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Beyond HLA-A*0201: New HLA-Transgenic Nonobese Diabetic Mouse Models of Type 1 Diabetes Identify the Insulin C-Peptide as a Rich Source of CD8+ T Cell Epitopes

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Type 1 diabetes is an organ-specific autoimmune disease characterized by T cell responses to β cell Ags, including insulin. Investigations employing the NOD mouse model of the disease have revealed an essential role for β cell-specific CD8+ T cells in the pathogenic process. As CD8+ T cells specific for β cell Ags are also present in patients, these reactivities have the potential to serve as therapeutic targets or markers for autoimmune activity. NOD mice transgenic for human class I MHC molecules have previously been employed to identify T cell epitopes having important relevance to the human disease. However, most studies have focused exclusively on HLA-A*0201. To broaden the reach of epitope-based monitoring and therapeutic strategies, we have looked beyond this allele and developed NOD mice expressing human β2-microglobulin and HLA-A*1101 or HLA-B*0702, which are representative members of the A3 and B7 HLA supertypes, respectively. We have used islet-infiltrating T cells spontaneously arising in these strains to identify β cell peptides recognized in the context of the transgenic HLA molecules. This work has identified the insulin C-peptide as an abundant source of CD8+ T cell epitopes. Responses to these epitopes should be of considerable utility for immune monitoring, as they cannot reflect an immune reaction to exogenously administered insulin, which lacks the C-peptide. Because the peptides bound by one supertype member were found to bind certain other members also, the epitopes identified in this study have the potential to result in therapeutic and monitoring tools applicable to large numbers of patients and at-risk individuals. The Journal of Immunology, 2012, 188: 5766–5775.

Type 1 diabetes is an organ-specific autoimmune disease in which T cell-mediated elimination of pancreatic islet β cells results in insulin insufficiency. Although the strong genetic determinant for disease susceptibility is the expression of predisposing class II MHC molecules (1), a series of studies have also uncovered an association with particular class I MHC alleles, including the common HLA-A*-0201 (2–11). This class I MHC association is not merely reflective of linkage disequilibrium with disease-promoting class II genes (4, 6, 9). Consistent with the idea that particular class I MHC molecules can foster diabetes development is the finding that CD8+ T cells specific for β cell Ags are present in the peripheral blood of type 1 diabetes patients (12). CD8+ T cells are also seen infiltrating the islets of new-onset and graft-recurrent type 1 diabetes patients, suggesting their contribution to β cell elimination (13–17). In the NOD mouse model of the disease, CD8+ T cells specific for β cell Ags are required pathogenic effectors (18) that have begun to demonstrate potential as therapeutic targets (19–24).

Peptide-based CD8+ T cell assays are showing promise as tools for the detection of β cell autoimmunity in recent-onset type 1 diabetes patients and islet transplant recipients, particularly when multiple T cell epitopes are simultaneously examined (25–27). Although it is true that the specific epitopes targeted can differ from one individual to another, it is clear from both NOD mouse models (28–30) and type 1 diabetes patients (25–27) that peptides derived from the β cell Ags insulin (Ins) and islet-specific...
glucose-6-phosphatase catalytic subunit-related protein (IGRP) are recognized by CD8+ T cells in most individuals. For example, in one study, 85% of recent-onset HLA-A*0201-positive patients showed T cell responses to the HLA-A*0201-binding peptide Ins L15–24, and one quarter showed responses to IGRP265–273 (27).

Although multiple β cell peptides have been identified as the antigenic targets of CD8+ T cells in type 1 diabetes patients, nearly all of these are recognized in the context of HLA-A*0201 (12). Although this is a common class I MHC molecule expressed in nearly 50% of certain ethnic groups (31), elucidation of the insulin and IGRP peptides recognized in the context of other human class I molecules would allow broader coverage of the patient population in terms of those that could benefit from the development of peptide-based predictive, diagnostic, and therapeutic strategies (32). In the past, we have used HLA-A*0201-transgenic NOD mouse models to identify epitopes of insulin and IGRP that are recognized by islet-infiltrating CD8+ T cells (28, 30, 33), and this strategy successfully identified epitopes (e.g., IGRP228–236, IGRP265–273, Ins B9–12, and Beckman Coulter), human β2m (TD99), H-2Kb (SPF-1.1), and H-2Db (K195). Splenocytes from NOD.IDCD and NOD mice were analyzed after staining with labeled Abs specific for murine CD4 (RM4-5), murine CD8α (53-6.7), and human CD8α (RPA-T8). All Abs for flow cytometry were obtained from BD Biosciences except where indicated.

