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Individual Nonobese Diabetic Mice Exhibit Unique Patterns of CD8$^+$ T Cell Reactivity to Three Islet Antigens, Including the Newly Identified Widely Expressed Dystrophia Myotonica Kinase[1]

Scott M. Lieberman,* Toshiyuki Takaki,* Bingye Han,‡ Pere Santamaria,‡ David V. Serreze,§ and Teresa P. DiLorenzo[2]*†

Spontaneous autoimmune diabetes development in NOD mice requires both CD8$^+$ and CD4$^+$ T cells. Three pathogenic CD8$^+$ T cell populations (represented by the G9C8, 8.3, and AI4 clones) have been described. Although the Ags for G9C8 and 8.3 are known to be insulin and islet-specific glucose-6-phosphatase catalytic subunit-related protein, respectively, only mimotope peptides had previously been identified for AI4. In this study, we used peptide/MHC tetramers to detect and quantify these three pathogenic populations among β cell-reactive T cells cultured from islets of individual NOD mice. Even within age-matched groups, each individual mouse exhibited a unique distribution of β cell-reactive CD8$^+$ T cells, both in terms of the number of tetramer-staining populations and the relative proportion of each population in the islet infiltrate. Thus, the inflammatory process in each individual follows its own distinctive course. Screening of a combinatorial peptide library in positional scanning format led to the identification of a peptide derived from dystrophia myotonica kinase (DMK) that is recognized by AI4-like T cells. Importantly, the antigenic peptide is naturally processed and presented by DMK-transfected cells. DMK is a widely expressed protein that is nonetheless the target of a β cell-specific autoimmune response. *The Journal of Immunology,* **2004, 173: 6727–6734.**

Autoimmune type 1 diabetes development is mediated by T cell destruction of the insulin-producing pancreatic β cells (1, 2). In the NOD mouse model, spontaneous autoimmune diabetes development requires both CD4$^+$ and CD8$^+$ T cells (3–7), with evidence suggesting that CD8$^+$ T cells are required for the initial stages of β cell destruction (6, 7). Several NOD-derived, β cell-autoreactive CD8$^+$ T cell clones have been reported (7–11); however, only three of these (designated G9C8, 8.3, and AI4) have demonstrated in vivo pathogenicity. The insulin $B_{15–23}$-reactive G9C8 clone (12), when activated in vitro, is capable of transferring disease to irradiated NOD mice (10). The 8.3 clone represents a prevalent population of islet-specific glucose-6-phosphatase catalytic subunit-related protein$_{206–214}$ (IGRP$_{206–214}$)[3]-reactive T cells present in NOD islets throughout disease development and progression to overt diabetes (7, 13–16). The pathogenicity of 8.3 is demonstrated by the accelerated rate of diabetes development observed in 8.3 TCR transgenic NOD mice (17), and by adoptive transfer studies (9, 18). The AI4 clone was isolated from the earliest detectable islet infiltrates (7), and AI4 TCR transgenic NOD mice were subsequently shown to develop accelerated disease, even in the complete absence of CD4$^+$ T cell help (19). AI4 has the unusual feature of recognizing both H-2K^d- and H-2D^b-bound peptides, although the peptides seen in each case do not appear to be the same (20). To date, only mimotope peptides for AI4, recognized in the context of H-2D^b, have been identified (20, 21).

In this study, we have used peptide/MHC tetramers to perform a detailed analysis of the three pathogenic CD8$^+$ T cell populations among T cells cultured from islets of individual NOD mice. Even within age-matched groups, individuals exhibited their own unique signature of β cell-specific autoreactivity. Thus, even within this inbred strain, spontaneous autoimmune responses show a variable developmental course. Screening of a recombinant peptide library in positional scanning format, followed by pattern searches of the mouse protein database, revealed the unexpected finding that one target recognized by the AI4-like T cell population is a naturally processed and presented peptide from a protein that is not β cell specific.

Materials and Methods

Mice

NOD/Lt mice were maintained by brother-sister mating. All NOD mice used for this work were bred at Albert Einstein College of Medicine; in this colony, 75% of females develop diabetes by 30 wk of age. NOD.AI4$\alpha\beta$ transgenic mice, which transgenically express the TCR of the β cell-autoreactive CD8$^+$ T cell clone AI4 (7), have been previously described (19).

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[3] Abbreviations used in this paper: IGRP. islet-specific glucose-6-phosphatase catalytic subunit-related protein; DMK, dystrophia myotonica kinase; MRCK, myotonic dystrophy kinase-related Cdc42-binding protein kinase; PLN, pancreatic lymph node.

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All mice were maintained under specific pathogen-free conditions and used in accordance with institutional guidelines for animal welfare.

Peptides

A positional scanning synthetic combinatorial peptide library (22) and the natural peptides listed in Table I were purchased from Mimotopes (Victoria, Australia). Mim (YFIENYLEL), MimA2 (YAIENYLEL), murine glutamic acid decarboxylase $65_{206-214}$ (TYEIAPVFV) (23), and TRL9 (TSPRNSTVL) peptides were synthesized by standard solid-phase methods using fluorenylmethoxycarbonyl chemistry in an automated peptide synthesizer (model 433A; Applied Biosystems, Foster City, CA) in the Laboratory for Macromolecular Analysis and Proteomics at Albert Einstein College of Medicine, and their identities were confirmed by mass spectrometry.

