The Selection of M3-restricted T Cells Is Dependent on M3 Expression and Presentation of *N*-formylated Peptides in the Thymus

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Summary

The major histocompatibility complex (MHC) class Ib molecule H2-M3 binds *N*-formylated peptides from mitochondria and bacteria. To explore the role of M3 expression and peptide supply in positive and negative selection, we generated transgenic mice expressing an M3-restricted TCR-α/β from a $CD8^+$ T cell hybridoma (D7) specific for a listerial peptide (LemA). Development of M3-restricted transgenic T cells is impaired in both β2-microglobulin–deficient and transporter associated with antigen processing (TAP)-deficient mice, but is not diminished by changes in the *H-2* haplotype. Maturation of M3/LemA-specific $CD8^+$ single positive cells in fetal thymic organ culture was sensitive to M3 expression levels as determined by antibody blocking and use of the *castaneus* mutant allele of M3. Positive selection was rescued in $TAP^{-/-}$ lobes by nonagonist mitochondrial and bacterial peptides, whereas LemA and a partial agonist variant caused negative selection. Thus, M3-restricted $CD8^+$ T cells are positively and negatively selected by M3, with no contribution from the more abundant class Ia molecules. These results demonstrate that class Ib molecules can function in thymic education like class Ia molecules, despite limited ligand diversity and low levels of expression.

Key words: major histocompatibility complex • thymic selection • cytotoxic T cell • transgenic mice • T cell receptor

The repertoire of $CD8^+$ T cells is shaped by both positive and negative selection in the thymus. Results from fetal thymic organ culture (FTOC)[1] in both β2-microglobulin (β2m)– and transporter associated with antigen processing (TAP)-deficient mice showed that both of these selection processes involve the recognition of self-peptide–MHC class I complexes by T cell receptors expressed on double-positive thymocytes (1, 2). A key factor that determines the fate of thymocytes is the avidity and affinity of the TCR–ligand interaction (3–7). Strong TCR peptide–MHC interactions lead to negative selection, and weak TCR peptide–MHC interactions lead to positive selection. Since both types of selection can be mediated by the same MHC molecule, the MHC-bound peptides are believed to play an important role in dictating the outcome of the selection process. Several studies showed that a degree of degeneracy exists between TCR peptide–MHC interactions during positive selection, suggesting that this type of flexibility might be required to generate a diverse T cell repertoire (8–10). Negative selection requirements appear more stringent, requiring antigenic or partial agonist peptides with only minor differences from the antigenic peptide (6).

Studies on the selection of $CD8^+$ T cells thus far have focused on class Ia–restricted T cells. However, little is known about the selection requirements of class Ib–restricted T cells. Class Ib molecules are less polymorphic and have lower expression levels than class Ia molecules. The peptide-binding specificities of class Ib molecules H2-M3, Qa-1, and HLA-E have been found to be more constrained than those of class Ia molecules (11–16). These properties may affect the contribution of class Ib molecules to thymic education. To explore this question, we generated transgenic mice expressing TCR-α/β restricted by H2-M3, a class Ib molecule with unique specificity for hydrophobic *N*-formylated peptides.

M3 presents *N*-formylated peptides from bacteria and

[1] *Abbreviations used in this paper:* APC, allophycocyanin; β2m, β2-microglobulin; B6, C57BL/6; B6.R9, B6.CAS3(R9); cas, *castaneus*; ES, embryonic stem; FTOC, fetal thymic organ culture; IF, immunofluorescence; SP, single positive; TAP, transporter associated with antigen processing; wt, wild-type.

mitochondria to $CD8^+$ CTLs (17, 18). M3 is nonpolymorphic in most strains of mice, and thus may have evolved to present this class of peptides, which are not presented by class Ia molecules (19). M3 was originally identified as the MHC molecule that presents allelic forms of the maternally transmitted factor (MTF) peptide, which is encoded at the NH_2 terminus of the mitochondrial *ND1* gene (20–22). Subsequently, an allelic variant of the mitochondrial *COI* gene was also found to be presented as a minor histocompatibility antigen by M3 (23). Additionally, M3 has been found to present three peptides from the intracellular pathogen *Listeria monocytogenes*, namely LemA (fMIGWII [24, 25]), Fr38 (fMIVIL [26]), and fMIVTLF (27). Adoptive transfer of M3-restricted, *Listeria*-specific CTLs protects animals from infection, suggesting that M3 plays an important role in the host defense against intracellular bacteria (28).

M3 is encoded at the telomeric end of the mouse *H-2* complex, and shares many conserved features with class Ia molecules, including interaction with CD8 (22, 29). *M3* message can be detected in most adult tissues and during early embryonic development, although at much lower levels than class Ia molecules (30). We have recently shown that the majority of M3 is retained intracellularly because of a lack of endogenous antigen supply (31). M3 is undetectable on the surface of thymocytes and thymic epithelial cell lines by staining with a monoclonal reagent. However, surface expression of M3 can be induced on antigen presenting cells by addition of exogenous *N*-formylated peptides. This expression pattern raises the question of the role of M3 in thymic education. Can a molecule with such a low level of expression contribute to selection of T cells restricted by this molecule? Furthermore, because the pool of endogenous mitochondrial peptides is limited both in amount and diversity (13 sequences, only 2 of which bind M3 with high affinity), M3-restricted T cells may have a very low possibility of autoreactivity. This might lessen the requirement for M3 to participate in negative selection.

In this study, we analyzed positive and negative selection in TCR transgenic animals bearing the D7 TCR specific for listerial peptide (LemA) in the context of M3. We show that M3 is the primary selecting element for M3-restricted T cells. Similar to class Ia-restricted T cells, positive selection and negative selection of M3-restricted T cells were peptide dependent. The essential role of M3 in the selection of a subset of pathogen-specific T cells indicates that MHC class Ib molecules play a significant role in T cell repertoire development and, consequently, in defense against microbial infection.

