HLA-A*0201-Restricted T Cells from Humanized NOD Mice Recognize Autoantigens of Potential Clinical Relevance to Type 1 Diabetes

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HLA-A*0201-Restricted T Cells from Humanized NOD Mice Recognize Autoantigens of Potential Clinical Relevance to Type 1 Diabetes

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In both humans and NOD mice, particular MHC genes are primary contributors to development of the autoreactive CD4+ and CD8+ T cell responses against pancreatic β cells that cause type 1 diabetes (T1D). Association studies have suggested, but not proved, that the HLA-A*0201 class I variant provides an important component of T1D susceptibility in humans. In this study, we show that transgenic expression in NOD mice of HLA-A*0201, in the absence of murine class I MHC molecules, is sufficient to mediate autoreactive CD8+ T cell responses contributing to T1D development. CD8+ T cells from the transgenic mice are cytotoxic to murine and human HLA-A*0201-positive islet cells. Hence, the murine and human islets must present one or more peptides in common. Islet-specific glucose-6-phosphatase catalytic subunit-related protein (IGRP) is one of several important T1D autoantigens in standard NOD mice. Three IGRP-derived peptides were identified as targets of diabetogenic HLA-A*0201-restricted T cells in our NOD transgenic stock. Collectively, these results indicate the utility of humanized HLA-A*0201-expressing NOD mice in the identification of T cells and autoantigens of potential relevance to human T1D. In particular, the identified antigenic peptides represent promising tools to explore the potential importance of IGRP in the development of human T1D. The Journal of Immunology, 2006, 176: 3257–3265.

T type 1 diabetes (T1D) in both humans and NOD mice is a multifactorial disease resulting from T cell-mediated autoimmune destruction of insulin-secreting pancreatic β cells. Although T1D is a polygenic disease, the primary susceptibility component in both organisms is particular combinations of MHC genes (reviewed in Refs. 1 and 2). Within the MHC, specific HLA-DQ and HLA-DR class II molecules provide a large component of T1D susceptibility in humans by mediating β cell-autoantibody CD4+ T cell responses. Similarly, the H-2Aβ7 class II variant provides an important component of T1D susceptibility in NOD mice. It is now clear that in addition to the pathogenic effects mediated by CD4+ T cells, T1D development also requires contributions from autoreactive MHC class I-restricted CD8+ T cells (3–8). Interestingly, the H-2Dβ and H-2Kβ class I molecules encoded within the H2β7 MHC haplotype of NOD mice are common variants also characterizing many strain lacking autoimmune pr- oclivity. However, efficient T1D development in NOD mice is dependent on expression of the class I genes characterizing the H2β7 haplotype (9–12). The ability of the H2β7-encoded class I variants to mediate the development of diabetogenic CD8+ T cell responses when expressed in NOD mice is due to interactive contributions from other susceptibility genes characterizing this strain (13, 14).

Association studies suggest certain MHC class I molecules, including the common variant HLA-A*0201 (hereafter designated HLA-A2.1), likely confer an additional risk factor for T1D development in humans when expressed in conjunction with particular class II susceptibility alleles, and perhaps other genes (15–21). Because it is difficult to directly assess the role of class I MHC molecules in T1D development in patients, we have used a “humanized” NOD mouse model to do so. Previously, we showed that T1D development is accelerated in NOD mice transgenically expressing the human HLA-A2.1 H chain (designated NOD.HLA-A2.1) (22). Furthermore, HLA-A2.1-restricted β cell-autoreactive CD8+ T cells can be isolated from islets of young transgenic NOD mice. However, NOD.HLA-A2.1 mice also express the murine class I MHC molecules H-2Kb and H-2Db, making it impossible to precisely define the ability of HLA-A2.1 alone to mediate autoreactive CD8+ T cell responses contributing to T1D development. To address this important issue, we used the chimeric monoclonal transgene construct designated HHD (23), which encodes human β2-microglobulin (β2m) covalently linked to the α1 and α2 domains of human HLA-A2.1, and the α3, transmembrane, and cytoplasmic domains of murine H-2Dβ. We transgenically introduced

**Abbreviations used in this paper:** T1D, type 1 diabetes; β2m, β2-microglobulin; IGRP, islet-specific glucose-6-phosphatase catalytic subunit-related protein; m, murine; h, human; DMK, dystrophia myotonica kinase; MFI, mean fluorescence intensity.
the HHDD construct into NOD mice and then crossed the transgenic mice to the NOD.β2mnull strain to eliminate expression of murine class I MHC molecules. In HLA-transgenic models, the frequency of CD8\(^+\) T cells restricted to human class I MHC molecules is often low due to the poor interaction between murine CD8 and the α3 domain of human class I MHC molecules (24). The presence of a murine α3 domain in HHDD molecules is designed to overcome this difficulty.

