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Mice Lacking H2-M Complexes, Enigmatic Elements of the MHC Class II Peptide-Loading Pathway

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Summary

We have generated mice lacking H2-M complexes, critical facilitators of peptide loading onto major histocompatibility complex class II molecules. A^b molecules in these mice matured into stable complexes and were efficiently expressed at the cell surface. Most carried a single peptide derived from the class II-associated invariant chain; the diverse array of peptides normally displayed by class II molecules was absent. Cells from mutant mice presented both whole proteins and short peptides very poorly. Surprisingly, positive selection of $CD4^+$ T cells was quite efficient, yielding a large and broad repertoire. Peripheral T cells reacted strongly to splenocytes from syngeneic wild-type mice, no doubt reflecting the unique peptide complement carried by class II molecules in mutant animals.

Introduction

The intracellular transport of major histocompatibility complex (MHC) class II molecules has been neatly adapted to favor their loading with peptides derived from extracellular proteins (Cresswell, 1994; Wolf and Ploegh, 1995). Shortly after their synthesis, class II α and β chains dimerize and associate with the invariant chain (Ii). Ii blocks most peptides from binding to class II molecules in the endoplasmic reticulum (ER) and directs their egress from the ER and targeting to endocytic compartments. In this acidic environment, Ii is dissociated from class II molecules, and peptides derived from proteins traveling the endocytic pathway are loaded in its place. Class II–peptide complexes are then transported to the cell surface, where they can be recognized by $CD4^+$ T cells.

During the past few years, some intriguing intricacies have been superimposed on this sketchy scenario, mainly reflecting a series of observations with mutant human lymphoblastoid cell lines. Pious and colleagues used immunoselection to isolate a set of mutant lines recognized normally by some anti–MHC class II monoclonal antibodies (MAbs) but ignored by others (Mellins et al., 1990). A similar phenotype could be engineered by transfecting class II genes into lymphoblastoid lines bearing large deletions within both copies of the MHC (Riberdy and Cresswell, 1992; Ceman et al., 1992). Although the class II molecules expressed by the two types of mutant line were of normal sequence, they were unusually unstable, dissociating into individual α and β chains in sodium dodecyl sulfate (SDS) at room temperature (Mellins et al., 1990; Riberdy and Cresswell, 1992), a property thought to signify improper peptide loading (Sadegh-Nasseri and Germain, 1991; Germain and Hendrix, 1991). Indeed, peptides eluted from these atypical class II molecules were strikingly enriched for a nested set of fragments derived from Ii, residues 81–104, also known as the class II–associated invariant chain peptides (CLIPs) (Riberdy et al., 1992; Sette et al., 1992). The mutant lines all had the same defect in antigen presentation: they could not present whole proteins to T cell hybridomas, but could present the corresponding peptides (Mellins et al., 1991; Riberdy and Cresswell, 1992; Ceman et al., 1992), a defect highly reminiscent of that exhibited by cells lacking Ii (reviewed by Cresswell, 1994).

These observations suggested the following, more detailed scenario: Ii and MHC class II molecules associate in the ER; Ii is gradually processed to CLIP during the intracellular transport of the complex and residence in endocytic compartments; CLIP is replaced by a diverse set of peptides, promoting a conformational change in the complex and heightened stability; finally, stable complexes composed of class II molecules and a heterogeneous set of peptides are released to the cell surface. The replacement of CLIP by other peptides and subsequent events evidently require a molecule absent from the mutant lymphoblastoid lines.

Identification of this molecule was not long in coming. Analysis of chromosome deletion mutants revealed that it was encoded by a locus in the class II region of the MHC, somewhere between *HLA-DP* and *HLA-DQ* (Mellins et al., 1991; Riberdy and Cresswell, 1992; Ceman et al., 1992). This interval included some recently discovered loci with suggestive properties: a gene with some homology to class II α genes and another similar to class II β genes were cloned from a human cDNA library (*HLA-DMa* and *HLA-DMb*) (Kelly et al., 1991); an α-like and two β-like genes were simultaneously isolated from a mouse library (*H2-Ma*, *H2-Mb1*, and *H2-Mb2*) (Cho et al., 1991b). It was not known at the time whether the products of the *Ma* and *Mb* genes serve a necessary function, nor even whether they were expressed. Nonetheless, the *M* genes were transfected into the mutant lymphoblastoid lines in an attempt to complement their deficiencies. The transfections proved highly effective; either the *Ma* or *Mb* gene was required for some lines (Morris et al., 1994; Fling et al., 1994), and both genes were required for others (Denzin et al., 1994). Thus, it was concluded that the Ma–Mb complex is the critical

facilitator of peptide loading absent from the mutant lines. This notion was supported by biochemical experiments showing that Ma and Mb form a heterodimeric complex and by a series of observations establishing that the complexes are localized in internal subcellular compartments, coincident with class II molecules, rather than at the cell surface (Sanderson et al., 1994; Karlsson et al., 1994; Denzin et al., 1994). Direct evidence that Ma–Mb performs a catalytic function, facilitating the dissociation of CLIP and the association of other peptides, has recently come from studies with soluble complexes (Sloan et al., 1995; Denzin and Cresswell, 1995; Sherman et al., 1995).

Although our understanding of these enigmatic elements has evolved rapidly, important issues remain unresolved. We have an appreciation of how Ma–Mb complexes operate in some mutant human lymphoblastoid lines, but can this be extrapolated to the murine system, or to an organismal setting? The human and murine molecules exhibit sufficient sequence similarity to suggest functional homology, but the absence of analogous mutant lines has precluded direct evaluation of the function of mouse H2-M complexes. Nor, as a consequence, has mouse CLIP been very well characterized. The few existing results on species-mismatched systems have been rather disconcerting. The properties of murine class II molecules in human mutant lines varied with the particular allele and line examined, but more often than not they achieved SDS stability or efficiently presented whole proteins (or did both) (Brooks et al., 1994; Stebbins et al., 1995). Clearly, a true murine system is required to generalize the observations on human mutant lines. We also need to extend the findings to other processes in which antigen presentation has been implicated, given that presentation by lymphoblastoid lines is known to be representative of that by some, but certainly not all, antigen-presenting cell (APC) types (Lanzavecchia, 1990). We are at present entirely ignorant of the role of Ma–Mb complexes in T cell education in the thymus or during the unfolding of a real immune response.

It seemed important, then, to generate a strain of mice lacking Ma–Mb complexes. We accomplished this by mutating the *H2-Ma* locus via homologous recombination in embryonic stem (ES) cells. Here we present a characterization of these animals, concentrating on the structure of MHC class II complexes, antigen presentation, and selection of the T cell repertoire.