Materials and Methods

Cloning of D7 TCR Transgenic Constructs. The following oligonucleotides were used in reverse transcription PCR on cDNA from clone CN.8 (28): Vα10cass 5′-AACGTCGCAGCTCTTTGCAC-3′, Vβ5.2cass 5′-AAGGTGGAGAGAGACAAAGGATTC-3′ (32), Alcon 5′-GATGTTTTACTGGTACACAG-3′, and Cβreverse 5′-TGTGCTTGGCCAGGGGTTCTT-3′. For cloning of the full VDJ regions from D7 hybridoma genomic DNA, the following primer pairs were used: Vα10lead-XmaI 5′-GACCCGGGCTTCTCACTGCCTAGCCATGAAGAGCCTGCTGAGCTCTCTG-3′ and JαD7intron-NotI 5′-CTTACGGCCGAGGAAGTACTGTCCTGAG-3′, and Vβ5.2lead-XhoI 5′-CCAGCATCTCGAGAAGAAGCATGTCTAAC-3′ and Jβ2.2intron-SacII 5′-GATGCCGCGGAGCTGTCCTGCTCTGAATATCTTC-3′. The amplified D7 TCR-α and -β DNA fragments were sequenced and cloned into the XmaI and NotI sites in TCR-αcass, and into the XhoI and SacII sites in TCR-βcass (TCR cassette vectors were provided by Drs. C. Benoist and D. Mathis, Harvard Medical School, Boston, MA [33]).

Generation of M3-restricted LemA-specific TCR Transgenic Mice (D7 Tg). The α and β TCR transgenic constructs were cotransfected with the neomycin resistance plasmid (pPNT-neo) into R1 embryonic stem (ES) cells (strain 129) by electroporation, as described previously (34). Transfected cells were selected by G418, and the TCR-α and -β transgenes were detected by PCR using Vα10lead-XmaI and JαD7intron-NotI, and Vβ5.2cas and Jβ2.2intron-SacII. Presence of both transgenes was confirmed by Southern blot with probes for the constant regions of TCR-α (35) and TCR-β (36) on HindIII– or EcoRI-digested genomic DNA, respectively. ES cells containing both TCR-α and -β transgenes were injected into C57BL/6 (B6) blastocysts to generate chimeric mice at the Gwen Knapp Center Transgenic Facility. Chimeric mice that contained high levels of $V\beta5^+Ly9.1^+$ cells in PBL were chosen as founders and bred with B6 mice to produce transgene-positive offspring. Three lines of transgenic mice were established. The development of the transgenic T cells in these three lines of mice is quite similar. Therefore, only one transgenic line was used for further breeding.

Mice. B6, B10.D2, $\beta2m^{-/-}$, $TAP^{-/-}$, and TCR-$\alpha^{-/-}$ mice were purchased from The Jackson Laboratory. B6.CAS3(R9) (B6.R9)—carrying a haplotype with *H2-K* through *H2-D* from B6 and the rest of *H2*, including M3, from *Mus musculus castaneus* (cas3)—was provided by Dr. Kirsten Fischer Lindahl (University of Texas, Southwestern Medical Center, Dallas, TX). Mice were housed in the conventional animal facility or in the barrier facility of the University of Chicago. During backcrosses, animals were typed for transgene by expression of Vβ5 on $CD8^+$ PBLs. Where Vβ5 expression was minimal, PCR of D7 α and β chains was performed as stated for ES cell typing. Changes in haplotype, as well as loss of functional β2m or TAP, were detected by loss of staining with FITC–anti-K^b. TCR-$\alpha^{-/-}$ status was assessed by expression of endogenous Vα2 on TCR-α/β^+ PBLs, and confirmed by Southern blot analysis of HindIII-digested genomic DNA with a probe for the TCR Cα region. To type $D7^+$B6.R9 animals, PBLs were incubated with 10 μM of LemA peptide for 4 h at 37°C, and stained for M3 surface expression by anti-M3 mAb (mAb130).

FTOCs. Fetal thymi were cultured according to procedures described by Ashton-Rickardt et al. (1). In brief, thymic lobes from gestational day 16.5 fetal mice were placed onto nitrocellulose filters (Millipore Corp.). Filters were placed in 2-cm-diameter dishes, then incubated for 10 d at 37°C in RPMI 1640 medium containing 10% fetal bovine serum (HyClone), 2 mM l-glutamine, 20 mM Hepes, 50 μM 2-ME, nonessential amino acids, sodium pyruvate, penicillin, and streptomycin (RPMI-10). The peptide and media were replenished every day where indicated.

Abs, M3-LemA Tetramer, and Peptides. The following mAbs were purchased from PharMingen: FITC–anti-CD8α, PE–anti-CD8α, PE–anti-Vα2, CyChrome–anti-CD4, FITC–anti-Vβ5, FITC–anti-K^b, FITC–anti-IA^b, biotin–anti-Ly9.1, and biotin–mouse anti–hamster IgG. Y3, anti-K^b; and B22, anti-D^b were

purchased from American Type Culture Collection. PE–M3-LemA tetramer and allophycocyanin–M3-LemA tetramer were prepared as described previously (37). mAb130 was purified as described previously (38). Synthetic peptides were purchased from Research Genetics. Peptide sequences were as follows: LemA, fMIGWII; $LemA_{5I\rightarrow A}$, fMIGW<u>A</u>I; $LemA_{6I\rightarrow A}$, fMIGW<u>A</u>I; Fr38, fMIVIL; ND1, fMFFINIL; <u>N</u>D4, fMLKIILP; and C<u>O</u>I, fMFINRWLFS. All peptides were >90% pure as determined by mass spectrometry. Peptides were dissolved in DMSO at concentrations of 1–20 mM.

