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Requirement for Both H-2D<sup>b</sup> and H-2K<sup>d</sup> for the Induction of Diabetes by the Promiscuous CD8<sup>+</sup> T Cell Clonotype AI4<sup>1</sup>

Toshiyuki Takaki, Scott M. Lieberman, Thomas M. Holl, Bingye Han, Pere Santamaria, David V. Serreze, and Teresa P. Di Lorenzo<sup>3</sup><sup>†</sup>

The NOD mouse is a model for autoimmune type 1 diabetes in humans. CD8<sup>+</sup> T cells are essential for the destruction of the insulin-producing pancreatic β cells characterizing this disease. AI4 is a pathogenic CD8<sup>+</sup> T cell clone, isolated from the islets of a 5-wk-old female NOD mouse, which is capable of mediating overt diabetes in the absence of CD4<sup>+</sup> T cell help. Recent studies using MHC-congenic NOD mice revealed marked promiscuity of the AI4 TCR, as the selection of this clonotype can be influenced by multiple MHC molecules, including some class II variants. The present work was designed, in part, to determine whether similar promiscuity also characterizes the effector function of mature AI4 CTL. Using splenocyte and bone marrow disease transfer models and in vitro islet-killing assays, we report that efficient recognition and destruction of β cells by AI4 requires the β cells to simultaneously express both H-2D<sup>b</sup> and H-2K<sup>d</sup> class I MHC molecules. The ability of the AI4 TCR to interact with both H-2D<sup>b</sup> and H-2K<sup>d</sup> was confirmed using recombinant peptide libraries. This approach also allowed us to define a mimotope peptide recognized by AI4 in an H-2D<sup>b</sup>-restricted manner. Using ELISPOT and mimotope/H-2D<sup>b</sup> tetramer analyses, we demonstrate for the first time that AI4 represents a readily detectable T cell population in the islet infiltrates of prediabetic NOD mice. Our identification of a ligand for AI4-like T cells will facilitate further characterization and manipulation of this pathogenic and promiscuous T cell population. *The Journal of Immunology, 2004, 173: 2530–2541.*

In both humans and NOD mice, type 1 diabetes (T1D) is an autoimmune disease that results from T cell-mediated destruction of insulin (INS)-producing pancreatic β cells, and involves complex interactions among developmental, genetic, and environmental factors (1). Although there are multiple susceptibility loci, the strong association of particular MHC class II molecules with disease has led to extensive investigation of CD4<sup>+</sup> T cells in T1D (2). However, several studies in NOD mice have documented the importance of pathogenic CD8<sup>+</sup> T cells in the initial stages of β cell destruction (3–6).

The CD8<sup>+</sup> T cells contributing to the earliest stages of islet inflammation represent several distinct antigenic specificities (7). The AI4 CD8<sup>+</sup> T cell clone, originally isolated from the islets of a 5-wk-old female NOD mouse (3), represents one of these β cell-autoreactive specificities. NOD mice transgenically expressing the AI4 TCR (designated NOD.AI4αβ) transgenic (Tg)) progress to overt diabetes significantly earlier than nontransgenic NOD mice (8). Strikingly, this accelerated diabetes development is also observed in NOD.scid.AI4αβ Tg, NOD.CD4<sup>−/−</sup>.AI4αβ Tg (8), and NOD.Rag<sup>−/−</sup>.AI4αβ Tg mice (7), all of which lack CD4<sup>+</sup> T cells. Hence, naive AI4 T cells are able to develop, mature, and mediate sufficient β cell destruction to cause accelerated disease in the complete absence of CD4<sup>+</sup> T cell help. AI4 represents the only diabetogenic CD8<sup>+</sup> T cell clone known to be capable of doing so. In NOD mice, two other pathogenic CD8<sup>+</sup> T cell clones, 8.3 and G9C8, have been identified. NOD mice transgenically expressing the diabetogenic 8.3 TCR (8.3-NOD) develop accelerated diabetes that is enhanced by CD4<sup>+</sup> T cell help, as the frequency and rate of disease onset are greatly reduced in 8.3-NOD mice lacking CD4<sup>+</sup> T cells (9). The other known pathogenic CD8<sup>+</sup> T cell clone, G9C8, has been shown to cause diabetes in the absence of CD4<sup>+</sup> T cell help, but these experiments involved transfer of previously activated G9C8 T cells into recipient mice; thus, their ability to develop and mature in the absence of CD4<sup>+</sup> T cell help is unknown (5).

8.3 recognizes an antigen peptide derived from islet-specific glucose-6-phosphatase catalytic subunit-related protein (IGRP<sup>206–214</sup>) while G9C8 recognizes an INS B chain peptide (INS-B<sub>14–23</sub>), with both clones being H-2K<sup>d</sup>-restricted (10, 11). We recently showed that AI4 recognizes a β cell peptide (still unidentified) that is distinct from these two (7). Thus, AI4 represents a third antigen specificity contributing to early β cell destruction in NOD mice. Crosses between NOD.AI4αβ Tg mice and NOD stocks congenic for a variety of MHC haplotypes were recently used to determine...
whether the development of pathogenic CD8+ T cells could be affected by MHC variants within haplotypes known to dominantly inhibit T1D (12). This work revealed that, when expressed on bone marrow-derived APC, MHC molecules within the H2old and H2ub haplotypes, including one or both of the H2old-encoded class II molecules, could influence AI4 T cell selection through the induction of thymic deletion or anergy. Thus, the AI4 TCR is capable of tolerogenic interactions with a number of different MHC molecules during T cell development. We now demonstrate that mature AI4 CTL also exhibit promiscuous recognition of multiple peptide/MHC complexes, and report the surprising finding that β cell coexpression of both H-2Dα and H-2Kα is required for their efficient recognition and destruction by AI4 T cells. Further, we have identified a synthetic ligand for AI4, and we have used this advance to demonstrate that, as has been previously shown for 8.3- and G9C8-like T cells, AI4-like T cells constitute a readily detectable population within the islets of prediabetic NOD mice. This brings to three the number of pathogenic CD8+ T cell populations in NOD mice that can now be manipulated and monitored.

