In the absence of HLA-DM or H-2M (20–22). Although the abundance of CLIP-class II complexes does not alter the level of surface class II expression, mice lacking H-2M (M<sup>−−</sup>) do have moderately reduced numbers of CD<sup>4+</sup> T cells in the thymus and periphery, reflecting the altered peptide repertoire displayed by class II molecules.

To investigate the in vivo functions of H-2M other than facilitating the removal of CLIP, we analyzed mutant mice lacking both II and H-2M (II<sup>−−</sup>M<sup>−−</sup>). A recent publication...
tion also describing these mice reported that maturation, transport, and surface expression of class II molecules was comparable in \( \text{Ii}^{-/-} \) and \( \text{Ii}^{-/-}\text{-M}^{-/-} \) splenocytes (23). Surprisingly enough in view of these data, CD4 \(^+\) T cell development in \( \text{Ii}^{-/-}\text{-M}^{-/-} \) mice was impaired profoundly compared to mice lacking either \text{Ii} or H-2M alone, a finding not explained by the published analyses of the mice (23). We hypothesized that the limited repertoire of class II-bound self peptides displayed by \( \text{Ii}^{-/-} \) cells, accounting for CD4 \(^+\) T cell development in \( \text{Ii}^{-/-} \) mice, is dramatically reduced in \( \text{Ii}^{-/-}\text{-M}^{-/-} \) mice because an \text{Ii}-independent H-2M function is required for the presentation of the majority of endogenous peptides. To assess the effect of H-2M on the loading of high-affinity endogenous self peptides, we generated a novel panel of T cells specific for self peptide-\( \text{I}-\text{A}^\beta \) complexes. We found that the presentation of the majority of these self epitopes was significantly reduced in the doubly mutant \( \text{Ii}^{-/-}\text{-M}^{-/-} \) APCs relative to \( \text{Ii}^{-/-} \) APCs. These studies demonstrate that even in the absence of \text{Ii}, H-2M can greatly enhance self peptide-class II complex formation, suggesting that it functions as a general chaperone in the peptide loading process. In addition, our data suggest that in the absence of \text{Ii}, surface class II molecules that internalize into or traffic through the endocytic pathway have access to the variety of antigenic peptides that are present in H-2M-containing early and late endocytic compartments.

Materials and Methods

Animals. Each of the mutant mice used in this study are of a mixed B6x129 (H-2\(^b\)) background, which expresses the MHC class II molecule \( \text{I}-\text{A}^\beta \). Thus, the phenotypes of \( \text{Ii}^{-/-} \) (4), \( \text{M}^{-/-} \) (19), or \( \text{Ii}^{-/-}\text{-M}^{-/-} \) mice were compared to that of wild-type B6x129 mice. \( \text{Ii}^{-/-} \) and \( \text{M}^{-/-} \) mice were bred to yield mice lacking both \text{Ii} and H-2M. All wild-type and mutant mouse strains were maintained under specific pathogen-free conditions at Vanderbilt University or University of Washington. \( \text{Ii}^{-/-} \) mice were provided by Dr. Elizabeth Bikoff (Harvard University, Cambridge, MA). MHC-II\(^{-/-} \) mice were purchased from Taconic Farms Inc. (Germantown, NY). Mice were used at 6–8 wk of age.

FACS\(^\text{a}\) Analyses. Analyses of T cell surface markers and MHC class II were performed as previously described (20). Experiments to assess cell surface binding of exogenous peptides using synthetic E\text{u}52-68 peptide and mAb YA\text{e} were done as previously described (24).