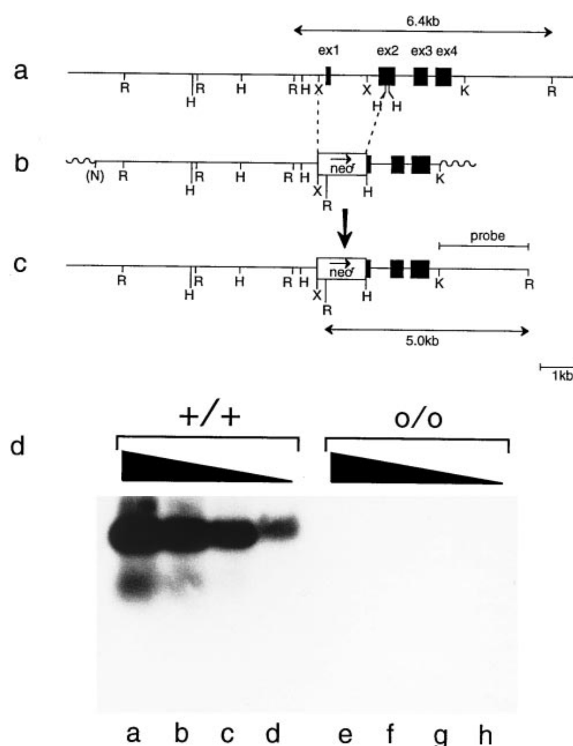

Figure 1. *H2-Ma* Gene Inactivation

(a) Organization of the wild-type *Ma* gene. Starting from the regional map of Cho et al. (1991a), it was determined by PCR amplification with primers in each of exons 1, 2, 3, and 4 (the sequence of the primers was based on the data of Cho et al. [1991b]). The location and boundaries of the exons were determined by sizing the PCR products and sequencing the exon–intron boundaries (data not shown). Exon 1 encodes the signal sequence, exon 2 the first external domain of the mature protein, exon 3 the second domain, and exon 4 the transmembrane region and cytoplasmic tail (yet another exon may encode a 3′ untranslated region, as in the human gene [Radley et al., 1994]). R, EcoRI; H, HindIII; X, XhoI; K, KpnI.

(b) The fragment used for targeting, with a neomycin resistance gene (open box) replacement. The NotI site (shown in parentheses) is artificial, derived from the original λ clone from which this pBSK subclone was derived.

(c) The modified locus resulting from homologous recombination. The probe used for Southern blots is indicated, as are the 6.4 and 5.0 kb EcoRI hybridizable fragments in wild-type and mutant DNA, respectively.

(d) *Ma* gene expression in total spleen RNA of wild-type (+/+) or mutant (0/0) littermates was assessed by semi-quantitative PCR. The amounts of template cDNA for RT–PCR correspond to 5×10^5, 5×10^4, 5×10^3, or 5×10^2 cells.

Results

Generation of $Ma^{-/-}$ Mice

Mice incapable of making the H2-M complex were produced by mutating the single *Ma* locus. A DNA fragment containing the entire *Ma* protein-coding sequence was cloned (Figure 1A), and a targeting vector was constructed by replacing all of exon 1 and part of exon 2 with the neomycin resistance gene (Figure 1B). It was electroporated into the P1 ES cell line, derived from a 129 mouse ($H2^b$) haplotype. G418-resistant clones were isolated and screened by Southern blotting (Figure 1C). Positive clones were injected into C57Bl/6 (B6) blastocysts, and chimeric offspring were mated to B6 females. Mice carrying the mutation in the heterozygous state ($Ma^{+/-}$) were inter-crossed to produce homozygous mutants ($Ma^{-/-}$).

To demonstrate that the alteration abolished expression of *Ma*, we performed polymerase chain reaction (PCR) analysis of splenocyte RNA from $Ma^{-/-}$ mice using primers spanning exons 3 and 4. We could not detect any RNA transcribed from the altered locus (Figure 1D).

Transport, Maturation, and Display of MHC Class II Molecules

Transport and maturation of class II molecules was assessed by biosynthetic labeling experiments. Splenocytes were pulse labeled with [^{35}S]methionine for 30 min,

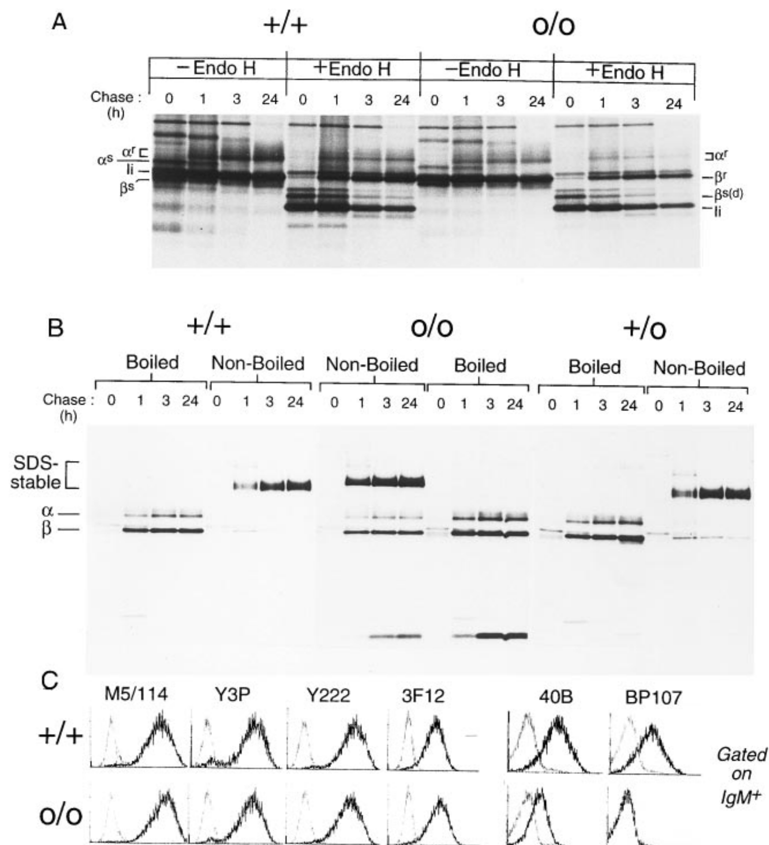

Figure 2. Normal Maturation and Transport of A^b Molecules in *Ma*-Deficient Mice

(A) Proper Golgi transport. Splenocytes from $Ma^{-/-}$ mice (0/0) or wild-type littermates (+/+) were pulse labeled with [^{35}S]methionine for 30 min, followed by a chase in cold methionine as indicated, and A^b complexes were then immunoprecipitated with M5-114. One half of the samples were digested with endo H prior to electrophoresis, as indicated. α^s and β^s indicate the position of the endo H–sensitive (high mannose) forms of Aα and Aβ chains before digestion; α^s(d) is the same after endo H digestion; α^r and β^r are endo H resistant.

(B) Assembly into SDS-stable complexes of altered mobility. A^b complexes were immunoprecipitated with Y-3P and resolved by SDS–PAGE, with or without boiling the samples prior to loading. Complexes that are stable in SDS at room temperature are bracketed; the positions of free α and β chains are indicated.