Materials and Methods

Mice

NOD/Lt mice (H2old MHC haplotype, i.e., Kb, Aα7, Eαnull, Dα) are maintained by brother-sister mating. Previously described T1D-resistant strains of lymphocyte-deficient NOD.scid.EμMv30/w mice (13) and class I MHC-deficient NOD.Rag1null mice (4) are all maintained at the N11 backcross generation. Previously described stocks (14, 15) of T1D-resistant NOD mice congenic for the H2old2 (Kb, Aαnull, Eαnull, Dα) or H2ub2 (Kb, Aα, Eαnull, Dα) haplotypes are designated NOD.H2old2 and NOD.H2ub2 and are maintained at the N21 or N15 backcross generation, respectively. T1D-resistant NOD mice congenic for a previously described (16) class II-deficient H2ub haplotype (Kb, Aαnull, Eαnull, Dα) are maintained at the N11 backcross generation (designated NOD.H2ub2nullmouse). A newly developed N10 backcross generation stock of NOD mice congenic for the ALR-derived H2ub2 haplotype (Kb, Aαnull, Eαnull, Dα) is partially T1D-resistant (female incidence of 35% at 25 wk of age, compared with 90% for standard NOD females at The Jackson Laboratory, Bar Harbor, ME). These NOD.H2ub2 mice were obtained from E. Leiter (The Jackson Laboratory) and will be described in detail elsewhere (E. Leiter, manuscript in preparation). NOD.scid mice have been previously described (12). Expression of the H-2Kα transgene does not alter the incidence of T1D development in these mice. NOD mice transgenically expressing the TCR of the β cell-autoantigenic CD8+ T cell clone AI4 (Vα8.2/Vβ2), and a substack congenic for a functionally inactive Rag1 gene (designated NOD.AI4αβ Tg or NOD.Rag1null AI4αβ Tg mice) have also been both previously described (7, 8). 8.3-NOD mice transgenically expressing the TCR of the β cell-autoantigenic CD8+ T cell clone AI4 (Vα8.2/Vβ2) (9), C57BL/6 mice congenic for the H2ub2 haplotype (Kb, Aαnull, Eαnull, Dα) derived from the DBA/2J strain were obtained from The Jackson Laboratory, and are designated B10.D2. All mice are maintained under specific pathogen-free conditions and used in accordance with institutional guidelines for animal welfare. All scid mice receive a sulfamethoxazole-trimethoprim mixture in their drinking water on alternate weeks to prevent infection by Pneumocystis carinii.

Cell lines

RMA-S/Ki (generously provided by M. Bevan, University of Washington, Seattle, WA) was derived from the TAP-deficient cell line RMA-S (21) and engineered to express H-2Kα in addition to the endogenous H-2Kk and H-2Dd. The TAP-deficient cell line T2 (22) was obtained from the American Type Culture Collection (Manassas, VA). T2-Ki2 (generously provided by J. Yewdell, National Institute of Allergy and Infectious Diseases, Bethesda, MD) was derived from T2 and engineered to express H-2Kk in addition to the endogenous HLA-A2.1. The NIT-1 β cell line, established from an adenoma that arose in an NOD mouse transgenically expressing SV40 T Ag under the control of a rat INS promoter, was maintained in a complete medium previously described (23). For IFN-γ treatment, NIT-1 cells were cultured overnight in this complete medium supplemented with 10 U/ml IFN-γ (murine recombinant; PeproTech, Rocky Hill, NJ).

Isolation of peptides from immunofluorescence-purified MHC class I

H-2Kd and H-2Dd molecules were immunofluorescence purified from 4 × 109 IFN-γ-treated NIT-1 pancreatic β cells using the mAbs SF1-1.1 (anti-H-2Kk) and 28-14-8 (anti-H-2Dd) (hybridomas from the American Type Culture Collection) and their associated peptides extracted as previously described (24). Peptide extracts were fractionated by reverse-phase HPLC as described (7).

Peptide libraries and synthetic peptides

Positional scanning combinatorial peptide libraries (25), mimotope candidate peptides (including YFIENYLYL, designated Mim), and alanine-substituted Mim peptides (including the F2A variant, designated MimA2) were purchased from Mimotopes (Clayton, Victoria, Australia). NOD-relevant peptide (NRP)-V7 (KYKNANVFL), INS-19 (G9 variant of murine INS-B13-25; LYLVCGERI), INS-BC (murine INS B25-33; FYTPMSREVR), murine glutamic acid decarboxylase (GAD)65 (GAD 65 220; GAD 65 220-234; TYEIAVPFFV), GAD65m (murine GAD65 mouse; SYQPLGDVK), and TRL9 (a negative control H-2Db-binding peptide; TSPRNSTVL) peptides were synthesized by standard solid-phase methods using fluorenylmethoxycarbonyl chemistry in an automated peptide synthesizer (model 433A; Applied Biosystems, Foster City, CA), and their identities were confirmed by mass spectrometry.

Pancreatic islet isolation and culture

Islet isolation by collagenase P perfusion of the common bile duct was used as previously described protocol (26). Briefly, the bile duct was cannulated and the pancreas perfused with collagenase P (Boehringer Mannheim, Mannheim, Germany). 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 (Workington Biochemical, Lakewood, NJ), and handpicked using a silanized micropipet under a dissecting microscope. Isolated islets were washed with FBS-containing HBSS, resuspended in RPMI 1640 medium supplemented with 10% FBS (HyClone, Logan, UT) and 50 U/ml recombinant human IL-3 (PeproTech), and cultured in 24-well (issuemeasured wells) at 37°C, 5% CO2 for 7 days. The incubation of NOD islets in the IL-2-supplemented medium allows for the expansion of β cell-autoreactive CD8+ T cells (27).

Splenocyte adoptive transfer

Aliquots of 1 × 107 splenic leukocytes isolated as described (28) from prediabetic female NOD.Rag1null AI4αβ Tg donors were injected i.v. into irradiated (700–750 rad from a 137Cs source) mice or nonirradiated scid mice as indicated in the figures. Recipient mice were monitored for diabetes development for up to 20 wk after transfer. At diabetes onset or the end of the observation period, recipient splenocytes were analyzed by multicolor flow cytometry for extent of AI4 T cell reconstitution.

Bone marrow transfer

Mice were lethally irradiated (1200–1400 rad from a 137Cs source) at 4–6 wk of age and reconstituted as previously described (29) with 5 × 106 bone marrow cells from prediabetic female NOD.Rag1null AI4αβ Tg donors. Recipient mice were monitored for diabetes development for up to 20 wk after reconstitution. At diabetes onset or at the end of the observation period, recipient splenocytes were analyzed by multicolor flow cytometry for extent of AI4 T cell reconstitution.

