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Posttranslational Regulation of I-E<sup>d</sup> by Affinity for CLIP<sup>1</sup>


Several MHC class II alleles linked with autoimmune diseases form unusually low stability complexes with CLIP, leading us to hypothesize that this is an important feature contributing to autoimmune pathogenesis. To investigate cellular consequences of altering class II/CLIP affinity, we evaluated invariant chain (Ii) mutants with varying CLIP affinity for a mouse class II allele, I-E<sup>d</sup>, which has low affinity for wild-type CLIP and is associated with a mouse model of spontaneous, autoimmune joint inflammation. Increasing CLIP affinity for I-E<sup>d</sup> resulted in increased cell surface and total cellular abundance and half-life of I-E<sup>d</sup>. This reveals a post-endoplasmic reticulum chaperoning capacity of Ii via its CLIP peptides. Quantitative effects on I-E<sup>d</sup> were less pronounced in DM-expressing cells, suggesting complementary chaperoning effects mediated by Ii and DM, and implying that the impact of allelic variation in CLIP affinity on immune responses will be highest in cells with limited DM activity. Differences in the ability of cell lines expressing wild-type or high-CLIP-affinity mutant II to present Ag to T cells suggest a model in which increased CLIP affinity for class II serves to restrict peptide loading to DM-containing compartments, ensuring proper editing of antigenic peptides.


M

Major histocompatibility complex class II molecules are polymorphic glycoproteins that are constitutively expressed on professional APCs, including dendritic cells (DC), B cells, and macrophages, and can be induced in other cell types by cytokines (1). Recognition of class II MHC/peptide complexes by TCR is critical for appropriate thymic selection, immune responses to pathogens and tumors, and the maintenance of peripheral tolerance. Epidemiological studies have revealed strong and statistically well-substantiated associations of MHC class II alleles with particular autoimmune diseases, including rheumatoid arthritis (RA), type I diabetes, multiple sclerosis, and celiac disease (2). As CD4<sup>+</sup> T cells are implicated in pathogenesis in these diseases and their animal models, it is postulated that class II disease associations are related to the role of MHC class II molecules in Ag presentation. However, the specific mechanisms that link the polymorphisms to autoimmune pathogenesis have remained elusive. A number of observations, including the association of certain class II alleles with multiple autoimmune diseases with unrelated Ags (2, 3), argue that a general feature of class II alleles plays an important role in determining their association with susceptibility to autoimmunity.

The major steps in the proper assembly, transport, and loading of class II molecules have been well-described and are influenced by three major accessory molecules: invariant chain (Ii), DM, and DO (reviewed in Refs. 3 and 4). Briefly, nascent class II molecules are assembled onto Ii trimers in the endoplasmic reticulum (ER), Ii directs the trafficking of these nonameric (αβ<sub>2</sub>)<sub>3</sub>Ii complexes out of the ER, through the Golgi apparatus, and into the endosomal class II-loading compartments. Different class II alleles vary in their dependence upon Ii for efficient assembly and egress from the ER (5). In endosomal compartments, Ii is degraded, leaving a nested set of peptides called CLIP as a surrogate ligand in the peptide-binding groove of the MHC class II molecule. Exchange of CLIP for a diverse array of peptides is promoted by the peptide-exchange catalyst DM (H-2M or H2-DM in mice, HLA-DM in humans), which is also thought to stabilize empty MHC class II intermediates and edit the repertoire of bound endosomal peptides, favoring stable peptide/MHC class II complexes. Another nonclassical class II molecule, DO (H-2O or H2-DO in mice, HLA-DO in humans), is a negative regulator of DM function expressed in a subset of APCs, including B cells, thymic medullary epithelial cells, and specific subsets of DC (4, 6–9). Reduced or absent DM function (for example, due to low DM expression or to coexpression of DO) results in the accumulation of class II/CLIP complexes.

Of the molecules implicated in general mechanisms of Ag presentation, CLIP is well positioned to be involved in mechanisms that link particular MHC class II polymorphisms to autoimmune disease. CLIP occupies the class II-binding groove much like antigenic peptides do, and different MHC class II alleles differ widely.
in their affinity for CLIP (10, 11). We previously compared RA-associated alleles of DR4 (DR*0401, *0404, and *0405) with closely related, non-RA-associated alleles (DR*0402 and *0403) and found that the disease-associated alleles form less stable complexes with CLIP both in vitro and in living cells (12). Evidence from others supports our hypothesis, as a disproportionate number of disease-associated alleles have been found to have low affinity for CLIP (reviewed in Ref. 3). The low affinity for CLIP leads these alleles to spontaneously release CLIP peptides, even in the absence of DM (12, 13).