Islet T cell tetramer analysis

Islet isolation by collagenase perfusion of the common bile duct was modified from a previously described protocol (24). Briefly, the bile duct was cannulated and the pancreas perfused with collagenase P (Roche, Indianapolis, IN). The inflated pancreas was removed and incubated at 37°C to digest exocrine tissue. Islets were washed and then resuspended in HBSS containing DNase I (Worthington Biochemical, Lakewood, NJ) and handpicked using a silanized micropipet. Isolated islets were resuspended in RPMI 1640 medium supplemented with 10% FBS (HyClone, Logan, UT) and 50 U/ml human rIL-2 (PeproTech, Rocky Hill, NJ), and cultured intact in 24-well tissue culture plates (~50 islets/well) for 7–9 days.

PE-conjugated MimA2/H-2D^b tetramers were obtained through the National Institute of Allergy and Infectious Diseases Tetramer Facility and titrated to determine optimal concentration. PE-conjugated NRP-V7/H-2K^d and INS-L9/H-2K^d tetramers were prepared as previously described (15, 16). MimA2 (YAIENYLEL) is a mimotope peptide recognized by AI4-like T cells (20). NRP-V7 (KYNKANVFL) is a mimotope peptide recognized by 8.3-like T cells (16, 25). INS-L9 (LYLVCGERL) is the G9L variant of murine insulin B_{15-23}. INS-L9 shows more stable binding to H-2K^d than insulin B_{15-23}, but both are recognized by the G9C8 clone (26). FITC-conjugated anti-CD8α was purchased from BD Pharmingen (San Diego, CA). Cells were incubated with tetramer and/or Ab in 96-well V-bottom plates at 4°C for 45 min. Samples were analyzed by flow cytometry using a FACSCalibur instrument and CellQuest software (BD Immunocytometry Systems, San Jose, CA). All samples were gated on live cells, as determined by propidium iodide labeling.

Cytotoxicity assay

AI4 CTL were generated by culturing splenocytes from NOD.AI4$\alpha\beta$ transgenic mice with IFN-γ-treated NIT-1 β cells (27) and IL-2, as described (28). CTL were used in 16-h ^{51}Cr release cytotoxicity assays to test for recognition of peptide-pulsed target cells at an E:T ratio of 40:1, as described (28). TAP-deficient RMA-S cells (29) were used as targets. Synthetic peptides or peptide library mixes were used at the concentrations indicated in the figures.

IFN-γ ELISPOT

ELISPOT plates (MAHA S45 10; Millipore, Billerica, MA) were coated with anti-murine IFN-γ Ab (R4-6A2; BD Pharmingen) and blocked with 1% BSA (Sigma-Aldrich, St. Louis, MO) in PBS. APC (mitomycin C-treated NOD splenocytes) were added at 2×10^4 cells/well and pulsed with 1 μM peptide. Cultured islet T cells were added at 2×10^4 cells/well, and plates were incubated at 37°C for 40 h. Secreted IFN-γ 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-indolylphosphate/NBT chloride substrate (Sigma-Aldrich, St. Louis, MO). For all incubation steps, plate bottoms were covered with aluminum foil to distribute heat uniformly across the plate, as described (30).

RT-PCR and molecular cloning

Total RNA was isolated from IFN-γ-treated NIT-1 β cells using the RNeasy kit (Qiagen, Valencia, CA) and mRNA purified using the Oligotex mRNA kit (Qiagen). First strand cDNA was synthesized using oligo(dT)$_{12-18}$ primers and Moloney murine leukemia virus reverse transcriptase, according to manufacturer's protocol (Invitrogen Life Technologies, Carlsbad, CA). For detection and cloning of full-length dystrophia myotonica kinase (DMK) cDNA (1.9 kb), sense (5′-AGCTTCCAACATGT CAGCCGAAGTG-3′) and antisense (5′-GAATTCTCAGGGGGCGAAG GTGG-3′) primers containing *Hind*III and *Eco*RI restriction sites (underlined), respectively, were used. For detection of full-length myotonic dystrophy kinase-related Cdc42-binding protein kinase β (MRCKβ) cDNA (5.6 kb), sense (5′-GCACCATGTCGGCCAAGG-3′) and antisense (5′-TCTATCTACAAACTGATTCTACAT-3′) primers were used. cDNA was amplified using *PfuTurbo* hotstart DNA polymerase (Stratagene, La Jolla, CA). For cloning of a 441-bp cDNA fragment of MRCKβ inclusive of exons 3–6, cDNA was amplified using KOD hotstart DNA polymerase (Novagen, Madison, WI) and sense (5′-TTTGGTGAGGTTGCTGTT GTC-3′) and antisense (5′-AGACTGAACAGTGCCATCAT-3′) primers corresponding to nt 259–279 and 699–680 of the murine MRCKβ coding sequence, respectively.

For cloning of full-length DMK and the 441-bp fragment of MRCKβ, blunt-ended RT-PCR products were gel purified using the QIAEX II gel extraction kit (Qiagen) and ligated to the cloning vector pPCR-Script Amp SK$^+$ (Stratagene). The identities of the inserts were confirmed by sequencing at the DNA Sequencing Facility of the Albert Einstein College of Medicine. Full-length DMK cDNA was subsequently removed by *Hind*III/*Eco*RI digestion and transferred to the expression vector pcDNA3.1$^+$ (Invitrogen Life Technologies).

