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Structural Factors Contributing to DM Susceptibility of MHC Class II/Peptide Complexes

Michael P. Belmares,* Robert Busch,† Kai W. Wucherpfennig, §§ Harden M. McConnell,* Elizabeth D. Mellins2†

Peptide loading of MHC class II (MHCII) molecules is assisted by HLA-DM, which releases invariant chain peptides from newly synthesized MHCII and edits the peptide repertoire. Determinants of susceptibility of peptide/MHCII complexes to DM remain controversial, however. Here we have measured peptide dissociation in the presence and the absence of DM for 36 different complexes of varying intrinsic stability. We found large variations in DM susceptibility for different complexes using either soluble or full-length HLA-DM. The DM effect was significantly less for unstable complexes than for stable ones, although this correlation was modest. Peptide sequence- and allele-dependent interactions along the entire length of the Ag binding groove influenced DM susceptibility. We also observed differences in DM susceptibility during peptide association. Thus, the peptide repertoire displayed to CD4+ T cells is the result of a mechanistically complicated editing process and cannot be simply predicted from the intrinsic stability of the complexes in the absence of DM. The Journal of Immunology, 2002, 169: 5109–5117.

The structural requirements for peptide binding to MHCII are well understood. The peptide occupies a groove at the membrane-distal end of the MHCII molecule, with both termini protruding from the groove (8–12). Peptide binding involves interactions between peptide side chains and specificity pockets lining the groove (with P1, -4, -6, and -9 interactions contributing the most). These interactions can vary due to differences in peptide sequence or allelic variation in the pocket residues, which explains differences in stability between peptide/MHCII complexes (13–16) and allele-specific sequence motifs of class II-associated peptides (3). Quantitative algorithms predict peptide/MHCII affinity from sequence (13–16), but are less successful at predicting epitopes recognized during immune responses. In addition to sequence-dependent peptide/MHC interactions, conserved hydrogen bonds between the peptide backbone and the MHCII molecule tether the peptide in the groove in a sequence-independent manner (8–11, 17), contributing critically to the overall stability of the complex (18–20).

Several other molecules are involved in endosomal peptide loading of MHCII molecules (4, 21, 22). Invariant chain (Ii) associates with nascent MHCII molecules, occupies the Ag binding groove, and prevents binding of other ligands. Assembly with Ii results in export of the complexes to endosomes, where cathepsins proteolyze Ii, leaving a nested set of Ii peptides spanning residues 81–104 (collectively termed CLIP, for class II associated Ii peptides) in the groove. HLA-DM promotes the exchange of CLIP for endosomal peptides. It also alters the repertoire of non-CLIP peptides that bind to MHCII molecules, affecting the recognition of exogenous (23) and alloantigens (24, 25), and introduces a bias toward DM-resistant complexes (peptide editing) (26). DM does not measurably bind peptides (R. Busch and M. P. Belmares, unpublished observations); rather, it binds MHCII molecules (27, 28) and prefers empty MHCII molecules (28), stabilizing them against loss of binding activity (29). In vitro, DM catalyzes the release of CLIP and other peptides in an enzyme-like fashion, with optimal activity at endosomal pH (30–34), accounting for CLIP release and peptide editing by DM.

Human and murine DM crystal structures revealed similarity to MHCII, except for lack of a binding groove (35, 36). Mutagenesis identified the DM interaction surface of HLA-DR as a lateral face, comprising residues on the membrane-proximal and peptide-binding domains near the peptide N terminus (37).

The structural parameters that determine the DM susceptibility of peptide/MHCII complexes remain unclear. Early studies suggested that unstable complexes are DM susceptible, whereas stable ones are resistant (30). Peptide length and destabilizing glycine and proline residues were reported to influence DM susceptibility (38, 39). However, another study found that dissociation of stable and unstable complexes was accelerated by DM to a similar extent (40). This suggested that DM disrupts the conserved hydrogen bond network, rather than side chain/pocket interactions. In contrast, our recent studies confirm that different peptide/DR complexes are differentially susceptible to DM (41). In another report...
stable occupancy of the P1 pocket rendered peptide/DR complexes DM resistant (42). All results are consistent with functional effects of DM on the peptide repertoire, although models derived from each dataset would make different predictions about the fate of any given peptide/DR complex. Here, we have attempted to identify structural determinants of DM susceptibility. We found that the intrinsic stability of peptide/DR complexes is a weak predictor of DM susceptibility and that interactions along the entire length of the peptide-binding groove contribute to DM susceptibility.

