Effect of Decreasing the Affinity of the Class II-Associated Invariant Chain Peptide on the MHC Class II Peptide Repertoire in the Presence or Absence of H-2M

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The class II-associated invariant chain peptide (CLIP) region of the invariant chain (Ii) directly influences MHC class II presentation by occupying the MHC class II peptide-binding groove, thereby preventing premature loading of peptides. Different MHC class II alleles exhibit distinct affinities for CLIP, and a low affinity interaction has been associated with decreased dependence upon H-2M and increased susceptibility to rheumatoid arthritis, suggesting that decreased CLIP affinity alters the MHC class II-bound peptide repertoire, thereby promoting autoimmunity. To examine the role of CLIP affinity in determining the MHC class II peptide repertoire, we generated transgenic mice expressing either wild-type human Ii or human Ii containing a CLIP region of low affinity for MHC class II. Our data indicate that although degradation intermediates of Ii containing a CLIP region with decreased affinity for MHC class II do not remain associated with I-Ab, this does not substantially alter the peptide repertoire bound by MHC class II or increase autoimmune susceptibility in the mice. This implies that the affinity of the CLIP:MHC class II interaction is not a strong contributory factor in determining the probability of developing autoimmunity. In contrast, in the absence of H-2M, MHC class II peptide repertoire diversity is enhanced by decreasing the affinity of CLIP for MHC class II, although MHC class II cell surface expression is reduced. Thus, we show clearly, in vivo, the critical chaperone function of II-2M, which preserves MHC class II molecules for high affinity peptide binding upon dissociation of Ii degradation intermediates. The Journal of Immunology, 2004, 172: 4142-4150.
peptide repertoire, one would need to compare the same allelic variant of MHC class II assembled in the presence of hIi containing a CLIP region of high and low affinity for MHC class II. In this report we describe transgenic mice expressing hIi molecules with CLIP regions of differing affinity for MHC class II. The MHC class II allele was fixed by backcrossing li transgenic mice onto a C57BL/6 (BL6) background, making it possible to examine the effect of CLIP affinity on the MHC class II peptide repertoire.

Previous reports have defined mutations in the CLIP region of human li (hli) that both increase and decrease the affinity of CLIP for HLA-DR1 (10). In this study we engineered the hli genomic locus to express these same li variants and made transgenic mice expressing either wild-type hli (hliWt) or mutant hli with its CLIP region (BAD-CLIP) engineered to have a low affinity for I-Ab (hliBAD-CLIP). To enable us to examine the role of CLIP:MHC class II affinity on generation of the MHC class II peptide repertoire, the transgenic mice were backcrossed onto an li−/− BL6 background. We show that although degradation intermediates of liBAD-CLIP do not remain associated with MHC class II, the repertoire of MHC class II-bound peptides remains as broad as that expressed in the presence of hliWt, as assessed by presentation of a CLIP region of high and low affinity for MHC class II. The comparable reactivity of mouse li to a panel of T cell hybridomas; the comparable reactivity of liBAD-CLIP, and control BL6 splenocytes; and the number of CD4+ T cells selected. However, in the absence of H-2M, the affinity of CLIP for MHC class II influences the diversity of the MHC class II peptide repertoire, as we were able to detect a diverse array of endogenous peptide:MHC class II complexes only in the presence of low affinity CLIP:MHC class II interactions. Interestingly, although the diversity of this repertoire was substantially greater than that generated in li−/− H-2M−/− hli mice, the stability of the complexes generated was dramatically reduced, as demonstrated by the decreased levels of MHC class II on li−/− H-2M−/− BAD-CLIP APCs and their increased availability for peptide loading. These results indicate that in wild-type animals the diversity of peptides bound by MHC class II is not dramatically altered by decreasing the affinity of CLIP for MHC class II. Furthermore, li−/− BAD-CLIP mice exhibit normal CD4+ T cell development and have no manifestations of autoimmunity. Together, these data argue against the hypothesis that the expression of li molecules containing a CLIP region of low affinity for MHC class II is a significant factor in susceptibility to autoimmunity. In addition, the data suggest that H-2M is critical for preserving the structural integrity of MHC class II molecules if the affinity of CLIP for MHC class II is not sufficient to maintain the interaction of MHC class II with li degradation intermediates. Therefore, we propose that H-2M acts as a chaperone protein to MHC class II molecules in the absence of the stabilizing effect of li degradation intermediates.