Transient transfection

COS-7 cells were transfected, using a DEAE-dextran protocol (31), with varying concentrations of DMK/pcDNA3.1$^+$ and 10 ng/ml pcDNAI/H-2D^b (generously provided by N. Shastri, University of California, Berkeley, CA), with varying concentrations of pcDNA3.1$^+$ and 10 ng/ml pcDNAI/H-2D^b, or with varying concentrations of DMK/pcDNA3.1$^+$ alone. Separate cultures were transfected with pcDNAI/H-2D^b alone and pulsed with varying concentrations of FNL9 (DMK$_{138-146}$) peptide as positive control (data not shown). Following coculture with AI4 CTL, T cell response was measured as IFN-γ release by ELISA using capture (R4-6A2) and detecting (biotinylated XMG1.2) anti-murine IFN-γ Abs purchased from BD

Table I. *Natural peptides with AI4-preferred TCR contact residues*[a]

Peptide Sequence	Peptide Abbreviation	Accession No.	Protein Source(s)
HEAESYMYL	HL9	Q60755	Calcitonin receptor precursor
FQDENYLYL	FNL9	P54265	DMK
		Q7TT50	MRCK β
FTDESYLEL	FSL9	P56960	Polymyositis/Scleroderma autoantigen 2
RLFENYIEL	RIL9	Q9D4H1	Exocyst complex component Sec5
QYLENYLWM	QM9	P97871	Aquarius
TNKENYTEL	TL9	Q8C6H8	Stromal cell-derived factor receptor 1
EVVESYMYL	EL9	Q8K2N2	Similar to 1,2-α-mannosidase IC (fragment)
RTSENYLEL	RLL9	Q80TB9	MKIAA1569 protein
VMLENYTHL	VML9	Q8C1H3	Similar to Kruppel-associated box zinc finger protein
DMHENYMEM	DM9	Q8BS74	X-linked lymphocyte-regulated complex
VTLENYTHL	VTL9	AAH53084	Hypothetical protein
YFIENYLEL	Mim		Original AI4 mimotope
YAIENYLEL	MimA2		A2-substituted AI4 mimotope

[a] This list contains the results of a ScanProsite search of Swiss-Prot and TrEMBL protein databases, limited to the taxon *Mus musculus,* for peptides with the AI4-preferred pattern X-X-X-E-[NS]-Y-[ILMTV]-[EHWY]-[LM], in which X represents any amino acid.

Pharmingen. Plates were developed with streptavadin-conjugated HRP and ABTS (Southern Biotechnology Associates, Birmingham, AL).

Results

Analysis of the pathogenic IGRP-reactive, insulin-reactive, and AI4-like CD8$^+$ T cell populations cultured from NOD islets

The incubation of intact islets from NOD mice in IL-2-supplemented medium allows for the expansion of β cell-autoreactive CD8$^+$ T cells, as their cognate Ags are naturally present within the culture system (15). This expansion allows sufficient numbers of cells to be obtained to permit reliable T cell analyses of individual mice. NRP-V7/H-2K^d and INS-L9/H-2K^d tetramers have previously been used to detect 8.3-like and G9C8-like T cells, respectively, in islet infiltrates (14, 16). We recently identified a tetramer reagent (MimA2/H-2D^b) that can similarly be used to detect AI4-like T cells (20). The tetramer reagents were used to characterize the relative proportions of these three known pathogenic CD8$^+$ T cell populations at various prodromal stages of diabetes development (5, 7, 11, and 15 wk old) in individual NOD mice. Islets from six nondiabetic, female NOD mice of each age were individually cultured in IL-2-supplemented medium. The expanded T cells from each culture were stained with FITC-conjugated anti-CD8 Ab and PE-conjugated tetramer, and analyzed by flow cytometry (Fig. 1). For these experiments, we defined tetramer-staining populations accounting for $\geq$1% of the CD8$^+$ cells as a tetramer$^+$ population. In separate experiments, we have found that a tetramer-staining population of this size invariably correlates with the detection of peptide-specific T cells using an IFN-γ ELISPOT assay (our unpublished data).

With the exception of one of the 5-wk-old animals, all mice had at least one tetramer$^+$ T cell population. However, the percentages of CD8$^+$ cells making up each of the tetramer$^+$ populations varied from individual to individual. Furthermore, nine mice had only one tetramer$^+$ population, nine had two, and five had all three. The number of tetramer$^+$ populations did not correlate with age. When all three tetramer$^+$ populations were present, the majority population was most commonly 8.3-like. Among the nine mice that had two tetramer$^+$ populations, most harbored both NRP-V7 and INS-L9 tetramer$^+$ populations, although their relative proportions varied considerably among individual mice. Thus, individual NOD mice demonstrate high variability among their islet-infiltrative CD8$^+$ T cell populations, both in terms of the number of tetramer$^+$ populations and the relative proportions of each within the islet infiltrate. Individual patterns of autoimmune activity were also reflected in the total percentage of CD8$^+$ T cells that could be accounted for by the sum of the three known pathogenic populations. Although other antigenic specificities exist among these infiltrates, the tetramer-staining profiles demonstrate that the G9C8-, 8.3-, and AI4-like T cell populations often account for a considerable proportion (up to 60%) of the CD8$^+$ T cells infiltrating islets of NOD mice, and insulin, IGRP, and the Ag targeted by AI4 CTL are, therefore, of relevance to understanding disease pathogenesis.

Defining AI4-preferred amino acids in TCR contact positions of H-2D^b-binding peptides

Our efforts to characterize the antigenic specificities of AI4 by analysis of peptides eluted from MHC of NOD-derived β cells are ongoing. To complement this biochemical approach, we recently screened a recombinant peptide library in positional scanning format to identify the residues preferred by AI4 at each position of the peptide. This work led to the identification of the AI4 mimotope peptide YFIENYLEL (designated Mim) (20, 21), and subsequent alanine substitution allowed the derivation of the superagonist peptide MimA2 (YAIENYLEL), which shows far superior binding to H-2D^b and is recognized by AI4 at lower concentrations (20). However, protein database searches using the mimotope sequences did not lead us to a natural counterpart.

