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*J Immunol* 2001; 166:4438-4445;
doi: 10.4049/jimmunol.166.7.4438
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Distinguishing Self from Nonself: Immunogenicity of the Murine H47 Locus Is Determined by a Single Amino Acid Substitution in an Unusual Peptide

Lisa M. Mendoza,* Gilbert Villaflor,* Peter Eden,2† Derry Roopenian, † and Nilabh Shastri3*  

Histocompatibility (H) Ags are responsible for chronic graft rejection and graft versus host disease in solid tissue and bone marrow transplantation among MHC-matched individuals. Here we defined the molecular basis of self-nonself discrimination for the murine chromosome 7 encoded H47 histocompatibility locus, known by its trait of graft-rejection for over 40 years. H47 encodes a novel, highly conserved cell surface protein containing the SCILLLYIVI (SI9) nonapeptide in its transmembrane region. The p7 isoleucine-to-phenylalanine substitution in SI9 defined the antigenic polymorphism and T cell specificity. Despite absence of the canonical consensus motif and weak binding to Db MHC I, both H47 peptides were presented to CTLs. However, unlike all the other known H loci, the relative immunogenicity of both H47 alleles varied dramatically and was profoundly influenced by neighboring H loci. The results provide insights into the peptide universe that defines self and the basis of histoincompatibility.  


Immune responses directed toward histocompatibility (H) Ags have been studied for over 80 years (1), and dozens of loci are known to be encoded by the autosomal, sex, and mitochondrial chromosomes (2–6). The H loci give rise to processed peptides that are presented to host T cells by the classical and nonclassical MHC class I molecules on the surface of donor cells (7, 8). Therefore, knowledge of H loci at the molecular level is key to understanding the mechanisms of immunogenicity and histoincompatibility.  

Until recently, the molecular definition of H Ags had remained elusive because of the technical challenges of identifying T cell-stimulating Ags (9). Only 10 unique antigenic precursors are known in mice, where H loci have been extensively studied by genetic, biochemical, and immunological methods and have served as models for the study of graft rejection and the immune system itself. Two of these (ND1, COI) are encoded by the mitochondrial (8, 10), another three (Smyc, Uty, and Dby) by the Y (11–13), and four others by autosomal chromosomes (14–17). For each of these H loci, the donor and host differ in transcriptional regulation or in polymorphic amino acid substitutions within the antigenic peptides, explaining why host CD8 T cell responses were induced to donor peptide-MHC complexes (11, 12, 16). Interestingly, if both the donor and the host expressed polymorphic H Ag peptide-MHC complexes, reciprocal CTL responses were elicited in most cases (8, 10, 14, 15), suggesting that the mere presence of distinct H peptides was sufficient for immunogenicity.  

Here we provide the molecular definition of the polymorphic murine H47 locus that encodes the CD8 T cell epitope of the classically defined H4 transfection locus first detected four decades ago (18, 19). Unlike previously identified H loci where single amino acid substitutions elicit bidirectional CD8 T cell responses, the immunogenicity of the H47 allelic products was profoundly influenced by other neighboring H loci. Thus, the results provide new insights into the unique peptides that allow self-nonself discrimination and the mechanisms of histoincompatibility.

Materials and Methods

Mice, immunizations, and cell lines  

Inbred mice were obtained from or bred at The Jackson Laboratory (Bar Harbor, ME) and have been described previously (19). Immunizations, generation of CTL lines, β-galactosidase (lacZ)-inducible T cell hybrids, and the maintenance of the cell lines has been described (14, 19, 20). Briefly, the H47-specific CTL was generated by immunizing the congenic B10.129-H46/Q/H47b (21M) with B10 splenocytes. All experiments were performed in compliance with the institutional Animal Use and Care Committee guidelines.  

T cell activation assays  

LacZ-inducible T cell hybrids were cocultured with APC that either were transfected with Ag cDNAs or were pulsed with exogenous peptides. The lacZ activity was measured as described previously (20). CTL lysis assays were performed with target cells, either 51Cr-labeled Con A-stimulated  

cDNA library and expression screens  

An EL4 cDNA library was screened in pools of ~50–100 CFU by transfection with recombinant plasmids as described previously (14). Briefly, cDNA pools were cultured, isolated, and screened in 96-well plates by transient transfection into recipient APCs cotransfected with the relevant MHC class I cDNA and B7-2 cDNA. The sequence of 1163 cDNA is

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Received for publication October 24, 2000. Accepted for publication January 23, 2001.

