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Selection of a Broad Repertoire of CD4$^+$ T Cells in H-2Ma$^{0/0}$ Mice

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Summary

According to past reports, H-2Ma$^{0/0}$ mice express a single major histocompatiblity complex class II molecule, A^b, heavily loaded with a single peptide derived from the invariant chain, CLIP. Despite the highly restricted diversity of the class II:peptide complexes expressed on thymic stromal cells in the mutant animals, a large and diverse population of CD4$^+$ T cells is positively selected. However, two important issues remained unresolved and are addressed here: Just how preponderant is CLIP occupancy of the class II molecules from H-2M$^{0/0}$ mice? How extensive and functionally competent is the CD4$^+$ population selected in the mutant animals? Our results argue that a single class II:peptide complex can select a very broad, though not complete, repertoire of CD4$^+$ T cells.

Introduction

The differentiation of T cells in the thymus includes a positive selection event whereby only those thymocytes whose T cell receptors (TCRs) can interact with major histocompatibility complex (MHC) molecules encountered on stromal cells are able to complete maturation. This is a well-established fact, accepted essentially universally (reviewed by Kisielow and von Boehmer, 1995). However, the definition of the precise ligand engaged by the TCR during positive selection has remained a prickly issue (reviewed by Benoist and Mathis, 1997). It is known that peptides residing in the groove of MHC molecules are somehow involved, but is not clear to what extent specific TCR:peptide contacts, versus less stringent TCR:MHC interactions, are determinant.

The first direct approach to this issue came with a wave of studies evaluating the effect of adding exogenous peptides to fetal thymic organ cultures (FTOCs) (Ashton-Rickardt et al., 1993, 1994; Hogquist et al., 1993, 1994; Sebzda et al., 1994). High occupancy of a particular MHC molecule with a chosen peptide was achieved by using FTOCs from mouse strains carrying mutations that interfere with the loading of class I molecules, and the evaluation of positive selection was facilitated by using cultures from crosses of the mutants with TCR transgenic strains. Positive selection of CD8$^+$ T cells was promoted most effectively by those peptides exhibiting minimal, or even no, structural divergence from the antigenic peptide capable of engaging the TCR on peripheral T cells; in general, peptides with very different sequences did not enhance selection. These findings appeared to support the notion that specific TCR:peptide contacts play a critical role in the selection process. However, some caution was called for because, first, only a few receptors, all class I–restricted, were assayed; second, while such systems could identify peptides capable of influencing the positive selection of a particular TCR, they did not actually reveal which peptide(s) was (were) responsible for their selection in vivo; and third, FTOCs from mutant animals created a situation in which selection was based on recognition of peptides displayed on abnormally low numbers of MHC molecules.

To circumvent these problems, several groups set out to develop an in vivo system focusing on MHC class II–mediated positive selection of CD4$^+$ T cells. In one set of experiments, mice that carry a transgene encoding a class II molecule (Aβ^b) covalently linked to a particular peptide (Eα(52–68)) were produced; when null mutations of the Aβ^b and invariant chain (Ii) genes were introduced into the transgenic strain, already bearing a natural mutation of the Eα gene, the animals expressed only Aα^bAβ^b class II molecules, all seemingly loaded with the Eα peptide (Ignatowicz et al., 1996; Fukui et al., 1997). A second series of experiments made use of mice lacking H-2M complexes, critical for loading of class II molecules with a diversity of peptides; these animals expressed normal numbers of Aα^bAβ^b molecules primarily filled with a peptide from Ii termed CLIP (although it remained unclear how dominant CLIP occupancy really was and how complete was the exclusion of the usual array of self-peptides) (Fung-Leung et al., 1996; Martin et al., 1996; Miyazaki et al., 1996). Results on the two types of engineered mice were almost surprisingly convergent: in both cases, significant numbers of CD4$^+$ T cells developed in the thymus and appeared in the periphery, at 20%–50% of the normal numbers (Fung-Leung et al., 1996; Ignatowicz et al., 1996; Martin et al., 1996; Miyazaki et al., 1996; Fukui et al., 1997); these cells expressed the full range of Vβ segments (Ignatowicz et al., 1996; Miyazaki et al., 1996; Fukui et al., 1997).

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At first glance, the observation that a large, diverse repertoire of $CD4^+$ T cells can be selected on an MHC molecule loaded with essentially a single peptide seems at odds with the exquisite specificity of positive selection suggested by results from the in vitro FTOC systems. However, little was reported about the precise make-up of the $CD4^+$ population that emerged in these newly engineered mouse strains. It is large and diverse, but it is not known whether it includes most, or just a limited subset of, TCR specificities. It also is not known whether this population is capable of responding to the majority, or just a particular fraction, of antigen challenges. The answers to these questions could critically influence our view of positive selection as a fundamentally specific or promiscuous process.

Here, we have taken a closer look at the positive selection of $CD4^+$ T cells in H-2M–deficient mice. As a prelude, further evidence in support of the contention that the class II molecules in these animals are essentially all loaded with CLIP is provided. Subsequently, we examine the precise constitution of the $CD4^+$ compartment and evaluate its response capabilities.

Results and Discussion

Evidence That MHC Class II Molecules from H-2M–Deficient Mice Are Predominantly Loaded with CLIP: Endogenous Antigen and Superantigen Presentation

Published studies on mice lacking H-2M molecules strongly suggested that the class II molecules in these animals are occupied almost exclusively with CLIP rather than with the usual diverse array of peptides (Fung-Leung et al., 1996; Miyazaki et al., 1996). Multiple lines of evidence were presented: the different mobilities of stable class II complexes from mutant (M^-) and wild-type (M^+) mice, the absence of detectable staining with certain anti-A^b monoclonal antibodies (MAbs), the ability of an anti-CLIP reagent to completely block staining with an anti-A^b MAb, and a strong one-way "syngeneic" mixed lymphocyte reaction (MLR) between cells from M^+ and M^- littermates. Although these observations seemed to argue a strong case, we sought additional substantiation of this important point.