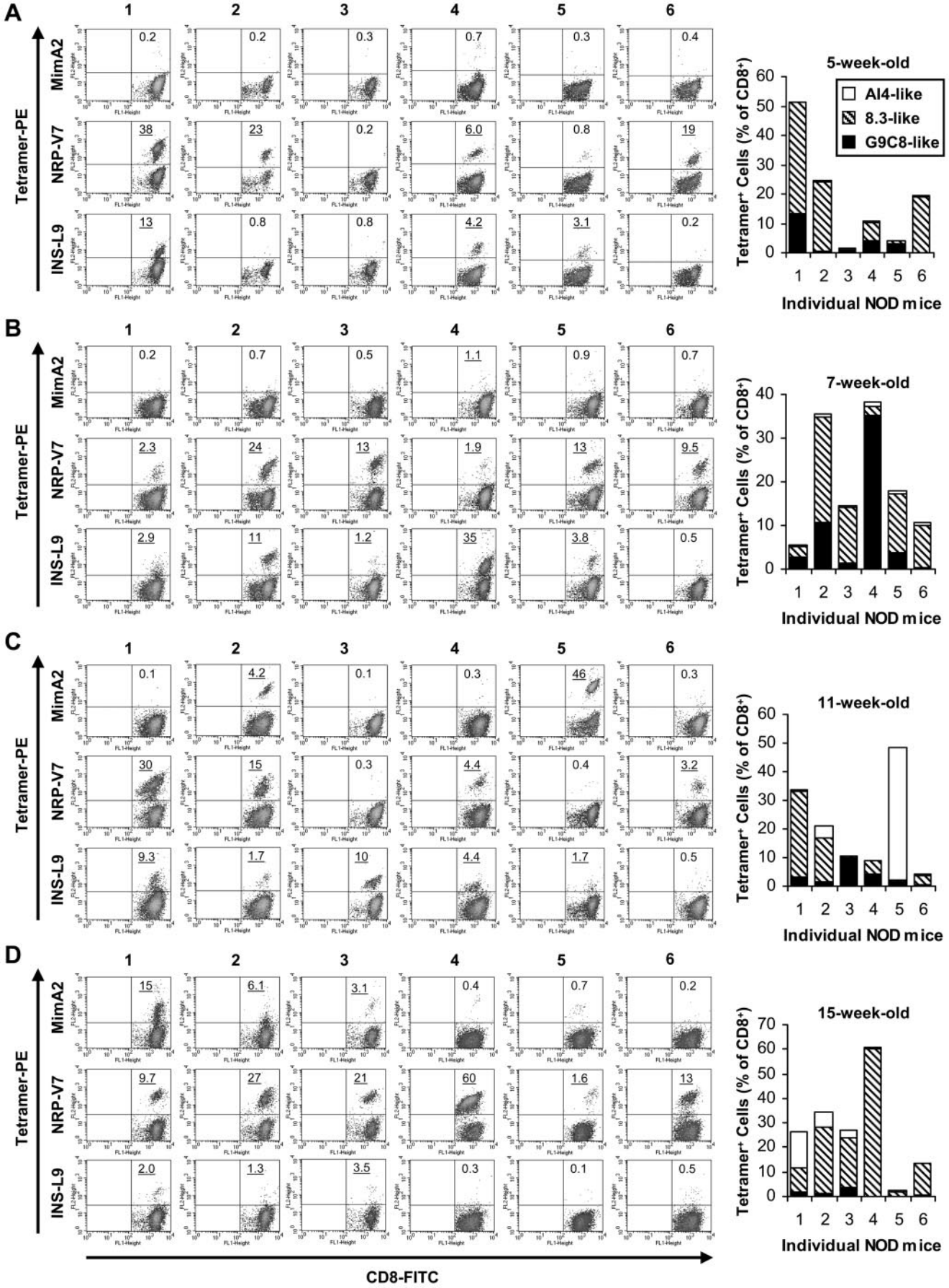
The IGRP peptide targeted by 8.3-like T cells (VYLKTNVFL) (14) is very similar in sequence to the superagonist 8.3 mimotope peptide NRP-V7 (KYNKANVFL) (25). These peptides have the same amino acids at positions 2 and 9, the H-2K^d-binding anchor positions (32), and at positions 4, 6, 7, and 8, which are predicted to be important in TCR recognition of H-2K^d-binding peptides (26). Thus, to identify an AI4-targeted Ag, we sought to better characterize AI4-preferred amino acids at the TCR contact positions of H-2D^b-binding peptides, i.e., positions 4, 6, 7, and 8 (33). To identify the amino acids preferred by AI4 CTL, we designed a synthetic combinatorial peptide library composed of 9-mers with fixed H-2D^b anchor residues N at position 5 and L at 9 (32). The library was composed of four sets of peptides (Fig. 2*A*), with each set consisting of multiple mixes in which a nonanchor position (O) was fixed. The amino acids used for the fixed positions were those that had elicited an AI4 response greater than the average for all 19 aa (cysteine excluded) fixed at that position in the original library screen used to identify the Mim peptide (20). All other nonanchor positions were similarly composed of equimolar mixes (X) of the amino acids that had previously elicited an AI4 response greater than the average for all 19 aa fixed at that position. The AI4 lysis response elicited by each of the peptide mixes is shown in Fig. 2*B*. Based on this data and subsequent repeated screens at different dilutions, we defined the AI4-preferred TCR contact amino acids of H-2D^b-bound peptides to be E at position 4; Y at position 6; I, L, M, T, and V at position 7; and E, H, W, and Y at position 8.