T Cell Assays. T cell hybrids specific for \( \text{I}-\text{A}^\beta \) bound to p2m 48–58 (4.1) and IgM 377–392 (77.1) peptides have been previously described (20). T cell hybrids specific for \( \text{I}-\text{A}^\beta \) bound to naturally processed epitopes of actin (15.10), Clp36 (3.5), amino aspartate aminotransferase (AAAT) (15.15), and low density lipoprotein (LDL) receptor (41.12) were generated after peptide immunization of H-2M-deficient mice (Dongre, A., S. Kovats, P. deRoos, and A.Y. Rudensky, manuscript submitted). Individual T cell hybrids subcloned by limiting dilution were tested for MHC restriction and peptide specificity using \( \text{I}^{-/-}\text{-APCs} \) T cell hybrids (2–10 \( \times \) 10\(^4\) well) were incubated with variable numbers of ex vivo splenocytes (depleted of red blood cells) of the indicated genotype (10\(^4–\)10\(^5\) well) for 20 h. Peptide specificity of the T cell hybrids was shown by addition of either cognate peptides or irrelevant peptides (10 \( \mu \)g/ml) to cultures of \( \text{I}^{-/-} \) splenocytes (10\(^5\)) and T cells

Synthetic Peptides and Peptide Binding Studies. Peptides were synthesized on an automated peptide synthesizer (Synergy 432; Applied Biosystems, Foster City, CA) using F-moc chemistry. The purity of peptides used was >90%. For binding assays, affinity purified I-A\(\beta\) (500 pm) was incubated with titrated amounts of inhibitor peptides (300 nm–700 \( \mu \)M) and biotinylated E\text{u}52-68 peptide (48 \( \mu \)M) for 16 h at 37°C in 0.1 M citrate-phosphate buffer, pH 5.0, containing 1% octylglucoside. After neutralizing pH, reaction mixtures were transferred into mAb Y3P-coated plates and incubated for 4 h at 4°C. After washing, plates were incubated for 2 h with Eu\(^3+\)-streptavidin (O.Y. W allac, Turku, Finland), then washed and read on a fluorometer (1234 DELFIA; O.Y. W allac) after adding fluorescence enhancement solution (O.Y. W allac).

Results

The absence of \text{Ii} chain and H-2M results in a Synergistic Reduction in CD4\(^+\) T Cell Development. Analyses of mice lacking \text{Ii} have revealed a significant reduction in CD4\(^+\) thymocytes, 20–30% of wild-type numbers, suggesting that efficient CD4\(^+\) thymic selection requires high levels of surface MHC class II molecules and/or the diverse array of endogenous peptides that are displayed by thymic epithelial cells in the presence of \text{Ii}. In contrast, in H-2M-deficient mice, which display wild-type levels of class II molecules predominantly occupied by a single peptide, CLIP, the number of CD4\(^+\) thymocytes is significantly higher than in \( \text{Ii}^{-/-} \) mice, ~30–50% of the number in wild-type mice.

To assess CD4\(^+\) T cell development in \( \text{Ii}^{-/-}\text{-M}^{-/-} \) mice, thymocytes and splenic T cells were assessed by flow cytometry for expression of CD4, CD8, and \( \alpha/\beta \) TCR. The total number of thymocytes did not vary significantly in the mice of various genotypes. Three electronic gates were used to analyze the presence of CD4\(^-\)CD8\(^b\) TCR\(^hi\) (Fig. 1, box 1), CD4\(^+\)CD8\(^b\) TCR\(^hi\) (Fig. 1, box 2), or CD4\(^+\)CD8\(^b\) TCR\(^lo\) (Fig. 1, box 3) thymocyte populations isolated from mice of five distinct genotypes (B6x129, \( \text{Ii}^{-/-}\), \( \text{M}^{-/-}\), \( \text{Ii}^{-/-}\text{-M}^{-/-}\), and MHC-II\(^{-/-}\)), shown as two parameter dot plots of CD8 versus TCR-\(\alpha/\beta\) (Fig. 1). The CD4\(^+\)CD8\(^b\) TCR\(^lo\) cells (box 3) represent a minor population in a wild-type thymus (Fig. 1A), which is more prominent in an \( \text{Ii}^{-/-}\) thymus, concomitant with a marked decrease in CD4\(^+\) TCR\(^hi\) SP thymocytes (Fig. 1B, box 2). In contrast, the M\(^/-\) thymus shows a 50% reduction in CD4\(^+\) TCR\(^hi\) single positive thymocytes (box 2) but no change in the intermediate (box 3) population (Fig. 1D). Notably, the M\(^/-\) thymus shows a dramatic reduction (approximately sixfold relative to the I\(^/-\) thymus) in the number of CD4\(^+\) TCR\(^hi\) cells (Fig. 1C), similar to that observed in mice lacking class II molecules (MHC-II\(^{-/-}\); Fig. 1E). In both I\(^/-\) and MHC-II\(^{-/-}\) mice, virtually all of the CD4\(^+\) thymocytes display low levels of TCR and have incomplete downregulation of CD8 (box 3). Thus, the partial block in positive selection of CD4 SP thymocytes observed in I\(^/-\) thymus is exacerbated by the additional loss of H-2M function in doubly mutant I\(^/-\text{-M}^{-/-}\) thymus.