First, we exploited a new mouse line that we recently developed by crossing the H-$2Ma^{0/0}$ strain with a strain carrying an $E\alpha^k$ transgene on the C57Bl/6 (B6) background. This line allows evaluation of the influence of H-2M on the loading of E, in addition to A^b, molecules (P. Wolf et al., submitted). Here, we made use of the availability of both a MAb (Yae [Murphy et al., 1989]) and a T cell hybridoma (BEα20.6 [Ignatowicz et al., 1995]) capable of specifically recognizing A^b molecules occupied with the Eα peptide 52–68. These reagents permitted us to directly assess the degree to which the M^- mutation influences the loading of this endogenous self-peptide. Figure 1A demonstrates that B cells from wild-type mice carrying the Eα transgene were readily stained with Yae, illustrating that a significant fraction of A^b molecules were loaded with the Eα(52–68) peptide. In contrast, no staining above background was observed with B cells from mutant animals expressing Eα. Figure 1B shows

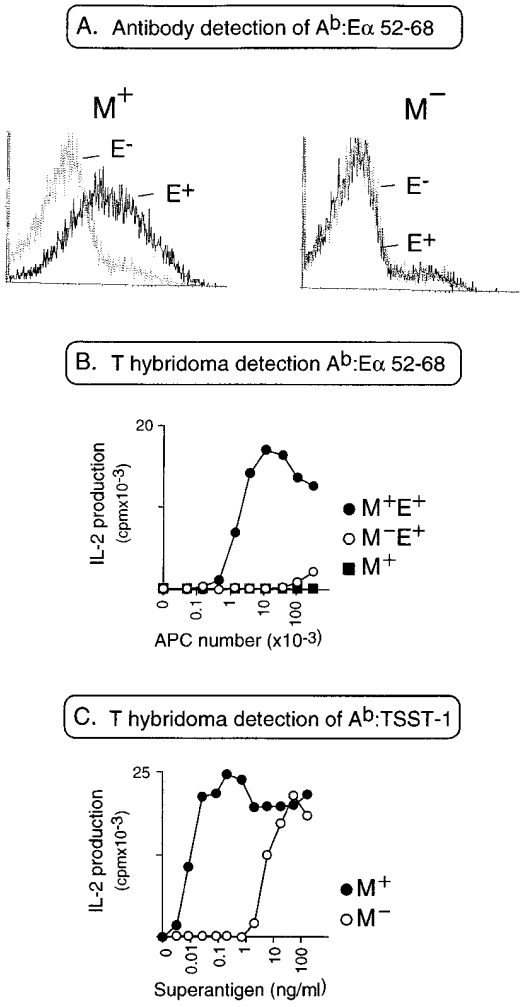

Figure 1. No Diversity of Natural Peptides Loaded onto A^b Molecules in M^- Cells

(A) The MAb YAe detects A^b molecules carrying the 52–68 peptide from Eα (Murphy et al., 1989). It was used to stain splenic B cells from M^+ or M^- mice, carrying or not an Eα-expressing transgene, as indicated. Light lines, control staining of E^- splenocytes; bold lines: staining of E^+ transgenics.

(B) The same peptide was detected by stimulation of the BEα20.6 T cell hybridoma (Ignatowicz et al., 1995), challenged with graded numbers of splenocytes from mice of the indicated genotypes.

(C) Presentation of the TSST-1 sAg, whose binding to MHC class II molecules is inhibited by the CLIP peptide. Splenocytes from M^+ or M^- mice were used as APCs in the presence of titrated amounts of TSST-1 and of the TSST-1-reactive Kox 15.8.1 T hybridoma.

entirely consistent results with the BEα20.6 T cell hybridoma: a clear response to antigen-presenting cells (APCs) from M^+E^+ mice but only very weak stimulation by M^-E^+ APCs. To quantitate the reduction in A^b:Eα(52–58) complexes, we performed parallel titrations with APCs not expressing Eα but supplemented with varying doses of exogenously added Eα(52–68) peptide (not shown). M^+E^+ cells behaved like Eα-negative cells supplemented with 10 μg/ml of peptide, while the M^-E^+ curve corresponded to approximately 20 ng/ml. Thus, the A^b molecules from mutant mice carried about 500-fold less of the endogenous Eα(52–68) peptide.