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This research was supported by grants from the National Institutes of Health (to N.S. and D.R.). L.M.M. was supported in part by a National Institutes of Health Training Grant, G.V. by the Howard Hughes Medical Institute Biology Fellows Undergraduate Award, and P.A.E. by a National Cancer Institute postdoctoral fellowship.

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Abbreviations used in this paper: H, histocompatibility; lacZ, β-galactosidase.
available from the National Center for Biotechnology Information GeneBank with the accession number AF335543.

**Expression constructs and peptides**

All plasmid constructs were in the expression vector pcDNA1 unless otherwise noted. Nested deletions were made by PCR of the 11C6 cDNA with a vector-specific forward primer and an 11C6-specific reverse primer H47aR4 (5'-TCAGAAGACGCTGCCTGGTCT-3'), H47aR7 (5'-TCACGCTGCTAAGGCTTACGTC-3') and H47aR8 (5'-TACAG TAGGATGCAGCTGAAAG-3') with Pfu polymerase (Stratagene, La Jolla, CA). PCR fragments were generated by RT-PCR were amplified with the H47aF1 forward with an internal BamHI site from clone for the TGF
tgATG ATGACGACAGCTGAAAG-3' and either the H47aR1 (5'-GC CTTAGCACTGCAAACTG-3') or H47aR4 primer. The genomic DNA PCR fragments presented in Fig. 4 were amplified with the H47F5 (5'- CGATTCCTGCAGCTGAGAG-3') forward and H47R5 (5'-ACAA TCAAGATGCTGACAG-3') reverse primers and sequencing directly. The hemagglutinin (HA) epitope (YPYDVPDYASL)-tagged H47 construct was generated by fusing an oligonucleotide cassette (coding strand, 5'-CTGGGGCTACACCTCGTCTGACGACAGCTGAAAG-3'); noncoding strand, 5'-CTAGAAGACGCTGCCTGGTCTGTTGTTGGG-3') with Pfu polymerase (Stratagene, La Jolla, CA). PCR fragments were generated by RT-PCR were amplified with the H47aF1 forward with an internal BamHI site from clone for the TGF
tgATG ATGACGACAGCTGAAAG-3' and either the H47aR1 (5'-GC CTTAGCACTGCAAACTG-3') or H47aR4 primer. The genomic DNA PCR fragments presented in Fig. 4 were amplified with the H47F5 (5'- CGATTCCTGCAGCTGAGAG-3') forward and H47R5 (5'-ACAA TCAAGATGCTGACAG-3') reverse primers and sequencing directly.

**Genetic mapping**

Southern blot RFLP analysis indicated in Fig. 4b was with genomic DNA purchased from The Jackson Laboratory DNA resource and probed with *Pst*I-labeled 1.2-kb 1163 DNA by standard methods. The Minigene constructs MFI10 (Met-SCILLYVI), MS91 (Met-SCILLYVI), and MSF90 (Met-SCILLYVI) were prepared by using oligonucleotide primers corresponding to the indicated sequences and followed by a labeling and probing step. Minigene construct MSX9 (Met-SCILLYVI1) was prepared as described above except X encoded any amino acid (X = N99c). The synthetic peptides Si09 (SCILLYVI), Si19 (SCILLYVI), SP (AS NENMETM), AEV8 (ANYDIEVC), and W9 (WMHEHLMIDLa) (12) were purified by HPLC and confirmed by mass spectrometry.

**RMA/S stabilization and biochemical analysis**

Peptide/MHC I binding was measured by RMA/S stabilization assay (14). For immunofluorescence microscopy, COS cells were transiently cotransfected with cDNAs expressing K b and H47-tagged at the C terminus. The Minigene constructs MFI10 (Met-SCILLYVI), MS91 (Met-SCILLYVI), and MSF90 (Met-SCILLYVI) were prepared by using oligonucleotide primers corresponding to the indicated sequences and followed by a labeling and probing step. Minigene construct MSX9 (Met-SCILLYVI1) was prepared as described above except X encoded any amino acid (X = N99c). The synthetic peptides Si09 (SCILLYVI), Si19 (SCILLYVI), SP (AS NENMETM), AEV8 (ANYDIEVC), and W9 (WMHEHLMIDLa) (12) were purified by HPLC and confirmed by mass spectrometry.