Analyses of peripheral T cells showed that the number of splenic CD4\(^+\) T cells in I\(^/-\text{-M}^{-/-}\) mice was reduced rel-
The absence of Ii chain and H-2M results in synergistic reduction of CD4⁺ T cell development. Thymocytes from (A) wild-type B6x129 and mutant (B) Ii⁻/⁻, (C) Ii⁻/⁻ M⁻/⁻, (D) M⁻/⁻, and (E) MHC-II⁻/⁻ mice were analyzed for expression of CD4, CD8, and TCR-αβ using flow cytometry. Shown are two parameter dot plots of CD8 versus TCR-α/β, in which three electronic gates are indicated, delineating the following populations: CD4⁻CD8⁺ TCR hi (box 1), CD4⁺CD8⁻ TCR hi (box 2), and CD4⁺CD8⁺ TCR lo (box 3). Also shown are the percentages of cells that fall within each box. Cells in boxes 2 and 3 are ≥97% CD4⁺. Splenocytes from wild-type and mutant mice were analyzed for expression of CD4, CD8, and TCR-αβ using flow cytometry. Shown are comparisons of TCR expression on (F) CD4⁺ T cells and (G) CD8⁺ T cells in the mice of the five genotypes, as indicated. Data are representative of analyses of four mice of each genotype.
These T cell hybrids were specific for the immunizing peptide sequences (24), or mice lacking the specific self-protein, e.g., IgM M−/− or β2m M−/− (20). These T cell hybrids were specific for the immunizing peptide sequence to 1-Aβ, as demonstrated by experiments in which exogenous cognate peptide was added to M−/− splenocytes (Fig. 4, bar graphs). Irrelevant peptides added to M−/− APCs did not stimulate the T cell hybrids (data not shown). The T cell hybrids also recognized endogenously formed peptide-I-Aβ complexes on wild-type splenocytes (Fig. 4, line graphs).

A comparison of the ability of wild-type and mutant APCs to form these endogenous peptide-I-Aβ complexes was obtained by incubation of variable numbers of ex vivo activated splenic APCs with a constant number of T cells in the absence of exogenously added antigen (Fig. 4, A–F).

The comparison of li−/− and li−/−M−/− APCs allows direct assessment of the role of H-2M, since the surface class II expression is equivalent on splenocytes from these two types of mice (Fig. 2). Each of the six epitopes was efficiently presented by li−/− splenocytes (Fig. 4). The responses of T cells specific for IgM, actin, AAT, and Cllsp36 epitopes were reduced by approximately fivefold, whereas the responses of T cells specific for β2m and LDLr epitopes were enhanced about twofold, relative to wild-type APCs. These data indicate that these endogenous peptides can be efficiently loaded in vivo onto class II molecules that traffic through cellular compartments via an li-independent route.