Identification of a natural peptide recognized by AI4-like T cells

To identify peptides found in murine proteins that might be recognized by AI4, we searched databases for proteins containing peptide sequences conforming to the pattern X-X-X-E-[NS]-Y-[ILMTV]-[EHWY]-[LM], in which X is any of the natural 20 aa. The amino acids included for the TCR contact positions 4, 6, 7, and 8 are based on the library screening data just described. H-2D^b-binding anchor residues N or S at position 5 and L or M at position 9 were included to restrict our search to H-2D^b-binding peptides (32) (our unpublished data). We used the pattern profile search algorithm ScanProsite (http://us.expasy.org/tools/scanprosite/) to search the Swiss-Prot and TrEMBL protein databases for nonamer peptides conforming to the AI4-preferred motif. The search was restricted to the taxon *Mus musculus*. The resulting peptides along with their protein source(s) are listed in Table I.

The natural peptides conforming to the AI4-preferred motif were tested for recognition by AI4 CTL at concentrations ranging from 0.1 nM to 1 μM. The majority of the peptides elicited no more of an AI4 response than the H-2D^b-binding, negative control peptide TRL9 (Fig. 3*A*). A minority of the peptides elicited an AI4 response at the highest concentrations tested. However, only one peptide (FNL9) elicited an AI4 response mirroring that obtained with the Mim peptide. The FNL9 peptide recognized by AI4 is found in two murine proteins, DMK (34) and MRCKβ (35).

Although these results were intriguing, it remained possible that recognition of the FNL9 peptide was unique to the AI4 clone and not representative of the AI4-like T cell population detectable in NOD islet infiltrates. To test whether the FNL9 peptide was recognized by the AI4-like T cell population, we assayed T cells cultured from NOD islets for their ability to secrete IFN-γ in response to peptide-pulsed target cells in an ELISPOT assay. As shown in Fig. 3*B*, FNL9 is indeed recognized by a detectable population of T cells cultured from the islets of NOD mice.



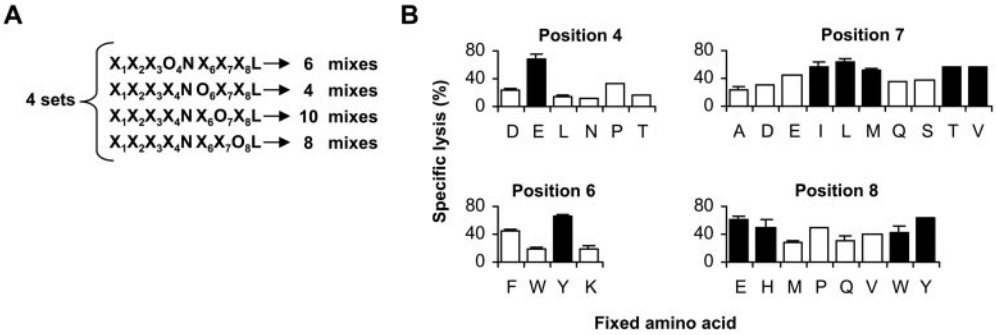


FIGURE 2. Definition of AI4-preferred TCR contact residues of H-2D^b-binding peptides. *A*, Schematic of the H-2D^b-binding peptide library consisting of 9-mers with fixed anchors N5 and L9. The library is composed of four sets, each defined by a fixed nonanchor position (O). Each set is composed of peptide mixes in which position O is fixed as one of the amino acids that previously elicited an AI4 response at that position, as described in the text. Each nonfixed nonanchor position X consists of an equimolar mix of the amino acids that had previously elicited an AI4 response when fixed at that position. X_1 represents an equimolar mixture of F, H, L, M, N, P, Q, S, T, W, and Y; X_2 represents an equimolar mixture of A, F, G, H, I, L, M, N, Q, S, V, and W; and X_3 represents an equimolar mixture of F, I, L, M, N, P, Q, S, and W. O_4 was individually fixed as D, E, L, N, P, or T, and X_4 represents an equimolar mixture of these same amino acids. O_6 was individually fixed as F, W, Y (or K as a negative control, as K was found to not be tolerated at position 6 in our previous work), and X_6 represents an equimolar mixture of F, W, and Y. O_7 was individually fixed as A, D, E, I, L, M, Q, S, T, or V, and X_7 represents an equimolar mixture of these same amino acids. O_8 was individually fixed as E, H, M, P, Q, V, W, or Y, and X_8 represents an equimolar mixture of these same amino acids. *B*, AI4 cytotoxic response toward RMA-S target cells pulsed with mixes from the H-2D^b-binding peptide library at a final concentration of 2 ng/ml. Based on this data and subsequent screens at different dilutions, we defined the AI4-preferred TCR contact amino acids (■) to be E at position 4; Y at position 6; I, L, M, T, and V at position 7; and E, H, W, and Y at position 8.