Cytotoxicity assays using intact islets as targets

NOD pancreatic islets (10/well) were allowed to adhere in 96-well plates during a 7–10 day incubation at 37°C in low-glucose DMEM (3). Adherent islets were then labeled with 5 μCi/well of 3H thymidine for 3 h at 37°C. Islets were washed and overlaid with 100 μl of medium containing various numbers of T cells from NOD.Rag1null AI4αβ Tg mice that had been pretreated for 72 h with the mimotope peptide YFIENYLYL at a concentration of 10 nM. For establishing E:T ratios, each islet was assumed to contain ~800 cells. A minimum of three wells were set up for each E:T ratio. Controls consisted of at least six wells of labeled NOD islets cultured in the absence of T cells. Following an overnight 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 100 μl of 2% SDS. The percentage of 3H thymidine release for each well was calculated by the formula [(supernatant cpm)/(supernatant cpm + SDS lysate cpm)] × 100. In turn, percentage of specific lysis was calculated by subtracting the percentage of 3H thymidine release from islets cultured with medium alone (i.e., spontaneous release) from the release by each well of islets cultured with a given number of T cells.

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Cytotoxicity assays for screening of HPLC fractions and peptide libraries

AI4 CTL were generated by culturing splenocytes from NOD.AI4aβ Tg mice with IFN-γ-treated NIT-1 cells and 12.5 Units IL-2 as described (7). NY.83 CTL were generated by culturing splenocytes from B6.NOD mice with mitomycin C-treated NOD splenocytes pulsed with 10 nM NRP-A7 peptide as described (7). CTL were used in 16-h 51Cr-release cytotoxicity assays to test for recognition of peptide-pulsed target cells at an E:T ratio of 40:1 as described (7). TAP-deficient cell lines RMA-S, RMA-S/K∗, T2, and B6 were used as targets. Synthetic peptides or peptide library mixes were used at concentrations indicated in the figures, and 1.6 × 10⁶ NIT-1 cell equivalents of peptide were used for HPLC fraction screening assays.

H-2Dβ stabilization assay

RMA-S cells, cultured overnight at 28°C, were pulsed with peptides in DMEM with 10% FBS for 1 h at 28°C, incubated at 37°C for 3 h, washed, stained with anti-H-2Dβ mAb 28-14-8, counterstained with FITC-conjugated polyclonal goat anti-mouse Ab (BD Pharmingen, San Diego, CA), and analyzed by flow cytometry. Data were calculated by subtracting mean fluorescence intensity of H-2Dβ on nonpeptide-pulsed cells from that on peptide-pulsed cells.

Tetramer staining and flow cytometry

PE-conjugated MimA2/H-2Dβ tetramers were obtained through the National Institute of Allergy and Infectious Diseases T-Cell MHC Facility and titrated to determine optimal concentration. PE-conjugated NRP-V7/H-2Kd and INS-L9/H-2Kd tetramers were prepared as previously described (30). INS-L9 (LYLVCGERL) is the G9L variant of murine INS-B 15-23. FITC-conjugated anti-CD8α Ab was purchased from BD Pharmingen. Cells were prepared to the appropriate density and incubated with tetramer and anti-CD8α Ab in 96-well V-bottom plates at 4°C for 45 min. Samples were analyzed by flow cytometry using a FACS Calibur instrument and CellQuest software (BD Immunocytometry Systems, San Jose, CA). All samples were gated on live cells as determined by propidium iodide labeling.

ELISPOT assay

ELISPOT plates (MAHA S45 10; Millipore, Billerica, MA) were precoated with anti-murine IFN-γ Ab (R4-6A2; BD Pharmingen) and blocked with 1% BSA (Fraction V; Sigma-Aldrich, St. Louis, MO) in PBS. APC (mitomycin C-treated NOD splenocytes) were added at 2 × 10⁵ cells/well and pulsed for 1 h with 1 μM of each peptide (MimA2, NRP-V7, INS-L9, INS-BC, GAD6529, and GAD6556). Cultured islet T cells were added at 2 × 10⁵ cells/well and plates were incubated at 37°C for 40 h. IFN-γ secretion was detected with a second biotinylated anti-murine IFN-γ Ab (XMG1.2; BD Pharmingen). Spots were developed using streptavidin-alkaline phosphatase (Zymed Laboratories, South San Francisco, CA) and 5-bromo-4-chloro-3-indolyl phosphate/NBT chloride substrate (Sigma-Aldrich).

Results

AI4 T cells target a β cell peptide in the context of H-2Dβ

AI4 was originally reported to be restricted to the MHC class I molecule H-2Kd, based on its cytotoxic activity against pancreatic islet cells from NOD-scid (expressing H-2Kd and H-2Dd) and B6 (I-Ek) mice (3). We have previously shown that AI4 CTL recognize an NOD-derived β cell peptide distinct from those targeted by the other diabetogenic CD8+ T cell clones 8.3 and G9C8 (7). This was done through mild acid stripping of the NOD-derived β cell line NIT-1, resulting in a mixture of β cell peptides eluted from both cell surface H-2Kd and H-2Dd combined. In this earlier work, we then fractionated the peptides by HPLC and performed 51Cr-release cytotoxicity assays using target cells expressing both H-2Dd and H-2Kd. However, in subsequent experiments, an epitope recognized by AI4 was surprisingly reconstituted using a target cell line lacking H-2Kd expression, but expressing H-2Dd (T.P.D., unpublished observations).

In an attempt to resolve the apparent contradiction between the islet cytotoxicity and epitope reconstitution data, we next assayed peptides eluted separately from H-2Kd and H-2Dd molecules immunoadfinity purified from NIT-1 β cells (Fig. 1). The positive peak previously noted in HPLC fraction 22 of the acid-stripped combined-peptide pool (7) was clearly reproduced when HPLC fractions of peptides purified from H-2Dd (Fig. 1A), but not H-2Kd (Fig. 1B), were assayed. A second active peak, possibly representing a modified version of the antigenic peptide detected in fractions 22 and 23, was also detected when the H-2Dd-eluted peptide fractions were examined (Fig. 1A). Thus, AI4 recognizes a peptide from NIT-1 β cells in an H-2Dd-restricted fashion.