In contrast, formation of the six distinct peptide-class II complexes by doubly mutant li−/−M−/− splenocytes was much more variable, demonstrating differential requirements for H-2M function in the absence of CIIP. Presentation of the IgM 377–392 epitope was profoundly diminished in the absence of both li and H-2M, a striking result since the IgM 377–392–I-Aβ complex is expressed very well in li−/− APCs (Fig. 4 A). Presentation of the β2m48–58 epitope was also significantly diminished in li−/−M−/− APCs (reduced ~10-fold relative to li−/− APCs; Fig. 4 B). As previously described (20), these two epitopes were presented at undetectable or very low levels by M−/− splenocytes (Fig. 4, A and B). Presentation of peptides derived from the cytosolic antigens AAT and Cllsp36 by li−/−M−/− splenocytes was diminished 5–10-fold relative to li−/− APCs (Fig. 4 E and F), while the already low level of presentation of the actin epitope by li−/− deficient cells was not profoundly affected by the absence of H-2M (Fig. 4 D). Interestingly, formation of LDLr486–501–I-Aβ complexes was essentially equivalent in li−/− and li−/−M−/− cells (Fig. 4 C), and slightly enhanced relative to wild-type cells, suggesting that this peptide preferentially binds to class II molecules via a mechanism independent of both li and H-2M. As expected, since the T cells specific for LDLr, actin,
AAT, and Clp36 peptides were generated in H-2M-deficient mice, these peptides were not presented by M\(^{-/-}\) splenocytes (Fig. 4, C-F).

**Discussion**

In this report, we demonstrate that H-2M functions in the in vivo loading of antigenic peptides onto class II molecules in a capacity that is distinguishable from its previously described role in CLIP removal. Using a panel of T cells specific for high-affinity endogenous self-peptides bound to the class II molecule I-A\(^d\), we found that for the majority of epitopes normally expressed in the absence of Ii, additional loss of H-2M function significantly reduced the ability of APCs to assemble these self-peptide-class II complexes. Thus, even in the absence of Ii, class II molecules traffic through the endocytic vesicles in which H-2M facilitates the binding of antigenic peptides. As a consequence of this deficient loading of self-peptides, CD4\(^+\) thymocyte development is profoundly impaired in Ii\(^{-/-}\)M\(^{-/-}\) mice, resulting in a peripheral CD4\(^+\) T cell population with uniformly low levels of TCR. Thus, we hypothesize that the normal diversity of class II-bound endogenous peptides displayed on antigen presenting cells, made possible by the disparate chaperone functions of both Ii and H-2M, is required for development of a complete repertoire of CD4\(^+\) T cells.

Each of the endogenous epitopes we studied were presented well to T cells in the absence of Ii. With the exception of the response to the LDLr epitope, self-antigen-specific T cell responses to Ii\(^{-/-}\)M\(^{-/-}\) APCs were significantly reduced relative to Ii\(^{+/+}\) APCs, suggesting that even in the absence of Ii, some endogenous peptide-class II complexes are formed in H-2M containing lysosomal compartments. There are two ways in which Ii-free class II molecules may enter the endocytic pathway in the absence of Ii (27). In view of this, the self-epitopes analyzed here may be generated efficiently and...
bind to class II in early or late endocytic compartments accessed by class II molecules internalized from the cell surface or after diversion from the trans-Golgi network. Although some of the high affinity self-peptides studied here were presented in the absence of Ii and H-2M, the dramatic reduction of mature CD4+ T cells in the Ii−/−M−/− mice indicates that the function of H-2M is generally required in vivo to promote the binding of most self-peptides to class II molecules.

H-2M may significantly increase the class II-bound repertoire of endogenous peptides in Ii−/−APCs by virtue of its ability to stabilize empty class II molecules or its capacity to mediate replacement of a limited set of unstable polypeptides with a larger repertoire of relatively stable peptides. Thus, it is perhaps unexpected that the absence of H-2M did not decrease the steady-state level of I-Aβ expression in Ii−/−M−/−APCs. However, since the majority of class II molecules in Ii−/− cells are unstably bound to low-affinity polypeptides, the minor proportion of class II molecules stably bound to high-affinity peptides in the presence of H-2M may readily assemble with available polypeptides in the absence of H-2M, thus maintaining an apparently similar level of surface class II expression. Alternatively, the absence of this minor population in Ii−/−M−/−APCs would not significantly affect total surface class II expression as measured by flow cytometry.