Materials and Methods

Mice

BL6 and BALB/c mice were purchased from The Jackson Laboratory (Bar Harbor, ME) and maintained under specific pathogen-free conditions at University of Washington. H-2M−/− mice (provided by L. van Kaer, Vanderbilt University, Nashville, TN), li−/− mice on a BL6 background (purchased from The Jackson Laboratory), and li−/− hli and li−/− BAD-CLIP transgenic mice, generated as described below, were bred and maintained under these same conditions. All procedures and care of the animals were in accordance with University of Washington guidelines.

Generation of hli and BAD-CLIP transgenic mice

Mutating five anchor residues of the hli CLIP region has been shown previously to decrease its affinity for HLA-DR1 (10). The previously described hli genomic cassette (8, 34, 35) was engineered to encode these same amino acids, and both the modified DNA (hliBAD-CLIP) as well as the original genomic hli construct (hliWt) were purified and injected into (BL6×DBA/2)F1×BL6 embryos. Transgene positive mice were crossed with li−/− mice on the BL6 background to generate the li−/− BAD-CLIP and li−/− hli transgenic lines.

Antibodies

The following Abs directed toward mouse cell surface Ags were purchased from eBioscience (San Diego, CA): FITC-conjugated anti-CD4, FITC-conjugated anti-CD8, and PE-conjugated anti-CD80. Biotin-conjugated Y3F1 (anti-I-Ab), biotin-conjugated YAe (anti-I-Ab:Es complex specific Ab), M/5114 (anti-I-Aβd, anti-I-Eβ), and PIN-1 (anti-human li) have been described previously (36–39).

Peptide loading and flow cytometry

Single-cell splenocyte suspensions were incubated in the presence of 10 μg/ml I-Eα peptide 52–62 (ASFAEQGALANIAVDKAA). Peptide binding was detected using the biotin-conjugated anti-I-Ab:Es complex-specific Ab, YAe, and was analyzed by flow cytometry.

The cell surface phenotypes of splenocytes and thymocytes were determined by four-color flow cytometry. Single-cell suspensions were depleted of erythrocytes, and 1 × 106 cells were incubated in the presence of conjugated Abs. Binding of biotin-conjugated Abs was detected by allophy- sumycin-conjugated streptavidin (BD Pharmingen, San Jose, CA). Data were collected using a FACSCalibur flow cytometer (BD Biosciences, Mountain View, CA) and were analyzed using CellQuest software (BD Biosciences). Typically 50,000 events were recorded for analysis.

Metabolic labeling and immunoprecipitation

Metabolic labeling was performed as described previously (40). Briefly, single-cell splenocyte suspensions were preincubated for 2 h at 37°C in cysteine/methionine-free RPMI 1640, pulsed for 40 min in the presence of 1 μCi/ml [35S]methionine/cysteine (Trans 35S-label; ICN Pharmaceuticals, Costa Mesa, CA), and chased for 0, 1, 3, and 6 h in the presence of unlabeled methionine (3 mM) and cysteine (16 mM). Labeled cells were lysed in the presence of a mixture of protease inhibitors (Roche, Indianapolis, IN), and lysates were precleared with protein G-Sepharose (Amersham Pharmacia Biotech, Piscataway, NJ) and then with 10 μg of normal rat IgG (Cattig Laboratories, Burbimage, CA) before precipitation with an Ab specific for hli, PIN-1, or MHC class II, M5/114. Precipitated proteins were either boiled in SDS-reducing buffer or resuspended in SDS-nonreducing buffer and were separated by 7.5–20% gradient SDS-PAGE. Gels were fixed, treated with Amplify (Amersham Pharmacia Biotech), and dried, and labeled proteins were visualized by exposure to BioMax MR film (Eastman Kodak, Rochester, NY).

Immunoblotting

Splenocytes were lysed in 0.5% Nonidet P-40, 0.15 M NaCl, 5 mM EDTA, and 50 mM Tris-HCl, pH 7.2, in the presence of a mixture of protease inhibitors, and debris was removed by centrifugation. The lysate was analyzed for protein content using Coomassie Plus Protein Assay Reagent (Pierce, Rockford, IL), and 40 μg total protein was boiled in SDS-reducing buffer and separated by 12% SDS-PAGE. The proteins were electrophoretically transferred onto nitrocellulose membrane, and this was probed with cell culture supernatant containing the anti-hli Ab PIN-1 diluted 1/2. Binding was detected using HRP-conjugated sheep anti-mouse Ig (Amer- sham Pharmacia Biotech) diluted 1/1000 and was visualized by chemiluminescence (ECL; Amersham Pharmacia Biotech).