These observations led us to propose that low affinity for CLIP is a critical feature of disease-linked MHC class II alleles and contributes to disease pathogenesis. We reasoned that, to influence an autoimmune disease in a whole organism, low CLIP affinity must have molecular consequences detectable at the level of individual APCs. Comparisons of different class II alleles with varying CLIP affinity are confounded by additional variations in the characteristics of the alleles, such as peptide-binding specificity and inherent dimer stability. Thus, we chose to introduce targeted mutations into the CLIP region of Ii constructs to vary CLIP affinity for a single disease-linked class II allele. I-Ed is a murine class II allele with extremely low affinity for wt CLIP (10, 11), and is associated with a spontaneous model of autoimmune joint inflammation which may share some features with human RA (A. Caton, unpublished observations). In this study, we identify mutations in Ii that increase the affinity of CLIP for I-E\*.

### Table 1. Effect of Ii mutations on persistence of I-E\*/CLIP coimmunoprecipitation

<table>
<thead>
<tr>
<th>Anchor Pocket</th>
<th>P1</th>
<th>P4</th>
<th>P6</th>
<th>P9</th>
</tr>
</thead>
<tbody>
<tr>
<td>wt CLIP anchor residue</td>
<td>M90</td>
<td>A93</td>
<td>P95</td>
<td>M98</td>
</tr>
<tr>
<td>Greatest effect</td>
<td>F,T,L</td>
<td>R</td>
<td>K</td>
<td>L,F</td>
</tr>
<tr>
<td>Moderate effect</td>
<td>F,G,L,V</td>
<td>G,I,L,V</td>
<td>F,L</td>
<td></td>
</tr>
</tbody>
</table>

*\(^{a}\) The single most effective mutant at each pocket is shown in bold. Mutations in the “moderate” category are listed from most to least effective.*

*\(^{b}\) M98L was included in this analysis as a control; it was expected to have no effect on CLIP affinity for I-E\*.*

### Vectors and transfections

Methods for site-directed mutagenesis, cloning, and retroviral transduction have been previously described (26). Briefly, mutant p31 marine II cDNAs were generated by site-directed mutagenesis, using overlap extension PCR and high-fidelity Pfu polymerase, with a pGEM-mi-p31 construct as the original template (gift of E. K. Bickoff, University of Oxford, Oxford, U.K.). II cDNAs were cloned into the retroviral vector pBMN-RES-Neo and verified by sequencing. Vectors were transfected into Phoenix-A cells by calcium phosphate precipitation. Phoenix-A cell supernatant containing retroviral particles was harvested and used to infect A20, 3A5, or L cells.

### Flow cytometry

Cells were stained on ice with FITC-conjugated 14-4-4S or isotype control. For cell surface FACS with L cells, propidium iodide was used to exclude dead/dying cells. For intracellular staining, fixation and permeabilization was performed using the Cytofix/Cytoperm kit (BD Biosciences/BD Pharmingen). Data were collected using a FACScan or FACSCalibur flow cytometer (BD Biosciences) and CellQuest Pro software (BD Biosciences), and were analyzed using FlowJo software (Tree Star). The mean fluorescence intensity (MFI) of isotype controls was routinely under 10. MFI of staining on cells expressing mutant II was normalized to the appropriate (untagged or 6xHis-tagged) wild-type (wt) control within the same experiment: 100% × MFI_{tagged}/MFI_{untagged} = MFI of mutant as a percentage of wt. Data represent staining of polyclonal populations from multiple independent transfections and selections.

### Immunoblotting and densitometry

Cells were harvested and solubilized in lysis buffer containing 1% Nonidet P-40, 50 mM Tris-HCl (pH 8.0) and complete protease inhibitors (Roche Diagnostics). Lysates were cleared of nuclear and cellular debris by centrifugation, and were precleared several times with combinations of normal mouse serum, rabbit-anti-mouse IgG (Zymed Laboratories), Pansorbin (a gel isolated Streptococcus cells; Calbiochem/EMD Biosciences), and protein A-Sepharose (Amersham Pharmacia Biotech). Samples were normalized either for starting cell number at time 0 or for counts following I-E\* IP, using a 1450 Microbeta beta counter (PerkinElmer/Wallac). I-E\* was immunoprecipitated by incubating lysates with protein A-Sepharose beads coated with 14-4-4S ascites or concentrated supernatant. Proteins were eluted from the beads by boiling in reducing SDS sample buffer and separated by SDS-PAGE. Gels were treated with Amplify (Amersham Biosciences), dried under vacuum, and exposed to radiography film (Kodak).