Biochemical experiments have shown that empty class II α/β dimers are partially unfolded at pH 4.0–5.5 and tend to form aggregates (28). HLA-DM is able to protect these α/β dimers from denaturation through a stable interaction (10, 15). In vivo, such chaperone activity would maintain more class II molecules in a peptide-receptive state in endocytic vesicles until they contacted antigenic peptides, eventually increasing the number of stable peptide-class II complexes at the cell surface. For the high affinity self-peptides we studied, it is unlikely that the editing function of H-2M was required to select for their stable binding. These peptides are able to efficiently displace low stability or affinity peptides or polypeptides bound to surface class II molecules on Ii−/− cells at neutral pH without the aid of H-2M (Fig. 2 and data not shown). Rather, H-2M may be primarily enhancing their presentation by virtue of its ability to bind to and prolong the half-life of empty class II α/β dimers at acidic pH in vivo, as suggested by the in vitro studies.

The ability of a particular peptide to bind productively to class II molecules in the absence of H-2M may depend on its unique complement of amino acid side chains, or the location and stability of the antigenic fragment within the various endocytic vesicles. Thus, H-2M-independent peptide binding would occur if the on-rate of the binding reaction is very high, or if the half-life of an antigenic peptide is greater than the half-lives of empty class II molecules, a condition that might exist in the less proteolytic early endocytic compartments that contain little or no H-2M (29–31). In this regard, it is of interest to note that the LDL receptor protein contains a targeting signal for early endosomes, which may be the site where the H-2M- and Ii-independent epitope of LDLr is generated (32).

Observations with singly mutant Ii−/− or M−/− mice have indicated that efficient positive selection of CD4+ T cells requires high levels of surface class II molecules and/or a diverse array of class II-bound endogenous peptides (3, 4, 17–19). Since the majority of surface class II molecules in Ii−/−APCs are apparently associated with unstably bound peptides or larger polypeptides, the profound impairment in CD4+ T cell development in Ii−/− mice suggests that this majority of class II molecules does not mediate positive selection. Rather, CD4+ T cells in Ii−/− mice are likely to be selected on the small minority of stable endogenous peptide-class II complexes that do reach the cell surface, albeit with a contracted repertoire of high-affinity endogenous peptides. The absence of H-2M function in Ii-deficient cells is likely to further decrease the diversity of the class II-bound repertoire and/or the number of functional endogenous peptides, which may be replaced with unstably bound polypeptides. In both of these scenarios, steady state surface class II expression would be similar to that of Ii−/− cells. Although distinguishing between these two possibilities is key to understanding the requirements for positive selection in these mice, we currently lack the means to quantitate the proportion of class II molecules occupied with high affinity peptides in Ii−/− cells. Nevertheless, these reductions in the number or diversity of functional peptides apparently puts their expression below a threshold level required for CD4+ positive selection in Ii−/−M−/− mice. In view of our data, the impairment of CD4+ thymic selection in M−/− mice may be due to the dramatic reduction in diversity of endogenous peptides bound to wild-type levels of class II molecules, although this impairment may be less marked than in Ii−/− mice due to the high density of the single CLIP peptide–class II complex.

In summary, our findings are consistent with the idea that H-2M evolved as the primary chaperone assisting in the loading of antigenic peptides onto MHC class II molecules in the late endocytic compartment, presumably by promoting the stability of empty class II α/β dimers. The facilitated dissociation of CLIP, and of other peptides that bind to class II molecules with a high dissociation rate, may be more specific manifestations of the general chaperone function of H-2M.

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