T cell hybridoma assays

The T cell hybridomas used have been described previously (14, 24). T cell hybridomas (5 × 104) were cocultured in flat-bottom, 96-well tissue culture plates with 1 × 105 splenocytes, and Ag was added at the indicated concentration. In the case of T cell hybridomas specific for endogenous Ags, the number of splenocytes cocultured with the fixed number of T cell hybridomas was titrated 3-fold from 3 × 105/well. After 24 h, culture supernatant was assayed for IL-2 using the CTL-L-2 indicator cell line and the colorimetric reagent Alamar Blue (Trek Diagnostic Systems, Cleveland, OH). The data are expressed as absorbance units (OD570–630).

CD4+ T cell isolation and mixed lymphocyte proliferation assays

To isolate highly purified populations of CD4+ T cells, lymph node cells were stained with magnetic anti-CD4 microbeads (Miltenyi Biotech, Auburn, CA) and positively selected over two columns on an automated magnetic cell separator (AutoMACS; Miltenyi Biotech). The purity of the samples was assessed by flow cytometric analysis of a small number of the
cells stained with FITC-conjugated anti-CD4. All positive fractions were >96% CD4<sup>hi</sup>

Splenocytes (5 × 10<sup>5</sup>) exposed to 2000 rad of irradiation were cocultured in triplicate with 1 × 10<sup>5</sup> Cd4<sup>+</sup> T cells, purified as described above. The cells were cultured in a flat-bottom, 96-well tissue culture plate for 96 h, and 0.5 μCi of [<sup>3</sup>H]thymidine (Amersham Pharmacia Biotech) well was added for the final 24 h of culture. To analyze proliferation, [<sup>3</sup>H]thymidine incorporation was measured using a 1205 Betaplate liquid scintillation counter (Wallac, Gaithersburg, MD).

**Results**

**Generation and characterization of transgenic mice**

Previous studies have shown that the affinity of the CLIP:MHC class II interaction exhibits haplotype variation (25, 26). However, as in these reports the affinity of the CLIP:MHC class II interaction was varied by altering the MHC class II allele, it remains undetermined whether this influences the MHC class II-bound peptide repertoire. We sought to determine the role of CLIP:MHC class II affinity on MHC class II peptide presentation by generating I-Ab mice transgenic for li molecules with different CLIP sequences and, thus, different MHC class II affinities.

Substitution of five key anchor residue amino acids in the CLIP region of hli that decrease the affinity of CLIP for HLA-DR1 has been described previously (10). These mutations also result in a >1000-fold decrease in the affinity of CLIP for I-Ab, as determined by competitive peptide binding assays (data not shown). Therefore, we engineered our hli genomic construct (8, 34) (Fig. 1A) to encode the same low affinity CLIP sequence (Fig. 1B) and generated transgenic mice expressing either hli<sup>WT</sup> or hli<sup>BAD-CLIP</sup>. Two wild-type hli founders and seven BAD-CLIP founders were generated and crossed with Ii<sup>−/−</sup> mice on the BL6 background to distinguish the effect of the transgene-encoded li molecules on the MHC class II presentation pathway from regulation by the endogenous mouse li.

Expression of protein in the transgenic mice was confirmed by immunoblotting with a human li-specific Ab, and li<sup>−/−</sup>BAD-CLIP and li<sup>−/−</sup>hli founders expressing comparable levels of hli protein were characterized further (Fig. 1C). Cell surface expression of MHC class II is substantially diminished in the absence of li (23, 24); however, both hli<sup>WT</sup> and hli<sup>BAD-CLIP</sup> were able to restore I-A<sub>b</sub> expression on B cells to levels comparable to those in BL6 control mice (Fig. 1D). Furthermore, the cell surface expression of MHC class II on li<sup>−/−</sup>hli and that on li<sup>−/−</sup>BAD-CLIP B cells were not dramatically different, indicating that the affinity of the CLIP:MHC class II interaction does not influence the level of total MHC class II at the cell surface.