(C) Normal A^b surface expression levels. Splenocytes were stained with anti-A^b MAbs, with an anti-IgM counterstain, and analyzed by cytofluorometry. A^b-specific staining on gated IgM$^+$ B cells is shown by the darker line, superimposed with negative control staining (faint line). Wild-type-level staining of mutant splenocytes detected with the four MAbs on the left was also observed with several other MAbs (Y-248, Y-237, Y-266, and Y-212; data not shown).

and the label was chased with cold methionine for various times. Lysates were prepared, immunoprecipitated with a polyclonal rabbit anti–MHC class I serum to control for comparable levels of ^{35}S incorporation, and then reprecipitated with M5/114, a MAb that recognizes A^b molecules regardless of their degree of maturation (Germain and Hendrix, 1991). Intracellular transport of class II molecules is normally accompanied by the maturation of N-linked carbohydrates, reflected as an increase in apparent molecular mass and by the acquisition of endonuclease H (endo H) resistance. As illustrated in Figure 2A, these modifications were already evident with splenocytes from both $Ma^{+/+}$ and $Ma^{-/-}$ mice after only 1 hr of chase. The degree and kinetics of maturation were indistinguishable for the two types of cells.

To evaluate the stability of class II molecules made in the absence of H2-M complexes, we precipitated the splenocyte lysates with the MAb Y-3P, which preferentially recognizes mature forms of A^b (Germain and Hendrix, 1991). Half of the precipitated material was incubated in SDS at room temperature, and the other half was dissociated by boiling. Figure 2B shows that SDS-stable A^b molecules of the molecular mass expected for compact dimers ($\sim$50 kDa) first appeared after 1 hr of chase in cells expressing H2-M and steadily increased in quantity with longer chase times, concomitant with a decrease in SDS-labile molecules. In cells devoid of H2-M, SDS-stable molecules appeared with similar kinetics, but exhibited a reduced mobility. The dichotomy in mobility was quite striking, as none of the mutant complexes was found at the position where most of the wild-type complexes migrated.

In the precipitates from $Ma^{-/-}$ cells, a small molecular mass species running just below the dye front was clearly visible after 1 hr of chase and increased in quantity with longer chase times. It was observed only at very low levels in precipitates from cells expressing *Ma*. Its identification will be discussed below.

Finally, we analyzed class II molecules displayed at the cell surface by staining with a panel of anti–class II MAbs (Figure 2C). Two types of pattern were observed. Most reagents, including M5/114, Y-3P, Y-222, and 3F12, stained immunoglobulin M positive (IgM$^+$) splenocytes from $Ma^{-/-}$ mice very effectively: labeling of mutant B cells with this class of MAb averaged 120% that of wild-type cells; immunohistological analysis of various tissues, including the thymus, gave similar results (data not shown). One of these MAbs (3F12) recognizes an epitope mainly on the class II α chain, while two others (M5/114 and Y-222) recognize the β chain (Landais et al., 1986). On the other hand, staining by two A^b-specific reagents, 40B and BP107, was markedly reduced on splenocytes from $Ma^{-/-}$ mice. The reduction was only partial for 40B, but staining by BP107 dropped to background levels.

Loading of Class II Molecules

Based on previous findings with lymphoblastoid lines lacking HLA-DM, we hypothesized that the low molecular mass species coprecipitating with A^b molecules in the pulse–chase samples from $Ma^{-/-}$ mice was CLIP. To test this notion, we labeled splenocytes from *Ii*-negative animals and *Ma*-positive and *Ma*-negative littermates

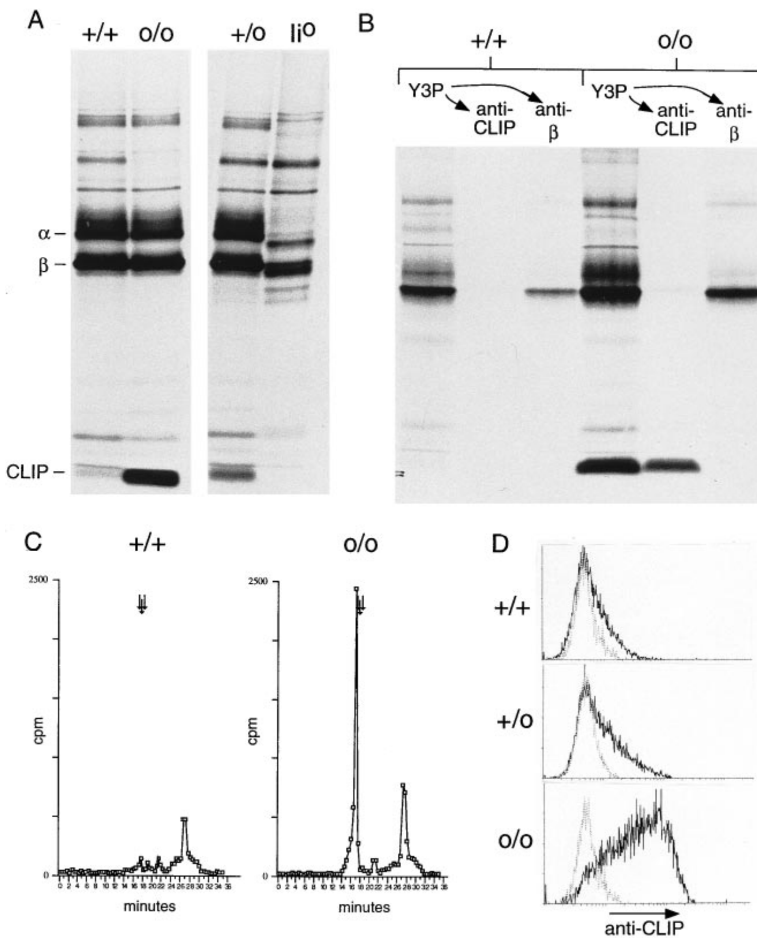

Figure 3. Large Amounts of CLIP Peptide Complexed with A^b Molecules of *Ma*-Deficient Mice

(A) Large increase of a discrete peptide associated with A^b complexes in $Ma^{-/-}$ mice. Splenocytes from homozygous (0/0), heterozygous (+/0), or wild-type (+/+) littermates and from an *Ii*-deficient mouse (Ii^0) were labeled with [^{35}S]methionine for 5 hr; A^b complexes were immunoprecipitated with Y-3P and resolved by SDS–PAGE after boiling. The position of the α and β chains is indicated, as is that of the low molecular mass species (CLIP) predominant in immunoprecipitates from mutant mice.

(B) The dominant peptide of A^b complexes in $Ma^{-/-}$ mice can be reprecipitated with anti-CLIP. Y-3P immunoprecipitates prepared as in (A) were denatured and reprecipitated with anti-CLIP or anti-Aβ antisera. The reprecipitated samples were resolved by SDS–PAGE after boiling, next to an aliquot of the starting Y-3P-precipitated material.