Assessment of diabetes development
Mice were monitored weekly for glucosuria with Diasstrix reagent strips for urinalysis (Bayer). Mice were considered diabetic when two positive measurements were obtained. The time of onset of disease was recorded as the date of the first of the two positive readings.

Cyclophosphamide treatment
Female mice (12–21 wk age) were treated with two injections of 200 mg/kg cyclophosphamide (Sigma-Aldrich) i.p. 2 wk apart (47, 48). Diabetes development was monitored weekly after the first injection.

Peptides
Peptide libraries containing all of the 8-mer, 9-mer, 10-mer, and 11-mer peptides that can be derived from murine preproinsulin 1, preproinsulin 2, or IGRP were synthesized by Mimotopes using their proprietary Truncated PepSet technology. Each library mixture contained four peptides with a common C terminus and 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 mM) were prepared in 50% acetone/H2O, and 40 μM (i.e., ~10 μM 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 mM) were prepared in DMSO, and 10 μM working stocks were obtained by dilution in PBS.

Islet-infiltrating T cells
Pancreatic islets were isolated from female mice by collagenase perfusion of the common bile duct and islet-infiltrating T cells were cultured as described (49). Briefly, islets were handpicked and cultured for 7 d in 24-well cell culture plates (≈50 islets/well) in RPMI 1640 medium supplemented with 10% FBS (Hyclone), 1 mM sodium pyruvate, nonessential amino acids, 28 μM 2-ME, and 50 U/ml recombinant human IL-2 (PeproTech).

IFN-γ ELISPOT
IFN-γ ELISPOT was performed as described (49) using 2 × 105 islet-infiltrating T cells as responders and 2 × 103 syngeneic splenocytes pulsed with 1 μM peptide as APCs. Spots were developed after incubation at 37°C for 40 h and were counted using an automated ELISPOT reader system (Autoimmun Diagnostika). Where indicated, CD8+ T cells were first purified by negative selection from cultured islet infiltrates using magnetic separation (Miltenyi Biotec).

Tetramer staining of islet-infiltrating T cells
PE-labeled HLA-A*1101 tetramers were prepared following standard procedures (50) using a chimeric H chain (α1 and α2 domains of HLA-A*1101, and α3, transmembrane, and cytoplasmic domains from H-2Kb) or a fully human HLA-A*1101 or HLA-B*0702 and used these mice to identify β cell peptides recognized in the context of these human class I MHC molecules. Our characterization of these new strains has revealed the insulin C-peptide to be a rich source of CD8+ T cell epitopes. Responses to these epitopes should be of particular utility for immune monitoring of type 1 diabetes patients and islet-transplant recipients, as they cannot reflect an immune reaction to exogenously administered insulin, which lacks the C-peptide. Furthermore, HLA-A*1101 and HLA-B*0702 are representative members of two different HLA supertypes, which are groupings of MHC molecules that have been defined on the basis of structural similarities in the peptide-binding groove (39–41). Members of a supertype show overlap in peptide-binding repertoires (40, 42), and the β cell peptides we identified using one supertype member were found to also bind certain other members of that supertype, potentially resulting in monitoring and therapeutic reagents relevant to large numbers of patients and at-risk individuals.

Materials and Methods

Derivation of NOD.mβ2mnull.hβ2m mice transgenic for HLA-A*1101 or HLA-B*0702
C57BL/6 mice expressing a chimeric HLA-A*1101 H chain (α1 and α2 domains of HLA-A*1101, and α3, transmembrane, and cytoplasmic domains from H-2Kb) or a fully human HLA-B*0702 H chain were obtained from C. David (Mayo Clinic) and have been described previously (43, 44). Both strains were backcrossed to NOD mice for at least 10 generations and fixed to homozygosity for markers of NOD Idd loci, resulting in the NOD.hCD8 strain.

