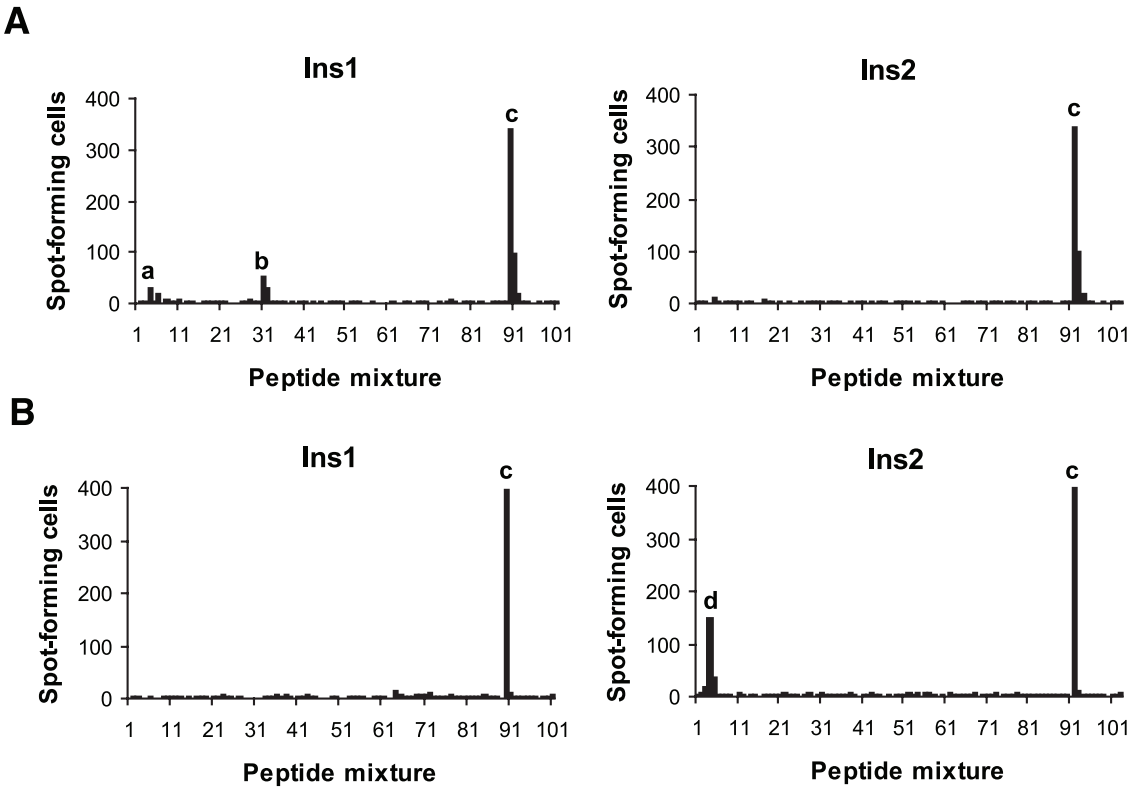


FIG. 1. Islet-infiltrating T-cells from NOD.β*2m*^{null}.*HHD* mice recognize multiple insulin peptides. Islets were cultured from 12-week-old (*A*) or 16-week-old (*B*) nondiabetic NOD.β*2m*^{null}.*HHD* mice. Islet-infiltrating T-cells were harvested and 2 × 10^4 cells/well were used to screen Ins1 and Ins2 peptide libraries by IFN-γ ELISPOT. T2 cells (2 × 10^4/well) pulsed with 1 μmol/l peptide served as antigen-presenting cells. Numbers of spot-forming cells per 2 × 10^4 cells are shown. Reactive mixtures are designated a–d.

(31–36). Indeed, in NOD mice, establishment of tolerance to insulin can lead to prevention of diabetes (37), as well as remission of established disease (38). Here we have found that multiple epitopes of insulin are recognized by islet-infiltrating HLA-A*0201–restricted T-cells in HLA transgenic NOD mice as young as 5 weeks of age and in the absence of immunization or exogenous insulin treatment. These epitopes have not been described previously. Because they are targeted by islet-infiltrating T-cells during spontaneous diabetes development, their potential disease relevance is high. Thus, they represent excellent candidates to explore as important T-cell targets in human type 1 diabetic patients.

RESEARCH DESIGN AND METHODS

Mice. NOD.β*2m*^{null} (7) and NOD.β*2m*^{null}.*HHD* mice (29) have been previously described and are maintained by brother-sister mating at the Albert Einstein College of Medicine. NOD.β*2m*^{null}.*HHD* mice express a monochain chimeric HLA-A*0201 molecule consisting of human β2-microglobulin covalently linked to the α1 and α2 domains of HLA-A*0201, followed by the α3 transmembrane and cytoplasmic portions of H-2D^b. Animal experiments were approved by the institutional animal care and use committee at Albert Einstein College of Medicine.

Peptides. Peptide libraries containing all of the 8mer, 9mer, 10mer, and 11mer peptides that can be derived from murine preproinsulin 1 and 2 were synthesized by Mimotopes (Raleigh, NC) using their proprietary Truncated PepSet technology. Each mixture in the libraries contained four peptides with a common COOH-terminus, but having a length of 8, 9, 10, or 11 residues. The four peptides in each mixture were present in approximately equimolar amounts. Concentrated peptide stocks (2.75 mmol/l) were prepared in 50% acetonitrile/H_2O, and 40 μmol/l (i.e., ~10 μmol/l for each peptide in the mixture) working stocks were obtained by serial dilution in PBS (pH 6.5). Individual peptides, having a purity of >90%, were obtained from Mimotopes. Concentrated stocks (10 mmol/l) were prepared in DMSO, and 10 μmol/l working stocks were obtained by dilution in PBS.

Diabetes assessment. Mice were monitored weekly for glucosuria with Diastix reagent strips (Bayer, Elkhart, IN) and were considered diabetic when glucosuria was observed.