(C) The dominant peptide of A^b complexes in $Ma^{-/-}$ mice coelutes with synthetic CLIP peptides on RP-HPLC. Splenocytes were labeled for 3 hr, followed by a 3 hr chase. Y-3P immunoprecipitates were denatured in acid and run on an RP-HPLC column, and the eluted peptides were detected by scintillation counting of fractions. A mix of unlabeled CLIP peptides was added to the samples and detected by ultraviolet absorption (arrows at the top of the panel).

(D) CLIP expression on the cell surface. Splenocytes were stained with MAb 30-2, which detects the CLIP peptide associated with A^b molecules (Eastman et al., 1996). CLIP-specific staining on gated IgM^+ B cells is shown on the histograms (dark line) superimposed with negative control staining (faint line).

for 5 hr, prepared lysates, and precipitated them with Y-3P (Figure 3A). There was a clear gradation in the amount of the low molecular mass band precipitated in the *Ma*-positive and *Ma*-negative samples: low in $Ma^{+/+}$, intermediate in heterozygotes, and very high in $Ma^{-/-}$. No such band was observed in samples from $Ii^{-/-}$ mice, which is consistent with the low molecular mass species being CLIP.

Definitive identification was made by two means. First, the Y-3P precipitates were dissociated by boiling in an SDS-containing solution and reprecipitated with antisera specific for either the Aβ chain or murine CLIP (Figure 3B). Recovery of Aβ was comparable for the $Ma^{+/+}$ and $Ma^{-/-}$ samples. Large amounts of the low molecular mass material were observed in the anti-CLIP reprecipitate of the $Ma^{-/-}$, but not the $Ma^{+/+}$, sample. Second, we acid-extracted peptides from the Y-3P precipitates of wild-type and mutant cells and subjected them to reverse-phase high pressure liquid chromatography (RP-HPLC) (Figure 3C). A sharp major peak of radioactivity was observed in the $Ma^{-/-}$ sample, its elution nearly coinciding with that of synthetic CLIP standards. This peak was considerably reduced in the $Ma^{+/+}$ sample. (The labeled material eluting later in both samples was in the range of whole proteins or large polypeptides and probably represented Aα, Aβ, or Ii molecules.)

The peak fraction from the $Ma^{-/-}$ sample was subjected to automated Edman degradation. It yielded spikes of radioactivity at cycles 6, 8, and 14, allowing unambiguous identification of the CLIP sequence 86, KPVSQMRMATPLLM . . . (data not shown). While the identification of these 14 N-terminal residues is solid, we cannot be certain of whether or not there are additional C-terminal residues. In a prior study, peptides eluted from splenic A^b molecules from wild-type animals included CLIP 86–100, i.e., with the same N-terminus but one residue longer (Rudensky et al., 1991).

Finally, a MAb directed against a synthetic human CLIP permitted us to visualize CLIP-loaded A^b molecules at the cell surface (Figure 3D). IgM^+ splenocytes from wild-type mice were stained, but only slightly above background levels; B cells from heterozygous mutant animals exhibited somewhat stronger staining; and cells from homozygous mutants were stained to very high levels, at least ten times higher than wild-type cells.

Antigen Presentation

As mentioned above, cells lacking HLA-DM or Ii generally present whole proteins very poorly, but are quite able to present the corresponding peptides. To see whether the *Ma* mutation resulted in faulty antigen presentation, we compared the ability of splenocytes from

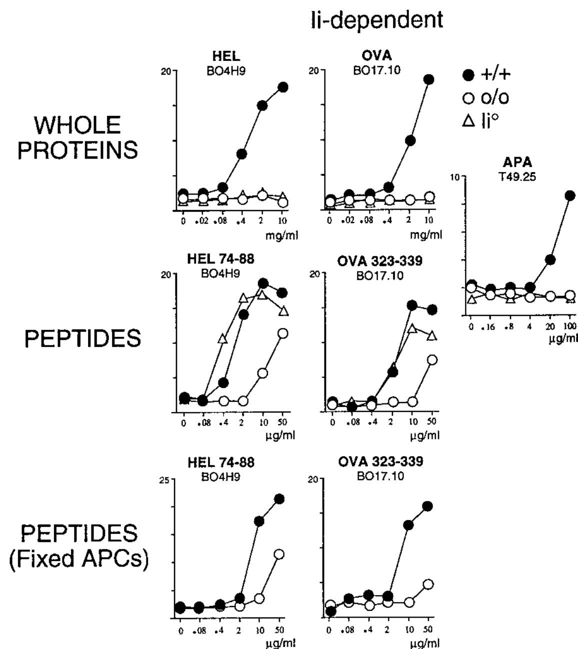

Figure 4. Antigen Presentation in *Ma*-Deficient Mice

Spleen cells from wild-type, *Ma*-deficient, or *Ii*-deficient (Ii^0) mice were used as APCs for T hybridoma stimulation, read out as interleukin-2 production (vertical scales are [^{3}H]thymidine incorporation by the CTLL indicator cells). Antigens were provided in the form of whole proteins (HEL, OVA, or apamin [APA], at top) or corresponding peptides (middle and bottom). Splenic APCs were fixed with paraformaldehyde in some assays (bottom).

wild-type, *Ma*-negative, and *Ii*-negative mice to present hen–egg white lysozyme (HEL) and ovalbumin (OVA) proteins and the corresponding peptides to T cell hybridomas. As shown in Figure 4 (top), neither $Ma^{-/-}$ nor $Ii^{-/-}$ splenocytes were able to present the whole proteins. The latter offered peptides as efficiently as wild-type cells, but, surprisingly, $Ma^{-/-}$ splenocytes exhibited a 10- to 20-fold reduction (Figure 4, middle). Poor peptide presentation was observed with either fresh or fixed $Ma^{-/-}$ splenocytes (compare middle and bottom panels in Figure 4), indicating that the observed defect was not dependent on peptide internalization and processing.

Another aspect of processing was evaluated by measuring the presentation of apamin. This 18 amino acid polypeptide requires reduction of a disulfide bridge, but not additional degradation, before it can be presented to the hybridoma T49.25 (Régnier-Vigouroux et al., 1988). Neither *Ma*- nor *Ii*-deficient splenocytes could present apamin to this hybridoma (Figure 4, right-most panel).

T Cell Differentiation

Having established that cells from $Ma^{-/-}$ mice displayed normal levels of MHC class II molecules predominantly loaded with CLIP, we wondered how such molecules would operate in the selection and maintenance of T cells. We therefore performed a cytofluorometric analysis of thymic and peripheral T cell compartments in *Ma*-positive and *Ma*-negative littermates and, for comparison, in *Ii*-negative animals.