**Degradation intermediates of li molecules with low affinity CLIP rapidly dissociate from MHC class II**

Although we observed that B cells from li<sup>−/−</sup>BAD-CLIP transgenic mice express normal cell surface levels of MHC class II, we wanted to determine whether the decrease in affinity of CLIP for MHC class II altered the repertoire of bound peptides. The degradation of li and subsequent exchange of CLIP for antigenic peptide are both critical events in determining the MHC class II peptide repertoire (14, 21, 22, 28); therefore, we analyzed the kinetics of li degradation in mice expressing hli with either wild-type or low affinity CLIP:MHC class II interactions.

Splenocytes from li<sup>−/−</sup>hli and li<sup>−/−</sup>BAD-CLIP mice were metabolically labeled and chased for 0, 1, 3, and 6 h. Protein lysates were immunoprecipitated with the hli-specific Ab, PIN-1, and analyzed by SDS-PAGE (Fig. 2A). The degradation of hli<sup>WT</sup> yielded a pattern of fragments of comparable size to those observed in APCs expressing mouse li (p18–22 and p12–14) (14, 15, 41), whereas hli<sup>BAD-CLIP</sup> degradation produced a more complex pattern of intermediates, with multiple bands of ~8–12 kDa. These observations demonstrate that decreasing the affinity of the CLIP: MHC class II interaction results in alternate degradation of li. We suggest that in li<sup>−/−</sup>BAD-CLIP APCs, although decreasing the affinity of CLIP for MHC class II does not affect the association of

![FIGURE 1](image-url)
full-length Ii with MHC class II, smaller Ii degradation intermediates are unable to remain associated with I-A^d. Previously unavailable enzyme sites in these dissociated Ii fragments would then be exposed, and a wider range of Ii degradation intermediates would be generated, as was observed.

To establish whether hIiBAD-CLIP degradation intermediates dissociated from MHC class II more readily than wild-type hIi fragments, splenocytes from both Ii^+/+ and Ii^−/−BAD-CLIP mice were metabolically labeled and chased for 0, 1, 3, and 6 h. I-A^d molecules were immunoprecipitated and analyzed by SDS-PAGE (Fig. 2B). As has been previously shown for mouse Ii (14, 15, 41), the p18–22 and p12–14 fragments of hIi^WT were found to associate with MHC class II, accumulating after 1 h of chase and being slowly degraded thereafter. However, no MHC class II-bound hIi^BAD-CLIP degradation intermediates could be detected. The full-length p31 and p41 isoforms of both hIi and BAD-CLIP were only the stability of the interaction of MHC class II with Ii affecting CLIP for MHC class II affects only the stability of the interaction of MHC class II with Ii fragments.

MHC class II peptide loading in the presence of Ii molecules with low affinity CLIP is increased only in the absence of H-2M

In addition to controlled degradation of Ii, H-2M is a critical regulator of the MHC class II presentation pathway, mediating efficient replacement of CLIP with antigenic peptides (21, 22). Given this function of H-2M and having observed that hIi^BAD-CLIP degradation intermediates readily dissociate from MHC class II, we hypothesized that MHC class II peptide loading in Ii^−/−BAD-CLIP APCs may be less dependent upon H-2M. To address this point, Ii^−/− and Ii^−/−BAD-CLIP transgenic mice were crossed with H-2M^+/+ animals (Ii^−/−H-2M^+/+hIi, and Ii^−/−H-2M^+/−BAD-CLIP, respectively). Splenocytes derived from H-2M^+/−mice express wild-type levels of MHC class II on their cell surface, whereas this is dramatically reduced on cells isolated from H-2M^−/−Ii^−/− double-deficient animals (23, 24). This diminished expression of MHC class II on cells lacking H-2M^−/−Ii^−/− was rescued almost completely by the expression of hIi^WT, but only partially by hIi^BAD-CLIP (Fig. 3A). These data, taken together with our observation that MHC class II levels are comparable on Ii^+/+ and Ii^−/−BAD-CLIP splenocytes, suggests that in the absence of Ii fragment association, H-2M plays a role in stabilizing MHC class II, either directly or indirectly via its role in Ii-independent peptide loading (23, 24).