Cell Preparations and Flow Cytometric Analysis. Thymocyte suspensions were prepared from cultured fetal thymi by mechanical desegregation in RPMI-10 media, and stained in immunofluorescence (IF) buffer (HBSS containing 2% fetal bovine serum and 0.1% NaN_3) using combinations of fluorescent-conjugated Abs for 30 min at 4°C. When staining involved M3-LemA tetramer, incubation time was extended to 1 h. The stained cells were analyzed by flow cytometry using a FACSCalibur™ (Becton Dickinson) with the CELLQuest™ software. Thymic stromal cell suspensions were prepared by digesting fetal thymi in 0.1% trypsin, 0.5 mM EDTA for 40 min at 37°C. Digestion was stopped by addition of IF buffer. After mechanical disruption of the lobe, cells were harvested and washed two times with IF buffer before cell surface staining experiments (10). Cells were stained with anti-M3 mAb (130) followed by biotinylated mouse anti–hamster IgG, and a third incubation with streptavidin-conjugated PE and FITC-anti–I-A^b. Staining with each reagent was performed for 30 min on ice in IF, followed by washing with the same buffer. The expression of M3 on I-A^b–positive thymic stromal cells was analyzed by FACS®.

CTL Assays. CTL effectors were established by culturing splenocytes of $D7^+TCR\text{-}\alpha^{-/-}$ mice at 5×10^6 cells/ml in RPMI-10 with 5 μM of LemA peptide for 3 d at 37°C. 1×10^6 target cells were incubated in RPMI-10 with or without 1 μM of LemA peptides for 18–20 h. Cells were washed free of excess peptide and labeled with 100 μCi [^{51}Cr]sodium chromate for 1 h at 37°C. Target cells (1×10^4 cells) were added to round-bottomed microtiter wells containing effector cells. After 4 h incubation at 37°C, 100 μl of supernatant from each well was removed and assayed for ^{51}Cr release. Percent specific lysis = (experimental − spontaneous release) × 100/(maximal release − spontaneous release). Limiting dilution analysis for CTL precursor frequency was performed on fetal thymic lobes after 10 d of culture. Four or five lobes from each treatment were pooled, and thymocytes were diluted to concentrations between 10,000 and 3 cells per well and incubated with 1×10^6 irradiated B6 stimulators per well, 10 U/ml IL-2, and 5 μM LemA peptide. Peptide was replenished at days 3 and 6. On day 7, ^{51}Cr-labeled LemA-coated L929 targets were added at 1×10^4 per well, and ^{51}Cr release was measured after 4 h.

Cytokine Assay. Splenocytes from $D7^+$ mice (5×10^5 cells per well) were cultured in round-bottomed microtiter wells in a final volume of 200 μl of RPMI-10, with 10 μM of various peptides. After 48 h, the culture supernatants were harvested and the levels of IFN-γ were quantitated by sandwich ELISA (PharMingen).

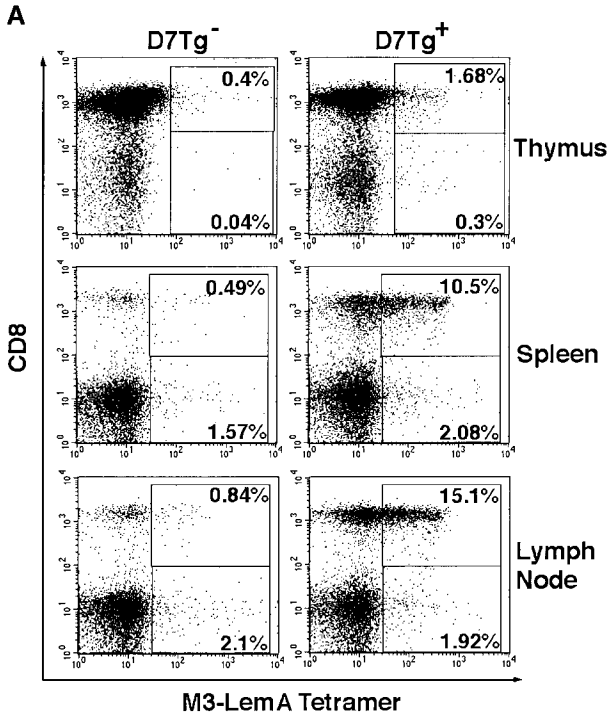
Results

Generation of Transgenic Mice Expressing an M3-LemA–specific TCR. The D7 hybridoma generated from CTL clone CN.8 was chosen for the construction of TCR transgenic mice (25, 28). This CTL is $CD8^+$ and specific for a listerial peptide, LemA (f-MIGWII), in the context of M3, and can confer protection from infection by adoptive transfer. Leader and J-C intron primers were designed and used to clone the entire coding sequence (Vα10.2JαD7 and Vβ5.2Jβ2.2) from D7 hybridoma DNA into the TCR cassette vectors (33). Three lines of D7 transgenic mice were generated and bred onto the B6 background. Lymphocytes isolated from thymus, spleen, and lymph nodes of D7 transgenic mice were stained with FITC–anti-CD8, PE–anti-CD4, and APC–M3-LemA tetramer to examine the development of $D7^+$ T cells. FACS® analysis showed that $D7^+$ T cells (M3-LemA tetramer positive) are highly enriched in the CD8 lineage (Fig. 1 A). Compared with nontransgenic controls, the transgenic mice showed an increase in percentage of $D7^+CD8^+$ cells (4-fold in thymus, 20-fold in spleen, and 18-fold in lymph node).

Splenocytes from transgenic animals were evaluated against nontransgenic littermates for antigen-specific responses against the hexameric LemA peptide. Nontransgenic splenocytes showed no reactivity to LemA peptide (data not shown). Splenocytes from D7 animals developed into M3-restricted, LemA-specific CTLs after 3 d in culture with LemA peptide. D7 CTLs specifically lysed LemA-coated L929 targets bearing the wild-type M3 allele ($M3^{wt}$) (Fig. 1 B). B10.CAS2 fibroblasts express a mutant *castaneus* allele of M3 ($M3^{cas}$) that does not present *N*-formylated peptides efficiently. LemA-coated B10.CAS2 targets were only slightly susceptible to lysis, whereas an $M3^{wt}$ transfectant of B10.CAS2 (TR8.4a) was recognized as efficiently as the L929 targets. These data suggest that D7 transgenic T cells preserve functional capacities and antigenic specificity of the original T cell clone.