Total thymocyte numbers were essentially the same in the three types of mice (data not shown). Typical staining profiles are presented in Figure 5A. The CD4/CD8 plots to the left of Figure 5A show that the $CD4^+CD8^-$ population was reduced in $Ma^{-/-}$ animals to about half the number of cells found in normal littermates. The reduction was not as extreme as that found in age-matched $Ii^{-/-}$ animals, which had about a quarter the normal number. Deficient positive selection of the $CD4^+CD8^-$ population in $Ii^{-/-}$ mice has been described previously and is known to involve phenotypic changes as well: residual levels of CD8 on most cells and slightly reduced expression of CD3 (Viville et al., 1993; Bikoff et al., 1993; Tourne et al., 1995). For both of these features, cells from *Ma*-negative mice exhibited a phenotype intermediate between those of cells from wild-type and *Ii*-negative animals (Figure 5A, left and center panels). A more discriminating view of the maturation of $CD4^+CD8^-$ cells came from correlating the expression of CD3 and the capacity to bind peanut agglutinin (PNA) (Figure 5A, right panels). This permits the distinction of two subpopulations: the most mature cells (gate K) are PNA-negative and express marginally higher CD3 than the less mature cells (gate L), which stain with PNA. In wild-type mice, the former predominated, while in *Ii*-negative animals there was a greater fraction of the latter, apparently arrested at that stage; hence a markedly lower K to L "maturation ratio" is observed. The cells in *Ma*-negative animals displayed an intermediate phenotype: fewer $CD4^+CD8^-$ cells, but a close to normal maturation ratio.

The total numbers of T cells in the lymph nodes and spleen were also very comparable in the three types of mice (data not shown). However, as illustrated in Figure 5B, the relative contributions of the different populations varied. The ratio of $CD4^+$ to $CD8^+$ cells was low in *Ma*-negative mice, although still higher than in age-matched *Ii*-negative animals (Figure 5B, left panels). The reduced ratios were due to both a decrease in numbers of $CD4^+$ cells and an increase in numbers of $CD8^+$ cells. The phenotype of the residual $CD4^+$ cells was somewhat different in the two types of mutant: they displayed normal levels of CD44 in $Ma^{-/-}$ mice, but many expressed high levels in $Ii^{-/-}$ animals (Figure 5B, right panels).

To characterize the T cell repertoire selected by the class II molecules in $Ma^{-/-}$ mice, we stained thymocytes and lymph node cells with a panel of anti-Vβ and anti-Vα reagents. The data from lymph node cells are presented in Table 1. Clearly, the *Ma*-negative animals expressed a very diverse repertoire, quite similar to that of *Ma*-positive littermates. All variable regions were represented in essentially normal proportions, with no overrepresentation by any particular Vβ or Vα. Some minor differences were seen, such as higher numbers of V$\beta5^+$ and V$\alpha2^+$ cells in the mutants, but further study will be required to elucidate their significance.

MLRs

The special peptide complement carried by class II molecules from $Ma^{-/-}$ mice begged the question of how T cells from these animals behave in the mixed lymphocyte reaction (MLR). Are they tolerant of the diverse set of self-peptides presented by class II molecules from normal mice?

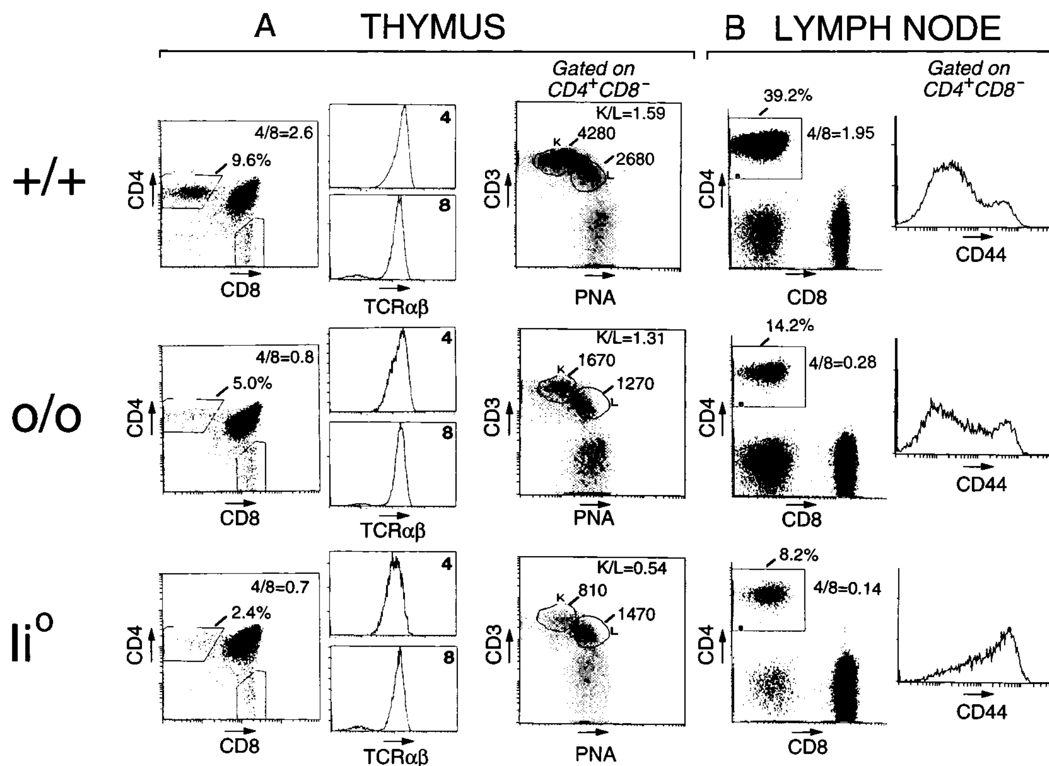

Figure 5. Partially Impaired Selection of CD4$^+$ Cells in *Ma*-Deficient Mice

(A) Thymocyte suspensions from the indicated mice were stained and analyzed by flow cytometry. The left panels display the CD4/CD8 profiles and the αβ T cell receptor (TCRαβ) histograms of CD4$^+$ or CD8$^+$ single-positives (4 and 8), gated as shown. *Ma* heterozygotes were indistinguishable from wild type in these experiments (data not shown). The values indicate the percentages of CD4$^+$CD8$^-$ cells in these thymi (an average of five different determinations) and the ratio of CD4 to CD8 single positives. In the panels to the right, the thymocytes were stained with reagents specific for CD3, CD4, and CD8 and with PNA–fluorescein isothiocyanate. The panels display the CD3–PNA profiles of gated CD4$^+$CD8$^-$ cells. The two subpopulations of CD4 single positives that can be split on this basis (low PNA, highest CD3 versus PNA positive, high CD3) are highlighted. The number of cells falling within these gates is indicated (of a total of 75,000 thymocytes analyzed) for a representative experiment. The K to L maturation ratio of CD4 single positives is also shown.

(B) To the left, CD4/CD8 profiles of lymph node cells are shown. The percentages of CD4$^+$ cells (an average of four to six experiments) and the average CD4 to CD8 ratio are shown. To the right, CD44 staining profiles of CD4$^+$CD8$^-$ cells are shown.