### Flow cytometry

Cells were washed in cytochrome/methionine-free DMEM (Invitrogen Life Technologies) to remove unlabeled cytosine and methionine. Cells were starved for 1–2 h (at 37°C, 5% CO\(_2\)) in starvation medium (Cys/Met-free DMEM plus 10% dialyzed FBS plus 2 mM t-glutamine), then labeled for 1 h (at 37°C, 5% CO\(_2\)) with 125 Ci/ml ExpreSS [\(^{35}\)S]labeling mix (PerkinElmer). Excess free radiolabel was removed by washing with complete medium (DMEM plus 10% FBS plus 2 mM t-glutamine), and cells were chased in complete medium for the designated times. Cells were lysed in buffer containing 1% Nonidet P-40, 50 mM Tris-HCl, 150 mM NaCl, and 5 mM EDTA (pH 8.0) and complete protease inhibitors (Roche Diagnostics). Lysates were cleared of nuclear and cellular debris by centrifugation, and were precleared several times with combinations of normal mouse serum, rabbit-anti-mouse IgG (Zymed Laboratories), Pansorbin (a gel isolated Streptococcus cells; Calbiochem/EMD Biosciences), and protein A-Sepharose (Amersham Pharmacia Biotech). Samples were normalized either for starting cell number at time 0 or for counts following I-E\* IP, using a 1450 Microbeta beta counter (PerkinElmer/Wallac). I-E\* was immunoprecipitated by incubating lysates with protein A-Sepharose beads coated with 14-4-4S ascites or concentrated supernatant. Proteins were eluted from the beads by boiling in reducing SDS sample buffer and separated by SDS-PAGE. Gels were treated with Amplify (Amersham Biosciences), dried under vacuum, and exposed to radiography film (Kodak).
in the sensitivity of the primary Abs being used for detection, all experiments included two gels run in parallel on the same day from the same lysates; one with high protein amounts loaded for detection of I-E\(^d\) (anti-I-E\(a\) antiseraum) and His-Ii (anti-Tetra-His), and one gel with low protein amounts loaded for detection of \(\beta\)-actin and total cellular Ii (In-1). Denitometry was performed using a Bio-Rad GS-710 densitometer and QuantityOne software (Bio-Rad).

T cell stimulation assays

D2-4-8 T hybridoma cells (10\(^5\)) were cultured with A20 or 3A5 transfectants (5 \times 10\(^5\)) and different concentrations of whole HEL (Sigma-Aldrich) or HEL 106–116 peptide, as indicated in the figure labels and legends. All cultures were done in duplicate. Culture supernatants were collected at 20–24 h, and assayed for IL-2 with the BD OptEIA Mouse IL-2 ELISA kit and BD OptEIA TMB substrate (BD Biosciences).

Statistical methods

For the analysis of FACS data in Figs. 2–5, normalized MFIs of wt and mutant li transfectants were compared using paired Student’s \(t\) tests (GraphPad Prism; GraphPad Software). Within each figure, we corrected for multiple comparisons across \(t\) tests using sequential Bonferroni adjustment (27). For titration analyses, MFI was modeled as a logistic response against the mean for each mutant, at each dose level, using a mixed-effect quasi-Newton method (28). A \(t\) test was used to compare the positions of the plateaus (at 5 \(\mu\)g) between groups. SEs for \(\delta\) between groups were calculated using a first-order \(\delta\) approximation (29). For Fig. 7, in preparation for analysis, data were smoothed by calculating the mean for each combination of peptide concentration and genotype within a plate (experiment). Analysis was then performed on these means. For 3A5, we compared the mean for wt against the mean for each mutant, at each dose level, using a mixed-effect model (30). Plate was modeled as a random factor to account for correlation among wells within an experiment. For A20, analysis used generalized estimating equations (GEE) (31), because data were too leptokurtic to meet normality assumptions of mixed models. Mixed model and GEE analyses were performed in SAS version 9.1 (SAS Institute).

Results

Identification of mutations in murine li that increase affinity of CLIP for I-E\(^d\)

Mutations in murine li that were likely to increase the affinity of CLIP for I-E\(^d\) were chosen based on predictions using crystal structures of I-E\(^d\) (14, 15) to model I-E\(^d\), and on published peptide-binding motifs (32–34). None of the wt CLIP anchors (M90, A93, P95, and M98) conformed to known I-E\(^d\)-binding motifs. The P1 and P9 methionine anchors in CLIP are thought to be moderate anchors for most class II alleles, leaving room for allele-specific improvement. Our random mutagenesis of DR (homolog of I-E) revealed that mutations which increased the affinity of DR3 for wt CLIP were centered around the P4 and P6 pockets (35). In addition, molecular modeling suggested that the reported strong association between wt CLIP and I-E\(^d\)/I-E\(^b\) molecules (36) was due to differences between I-E\(^d\)/I-E\(^b\) centered around the P4 and P6 pockets (data not shown). Based on these observations, we made selected mutations at P1, P4, P6, and P9 (Table I) and tested these empirically, as the relative contributions of specific anchors are often context dependent (37). Single amino acid mutations were incorporated into cDNA constructs for the p31 isoform of murine li. Most constructs (specified in figures) included a C-terminal 6xHis tag for subsequent differentiation between endogenous and transfected li in cell lines. Mutant li constructs and wt control constructs were expressed in A20 (a murine B cell line with H-2\(d\) haplotype) and 3A5 (a DM-deficient derivative of A20), and in L cells (a murine fibroblast cell line that does not express DM) transfected with I-E\(^d\). In each of these cell lines, transfected li must compete with endogenous li. Expression of endogenous li in L cells varies with the sublines; the L cells used in this study express endogenous li at levels comparable to those found in A20 and 3A5 (data not shown).