**Results**

**Expression cloning of the CTL-defined H47 cDNA**

To identify the T-cell stimulating H47 gene product, we generated the lacZ-inducible, T cell hybrid 1AZ by fusing the H47-specific CTL line (19) with the lacZ-inducible fusion partner, BWZ.36/ CD8α (20). Similar to the parental 1A line, the 1AZ hybridoma recognized B6 spleen cells as well as EL4, a thymoma cell line of B6 origin. Furthermore, this recognition was specifically inhibited by anti-D b mAbs (not shown). The lacZ-inducible 1AZ T cell hybrid was used as a probe to screen for the D b-restricted Ag gene in an EL4 cDNA library (14, 22). The cDNA pool, 224-11C, was identified by its ability to stimulate the 1AZ T cell hybrid when transfected into LMK-recipient cells (Fig. 1a). The response of the 1AZ hybrid to pool 224-11C was dose dependent and occurred only when the D b but not K b MHC was cotransfected. The 224-11C cDNA pool was further subdivided into individual colonies by transforming bacteria and several cDNAs that stimulated the 1AZ hybrid were identified (Fig. 1b). One of these clones, designated 1163, stimulated 1AZ when cotransfected with the D b MHC I but not with the irrelevant K b MHC I cDNA (Fig. 1c). We conclude that 1163 cDNA encodes a D b-restricted antigenic activity recognized by the 1AZ T cell hybrid.

The 1163 cDNA maps to the H47 locus on chromosome 7

H47 encodes a CD8 T cell epitope and is part of the H4 complex on chromosome 7 based on tissue-graft rejection and in vitro CTL reactivity assays (19, 23). Genetic mapping was performed to establish whether the 1163 cDNA maps to the chromosome 7-encoded H47 locus, a gene that regulates the expression of H47 or a gene encoding an unrelated but antigenically cross-reactive Ag. Southern blot RFLP analysis of XbaI-digested genomic DNA from mouse strains previously typed for H47 Ag polymorphisms were probed with the 1163 cDNA. Unique 4- and 0.4-kb RFLP bands were found in the H47 strain B10 in contrast to 1.8- and 5-kb bands in the prototypic H47 strain 129/J (Fig. 1d). Most significantly, RFLP patterns of DNAs from 21M (B10.129-H46/147H7) mice the congenic segment of which spans the H4 complex and the recombinant strain RC6 (B10.129-H47) that retains H47 but not H46 were identical to that of the 129/J strain. This demonstrated that the 1163 cDNA probe identified a gene in the H4 congenic interval. Higher resolution mapping consisting of RFLP analysis of a (129/J × B10) × 129/J backcross panel previously typed for the H47 Ag polymorphisms was performed with the 1163 cDNA. Unique 4- and 0.4-kb RFLP bands were found in the H47 strain B10 in contrast to 1.8- and 5-kb bands in the prototypic H47 strain 129/J (Fig. 1d). Most significantly, RFLP patterns of DNAs from 21M (B10.129-H46/147H7) mice the congenic segment of which spans the H4 complex and the recombinant strain RC6 (B10.129-H47) that retains H47 but not H46 were identical to that of the 129/J strain. This demonstrated that the 1163 cDNA probe identified a gene in the H4 congenic interval. Higher resolution mapping consisting of RFLP analysis of a (129/J × B10) × 129/J backcross panel previously typed for the H47 Ag (19) as well as typing of The Jackson Laboratory mapping panel with the 1163 cDNA (Fig. 1e), indicated that the H47 Ags and the 1163 cDNA cosegregated and colocalized within the H4 complex 0.9 ± 0.6 cm distal to the pink eye dilution locus, p. Consistent with this map position, simple sequence length polymorphism analysis of The Jackson Laboratory interspecific BSS mapping panel (http://www.jax.org/resources/documents/cndata/) with 94 (B6 × SPRET/Ei) × SPRET/Ei backcross mice mapped the 1163 cDNA near p and 1.06 ± 0.6 cm from Pcsk6 and Tjp1 (data not shown). Together the results proved that the 1163 cDNA was derived from the H47 locus.