Figure 6A depicts a representative MLR experiment, illustrating that lymph node T cells from homozygous *Ma*-mutant mice were unusually good responders. Most striking was their strong reaction to syngeneic APCs from B6 mice, quite in contrast with T cells from heterozygous *Ma* mutants, *Ii* mutants, and wild-type animals, which made essentially no response (Figure 6A, left). This difference was not due to segregating minor histocompatibility loci because three out of three *Ma*-negative mice exhibited the "super-responder" phenotype, while six of six paired *Ma*-positive littermates failed to respond. In addition, responses of $Ma^{-/-}$ cells to $H2^b$ stimulators from 129 mice were equally strong (data not shown). Since challenges were performed in the presence of anti-CD8 MAbs, and because T cells from *Ma*-mutant mice did not react to APCs from B6 mutant animals lacking MHC class II molecules (Figure 6A, center), the response emanated from CD4$^+$ T cells reacting to class II molecules. T cells from $Ma^{-/-}$ mice also showed an increased response to allostimulation by APCs from B10.BR mice, although the increase vis-à-vis T cells from $Ma^{+/+}$ mice was modest (Figure 6A, right). Thus, to T cells from *Ma*-negative mice, a syngeneic A^b molecule loaded with the usual spectrum of peptides was as stimulatory as an allogeneic A^k molecule, if not more so.

Figure 6B shows that the impressive syngeneic MLR is one way. Splenocytes from $Ma^{-/-}$ mice were unable to stimulate lymph node T cells from B6 animals. As expected, neither could spleen cells from $Ma^{+/+}$ littermates, confirming the view that segregating minor histocompatibility loci were not making a major contribution. Splenocytes from *Ma*-negative mice could, however, stimulate allogeneic T cells (data not shown).

Discussion

Presentation of Proteins and Peptides by APCs from $Ma^{-/-}$ Mice Is Defective

Splenocytes from *Ma*-mutant mice were unable to present whole proteins to T cell hybridomas derived from wild-type animals, a property shared with HLA-DM-deficient human lymphoblastoid lines (Mellins et al., 1991; Riberdy and Cresswell, 1992; Ceman et al., 1992). This defect almost certainly reflects an inability to strip MHC class II molecules of CLIP in the endocytic pathway and replace them with peptides processed from internalized proteins. It is worth stressing that this represents the

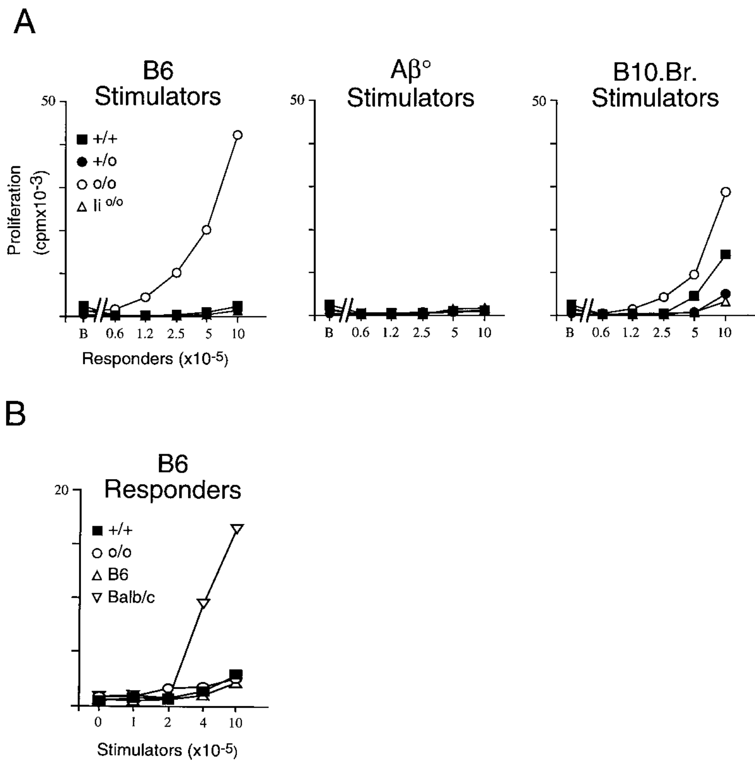

Figure 6. High Reactivity of T Cells from *Ma*-Deficient Mice against Self–Class II MHC of Wild-Type Mice

(A) MLR responders were titrated lymph node cells from various mice, as indicated, stimulated with X-irradiated spleen cells from MHC-matched B6 or Aβ-deficient (Aβ°) mice or from MHC-mismatched B10.BR ($H2^k$) mice. In this representative experiment, 2 μg of anti-CD8 MAb was added to the culture to block responses directed against MHC class I molecules.

(B) MLR was performed as above, with B6 lymph node responders and X-irradiated spleen cells from various mice as stimulators.

examination of an entirely species-matched murine system: mouse H2-M complexes removing mouse CLIP from mouse class II molecules. Class II molecules and their accessories, such as Ii and M, have coevolved, so it would not be entirely surprising to obtain results different from those derived from species-mismatched systems. Indeed, most (though not all) assays of whole protein presentation by murine class II molecules in HLA-DM-deficient human cell lines showed little reduction in efficiency (Riberdy et al., 1992; Brooks et al., 1994; Stebbins et al., 1995). Although the A^b allele was not assayed in these studies, they did include analyses of alleles with high or low affinity for CLIP.

Human lymphoblastoid cells lacking HLA-DM (Mellins et al., 1991; Riberdy and Cresswell, 1992; Ceman et al., 1992) and cells without Ii (Cresswell, 1994) are able to present peptide antigens to T hybridomas efficiently because, not requiring processing, peptides can be loaded directly onto class II molecules at the cell surface. Somewhat surprisingly, splenocytes from *Ma*-mutant mice were poor presenters of peptides. This was true of either fresh or fixed cells, indicating that loading at the cell surface was probably affected, rather than some process dependent on internalization and further processing. Thus, the poor presentation was probably due to inefficient replacement of CLIP by antigenic peptides. Given reports that the affinity and kinetics of CLIP binding vary widely with class II alleles (Avva and Cresswell, 1994; Sette et al., 1995; Liang et al., 1995; Malcherek et al., 1995), one might predict that the defect in peptide presentation will not be observed when *Ma*-mutant mice expressing other alleles are examined and