To screen the li mutants for increased affinity for I-E\(^d\), we used a pulse-chase assay in 3A5 cells. In the absence of DM, class II/CLIP complexes are expected to accumulate. However, because I-E\(^d\) has extremely low affinity for wt CLIP, very little CLIP is coimmunoprecipitated (co-IP) with I-E\(^d\), and the CLIP band vanishes rapidly as CLIP spontaneously dissociates from I-E\(^d\) (Fig. 1, (a)).
The addition of a single high-affinity mutation at the P9 pocket (M98K) results in a clear increase both in the maximal amount of I-\textsuperscript{E}\textsuperscript{d}-associated CLIP, and in the duration of I-\textsuperscript{E}\textsuperscript{d}/CLIP association (Fig. 1, center). The effects of the 14 single amino acid mutations on persistence of I-\textsuperscript{E}\textsuperscript{d}/CLIP co-IP are summarized in Table I. The effects of individual mutations on CLIP retention by I-\textsuperscript{E}\textsuperscript{d} are cumulative, as Ii constructs containing two or three mutations incrementally increase accumulation and retention of CLIP (Fig. 1, right, and data not shown). Thus, our single, double, and triple Ii mutants constitute a panel of Ii mutants with a wide range of CLIP affinity for I-\textsuperscript{E}\textsuperscript{d}. Because this assay uses radioactively labeled methionine for detection, and both P1 and P9 mutations remove a methionine label from CLIP, this assay substantially underestimates the amount of mutant CLIP on these gels. Parallel pulse-chase experiments in A20 cells (DM\textsuperscript{+}) do not show significant accumulation of mutant CLIP peptides (data not shown), suggesting that these mutants remain DM-susceptible and are within a physiologically relevant range of CLIP affinity.

Increased CLIP affinity for I-\textsuperscript{E}\textsuperscript{d} results in increased cell surface levels of I-\textsuperscript{E}\textsuperscript{d}

To begin studying the effects of varying CLIP affinity on I-\textsuperscript{E}\textsuperscript{d} in a cellular context, we examined the level of cell surface I-\textsuperscript{E}\textsuperscript{d} expression in the presence of wt (low CLIP affinity) vs mutant (high CLIP affinity) Ii by flow cytometry. The Ii mutants that caused the greatest increase in CLIP association with I-\textsuperscript{E}\textsuperscript{d} in pulse-chase experiments (Table I) also resulted in increased cell surface staining with the monoclonal anti-I-\textsuperscript{Ea}Ab 14-4-4S in transfectants of the DM-deficient B cell line 3A5 (Fig. 2). The effects of individual mutations were again found to be cumulative, with an apparent plateau in the extent of the effect (Fig. 3). Furthermore, the increase in cell surface I-\textsuperscript{E}\textsuperscript{d} in the presence of high-CLIP-affinity Ii is independent of the 6xHis tag (Fig. 3, A vs B) and of the particular host cell line (Fig. 3, A vs C).

The binding of some mAbs to cell surface class II molecules is influenced by the bound peptide repertoire and the conformation of the class II molecules. Thus, increased binding of 14-4-4S could reflect either an increase in abundance of I-\textsuperscript{E}\textsuperscript{d} molecules at the cell surface or change in conformation that increases affinity for 14-4-4S. To differentiate between these two scenarios, we measured levels of I-\textsuperscript{E}\textsuperscript{d} binding with titrated amounts of Ab. If differences in 14-4-4S binding reflect a change in Ab affinity for I-\textsuperscript{E}\textsuperscript{d} without a change in the number of binding sites, then these differences should be overcome by saturating levels of Ab. If, however, the number of I-\textsuperscript{E}\textsuperscript{d} molecules is increased by the Ii mutation, we would expect to observe different plateau levels of staining with saturating amounts of Ab. Titration showed that the observed differences in 14-4-4S staining cannot be overcome by addition of excess Ab (Fig. 3D), indicating that increased affinity of CLIP for I-\textsuperscript{E}\textsuperscript{d} results in increased abundance of I-\textsuperscript{E}\textsuperscript{d} at the cell surface. Increased cell surface I-\textsuperscript{E}\textsuperscript{d} correlated with increased I-\textsuperscript{E}\textsuperscript{d}/CLIP affinity and was not simply a result of increased expression of the transfected Ii mutant. Transfection with wt or 6xHis tagged wt Ii did not increase cell surface I-\textsuperscript{E}\textsuperscript{d} compared with untransfected cells or cells transfected with control vectors lacking Ii (data not shown).

The same Ii mutations that increased I-\textsuperscript{E}\textsuperscript{d} expression in DM-negative cells (3A5 and L cells, Figs. 2 and 3) also did so in DM-expressing A20 cells (Fig. 4) across multiple transfections/selections and in both the presence and absence of a 6xHis tag. The effects of varying CLIP affinity on cell surface I-\textsuperscript{E}\textsuperscript{d} expression levels were more modest in the presence of DM (Fig. 2 vs Fig. 4). For example, for M98K, MFI = 141% of wt (mean MFI from all experiments) in 3A5 vs 113% of wt in A20. Cumulative effects of multiple CLIP mutations were not detected in DM\textsuperscript{+} cells.