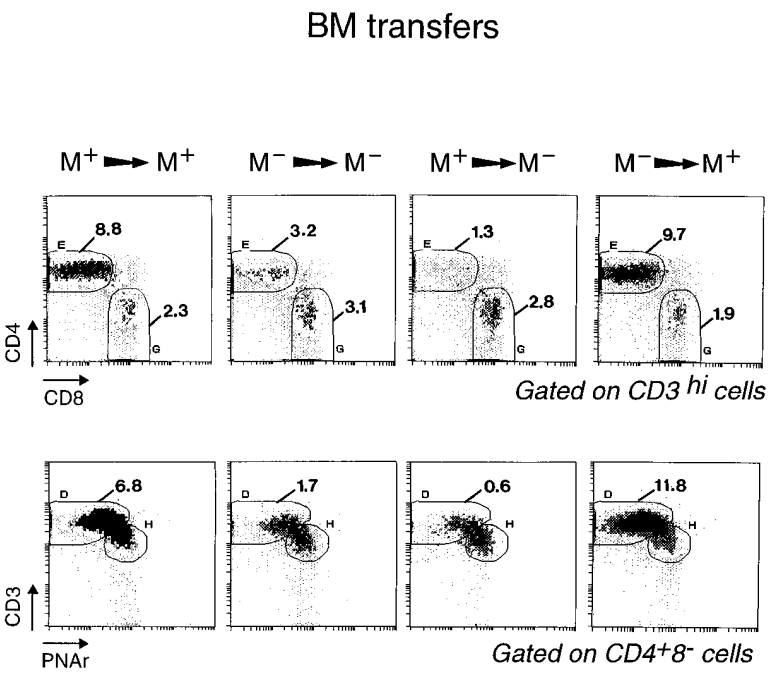

Figure 2. Deletion of Thymocytes Selected on M− Stroma, upon Encounter with M+ Cells of Hematopoietic Origin

Radiation chimeras were constructed by transferring bone marrow from M+ or M− littermates into lethally irradiated recipients, also of M+ or M− phenotypes, as indicated. Six weeks after reconstitution, thymocyte suspensions were analyzed by four-color cytofluorimetry, staining for CD3, CD4, CD8, and the PNAr. (Top) CD4/CD8 profiles gated on CD3hi cells. (Bottom) CD3/PNAr profiles of CD4+CD8− cells. The proportion (percentage) of the indicated populations within all thymocytes is shown for this representative experiment (total thymocyte numbers did not differ significantly among the different combinations).

Second, we studied the effect of the H-2Ma0/0 mutation on presentation of the superantigen (sAg) toxic shock syndrome toxin-1 (TSST-1). Crystallographic (Kim et al., 1994) and mutagenesis (Thibodeau et al., 1994) data provided strong evidence that TSST-1 binding to MHC class II molecules (and its subsequent presentation to T cells) was heavily influenced by the particular peptide residing in the groove of the class II molecule. Most relevant here, lymphoblastoid cells lacking HLA-DM, and thereby displaying class II molecules (DR or Ab) highly occupied with CLIP, could not effectively bind TSST-1 and present it to T cell hybridomas; however, when CLIP was replaced with other peptides, TSST-1 could provoke strong stimulation (von Bonin et al., 1995; Wen et al., 1996). Thus, we compared the ability of APCs from mutant and wild-type mice to present TSST-1 to the T cell hybridoma Kox 15.8.1. As indicated in Figure 1C, this hydridoma reacted very poorly to TSST-1 presentation by APCs from M− mice, reduced by about 500-fold compared with those from M+ littermates.

These two approaches assayed different parameters: in the first case, exclusion of an endogenous self-peptide that is normally an important component of the peptide constituency of class II molecules; in the second, steric hindrance by CLIP of the binding of a sAg. Yet the two suggested strikingly similar conclusions: as many as 99.8% of the Ab molecules expressed on APCs from H-2M−deficient mice are loaded with CLIP.

Further Evidence That Class II Molecules from Mutant Mice Display Highly Restricted Peptide Diversity: CD4+ T Cell Compartments in Bone Marrow Chimeras

A completely independent strategy for evaluating peptide heterogeneity was based on the previous observation of a striking one-way syngeneic MLR between cells derived from H-2M–negative and H-2M-positive littermates: lymph node CD4+ T cells from M− mice responded vigorously to APCs from M+ animals, but the converse was not true (Fung-Leung et al., 1996; Miyazaki et al., 1996). It was proposed that the CD4+ population from mutant mice had not been purged of reactivities directed at Ab molecules loaded with a diversity of self-peptides because these cells had encountered only Ab:CLIP complexes in the thymus, in particular on stromal cells of haematopoetic origin in the medulla, thought to be primarily responsible for clonal deletion. On the other hand, the CD4+ compartment from wild-type mice had lost all reactivity to Ab:CLIP complexes because the cells had been exposed to them, among a multitude of other class II:peptide complexes, on thymic stromal cells, resulting in clonal deletion or another form of tolerance induction.

To substantiate this notion, we produced a set of bone marrow chimeras: M+ or M− mice were irradiated and injected with bone marrow cells from M+ or M− animals; 5–6 weeks later, lymphoid organs were removed from the reconstituted animals and the CD4+ T cell compartments analyzed. The top row of Figure 2 shows CD4/CD8 profiles of thymocytes from the four types of bone marrow chimeras; the bottom row presents CD3/peanut agglutanin receptor (PNAr) profiles of the CD4+ population, which permit the most accurate quantitation of completely mature cells (CD3+PNAr^{lo}, delineated in the D gate). The M+→M+ and M−→M− control chimeras gave the expected results, the latter showing a reduced contribution of mature (CD3+PNAr^{lo}) CD4+CD8− cells of about 4-fold. When thymocytes were positively selected on H-2M–deficient epithelial cells and then confronted with H-2M–expressing hematopoetic cells (M+→M−), only about one third as many mature CD4+CD8− cells developed as in animals lacking H-2M in all thymic stroma. In contrast, when thymocytes were positively

selected on H-2M–positive epithelial cells and then encountered H-2M–negative hematopoetic cells, the contribution of mature $CD4^+CD8^-$ cells was enriched 1.7-fold.

These data are fully consistent with the proposed interpretation of the one-way syngeneic MLR. A large fraction (two thirds) of thymocytes positively selected on A^b:CLIP complexes is reactive to A^b molecules loaded with diverse self-peptides; these cells escape the thymus in M^- mutants or $M^-\rightarrow M^-$ chimeras but are clonally deleted when confronted with M^+ hematopoetic cells. On the other hand, clonal deletion of cells positively selected on heterogeneously loaded A^b molecules is restricted to those reactive to A^b:CLIP complexes in $M^-\rightarrow M^+$ chimeras, leading to an overproduction of mature $CD4^+CD8^-$ cells. This pair of findings underlines the poor overlap between the peptide complements of the class II molecules in M^+ and M^- animals, arguing that the usual self-peptides are excluded from the latter.