DMK is expressed by β cells and is recognized by AI4 in an H-2D^b-restricted manner

Neither DMK nor MRCKβ is β cell specific, as both have been shown to be expressed in most tissues tested (34–37). However, expression in pancreatic β cells has not been documented. We isolated mRNA from NOD-derived NIT-1 β cells, because we know that these cells generate H-2D^b-bound peptides recognized by AI4 T cells (20). By RT-PCR, we detected both DMK and MRCKβ expression (Fig. 4*A*). RT-PCR of NOD islet RNA confirmed that DMK and MRCKβ are also both expressed in islets (our unpublished data). Sequencing of the appropriate regions of DMK and MRCKβ confirmed that, like the GenBank sequences (accession numbers NM_032418 and NM_183016, respectively), the NOD-derived cDNAs also encode the FNL9 peptide. We then transiently transfected COS-7 cells with varying concentrations of a full-length DMK expression construct, or vector alone, in combination with an H-2D^b expression construct, cocultured them with AI4 CTL, and monitored AI4 recognition by IFN-γ ELISA. Targets transfected with DMK, but not vector alone, elicited a dose-dependent AI4 CTL response (Fig. 4*B*). This recognition was H-2D^b dependent, as DMK transfection without H-2D^b resulted in no AI4 response. Thus, DMK represents a widely expressed protein, also expressed in β cells, which can be targeted by AI4 CTL. Whether the FNL9 peptide can also be processed from the related protein MRCKβ is under investigation.

Discussion

In NOD mice, three pathogenic β cell-autoreactive CD8$^+$ T cell clones have been identified (9, 10, 17–19). The G9C8 and 8.3 clones have previously been shown to represent detectable populations of CD8$^+$ T cells within islet infiltrates of NOD mice by peptide/MHC tetramer analysis (12, 14–16). We recently identified mimotope peptides recognized by AI4 CTL and used peptide/H-2D^b tetramers to demonstrate that AI4-like T cells are also detectable in islet infiltrates (20, 21). The simultaneous analysis of all three of these T cell populations within islet infiltrates of individual NOD mice has not previously been reported. In this study, we have found that, even within a given age group, the number of tetramer$^+$ populations present within infiltrates from individual mice varied, as did the relative proportions of each of the tetramer$^+$ populations (Fig. 1). Thus, individual NOD mice, even those of the same age, exhibit unique patterns of CD8$^+$ T cell reactivity to β cell Ags. These findings may be due, in part, to the fact that development of diabetes in NOD mice is not completely synchronous. Arguing against this explanation is the observation

FIGURE 1. Individual NOD mice exhibit distinct patterns of CD8$^+$ T cell reactivity to three islet Ags. Islets were isolated from individual female NOD mice and cultured in the presence of IL-2. The resulting cells were stained with FITC anti-CD8 and PE tetramer, as indicated, and analyzed by flow cytometry. Profiles of islet-infiltrating T cells are shown for 5 (*A*)-, 7 (*B*)-, 11 (*C*)-, and 15 (*D*)-wk-old mice. Each vertical set of three density plots represents staining of cells cultured from an individual mouse. All samples are gated on CD8$^+$ cells, and the numbers in each *upper right quadrant* represent percentage of CD8$^+$ cells staining with the indicated tetramer. Underscores designate tetramer$^+$ populations (i.e., those representing $\geq$1% of CD8$^+$ cells). MimA2, YAIENYLEL/H-2D^b tetramer (stains AI4-like T cells); NRP-V7, KYNKANVFL/H-2K^d tetramer (stains 8.3-like T cells); INS-L9, LYLVCGERL/H-2K^d tetramer (stains G9C8-like T cells). Graphical summaries of the flow cytometry data are presented at the *right* of each panel. The culture of intact islets has been reported to permit the expansion of β cell-autoreactive CD8$^+$ T cells (15). This was verified in a preliminary experiment in which islets from four 11-wk-old NOD mice were pooled to obtain sufficient numbers of cells to perform peptide/MHC tetramer analyses both directly ex vivo and after 7 days of culture. As expected, we observed an expansion of both the 8.3-like and AI4-like T cell populations from 4.5 and 1.7% of CD8$^+$ T cells on day 0 to 21 and 14% on day 7, respectively. Although some selection may occur during the culture period, resulting in a change in the absolute ratio between any two of the different T cell populations, we consistently find that the rank order of prevalence of the different T cell populations remains stable from days 0 to 7 (data not shown). Furthermore, islet preparations that are split in half on day 0 and analyzed independently on day 7 reveal identical rank orders of prevalence (data not shown).