Both H-2Kd and H-2Dd molecules are required for AI4 T cells to cause diabetes

To test whether AI4 CTL are also restricted to H-2Dd in terms of their in vivo effector function, we used a splenocyte transfer model of disease. Splenocytes were isolated from prediabetic female NOD.Rag1null, AI4αβ Tg donors and transferred to a variety of mouse strains as indicated (Fig. 2). NOD.Rag1null, AI4αβ Tg mice were used as the AI4 T cell source, because T cells from these mice can only express the Tg AI4 TCR, due to their inability to rearrange endogenous TCR genes. As expected, at 20 wk posttransfer, the majority of standard NOD recipients had developed disease (Fig. 2A). Surprisingly, however, congeneric strains expressing H-2Dd in the absence of H-2Kd (NOD.H2dβ and NOD.H2nb1) remained disease-free, as did NOD.H2nb1 recipients, which express H-2Kd in the absence of H-2Dd. Disease could only be transferred to recipients homozygously expressing both H-2Kd and H-2Dd (NOD, NOD-Kb Tg, and B6.H2nb1). Failure to transfer disease did...
not correlate with the overall genetic T1D resistance of the recipient strains. For example, 100% of the B6.H2\textsuperscript{g7} recipients developed diabetes upon AI4 T cell transfer, even though unmanipulated B6.H2\textsuperscript{g7} mice are T1D-resistant. Also, although the NOD.H2\textsuperscript{gx} strain is only partially genetically resistant to spontaneous diabetes, none of these recipients of AI4 T cells developed disease. Failure to transfer disease did not correlate with the presence of non-NOD class II MHC molecules, as the only class II molecule expressed by NOD.H2\textsuperscript{gx} recipients is H-2A\textsuperscript{g7}, yet they did not develop disease upon AI4 transfer. The presence of the non-NOD class I MHC molecule H-2K\textsuperscript{d} also cannot explain the failure to transfer disease to NOD.H2\textsuperscript{b} and NOD.H2\textsuperscript{nb1} recipients, as disease was efficiently transferred to the NOD-K\textsuperscript{b} Tg strain.

Our finding that disease could only be transferred to recipients expressing both H-2K\textsuperscript{d} and H-2D\textsuperscript{b} is most consistent with the idea that efficient destruction of islets by AI4 requires recognition of two peptide/MHC complexes, one containing H-2D\textsuperscript{b} and the other H-2K\textsuperscript{d}. Individually, each of the complexes is apparently of too low an abundance on the surface of /H9 cells to trigger sufficient AI4 T cell activation for T1D development, but together they provide a sufficient antigenic stimulus for disease induction. To further explore this idea, we next transferred AI4 splenocytes to (NOD x NOD.H2\textsuperscript{b})F\textsubscript{1}, (NOD x NOD.H2\textsuperscript{gx})F\textsubscript{1}, or (NOD x B10.D2)F\textsubscript{1} recipients, all of which express both H-2K\textsuperscript{d} and H-2D\textsuperscript{b}. However, all of the F\textsubscript{1} mice are heterozygous for either H-2K\textsuperscript{d} or H-2D\textsuperscript{b}, thus presumably leading to a decrease in the abundance of the corresponding antigenic peptide/MHC complexes recognized by AI4.

Interestingly, despite efficient AI4 T cell reconstitution, diabetes did not develop in any of the F\textsubscript{1} recipients. This finding is consistent with the hypothesis that the AI4 ligands are of low abundance, and that heterozygous expression of H-2K\textsuperscript{d} or H-2D\textsuperscript{b} reduces them to a level that is insufficient to lead to \beta cell elimination by AI4. This is consistent with the earlier suggestion that low-avidity interactions between T cells and /H9 cells may not be pathogenic (27).

Although NOD.H2\textsuperscript{nb1}, NOD.H2\textsuperscript{b}, and NOD.H2\textsuperscript{gx} mice were poorly reconstituted with AI4 T cells (Fig. 2A), perhaps due to allogeneic rejection of the transferred cells or regulatory effects from recipient lymphocytes, we next did splenocyte transfers to scid recipients (Fig. 2B). Here again, no diabetes development was observed if either H-2K\textsuperscript{d} or H-2D\textsuperscript{b} were expressed alone. Unfortunately, however, B6-scid recipients were poorly reconstituted, perhaps due to NK cell-mediated rejection of the transferred cells or a requirement for H-2K\textsuperscript{d} for homeostatic expansion (31) by AI4.

In an attempt to minimize the problems of poor reconstitution due to rejection or lack of expansion, we next used a bone marrow transfer model of disease. Bone marrow from prediabetic female NOD.Rag1null.AI4\textalpha\beta Tg donors was reconstituted into lethally irradiated mice as indicated (Table I). In all recipients, AI4 T cells represented at least 30% of CD8\textsuperscript{+} splenocytes at the time of T1D
development or at 20 wk posttransfer. As before, diabetes development was only observed in recipients that homozygously expressed both H-2Kd and H-2Dd. Note that heterozygous expression of H-2Kd led to failure to transfer disease in (NOD × NOD.H2a-Kb) F1 mice, again supporting the idea that abundances of the AI4 ligands are low, and heterozygous expression results in an insufficient level of β cell destruction by AI4 for T1D to develop. Importantly, both NOD.H2a-Kb and (NOD × NOD.H2a-Kb) F1 recipients did not develop disease. This suggests that the presence of non-NOD MHC class II molecules in NOD.H2d and NOD.H2abi mice is not responsible for the lack of disease development in these recipients.

We also used in vitro islet-killing assays to examine more directly the MHC requirements for AI4 T cell recognition of β cells (Fig. 3). These experiments confirmed the necessity for both H-2Kd and H-2Dd expression on the β cell surface for efficient killing by AI4 T cells. Interestingly, if we added a mimotope peptide recognized by AI4 in the context of H-2Dd (described below), AI4 T cells could efficiently kill islet β cells in the presence of only H-2Dd, indicating that these islets are not intrinsically resistant to CTL lysis.

AI4 T cells exhibit promiscuous peptide recognition behavior

Because our ongoing attempts to identify AI4’s target Ag(s) by sequence analysis of peptides eluted from purified MHC or by cDNA expression cloning have not yet been successful, we used positional scanning synthetic combinatorial peptide libraries to identify mimotope peptides recognized by AI4 CTL. For T cells of unknown antigenic specificities, mimotope peptides have proven their utility. For example, mimotope peptides were used to demonstrate the importance of the diabetogenic 8.3-like T cell population even before IGRP was identified as its Ag (27, 30). Mimotope peptides can also be used to study thymic selection of specific T cells and may even help to identify natural peptide ligands.