Flow cytometric analysis of splenocytes
Single-cell spleen suspensions were obtained by gentle grinding between frosted slides and passing through a 40-μm cell strainer. Splenocytes from NOD.mβ2mnull.hβ2m.HLA-A11, NOD.mβ2mnull.hβ2m.HLA-B7,

and murine β2m-expressing NOD.HLA-A11 mice were analyzed by multicolor flow cytometry after staining with labeled Abs to class I HLA H chains (B9.12.1; Beckman Coulter), human β2m (TD99), H-2Kb (SPF-1.1), and H-2Db (K195). Splenocytes from NOD.hCD8 and NOD mice were analyzed after staining with labeled Abs specific for murine CD4 (RM4-5), murine CD8α (53-6.7), and human CD8α (RPA-T8). All Abs for flow cytometry were obtained from BD Biosciences except where indicated.

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peptide was co-incubated at room temperature with 1 μM to 1 mM purified MHC in the presence of a mixture of protease inhibitors and 1 mM β2m. Following a 2-d incubation, MHC-bound radioactivity was determined by capturing MHC/peptide complexes on W6/32 (anti-class I) Ab-coated Lumitrac 600 plates (Greiner Bio-One), and measuring bound cpm using the TopCount microscintillation counter (Packard Instrument). In the case of competitive assays, the concentration of peptide yielding 50% inhibition of the binding of the radiolabeled peptide was calculated. Under the conditions used, where [MHC] and IC50 > [MHC], the measured IC50 values are reasonable approximations of the true Kd values (53, 54).

Each competitor peptide was tested at six different concentrations covering a 100,000-fold dose range, and in three or more independent experiments. As a positive control, the unlabeled version of the radiolabeled probe was also tested in each experiment.

Results

**NOD.mβ2mnull-hβ2m mice transgenic for HLA-A11 or HLA-B7 are diabetes-susceptible to varying degrees**

Splenocytes from NOD.mβ2mnull-hβ2m mice transgenic for HLA-A11 or HLA-B7 were analyzed by flow cytometry, which confirmed the cell-surface expression of the human β2m and HLA molecules (Fig. 1A, 1B). As expected, expression of the endogenous NOD class I MHC molecules H-2Kd and H-2Db was also observed (Fig. 1A, 1B).

Both the NOD.mβ2mnull-hβ2m.HLA-A11 and NOD.mβ2mnull-hβ2m.HLA-B7 mice were diabetes-susceptible, albeit to varying degrees (Fig. 2A, 2B) and far less so than standard NOD mice, which had a diabetes incidence of 90% (Fig. 2C). As had been reported previously (45), even non-HLA–transgenic NOD mice transgenic for HLA-A11 or HLA-B7 express the transgenic HLA molecule in addition to the endogenous murine HLA-B7 is poorly expressed in the absence of human β2m (45). NOD.HLA-A11 mice similarly harbor HLA-B7 and HLA-A11 restricted T cells specific for this β cell Ag, we used cultured islet-infiltrating T cells to screen, by IFN-γ ELISPOT assay, a peptide library containing all possible 8– to 11-mer sequences that can be derived from murine IGRP. The library was offset by one residue, and the peptide mixtures in the library contained four sequences with a common C terminus, but having a length of 8, 9, 10, or 11 residues. As shown in Fig. 3A, a strong T cell response was observed to peptide mixture 273. We next synthesized and tested the individual 8-, 9-, 10-, and 11-mer peptides comprising mixture 273 and found that the 9-mer peptide IGRP272–280, having the sequence AINSEFRL, was the minimal epitope recognized by T cells (Fig. 4A). This sequence is consistent with the peptide-binding motif reported for HLA-A11, that is, L, M, I, V, S, A, T, G, or N at position 2, and K or R at the C terminus (57). In-