Materials and Methods

Expression and purification of recombinant MHCII molecules

A cDNA for soluble DRB1*0404 was engineered as described previously for DR*0402 (41). Soluble HLA-DR4 (∗0401, ∗0402, and ∗0404) molecules were produced as previously described (30, 41). Soluble HLA-DR*1501 (DR2b) with a covalently linked myelin basic protein (MBP) peptide was generated as previously described (43, 44) and expressed in insect cells, using a 2-ml Sephadex G-50 super

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nal purification of preloaded complexes (0.62/H9262 complexes (MBP f84

5110 DM-MEDIATED PEPTIDE EXCHANGE

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Vegetation Software, Reading, PA).
Table I. Dissociation half-lives of soluble peptide/MHCII complexes in the absence (t_{1/2, in}) and presence of 0.25 μM soluble HLA-DM (t_{1/2, obs}) and the calculated dissociation enhancement (t_{1/2, obs}/t_{1/2, in}).

<table>
<thead>
<tr>
<th>Soluble DR</th>
<th>Peptide</th>
<th>Sequence</th>
<th>t_{1/2, in} (h) Intrinsically</th>
<th>t_{1/2, obs} (h) + dDM</th>
<th>Dissociation Enhancement t_{1/2, obs}/t_{1/2, in}</th>
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<td>0401</td>
<td>Human MBP f_{64-99}</td>
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<td>1.3</td>
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<td>0.16</td>
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<td>0.99</td>
<td>19</td>
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<tr>
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<td>97</td>
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<td>Human HCgp-39 f_{262-276}</td>
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<td>1.1</td>
<td>97</td>
</tr>
<tr>
<td></td>
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<td>180^b</td>
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<td></td>
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<td>600^g</td>
<td>2.7^h</td>
<td>230</td>
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</tbody>
</table>

- The P1 anchor residue of each peptide is underlined. The first column lists the MHCII allele. The second and third columns provide the name and sequence of the peptide used to form a complex with the MHCII. In most cases the dissociation half-lives of the complexes were calculated from single-exponential fits of the fraction of complex remaining after various times (cf Fig. 1). Dissociation experiments were continued until at least 87.5% of the complex dissociated (i.e., for at least three half-lives), unless indicated. The dissociation experiments were performed in PBS/citrate buffer, pH 5.3, at 37°C. Half-lives are averages of at least two independent experiments, and typical variations in t_{1/2} are <20%. The smallest and largest dissociation enhancements for each allele are underlined. Hb, hemoglobin.
- Data from Ref. 41. The half-lives of complexes were corrected for differences in DM activity between Ref. 41 and the present report.
- The P1 anchor residue of each peptide is underlined. The second column lists the MHCII allele. The second and third columns provide the name and sequence of the peptide used to form a complex with the MHCII. In most cases the dissociation half-lives of the complexes were calculated from single-exponential fits of the fraction of complex remaining after various times (cf Fig. 1). Dissociation experiments were continued until at least 87.5% of the complex dissociated (i.e., for at least three half-lives), unless indicated. The dissociation experiments were performed in PBS/citrate buffer, pH 5.3, at 37°C. Half-lives are averages of at least two independent experiments, and typical variations in t_{1/2} are <20%. The smallest and largest dissociation enhancements for each allele are underlined. Hb, hemoglobin.
- Data from Ref. 41. The half-lives of complexes were corrected for differences in DM activity between Ref. 41 and the present report.
- Data from Ref. 49.
- The fit of the dissociation kinetics may be slightly improved by a double-exponential function.
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In conclusion, we have shown that the rate of DM-dependent dissociation of complexes is strongly dependent on the identity of the peptide/DR complex. The intrinsic stability of the complexes was distributed relatively evenly over 3 orders of magnitude (Table I and Fig. 3), ranging from minutes to weeks. Thus, the choice of complexes did not introduce undue bias in this regard. Interestingly, DM susceptibility also varied over 3 orders of magnitude in this assay (Table I and Figs. 1 and 3). Thus, the magnitude of the DM effect depended strongly on the identity of the peptide/DR complex.
Dependence of DM susceptibility on intrinsic stability