In this study, we show that NOD.β2mnull.HHDD mice are T1D-susceptible. This demonstrates that when expressed in the proper context, HLA-A2.1 alone can mediate sufficient β cell-autoreactive CD8\(^+\) T cell responses to elicit T1D. Studies were also conducted to determine whether NOD.β2mnull.HHDD mice allow identification of candidate β cell autoantigens that could be tested in future clinical studies as possible targets of pathogenic CD8\(^+\) T cells in HLA-A2.1-expressing T1D patients. CD8\(^+\) T cells isolated from NOD.β2mnull.HHDD mice were found to lyse human HLA-A2.1-positive islet cells. A subset of these T cells recognize the β cell Ag islet-specific glucose-6-phosphatase catalytic subunit-related protein (IGRP), previously identified as an important target of CD8\(^+\) T cells in standard NOD mice (25, 26). We have mapped three novel epitopes of IGRP that are recognized by HLA-A2.1-restricted T cells in NOD.β2mnull.HHDD mice. Importantly, one of these antigenic peptides is conserved between mouse and human IGRP, and the other two are similar. Therefore, humanized NOD.β2mnull.HHDD mice can be used to identify HLA-A2.1-restricted T cells and β cell autoantigens potentially relevant to human T1D.

**Materials and Methods**

**Mice**

NOD.Ltdvs mice are maintained at The Jackson Laboratory by brother-sister mating. Currently, diabetes develops in 90% of female and 63% of male NOD.Ltdvs mice by 30 wk of age. NOD.β2mnull (3) and NOD.Rag1null mice (27) have been previously described. The HHDD transgene, provided by F. Lemmeron (Institut Pasteur, Paris, France), has been described (23). The transgene was injected directly into NOD zygotes, and founder carrying the HHDD transgene were identified by PCR using forward primer 5’-CTTCTATCGAGTGGGCTAC-3’ and reverse primer 5’-CGGTTAGCTCTGGTGG-3’. Cell surface expression of transgenic HLA-A2.1 molecules in NOD.HHDD mice was confirmed by flow cytometry using FITC-conjugated mAb CR11-351 (28), provided by V. Engelland (University of Virginia, Charlottesville, VA). To eliminate expression of murine class I MHC molecules, the HHDD transgene was transferred to the previously described NOD.β2mnull background (3). The β2mnull mutation was fixed to homozygosity by backcrossing F₂ progeny to NOD.β2mnull mice, then intercrossing to establish the NOD.β2mnull.HHDD line. The HHDD transgene also was transferred to the NOD-Scid.Envβ2mnull background (29) and fixed to homozygosity (designated NOD-Scid.HHDD). All mice were housed under specific-pathogen-free conditions. All experiments involving mice were performed in compliance with federal laws and institutional guidelines and have been approved by an institutional animal care and use committee.

**Assessment of T1D and insulitis development**

T1D development was defined by glycosuric values of ≥3 as assessed with Ames Diastix (supplied by Miles Diagnostics). Rates of diabetes development in the indicated experimental groups were assessed for statistically significant differences by Kaplan-Meier life table analysis using Statview 4.5 (Abacus Concepts).

Insulitis levels were assessed in nondiabetic 8-wk-old female NOD and NOD.β2mnull.HHDD mice. Pancreata from these mice were fixed in Bouin’s solution, and sectioned at three nonoverlapping levels. Granulated β cells were stained with aldehyde fuchsin and leukocytes with an H&E counterstain. Islets (at least 20 per mouse) were individually scored as follows: 0, no lesions; 1, peri-insular leukocytic aggregates, usually periductal infiltrates; 2, <25% islet destruction; 3, >25% islet destruction; and 4, complete islet destruction. An insulitis score for each mouse was obtained by dividing the total score for each pancreas by the number of islets examined. Data are presented as mean insulitis scores ± SEM for each strain.

**Flow cytometric analysis of splenic leukocyte populations**

Single-cell suspensions of splenocytes were analyzed by multicolor flow cytometric analysis. Total B lymphocytes were detected by staining with FITC-conjugated pan-B mouse IgG (Southern Biotechnology Associates). Total T lymphocytes were detected by staining with FITC-conjugated CD3ε-specific mAb 145-2C11. Total T cells were then further characterized for CD4 expression using the mAb GK1.5 conjugated to the red fluoroscent tag Cy3.18-Osu (Cy3; Biological Detection Systems), or for CD8 expression with the mAb 53-6.2 conjugated to PE whose red fluorescence intensity can easily be distinguished from that of Cy3. CD8\(^+\) T cell populations were also analyzed by staining with PE-conjugated TCR β-chain-specific mAb RB4-68C5 and FITC-conjugated CD8-specific mAb 53-6.2. Expression of murine MHC class I molecules was assessed by staining with FITC-conjugated H-2K\(^+\) specific mAb SF1-1.1, FITC-conjugated H-2D\(^+\) specific mAb KH95, and FITC-conjugated pan murine class I MHC mAb M1/42. Expression of transgenic HLA-A2.1 molecules was assessed by staining with FITC-conjugated mAb BB7.2.