FIGURE 3. Effects of individual Ii mutations on cell surface I-\textsuperscript{E}\textsuperscript{d} staining are cumulative and reflect a change in cell surface abundance of I-\textsuperscript{E}\textsuperscript{d}.

Site-directed mutagenesis was used to generate Ii constructs with multiple CLIP mutations favorable for binding I-\textsuperscript{E}\textsuperscript{d}. A–C, 3A5 cells transfected with untagged (A) or 6xHis-tagged (B) Ii constructs and L cells plus I-\textsuperscript{E}\textsuperscript{d} transfected with untagged Ii constructs (C) were stained with 14-4-4S-FITC. MFI of cells expressing mutant Ii is normalized to the appropriate (untagged or 6xHis-tagged) wt control within each experiment. Statistical significance is determined by paired Student’s t test. In 3A5 (A and B), p < 0.05 for all wt vs mutant comparisons and for all M98K single-mutant vs double- or triple-mutant comparisons. For double- vs triple-mutant comparisons, only His-FK vs His-FRK is statistically significant (p = 0.0165). All of these differences are statistically significant even after correction for multiple comparisons. In L cells plus I-\textsuperscript{E}\textsuperscript{d} (C), p < 0.05 for wt vs FKK, wt vs FRK, and K vs FK; and p = 0.0056 for K vs FRK (all statistically significant after correction for multiple comparisons). p < 0.05 for wt vs FK and for K vs FK (not significant after correction for multiple comparisons). D, 3A5 cells were stained with titrated amounts of 14-4-4S-FITC. Shown is one experiment representative of two experiments with the His-M90F-M98K double mutant and several experiments with P1, P4, and P9 single mutants. Titration curves were fit by nonlinear regression. A t test was used to compare the positions of the plateaus (at 5 μg) between His-wt and His-M90F-M98K titrations (p < 0.0001). Statistical analysis was not performed on titrations for single mutants.

The cell surface increase in I-\textsuperscript{E}\textsuperscript{d} in the presence of high-affinity CLIP is associated with an increase in total cellular abundance of I-\textsuperscript{E}\textsuperscript{d}

At least two distinct mechanisms could explain the increase in cell surface levels of I-\textsuperscript{E}\textsuperscript{d} in the presence of high-affinity CLIP. Tightly bound, high-affinity CLIP mutants could induce a more mature
conformation of I-E\(^d\) that is preferentially exported to the cell surface, causing an altered distribution of I-E\(^d\) molecules between intracellular and cell surface compartments. Alternatively, increased CLIP affinity could result in an increase in total cellular (and surface) levels of I-E\(^d\).

Detection of total cellular levels of I-E\(^d\) in 3A5 transfectants by combined cell surface and intracellular FACS (Fig. 5A) and by Western blotting of whole cell lysates (Fig. 5, B and C) revealed an increase in total cellular levels of I-E\(^d\) in the presence of high-affinity CLIP mutants. The increase was on the order of 10–40% by FACS for single and double mutants, and 1.6- to 4-fold by Western blot for triple mutants. It appears that the change in total cellular I-E\(^d\) is at least as robust as the change in cell surface levels of I-E\(^d\), if not more so.

The half-life of I-E\(^d\) is increased in the presence of high-affinity CLIP mutants

The increase in steady-state abundance of I-E\(^d\) is likely to be the result of an increase in the half-life of I-E\(^d\). To test whether this is true, we treated 3A5 transfectants with CHX to block de novo protein synthesis and assessed the level of surviving I-E\(^d\) at various time points by Western blot. In the presence of wt Ii (low-affinity CLIP), the amount of I-E\(^d\) in CHX-treated cells begins to decline after 8 h (Fig. 6, left). However, in the presence of an Ii triple mutant with high CLIP affinity, the amount of I-E\(^d\) remains constant over at least 22 h (Fig. 6, right), indicating that high-affinity CLIP mutants protect against I-E\(^d\) turnover. Over the same period of observation, β-actin levels remain constant, and total cellular Ii turns over at a similar rate in both cell lines, confirming that the effect is specific to I-E\(^d\). Steady-state levels of 6xHis-tagged Ii (0 h CHX treatment) were lower in triple mutant transfectants than in wt transfectants (also shown in Fig. 5). Densitometry reveals a modest increase in the rate of turnover of triple mutant 6xHis-Ii compared with wt (Fig. 6). This increase in rate of turnover may be sufficient to explain the differences in steady-state 6xHis-Ii levels.

FIGURE 4. The effect of CLIP affinity on cell surface I-E\(^d\) is less robust in the presence of DM. A20 cells transfected with mutant li constructs with (A) or without (B) a 6xHis tag were stained with 14-4-4S-FITC. MFI of staining on cells expressing mutant li is normalized to the appropriate (untagged or 6xHis-tagged) wt control within the same experiment. Data represent staining of polyclonal populations from multiple independent transfections and selections. Statistical significance was determined by paired the Student t-test: *, p < 0.05; **, p < 0.01; ***, p < 0.001. With sequential Bonferroni correction for multiple comparisons, the difference between triple mutant M90F-A93R-M98K and wt is not significant.