**H47 is a membrane-bound cell surface protein**

The H47 cDNA encoded a novel 188-residue protein with no exact matches in the current nonredundant sequence databases (Fig. 2a; http://www.ncbi.nlm.nih.gov/blast/l). The closest relative of H47+, with a striking 64% amino acid identity, was a hypothetical protein from the human adrenal gland (accession number AF157317). Hydrophaticity analysis of predicted H47 amino acid sequence suggested that it contained a putative transmembrane region, Tm (Fig. 2a). To test this prediction, we used the cDNAs in an in vitro translation assay including [35S]cysteine and microsomes. The H47 cDNA yielded a 24-kDa band, close in size to the predicted 21-kDa product that was membrane-bound, as it associated with the microsomal pellet (Fig. 2b). To determine whether
this polypeptide was translocated into the lumen of the microsomes, the pellet was treated with proteinase K, which resulted in a reduction of the product size to a single 5.5-kDa fragment. Disruption of the membranes with Triton X-100 in the presence of proteinase K caused a further reduction in band intensity and size, indicating that the 5.5-kDa fragment was protected from hydrolysis in intact microsomes. The protease-sensitive 18-kDa and protease-resistant 5.5-kDa fragments correspond closely to those expected if the N-terminal 48-residue peptide, including the transmembrane residues 29–48, were sequestered within the microsomal membranes, and the C-terminal residues 49–188 were in the cytoplasm and accessible to proteinase K. These results directly confirmed the predictions of the sequence analysis and showed that H47 is a type I transmembrane protein.

To further confirm the membrane association and to establish the subcellular location, the H47 protein was tagged at its C terminus with an influenza HA epitope. COS cells were cotransfected with the HA-tagged construct and the Kb cDNA construct. Immunohistology of transfected cells clearly showed that the HA-tagged H47 was largely colocalized with Kb MHC both in intracellular membranes and on the cell surface (Fig. 2c). We conclude that H47 is an integral membrane protein that is expressed on the cell surface.

The SCILLYIVI nonpeptide defines the antigenic activity of the H47b locus

To define the self-nonself relationship between the donor and host strains, we first identified the minimal antigenic peptide encoded by the H47b cDNA by deletion analysis (Fig. 3a). Positive response to the deletion construct ΔR4 mapped the 1AZ stimulating activity to the first 229 nucleotides of clone 1163 (Fig. 3b). Surprisingly, this region did not contain a sequence with the canonical Db consensus motif xxxx[N][x][L, I, M] (24). Differential responses to additional deletion constructs ΔR7 and ΔR8 further narrowed the antigenic activity to be contained within or to overlap residues 44 and 60 (Fig. 3c). Minigenes encoding either the decamer peptide FSCILLYIVI (FI10) or the nonamer peptide SCIL...
LYIVI (SI9) and an additional methionine codon for translational initiation were then tested for their ability to stimulate the 1AZ hybrid. Both minigenes stimulated strong and comparable 1AZ T cell responses (Fig. 3d). We conclude that the 1AZ T cell-stimulating activity was defined by the noncanonical SI9 nonapeptide contained within the transmembrane region (Fig. 2a).

A single Ile→Phe substitution defines antigenic polymorphism of the H47 locus

Northern blot analysis of the donor B6 (H47⁺) and host 129/J (H47⁻) mRNA with a 1163 cDNA probe confirmed that the H47 genes were transcribed in both strains (data not shown). To determine the molecular basis of the allelic polymorphism, B6 (H47⁺), 129/J (H47⁻), and BALB/c (H47⁰) mRNA as well as Spretus (H47⁺) genomic DNA were used as template for PCR with H47-specific primers. The PCR products were cloned into an expression vector, transfected into recipient APCs with Db MHC I cDNA, and tested for their ability to stimulate the 1AZ T cell. All the constructs, except that from the H47⁻ 129/J strain, stimulated the 1AZ T cell hybrid (Fig. 4a). Nucleotide sequence of the 129/J construct revealed that a single nucleotide change had resulted in the amino acid substitution from Ile to Phe at the p7 residue of the SI9 peptide. Remarkably, nucleotide sequences of this region in 15 other mouse strains showed that all, including the evolutionarily distant Mus spretus- and Mus castaneus-derived strains, could be typed as H47⁺ or H47⁻ by the presence of the Ile or the Phe residue, despite other nucleotide sequence differences that were reflected in their distinct RFLP band patterns (Fig. 4b). Thus, the SI9/FI9 coding sequences appear to be conserved in the suborder Mus.