Having observed that Ii degradation and H-2M dependence, two factors influencing the MHC class II pathway, were altered by decreasing the affinity of CLIP for MHC class II, we wanted to
study the effect of CLIP affinity for MHC class II on the presentation of high affinity endogenous peptide:MHC class II complexes on the cell surface of APCs. As an indirect measure of this, we assessed the availability of MHC class II molecules for binding of exogenous provided peptide. Splenocytes isolated from li−/− hli and li−/− BAD-CLIP mice sufficient for and deficient in H-2M, were incubated in the presence of OVA peptide, and MHC class II peptide binding was detected using an OVA peptide-specific Ab. Peptide loading of MHC class II on both li−/− hli and li−/− BAD-CLIP splenocytes was comparable to that observed on control BL6 cells (Fig. 3B). However, OVA peptide loading of MHC class II on li−/− H-2M−/− BAD-CLIP splenocytes was substantially greater than that on li−/− H-2M−/− hli splenocytes, which expressed levels of OVA-I-A^b complexes comparable to those on control H-2M−/− splenocytes expressing wild-type mouse li (Fig. 3C). Thus, the differences we observed in the presence of H-2M in degradation of li molecules with differing affinities for MHC class II do not impact the binding of OVA peptide to MHC class II. However, in the absence of H-2M, the affinity of CLIP strongly influences the binding of exogenous peptide, and the increased availability of MHC class II molecules for peptide loading in li−/− H-2M−/− BAD-CLIP mice suggests that H-2M plays an important role in generating stable peptide:MHC class II complexes if li fragments dissociate from MHC class II.

MHC class II presentation of endogenous Ags is enhanced in H-2M-deficient splenocytes if li has a low affinity CLIP

We further investigated the effect of decreasing the affinity of CLIP for MHC class II on peptide presentation by evaluating the ability of splenocytes derived from li−/− hli, li−/− BAD-CLIP, li−/− H-2M−/− hli, and li−/− H-2M−/− BAD-CLIP mice to present Ag to a panel of T cell hybridomas. Presentation of a control peptide, as assessed by T cell hybridoma stimulation, yielded results similar to those achieved by flow cytometry using a peptide-I-A^b complex-specific Ab (Fig. 3, B and C). Splenocytes derived from li−/− hli and li−/− BAD-CLIP mice were equally efficient at peptide presentation, whereas li−/− H-2M−/− BAD-CLIP cells elicited a much greater T cell response than li−/− H-2M−/− hli splenocytes (Fig. 4A). These data indicate that MHC class II molecules on li−/− H-2M−/− BAD-CLIP splenocytes are readily available for exogenous peptide binding, providing further evidence to suggest that the formation of stable endogenous peptide:MHC class II complexes in the presence of a low affinity CLIP requires H-2M.

Splenocytes isolated from li−/− hli and li−/− BAD-CLIP mice exhibited equivalent capacities to present both exogenously provided intact protein Ag and endogenous Ags (Fig. 4, A and B), indicating that in the presence of H-2M, the affinity of CLIP for MHC class II does not affect presentation of this panel of Ags. However, although li−/− H-2M−/− hli splenocytes were unable to elicit presentation of exogenous protein Ags, li−/− H-2M−/− BAD-CLIP cells were able to present a T cell epitope derived from one Ag, but not from a second Ag (Fig. 4A). Furthermore, li−/− H-2M−/− BAD-CLIP splenocytes were able to efficiently present peptides derived from two endogenous Ags, IgM and β2-microglobulin (β2m), whereas li−/− H-2M−/− hli splenocytes were incapable of this (Fig. 4B). In contrast, presentation of a third peptide derived from an endogenous Ag, CD22, was not detected for either li−/− H-2M−/− BAD-CLIP or li−/− H-2M−/− hli (Fig. 4B). These data indicate that in the presence of higher affinity CLIP:MHC class II interactions (lihli), the generation of peptide:MHC class II complexes requires H-2M. However, if the affinity of CLIP for MHC class II is substantially reduced (liBAD-CLIP), and the low affinity CLIP fragments spontaneously dissociate from MHC class II, formation of some peptide:MHC class II complexes requires H-2M, whereas others are able to load independently of H-2M. Thus, these results provide further evidence to indicate that H-2M acts not only to catalyze the release of CLIP from the MHC class II peptide-binding groove, but also influences peptide loading independently of li. Presentation of IgM and β2m epitopes by li−/− H-2M−/− BAD-CLIP splenocytes was less efficient than li−/− hli and li−/− BAD-CLIP presentation, presumably as a result of the decreased levels of MHC class II on these cells (Figs. 1D and 3A).