Culture of islet-infiltrating T-cells. Islets were isolated from female NOD.β*2m*^{null}.*HHD* mice by collagenase perfusion of the common bile duct as described (29). Islets were handpicked and cultured for 7 days in 24-well tissue culture plates (~50 islets/well) in RPMI medium supplemented with 10% FBS (Hyclone, Logan, UT) and 50 units/ml recombinant human IL-2 (PeproTech, Rocky Hill, NJ).

Interferon-γ enzyme-linked immunospot assay. Interferon (IFN)-γ enzyme-linked immunospot (ELISPOT) was performed as previously described (29). Spots were counted using an automated ELISPOT reader system (Autoimmun Diagnostika, Strassberg, Germany). For select experiments, CD8$^+$ T-cells were purified by negative selection from cultured islet infiltrates using magnetic separation (Miltenyi Biotec, Bergisch Gladbach, Germany).

HLA-A*0201 binding assay. Peptide binding to HLA-A*0201 was determined as described (39). Briefly, T2 cells were incubated for 18 h at 26°C. Cells were then incubated with peptide or PBS and human β2-microglobulin (Sigma-Aldrich, St. Louis, MO) for 20 h at 37°C, then incubated at 50°C for 3 min. Cells were stained with anti–HLA-A2 mAb BB7.2 (BD Biosciences Pharmingen, San Jose, CA) and analyzed by flow cytometry.

In vivo cytotoxicity assay. Splenocytes were prepared from NOD or NOD.β*2m*^{null}.*HHD* mice and divided into three groups. Cells were labeled with 0.5 or 5 μmol/l carboxyfluorescein diacetate succinimidyl ester (CFSE) (Molecular Probes, Carlsbad, CA) or with 10 μmol/l CellTracker Orange (Molecular Probes) for 10 min at 37°C. Labeling was stopped by quenching with complete Dulbecco's modified Eagle's medium on ice for 5 min. After washing, cells were pulsed with 1 μmol/l peptide for 1 h at 37°C. For experiments using NOD mice, control NOD splenocytes (5 μmol/l CFSE) were pulsed with an irrelevant peptide (the HLA-A*0201-binding hepatitis C virus core peptide 132–140; DLMGYIPLV). Target splenocytes labeled with 10 μmol/l CellTracker Orange were pulsed with the I9 variant of Ins1/2 B15–23 (LYLVCGERI), while targets labeled with 0.5 μmol/l CFSE were pulsed with IGRP 206–214 (VYLKTNVFL). For experiments using NOD.β*2m*^{null}.*HHD* mice, control NOD.β*2m*^{null}.*HHD* splenocytes (5 μmol/l CFSE) were pulsed with the irrelevant peptide IGRP 206–214. Target cells labeled with 10 μmol/l CellTracker Orange were pulsed with Ins1 B5–14, while targets labeled with

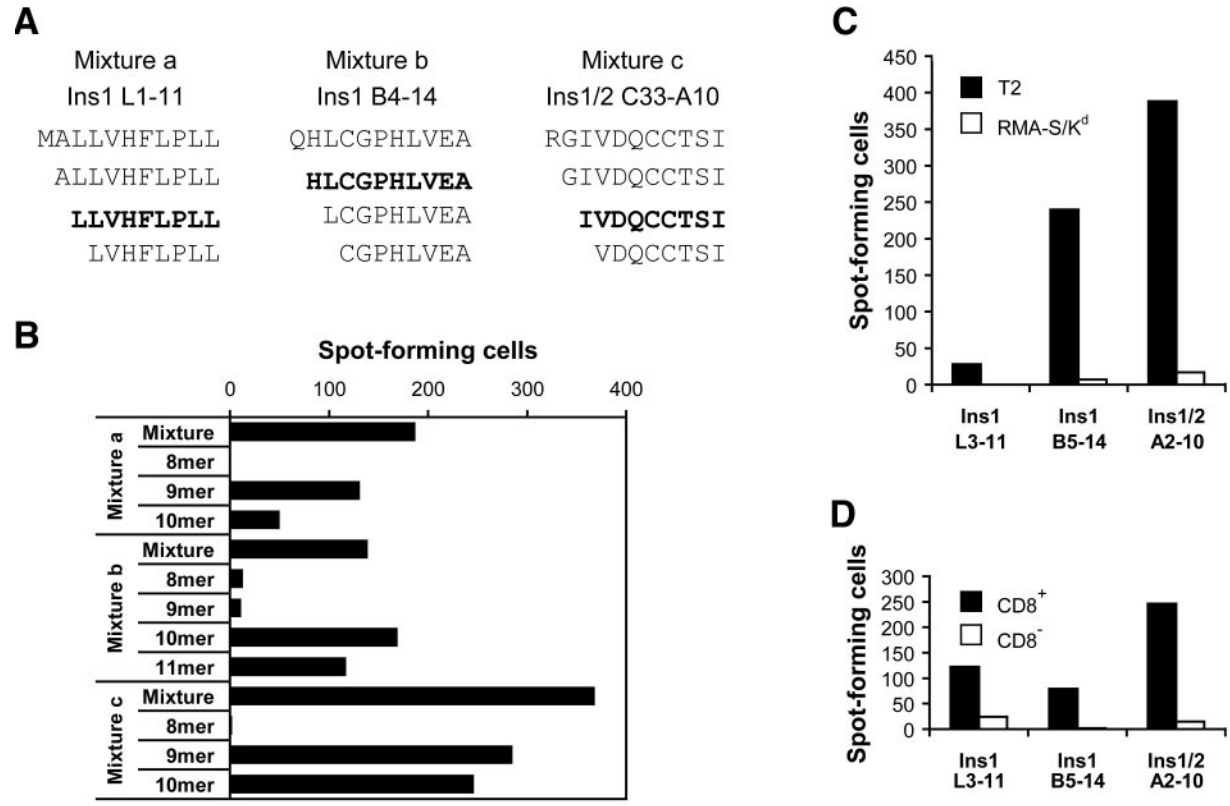


FIG. 2. Identification of the minimal epitopes present in peptide mixtures a–c. *A*: The peptides comprising library mixtures *a–c* (see Fig. 1) are indicated. The minimal epitopes identified in *B* are shown in bold. *B*: Islet-infiltrating T-cells from NOD.β*2m*^{null}.*HHD* mice were examined for reactivity to the indicated peptide mixtures and their components using IFN-γ ELISPOT. *C*: T2 or RMA-S/K^d cells were pulsed with the indicated peptides, washed, and then used as antigen-presenting cells to confirm that the identified minimal epitopes were recognized in the context of HLA-A*0201. *D*: Islet-infiltrating cells were sorted for CD8 positivity and tested for reactivity to the peptides. The CD8$^+$ fraction was ≥93% CD8$^+$ T-cells, whereas the negative fraction contained 9–13% CD8$^+$ cells. Low levels of reactivity in the CD8$^-$ fraction probably represent this low percentage of contaminating CD8$^+$ T-cells. In *B–D*, numbers of spot-forming cells per 2 × 10^4 cells are indicated.