**Cytotoxicity assays using intact islets as targets**

Human pancreas that were not allocated for whole organ transplantation were obtained from organ procurement organizations (Center for Organ Recovery and Education, PA, and National Organ Procurement Interchange, Philadelphia, PA). Pancreata were harvested using standard multiorgan recovery techniques. Pancreatic islets were isolated using the semiautomated method described previously (30) with minor modifications. Pancreas dissociation was performed using multiple lots of Liberase (Liberase-HI, Roche). Enzymes were reconstituted and dissolved in cold 4°C HBSS. Before being used for immunocytochemical or filtration device, pancreas were intraduotally injected with enzyme solution in a recirculation system designed by Rajotte and colleagues (31). Islets were purified with a COBE 2991 cell separator using discontinuous Euro-Ficol gradients; the purity (islets/whole tissue) was assessed by di-thizone staining, as previously described (32). Preliminary HLA typing was performed by the laboratory of the local donor hospital (HLA-A*02/A*68 sample) or the typing laboratory of the Allegheny General Hospital (Pittsburgh, PA) (HLA-A*11/A*13 sample). High-resolution HLA typing was performed using the PEL-FREEZ SSP UniTray PCR-based method for specific HLA class I alleles as per the manufacturer’s directions (Dynal Biotech).

Mouse islets were isolated from female NOD-Scid.HHDD and NOD.Rag1null animals using a published collagenase inflation method (33). Islets were hand-picked for purity in HBSS under a dissecting microscope after isolation on a Histopaque 1119 (Sigma-Aldrich) gradient.

Cytotoxicity assays using intact islets as targets were performed as described (13, 34). Briefly, human, NOD-Scid.HHDD, and NOD.Rag1null pancreatic islets (10 islets/well) were allowed to adhere in 96-well plates during a 10-day incubation at 37°C in low-glucose DMEM. Adherent, monolayered islets were then labeled with 5 μCi/well of \(^{33}\)Cr for 3 h at 37°C. Islets were washed and cultured overnight in 100 μl of medium containing various numbers of cultured islet-infiltrating T cells from NOD.β2mnull.HHDD mice. For establishing E:T ratios, each islet was assumed to contain 750 cells. A minimum of three wells were established for each E:T ratio. Spontaneous release controls consisted of nine wells of labeled islets from each donor cultured in the absence of T cells. Following a 20-h incubation at 37°C, the radioactivity in two fractions from each well was measured. The first fraction was the culture supernatant, and the second was obtained by solubilizing the remaining islets in 200 μl of 2% SDS. The percentage of \(^{33}\)Cr release for each well was calculated by the formula ((supernatant cpm)/(supernatant cpm + SDS lysate cpm)) × 100%. This allowed us to normalize for the fact that due to variation in the sizes of individual islets, the total levels of \(^{33}\)Cr incorporation in each well could differ greatly. In turn, the percent-specific cytotoxicity was calculated by subtracting the percent spontaneous release from the release by each well of islets cultured with a given number of T cells. Percent-specific cytotoxicities against the different islet types were assessed for statistically significant differences by Bonferroni/Dunn analysis using SuperANOVA (Abacus Concepts).

**IGRP peptide library and individual peptide analysis**

A peptide library containing all of the 8-, 9-, 10-, and 11-mer peptides that could be derived from mouse IGRP was synthesized by Mimotopes using their proprietary Truncat PepSet technology. Each peptide mixture in the library contained four peptides with a common C terminus, but having a length of 8, 9, 10, or 11 residues. The four peptides in each mixture were

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present in approximately equimolar amounts. A library of 348 peptide mixtures was synthesized to cover the 355 aa of the IGRP protein. Concentrated peptide stocks (2.75 mM) were prepared in 50% acetonitrile/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). Murine (m) IGRP206–214 (VYLKTNVF), mIGRP226–236 (RLFGLDLLWSV), mIGRP227–236 (LF GIDL LLL SV), mIGRP228–236 (FGIDL LLSV), mIGRP229–236 (VLFGLLGFAI), mIGRP233–345 (ALIPYCVHM), human (h) IGRP233–245 (LNNEDLLLWSV), hIGRP (FAIPYVS VHS), and Flu-MP546–556 (GLI GFVF TFL) were synthesized by standard solid-phase methods using Fmoc chemistry in an automated peptide synthesizer (model 433A; Applied Biosystems), and their identities were confirmed by mass spectrometry. Concentrated stocks (10 mM) were prepared in DMSO, and 10 μM working stocks were obtained by dilution in PBS.