The CD4+ T Cell Repertoire in H-2Ma–Deficient Mice: TCR Sequences

In our original description of H-2M–negative mice, we reported that these animals have a polyclonal $CD4^+$ T cell repertoire with minor differences in use of Vβ or Vα segments (Miyazaki et al., 1996). These data have since been confirmed with more animals (data not shown). It is worth emphasizing that, contrary to what has been suggested (Lucas and Germain, 1996), M^- and M^+ animals showed no difference in use of the Vβ14 segment, at an average of 9.7% versus 10.2% of $CD4^+$ cells in the six animals examined.

It remained possible, however, that, the $CD4^+$ cells in mutant mice used a restricted set of TCRs with features in the complementary determining region-3 (CDR-3) reflecting positive selection on CLIP. Therefore, we sequenced a number of Vβ6, Vβ8, and Vα1 segments from $CD4^+$ lymph node cells from M^+ and M^- mice (Figure 3). All of the sequences in both wild-type and mutant mice were unique. The TCRs from mutant animals showed normal diversity, with no obvious peculiarities in CDR-3 length distribution, overall amino acid composition, or amino acid choice at particular positions.

These results substantiate our previous contention that the $CD4^+$ T cells in H-$2Ma^{0/0}$ mice are polyclonal and show no obvious structural "imprint" of positive selection on CLIP. They are reminiscent of findings on $CD4^+$ cells selected in mice expressing only A^b:Eα(52–68) class II complexes—again polyclonal with no distinguishing features in the TCR CDR-3 regions (Ignatowicz et al., 1996).

The CD4+ T Cell Repertoire in Mutant Mice: Response Capabilities

To analyze the breadth of the $CD4^+$ T cell repertoire in H-$2Ma^{0/0}$ mice, we evaluated their ability to respond to different antigenic challenges. As an initial test, we injected various A^b-restricted peptides into wild-type and mutant littermates, removed the lymph nodes 10 days later, and measured T cell proliferation to added peptide. Dose–response curves from a typical experiment are shown in Figure 4A, and data from multiple assays are

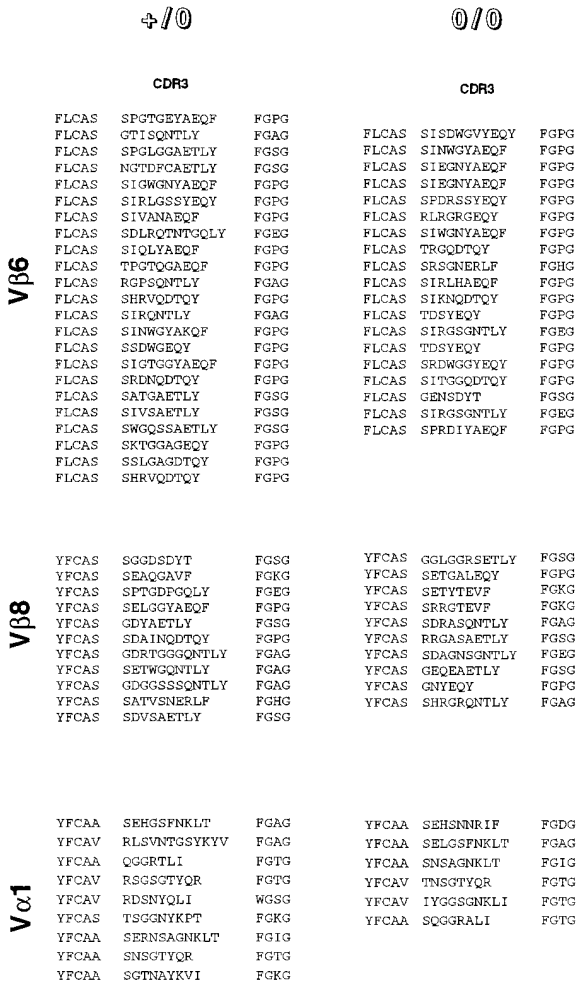

Figure 3. Junctional Diversity of TCRs Selected on a Unique Peptide

Junctional (CDR3) sequences were determined after PCR amplification of Vβ6, Vβ8.3, and Vα1-containing mRNA from sorted $CD4^+$ lymph node cells. The deduced amino acid sequences are shown, aligned along the boundaries of the CDR3 motif.

summarized in Figure 4B. The M^- mice responded to all of the four peptides injected, at least as well as, and often better than, M^+ animals.

To assay additional facets of T cell responsiveness, we injected whole keyhole limpet hemacyanin (KLH) into M^+ and M^- littermates, quantitated the primary immunoglobulin G (IgG) antibody response after 10 days, boosted, and measured the secondary IgG response (Figure 5). No primary response was observed in the mutant animals; there was a clear but reduced secondary response. This was a very stringent in vivo assay of reactivity, requiring processing of KLH to the relevant A^b-restricted peptide(s), priming of T cells, and T–B cell collaboration.