0.5 μmol/l CFSE were pulsed with Ins1/2 A2–10. After washing with PBS, cells were mixed in equal proportions and 1.5 × 10^7 total cells in 200 μl injected intravenously into NOD.β*2m*^{null} (control) and NOD (8–14 weeks old) or NOD.β*2m*^{null}.*HHD* (15 weeks old) mice. Pancreatic lymph nodes were removed 16 h later, and single-cell suspensions were prepared and analyzed by flow cytometry on a FACSCalibur (BD Biosciences) after addition of 7-AAD (aminoactinomycin D) (BD Biosciences). Data shown are gated on live cells and uncompensated for better identification of the populations of interest. Gating was done for the NOD.β*2m*^{null} mouse and applied to all other mice in the experiment. Percent specific lysis was calculated as: {[(control population × A) − target population]/(control population × A)} × 100, where A = target population/control population in the NOD.β*2m*^{null} mouse. Islets were prepared from the same mice for propagation of infiltrating T-cells for 7 days, and IFN-γ ELISPOT was performed as described above. For data representation, the number of spots in wells with PBS was subtracted from those with peptide. For select experiments, CD8$^+$ T-cell depletion was achieved by intraperitoneal injection of NOD.β*2m*^{null}.*HHD* mice with 0.25 mg of the depleting anti-CD8 mAb YTS169. The in vivo cytotoxicity assay was performed 7 days later, when peripheral blood CD8$^+$ T-cells were depleted by 84 ± 4% (n = 5).

RESULTS

The type 1 diabetes–susceptible NOD.*β2m*^{null}.*HHD* stock expresses a monochain chimeric HLA-A*0201 molecule but no murine class I MHC molecules (29). To investigate whether CD8$^+$ T-cell responses to insulin-derived peptides develop spontaneously in NOD.*β2m*^{null}.*HHD* mice during the pre-diabetic period, we used IFN-γ ELISPOT analysis and islet-infiltrating T-cells to screen peptide libraries representing murine Ins1 and Ins2. The libraries consisted of mixtures of four peptides each, with all four having a fixed COOH-terminus and ranging in length from 8 to 11 residues. As the libraries were offset by one, they contained every possible 8- to 11-mer peptide that can be derived from Ins1 and Ins2. Four peptide mixtures exerted antigenic activity (designated a–d in Figs. 1 and 2*A*). We tested T-cells for reactivity to individual components of the mixtures and identified Ins1 L3–11, Ins1 B5–14, and Ins1/2 A2–10 as the minimal epitopes present in mixtures a, b, and c, respectively (Fig. 2*B*). Reactivity to mixture d (spanning Ins2 L1–11) proved to be extremely rare. Consequently, we were unable to definitively identify the minimal epitope responsible for the activity of this mixture.

NOD.*β2m*^{null}.*HHD* mice express HLA-A*0201 but not the murine class I MHC molecules H-2K^d and H-2D^b encoded by their *H2*^{g7} MHC haplotype (29). Nonetheless, to confirm that recognition of the antigenic insulin peptides was dependent on HLA-A*0201, we performed ELISPOT analysis using either T2 (expressing HLA-A*0201) or RMA-S/K^d cells (expressing H-2K^d, H-2D^b, and H-2K^b) as antigen-presenting cells. These experiments confirmed that peptide recognition was dependent on HLA-A*0201 (Fig. 2*C*). We also verified that CD8$^+$ T-cells are responsible for recognition of the peptides by purifying CD8$^+$ T-cells from the islet infiltrates and using both the CD8$^+$ and CD8$^-$ cell fractions as effectors. As shown in Fig. 2*D*, peptide-reactive cells were highly enriched in the CD8$^+$ T-cell fraction.

Next, we monitored T-cell reactivity to Ins1 L3–11, Ins1 B5–14, and Ins1/2 A2–10 in nondiabetic 12- to 13-week-old