We screened a peptide library designed to bind H-2Dd, namely, peptides of 9 aa with anchors N at position 5 and L at position 9 (32). The library is composed of seven peptide sets (one for each nonanchor position), each containing 19 different peptide mixes. In each mix, 1 aa is fixed at a nonanchor position; thus, the 19 mixes in each set cover all natural amino acids (cysteine excluded) at the specified position. The six remaining nonfixed positions in each mix are composed of equimolar amounts of the 19 aa under consideration. Using this positional scanning format, the potential contribution of each of the 19 aa to T cell recognition can be evaluated at each position of the peptide individually, and, ideally, a dominant amino acid can be identified for each nonanchor position (33). With three of the nine positions of the peptide fixed as single amino acids, and an equimolar mixture of the 19 amino acids at each of the other six positions, each peptide mix is composed of 196, or 4.7 × 1014, different peptides. A positive response to any one mix is likely due to T cell recognition of multiple peptides within that mix, and suggests that the fixed nonanchor amino acid in that mix is important for T cell recognition. Screening of our H-2Dd-binding peptide library in a 51Cr-release cytotoxicity assay with AI4 CTL and a total peptide concentration of 60 μg/ml per mix resulted in the activity profile shown in Fig. 4A. AI4 showed

![Figure 3](http://www.jimmunol.org/)

**FIGURE 3.** AI4 T cells only show in vitro cytotoxic responses to islets expressing both H-2Kd and H-2Dd. Splenocytes from NOD.Rag1null.AI4αβ Tg mice were cultured for 72 h in the presence of the AI4 mimotope peptide YFIENYLEL (10 nM) and 50 U/ml IL-2. Effector AI4 T cells were seeded at the indicated E:T ratios into wells containing 51Cr-pulsed islets from the indicated mice. Percentage of specific lysis was determined as described in Materials and Methods. Two single data points for the E:T ratio of 50 indicate cytotoxic responses to the NOD.H2abi (○) and NOD.H2d (▲) islets pulsed with the mimotope peptide.

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Table 1. Both H-2Kd and H-2Dd are required for recipient mice to develop diabetes after transfer of bone marrow from NOD.Rag1null.AI4αβ Tg mice

<table>
<thead>
<tr>
<th>Recipient</th>
<th>MHC Class I Alleles</th>
<th>MHC Class II Alleles</th>
<th>Percentage Diabetes Incidence (%)</th>
<th>Percentage of AI4 TCR⁺ Cells among CD8⁺ Splenocytes⁺</th>
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<tbody>
<tr>
<td>NOD (n = 13)</td>
<td>Kd, Db</td>
<td>Aβ, Eαnull</td>
<td>53.8</td>
<td>49.0 ± 6.6</td>
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<tr>
<td>NOD.Kd Tg (n = 8)</td>
<td>Kd, Db, Tg Kb</td>
<td>Aβ, Eαnull</td>
<td>87.5</td>
<td>30.5 ± 5.0</td>
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<tr>
<td>NOD.H2a (n = 7)</td>
<td>Kd, Db</td>
<td>Aβ, Eαnull</td>
<td>0</td>
<td>75.2 ± 3.5</td>
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<tr>
<td>NOD.H2abi (n = 5)</td>
<td>Kd, Db</td>
<td>Aβ, Eαnull</td>
<td>0</td>
<td>66.4 ± 0.71</td>
</tr>
<tr>
<td>NOD.H2abi (n = 9)</td>
<td>Kd, Db</td>
<td>Aβ, Eαnull</td>
<td>0</td>
<td>40.0 ± 6.9</td>
</tr>
<tr>
<td>NOD.H2abi (n = 4)</td>
<td>Kd, Db</td>
<td>Aβ, Eαnull</td>
<td>0</td>
<td>77.4 ± 5.1</td>
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<tr>
<td>(NOD × NOD.H2abi) F1 (n = 4)</td>
<td>Kd, Db, Tg Kb</td>
<td>Aβ, Eαnull</td>
<td>0</td>
<td>70.2 ± 2.2</td>
</tr>
</tbody>
</table>

* All recipient mice were followed for diabetes development for up to 20 wk after bone marrow transfer.

⁺ Splenocytes were assessed for proportions of AI4 TCR-positive (Vα8) CD8⁺ T cells by costaining with Vα8- and CD8-specific Abs at diabetes onset or at the end of the observation period (20 wk after bone marrow transfer).
high reactivity to most amino acids fixed at positions 1, 2, 3, 7, and 8. When peptide position 6 was fixed, AI4 tolerated only large hydrophobic amino acids with aromatic side chains (F, W, or Y). The AI4 response to amino acids fixed at position 4 was also somewhat restricted.

The initial peptide concentration of 60 \( \mu g/ml \) was chosen based on previous peptide library screens, such as was used to identify the mimotope peptide recognized by 8.3 CTL (34). However, the library used in that study was designed slightly differently, in that each fixed position consisted of an equimolar mix of two similar amino acids rather than an individual amino acid as in our library. To test whether the concentration was appropriate with our library design, we screened a peptide library designed to bind H-2K\(^d\) with H-2K\(^d\)-restricted 8.3 CTL. This library was similar to our H-2D\(^b\)-binding peptide library except for the differences in fixed anchor residues. Upon screening this H-2K\(^d\)-binding peptide library with 8.3 CTL at a concentration of 60 \( \mu g/ml \), we observed a dramatically
different activity profile (Fig. 4B) than that seen when we screened the H-2D\(^b\) library with AI4 CTL. Whereas AI4 responded to almost all mixes tested (Fig. 4A), the 8.3 response resulted in clear dominant amino acid contributions at six of seven positions tested, and the one position that did not show obvious dominance among the amino acids fixed at that position (position 1) resulted in a profile with no activity, rather than with activity toward all mixes (Fig. 4B). In fact, only one or two mixes elicited clearly positive 8.3 lysis responses for positions 3, 4, 5, 6, 7, and 8. Importantly, our results agree with the sequences of the superagonist 8.3 mimotope NRP-V7 (KYNKANVFL) (30) and the original 8.3 mimotope NRP (KYNKANWFL) (34) at all positions other than position 1.