Development of $D7^+$ T Cells Is MHC Unrestricted and TAP and β2m Dependent. Because M3 is nonpolymorphic and expressed at low levels, it is possible that M3-restricted T cells are selected on the more abundant class Ia molecules H-2K, D, or L. We bred the D7 transgenes onto the B10.D2 background which bears the $H\text{-}2^d$ class Ia molecules to see if the selection and development of M3-restricted T cells was dependent on a particular class Ia allele. Staining with M3-LemA tetramers showed that $D7^+CD8^+$ T cell development is unimpaired in the B10.D2 background (Fig. 2 A). To eliminate the possibility that class Ia interaction with endogenous α chains contributes to positive selection, we bred the transgene onto the $TCR\text{-}\alpha^{-/-}$ background. The $CD8^+$ population in $D7^+TCR\text{-}\alpha^{-/-}$ mice is exclusively positive for M3-LemA tetramer staining (Fig. 2 B), showing that the D7 TCR is sufficient for selection. The development of transgenic T cells is impaired in $TCR\text{-}\alpha^{-/-}$ $TAP^{-/-}$ and $\beta2m^{-/-}$ mice (Fig. 2 B). These data suggest that the selection and development of $D7^+$ T cells requires the presentation of peptide on a class I molecule. Both TAP and β2m have been shown to be important for the expression of M3 on the cell surface (31, 39, 40).

M3 Expression Is Required for Development of $D7^+CD8^+$ Single Positive Thymocytes. The lack of *H-2* restriction and the TAP and β2m dependence of $D7^+$ T cell development suggested that M3 expression might be required for positive selection. We analyzed the selection of the $D7^+$ T cells



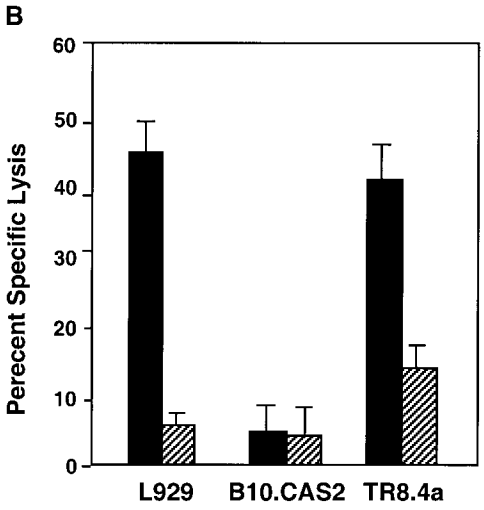


Figure 1. D7 transgenic mice develop increased numbers of $CD8^+$ cells that are M3 restricted and LemA specific. (A) Thymus, spleen, and lymph node cells from nontransgenic ($D7Tg^-$) and D7 transgenic ($D7Tg^+$) B6 animals were stained with FITC–anti-CD8 and APC–M3-LemA tetramers. Percentages of D7 TCR^+ cells are indicated from a representative experiment. (B) Splenocytes from $D7^+TCR\alpha^{-/-}$ mice were incubated at 5×10^6/ml with 1 μM LemA peptide for 3 d. Ficoll-purified effector cells were tested for cytolytic activity against L929, B10.CAS2 fibroblasts, and an $M3^{wt}$ transfectant (TR8.4a) in the presence (black bars) or absence (hatched bars) of 1 μM LemA peptide at an E/T ratio of 10:1.

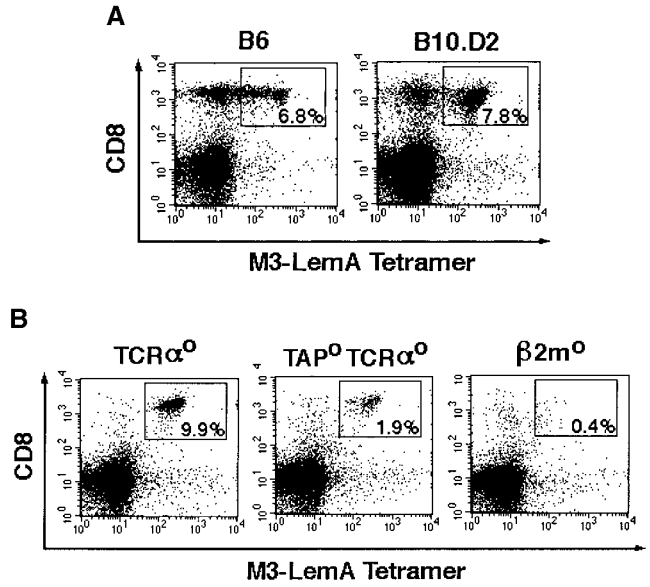


Figure 2. Development of $D7^+$ T cells is β2m and TAP dependent, but MHC unrestricted. Splenocytes from wild-type (A) and mutant animals (B) of the indicated backgrounds were stained with FITC–anti-CD8 and with an APC-conjugated M3-LemA tetramer to assess development of M3-LemA–specific T cells. The percentage of double positive cells is indicated for each animal.

in FTOC in order to manipulate TCR access to M3 with an mAb (mAb130) that blocks M3-restricted CTL responses (31). Fetal thymic lobes were harvested from gestational day 16.5 $D7^+TCR\text{-}\alpha^{-/-}$ animals, and incubated either with anti-M3 or a control hamster Ab at 50 μg/ml for 10 d. Additional lobes were incubated with anti–class Ia Abs or a control mouse Ab. Fig. 3 shows representative reduction in the percentage of $CD8^+$ single positive ($CD8^{sp}$) thymocytes in anti-M3–treated lobes. The average percent reduction was ~80% (from average 20% reduced to 4%). Treatment with Ab to class Ia molecules had no effect, in agreement with our data for $D7^+$ T cell development in different *H-2* haplotypes. $CD8^{sp}$ thymocytes expressed Vβ5 at high levels (data not shown), which is indicative of antigen specificity in the $TCR\text{-}\alpha^{-/-}$ background.