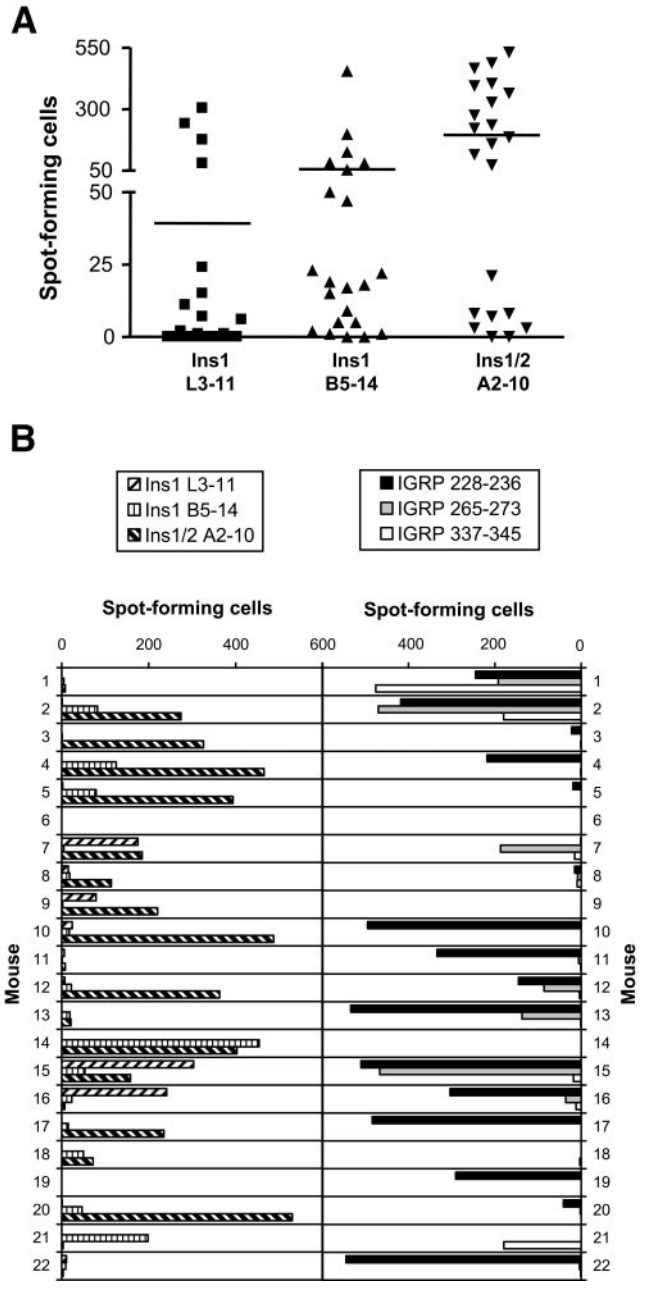


FIG. 3. Characterization of T-cell responses to insulin and IGRP in NOD.β*2m*^{null}.*HHD* mice. *A*: Islet-infiltrating T-cells were cultured individually from 22 nondiabetic female NOD.β*2m*^{null}.*HHD* mice (12–13 weeks old), and their reactivity to the indicated peptides was monitored by IFN-γ ELISPOT. Numbers of spot-forming cells per 2 × 10^4 cells are depicted. Horizontal bars indicate means. *B*: The same T-cell cultures used in *A* were simultaneously tested for reactivity to the indicated insulin and IGRP peptides.

NOD.*β2m*^{null}.*HHD* mice (Fig. 3*A*). These analyses revealed that Ins1/2 A2–10 is the immunodominant HLA-A*0201–restricted insulin epitope among mice in this age-group. We previously reported that HLA-A*0201–restricted islet-infiltrating T-cells from NOD.*β2m*^{null}.*HHD* mice recognize three peptides derived from the β-cell antigen IGRP (29). When we compared T-cell responses to insulin and IGRP in multiple 12- to 13-week-old NOD.*β2m*^{null}.*HHD* mice, four patterns were observed (Fig. 3*B*). Most mice responded to both insulin and IGRP (e.g., mouse 2), although a minor subset responded to only insulin (e.g., mouse 9) or only IGRP (e.g., mouse 19). A

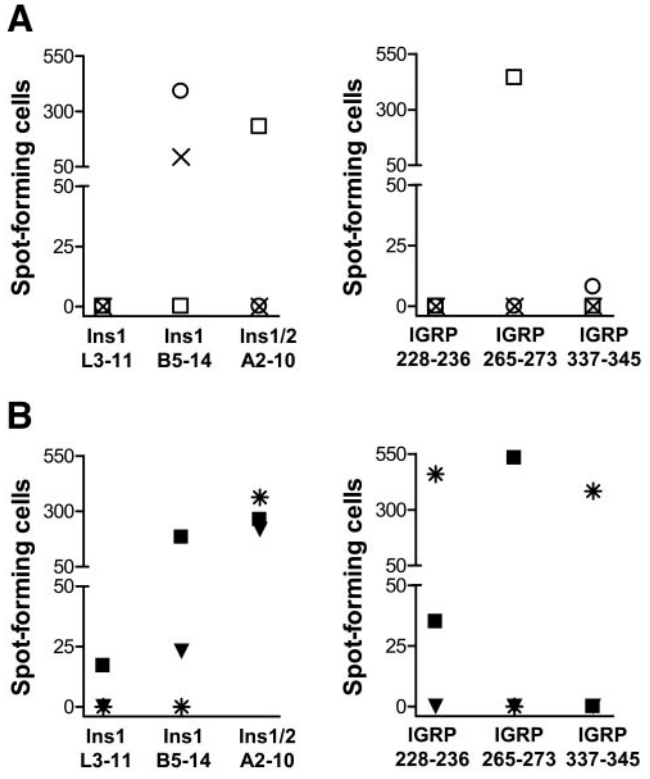
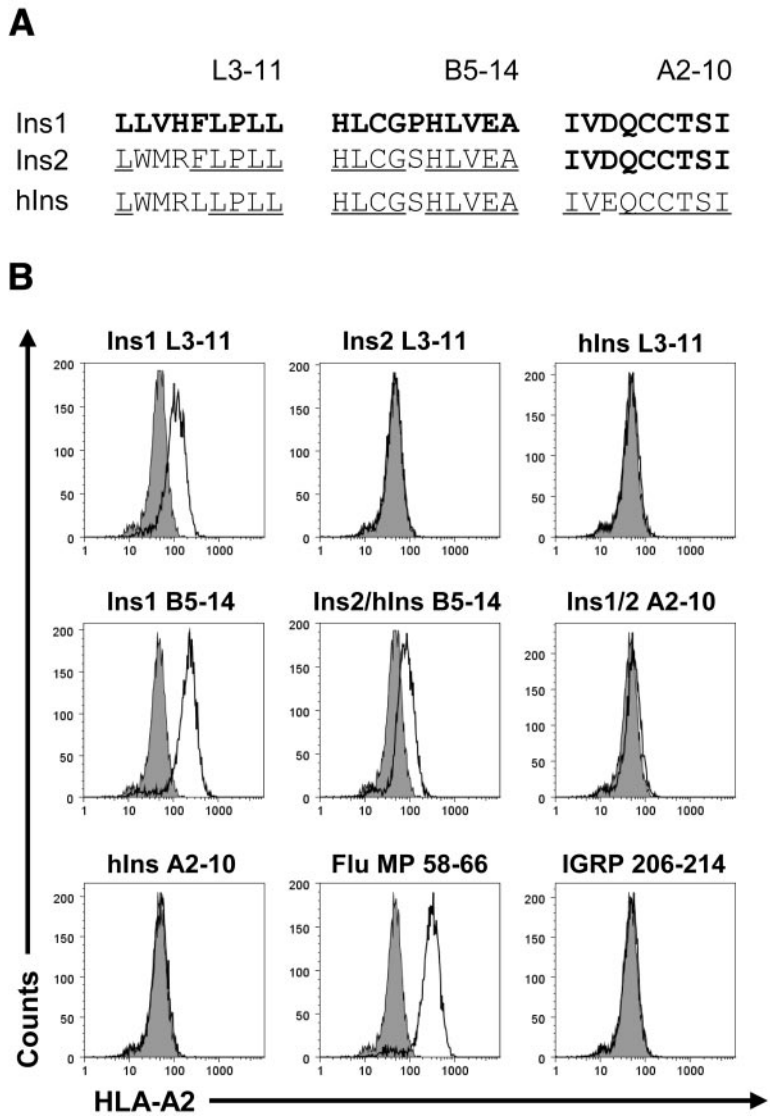