Considering our splenocyte and bone marrow transfer data (Fig. 2, and Table I), along with our in vitro islet-killing results (Fig. 3), all of which suggested that AI4 recognizes β cell peptides in the context of both H-2D\(^b\) and H-2K\(^d\), we next screened the H-2K\(^d\)-binding peptide library with AI4 CTL to independently test whether AI4 can interact with peptide/H-2K\(^d\) complexes. The results showed that an AI4 response could indeed be elicited by H-2K\(^d\)-bound peptides (Fig. 4C), although, in this case, the responses were not as robust as those observed in the H-2D\(^b\)-binding peptide library screen (Fig. 4A). However, while not as robust as the H-2D\(^b\)-mediated responses, AI4 also responded to H-2K\(^d\)-bound peptides in a promiscuous fashion. For the sake of comparison, we also tested the H-2D\(^b\)-binding peptide library with 8.3 CTL, but we could not detect any reproducible significant responses in this screening (Fig. 4D). Taken together, these results indicate that AI4 is a promiscuous clonotype. Although degeneracy in Ag recognition by TCRs is now well accepted (35, 36), the extreme promiscuity demonstrated in this study by AI4 is clearly not characteristic of all autoreactive T cells, as diabetogenic 8.3 CTL exhibited a far more restricted response.

Identification of mimotope peptides recognized by AI4 CTL in the context of H-2D\(^b\)

Because our data suggested that AI4 recognizes β cell peptide in the context of H-2D\(^b\) (Fig. 1A), we continued to screen the H-2D\(^b\)-binding peptide library to identify a mimotope peptide recognized by AI4. To do this, we reduced the peptide concentration by 250-fold dilution (0.24 \(\mu\)g/ml) compared with the initial screen (60 \(\mu\)g/ml). Even at this greatly reduced concentration (Fig. 5A), the AI4 response was still more broad than that seen when 8.3

\[\text{FIGURE 5. Identification of a mimotope peptide recognized by AI4 in the context of H-2D}^b. A, AI4 cytotoxic response toward RMA-S target cells pulsed with peptide mixes from the H-2D\(^b\)-binding peptide library at a final total peptide concentration of 0.24 \(\mu\)g/ml. Lysis of target cells by AI4 in the absence of peptide was 7%. Nineteen different peptide mixes were tested for each amino acid position, and 1 aa was fixed at each nonanchor position in each mix (A, D, E, F, G, H, I, K, L, M, N, F, Q, R, S, T, Y, W, and Y, in order from left to right). Each black bar represents the cytotoxic response toward the corresponding peptide mix. Peptide recognition was determined by \(^{51}\)Cr-release assay at an E:T ratio of 40. Letters denote amino acids chosen to be included in candidate mimotope peptides. B, AI4 cytotoxic response toward RMA-S target cells pulsed with varying concentrations of candidate mimotope peptides or an H-2D\(^b\)-binding negative control peptide (TRL9). The cytotoxic activity of the T cells was determined by \(^{51}\)Cr-release assay at an E:T ratio of 40.\]
CTL were used to screen the H-2K\(^d\) library at 60 \(\mu g/ml\) (Fig. 4B). Despite the lack of single dominant amino acids for each position, however, this screen did result in a smaller set of AI4-preferred amino acids at each position, compared with the initial screen. Again, AI4 demonstrated a clear preference for F, W, or Y at position 6 (Fig. 5A). In addition, E was dominant at position 4 (Fig. 5A). To choose amino acids for each of the other nonanchor positions to include in our candidate mimotope peptides, we considered reproducibility among the top responders in multiple screens. However, we avoided combinations of residues that would result in highly hydrophobic peptides, as their lack of solubility in culture medium would make them difficult to assay. Using these criteria, three mimotope peptide candidates, differing only at position 6, were chosen: YFIENFLEL, YFIENWLEL, and YFIENYLEL. These peptides were synthesized and tested for recognition by AI4 CTL (Fig. 5B). All peptides demonstrated dose-dependent recognition by AI4, compared with an irrelevant H-2D\(^b\)-binding peptide, TRL9 (TSPRN-STVL) (37). However, YFIENYLEL demonstrated the best response from AI4 CTL, as it elicited greater specific lysis at concentrations of 10 and 1 nM compared with the other candidate peptides. Thus, YFIENYLEL (Mim) is the most active AI4 mimotope peptide of the three examined. Recognition of Mim was restricted to H-2D\(^b\), as AI4 did not lyse Mim-pulsed T2-K\(^d\) cells (data not shown).

To better understand the contributions of each amino acid position within this AI4 mimotope peptide, we tested a set of alanine-substituted mimotope peptides (Fig. 6A). As expected, A substituted for Y at position 6 (designated A6 in Fig. 6) and A substituted for E at position 4 (A4 in Fig. 6) resulted in significantly lower responses compared with the original Mim peptide. Similarily, the responses toward A7- and A8-substituted peptides were decreased compared with the original Mim peptide response. Based on crystallographic data, amino acids at positions 4, 6, 7, and 8 of peptides bound to H-2D\(^b\) are expected to point away from the MHC molecule, and thus, are expected to contact the TCR (38, 39). Strikingly, the A2 substitution resulted in a peptide that elicited a near maximal response at all concentrations tested (as low as 0.1 nM). This response was confirmed to be dose-dependent upon screening lower concentrations, with half-maximal activity at a concentration of 10 pM (Fig. 6B). Position 2 is not expected to be a key TCR contact residue, so we hypothesized that alanine at position 2 allows for a more stable peptide-MHC interaction. To evaluate this possibility, we tested the ability of these alanine-substituted mimotope peptides, along with the original Mim peptide, to bind to H-2D\(^b\) in an MHC stabilization assay. The A2-substituted mimotope peptide clearly demonstrated a much greater ability to stabilize H-2D\(^b\) (Fig. 6C). It is possible that steric interactions involving the bulky F side chain at position 2 in the original and all other alanine-substituted mimotope peptides hindered their ability to bind MHC as tightly as the A2-substituted mimotope peptide. Interestingly, F appeared favored over A when the position 2 peptide mixes of the H-2D\(^b\)-binding peptide library were tested (Figs. 4A and 5A). This suggests that the number of individual peptides recognized by AI4 within the mix where position 2 is fixed as A may be limited, whereas the number of peptides recognized by AI4 within the mix where position 2 is fixed as F may be higher.