**Identification of HLA-A11–restricted epitopes of IGRP and insulin**

We previously showed that IGRP is an important antigenic target of pathogenic CD8+ T cells in both standard (56) and HLA-A2–transgenic NOD mice (30). To determine whether NOD.mβ2mnull-hβ2m.HLA-B7 mice, similarly harbor HLA-A11–restricted T cells specific for this β cell Ag, we used cultured islet-infiltrating T cells to screen, by IFN-γ ELISPOT assay, a peptide library containing all possible 8– to 11-mer sequences that can be derived from murine IGRP. The library was offset by one residue, and the peptide mixtures in the library contained four sequences with a common C terminus, but having a length of 8, 9, 10, or 11 residues. As shown in Fig. 3A, a strong T cell response was observed to peptide mixture 273. We next synthesized and tested the individual 8-, 9-, 10-, and 11-mer peptides comprising mixture 273 and found that the 9-mer peptide IGRP272–280, having the sequence AINSEFRL, was the minimal epitope recognized by T cells (Fig. 4A). This sequence is consistent with the peptide-binding motif reported for HLA-A11, that is, L, M, I, V, S, A, T, G, or N at position 2, and K or R at the C terminus (57). In-

**FIGURE 1.** NOD.mβ2mnull-hβ2m mice transgenic for HLA-A11 or HLA-B7 express the transgenic HLA molecule in addition to the endogenous NOD class I MHC molecules H-2Kd and H-2Db. Splenocytes from (A) NOD.mβ2mnull-hβ2m.HLA-A11, (B) NOD.mβ2mnull-hβ2m.HLA-B7, or (C) NOD.HLA-A11 mice (expressing murine β2m) were analyzed by flow cytometry for cell surface expression of the indicated molecules. Open histogram, unstained; filled histogram, stained.
Interestingly, the peptide overlaps the HLA-A2–binding epitope IGRP265–273. Note that recognition of the 10- and 11-mer peptides from mixture 273 (Fig. 4A) most likely reflects proteolytic generation of the 9-mer peptide during the assay period. However, it cannot be ruled out that these longer peptides represent epitopes distinct from the 9-mer.

Peptide libraries representing the two insulin proteins expressed in mice, that is, Ins1 and Ins2, were screened similarly using islet-infiltrating T cells pooled from two independent sets of NOD.mβ2mnull.hβ2m.HLA-A11 mice (Fig. 3B, 3C). Several peptide mixtures elicited positive responses, some of which were observed with only one of the T cell pools (e.g., Ins281–82), and some of which were in common to both pools (e.g., Ins179). We identified Ins1 C21–30 (TLALEVARQK) as the minimal epitope present in mixture Ins179, and Ins2 C23–32 (TLALEVAQQK) was found to be the minimal epitope present in mixture Ins281 (Fig. 3D).

**FIGURE 2.** NOD.mβ2mnull.hβ2m mice transgenic for HLA-A11 or HLA-B7 are diabetes susceptible to varying degrees. (A–C) Female HLA-transgenic mice of the indicated strains and their corresponding nontransgenic littersmates were monitored weekly for diabetes development. (D) Female mice of the indicated strains (12–21 wk of age) were treated with two injections of 200 mg/kg cyclophosphamide i.p. 2 wk apart. Diabetes incidence was monitored weekly after the first injection. The horizontal axis indicates weeks following the first injection.
Although these two peptides differ at only a single position, their status as independent epitopes is suggested by the finding that some T cell pools responded only to mixture Ins179 and not to Ins281 (Fig. 3B, 4B).

When screening peptide libraries designed as were ours, a doublet of reactivity is sometimes seen. In this case, the true epitope is generally present in the first positive peptide mixture, but reactivity is sometimes observed to the next mixture due to proteolytic generation of the epitope from a longer peptide during the assay period. This likely explains the reactivity observed to Ins2 mixtures 81 and 82, as we did not see reactivity to one but not the other in any of our experiments (Fig. 3C and data not shown).