FIG. 4. Young NOD.β*2m*^{null}.*HHD* mice show T-cell responses to both insulin and IGRP peptides. Islet-infiltrating T-cells were cultured individually from three nondiabetic female 5-week-old (*A*) or 8-week-old NOD.β*2m*^{null}.*HHD* mice (*B*), and their reactivity to the indicated insulin and IGRP peptides was monitored by IFN-γ ELISPOT. Numbers of spot-forming cells per 2 × 10^4 cells are indicated. For each mouse, the same symbol is used in the left and right panels of the figure.

single mouse (mouse 6) responded to neither antigen. These findings support our previous assertion that individuals exhibit distinct patterns of reactivity to islet antigens (40).

Because female NOD.*β2m*^{null}.*HHD* mice can begin to show signs of overt diabetes as early as 12 weeks of age, the process of β-cell destruction can be well advanced by this time. It was therefore of interest to know whether HLA-A*0201–restricted T-cell responses to insulin epitopes were also present in the early pre-diabetic period, which would suggest an important role for these CD8$^+$ effectors in the initiation of the pathogenic process. Thus, we examined 5- and 8-week-old female NOD.*β2m*^{null}.*HHD* mice and found that insulin-specific T-cell responses are indeed present in these younger age-groups (Fig. 4). T-cell responses to IGRP peptides in young mice were also noted.

Ins1 B5–14 and Ins1/2 A2–10 differ from their human counterparts by only a single residue (Fig. 5*A*). For the purpose of evaluating the potential translation of our epitope mapping studies to human type 1 diabetes, it was important to determine whether human (h) Ins B5–14 and A2–10 could also be recognized by T-cells in the context of HLA-A*0201. To investigate Ins2/hIns B5–14 as a potential antigen in type 1 diabetes, we investigated the ability of this peptide to be recognized by islet-infiltrating T-cells isolated from multiple NOD.*β2m*^{null}.*HHD* mice. As shown in Table 1, a subset of the mice that responded to Ins1 B5–14 also showed some, albeit usually less vigorous, reactivity to the Ins2/hIns version. In these cases, reactivity to the Ins2/hIns peptide may represent cross-reaction



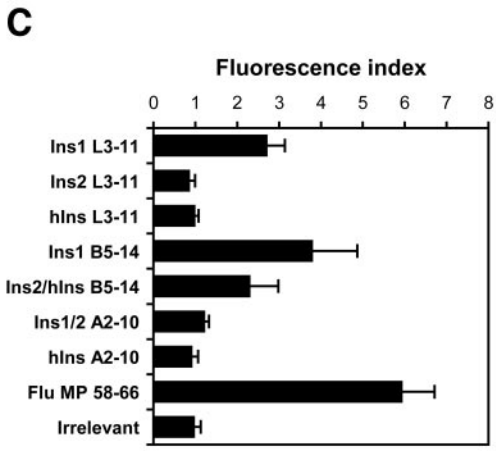


FIG. 5. Determination of the HLA-A*0201 binding capability of Ins L3–11, B5–14, and A2–10. *A*: The antigenic peptides identified in our library screens are shown in bold. Residues conserved in the Ins2 or human equivalents of these peptides are underlined. *B*: T2 cells were incubated in the presence or absence of the indicated peptides and stained for HLA-A*0201 using fluorescein isothiocyanate–conjugated BB7.2. Flu MP 58–66 is a well-characterized HLA-A*0201–binding peptide and was used as a positive control. The H-2K^d–binding peptide IGRP 206–214 was used as a negative control. Filled histograms, no peptide; open histograms, plus peptide. In *C*, fluorescence indexes were defined as the mean fluorescence intensity of the experimental sample divided by the mean fluorescence intensity in the absence of peptide. Bars represent the mean ± SD of two separate experiments.

by Ins1-reactive T-cells or it may represent T-cells specific only for Ins2/hIns present in the islet infiltrates. The reactivity profile of mouse 2 (Table 1), which responded strongly to Ins2/hIns B5–14 and only minimally to its Ins1 equivalent, indicates that Ins2/hIns B5–14 can be independently antigenic in the context of HLA-A*0201. However, in the case of the other mice, it is not possible to discriminate between the two possibilities, which are not mutually exclusive, and it remains possible that in some instances, T-cells can cross-react with both peptides. We

TABLE 1
Recognition of hIns B5–14 and A2–10 by islet-infiltrating T-cells from NOD.β*2m^{null}.HHD* mice

	Spot-forming cells*			Spot-forming cells*	
Mouse	Ins1 B5–14	Ins2/hIns B5–14	Mouse	Ins1/2 A2–10	hIns A2–10
1	82	2	1	217	0
2	5	281	2	261	96
3	125	67	3	282	105
4	79	2	4	519	204
5	63	2	5	252	87
6	302	0			
7	183	20			
8	168	14			
9	216	1			
10	315	8			
11	170	6			

*Islet-infiltrating T-cells were cultured individually from nondiabetic female NOD.β*2m^{null}.HHD* mice, and their reactivity to the indicated peptides was measured by IFN-γ ELISPOT. Numbers of spot-forming cells per 2×10^4 cells are indicated.