To directly test the functional significance of the p7 Ile-to-Phe substitution within the antigenic peptide, an expression construct encoding the H47⁻ allelic peptide SCILLYFVI (SFI9) was directly compared with the construct encoding H47⁺ SI9 in an endogenous presentation assay. Only the M-[SI9] stimulated the 1AZ T cell hybrid, and the response to the M-[SFI9] construct was virtually indistinguishable from vector alone (Fig. 4c). To rule out the possibility that the inability of the M-[SFI9] minigene to stimulate 1AZ was attributable to inefficient processing of this antigenic precursor, we tested synthetic peptides in an exogenous presentation assay for their ability to stimulate the 1AZ hybrid. Again, the SI9 peptide was recognized by the 1AZ T cells, and at a 1000-fold lower concentration than the allelic SFI9 peptide (Fig. 4d). These results directly confirmed the assignment of the antigenic activity to the SI9 peptide and demonstrated that the single amino acid substitution between the two alleles was sufficient to determine T cell specificity.

We determined whether the inability of the SFI9 peptide to stimulate the 1AZ T cell was attributable to its inability to bind to the Db class I MHC molecule or an effect on TCR recognition. Despite
the lack of a $D^b$ binding consensus motif, both peptides specifically stabilized the $D^b$ MHC I molecule on the surface of RMA/S cells (Fig. 4e). Interestingly, the SFI9 peptide was apparently 5-fold more effective than the SII9 peptide. However, both H47 peptides were far less efficient than the influenza NP or the HY-derived WI9 peptides, both of which conform to the $D^b$ consensus motif (Fig. 4e). Conversely, neither SII9 nor SFI9 peptides stabilized the $K^b$ MHC I molecule that was stabilized by a $K^b$-restricted peptide AFV8 (Fig. 4f). We conclude that the antigenic polymorphism at the H47 locus was attributable to a single amino acid substitution within poor $D^b$ binding S[X,I,F]I9 peptides that determined the ability of T cells to recognize the peptide/$D^b$ complex.

The $p7$ polymorphism influences TCR specificity

To define the stringency with which the T cell specificity was influenced by the polymorphic amino acid substitution in the H47 alleles, we generated M-S[X,I,F]I9 minigene constructs encoding the minimal H47+ antigenic peptide SCILLYIVI with a redundant p7 codon, NNG/C (N = A, C, G or T). This allowed 32 different codons at p7 and included all 20 amino acids designated as X. Individual plasmid DNAs from the M-S[X,I,F]I9 panel were tested for their ability to stimulate the 1AZ hybrid. The frequency of individual clones that could stimulate the 1AZ T cell was higher than the 1:16 expected if the naturally occurring isoleucine was the only amino acid that could be recognized by T cells at p7. To determine which amino acids were compatible with TCR recognition, several positive clones from the primary screen were sequenced and the p7 codon was found to specify several amino acids including valine, leucine, alanine, and methionine (Fig. 5a). However, in a stringent DNA titration test of these constructs for their antigenic activity we found that the natural isoleucine residue could only be effectively substituted with the valine and to a 10-fold lower extent with the leucine residue (Fig. 5b). The response to Ala and Met substitutions remained below the detection limit. Because most amino acid substitutions were detrimental to TCR recognition, we conclude that the p7 residue was a key determinant of TCR specificity.
Immunogenicity of H47 alleles is profoundly influenced by neighboring H loci