Table 1. T Cell Repertoire Variable Region Usage

		Vβ2	Vβ4	Vβ5	Vβ6	Vβ7	Vβ8	Vβ10	Vβ11	Vβ12	Vβ14	Vα2
		6.8	6.1	2.3	7.2	1.3	19.7	3.6	4.4	3.7	9.1	9.8
CD4	+/−	6.8	6.7	1.6	7.1	1.4	20.0	3.5	4.0	2.5	10.3	13.9
		6.4	6.0	1.9	7.5	1.5	17.9	3.4	4.2	2.5	8.4	14.6
		5.5	7.6	5.4	6.5	1.3	18.2	2.1	4.9	3.4	11.2	10.5
	−/−	5.1	9.0	4.4	7.1	0.9	16.6	5.9	5.3	4.0	18.6	8.1
		4.7	7.9	4.3	8.0	1.6	14.5	2.6	4.9	2.9	11.3	8.9
		5.9	3.5	13.3	5.6	4.4	17.4	4.1	5.8	2.7	2.5	6.9
CD8	+/−	5.3	5.7	9.4	5.7	4.4	18.8	4.1	5.6	2.3	2.4	8.1
		4.9	3.6	9.8	6.5	5.1	17.0	4.2	6.5	2.3	2.7	8.3
		5.7	3.6	17.1	6.7	3.9	16.5	3.0	5.7	2.8	6.8	9.0
	−/−	4.8	4.2	17.6	5.6	3.7	19.0	6.1	6.3	3.2	6.8	7.5
		4.5	3.6	14.5	7.4	4.7	17.4	3.0	6.2	3.0	6.4	8.3

Heterogeneous T cell repertoire in *Ma*-deficient mice. Percentage of gated $CD4^+$ cells expressing each of the variable regions, compiled from several different experiments. V region usage frequencies in the $CD8^+$ population were indistinguishable in mutant and wild-type littermates (data not shown). Results from +/− mice were similar to those of +/+ controls (data not shown).

will be found once human cells expressing more HLA class II alleles have been analyzed.

How Much CLIP?

To evaluate the data on T cell selection in $Ma^{-/-}$ mice, it is important to estimate the fraction of mature class II molecules loaded with CLIP versus other peptides. Several arguments lead us to conclude that the vast majority, if not essentially all, of the A^b molecules in the mutant mice carried CLIP, with few or none of the peptides normally found in class II molecules from wild-type animals. First, gels like that in Figure 2B indicated that the mobility of the SDS-stable complexes from $Ma^{-/-}$ splenocytes did not overlap with that of the bulk of complexes from $Ma^{+/+}$ cells. Upon dissociation at high temperature, the SDS-stable dimers from mutant mice gave rise to a massive amount of CLIP, almost invisible after similar treatment of dimers from wild-type animals. We suggest that class II–CLIP complexes have a reduced mobility, as has been observed for certain other class II–peptide complexes (Sadegh-Nasseri and Germain, 1991) and that essentially all of the SDS-stable complexes in cells from $Ma^{-/-}$ animals carried CLIP, with no significant amount of the diverse complement of peptides normally found in SDS-stable complexes. Second, radiometric analysis of gels like that in Figure 3A led to estimates of between 55% and 75% CLIP occupancy. However, this is almost certainly an underestimate: there was probably dissociation and loss of peptide in the course of immunoprecipitation; there was probably also a significant loss of peptide in the CLIP size range in preparation of the gels for autoradiography, compared with little or no loss of intact class II chains. Third, the absence of detectable BP107 staining of B cells from $Ma^{-/-}$ mice was in stark contrast to the normal, even elevated, level of staining with other anti–class II reagents. Had a substantial fraction of A^b molecules carried the usual peptides, some staining with BP107 should have been detected.

The fourth and perhaps most compelling factor, since it relates to what T cells actually see, was the strong one-way syngeneic MLR. Lymph node T cells from *Ma*-negative mice reacted strongly to stimulation by splenocytes from B6 animals, even more strongly than they did to cells from allogeneic B10.BR animals. In contrast, splenocytes from *Ma*-negative mice could not stimulate lymph node T cells from B6 animals. The simplest interpretation of such a response pattern is that the class II molecules in $Ma^{-/-}$ mice carry little other than CLIP, given that negative selection is a very sensitive screening process. In the thymi of mutant animals, thymocytes would see mostly (or perhaps only) class II–CLIP complexes, and so the emerging repertoire would remain unpurged of many self-specificities because the relevant MHC–peptide complexes were not encountered. Since they are also not displayed in the periphery, the repertoire would remain untolerized. T cells with these self-specificities would, however, be strongly stimulated by APCs from normal animals displaying a diverse complement of class II–peptide complexes. On the other hand, splenocytes from mutant mice would display predominantly class II–CLIP complexes and thus would not be able to stimulate peripheral T cells of normal $H2^b$ mice.

Together, these data argue that a very high proportion of class II molecules in mutant mice was loaded with CLIP. Kropshofer et al. (1995) have recently presented evidence for self-release of human CLIP from HLA-DR: the 81–89 region destabilized binding of the 90–103 stretch, promoting self-removal that did not require HLA-DM. These data fit well with prior observations that treatment of HLA-DR–peptide complexes with acidic pH provoked release of CLIP, but only the longer species (Urban et al., 1994). Self-release probably explains why only about 50% of the class II molecules in DM-deficient cell lines carry CLIP (Riberdy et al., 1992; Sette et al., 1992). One might have expected to see a similar phenomenon in $Ma^{-/-}$ mice. This did not appear to happen, probably because the CLIP peptide bound to A^b in the mutant started at position 86 and thus lacked most of the sequence required for self-removal. The murine system may have forfeited this option owing to more extensive cleavage of Ii, or we may find that different alleles carry longer CLIPs and are thereby more susceptible to self-release.

$Ma-/-$ Mice Have a Diverse T Cell Repertoire

How does this major perturbation of the array of peptides carried by class II molecules affect selection of the T cell repertoire? As discussed above, negative selection was markedly altered, permitting the emergence of cells reactive with syngeneic A^b carrying the full panoply of peptides.

The role of peptides in the positive selection of T cells is still the subject of considerable debate (for review and references, see Jameson et al. [1995]). Three possibilities have been considered: that peptides play no role; that they are required for the generation or maintenance of stable MHC molecules, but do not contribute directly to the specificity of T cell recognition (only by not interfering); or, third, that peptides are directly recognized by T cells and are a major contributor to specificity. Recent evidence based on adding peptides to fetal thymus organ cultures supported the third possibility, but this approach was subsequently challenged. In addition, all of these studies focused on positive selection mediated by MHC class I molecules, leaving us almost completely ignorant of the role of peptides in class II–driven selection.

In spite of their preponderant loading with CLIP and their lack of the usual peptides, class II molecules in $Ma^{-/-}$ mice were surprisingly effective at selecting T cells. The numbers of $CD4^+CD8^-$ cells were only slightly reduced, considering either the intermediate or fully mature populations, and the selected cells displayed a very diverse set of receptors, showing no evidence of grossly skewed $V\beta$ or $V\alpha$ usage. This observation might be interpreted to support a purely structural role for peptide in positive selection, CLIP being sufficient to stabilize mature forms of class II molecules at the cell surface. Alternatively, selection might be promiscuous, and the CLIP peptide might be able to select a wide range of receptors. (Might CLIP be a major selecting peptide in normal mice as well?) To resolve this issue, it will be

necessary to assay the functional capabilities of this repertoire, as well as to establish exactly how much class II molecules displaying non-CLIP peptides contribute to its selection. Recent reports have claimed that very low levels of class II molecules can select a normal repertoire (Naujokas et al., 1995; Shachar et al., 1995); however, we have not been able to confirm this in very similar experiments (data not shown), nor is such a claim consistent with the often surprising sensitivity of positive selection to class II expression levels in existing T cell receptor transgenic systems (e.g., Berg et al., 1990).