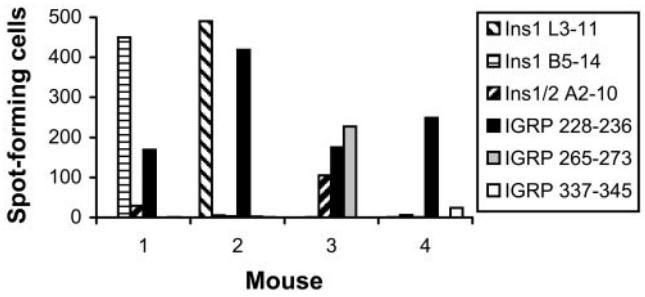


FIG. 6. The antigenic insulin and IGRP peptides identified using T-cells from pre-diabetic NOD.β*2m*^{null}.*HHD* mice are also recognized after the onset of overt disease. Islet-infiltrating T-cells were cultured individually from four diabetic female NOD.β*2m*^{null}.*HHD* mice, and their reactivity to the indicated peptides was monitored by IFN-γ ELISPOT. Numbers of spot-forming cells per 2×10^4 cells are indicated.

next investigated whether T-cells that recognize Ins1/2 A2–10 could cross-react with the human equivalent of this peptide, which differs by a single conservative substitution (Fig. 5*A*). Four of the five T-cell cultures examined showed appreciable cross-reaction (Table 1), suggesting that, because of the structural similarities of the peptide/MHC complexes, T-cells recognizing Ins1/2 A2–10 can also recognize hIns A2–10. Taken together, these results indicate that hIns B5–14 and A2–10 can be presented by HLA-A*0201 and may be relevant to human type 1 diabetes. Experimental proteasomal cleavage sites within human insulin have been reported at the COOH-termini of both of these peptides (41), lending further support to their potential relevance in humans.

A cell-based MHC stabilization assay was next used to probe the ability of the antigenic peptides identified here to bind to HLA-A*0201. Ins1 L3–11 and Ins1 B5–14 showed readily detectable binding to HLA-A*0201 (Fig. 5), although neither peptide bound as well as the positive control peptide (Flu MP 58–66). Ins2/hIns B5–14 also showed measurable binding. In contrast, Ins2 and hIns L3–11, which differ from Ins1 L3–11 by three or four residues, respectively, did not show detectable binding. Interestingly, Ins1/2 A2–10, the immunodominant insulin epitope, did not bind detectably to HLA-A*0201, nor did its human counterpart, although both peptides are readily recognized by HLA-A*0201–restricted T-cells (Fig. 3 and Table 1). A likely explanation for the inefficient binding of the A2–10 peptides is that they lack preferred residues at both of the HLA-A*0201 anchor positions, i.e., L or M at position 2 and V or L at the COOH-terminus (42). This result is reminiscent of Ins1/2 B15–23, which is targeted by H-2K^d–restricted CD8$^+$ T-cells in standard NOD mice, yet binds very poorly to H-2K^d (36,43).

The ability of hIns B5–14 and A2–10 to be recognized by HLA-A*0201–restricted T-cells (Table 1) suggests they may be excellent candidates for applicability to humans, e.g., to form the basis of a peptide-based intervention or for trial monitoring of new-onset patients undergoing intervention therapies. However, we identified these epitopes using T-cells from pre-diabetic NOD.*β2m*^{null}.*HHD* mice. To confirm that they are also targeted after the onset of overt disease, we isolated T-cells from the residual islets of diabetic female NOD.*β2m*^{null}.*HHD* mice that had not received exogenous insulin therapy (Fig. 6). We found that each of the insulin epitopes identified here was recognized by at least one diabetic mouse. This suggests the potential applicability of the human counterparts of these CD8$^+$ T-cell epitopes for the development of peptide-based intervention or monitoring strategies in both pre-diabetic and new-onset type 1 diabetic patients.

Our ELISPOT results indicated that T-cells capable of secreting IFN-γ in response to insulin peptides are present in the islets of NOD.*β2m*^{null}.*HHD* mice. The pathogenicity of these T-cells would be further supported if they were shown to be cytotoxic in vivo. To address this, we modified an in vivo assay, previously used to demonstrate cytotoxicity after immunization with a peptide (44,45), to instead examine the ability of spontaneously arising autoreactive T-cells to kill target cells presenting insulin peptides. In our assay, three populations of splenocytes are differentially labeled with fluorescent dyes, allowing their detection by flow cytometry. Two of the populations are pulsed with target peptides and a third with an irrelevant peptide. After mixing, the cells are injected into NOD.β*2m*^{null} mice, which lack CD8$^+$ T-cells and therefore act as a negative control, and into recipient autoimmune-prone mice. Sixteen hours later, pancreatic lymph nodes, where injected cells would be expected to encounter β-cell autoantigen-specific T-cells, are retrieved and cells are analyzed by flow cytometry. Lysis of a population can be detected as a decrease in the ratio between the target and control populations in the autoimmune-prone mice compared with the ratio in NOD.β*2m*^{null} mice. To demonstrate the assay and its applicability, we performed an experiment with well-established targets of CD8$^+$ T-cells in NOD mice, i.e., IGRP 206–214 (40) and Ins1/2 B15–23 (36). As seen in Figs. 7*A* and *B*, specific lysis of populations pulsed with both target peptides could be detected. These results were further validated by the finding that peptide reactivity of T-cells cultured from the islets of the same mice, measured by IFN-γ ELISPOT (Fig. 7*C*), correlated with the cytotoxicity observed in the pancreatic lymph nodes. For example, NOD Mouse 4 showed high in vivo cytotoxicity against IGRP 206–214-bearing targets and a strong response to this peptide by islet-infiltrating T-cells. To our knowledge, this is the first time that an in vivo cytotoxicity assay has been used to monitor the activity of spontaneously arising autoreactive T-cells.