FIGURE 5. Increased affinity of CLIP for I-E\(^d\) is associated with an increase in total cellular abundance of I-E\(^d\). A, Total cellular levels of I-E\(^d\) in 3A5 transfectants were assessed by combined cell surface and intracellular FACs with 14-4-4S-FITC. MFI of staining on cells expressing mutant li is normalized to the wt control within the same experiment. Statistical significance is determined by paired the Student t-test: **, p < 0.01; ***, p < 0.001. All three comparisons show statistically significant differences even after correction for multiple comparisons. B, Western blotting was used to detect steady-state total cellular levels of I-E\(^d\) in titrated amounts of whole cell lysates from 3A5 transflectants. In-1 was used to detect total levels of endogenous and transfected li. Anti-Tetra-His was used to detect levels of transfected li. This experiment is one representative of several experiments. Results are consistent in the presence of both tagged and untagged li, comparing wt to M90F-A93R-M98K and M90F-A93K-M98K (data not shown). C, Densitometry of the bands shown in B.
Increased CLIP affinity impedes presentation of exogenous peptide, but not presentation of peptide processed from whole Ag

Increased cell surface levels of class II molecules on APCs are generally thought to be associated with increased capacity to stimulate T cells (38 - 40). However, despite considerable increases in cell surface I-E\(^\dagger\), presentation of an exogenously added, I-E\(^\dagger\)-restricted HEL peptide by 3A5 (DM\(^\dagger\)) transfectants to a T cell hybridoma is reduced in the presence of all but one (M90F) of the high-affinity CLIP mutants (Fig. 7, A - C). This effect is more pronounced at low, more physiologically relevant peptide concentrations, and can be overcome by the addition of excess peptide. In A20 (DM\(^\dagger\)) cells, effects of CLIP mutations on exogenous peptide presentation are diminished (Fig. 7D). The presentation of the same peptide from exogenously added whole HEL that must be processed by the APC is not impeded by the presence of high-affinity CLIP mutants (Fig. 7E). The whole Ag -presentation assays in A20 confirm that DM is able to mediate adequate exchange of the high-affinity mutant CLIP to allow presentation of antigenic peptides. As expected, 3A5 cells are unable to present this HEL peptide from whole HEL, because it is a DM-dependent epitope (data not shown).

### Discussion

The first goal of this study was to identify mutations that increase the strength of binding of CLIP to I-E\(^\dagger\) within a physiological range, where CLIP remains DM susceptible. We found that substitutions at the P1, P4, and P9 residues of CLIP (in the context of full-length Ii) had the most significant effects. Our results are consistent with published peptide-binding motifs (32 - 34) and with predictions from sequence and structural similarities and differences between I-E\(^\dagger\) and I-E\(^\dagger\) (our molecular modeling and Refs. 16 and 34). I-E\(^\dagger\) uses the common I-E \(\alpha\)-chain and has a \(\beta\)-chain with 92% sequence homology to that of I-E\(^\dagger\), a molecule whose structure has been solved (14, 15, 41). The P1 pockets of I-E\(^\dagger\) molecules are generally large, deep, and hydrophobic (37), preferring aromatic and aliphatic anchor residues. The P1 pockets of I-E\(^\dagger\) and I-E\(^\dagger\) differ only at \(\beta^{87}\); \(\beta^{87}\)F in I-E\(^\dagger\) reduces the pocket volume, while \(\beta^{87}\)S in I-E\(^\dagger\) leaves a pocket that accommodates large aromatic peptide anchors (Ref. 34, illustrated in Fig. 8A). Accordingly, we observed some increase in CLIP affinity for I-E\(^\dagger\) with aliphatic P1 mutants M90I and M90L, and a greater increase with the aromatic P1 mutant M90F (Table I). At the P9 pocket, I-E\(^\dagger\) and I-E\(^\dagger\) are essentially identical (Ref. 34 and our modeling). The I-E\(^\dagger\) P9 pocket is a long tunnel culminating in \(\beta^{9}\)E, which generally forms a salt bridge with the P9 residue (almost exclusively lysine) of bound peptides (15). The dramatic effect of the P9 CLIP mutant M98K is likely attributable to the formation of a similar salt bridge with I-E\(^\dagger\) (Fig. 8C). At the I-E\(^\dagger\) P4 pocket, aliphatic residues are favorable, but positively charged anchors are preferred (32 - 34). Accordingly, we found that A93R and A93K were more effective at P4 than A93I and A93F. Our modeling predicts that a positively charged P4 anchor is likely to form a salt bridge with a negatively charged residue (\(\beta^{70}\)D) at the side of the P4 pocket (Fig. 8B). We did not detect improvement in CLIP binding to I-E\(^\dagger\) for the four tested P6 mutations. Although a motif can be defined for the I-E\(^\dagger\) P6 pocket (32 - 34), many naturally occurring I-E\(^\dagger\) ligands adhere to binding motifs at the P1, P4, and P9 pockets, but not at P6 (34). It is likely that the P6 proline of wt CLIP is already acceptable, and it may also help maintain the polyproline type II helical conformation of class II-associated peptides. The favorable mutations at P1, P4, and P9 all yield similar phenotypes, suggesting that increasing CLIP affinity has common consequences, regardless of the pocket involved.