Peptide/MHC tetramers composed of YAIENYLEL/H-2D\(^b\) complexes were shown to specifically stain splenocytes from NOD.AH4\(\alpha\)Tg mice, with minimal staining of nontransgenic NOD splenocytes (Fig. 6D). Thus, YAIENYLEL (MimA2) is a superagonist mimotope peptide recognized by the diabetogenic AH4 T cell clone, and MimA2/H-2D\(^b\) tetramers may be used to detect AI4-like T cells.

Characterization of the AI4-like T cell population in NOD islet infiltrates

Until now, AI4 was only known to be present within the islet infiltrates of the one nondiabetic 5-wk-old NOD mouse from...
which it was originally isolated (3). Whether the AI4 clonotype represents a population of T cells detectable in islet infiltrates of other NOD mice was unknown. The use of MimA2/H-2D\(^{\beta}\) tetramers allowed us to address this issue. To do this, we cultured islets from four non-diabetic, 11-wk-old, female, NOD mice for 7 days in the presence of 50 U/ml IL-2. The expanded T cells were stained with FITC-conjugated anti-CD8 Ab and PE-conjugated tetramers, and analyzed by flow cytometry. In addition to the MimA2/H-2D\(^{\beta}\) tetramer, NRP-V7/H-2K\(^{d}\) and INS-L9/H-2K\(^{d}\) tetramers were included to detect the IGRP\(_{206-214}\)reactive 8.3-like (11) and INS-B\(_{15-23}\)reactive G9C8-like CD8\(^{+}\) T cell populations (10), respectively. Of the CD8\(^{+}\) cells, 8% were stained with the MimA2/H-2D\(^{\beta}\) tetramer, clearly indicating that AI4-like T cells constitute a detectable population in the islet infiltrates of NOD mice (Fig. 7A). In addition to the AI4-like T cells, IGRP-reactive T cells accounted for 41% of the CD8\(^{+}\) cells, and INS-reactive T cells accounted for 3%. Thus, >50% of the CD8\(^{+}\) T cells are accounted for with these three antigenic specificities.

Detecting Ag specific CD8\(^{+}\) T cell populations with peptide/MHC tetramers is a widely used method. However, it is important to know, in addition, if the tetramer-positive cells are functional.

We thus set out to determine whether the tetramer-positive CD8\(^{+}\) cells isolated from NOD islets were able to produce IFN-\(\gamma\) in response to peptide. To do this, we incubated an aliquot of the same T cells used for the tetramer analysis in IFN-\(\gamma\) ELISPOT plates along with peptide-pulsed APC. Importantly, the ELISPOT data agreed with the tetramer staining (Fig. 7B). Spots were detectable in response to NRP-V7 (8.3-like T cells), MimA2 (AI4-like), and INS-L9 (G9C8-like). The relative numbers of spots detected for each of these were consistent with the tetramer staining data. No spots were detected in response to a second INS peptide (INS-BC), proposed to be a potentially important CD8\(^{+}\) T cell epitope in NOD mice (40), or to either of two H-2K\(^{d}\) binding GAD65 peptides previously shown to be recognized by NOD splenocytes (41). Thus, the pathogenic AI4-like, 8.3-like, and G9C8-like T cells detected in islet infiltrates with tetramers represent functional IFN-\(\gamma\)-producing CD8\(^{+}\) T cell populations.

Preferred residues in H-2K\(^{d}\)-binding peptides recognized by AI4

We have defined an H-2D\(^{\beta}\)-binding AI4 mimotope peptide, but data presented above also suggested the existence of a peptide(s) recognized by AI4 in the context of H-2K\(^{\beta}\) (Figs. 2, 3, and 4C, and Table I). Next, we used a more elaborate screening of the H-2K\(^{d}\)-binding peptide library with AI4 CTL to obtain further evidence for the existence of such a peptide. For this H-2K\(^{\beta}\) screening, we used both T2 and T2-K\(^{d}\) as APC to insure that responses seen to peptide-pulsed T2-K\(^{d}\) cells were indeed dependent on this class I variant. At a 10-fold dilution (6 \(\mu\)g/ml) of the peptide concentration used for the initial screening depicted in Fig. 4C, it remained difficult to choose a dominant amino acid for each position using T2-K\(^{d}\) as APC (Fig. 8A). However, now AI4 did demonstrate a preference for G, H, or W at position 6, and the recognition profile became more clear at positions 5, 7, and 8. In contrast, AI4 did not show any significant response when T2 cells were used as APC (Fig. 8B). Taken together, our results indicate that AI4 is a promiscuous clonotype capable of productively interacting with both H-2K\(^{\beta}\) and H-2D\(^{\beta}\) peptide/MHC complexes.

Discussion

AI4 is one of a panel of \(\beta\) cell-autoreactive CD8\(^{+}\) T cell clones that we previously isolated from the earliest insulitic lesions of young NOD mice (3). It is able to mediate \(\beta\) cell destruction sufficient to cause overt diabetes development even in the complete absence of CD4\(^{+}\) T cell help or CD8\(^{+}\) T cells of other antigenic specificities (8). In this study, we have shown, using ELISPOT and peptide/MHC tetramer analyses, that AI4 represents a clearly measurable population among islet-infiltrating T cells in NOD mice (Fig. 7).

We recently reported that the H-2A\(^{nb1}\) and/or H-2E\(^{kb}\) class II MHC molecules encoded by the diabetes-protective H2\(^{nb1}\) haplotype can mediate the negative selection of AI4 when expressed on bone marrow-derived APC, and that this clonotype is anergized when developing in the presence of H-2K\(^{\beta}\) class I molecules (12). Heterozygous expression of the H2\(^{nb}\) haplotype during AI4 T cell development results in reduced CD8 expression and functional impairment (12). Thus, the AI4 TCR demonstrates remarkable promiscuity during T cell development. In this report, we have presented several independent lines of evidence indicating that mature AI4 CTL can interact with both H-2D\(^{\beta}\) and H-2K\(^{d}\), and that expression of both of these molecules is required for efficient recognition and destruction of islet \(\beta\) cells. Thus, the AI4 clonotype exhibits promiscuous behavior both during the selection process and while exerting its effector function.