In the absence of H-2M, a low affinity CLIP promotes diversity in the repertoire of MHC class II-bound peptides

CD4+ T cells from H-2M−/− mice respond vigorously to BL6 splenocytes (23, 24, 42), indicating that the repertoire of MHC class II-bound peptides presented in H-2M-deficient animals differs dramatically from that in wild-type control mice. We used this approach to further investigate the repertoire of peptides bound by MHC class II in the presence of CLIP with low affinity for MHC class II, comparing the ability of splenocytes from mice expressing different li molecules to stimulate CD4+ T cells isolated from H-2M−/− mice. BL6, li−/− hli, and li−/− BAD-CLIP splenocytes all stimulated comparable, strong proliferative responses by H-2M−/− CD4+ T cells (Fig. 5A), suggesting that in the presence of H-2M, the diversity of the repertoire of peptides presented by MHC class II is not affected by the affinity of CLIP for MHC class II. Furthermore, the diversities of peptides presented by these splenocyte populations are likely to overlap substantially, as CD4+ T cells isolated from BL6, li−/− hli, and li−/− BAD-CLIP mice exhibited no response to the same panel of stimulator cells (data not shown).
Interestingly, the proliferative response of H-2M^{−/−} CD4^{+} T cells to splenocytes derived from Ii^{+/−} BAD-CLIP mice was well above the background levels of stimulation induced by Ii^{+/−} hIi splenocytes and approximately half the response generated to BL6 cells (Fig. 5B). These data suggest that a diverse array of peptide:MHC class II complexes is generated in the absence of H-2M only if the affinity of CLIP for MHC class II is substantially reduced. Further evidence for this was provided by...
the phenotype of CD4+ T cells isolated from li−/−H-2M−/−hli and li−/−H-2M−/−BAD-CLIP mice. li−/−H-2M−/−hli CD4+ T cells proliferated weakly, but distinctly, in the presence of both BL6 and li−/−H-2M−/−BAD-CLIP splenocytes, whereas li−/−H-2M−/−BAD-CLIP CD4+ T cells did not respond in this manner (Fig. 5B). This inability of li−/−H-2M−/−BAD-CLIP CD4+ T cells to respond was not an intrinsic inability to proliferate, as allogeneic stimulation provoked comparable responses by li−/−H-2M−/−hli and li−/−H-2M−/−BAD-CLIP CD4+ T cells (data not shown). Thus, a diverse repertoire of peptide:MHC class II complexes can be generated in the absence of H-2M if the affinity of CLIP for MHC class II is reduced.

**In the absence of H-2M, a low affinity CLIP partially restores CD4+ T cell selection**

The diversity and level of expression of endogenous peptide:MHC class II complexes have been shown to determine the number of CD4+ T cells selected, with only ~30% of the normal number of CD4+ T cells present in li−/− mice, which exhibit a decrease in both the diversity and the abundance of such complexes (23, 24, 35). The expression of hliWT and hliBAD-CLIP fully restored the development of CD4+ T cells in li−/− mice to levels observed in BL6 control mice expressing wild-type mouse Ii (Fig. 6A), and the numbers of CD4+ T cells and CD8+ T cells in the spleen and thymus of li−/−hli and li−/−BAD-CLIP were not significantly different from those in wild-type animals (data not shown). These data provide further evidence to suggest that in the presence of H-2M, the diversity of the MHC class II peptide repertoire was restored to wild-type levels. Additional support for this comes from the observation that despite monitoring these mice for anti-nuclear autoantibodies, lymphoproliferation, and gross organ pathology for up to 1 year, we observed no signs of autoimmunity (data not shown). The presence or absence of antinuclear Abs was assessed by indirect immunofluorescence analysis of sera on HEp-2 slides (Bio-Rad), all samples tested were negative, and lymphoproliferation was determined by examination of spleen and lymph node cellularity.

In contrast to H-2M-sufficient animals, CD4+ T cell selection in li−/−H-2M−/−hli mice was barely above the level observed in li−/−H-2M−/− mice, whereas the expression of BAD-CLIP in li−/−H-2M−/− animals restored CD4+ T cell numbers to ~60% normal levels (Fig. 6B). These observations are consistent with the proliferation data described above and suggest that in the absence of H-2M, a diverse array of peptide:MHC class II complexes can be generated if CLIP has a decreased affinity for MHC class II.