Propagation of islet-infiltrating T cells

Islet isolation by collagenase perfusion of the common bile duct was modified from a previously described protocol (35). Briefly, the bile duct was cannulated and the pancreas perfused with collagenase P (Roche). The inflated pancreas was removed and incubated at 37°C to digest exocrine tissue. Following dispersion of digested tissue and three washes with HBSS, islets were resuspended in HBSS containing DNaSe I (Worthington Biochemical) and handpicked using a siliconized micropipet under a dissecting microscope. Isolated islets were washed with 2% FBS in HBSS, resuspended in RPMI 1640 medium supplemented with 10% FBS (HyClone) and 50 U/mL recombinant human IL-2 (PeproTech) and cultured in humidified air, then transferred into V-bottom 96-well culture plates and incubated at 37°C for 40 h. IFN-γ secretion was detected with a second, biotinylated anti-murine IFN-γ mAb (XMG1.2; BD Pharmingen). Spots were developed using streptavidin-alkaline phosphatase (Zymed Laboratories) and 5-bromo-4-chloro-3-indolyl-phosphate/NBT chloride substrate (Sigma-Aldrich) and counted using an automated ELISPOT reader system (Autoimmun Diagnostika).

**Elispot**

ELISPOT plates (MAHA S45 10; Millipore) were precoated with anti-murine IFN-γ mAb (R4-6A2; BD Biosciences/BD Pharmingen) and blocked with 1% BSA (Fraction V; Sigma-Aldrich) in PBS. APC (mitomycin C-treated T2 cells; American Type Culture Collection) were added at 2 × 10⁵ cells/well and pulsed with 1 μM peptide. Cultured islet-infiltrating T cells were added at 2 × 10⁴ cells/well, and plates were incubated at 37°C for 40 h. IFN-γ secretion was measured as IFN-γ ELISPOT spots (mean insulitis score, 2.26 ± 0.27; BD Pharmingen). Transgenic HLA-A2.1 mice in the absence of murine MHC class I molecules (40). Although female nontransgenic NOD mice remained completely free of disease as previously reported (3–6), 55% of HHD transgenic females developed T1D by 30 wk of age (p = 0.006; Fig. 2C). Pancreata from 8-wk-old NOD mice were examined histologically for insulin levels (scale 0–4). This analysis indicated similar levels of leukocytic infiltration in NOD β2mnull/HHD mice (mean insulin score, 2.26 ± 0.50; n = 5) and NOD islets (2.01 ± 0.27; n = 4). Although female nontransgenic NOD mice remained completely free of disease as previously reported (3–6), 55% of HHD transgenic females developed T1D by 30 wk of age (p = 0.006; Fig. 2C). Pancreata from 8-wk-old NOD mice were examined histologically for insulin levels (scale 0–4). This analysis indicated similar levels of leukocytic infiltration in NOD β2mnull/HHD mice (mean insulin score, 2.26 ± 0.50; n = 5) and NOD islets (2.01 ± 0.27; n = 4). Although female nontransgenic NOD mice remained completely free of disease as previously reported (3–6), 55% of HHD transgenic females developed T1D by 30 wk of age (p = 0.006; Fig. 2C). Pancreata from 8-wk-old NOD mice were examined histologically for insulin levels (scale 0–4). This analysis indicated similar levels of leukocytic infiltration in NOD β2mnull/HHD mice (mean insulin score, 2.26 ± 0.50; n = 5) and NOD islets (2.01 ± 0.27; n = 4).
NOD.β2mnull.HHD mice generate T cells that can lyse HLA-A2.1-positive human pancreatic islets

T cells were propagated from the islets of NOD.β2mnull.HHD mice as described in Materials and Methods. Comparable percentages of CD8+ T cells were observed in cultures derived from NOD.β2mnull.HHD (68.96 ± 23.16%; n = 12) and NOD islets (67.27 ± 16.15%; n = 6). All the CD8+ T cells propagated from the islets of NOD.β2mnull.HHD mice must be HLA-A2.1-restricted, because murine MHC class I molecules are not expressed in this strain. Thus, we asked whether NOD.β2mnull.HHD T cell cultures would exhibit cytotoxicity against HLA-A2.1-positive human pancreatic islets.