However, careful examination of our peptide library screening results revealed that the doublet of reactivity observed for Ins1 mixtures 79 and 80 (Fig. 3B) likely represents two independent epitopes, as the T cells tested in Fig. 3C responded to mixture 79 but not mixture 80. To confirm that mixture 80 contained an epitope independent from Ins1 C21–30 (the peptide responsible for reactivity to mixture 79), we identified another T cell pool that showed reactivity to both mixtures, and we screened it against the individual peptides comprising these mixtures. If reactivity to mixture 80 was due to proteolytic cleavage of a peptide generating Ins1 C21–30, we would have expected the 11-mer of this mixture to give the strongest response. However, we found instead that the...
10-mer was the minimal epitope responsible for the reactivity observed to mixture Ins1 80 (Fig. 5). Taken together with our peptide library screening results (Fig. 3B, 3C), these findings confirm the 10-mer peptide Ins1 C22–31 as an independent epitope.

T cells cultured from islets of NOD.mβ2mnull.hβ2m mice according to our protocol generally contained nearly 90% CD8+ T cells (Fig. 4D). Nonetheless, to confirm that CD8+ T cells were indeed responsible for the IFN-γ responses we observed, we magnetically separated CD8+ and CD8- populations and tested them for reactivity to the predominant epitope peptides, which are also expressed in the NOD.mβ2mnull.hβ2m.HLA-A11 mice (Fig. 1A). When we stained islet-infiltrating T cells from these mice with peptide/HLA-A11 tetramers (con-

![Image](file)

**FIGURE 5.** Ins1 C21-30 and Ins1 C22-31 are independent C-peptide–derived T cell epitopes in NOD.mβ2mnull.hβ2m.HLA-A11 mice. Islet-infiltrating T cells from a diabetic female NOD.mβ2mnull.hβ2m.HLA-A11 mouse were tested by IFN-γ ELISPOT for reactivity to peptide mixtures Ins130 and Ins140 as well as the individual peptides making up those mixtures. The peptide sequences are shown next to their corresponding bars, and the minimal epitopes for each mixture are shown in bold. Similar results were obtained using islet-infiltrating cells from nondiabetic mice (data not shown).

Table I. MHC binding capacity of mouse peptides recognized by islet-infiltrating HLA-restricted T cells in HLA-A*1101-transgenic NOD mice and their human equivalents

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*Bold and underlined letters indicate amino acid identity between murine and human peptides.

**Epitope mapping using HLA-B7–transgenic mice**

Because the low spontaneous disease incidence observed in the HLA-B7–transgenic NOD.mβ2mnull.hβ2m mice (Fig. 2B) made it difficult to collect sufficient numbers of islet-infiltrating T cells, it was not possible to do the extensive screens and characterizations carried out for the HLA-A11–transgenic mice. However, given our identification of three C-peptide–derived epitopes in the HLA-A11–transgenic mice, we used islet-infiltrating T cells from the HLA-B7–transgenic mice to perform a limited screen of the Ins1 peptide library that was restricted to the C-peptide and its flanking regions. Remarkably, mixture 73 elicited a positive T cell response (Fig. 6). Based on the peptide binding motif described for HLA-B*0702 (P at position 2, and L at the C terminus) (58), the 10-mer peptide in this mixture (Ins1 C15–24) is the minimal epitope responsible for the activity observed. Consistent with this idea is our finding that the 10-mer peptide Ins1 C15–24 bound very well to HLA-B*0702 (IC50 of 4.4 nM in a competitive binding assay; Table II).

The identified epitopes bind multiple members of the corresponding HLA supertype

Members of an HLA supertype show overlap in peptide-binding repertoires (40, 42). To determine whether the epitopes we identified using HLA-A11–transgenic NOD mice were capable of binding other alleles of the A3 supertype, we performed competitive peptide-binding assays under conditions where the measured IC50 is a reasonable approximation of the true KD (Table I). Marine IGRP272-280 exhibited strong to intermediate binding (IC50 < 500 nm) not only to HLA-A*1101, but also to three other A3 supertype members (A*0301, A*3101, and A*6801). Additionally, binding to other A3 supertype alleles was detectable, albeit with weak affinity (IC50 between 500 and 5000 nM; A*3301 and A*6601). Importantly, human IGRP272-280 also showed binding to A*1101 and to certain other members of the A3 supertype, albeit to varying degrees and generally lower affinity, perhaps reflective of the fact that it bears a less optimal A3 supertype motif. In the case of the murine insulin epitopes we identified and their human equivalents, some of these bound multiple members of the corresponding HLA supertype also (Tables I, II).