**Discussion**

MHC class II presentation of a diverse array of peptides is required for CD4+ T cell selection and is a tightly regulated process that requires that MHC class II maturation and Ag degradation occur in the same or interconnected intracellular compartments (1, 18). The importance of the cellular proteins involved in this process, in particular Ii and H-2M, is highlighted by the observation that null mutations in the genes encoding these proteins substantially alter the repertoire of peptides presented by MHC class II and hence impact CD4+ T cell selection (2–4, 21, 22). One critical function of li is that the CLIP region of the protein interacts with the MHC class II peptide-binding groove, preventing inappropriate peptide loading early in the endocytic pathway (7–9). Interestingly, the nonpolymorphic li protein mediates this effect despite extensive polymorphism between MHC class II haplotypes. Although it is clear that these polymorphisms influence the affinity of the CLIP: MHC class II interaction (25, 26), the effect that this has on the repertoire of peptides bound by MHC class II molecules is poorly understood. One recent study suggested that the repertoire of MHC class II-bound peptides may be altered by decreasing the affinity of CLIP for MHC class II, as RA-associated HLA-DR alleles formed less stable complexes with CLIP than non-RA-associated alleles (29). However, to clearly define the effects of different CLIP:MHC class II affinities on the repertoire of peptides bound by MHC class II, it is necessary to fix the MHC class II allele and vary the affinity of CLIP for this allele. Therefore, we report in this study the generation of transgenic mice expressing either hliWT or a mutated hli in which the CLIP region had been engineered to encode the same sequence previously shown to have a low affinity for HLA-DR1 (10) and I-Aβ (data not shown). We show that the expression of both hliWT and hliBAD-CLIP in mice backcrossed onto an li−/−BL6 background restores the cell surface levels of I-Aβ to normal, indicating that both molecules are able to facilitate the chaperone and targeting functions of li. We analyzed the kinetics of li processing and observed that hliBAD-CLIP degradation differed from that of hliWT as a result of the inability of li degradation intermediates to remain associated with I-Aβ. Despite these differences in li degradation, the presentation of both endogenous and exogenous Ags by transgenic splenocytes was normal, as were both the number of CD4+ T cells selected and their reactivity. Therefore, although decreasing the affinity of CLIP for MHC class II lead to the dissociation of li degradation intermediates from MHC class II, we were unable to discern any differences in the repertoire of MHC class II-bound peptides.

The exchange of MHC class II-bound CLIP for a diverse array of peptides is catalyzed by H-2M (19–24). Dependence upon H-2M for the generation of a diverse repertoire of MHC class II-bound peptides exhibits haplotype variation, and although there is some correlation between CLIP:MHC class II affinity and the requirement for H-2M, the precise relationship between these two factors is ill-defined (30–33). When backcrossed onto an li−/−H-2M−/−I-Aβ background, the diversity of the MHC class II-bound peptide repertoire of transgenic mice expressing hliBAD-CLIP was dramatically greater than that of hli animals, as assessed by the presentation of endogenous Ag to a panel of T cell hybridomas as well as the number of CD4+ T cells selected and their reactivity. These results indicate that decreasing the affinity of CLIP for MHC class II decreases the requirement for H-2M to mediate the loading of a diverse array of peptides into the MHC class II peptide-binding groove. This decreased dependence upon H-2M for peptide loading is probably a result of the spontaneous dissociation of li degradation intermediates from MHC class II and, hence, increased availability of the peptide-binding groove. As the affinity of CLIP for MHC class II increases, li degradation intermediates remain associated with MHC class II, and hence, H-2M is required to remove CLIP and make the binding groove available to peptides.

The identity of the peptides bound by MHC class II in li−/−H-2M−/−BAD-CLIP animals was not studied extensively. However, although we detected presentation of peptides derived from the endogenous Ags, IgM and β2-m, and the exogenous Ag, hen egg lysozyme (HEL), we were unable to detect the presentation of epitopes derived from CD22 and chicken OVA (Fig. 4B), indicating that at least some peptides require H-2M for presentation in the context of MHC class II. We believe that there are two possible explanations for this observation. Firstly, loading of these peptides may be mediated by H-2M. Secondly, as hliBAD-CLIP degradation intermediates dissociate rapidly from MHC class II, it is possible that the peptide-binding groove becomes accessible for peptide loading earlier in the endocytic pathway than when these peptides are generated. Thus, the spatial overlap between peptide generation and peptide-binding groove availability would determine the
MHC class II-peptide repertoire. Our observations indicate that the repertoire of peptides bound by MHC class II molecules in li/~ H-2M/~BAD-CLIP mice differs from that present in wild-type animals. However, CD4+ T cells isolated from li/~H-2M/~BAD-CLIP mice do not respond to stimulation by BL6 splenocytes, suggesting that although we were able to detect some differences in the MHC class II-bound peptide repertoire, the majority of peptides presented by li/~H-2M/~BAD-CLIP and BL6 APCs are overlapping.