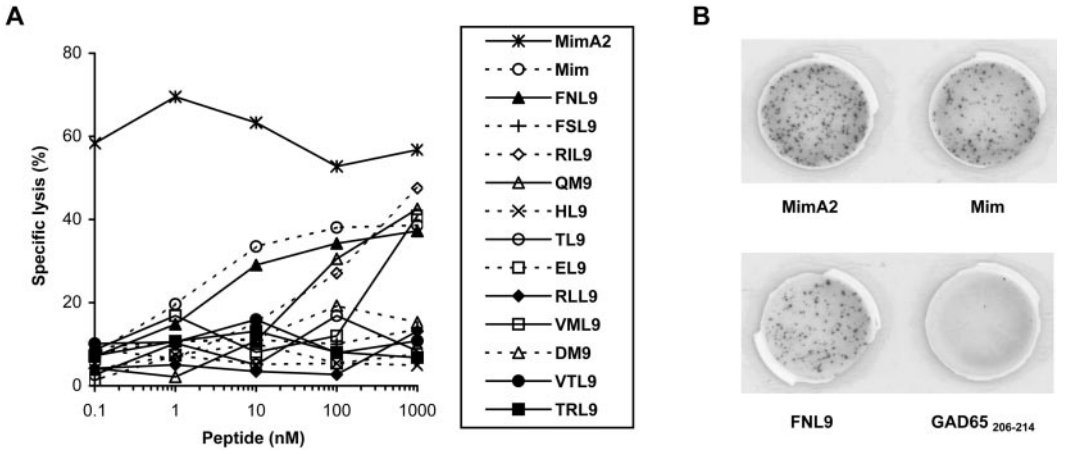


FIGURE 3. Identification of a natural peptide recognized by AI4-like CTL. *A*, AI4 cytotoxic response toward RMA-S target cells pulsed with varying concentrations of Mim, MimA2, natural peptides with AI4-preferred TCR contact residues (abbreviations as listed in Table I), or an H-2D^b-binding negative control peptide (TRL9). Each data point represents the average of two determinations; SDs were ≤8% specific lysis in all cases. Results shown are representative of three independent experiments. *B*, IFN-γ release response of T cells cultured from NOD islets toward peptide-pulsed target cells. T cells cultured from islets pooled from four 11-wk-old nondiabetic female NOD mice were incubated with target cells pulsed with the indicated peptides, and IFN-γ release was measured by ELISPOT.

that the 5-wk-old mice, although all in the first stages of the insulitic process, nonetheless also demonstrated unique signatures of autoimmune activity. Furthermore, in independent studies, we found that a correlation exists between the number of T cells derived per islet on day 7 and the degree of inflammation measured histologically (data not shown). However, for the mice examined in Fig. 1, we observed no correlation between the cell yield per islet and the percentage of tetramer-positive cells of any of the three populations (data not shown). Thus, we do not believe that the unique reactivity profiles of each animal simply reflect different stages of inflammation. An alternative explanation is that several antigenic specificities may be responsible for the initiation and progression of β cell destruction, and that in any individual mouse the autoimmune response may be dominated by T cells recognizing any one of these Ags. However, it is important to keep in mind that, based on the diabetes incidence in our NOD colony, ~25% of the individuals analyzed in Fig. 1 would not have developed diabetes by 30 wk of age. Thus, we cannot say that all of the patterns of autoimmune activity depicted in Fig. 1 are necessarily pathogenic ones. In this regard, individual 5 in the 15-wk-old group is of particular interest. Due to considerable islet destruction by 15 wk of age, the average number of islets isolated per animal in this age group was 141 ± 59 (compared with 238 ± 26 for the 7-wk-old group). However, mouse 5 yielded both the highest number of islets per individual (248) and the lowest number of T cells per islet among the 15-wk-old group. These data suggest that this was most probably a diabetes-resistant animal. Importantly, it was only weakly positive for NRP-V7/H-2K^d (<2% of CD8$^+$ cells) and negative for both INS-L9/H-2K^d and MimA2/H-2D^b. These observations further suggest the importance of the IGRP-, insulin-, and DMK-reactive T cell populations in the pathogenesis of diabetes in NOD mice.

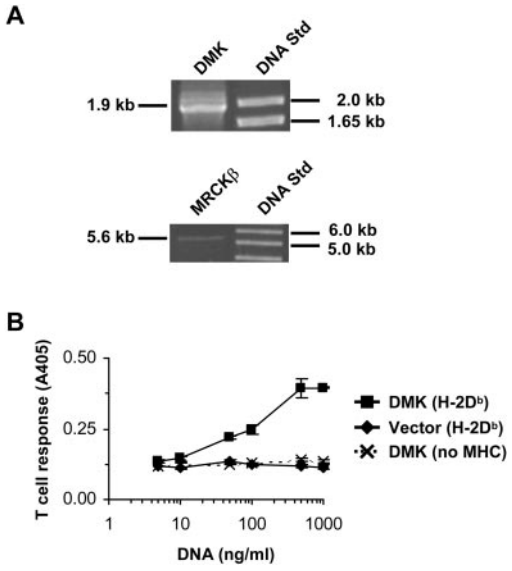


FIGURE 4. DMK is expressed in NIT-1 β cells and recognized by AI4 CTL in an H-2D^b-restricted manner. *A*, RT-PCR of mRNA purified from IFN-γ-treated NIT-1 β cells was performed, as described in *Materials and Methods*, using primers designed to amplify full-length DMK (1.9 kb) and MRCKβ (5.6 kb) cDNA. DNA Std, 1-kb Plus DNA Ladder (Invitrogen, Carlsbad, CA). *B*, COS-7 cells were transiently transfected with 10 ng/ml H-2D^b expression construct (solid lines) along with varying concentrations of a DMK expression construct or vector alone, as indicated, and cultured with AI4 CTL. COS-7 cells transiently transfected with varying concentrations of a DMK expression construct, but no MHC construct (broken line), were separately cultured with AI4 CTL. T cell response was measured as IFN-γ release by ELISA, and is reported as absorbance at 405 nm (A405).

Our previous work indicated that AI4 targets two perhaps related, but as yet unidentified, NIT-1 β cell peptides eluted from the class I MHC molecule H-2D^b (20). In this study, we have identified an H-2D^b-binding peptide (FNL9) that is recognized not only by the original AI4 T cell clone (Fig. 3*A*), but also by T cells cultured from the islets of NOD mice (Fig. 3*B*). In addition, we have demonstrated that DMK, which includes the FNL9 sequence and is expressed in both the NIT-1 β cell line and islets, is recognized by AI4 CTL in an H-2D^b-restricted manner (Fig. 4). We and our collaborators are currently using a biochemical approach (14) to determine whether FNL9 is one of the peptides responsible for AI4 activity toward H-2D^b-eluted NIT-1 β cell peptides.