Finally, we compared the responses of mutant and wild-type mice to virus infection. M^- animals were perfectly capable of surviving challenge with lymphocytic choriomeningitis virus (LCMV) (data not shown). M^+ and M^- littermates were infected intravenously with LCMV, and 12 days later the proliferative response of $CD4^+$ T

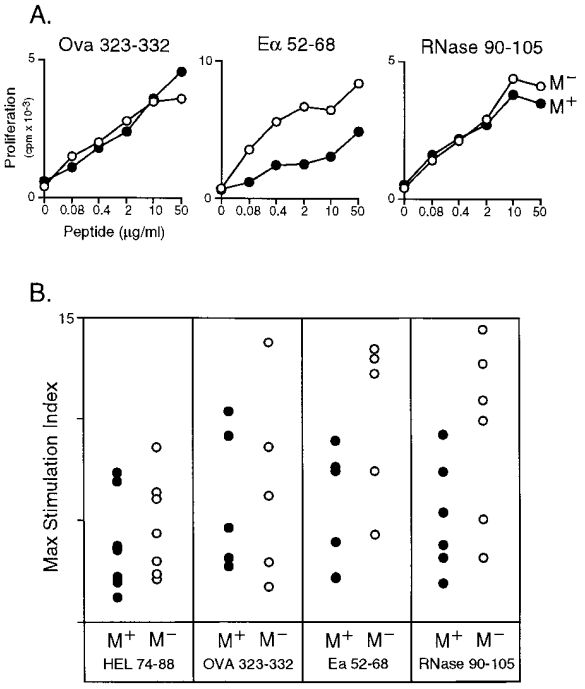

Figure 4. Capacity to Mount Proliferative Responses to Immunization with Foreign Peptides in M− Mice

(A) Littermates of the indicated phenotypes were immunized subcutaneously with peptides in CFA. Ten days later, lymphocytes from the draining lymph nodes were challenged in vitro with graded doses of the same peptide. Proliferation was measured by incorporation of [3H]thymidine after 3 days in culture.

(B) Data were compiled from several such experiments. Each point represents the maximum stimulation index for an individual mouse.

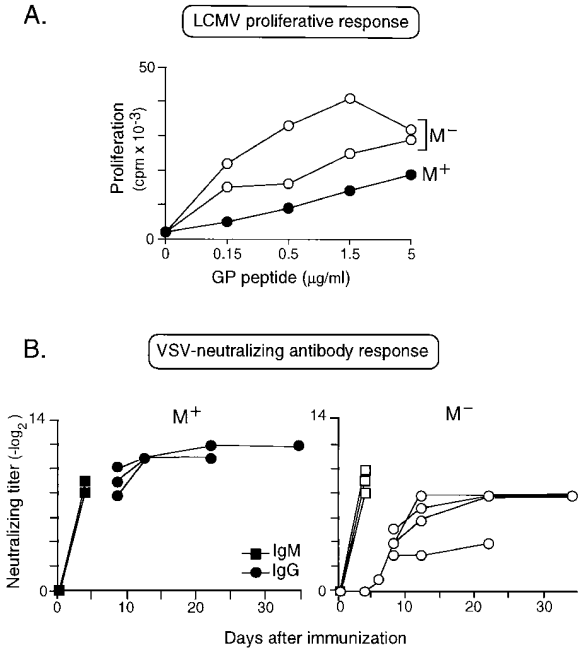

Figure 6. Capacity to Mount MHC Class II–Restricted Antiviral Responses in M− Mice

(A) Littermates of the indicated phenotypes were infected with LCMV, and the recall proliferation of purified CD4+ splenocytes was assayed in vitro after 12 days, in response to graded doses of the immunodominant gp 61–80 presented by M− APCs.

(B) Littermates of the indicated phenotypes were infected with VSV, and neutralizing antibody titers determined at different times. Each curve represents an individual mouse. All IgG titers were obtained with reduced serum, while IgM titers are from nonreduced sera (see Experimental Procedures).

cells to the hemaglutanin glycoprotein (gp) peptide 61–80, a dominant epitope in H-2b haplotype mice, was measured. As illustrated in Figure 6A, CD4+ cells from M− animals clearly responded, actually better than those from M+ animals.

The mutant mice also survived challenge with vesicular stomatitis virus (VSV) (data not shown). A convenient measure of VSV-reactive CD4+ T cells is quantitation of the IgM-to-IgG isotype switch normally exhibited by

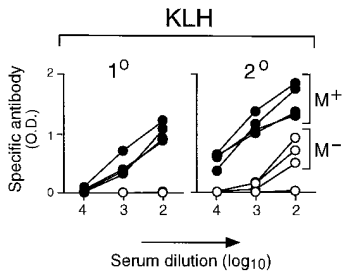

Figure 5. Help-Dependent Antibody Responses in M− Mice

Primary (1°) or secondary (2°) KLH-specific IgG responses were evaluated by enzyme-linked immunosorbent assay after subcutaneous immunization with KLH. The values shown for this representative experiment are the optical density readings at different serum dilutions, for littermates of the indicated phenotypes.

VSV-neutralizing antibodies 6–8 days after virus infection, the former being independent of CD4+ T cell help and the latter entirely help-dependent. As indicated in Figure 6B, the VSV-neutralizing antibodies that appeared in mutant mice did show the usual isotype switch, although the titers of IgG antibodies were reduced compared with wild-type controls.

Given the previously documented incompetence of APCs from H-2M–deficient mice to present exogenously added protein antigens to T cell hybridomas in vitro and the poor ability of added peptides to displace CLIP from A^b molecules and be presented in an in vitro assay (Martin et al., 1996; Miyazaki et al., 1996), the capacity of mutant animals to mount in vivo responses is astonishing. Also surprising are the different results obtained in helper assays ($M^- << M^+$) versus direct assays of T cell proliferation ($M^- \geqslant M^+$). A possible explanation might be that different APCs are responsible for presentation in the various contexts. For example, dendritic cells have special mechanisms of antigen internalization and show distinct features of MHC class II molecule trafficking (reviewed by Lanzavecchia, 1996) and appear to be the favored APCs after injection of antigen in adjuvant (Guery et al., 1996) and after LCMV infection (Oxenius et al., 1996). In contrast, B cells and macrophages might be the primary APCs in helper and in vitro assays (and cortical epithelial cells during positive selection).