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*Recently reported as an HLA-A3-restricted epitope in humans (26).*
FIGURE 6. Islet-infiltrating T cells from NOD.mβ2mnull.hβ2m.HLA-B7 mice recognize a C-peptide–derived insulin epitope. Islet-infiltrating T cells from four 15- to 24-week-old female NOD.mβ2mnull.hβ2m. HLA-B7 mice were used to conduct a partial screen of an Ins1 peptide library (C-peptide and flanking regions; mixtures 52–84) by IFN-γ ELISPOT using syngeneic splenocytes pulsed with 1 μM peptide as APCs. The number of the reactive mixture is indicated, and the individual peptides making up that mixture are shown. The predicted minimal epitope is shown in bold.

NOD.hCD8 mice are diabetes-susceptible

Our mapping of HLA–A11–restricted epitopes using our HLA-transgenic NOD.mβ2mnull.hβ2m mouse was likely aided by the chimeric nature of the transgenic HLA-A11 molecule in which the α3 domain was derived from the murine class I MHC molecule H-2Kb. This would be expected to facilitate HLA–A11–restricted T cell responses, as murine CD8 interacts preferentially with the murine α3 domain compared with the human α3 domain (59, 60). Future epitope mapping studies employing NOD mice transgenic for fully human class I MHC chains would thus likely benefit from the coexpression of human CD8. To this end, we took advantage of a previously described strain of C57BL/6 mice transgenically expressing human CD8 under the control of the murine T cell-specific p56ck proximal promoter (46). We used these mice to introduce human CD8 to NOD mice, backcrossed for at least 10 generations, and then analyzed T cell populations and diabetes incidence in the newly developed NOD.hCD8 strain. As reported for the C57BL/6 mice expressing human CD8 (46), the murine CD4+/murine CD8+ T cell ratio was unaltered by the expression of human CD8, and human CD8 was expressed on both murine CD4+ and murine CD8+ T cells (Fig. 7A). Diabetes development in NOD.hCD8 females was as observed for NOD mice (Fig. 7B). This supports the idea that introduction of hCD8 to NOD models transgenic for fully human class I MHC chains will increase their usefulness for epitope mapping studies. Such work is already in progress in our laboratory.

Discussion

Although multiple β cell peptides have been identified as the antigenic targets of CD8+ T cells in type 1 diabetes patients, nearly all of these are recognized in the context of HLA-A*0201 (12). Thus, they are only relevant to a subset of patients (32). Although the gene frequency of HLA-A*1101, used in this study, is generally lower than that of HLA-A*0201, for example, 7 and 27% among the white population in the United States, respectively (61), HLA-A*1101 is a representative member of the A3 supertype, which has a phenotypic frequency of 38–53%, depending on the ethnic group examined (39). This compares favorably to the phenotypic frequency of 39–46% for the A2 supertype (39), of which HLA-A*0201 is a member. In addition to HLA-A*1101, other A3 supertype members include A*0301, A*3101, A*3301, and A*6801 (39). The A3 supertype is characterized by a B-pocket specificity (position 2 of the peptide) for the amino acids A, I, L, M, V, S, or T, and an F-pocket specificity (peptide C terminus) for R or K (39). Importantly, this supertype has been functionally verified, meaning that it has been established that a peptide that binds one member of the supertype will likely bind other members as well (40). This led us to hypothesize that antigenic β cell peptides identified using the HLA-A11–transgenic NOD mice would have relevance to humans expressing other members of the A3 supertype. This has now been confirmed by a very recent report that identified the human equivalent of an epitope we mapped using our mice (i.e., human Ins C23–32, and having the sequence PLAEGSLQ; Table I) as being recognized in the context of HLA-A3 in type 1 diabetes patients (26). This finding, coupled with our MHC binding results (Table I), supports the idea that the other epitopes we identified using the HLA-A11–transgenic NOD mice should also be examined for their relevance in patients.