When dissociated islet cells are used as targets in 51Cr-release cytotoxicity assays, they exhibit a high degree of spontaneous death that can complicate data interpretation. To overcome this problem, we recently developed an alternative assay in which intact islets are allowed to adhere and monolayer in a 96-well plate and are then used as targets (13, 34). Maintenance of cell-cell contact greatly enhances cell survival and leads to low spontaneous 51Cr-release values. Allowing the islets to adhere and monolayer increases the number of β cells that are accessible to effector T cells. Using this assay, we measured the cytotoxic activity of NOD.β2mnull.HHD T cell cultures against HLA-A2.1-positive or negative human pancreatic islets (Table II). As further controls, cytotoxic activity was also assessed against NOD-scid.HHD (H-2Db, H-2Kd, and H-2Kd-specific) and NOD.Rag1null (H-2Db, H-2Kd) islets. HLA-A2.1-restricted cytotoxicity against both human and mouse islets was observed. Although these results do not demonstrate the extent of overlap, they indicate that at least some subset of the antigenic peptides targeted by diabetogenic HLA-A2.1-restricted T cells are common to both mouse and human β cells. Although cultured islet-infiltrating T cells from NOD.β2mnull.HHD mice can recognize islet Ags, they were not able to recognize Con A blasts generated from HLA-A2-positive or HLA-A2-negative humans (data not shown). These collective results indicate that the cytotoxicity observed against HLA-A2.1-positive human islets is both HLA-A2.1-restricted and islet-specific.

IGRP peptides are targeted by HLA-A2.1-restricted T cells from NOD.β2mnull.HHD mice

Residues 206–214 of IGRP (IGRP206–214) represent the Ag recognized by the NOD-derived diabetogenic CD8+ T cell clone 8.3 (25). The pathogenicity of IGRP206–214-reactive T cells is well-established (41–45), and ~20–30% of CD8+ T cells in NOD pancreatic islets recognize this peptide (25). Previously, using a peptide-MHC tetramer reagent, we tested reactivity to IGRP206–214 among islet-infiltrating T cells at various prodromal stages of TID development (5, 7, 11, and 15 wk of age) in individual NOD mice (46). We detected an IGRP206–214 response in 67–100% of mice in all age groups tested. This response was more frequent than that to the other autoantigens examined, i.e., insulin and dystrophia myotonica kinase (DMK). Importantly, the human IGRP open reading frame is identical in length to murine IGRP and is highly homologous (85% identity; 93% similarity), though a T cell response against IGRP in human T1D patients has not yet been described. For these reasons, we used NOD.β2mnull.HHD mice to investigate IGRP as a possible source of HLA-A2.1-binding peptides targeted by diabetogenic CD8+ T cells.

To determine whether CD8+ T cells from NOD.β2mnull.HHD mice recognize IGRP peptides, we transiently transfected COS-7 cells with varying concentrations of a full-length murine IGRP cDNA expression construct in combination with an HHD (H-2Db), or H-2Kd cDNA expression construct. Transfected COS-7 cells were assessed for their ability to stimulate T cells propagated from the islets of NOD.β2mnull.HHD mice as evidenced by IFN-γ secretion (Fig. 3A). T cell responses were only elicited in a dose-dependent manner when target T cells were propagated with both the IGRP and HHD constructs. These results demonstrate that at least some portion of the HLA-A2.1-restricted islet-infiltrating T cells in NOD.β2mnull.HHD mice recognize an IGRP-derived peptide(s). In addition, the inability of islet-infiltrating T cells from NOD.β2mnull.HHD mice to recognize kidney-derived COS-7 cells transfected with the HHD construct alone further demonstrates that...
the response of these effectors against human HLA-A2.1-positive islets shown in Table II is cell type-specific.

To identify the IGRP epitopes recognized by CD8\(^+\) T cells from NOD.\(^2\)mnull.HHD mice, we screened a peptide library containing all possible 8- to 11-mer sequences that can be derived from murine IGRP. The library consisted of peptide mixtures each containing four sequences with a common C terminus, but having a length of 8, 9, 10, or 11 residues. T cells were cultured from NOD.\(^2\)mnull.HHD islets and their responsiveness was measured by IFN-γ ELISPOT assay using HLA-A2.1-expressing human T2 cells as APC. Responses were observed to peptide mixtures 229 and 338–340 (Fig. 3B). Islet-infiltrating T cell cultures contained, on average, 70% CD8\(^+\) T cells. However, to ensure we were not detecting CD4\(^+\) T cell responses, the peptide library was screened again using purified CD8\(^+\) T cells cultured from the islets of an independent group of NOD.\(^2\)mnull.HHD mice. These CD8\(^+\) T cells responded to mixtures 229 and 266 (Fig. 3C), while purified islet-derived CD8\(^+\) T cells from a third group of mice responded to mixtures 229, 266, and 338 (data not shown).