$D7^+$ T Cells Are Inefficiently Selected on an $M3^{cas}$ Allele. We bred the D7 transgene onto an *H-2* recombinant background B6.R9, which expresses $M3^{cas}$, to detect any effect of reduced M3 expression on thymic development. The *castaneus* allele of *M3* contains an amino acid substitution ($Leu_{95} \rightarrow Gln$) that results in reduced recognition by M3-specific CTLs (30). This substitution might affect M3 surface expression, as we were unable to detect M3 by IF staining on the surface of B6.R9 splenocytes (data not shown). Fig. 4 shows the relative efficacy of $M3^{wt}$ (B6) and $M3^{cas}$ (B6.R9) in selection of $D7^+$ T cells. In FTOC of $D7^+$ B6 lobes, antigen-specific $CD8^{sp}$ thymocytes account for 13.4% of total thymocytes (57.8% of 23.2%). In comparison, $D7^+$ B6.R9 lobes have a reduced total percentage of $CD8^{sp}$ cells (11.5%), and only a minor proportion of these cells are antigen specific (15.9%). Thus, the total percentage of $D7^+CD8^{sp}$ thymocytes in the B6.R9 background is reduced by ~85% (from 13.4 to 1.8%). The percentage of $D7^+CD8^{sp}$ thymocytes in the B6.R9 thymic lobes is reduced when anti-M3 Ab is present during FTOC ($66 \pm 16\%$ reduction, $n = 6$; data not shown). This indicates that the small percentage of $D7^+CD8^{sp}$ cells in the

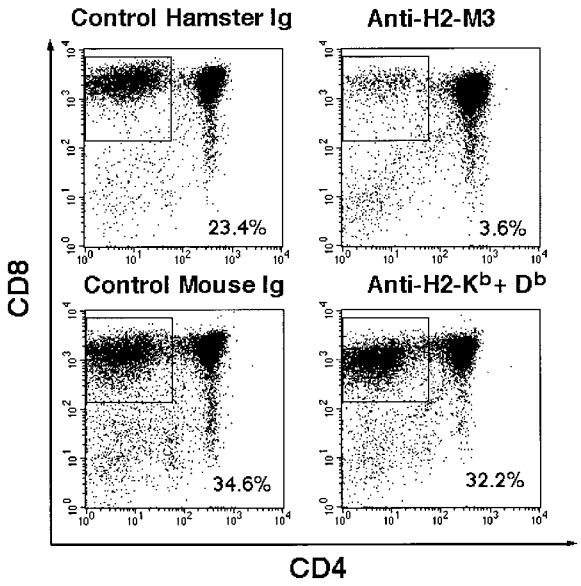


Figure 3. Development of D7$^+$ T cells in FTOC is M3 dependent. Thymic lobes were harvested from gestational day 16.5 D7$^+$TCR$\alpha^{-/-}$ mice. One lobe from each animal was incubated with Ab against either M3 or H-2K^b and H-2D^b. The remaining lobe was incubated with an appropriate control Ab. After 10 d, lobes were harvested and stained with CyChrome–anti-CD4, PE–anti-CD8α, and FITC–anti-Vβ5 to assess maturation of CD8^{sp} transgenic thymocytes. All CD8^{sp} thymocytes were Vβ5$^+$. The average reduction in CD8^{sp} generation after incubation with anti-M3 was 70–85%. Ab against class Ia molecules had no effect. Results are representative from a total of nine pairs of thymic lobes.

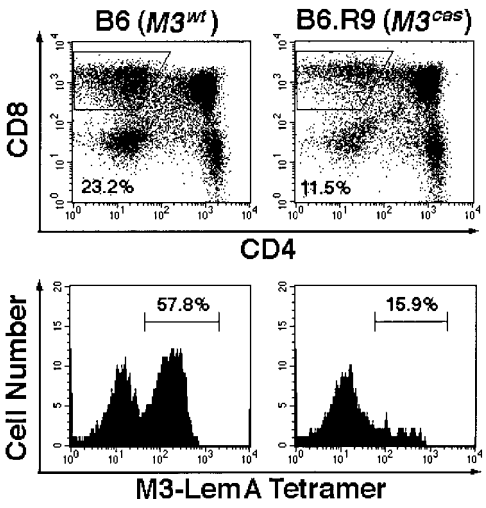


Figure 4. The M3^{cas} allele does not efficiently select D7$^+$CD8^{sp} thymocytes. Gestational day 16.5 thymic lobes were harvested from D7 transgenic animals in either the B6 (*M3^{wt}*) or B6.R9 (*M3^{cas}*) backgrounds, and incubated for 10 d to assess the development of D7$^+$ T cells on different *M3* alleles. Thymocytes were stained with PE–anti-CD4, FITC–anti-CD8, and with APC–M3-LemA tetramer. The percentage of CD8^{sp} cells is reduced in the B6.R9 background (top). The surface expression of D7$^+$ TCR on gated CD8^{sp} cells, detected by M3-LemA tetramer, was displayed as a histogram (bottom). The numbers represent the percentage of CD8^{sp} cells which are M3-LemA tetramer positive. Results are representative from two B6 thymic lobes and two B6.R9 lobes.

B6.R9 thymus develop in an M3-dependent manner, despite the low level of expression of M3^{cas}.

Positive Selection of the D7 TCR Is Induced by N-formylated Peptides. M3 binds *N*-formylated peptides with 100–1,000-fold greater affinity than nonformylated peptides (12). Mitochondria are the only source of these peptides in mammalian cells. In a previous study, we showed that the mitochondrial peptides ND1 and COI bind M3 with higher affinity than the 11 remaining mitochondrial peptides (31). We also found that Fr38, a peptide from *Listeria monocytogenes*, which is not recognized by the D7 hybridoma, binds M3 with high affinity. To determine whether these peptides can induce increased surface expression of M3 on TAP$^{-/-}$ thymic stromal cells, we cultured fetal thymic lobes from TAP$^{-/-}$ mice with or without 20 μM peptide for 10 d, and harvested the cells to stain with anti–class II and anti-M3 Abs. Fig. 5 shows that ND1, COI, and Fr38 increase surface expression of M3 on I-A^{b+} thymic stromal cells from TAP$^{-/-}$ mice. M3 expression is low on thymic stromal cells from B6 lobes as well (data not shown), staining at a similar level as cells from DMSO-treated TAP$^{-/-}$ thymi in the absence of exogenous *N*-formylated peptide. The induced increase in expression of M3 is similar for these three peptides.