This last point brings up the intriguing differences in the T cell compartments of $Ma^{-/-}$ and $Ii^{-/-}$ mice. A reduction in the thymic $CD4^{+}CD8^{-}$ population was seen in both types of mutants, but the defects seemed distinct. In *Ii*-negative animals, the decrease was more pronounced, and differentiation seemed to be severely affected at the $CD4^{+}CD8^{lo}$ to $CD4^{+}CD8^{-}$ transition, a maturation step considered to require continued engagement between the T cell and MHC molecules on stromal cells (Chan et al., 1993). In *Ma*-negative animals, the decrease was less severe, and the transition to the fully mature state seemed largely unimpaired. It is not clear which of the differences between class II molecules in the two mutants (surface density, conformation, or peptide complement) underlie(s) the different maturation patterns, but these observations imply distinct recognition requirements for maturing $CD4^{+}CD8^{-}$ cells at various points during the selection process: the selecting signal(s) might be qualitatively or quantitatively different for the initial entry into the pathway versus graduation to a fully mature compartment.

Perspectives

This view of mice devoid of H2-M molecules has provided novel information and will open avenues of experimentation concerning several points: the role of H2-M in the maturation and function of MHC class II molecules and the effects of a dominant peptide on the selection and response capacity of the T cell repertoire.

Experimental Procedures

Mice

B6, 129/Sv, B10.Br, Balb/c (Jackson Laboratory), A^{b}-deficient (Cosgrove et al., 1991), and *Ii*-deficient mice (Viville et al., 1993) were maintained in a conventional facility.

Disruption of the *H2-Ma* Gene

An 18 kb genomic λ clone (GF11) including the whole *Ma* protein-coding sequence was isolated from a D3 ES cell library probed with an *Ma* cDNA fragment cloned into the EcoRI site of pBluescript (pDM1, position 34–650; numbering as per Cho et al. [1991b]). A targeting vector was constructed from a 9.1 kb NotI–KpnI fragment subcloned by replacing a 1.8 kb XhoI–HindIII fragment (including exon 1 and a portion of exon 2) by an XhoI–HindIII fragment of pMC1-neo-poly(A). NotI-linearized DNA was electroporated into ES cells (129/Sv P1 line), and clones were selected as described elsewhere (Miyazaki et al., 1996). Three homologous recombinants were detected in 117 G418-resistant colonies by Southern blotting (EcoRI digested and probed with the 2.2 kb KpnI–EcoRI fragment shown in Figure 1). ES clone KV7 was injected into blastocysts, and chimeras were mated to B6 females. Offspring with the mutation were inter-crossed to produce −/− mice, as well as −/+ and +/+ control littermates. Mice were typed by Southern blot analysis, as above.

RT–PCR

Ma expression in total splenocyte RNA was determined by reverse transcription–PCR (RT–PCR) as described elsewhere (Miyazaki et al., 1996) with titrated amounts of splenocyte cDNA template, with primers at positions 406–426 and 782–803. We blotted and probed 10% of each reaction with *Ma* cDNA.

Serological Reagents and Flow Cytometry

For descriptions of anti-A^{b} MAbs, see Landais et al. (1986) and Viville et al. (1993). For secondary immunoprecipitations, we used polyclonal reagents against the cytoplasmic tail of A^{b} and a peptide covering the murine CLIP sequence (N. B., unpublished data). CLIP bound to surface A^{b} molecules was detected with MAb 30-2 (Eastman et al., 1996); this reagent cannot be used for biochemical purification of class II–murine CLIP complexes. Reagents and conditions for staining T cells have been described previously (Chan et al., 1993).

Peptides and Antigens

Peptides used were as follows: mouse Ii 81–104 and shorter variants 81–103, 83–104, and 83–103; HEL 74–88; chicken OVA 323–339. All peptides were synthesized by standard chemistry and HPLC purified. HEL, OVA, and apamin were obtained from Sigma; whole HEL was repurified by ultrafiltration (Centricon) before use with Ii-independent hybridomas.

Immunoprecipitations and SDS–PAGE Analysis

Spleen cells were labeled with [^{35}S]methionine as indicated in the figure legends and lysed in NP-40. Lysates were precleared four times, and aliquots (representing identical cell equivalents for each extract) were immunoprecipitated with specific or control antibodies and protein A–agarose and eluted in reducing SDS sample buffer (for 5 min at 95°C versus 20 min at 20°C to detect SDS-stable heterodimers). All samples were analyzed by 12.5% SDS–polyacrylamide gel electrophoresis (SDS–PAGE) under reducing conditions. For reprecipitation, Y-3P immunoprecipitates were denatured in 1% SDS at 95°C, diluted 20-fold, and reprecipitated with anti-CLIP or anti-A^{b} antiserum. For endo H digestion, precipitates were denatured in 0.5% SDS, 1% β-mercaptoethanol for 5 min at 95°C and incubated for 60 min at 37°C without or with recombinant endo Hf (New England Biolabs) at 50,000 U/ml.

Peptide Analysis of A^{b} Complexes

Y-3P precipitates from lysates of ^{3}H-labeled spleen cells were extracted in 0.1% TFA at 20°C, spiked with a mixture of synthetic CLIP (see above) as internal standards, and applied to an RP-HPLC column (DeltaPak C18, 39 × 150 mm; Waters). Elution was as follows: 5% acetonitrile in 0.1% TFA for 5 min and then a 5%–95% acetonitrile gradient for over 30 min at 1 milliliter per minute. CLIP standards were detected by absorbance at 214 nm. Fractions (0.5 ml) were collected, and aliquots were counted by liquid scintillation. The major radioactive HPLC peak was subjected to automated Edman degradation (performed by the Biopolymers Laboratory at the Massachusetts Institute of Technology), and fractions were counted.

Antigen Presentation Assays

T hybridomas and stimulation protocols were as described previously (Viville et al., 1993). When necessary, spleen cells were fixed by incubation with 0.5% paraformaldehyde in PBS for 30 min and quenched with 0.2 M glycine in PBS.

MLRs

Responders (total lymph node cells) were cultured with stimulators (X-irradiated total spleen cells) at various ratios for 4 or 5 days in 96-well plates in 250 μl of DMEM supplemented with 10% fetal calf serum and 50 μM β-mercaptoethanol. Proliferation was assessed by [^{3}H]TdR incorporation in the last 16 hr of culture.

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