In addition, the mode of in vivo delivery might enable the maintenance of high local concentrations of antigen. Upon viral infection, for example, high local antigen concentrations are reached upon lysis of infected cells, and this has already been demonstrated to overcome certain MHC class II antigen presentation deficiencies (such as those in Ii-deficient mice) (Battegay et al., 1996; Oxenius et al., 1997).

No matter what the explanation is eventually found to be, one conclusion is clear: the repertoire of $CD4^+$ T cells in H-$2Ma^{0/0}$ mice is broad, capable of responding effectively to all of the seven antigens assayed.

Participation of Particular Specificities in the Repertoire Selected in H-2M–Deficient Mice

The sequencing and response data indicated that the repertoire of $CD4^+$ T cells in mutant mice was very broad. It was still possible, however, that not all TCR specificities were included. To assay the positive selection of T cells expressing particular TCR specificities, we examined the behavior of thymocytes expressing three different transgene-encoded receptors in the presence of the H-2Ma null mutation. The TCR from the AND line recognizes the moth cytochrome c (MCC) peptide 88–103 in the context of E^k, but can be positively selected either on E^k or A^b molecules (Kaye et al., 1989, 1992); the SMARTA TCR transgenic line was derived from an A^b-restricted clone reactive to the dominant LCMV gp epitope 61–80; and the TCR from the DEP line sees the human C-reactive protein epitope 87–102 in an A^b-restricted fashion (Klein et al., 1995; Döffinger et al., 1997).

Figure 7A shows three-color stains of thymocytes from M^+ and M^- littermates carrying the AND TCR transgenes, produced by crossing the H-$2Ma^{0/0}$ mutant and AND TCR transgenic lines. Far fewer $CD4^+CD8^-$ cells were found in mice lacking H-2M, and most of those that did appear had reduced levels of the transgene-encoded $V\alpha11$ chain. The reduction was due not to endogenous sAg-mediated negative selection (see Experimental Procedures), but most likely to a failure of positive selection. Analogous results were obtained in the transfer experiments presented in Figure 7B, in which bone marrow precursors from AND, SMARTA, or DEP TCR transgenic mice were introduced into irradiated M^+ or M^- mice. In all three cases, the $CD4^+CD8^-$ compartment developed efficiently in the M^+ recipients but was almost absent in the M^- recipients (while total thymocyte numbers were comparable in both types of mouse).

These results indicate that T cells expressing particular TCR specificities may be essentially absent from the $CD4^+$ population that emerges in H-$2Ma^{0/0}$ mice. That such exclusion might be frequent is suggested by the finding that all of the three receptors tested were poorly selected in the mutants.

Conclusion

T cells differentiating in the thymus of an H-2M–deficient mouse encounter a monotonous array of MHC class II:peptide ligands on stromal cells, essentially all of them A^b:CLIP complexes. Yet, positive selection proceeds effectively, producing 20%–50% the normal number of $CD4^+$ T cells, which express heterogenous T cell receptors and are capable of responding to all antigens tested. So far, we have been unable to detect a clear "imprint" of frequent selection on CLIP on the population of $CD4^+$ cells that emerges. They did not show an extraordinary use of $V\beta$ segments or CDR-3 sequences. They also did not exhibit a special proclivity to recognize CLIP, since none of 77 hybridomas derived after polyclonal stimulation of $CD4^+$ lymph node cells were able to respond to CLIP or analogs bearing single amino acid substitutions at potential TCR contact sites (data not shown). In short, the $CD4^+$ repertoire of mutant mice seems at first glance surprisingly normal—large, diverse, and broad. However, it is not complete. There are never more than half of the normal number of $CD4^+$ cells selected, and cells displaying particular TCRs can be essentially excluded. It is not clear whether lack of selection of the three TCRs so far assayed merely reflects chance, or whether it indicates that the repertoires selected in the wild-type and mutant mice are largely nonoverlapping.

This picture of "promiscuity within limits" is different from the one derived from the original FTOC experiments, which argued that positive selection is fundamentally a very specific process (Ashton-Rickardt et al., 1994; Hogquist et al., 1994; Sebzda et al., 1994). However, it is similar to the view that emerged from a study using adenovirus-mediated delivery of neopeptides to the thymus (Nakano et al., 1997). Here, too, positive selection seemed lax, with a single peptide able to select a range of responses and a single response selectable by multiple, highly divergent peptides. Once again, however, the laxity appeared to have limits because the precise repertoire of T cells that emerged was dictated by the particular peptide driving selection. A certain promiscuity to the selection process is also consistent with findings on transgenic mice expressing only A^b:$E\alpha$(52–68) class II complexes (Ignatowicz et al., 1996; Fukui et al., 1997) and with results from one of the FTOC experiments (Pawlowski et al., 1996).

One way to reconcile the conflicting data might be to consider that positive selection of a given cell occurs if it has received a set quantity of stimulus. The required threshold could be reached by a few high-affinity (mainly TCR:peptide) or many low-affinity (mainly TCR:MHC) contacts or by some combination of the two. Different experimental systems might favor one or the other type of interaction; for instance, the low levels of MHC molecules largely occupied by a single peptide in FTOCs could well highlight specific TCR:peptide contacts. In vivo, the population of $CD4^+$ cells that arises would be a complex mixture displaying both fastidious and flexible TCRs, the difference being whether the cells were more tuned to "MHC-ness" or "peptidity" in the thymus.