Next, we screened the IGRP peptide library using T cells propagated from NOD.HHD mice that express endogenous H2\(^b\)\(^*\)-encoded class I MHC molecules (H-2D\(^b\) and H-2K\(^d\)) in addition to the transgenic HLA-A2.1 variant (Fig. 3D). As expected, we detected a strong response to peptide mixtures 206–210, corresponding to the H-2K\(^d\)-binding IGRP206–214 epitope. In contrast, T cells from NOD.\(^2\)mnull.HHD mice do not respond to IGRP206–214 (Fig. 3, B and C), consistent with the absence of H-2K\(^d\) expression in this strain. In addition to mixtures 206–210, T cells from NOD.HHD mice also released IFN-γ in response to peptide mixture 229. In separate screens using T cells from other NOD.HHD mice, responses to mixtures 266 and 338 were also observed (data not shown). These results support our hypothesis that acceleration of T1D in NOD.HHD mice can be attributed, at least in part, to addition of HLA-A2.1-restricted CD8\(^+\) T cell responses to those elicited by murine MHC class I molecules.

Screening of the peptide library using islet-infiltrating T cells from NOD.\(^2\)mnull.HHD mice (Fig. 3, B and C) detected multiple positive peptide mixtures (229, 266, and 338–340). As mentioned, each peptide mixture consists of four sequences having a common C terminus and varying in length from 8 to 11 residues (Fig. 4A). In mixtures 266 and 338, the 9-mer peptides agreed most closely with the peptide-binding motif described for HLA-A2.1 (i.e., most commonly 9-mers having L or M at position 2 and V or L at position 9) (47). Thus, we predicted that the 9-mer peptides mIGRP337–345 were responsible for the activity observed with mixtures 266 and 338, respectively. In the case of peptide mixture 229, the 9-mer peptide did not have a preferred residue at position 2. To determine the antigenic target in this mixture, we synthesized all four of the peptides contained within it. T cell reactivity to the 9-mer peptides from mixtures 266 and 338, and to all four peptides from mixture 229, was then assayed (Fig. 4B). Each graph shows a separate experiment using islet-infiltrating T cells cultured from individual 12-wk-old female NOD.\(^2\)mnull.HHD mice. In these experiments, one mouse showed a response to peptide mixtures 229 and 266, while the other showed a response to mixtures 266 and 338. The results clearly indicated the 9-mer peptides from mixtures 266 and 338 were antigenic. In the case of mixture 229, the 9-mer peptide was the minimal epitope. Recognition of the 10- and 11-mer peptides from this mixture suggests a proteolytic event generated the 9 mer during the assay period. Similarly, the response observed to mixtures 339 and 340 in the original screening likely reflects generation of the mIGRP337–345 9-mer peptide from longer sequences in each mixture.

### Table I. Splenocyte composition of NOD.\(^2\)mnull.HHD mice

<table>
<thead>
<tr>
<th></th>
<th>% B Cells</th>
<th>% CD4 Cells</th>
<th>% CD8 Cells</th>
<th>H-2D(^b) MFI</th>
<th>H-2K(^d) MFI</th>
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<tbody>
<tr>
<td>NOD</td>
<td>41.69 ± 0.77</td>
<td>31.04 ± 0.95</td>
<td>19.92 ± 0.44</td>
<td>40.25 ± 3.20</td>
<td>143.52 ± 6.24</td>
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<tr>
<td>NOD.(^2)mnull.HHD</td>
<td>46.36 ± 1.25</td>
<td>44.17 ± 1.16</td>
<td>2.59 ± 0.17</td>
<td>7.04 ± 0.54</td>
<td>5.96 ± 0.29</td>
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</table>

### Table II. Cultured islet-infiltrating T cells from NOD.\(^2\)mnull.HHD mice show cytotoxic activity against HLA-A2.1-positive human pancreatic islets

<table>
<thead>
<tr>
<th>E:T Ratio</th>
<th>HLA-A<em>0201/A</em>68 human islets</th>
<th>HLA-A<em>11/A</em>13 human islets</th>
<th>NOD-scid.HHD islets</th>
<th>NOD.Rag1null islets</th>
</tr>
</thead>
<tbody>
<tr>
<td>50:1(^a)</td>
<td>21.8 ± 1.6(^c)</td>
<td>2.4 ± 1.1</td>
<td>18.4 ± 2.7(^d)</td>
<td>1.4 ± 0.7</td>
</tr>
<tr>
<td>10:1(^a)</td>
<td>14.7 ± 0.5(^c)</td>
<td>2.8 ± 1.2</td>
<td>13.2 ± 3.1(^e)</td>
<td>0.6 ± 0.4</td>
</tr>
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</table>

\(^a\) Data represent the percentage of specific cytotoxicity exerted by the indicated target islets by cultured islet-infiltrating T cells from NOD.\(^2\)mnull.HHD mice. T cells from five 12- to 14-wk-old nondiabetic mice were pooled and used as effectors. CD8\(^+\) T cells were isolated from islets cultured in medium alone (i.e., spontaneous release) for each target was measured in nine independent wells to calculate specific cytotoxicity as described in Materials and Methods. Spontaneous release values were as follows: HLA-A*0201/A*68 human islets, 9 ± 0.4%; HLA-A*11/A*13 human islets, 20 ± 2.0%; NOD-scid.HHD islets, 10 ± 1.5%; NOD.Rag1null islets, 17 ± 1.3%.