Another goal of our study was to elucidate the effects of increased CLIP affinity on class II in APCs. Our data show that high-affinity CLIP mutants protect against molecular turnover. This result reveals a biochemical basis for the chaperoning effect of high-affinity CLIP, consistent with the importance of peptide occupancy for class II stability and longevity (42 - 46). Ii mutants that caused the greatest stabilization of CLIP/I-E\(^\dagger\) complexes also resulted in increased cell surface and total cellular abundance of I-E\(^\dagger\). The mechanism for this chaperoning and increase in abundance of class II by high-affinity CLIP mutants is most likely very simple: increased affinity of CLIP for I-E\(^\dagger\) likely reduces spontaneous CLIP dissociation, protecting against the subsequent inactivation and loss of empty or unstably loaded I-E\(^\dagger\) molecules. The major advantage of CLIP over other potentially sufficient affinity peptides, which are thought to be present in limiting amounts in class II-loading compartments (44 - 48), is that it is preloaded in the ER in the context of full-length Ii. In the absence of DM, the increase in total I-E\(^\dagger\) was at least as large as the increase in cell surface I-E\(^\dagger\), arguing against redistribution of I-E\(^\dagger\) from intracellular compartments to the cell surface as a major effect.
and concentration, IL-2 production is normalized to wt within the same experiment.

**B–D.** Summary of data from multiple experiments. For each Ag concentration, IL-2 production is normalized to wt within the same experiment.  

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A simple model would predict that a reduction in I-E\textsuperscript{d} abundance in the presence of low-affinity CLIP would become apparent shortly after Ii degradation and CLIP generation. This would be consistent with a study showing that a mutant I-A\textsuperscript{d} molecule which is unable to stably bind peptide due to disruption of the hydrogen-bonding network near the P1 and P2 pockets is rapidly degraded in the endosomes upon loss of Ii (49). However, when de novo synthesis was inhibited with CHX, we observed a drop in the remaining I-E\textsuperscript{d} in the presence of wt CLIP only after the 8-h time point (Fig. 6), which is beyond the expected time frame for CLIP generation (Refs. 49 and 50 and Fig. 1) and endosomal residence (51). This may reflect a delay in degradation of I-E\textsuperscript{d} that is inactivated and/or aggregated shortly after CLIP generation. This scenario is supported by the fact that a pool of aggregated class II can be detected at steady-state in a B cell line (52). Alternatively, varying CLIP affinity may affect cell surface and recycling populations of class II, rather than nascent/endosomal class II. APC appear to discriminate between stable and unstable conformations of class II (45). The rescue of class II by high-affinity CLIP mutants and other high-affinity peptides could occur by providing sufficient stabilization to escape selective reinternalization and degradation of unstable class II.

Our ability to detect effects of mutant CLIP despite high levels of endogenous Ii suggests 1) that high-CLIP-affinity mutant Ii successfully competes with endogenous wt Ii for assembly with I-E\textsuperscript{d}, and 2) that CLIP may contribute directly to early steps in the assembly of Ii with nascent class II molecules. Levels of total cellular Ii were indistinguishable in transfected and untransfected A20, 3A5, and L cells (data not shown), suggesting that the amount of transfected Ii is low compared with endogenous Ii. Interestingly, we detected reduced steady-state levels and increased rate of turnover of 6xHis triple mutant Ii (Figs. 5 and 6, and data not shown). This may reflect the preferential assembly of high-CLIP-affinity Ii with nascent class II, and a subsequent increase in degradation as these Ii arrive in endosomes. Ii mutants M90L and M98L, which have \~{}24- and \~{}7-fold lower CLIP affinity for I-A\textsuperscript{d} than wt Ii, respectively (53), do not appear to compete effectively with endogenous wt Ii for assembly with I-A\textsuperscript{d} (data not shown). Within single APC, alleles with differing affinity for CLIP may compete for interaction with Ii in the ER, possibly contributing to allelic variation in abundance.