Using this system, we asked whether in vivo cytotoxicity could be detected against Ins1 B5–14 and Ins1/2 A2–10 in NOD.*β2m*^{null}.*HHD* mice (Figs. 7*D* and *E*). Indeed, we found that T-cells reactive with both epitopes are cytotoxic in vivo. These findings also correlate well with IFN-γ ELISPOT results using T-cells propagated from the islets of the same mice (Fig. 7*F*). To confirm that CD8$^+$ T-cells are required for cytotoxicity, we also performed the assay in five CD8$^+$ T-cell–depleted NOD.*β2m*^{null}.*HHD* mice. The average lysis observed in these mice was only 1.5% of that seen in nondepleted animals (data not shown). Thus, Ins1 B5–14 and Ins1/2 A2–10 are CD8$^+$ T-cell targets during the HLA-A*0201–restricted pathogenic autoimmune response in vivo.

DISCUSSION

CD8$^+$ T-cells reactive to several islet antigens, including insulin and IGRP, have been detected in the peripheral blood of type 1 diabetic patients directly ex vivo (23–28). The finite, and often small, amount of blood available from subjects requires investigators to limit the number of peptides that can be examined for T-cell reactivity. Thus, several criteria have been developed to select candidate peptides for these investigations. These include predicted or experimental binding to an HLA molecule of interest