\(^b\) The MFI values represent the mean of three independent experiments ± SEM.

\(^c\) Data represent the percentage of specific cytotoxicity exerted by the indicated target islets by cultured islet-infiltrating T cells from NOD.\(^2\)mnull.HHD mice. T cells from five 12- to 14-wk-old nondiabetic mice were pooled and used as effectors.

\(^d\) Differences between NOD.scid.HHD and NOD.Rag1null islet lines tested at the same E:T ratio (p < 0.001).

\(^e\) Significant differences from cytotoxicity against NOD.\(^2\)mnull.HHD islets tested at the same E:T ratio (p < 0.0001).

\(^f\) Three independent wells were set up to calculate average and SEM.

\(^g\) Differences between NOD.scid.HHD and NOD.Rag1null islet lines tested at the same E:T ratio (p < 0.001).

\(^h\) Differed significantly from cytotoxicity against NOD.\(^2\)mnull.HHD islets tested at the same E:T ratio (p = 0.0001).
To confirm the three newly identified IGRP peptides were indeed recognized in the context of HLA-A2.1, we assayed them by ELISPOT using T2 cells (HLA-A2.1) or RMA-S/Kd cells (H-2Db, H-2Kb, and H-2Kd) as APC (Fig. 4C). APC were washed twice after incubation with the peptides to minimize the quantity of free peptide available for binding to HLA-A2.1 molecules on the responding T cells and subsequent presentation independent of the APC. Clearly, T cell responses were much better when T2 cells were used as APC, though some response likely due to presentation by the T cells themselves was noted for mIGRP228-236 and mIGRP265-273. These results confirmed that recognition of the IGRP peptides occurs in an HLA-A2.1-restricted manner.

Individual NOD.β2mnull.HHD mice exhibit distinct patterns of CD8+ T cell reactivity to IGRP peptides

As shown in Fig. 3, B and C, and Fig. 4B, islet-infiltrating T cells exhibited different patterns of recognition to the three HLA-A2.1-binding IGRP peptides. To investigate this in more detail, we independently propagated T cells from the islets of 16 non-diabetic 12- to 13-week-old female NOD.β2mnull.HHD mice. Responses to the three peptides were measured by IFN-γ ELISPOT assay (Fig. 5A). As expected, the response to each IGRP epitope varied among mice; however, all exhibited a T cell response at least one of the peptides. When the data are summarized as in Fig. 5B, it is clear that peptide 228-236 is the immunodominant HLA-A2.1-binding epitope of mIGRP.

Characterization of the human counterparts of HLA-A2.1-binding murine IGRP peptides

Up to nearly one-tenth of all CD8+ T cells propagated from the islets of NOD.β2mnull.HHD mice are IGRP-reactive. This robust response to IGRP suggested recognition of IGRP peptides may have contributed to the cytotoxicity against HLA-A2.1-positive human islet cells demonstrated in Table II. To investigate this possibility, we compared the sequences of the antigenic mIGRP peptides with the corresponding regions of the human protein (Fig. 6A). IGRP265-273 was identical in both the murine and human molecules. The sequences of IGRP228-236 and IGRP337-345 differed in two amino acids each. Next, we compared the ability of the peptides to bind to HLA-A2.1 using an MHC stabilization assay (Fig. 6B). All three murine IGRP peptides demonstrated HLA-A2.1 binding, as expected. hIGRP228-236 also bound well to HLA-A2.1. However, we were unable to detect any binding of hIGRP337-345, perhaps due to the change of the anchor residue at position 2 from L to F (47). We then tested the ability of islet-infiltrating T cell cultures containing cells that recognized mIGRP228-236 or mIGRP337-345 to also recognize the corresponding hIGRP peptides. Probably due to its inability to bind HLA-A2.1, hIGRP337-345 was not recognized by T cells responding to the murine sequence (data not shown). In contrast, three of the eight T cell cultures derived from 12-week-old NOD.β2mnull.HHD mice that exhibited reactivity to mIGRP228-236 also clearly contained effectors capable of recognizing the human version of this peptide (Fig. 6C). There were no examples of cultures having reactivity to hIGRP228-236, but not its murine counterpart. These collective results suggest that a subset of T cells in certain cultures that recognize mIGRP228-236 are cross-reactive against the corresponding human peptide.

hIGRP228-236 and hIGRP337-345 (conserved between human and mouse) bind HLA-A2.1. Bulk T cell populations from NOD.β2mnull.HHD mice recognize both human HLA-A2.1-positive islets and these peptides. Taken together, these results suggest hIGRP228-236 and hIGRP337-345 may be presented by human HLA-A2.1-positive islets. Recognition of these IGRP peptides may account, at least in part, for the cytotoxic activity of CD8+ T cells from NOD.β2mnull.HHD mice against human HLA-A2.1-positive islets. Formal testing of this hypothesis will require the future derivation of clonal T cell populations specific for each of the IGRP peptides.