Because of the ability of these *N*-formylated peptides to stabilize surface expression of M3 on thymic stromal cells, we analyzed the role of each peptide in positive selection. Fig. 6 A shows representative staining of thymocytes developed in FTOC of D7$^+$TCR-$\alpha^{-/-}$TAP$^{-/-}$ thymic lobes incubated with or without 20 μM peptide for 10 d. Although each peptide was able to stabilize surface expression of M3, the increase in positive selection of CD8^{sp} thymocytes varied between peptides. Fig. 6 B displays the percentage increase in CD8^{sp} thymocytes between pairs of lobes from each animal. The average increase in CD8^{sp} cells was approximately twofold for ND1 (1.85 ± 0.65, $n = 14$) and COI (1.78 ± 0.60, $n = 20$), and approximately threefold for Fr38 (2.86 ± 1.15, $n = 16$). ND4 is a mitochondrial peptide that binds to M3 weakly and is very inefficient at stabilization of M3 on the cell surface. Incubation with ND4 caused no increase in positive selection of transgenic CD8^{sp} thymocytes (1.05 ± 0.31, $n = 6$).

The ability of *N*-formylated peptides to increase development of functional CTL precursors was assessed by a limiting

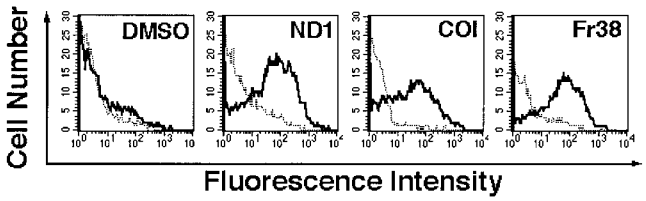


Figure 5. *N*-formylated peptide increases surface expression of M3 on TAP$^{-/-}$ thymic stromal cells. Gestational day 16.5 thymic lobes from TAP$^{-/-}$ animals were incubated with or without 20 μM peptide for 10 d. Thymic stromal cells were harvested as described in Materials and Methods. Cells were stained with FITC-anti–I-A^b and either anti-M3 or a control hamster Ab, followed by biotinylated anti–hamster Ig and PE-streptavidin. Histograms display the fluorescence intensity of staining with either anti-M3 (solid line) or the control Ab (dotted line) on I-A^b–positive cells.

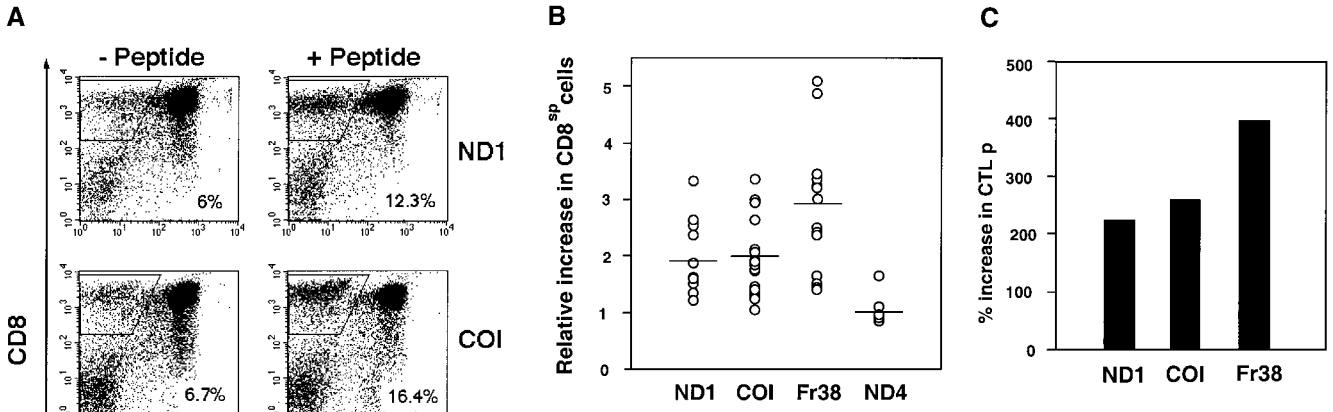


Figure 6. $D7^+CD8^{sp}$ development is peptide dependent. (A) Thymic lobes from $D7^+TAP^{-/-}TCR\alpha^{-/-}$ mice were harvested on gestational day 16.5. One lobe from each animal was incubated with 20 μM of the indicated *N*-formylated peptide from mitochondria (ND1 and COI) or *L. monocytogenes* (Fr38). The matching lobe was incubated with an appropriate concentration of solvent alone. After 10 d of culture, thymocytes were stained with CyChrome–anti-CD4, PE–anti-CD8α, and FITC–anti-Vβ5. All $CD8^{sp}$ thymocytes were Vβ5 positive. The plots are representative of experiments from several animals, with the percentage of $CD8^{sp}$ thymocytes indicated. (B) Relative increase in $CD8^{sp}$ cells was calculated for each pair of lobes as follows: % of $CD8^{sp}$ cells in the peptide-treated lobe/% of $CD8^{sp}$ cells in the control lobe. Circles represent pairs of thymic lobes, and the horizontal bar indicates the mean value. For ND1, $n = 14$; for COI, $n = 20$; for Fr38, $n = 16$; and for ND4, $n = 6$. No significant differences in the total number of thymocytes were observed between peptide-treated lobes and controls. (C) Increase in functional development in peptide treated lobes was calculated in a limiting dilution CTL development assay. After 10 d of culture as above, four or five lobes were pooled, and thymocytes were diluted in series ranging from 10,000 to 3 cells per well and incubated with B6 stimulators, IL-2, and LemA peptide. On day 7, ^{51}Cr-labeled, LemA-coated RMA cells were used as targets for CTL assay. Percent increase in CTL precursor (CTL p) frequency is calculated as follows: [(number of positive wells from peptide-treated lobes/number of positive wells from the control lobes) − 1] × 100. Wells were considered positive if the total counts released were 3 SD above the spontaneous release value.

dilution assay. Thymocytes from peptide-treated lobes from $D7^+TCR\text{-}\alpha^{-/-}TAP^{-/-}$ animals were compared with the control partner lobes. The pooled lobes from each group were diluted to concentrations between 10,000 and 3 per well and incubated with LemA peptide and irradiated stimulators to allow the development of CTLs. Each peptide induced at least a threefold increase in functional CTL precursors (Fig. 6 C), indicating effective positive selection by two mitochondrial peptides and one bacterial sequence.