When the transgenic HLA-A11 molecules expressed in NOD mice contained murine β2m, diabetes incidence was much higher than that observed when human β2m was expressed instead (Fig. 1A, 1C). Nonetheless, we chose to conduct our epitope mapping studies in the context of human β2m, so that the transgenic HLA molecules would resemble their human counterparts as closely as possible. For example, HLA class I H chains associated with murine β2m appear to take on an altered structure (as measured by Ab reactivity) compared with those associated with human β2m (62). This suggests that the peptide-binding repertoire might also be altered. Interestingly, we searched for T cell responses to our newly identified HLA-A11–binding epitopes IGRP 272–280 and Ins1 C21–30 using islet-infiltrating T cells derived from 13 NOD.HLA-A11 mice (expressing murine β2m), and we were unable to detect reactivity (data not shown). Although this might reflect altered peptide binding, it could instead, or in addition, be due to an altered T cell repertoire. Murine class I MHC molecules containing human β2m are apparently nondiabetogenic and fail to support the development of β cell–specific T cell responses (45). Thus, in the NOD.mβ2mnull.hβ2m.HLA-A11 strain, β cell–specific T cells restricted to the transgenic HLA molecules may enjoy reduced competition from those restricted to the murine class I MHC molecules.

Despite the diabetes-protective effect of hβ2m in NOD.mβ2mnull.hβ2m mice alluded to immediately above (45), diabetes susceptibility is restored when the HLA-A2 H chain is also expressed (63). In contrast, expression of either the HLA-A11 or the HLA-B7 H chain in NOD.mβ2mnull.hβ2m mice is insuf-
whereas HLA-A11 and HLA-B7 are not (4, 6). HLA-A2 is a susceptibility allele for type 1 diabetes in humans. This intriguing phenomenon may be related to the finding that fore or between dibasic residues, its involvement in the generation of viral epitope (ELFSYLIEK) has recently been reported (64). In that independent generation of the C terminus of an HLA-A3–restricted candidate T cell epitopes in human studies. Proteasome-inde-pendent generation of the C terminus of an HLA-A3–restricted of four such peptides in our work (Ins1 C21–30, Ins2 C23–32, and Ins1 C22–31 for HLA-A11, and Ins1 C15–24 for HLA-B7) (Fig. 8) establishes the C-peptide as a rich source of disease-relevant C#8 T cell epitopes. In humans, reactivities to C-peptide epitopes may be particularly informative as they cannot represent an immune response to exogenously administered insulin.

It is possible that the C termini of some of the peptides identified in this study (Ins1 C21–30 and Ins2 C23–32) may be generated independently of the proteasome. This is an important point, as protea-soomal cleavage is often taken as a prerequisite for the examination of candidate T cell epitopes in human studies. Proteasome-inde-pendent generation of the C terminus of an HLA-A3–restricted viral epitope (ELFSYLIEK) has recently been reported (64). In that study, the peptide C terminus was instead found to be generated by the cytosolic endopeptidase nardilysin. As nardilysin cleaves before or between dibasic residues, its involvement in the generation of Ins1 C21–30 and Ins2 C23–32 (Fig. 8) should be consid-ered. Similar to HLA-A*0201 and A*1101, B*0702 is also a member of a well-characterized and functionally verified HLA supertype, that is, the B7 supertype (42). Although in this study we were only able to conduct limited epitope mapping studies using the NOD.

This intriguing phenomenon may be related to the finding that HLA-A2 is a susceptibility allele for type 1 diabetes in humans, whereas HLA-A11 and HLA-B7 are not (4, 6).

Spontaneous CD8 T cell responses to only a limited number of epitopes entirely contained within the insulin C-peptide (hIns C20–28 for HLA-A2 and HLA-A3, and hIns C23–32 for HLA-A3) have been reported previously (Fig. 8) (25, 26). Our identification of four such peptides in our work (Ins1 C21–30, Ins2 C23–32, and Ins1 C22–31 for HLA-A11, and Ins1 C15–24 for HLA-B7) (Fig. 8) establishes the C-peptide as a rich source of disease-relevant C#8 T cell epitopes. In humans, reactivities to C-peptide epitopes may be particularly informative as they cannot represent an immune response to exogenously administered insulin.