FIGURE 3. Cultured islet-infiltrating T cells from NOD.β2mnull.HHD mice recognize murine IGRP peptides. A, COS-7 cells were transiently transfected with varying concentrations of a murine IGRP cDNA expression construct and 1 µg/ml of an HHD (●), H-2Dq (○), or H-2Kd (□) cDNA expression construct. Cultured islet-infiltrating T cells from 12-week-old female NOD.β2mnull.HHD mice were added, and T cell response was measured as IFN-γ release by ELISA. T cells did not respond to COS-7 cells cotransfected with the murine IGRP expression construct and pcDNA3.1, nor did they respond to COS-7 cells cotransfected with the HHD expression construct and pcDNA3.1 (data not shown). B, Islets from four 10-week-old female NOD.β2mnull.HHD mice; C, four 11-week-old female NOD.β2mnull.HHD mice; or D, five 12-week-old female NOD.HHD mice were pooled to obtain sufficient numbers of T cells to screen the murine IGRP peptide library. Islets were cultured in the presence of IL-2, and islet-infiltrating T cells were harvested after 7 days. For the experiment depicted in C, CD8+ T cells were purified from the culture. Human T2 cells were used as APC and T cell response was measured by IFN-γ ELISPOT assay.
factors for T1D development (15–21). The frequency of high-risk class II alleles, (17). Also, among an independent group of U.S. children having associated with increased risk for developing islet autoimmunity U.S.-based study indicated the development of autoantibodies and progression to T1D before 5 CD8 these epidemiological studies suggested HLA-A2.1-restricted the development of anti-islet autoimmunity in early life. Although even within age-matched groups, individuals exhibit variability in reactivity to the three islet Ags IGRP, insulin, and DMK (46).

Discussion
T1D is clearly associated with specific class II MHC alleles (1, 2). However, association studies have revealed that particular class I MHC alleles, including HLA-A2.1, likely represent additional risk factors for T1D development (15–21). The frequency of HLA-A2.1 in Finnish children carrying high-risk class II MHC alleles who develop T1D is higher than in those remaining disease-free (15). A Finnish children carrying high-risk class II MHC alleles who may confer an increased risk for autoimmunity is dominated by T cells recognizing a unique subset of antigenic epitopes.

IGRP is an important target of pathogenic CD8+ T cells in NOD mice (25, 26, 46). An examination of islet infiltrates in individual NOD mice revealed T cells responding to IGRP206–214/H-2Kd are present in nearly all mice examined, including those as young as 5 wk of age (46). Recently, additional IGRP epitopes targeted by CD8+ T cells in standard NOD mice were reported (26). We now show that IGRP antigenicity is not limited to peptides recognized by T cells restricted to murine MHC class I molecules, but extends also to those seen in the context of the human MHC class I molecule HLA-A2.1 (Fig. 3, A and C). Importantly, HLA-A2.1-restricted T cell responses to IGRP peptides can be detected in NOD,β2mnull.HHD mice as young as 5 wk of age (data not shown). Our results provide candidate peptides that could allow the potential importance of IGRP in human T1D to be tested in future clinical studies. Of course, it is possible such future investigations could indicate that the candidate IGRP peptides identified in the current study are not targeted as frequently by pathogenic CD8+ T cells in NOD,β2mnull.HHD mice. Thus, we have also begun to explore HLA-A2.1-restricted T cell responses to other β cell Ags in...
A2.1-restricted islet-infiltrating T cells in NOD.

Disease-relevant peptides. Multiple pathogen and tumor Ags recognized by HLA-A2.1-restricted T cells and here has permitted identification of some of the β cell peptides they recognize. We believe this to be the first case in which HHD-expressing mice were used to identify antigenic peptides bound to human MHC class I molecules that are targeted in a natural autoimmune disease process. Importantly, T cells isolated from NOD,β2mnull/HHD mice can recognize human HLA-A2.1-positive islets (Table II). This indicates that murine and human HLA-A2.1-positive islets present common disease-relevant peptides. Multiple pathogen and tumor Ags recognized by HLA-A2.1-restricted T cells in HHD mice have been shown to be targets for HLA-A2.1-restricted T cells in patients (23, 53–55). Thus, human counterparts of the antigenic β cell peptides identified through the use of NOD,β2mnull/HHD mice are prime candidates for exploration in future clinical studies as targets of pathogenic T cells in T1D patients.

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Disclosures

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