Variation in CLIP affinity for I-E\textsuperscript{d} also affects the efficiency of presentation of an I-E\textsuperscript{d}-restricted HEL epitope. For presentation of endogenous HEL peptide by DM-null cells, the difficulty of displacing tightly bound CLIP outweighs the benefit of the increase in available I-E\textsuperscript{d} molecules. However, there may be a “window” of intermediate CLIP affinity (P1 single mutant M90F) in which CLIP affinity is high enough to stabilize and increase cell surface levels of I-E\textsuperscript{d}, but still low enough to allow efficient peptide exchange, so that the outcome of the peptide presentation assay reflects the increased I-E\textsuperscript{d} at the cell surface. The inhibition of peptide presentation by high-affinity mutant CLIP is almost completely masked by the presence of DM. In DM\textsuperscript{+} APC, the majority of I-E\textsuperscript{d} molecules are loaded with a DM-edited repertoire of high-affinity peptides, and only the cohort of molecules that stochastically escapes DM editing may be affected by the modulation in CLIP affinity. In the presentation of peptide from whole HEL, there also may be a “window” of intermediate CLIP affinity (M98K and M90F-M98K), in which CLIP affinity is sufficiently high to preserve high I-E\textsuperscript{d} levels before interaction with DM, and sufficiently low to allow for efficient CLIP removal by DM in favor of antigenic peptides. Notably, the apparent windows for optimal presentation from peptide and from whole Ag are offset, such that with increasing affinity within a limited range, cell surface peptide exchange is minimized just as presentation of a DM-selected epitope is optimized. This trend suggests that increasing CLIP affinity for class II serves to restrict peptide loading to DM-containing compartments, ensuring proper editing of antigenic peptides. This model is consistent with a previous study which demonstrated a modest increase in the diversity of the I-A\textsuperscript{b}-
restricted peptide repertoire on splenocytes from cells with mutant low-CLIP-affinity Ii, as compared with wt high-CLIP-affinity Ii, even in the presence of DM (54).

We expect that class II alleles will be differentially affected by variation in CLIP affinity. Gautam et al. (55) examined the fate of I-A<sup>d</sup> and I-A<sup>β</sup> in the presence of mutant Ii with increased CLIP affinity for each class II allele. The high-CLIP-affinity Ii mutants moderately impaired Ag presentation from exogenous peptide and whole Ag. Honey et al. (54) used a reverse approach, choosing a high-CLIP-affinity allele, I-<sup>Aβ</sup>, and creating a low-CLIP-affinity mutant Ii. They noted an effect on cell surface I-A<sup>β</sup> levels in the absence of DM, although they did not elucidate the mechanistic basis of this phenotype, as we have done here. They also observed changes in peptide and whole Ag presentation and overall peptide repertoire diversity which, though modest, could be sufficient to perturb carefully regulated thresholds for T cell stimulation. Differential allelic susceptibility to effects of CLIP affinity is probably determined by several factors, including inherent dimer stability and efficiency of DM interaction. We would expect to see the greatest consequences of CLIP affinity in class II populations that have low inherent dimer stability and/or reduced DM interaction, either due to allelic variation in affinity for DM or to altered DM expression, localization, or regulation by DO in different APC.

These observations suggest numerous possible mechanisms for an influence of CLIP/class II affinity on susceptibility to autoimmunity. The Fathman and Nepom groups (56, 57) have proposed models in which the instability of type 1 diabetes-associated class II alleles (I-<sup>Aβ</sup> in NOD mice and DQ<sub>8</sub> or DQ<sub>3.2</sub> in humans) leads to reduced class II/peptide ligand density during T cell selection, which in turn leads to compensatory selection of T cells with particularly high affinity for self peptides. The reduced abundance and half-life of class II alleles in the presence of low-affinity CLIP could create a similar situation. Reduced class II levels due to low CLIP affinity could also impair thymic selection of regulatory T cells, which is thought to be dependent on strong recognition of self-peptide/MHC (58). Reduced CLIP affinity may also allow increased access of class II molecules to nonconventional, non-DM-regulated pathways of peptide loading, altering the peptide repertoire and possibly lowering the overall stability of the class II/peptide complexes, thus increasing the susceptibility of those complexes to further unsupervised peptide exchange at the cell surface. It is tempting to speculate that such cell surface exchange could contribute to presentation of cryptic self Ags, epitope spreading, and/or initiation of an autoreactive immune response if an extracellular source of peptides became available. Inflammation and tissue damage are likely to provide both the proteolytic environment to make peptides available and a reduced pH that assists peptide exchange (Refs. 59–62 and references therein). As the effects of CLIP affinity on class II abundance are more robust in the absence of DM, the impact of varied CLIP affinity on immune responses will be highest in cells with limited DM activity. In nonprofessional APC in which class II expression is induced by IFN-γ, failure of class II and DM to colocalize in peptide-loading compartments can allow class II to escape DM editing and chaperoning despite adequate expression levels of DM (63). Coexpression of DO can also reduce DM function. DO is found in naive B cells, specific immature DC subsets, medullary thymic epithelial cells, and in cells lining Hassall’s corpuscles, a thymic structure involved in selection of regulatory T cells (6, 8, 9, 64, 65). These cells are involved in thymic selection and maintenance of peripheral tolerance, suggesting that variation in CLIP affinity also may be influential in establishment and maintenance of tolerance. Finally, multiple experimental systems suggest that prolonged or increased presence of CLIP may favor a Th2 response over a Th1 response (66–68), a skewing expected to be protective against many autoimmune diseases.

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Disclosures

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References