Negative Selection Is Efficiently Mediated by M3. Because of the low level of expression of M3 compared with class Ia molecules, we sought to determine if negative selection was effectively mediated by M3. Thymic lobes from $D7^+$ $TCR\text{-}\alpha^{-/-}$ mice were incubated with or without 20 μM LemA peptide for 10 d, and cells were stained for CD4, CD8, and either anti-Vβ5 or M3-LemA binding. Both the percentage of $CD4^+CD8^+$ double positive cells and the percentage of $CD8^{sp}$ cells were significantly reduced (Fig. 7). Double positive cells were reduced by 70–85%, and $CD8^{sp}$ cells were reduced by 40–60%. The remaining $CD8^{sp}$ cells in peptide-treated lobes had reduced TCR expression as assessed by either Vβ5 staining or M3-LemA staining (data not shown). In addition, total cell counts were also reduced in peptide-treated lobes ($2.4 \pm 0.23 \times 10^5$ cells/lobe without peptide, $1.2 \pm 0.3 \times 10^5$ cells/lobe with 20 μM LemA). A similar effect was observed when lobes were incubated with a LemA variant ($LemA_{6I\rightarrow A}$, fMIGWIA), a weak agonist which stimulates $D7^+$ T cells minimally. Percentage reduction in $LemA_{6I\rightarrow A}$ treated lobes was ~80% for $CD4^+CD8^+$ double positive, and 20% for $CD8^{sp}$ (data not shown). In contrast to LemA and $LemA_{6I\rightarrow A}$, the peptides that promote positive selection (ND1, COI, and Fr38) elicit no response from $D7^+$ T cells, even at 10 μM concentrations (Fig. 8). Thus, M3 is capable of effective negative selection even with weak agonist peptides.

Discussion

To determine whether MHC class Ib molecules can contribute to thymic education in the same manner as class

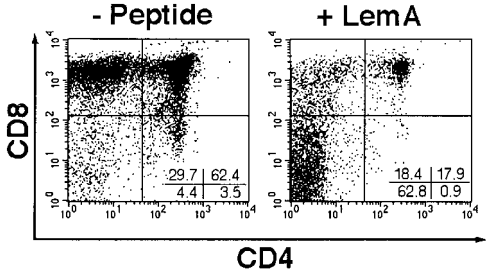


Figure 7. M3 efficiently mediates negative selection. Gestational day 16.5 thymic lobes from $D7^+TCR\alpha^{-/-}$ mice were harvested and incubated in the presence or absence of 20 μM LemA peptide. After 10 d, thymocytes were stained with CyChrome–anti-CD4, PE–anti-CD8, and FITC–anti-Vβ5. Percentages are indicated in quadrants. Results are representative from a total of four pairs of thymic lobes.

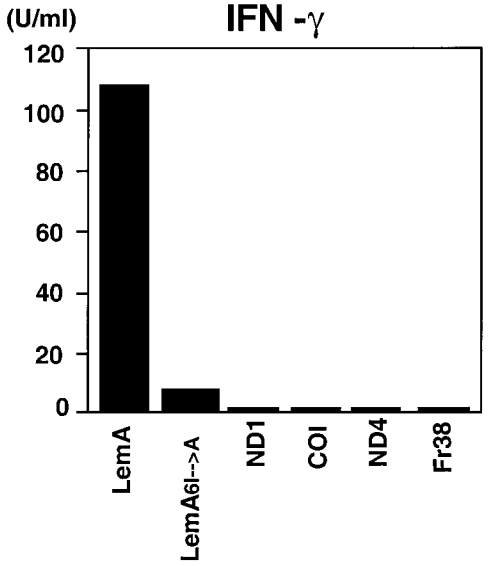


Figure 8. D7 splenocytes respond to LemA and $LemA_{6I\rightarrow A}$, but not to mitochondrial peptides. Splenocytes from a $D7^+$ B6 animal were incubated with 10 μM of the indicated peptides at 5×10^5 cells per well in a 96-well plate. After 2 d of culture, supernatants were harvested for analysis of IFN-γ secretion by ELISA.

Ia molecules, we analyzed positive and negative selection in TCR transgenic animals bearing the D7 TCR-α/β specific for the listerial peptide LemA in the context of M3. M3 plays the dominant role in positive selection of the D7 TCR, as shown by the impaired development of transgenic thymocytes in the presence of anti-M3 blocking Ab, or in the presence of the *castaneus* mutant M3 allele. Although selection was significantly diminished in the B6.R9 ($M3^{cas}$) background, incubation with anti-M3 Ab further reduced the percentage of $D7^+CD8^{sp}$ thymocytes, suggesting that $M3^{cas}$ is capable of selecting some $D7^+$ T cells. This is consistent with the ability of B6.R9 animals to develop allogeneic responses against $M3^{wt}$ molecules (41, 42). No apparent contribution is required from class Ia molecules, as suggested by the development of transgenic T cells in an MHC-unrestricted fashion. Peptide is required for positive selection of D7 TCR, as shown by the differential rescue of $CD8^{sp}$ development in $TAP^{-/-}$ fetal thymic lobes by four peptides from mitochondria and bacteria. The concentration of peptide (20 μM) used in these FTOCs increased the surface expression of M3 on thymic stromal cells significantly over background levels. Because of the hydrophobicity of M3-binding peptides, higher concentrations of peptide caused precipitation. Negative selection is also mediated by addition of the antigenic peptide to TCR-$\alpha^{-/-}$ FTOCs. Thus, M3 is responsible for both positive and negative selection of M3-restricted T cells.