The level of MHC class II expressed on li/~H-2M/~BAD-CLIP B cells was substantially reduced compared with that on li/~BAD-CLIP cells from H-2M-sufficient mice (Figs. 1D and 3A, and data not shown) and control H-2M/~ cells that have been shown previously to express wild-type levels of MHC class II (21, 22), indicating that H-2M enhances cell surface levels of MHC class II only when Ii degradation intermediates contain CLIP of low affinity for MHC class II. This suggests that in the absence of H-2M, although a decrease in the affinity of the CLIP:MHC class II interaction enables diverse peptide loading, the stability of the peptide:MHC class II complexes formed is dramatically reduced compared with that of complexes generated in the presence of H-2M. Further evidence to support this hypothesis comes from the observation that loading of oxogenous peptide, as determined by both Ag presentation to a T cell hybridoma and flow cytometry using a peptide-I-Ab complex-specific Ab, is greatly enhanced in li/~H-2M/~BAD-CLIP cells compared with that in control cells. Taken together, our data suggest that although H-2M is not required for MHC class II loading of peptides, it plays a critical role in ensuring that stable peptide:MHC class II complexes are generated if the affinity of CLIP for MHC class II is insufficient to maintain the association of Ii degradation intermediates with MHC class II. Thus, we propose that H-2M acts as a chaperone molecule, either stabilizing MHC class II molecules upon dissociation of Ii fragments by preventing its proteolysis or maintaining the peptide-binding groove in a conformation available for peptide loading.

Previous studies analyzing li/~H-2M/~ mice have suggested an Ii-independent role for H-2M in generating diversity in the repertoire of peptides bound by MHC class II (23, 24); however, in these mice MHC class II molecules are not efficiently targeted to the endosomal pathway. Therefore, as targeting to the endocytic pathway is not disrupted in the animals reported in this study, our data illuminate more clearly than previously the critical chaperone role of H-2M in determining the MHC class II-bound peptide repertoire beyond its ability to catalyze the release of CLIP from the MHC class II peptide-binding groove. These in vivo data are in agreement with a recent report analyzing the H-2M dependence of MHC class II peptide presentation in I-A^b-expressing, transformed cell lines (33).

The observation that the dependence of MHC class II peptide loading upon H-2M exhibits allelic variation that correlates with decreased affinity of CLIP (30–33) combined with the report that RA-associated HLA-DR molecules have lower affinity for CLIP than non-RA-associated HLA-DR molecules (29) led to the hypothesis that in the presence of li containing low affinity CLIP, peptide loading may escape editing by H-2M and thus autoantigenic peptide presentation may occur. However, we were unable to perceive any difference in the peptide repertoire of I-A^b assembled in the presence of hi^BAD-CLIP and hi^WT, and we observed no clinical signs of autoimmunity in our li/~H-2M/~BAD-CLIP mice. Therefore, in the context of I-A^b, the affinity of CLIP for MHC class II alone cannot determine the susceptibility of an individual to developing autoimmunity.

In conclusion, we have shown in this study that although decreasing the affinity of CLIP for MHC class II eliminates the association of Ii degradation intermediates with I-A^b, the repertoire of peptides presented by MHC class II is not substantially altered. Furthermore, we were unable to distinguish signs of autoimmunity in mice expressing hi^BAD-CLIP, indicating that decreased affinity of CLIP for MHC class II may not be a factor in susceptibility to autoimmunity. In the absence of H-2M, reducing the affinity of the CLIP:MHC class II interaction enhances the diversity of the MHC class II peptide repertoire compared with that of control li/~H-2M/~hi or H-2M/~ cells. Despite the increase in MHC class II peptide diversity in li/~H-2M/~BAD-CLIP cells, the cell surface expression of MHC class II is reduced, implicating H-2M as a critical chaperone molecule upon Ii fragment dissociation from MHC class II.

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