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Figure 7. NOD.hCD8 mice exhibit a normal murine CDP8 T cell ratio and are diabetes-suscep-tible. (A) Splenocytes from NOD and NOD.hCD8 mice were analyzed by flow cytometry for cell surface expression of the indicated markers. Numbers denote the percentage of cells in the corresponding quadrants. (B) Female NOD and NOD.hCD8 mice were monitored weekly for diabetes development. The two incidence curves were not statistically different (p = 0.48; log rank test).

Figure 8. CD8 T cell epitopes of insulin contained within the C-peptide and targeted by spontaneous T cell responses. An alignment of murine insulin I and insulin 2 and human insulin is shown. Promhoerone convertase cleavage sites are denoted by arrows, and residues trimmed by carboxypeptidase E are depicted in black. C-peptide epitopes identified in the current study are indicated with solid brackets, while those identified in patients and reported elsewhere (25, 26) are marked with dashed brackets. The restricting MHC is noted in each case.

m#2mnull.h#2mnull.HLA-B7 mice, we did successfully identify Ins1 C15–24 as an Ag (Fig. 6). Importantly, the human counterpart of Ins1 C15–24 (i.e., Ins C15–26) is capable of binding to B*0702 with high affinity (Table II). To our knowledge, only one other HLA-B7–binding epitope derived from a β cell Ag (Ins L4–13) has been identified to date (26). As the B7 supertype has a phenotypic frequency of 43–57% (39), further investigation is clearly warrant-ed. We anticipate that introduction of human CD8 from our newly developed NOD.hCD8 strain (Fig. 7) to the HLA-B7–transgenic NOD mice will further increase their utility for Ag discovery experiments (59, 60).

Although we have not yet proven directly that the T cell reactive-tivities we have identified in this study are pathogenic, their presence within the pancreatic islets prior to disease development (Figs. 3A, 3B, 4A–C, 6 and data not shown) certainly supports this idea, as does their secretion of IFN-γ in response to Ag stimulation. Also, we have previously shown, using both standard and HLA-A2–transgenic NOD mice, that T cell specificities identified using islet-infiltrating cells and IFN-γ ELISPOT assays do indeed exhibit cytoxicity in vivo (28). In addition to using islet-infiltrating cells to identify disease-relevant Ags, our MHC bind-ing results suggest that it is also important to look for candidate epitopes in an unbiased way, as we have done, rather than limit candidates to those that bind well to MHC. As seen in Tables I and II, the mouse epitopes we identified bound to MHC with a wide range of affinities. Despite these advantages of our epitope mapping approach, one drawback is that some of the peptides identified may not be relevant to the human disease due to sequence differences between the mouse and human versions. For example, human preproinsulin and IGRP are only ∼80% identical to their mouse counterparts. Ideally, then, human autoantigens would be introduced into HLA-transgenic NOD mouse models in the future. The establishment of NOD mice transgenically expressing human IGRP in β cells is an important first step toward this goal (65).

T cell epitope mapping continues to be a major research effort for a number of laboratories, and this interest is due, at least in part, to the potential of such work to identify peptides that could be useful for the development of preventive or therapeutic strategies. As suggested by a recent report (21), it is possible that manipulation of multiple specificities will be beneficial, if not required, so the more that is known about the relevant epitopes, the more successful the field is likely to be. Identification of new epitopes will also increase the utility of T cell assays, which have begun to show promise as tools for immune monitoring in type 1 diabetes, for example, to identify β cell autoimmunity in patients (25), to predict islet graft rejection (26, 27), or to confirm the immuno-logical efficacy of intervention therapies regardless of their clinical outcome (66), information that could be used to guide sub-
sequent clinical trials. When the A2, A3, and B7 supertypes are considered together, the population coverage provided ranges from 83 to 88%, depending on the population examined (39). Thus, increased knowledge of the β cell peptides recognized in the context of representative supertype members should result in immunological tools relevant to large numbers of individuals.

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Disclosures

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References