It is of interest that M3 is a potent positive selecting element, despite surface expression levels in the thymus that are undetectable by IF staining in wild-type animals (31). Because M3 can bind only two mitochondrial peptides with high affinity (31, 42), it is likely that the majority of M3 molecules on the cell surface are occupied by these ligands. The remaining mitochondrial ligands may also contribute, depending on the balance of affinity and abundance, which is currently unexplored. By comparison, the pool of 10^5–10^6 class Ia molecules on each cell presents as many as 10^3–10^4 different self-peptides (43). Although the expression level of M3 is about one to two orders of magnitude lower than that of class Ia molecules, the total number of identical M3 self-peptide complexes such as M3–ND1 or M3–COI complexes may be comparable to the number of any given class Ia–peptide complex, because of the greater numbers of ligands competing for presentation on class Ia molecules. A similar argument can be made in comparing low levels of M3 in the wild-type animal to low levels of class Ia in $TAP^{-/-}$ animals. We have shown previously that M3 behaves in a wild-type background as a class Ia molecule does in a $TAP^{-/-}$ background because of a limited supply of endogenous peptides. However, class Ia molecules expressed at low levels in $TAP^{-/-}$ animals are not sufficient to support positive selection (1). It is possible that even in the absence of TAP, the supply of peptides available to class Ia molecules in the endoplasmic reticulum still has sufficient diversity to dilute any particular MHC–peptide combination below the minimum level required for positive selection. Additionally, we have shown that M3–peptide complexes on the cell surface are more long-lived than class Ia complexes (31). The combined effect of limited ligand diversity and surface stability may explain the ability of M3 to select M3-restricted T cells.

The peptides used in these experiments differed in their ability to promote positive selection. Incubation with listerial Fr38 peptide increased $CD8^{sp}$ development more effectively than the self-derived mitochondrial peptides. Correspondingly, the percentage of $CD8^{sp}$ cells in Fr38-treated $D7^+$TCR-$\alpha^{-/-}TAP^{-/-}$ lobes was, on average, greater than that in $D7^+$TCR-$\alpha^{-/-}$ lobes (Fig. 6 A and Fig. 7). Because the ability to bind and stabilize M3 is not significantly different between ND1 or COI and Fr38, this suggests that peptide sequence can impact M3-restricted selection. An explanation for the efficiency of Fr38 at enhancing LemA-specific TCR selection may be provided by the nature of the M3 peptide binding groove. In the crystal structure of M3, the second residue is partially exposed to the TCR contact surface, while the side chains of residues three and four are buried. The position five side chain points up, and then a position six residue would be buried (13). Fr38 and LemA share the second amino acid (Ile) and a similar fifth residue (Ile in LemA, Leu in Fr38), whereas ND1 and COI share phenylalanine as the second residue, and have a fifth residue which is dissimilar to LemA (Asn in ND1 and Arg in COI). Notably, we found that a $LemA_{5I\rightarrow A}$ variant with a substitution at position five is a nonagonist (Chiu, N.M., and C.-R. Wang, unpublished results), indicating that this residue may be an important contact residue for the D7 TCR. However, as is the case for class Ia–restricted selection (9, 10), sequences lacking similarity to the antigenic peptide, i.e., ND1 and COI, are also able to promote positive selection, although not to wild-type levels.

Although M3 has 100–1,000-fold greater affinity for *N*-formylated over unformylated peptides (12), a potential role of unformylated peptide–M3 complex in thymic selection cannot be ruled out. Unformylated peptides can stimulate CTLs when present at high concentrations (12). However, even at high concentrations, unformylated peptides do not stabilize M3 on the cell surface. It remains possible that some unformylated peptide(s) with low affinity for M3 is present in such high abundance that it can contribute to positive selection. Positive selection of class Ia-restricted $CD8^{sp}$ thymocytes has been shown to be effective even with low affinity self-peptides (10). We are currently attempting to elute naturally processed self-ligand from M3 by immunoaffinity chromatography. This may provide insight into the proportion of M3 molecules on the cell surface, which may contain nonmitochondrial peptides.

The limitation in diversity of peptide bound to M3 has implications for the breadth of M3's role in selection. A diverse peptide supply has been shown to be important for the restoration both of normal amounts of positive selection and of a broad repertoire of class I–restricted responses (1, 2). This has also been shown to be the case in $H\text{-}2M^{-/-}$ animals and "single peptide" MHC transgenic mice for class II–restricted responses (44, 45). Since M3 has such a small repertoire of self-ligands, the diversity and total number of M3-restricted T cells may be reduced as a consequence. That the contribution of class Ib molecules to positive selection has a limit is demonstrated by the reduced numbers of $CD8^+$ cells in K^bD^b double-knockout animals (46).

It has been proposed that the selective advantage of presenting numerous foreign peptides has driven the polymorphism of class Ia molecules, but that the mechanisms for self-tolerance provide pressure of an opposite type. After thymocytes rearrange the antigen receptor genes to randomly generate diversity, a large proportion of thymocytes is negatively selected. An increase in numbers of polymorphic class Ia molecules, while expanding the range and diversity of foreign peptides that can be presented, may eventually decrease the responding TCR repertoire by a greater factor (47). This would occur if the range of self-peptides presented also increases and removes an additional subset of the TCR repertoire. A less polymorphic molecule may evolve a specialized function, for instance, presentation of a commonly encountered pathogenic epitope. The conservation of such a sequence serves to present the pathogen efficiently, while the limited polymorphism allows a reliably small set to be removed from the T cell repertoire. This strategy is particularly effective if the pathogenic epitope is never, or rarely, found in the repertoire of self-antigens. Human CD1 molecules exemplify this strategy by presenting bacterial lipids to T cells (48, 49). M3 also follows this strategy by presenting low amounts of a small set of self-peptides, allowing positive selection, while responding to a class of bacterial *N*-formylated peptide antigens that are present in large amounts exclusively during an infection. Therefore, selection of M3–restricted T cells can serve as a model for other nonpolymorphic class Ib molecules with restricted ligand specificity.

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