Reciprocal immunizations were conducted to determine the relative immunogenicity of the H47 allelic homologues. Immunization of 21M (H47b) mice with spleen cells of its congenic partner B10 (H47a) generated CTLs that specifically lysed the donor B10 but not host 21M cells as effectively as the H47 a-specific 1A CTL line (Fig. 6, a and b). Both the bulk and the 1A CTLs also lysed targets incubated with the H47a peptide SII9 but did not recognize targets incubated with the H47b peptide SFI9 (Fig. 6, c and d). In contrast, reciprocal immunization of B10 (H47a) mice with 21M (H47b) cells generated vigorous CTL responses which lysed the donor 21M target cells (Fig. 6, e and f) but failed to lyse the corresponding SFI9 peptide-coated target cells (Fig. 6, g and h). The SFI9/Db-specific lytic activity also was barely detectable in CTLs generated in 21M mice grafted with B10 skin. However, after in vitro restimulation of these CTLs in the presence of SFI9 peptide CTLs specific for the SFI9/Db complex were detected and were found to be as effective as SII9/Db-specific CTLs in their lytic activity (Fig. 6, i–l). Therefore, we conclude that both H47 alleles can be immunogenic but the immunogenicity of H47b is profoundly influenced by the existence of at least one additional H locus encoded within the congenic region of 21M mice.

Discussion

By using lacZ-inducible T cells as a probe, we isolated the cDNA clone that revealed the molecular identity of the murine H47 histocompatibility locus first detected by Snell and his colleagues by genetic analysis four decades ago (18). We identified the H47 gene product that is expressed on the cell surface and its processed antigenic peptide, as well as the molecular basis for its antigenic polymorphism. By defining the molecular basis for self-nonself recognition the findings provide insights into the immunological functions of H loci.

Several different approaches, including positional cloning, biochemical purification, expression cloning, and mimotope development, have been used to identify T cell-stimulating histocompatibility Ags (25, 26). Among these, the expression cloning approach has proved to be particularly powerful because it does not require any prior assumptions of genomic location and reveals not only the identity of the antigenic peptide, but that of the precursor gene as well (27). The H Ag cDNA as shown here is an essential tool for defining the underlying genetic mechanisms of polymorphism as well as structural and functional analysis of the precursor protein. Notably, despite repeated attempts, we failed to detect the extremely hydrophobic SII9 peptides in cell extracts (not shown). In our hands, this failure is unique to H47 and can be accounted for by the low (<0.5%) recovery of the SII9 peptide in HPLC fractionated cell extracts that were spiked with known amounts of...
the synthetic peptide (not shown). Because the biochemical purification approach depends on the ability to detect the antigenic activity in cell extracts, hydrophobic peptides may not be accessible to this method. Note also that like the H13 peptides we had identified earlier (14), the Db-presented H47 peptides do not conform to the Db peptide binding motif xxxxx[Nxx][II,L,M] (24) and thus would be inaccessible to the mimotope approach as well (26).

The high hydrophobicity of the S[LI,F]9 peptides is a consequence of their location within the transmembrane region of its precursor protein (Fig. 2). This location is most unusual among peptides presented by classical MHC class I molecules (28). However, presentation of hydrophobic materials to T cells is characteristic of nonclassical MHC I molecules; N-formylated peptides are presented by H2-M3 (8), signal peptides by Qa1 (29), and lipids by CD1 molecules (30, 31). Thus, the presentation of the transmembrane H47 peptides by Dβ may be analogous to the presentation of signal peptides by Qa1 molecules. Interestingly, the hydrophobic signal peptides presented by Qa1 serve as ligands for both the αβ TCR of CD8 T cells as well as the NKG2A,C,E receptors of NK cells (29, 32, 33). Recently, it was discovered that the H60 histocompatibility Ag that we had earlier identified by its ability to elicit CD8 T cell responses (16) is also a ligand for the NKG2D receptor (34, 35). Therefore, it is conceivable that the highly conserved S[LI,F]9/Dβ complexes or the H47 gene product could play a role in self-nonself discrimination beyond eliciting CD8 T cell responses.

The difference in relative immunogenicity of the H47 alleles was striking. Both the H47β (SII9) and H47β (SF19) peptides were presented by Dβ MHC and were recognized by CD8 T cells. However, although CTLs specific for SII9/Dβ were readily elicited after immunization of 21M (H47β) mice with congeneric B10 (H47β) cells, CTLs specific for SF19/Dβ complex were rather difficult to elicit in B10 mice responding to 21M cells (Fig. 6). Instead, the B6 anti-21M CTLs were specific for antigens presented by Db MHC and were recognized by CD8 T cell responses.

Acknowledgments
We are grateful to F. Gonzales, G. Christianson, T. Sproule, and T. Wu for technical assistance, and Dr. M. Foster for her critical comments on the manuscript.

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