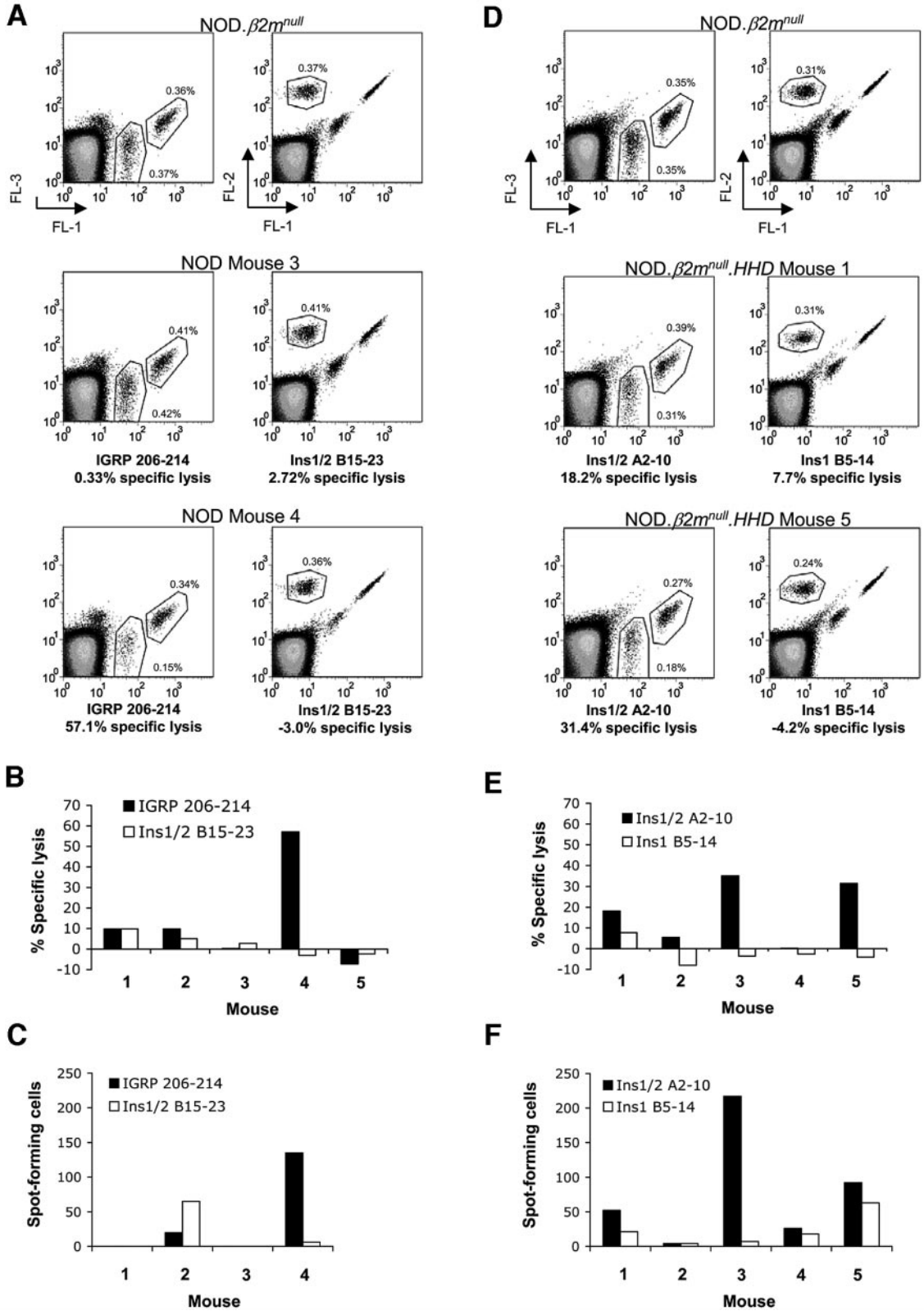


FIG. 7. Cells presenting Ins1 B5–14 and Ins1/2 A2–10 are lysed in vivo in NOD.β*2m^null^.HHD* mice. Donor splenocytes from NOD mice (*A–C*) were labeled with 5 μmol/l CFSE (FL-1^{high}), 0.5 μmol/l CFSE (FL-1^{low}), or 10 μmol/l CellTracker Orange (FL-2^{high}). The populations were pulsed with an irrelevant peptide, IGRP 206–214, or Ins1/2 B15–23, respectively. Cells were mixed and injected into NOD.β*2m^null^* and NOD mice. Pancreatic lymph nodes were removed 16 h later and analyzed by flow cytometry. Representative mice are shown in *A*. Specific lysis is reflected by a decrease in the ratio between the target and control populations in NOD mice compared with the ratio in NOD.β*2m^null^* mice. *B*: Specific lysis was calculated for all mice in the experiment represented in *A*. *C*: Islet-infiltrating T-cells were cultured from the same mice, and IFN-γ ELISPOT was performed. Numbers of spot-forming cells per 2 × 10^4 cells are indicated. The islet culture from Mouse 5 did not yield any T-cells. In *D–F*, splenocytes from NOD.β*2m^null^.HHD* mice were labeled with 5 μmol/l CFSE, 0.5 μmol/l CFSE, or 10 μmol/l CellTracker Orange and pulsed with an irrelevant peptide, Ins1/2 A2–10, or Ins1 B5–14, respectively. Cells were injected into NOD.β*2m^null^* and NOD.β*2m^null^.HHD* mice. Representative mice are shown in *D*, and specific lysis for all mice is given in *E*. *F*: IFN-γ ELISPOT results using islet-infiltrating T-cells from the mice in *D* and *E*.

(23–28,41), predicted or experimental evidence for proteasomal cleavage at the peptide's COOH-terminus (26–28,41), and immunogenicity when administered to an HLA transgenic mouse (23,41). These criteria, often used in combination, have been helpful in allowing the identification of candidate peptides that were subsequently shown to be recognized by peripheral T-cells of type 1 diabetic patients directly ex vivo. Our approach, whereby islet-infiltrating T-cells from diabetes-susceptible HLA transgenic NOD mice are used to identify candidate peptides, has also successfully predicted epitopes relevant to type 1 diabetes in humans (23,29) and presents several advantages. The candidate peptides identified here using spontaneously arising islet infiltrates are naturally processed (albeit by mouse β-cells that may process antigens for presentation differently from human β-cells), since T-cell reactivity to them is detected in the absence of immunization. As they are recognized by T-cells in the islet infiltrate, they are very likely to be disease relevant. Also, the peptides are known to be capable of binding the HLA molecule of interest in an immunologically relevant manner, regardless of the quality of binding revealed by a cell-based or biochemical assay. Importantly, Ouyang et al. (24) recently reported that the experimental binding affinity of β-cell peptides to HLA-A*0201 correlated inversely with the magnitude of the T-cell responses observed to those peptides in type 1 diabetic patients. T-cells reactive to weakly binding self-peptides may escape deletion in the thymus because of poor presentation. The fact that the candidate insulin epitopes reported here were not previously identified as peptides of interest using the aforementioned criteria demonstrates that our strategy is an important and useful complement to those that have been described by others. Furthermore, as shown by this work, our approach has the advantage that it allows us to determine whether the identified peptides are not only presented by HLA-A*0201 and elicit an IFN-γ response, but also whether they are targeted by pathogenic T-cells in vivo.

In NOD mice, *Ins2* is expressed in the thymus, while *Ins1* is not (46). The finding that *Ins2*-deficient NOD mice show accelerated disease led to speculation that thymic expression of *Ins2* permits negative selection of at least some pathogenic insulin-reactive T-cells (34,47). In the case of $CD4^+$ T-cells, evidence in support of this notion has been reported (47). Our studies in the NOD.β$2m^{null}$.*HHD* mice are consistent with the idea that Ins2-reactive $CD8^+$ T-cells are also eliminated by central tolerance. For example, two of the epitopes identified here (i.e., Ins1 L3–11 and Ins1 B5–14) are unique to Ins1. Further, in contrast to Ins1 B5–14, reactivity to the Ins2 version of this peptide was rare (Table 1), although the two differ by only a single residue and both show detectable binding to HLA-A*0201 in a cell-based assay (Fig. 5). Collectively, these findings are suggestive of negative selection of $CD8^+$ T-cells capable of recognizing Ins2 peptides. Although reactivity to A2–10, an epitope shared between Ins1 and Ins2, was detected in nearly all mice examined (Fig. 3), this peptide does not bind well to HLA-A*0201, at least as measured in our cell-based assay (Fig. 5). Similarly, B15–23 is shared between Ins1 and Ins2, is targeted by islet-infiltrating H-$2K^d$–restricted $CD8^+$ T-cells in standard NOD mice (36), and shows poor class I MHC binding (43). T-cells reactive to A2–10 and B15–23 likely escape central tolerance despite thymic expression of Ins2, as the peptides are unlikely to be well presented in the thymus because of poor MHC binding. In humans, the regulatory region of the insulin gene is polymorphic, with different alleles directing thymic expression to varying degrees (48,49). It is likely that the set of insulin peptides targeted in individual type 1 diabetic patients will also vary depending on the extent of thymic expression conferred by the insulin allele(s) that they carry.

We previously reported that, during the pre-diabetic period, individual NOD mice exhibit their own unique patterns of $CD8^+$ T-cell reactivity to different islet antigens, with a given mouse responding to as few as zero or as many as all three of the antigens examined (40). We now document parallel findings for the HLA-A*0201–restricted T-cell response in NOD.β$2m^{null}$.*HHD* mice. Whereas most mice responded to both insulin and IGRP, some responded to only one of the antigens and others to neither (Fig. 3*B*). Even among mice with insulin-responsive $CD8^+$ T-cells, there was variability in the number and identity of the targeted insulin peptides. Diabetic NOD.β$2m^{null}$.*HHD* mice also showed variation in their reactivity to peptides derived from insulin and IGRP (Fig. 6). Interestingly, all diabetic mice did not respond to all of the epitopes, as might have been predicted considering the phenomenon of epitope spreading. Such variation, even among inbred mice, strongly suggests that individual human type 1 diabetic patients will also show unique reactivity patterns to β-cell antigens. These results emphasize the importance of a more complete characterization of the epitopes targeted by $CD8^+$ T-cells in patients, since a diagnostic, monitoring, or therapeutic reagent based on a single one is unlikely to offer wide population coverage. Recent work indicates that the peptides targeted during the spontaneous development of the disease may also be important in the context of islet graft rejection (23,24, 26,28,50), providing an additional impetus for careful and continued epitope identification and validation.

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