An ancillary point from our data concerns the influence of negative selection on the emerging repertoire. We found that two thirds of the $CD4^+$ thymocytes selected on A^b:CLIP complexes were deleted when confronted with hematopoetic cells expressing A^b molecules occupied with diverse self-peptides. This is precisely the fraction of self-reactive cells estimated by Ignatowicz et al. (1996) from the proportion of hybridomas from

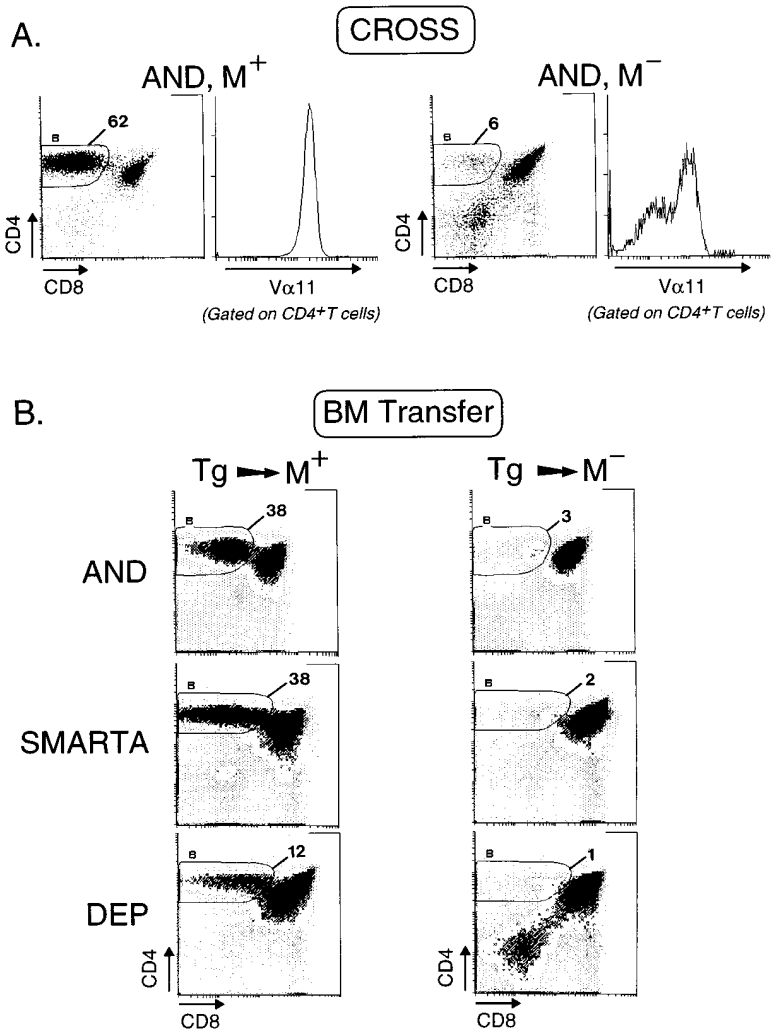

Figure 7. Positive Selection of Cells Carrying Individual TCRs in M− Mice

(A) The Ma knockout mutation was crossed onto the AND TCR transgenic line. After intercrossing, thymocytes from transgene-positive mice, M^+ or M^-, were analyzed by cytofluorimetry, staining for CD4, CD8, and the transgene specific Vα11 chain. (Left) CD4/CD8 profiles of total thymocytes; the proportion (percentage) of $CD4^+CD8^-$ cells is indicated. (Right) Vα11 expression of $CD4^+$ cells, gated as shown.

(B) M^+ or M^- littermates were reconstituted, after lethal irradiation, with bone marrow from transgenic donors carrying one of the three transgenes shown. Six weeks after reconstitution, thymocyte suspensions were analyzed by cytofluorimetry after staining for CD4, CD8, and TCR. The CD4/CD8 profiles for both types of recipients are shown; the proportion (percentage) of $CD4^+CD8^-$ cells is indicated (essentially all of these expressed high levels of the transgenic Vα chain, not shown).

transgenic mice expressing A^b:Eα(52–68) complexes that react to A^b molecules from wild-type animals. It also is similar to the fraction of self-reactive cells estimated from a 1.7-fold augmentation of the $CD4^+$ T cell compartment when MHC-negative bone marrow precursors were used to reconstitute irradiated MHC-positive recipients (van Meerwijk et al., 1997). In contrast, all of these estimates are much larger than the 5% value obtained using animals that were thought to express class II molecules in cortical epithelial cells but not medullary stroma (Laufer et al., 1996). They also exceed the estimate of self-MHC reactivity in the preselection repertoire (~5%) (Zerrahn et al., 1997), but this difference may just reflect an enrichment of potential self-reactivity after positive selection. Thus, the bulk of evidence supports the idea that a large fraction of the thymocytes capable of being positively selected succumb to negative selection. To complete the picture, we need to quantitate the proportion of thymocytes that can be positively selected.