DMK is a serine/threonine protein kinase expressed in most tissues examined (34). The DMK gene is composed of 15 exons that give rise to multiple differentially spliced variants in humans and

mice (38). Intracellularly, DMK may be localized to the endoplasmic reticulum, cytosol, or mitochondria, depending on the splice variant expressed (39). Although the exact function of DMK is unknown, studies have demonstrated a variety of roles for the protein depending on the cell type examined (40–43). Prior interest in understanding the function of DMK stemmed from its putative role in myotonic dystrophy (37). DMK has not previously been implicated as an autoantigen in NOD mice or in type 1 diabetes patients.

DMK does not represent the first β cell Ag targeted in diabetes that is expressed outside of β cells (44). For example, glutamic acid decarboxylase is expressed in several cell types in addition to β cells, and is frequently targeted by autoantibodies in type 1 diabetes patients (45). Even widely expressed proteins have been implicated as autoantigens in certain organ-specific autoimmune diseases (46–53). For example, the ubiquitously expressed protein glucose-6-phosphate isomerase has been identified as the target of pathogenic $CD4^+$ T cells in mice developing a rheumatoid arthritis-like joint pathology (52).

In NOD mice, a wave of physiological β cell death has been reported to occur at ~2 wk of age, just before T cell activation in the pancreatic lymph nodes (PLN) and the first signs of insulitis, and has been implicated as a potential initiating event in diabetes development (54). In this scenario, apoptotic β cells are engulfed by dendritic cells in the pancreas and brought to the PLN, where β cell Ags are then presented to T cells. This model is partly based on independent studies demonstrating that diabetogenic $CD4^+$ (BDC2.5) or $CD8^+$ (8.3) T cell clones proliferate in PLN during this time period (i.e., at ~3–4 wk), but earlier responses can be detected if β cell apoptosis is induced by treatment with the β cell toxin streptozotocin (55–57). Furthermore, 8.3 proliferation in PLN was shown to occur normally in the absence of CTL-mediated β cell destruction, suggesting that the presentation of β cell Ags for the initial priming of autoreactive T cells may result from physiological β cell death rather than T cell-mediated β cell destruction (58). Interestingly, however, other studies have shown that the proliferation of β cell-reactive T cells in the PLN is, in some instances, an abortive tolerogenic, rather than a pathogenic, process (59, 60). This might explain the finding that the induction of limited β cell apoptosis can enhance the process of T cell tolerance induction in PLN (61).

Although widely expressed, it is possible that a pathogenic autoimmune response to DMK occurs in the PLN, but not in other lymph nodes, due to β cell apoptosis. In support of this, B cells (and, thus, presumably T cells) recognizing the ubiquitously expressed autoantigen glucose-6-phosphate isomerase were recently reported to be initially activated only in the lymph nodes specifically draining the affected joints in a murine model of rheumatoid arthritis (62). T cells activated in certain secondary lymphoid organs, e.g., cutaneous or mesenteric lymph nodes, are instructed to express tissue-specific homing molecules, including adhesion molecules and chemokine receptors, that will cause the activated T cells to migrate preferentially to the skin or gut, respectively (63). If a similar process occurred in the PLN, and if DMK were only presented in the PLN, this could result in the migration of DMK-reactive T cells preferentially to the pancreas. Such a phenomenon would explain how a widely expressed Ag could be the target of a β cell-specific autoimmune response. Although there is no published evidence suggesting that T cells activated in the PLN will preferentially home to the pancreas if their cognate Ag is also expressed elsewhere, this remains a formal possibility. Alternative explanations should also be considered. Perhaps due to tissue-specific differences in expression or turnover, the FNL9 peptide is only presented by β cells, but not other cell types, in sufficient quantities to allow for T cell recognition, although DMK is widely expressed. It is also possible that the combination of adhesion molecules and chemokines in the NOD islet environment preferentially attracts activated T cells to this site, including those that are FNL9 reactive, and for this reason, autoimmune destruction of other DMK-expressing tissues is not observed. Although NOD mice can mount autoimmune responses to a variety of restricted and ubiquitous proteins (44), overt autoimmune destruction is only observed in select organs (i.e., pancreas, thyroid, and salivary glands). However, cryptic autoimmune diseases can become manifest in NOD mice whose immune systems are manipulated in some way. For example, B7-2-deficient NOD mice are diabetes resistant, but instead develop spontaneous autoimmune peripheral polyneuropathy (64). Although NOD mice have demonstrated immune responses to proteins expressed in neurons and Schwann cells, leading to damage of these cells in the vicinity of the islet (65, 66), peripheral nerve damage has not been reported in standard NOD mice. Similarly, an early lethal $CD8^+$ T cell-mediated autoimmune myositis develops in NOD mice transgenically made Th1 cytokine deficient (67), although myositis is only rarely observed in nontransgenic NOD mice. Thus, for reasons that are still unclear, autoimmune activity is focused mainly on the islet in unmanipulated NOD mice, although some of the targeted Ags are expressed in Schwann cells or neurons or are ubiquitously expressed.

As DMK is highly expressed in muscle, it is tempting to speculate that the myositis observed in Th1 cytokine-deficient NOD mice is due to AI4-like T cells. If experimental evidence supports this notion, the AI4/myositis system would provide an excellent tool for better understanding why organ-specific disease occurs in the context of a loss of tolerance to widely expressed Ags.

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