Our results obtained using three different in vivo disease transfer models (Table I, and Fig. 2) all indicate that AI4 must interact with
both an H-2D\textsuperscript{b} and an H-2K\textsuperscript{d} complex to efficiently destroy islet \( \beta \) cells. An alternative explanation for our disease transfer results is that non-NOD MHC molecules in certain of the recipients induced peripheral tolerance and rendered AI4 T cells unable to cause T1D. However, we do not favor this idea, as our in vitro islet cytotoxicity assays (Fig. 3), using AI4 CTL generated from NOD.Rag\textsuperscript{\textit{Lmo}}Tg splenocytes, demonstrate that AI4 can only kill \( \beta \) cells that express both H-2K\textsuperscript{d} and H-2D\textsuperscript{b}. Further, we previously reported that NOD.Rag\textsuperscript{\textit{Lmo}}Tg splenocytes are not stimulated in vitro by splenic APC from NOD.H\textsuperscript{2b} or NOD.H\textsuperscript{2b}.Ab\textsuperscript{10} mice (12), suggesting that peripheral tolerance induction by the H\textsuperscript{2b} or H\textsuperscript{2b}.Ab\textsuperscript{10} MHC haplotypes is unlikely.

Our ability to detect a peak of activity when screening peptides eluted from purified H-2D\textsuperscript{b} but not H-2K\textsuperscript{d} molecules of NIT-1 \( \beta \) cells (Fig. 1) is not necessarily at odds with our conclusion that AI4 must interact with both an H-2D\textsuperscript{b} and an H-2K\textsuperscript{d} complex to cause disease. The abundance of the antigenic peptide eluted from H-2K\textsuperscript{d} molecules of NIT-1 cells might simply be too low to elicit an AI4 response in our assay system. Alternatively, the NIT-1 cell line might no longer express the \( \beta \) cell protein from which the antigenic H-2K\textsuperscript{d}-binding peptide is derived, due to altered differentiation brought about by its transformation by SV40 T Ag (23) or repeated passage in culture. For example, expression of the \( \beta \) cell Ags recognized by two different diabetogenic CD4\textsuperscript{+} T cell clones is rapidly lost from \( \beta \) cell adenoma cells upon growth in culture (42). Screening of the synthetic H-2K\textsuperscript{d}-binding peptide library clearly indicates that AI4 is able to productively interact with H-2K\textsuperscript{d} complexes (Figs. 4C and 8).

TCR cross-reactivity or promiscuity (i.e., the ability of a single TCR to interact with more than one ligand) is now a well-documented phenomenon. The current understanding of the processes of T cell-positive selection (43) and homeostasis (31) indicates that some degree of cross-reactivity is a requirement for T cell development and survival. In some cases, positive selection has been shown to be mediated by the same MHC molecule to which the T cell is restricted in terms of its recognition of a foreign peptide, but the selectin peptide is a different (self) peptide (43). Besides these and multiple other examples (35, 36) of the ability of a TCR to recognize different peptides bound to the same MHC molecule, there are also examples of recognition of the same peptide presented by two different MHC molecules (44–50). In addition, the frequency of alloreactive T cells, which can respond to two different MHC molecules (one self and one non-self), is high (51–53). However, examples of recognition of two different peptides bound to two different self MHC molecules, as proposed in this study for AI4, are quite limited (54–56). They include a recent report that negative selection of a particular CD8\textsuperscript{+} T cell clonotype requires recognition of both an MHC class I and an MHC class II molecule (56). Thus, there is some precedent, albeit limited, for our finding that AI4 can respond to \( \beta \) cell peptides bound to H-2D\textsuperscript{b} as well as H-2K\textsuperscript{d} self MHC molecules. We are not aware of any other example of a T cell that requires the simultaneous recognition of two different MHC molecules in order for its effector function to become apparent.

Although the importance of CD8\textsuperscript{+} T cells in T1D is becoming more widely recognized (57), our knowledge of their antigenic specificities remains incomplete. Besides IGRP\textsubscript{206–214} and INS-B\textsubscript{12–23}, which are targeted by 8.3 and G9C8, respectively (10, 11), no other natural peptide targets for islet-infiltrating CD8\textsuperscript{+} T cells in NOD mice have been identified to date. Three other candidate peptides of potential relevance have previously been described (40, 41). GAD65\textsubscript{206} and GAD65\textsubscript{546} have both been shown to bind H-2K\textsuperscript{d} and to permit the generation of peptide-specific IFN-\( \gamma \)-producing CTL from NOD splenocytes (41). However, the ability of these GAD-reactive CD8\textsuperscript{+} T cells to kill \( \beta \) cells was not evaluated. Compelling, though indirect, evidence for the importance of a third

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**FIGURE 8.** AI4 T cells can recognize peptides in the context of H-2K\textsuperscript{d}. AI4 cytotoxic responses toward T2-K\textsuperscript{d} (A) or T2 (B) target cells pulsed with peptide mixes from the H-2K\textsuperscript{d}-binding peptide library at a final total peptide concentration of 6 \( \mu \)g/ml. Lysis of T2-K\textsuperscript{d} and T2 cells by AI4 in the absence of peptide was 21.6 and 13.5\%, respectively, and has been subtracted. Nineteen different peptide mixes were tested for each amino acid position, and 1 aa was fixed at each nonanchor position in each mix (A, D, E, F, G, H, I, K, L, M, N, P, Q, R, S, T, V, W, and Y, in order from left to right). Each black bar represents the cytotoxic response toward the corresponding peptide mix. Letters denote the AI4-preferred amino acids at the indicated positions based on those mixes eliciting the highest AI4 responses. No lysis was observed when peptide-pulsed target cells were cultured in the absence of T cells. The cytotoxic activity of T cells was determined by \( ^{51} \text{Cr} \)-release assay at an E:T ratio of 40.
H-2K\(^{b}\)-binding peptide, INS-BC, has also been previously provided (40). Using T cells propagated from islet infiltrates and IFN-\(\gamma\)-ELISPOT (Fig. 7B), we were unable to detect responses to GAD65\(_{596}\), GAD65\(_{646}\), or INS-BC. However, we only tested T cells from a pool of islets from 11-wk-old NOD mice, so it is still possible that these peptides are targets of islet-infiltrating T cells in mice of other ages. Our combined use of tetramers for the 8.3-,


15. Wong, F. S., I. Visintin, L. Wen, R. A. Flavell, and C. A. Janeway, Jr. 1996. CD8+ T cells from a pool of islets from 11-wk-old NOD mice, so it is still possible that these peptides are targets of islet-infiltrating T cells in mice of other ages. Our combined use of tetramers for the 8.3-,

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