Experimental Procedures

Mice

H-2M–deficient mice (Miyazaki et al., 1996) were maintained on a mixed 129×B6 genetic background, usually bred as balanced homozygote × heterozygote crosses. In all experiments, M^+ controls were littermates, either heterozygous for the mutation or fully wild-type. To introduce a functional E molecule, we crossed them with the Eα16 transgenic line (Lemeur et al., 1985) carried on a B6 background. To avoid interference by endogenous sAgs from the 129 genomes, capable of deleting Vβ3-positive T cells, we set up crosses of AND to H-$2M^{0/0}$ mice with breeders selected for high expression of Vβ3. The AND transgenic line (a gift from S. Hedrick) expresses an MCC(88–103)-reactive TCR that is E-restricted but that can be positively selected on either E or A^b class II molecules (Kaye et al., 1989, 1992); it was maintained on the B6 background. To prevent interference from Vβ3-deleting endogenous sAgs from the 129 genome, crosses between the AND and H-2M–deficient lines were set up with breeders selected for high expression of Vβ3. The SMARTA line expresses an A^b-restricted TCR specific for the LCMV gp peptide 61–80, and its TCR is composed of the Vα2 and Vβ8.3 variable regions. The DEP line expresses an A^b-restricted TCR from a T cell clone specific for human C-reactive protein epitope 87–102 (Klein et al., 1995; Döffinger et al., 1997). All mice were maintained in a conventional animal facility, under filter tops after irradiation and reconstitution, according to EEC guidelines.

Antibodies and Flow Cytometry

The MAb YAe (a gift from C. Janeway and D. Murphy) detects A^b molecules carrying the 52–68 peptide from Eα (Murphy et al., 1989). Its binding was revealed by secondary staining with fluorescein isothiocyanate–conjugated anti-mouse IgG(Fc) on B cells counterstained with Texas Red–congugated anti-mouse IgM (Jackson Immunoresearch). The reagents and protocols for flow cytometry of T cells have been detailed previously (Chan et al., 1993). The AND

TCR was detected by a combination of anti-Vβ3 and anti-Vα11 MAbs, the SMARTA TCR by anti-Vβ8.3 and anti-Vα2 reagents, and the DEP TCR by anti-Vα11 and anti-Vβ5 MAbs.

Antigen Presentation Assays

The T cell hybridoma BEα20.6 (a gift from P. Marrack) reacts to Eα peptide 52–68 presented by A^b (Ignatowicz et al., 1995). It was challenged by graded numbers of splenocytes in a 96-well plate, in 225 μl of Dulbecco's modified Eagle's medium (DMEM) supplemented with 1 mM glutamine, 50 μM β-mercaptoethanol (β-ME), and penicillin/streptomycin. Twenty-four hours later, interleukin-2 (IL-2) secretion in 50 μl of the supernatants was measured using the IL-2–dependent CTLL cell line, and read out as [^{3}H]thymidine incorporation by the indicator cells. For standardization curves for the prevalence of the Eα(52–68) peptide at the surface of splenic APCs, we used splenocytes from B6 mice, similarly titrated (from 50 to 3×10^5) and supplemented with synthetic Eα(52–68) peptide at different concentrations (from 30 to 0.001 μg/ml). The Kox 15.8.1 T hybridoma (a gift from R. Sekaly) expresses Vβ15 and reacts to the sAg TSST-1 presented by A^b (Thibodeau et al., 1994). Its response to graded doses of TSST-1 (Toxin Technology) was detected as IL-2 production as described above.

Radiation Chimeras

To construct bone marrow chimeras, recipient mice were lethally irradiated (9 Gy from a cobalt source). Twenty-four hours later, they were injected intravenously with 10^7 bone marrow cells and depleted of mature T and B lymphocytes by treatment with anti-CD4 (RL-172) and anti-CD8 (31M) plus complement (LoTox, Cedarlane). Reconstituted mice were kept on acidified water. The reconstitution of their thymi and lymphoid organs was evaluated 5–6 weeks after transfer.

Immune Responses

For proliferation assays, mice were primed in the footpad with 50 μg of peptide in 30 μl of complete Freund's adjuvant (CFA). Ten days later, draining popliteal lymph node cells were suspended in 250 μl of complete medium in 96-well plates, in the presence of graded concentrations of peptide. Proliferation was detected by incorporation of [^{3}H]thymidine during the last 12 hr of a 72 hr culture period. For antibody responses, mice were immunized subcutaneously with 50 μg of KLH in CFA and bled 10 days later. They were boosted intraperitoneally with 10 μg of antigen (in phosphate-buffered saline) on day 21 and bled again on day 28 to measure secondary responses. Anti-KLH antibodies were detected by enzyme-linked immunosorbent assay as described (Lemeur et al., 1985).

Antiviral Responses: T Cell Proliferation

Mutant and control mice were infected intravenously with 200 pfu of LCMV. After 12 days, $CD4^+$ T cells were purified from spleen cell suspensions by MACS sorting according to the protocol of the supplier (Miltenyi Biotec, Germany). $CD4^+$ T cells (1×10^5) were incubated for 3 days in 96-well plates with varying amounts of P13 peptide (LCMV gp 61–80) with 7×10^5 irradiated (2000 cGy) M^- splenocytes as APCs. Proliferation was assessed by incorporation of [^{3}H]thymidine (25 μCi/well).

Antiviral Responses: Serum Neutralization Test

Neutralizing titers of sera were determined as described (Roost et al., 1990). Sera were prediluted 40-fold in supplemented minimal essential medium and heat-inactivated for 30 min at 56°C. Serial 2-fold dilutions were mixed with equal volumes of virus diluted to contain 500 pfu/ml. The mixture was incubated for 90 min at 37°C in 5% $C0_2$. One hundred microliters of the serum–virus mixture was transferred onto Vero cell monolayers in 96-well plates and incubated for 1 hr at 37°C. The monolayers were then overlaid with 100 μl of DMEM containing 1% methyl cellulose. After incubation for 24 hr at 37°C, the overlay was removed and the monolayer was fixed and stained with 0.5% cristal violet. The highest dilution of the serum that reduced the number of plaques by 50% was taken as the neutralizing titer. To determine IgG titers, undiluted serum was first pretreated with an equal volume of 0.1 M β-ME in saline (Scott and Gershon, 1970).

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