To investigate the relationship between intrinsic stability (as measured by $k_{\text{in}}$) and DM susceptibility, we plotted $k_{\text{in}}$ against $k_{\text{obs}}$ on a logarithmic scale (Fig. 3A). A significant correlation was observed between intrinsic stability and stability in the presence of DM ($p < 0.0001$). However, the intrinsic stability of any one complex was a poor predictor of DM susceptibility, as the correlation coefficient was only modest ($r = 0.693$). Greater than 3-fold (0.5 log) deviations from the best-fitting straight line were seen in seven of 36 (19%) complexes. This is highly unlikely to reflect experimental error, which was $<20\%$ for most complexes. In one particularly striking instance, an extremely stable complex, DR*0401/HC gp39262–276, was almost entirely resistant to DM. (This complex was treated as an outlier in statistical analyses.) Interestingly, the slope of the best-fitting straight line was 0.47 (95% confidence interval, 0.30–0.64). In contrast, a slope of 1

**FIGURE 1.** Representative dissociation kinetics. The dissociation of f-MBP84–99/HLA-DR*0402 (A), f-MBP84–99/HLA-DR*0401 (B), murine hemoglobin64–76 V67I I68V/HLA-DR*0402 (C), and human HLA-DM (D) were measured at 37°C, pH 5.3 with (○) or without (□) 0.25 μM soluble HLA-DM (see Materials and Methods). The fluorescence value at time zero was arbitrarily set at 1. Each solid line is a fit of the data to a single exponential model. Excess of an unlabeled peptide competitor was added to prevent f-peptide rebinding to MHCII. The insets in A and C show dissociation kinetics at early times.

**FIGURE 2.** Linear dependence of the dissociation rate constant on HLA-DM concentration. The dissociation rate constant ($k$) of various f-peptide/MHCII complexes was measured as a function of DM concentration. The solid lines are best fits of the data for each complex to $k_{\text{obs}} = k_{\text{in}} + k_{\text{DM}}[\text{DM}]$, where $k_{\text{in}}$ is the measured rate constant of the complex in the absence of DM, $k_{\text{DM}}$ is the best-fit slope, and [DM] is the concentration of DM. The complexes are MBP84–99/DR*0404 (A), HC gp39262–276/DR*0402 (B), hemoglobin mutant (f-GKKIVTAFNEGLK)/DR*0402 (C), murine HLA DR2b (D), and MBP84–99/DR*0401 (E).
would have been expected if all complexes were equally susceptible to DM: for $k_{obs}/k_{in}$ = constant, a log/log plot would obey the equation, log $k_{obs} = \log k_{in} +$ constant. The implication is that, on the average, DM susceptibility (as represented by the vertical distance between the observed log $k_{obs}$ values and a straight line of slope 1 through the origin; see Fig. 3) increases with increasing complex stability (or with decreasing values for slope 1 through the origin; see Fig. 3) increases with increasing $k_{t}$. Extrapolation of this line suggests that a complex with an intrinsic lifetime ($t_{1/2}$ $k_{in}$) of 1.24 min should not be affected substantially by DM under these experimental conditions. We conclude that intrinsic stability only modestly predicts DM susceptibility and that other factors are likely to be involved as well.

**Role of allelic polymorphism and N/C-terminal peptide overhangs**

To search for other factors that predict DM susceptibility, we investigated subsets of these data, stratified by MHCII allele (Fig. 3B), the length of N- or C-terminal overhangs (Fig. 3, C and D, respectively), and CLIP vs non-CLIP peptides (not shown). Overhangs were assigned based on alignment of peptides to known binding motifs for the different MHCII alleles and were verified in some instances by mutating putative P1 anchor residues (M. Belmares, unpublished observations; cf Table I). No significant differences were detected between MHCII alleles (Fig. 3B), peptides of varying N termini (Fig. 3C), or CLIP and non-CLIP peptides ($p > 0.05$ for all comparisons, excluding the outlier). There was a suggestion that C-terminal extensions influence the relationship between intrinsic stability and DM susceptibility ($p = -0.05$ for all comparisons, excluding the outlier). The interpretation of this putative effect is in doubt, because many peptides with long C-terminal overhangs also happened to have extended N termini.

Individual comparisons between closely related peptides differing only in their N-terminal overhangs also failed to reveal strong effects on DM susceptibility. For instance, no differences were observed when N-terminally extended vs short human CLIP peptides were compared or when a nonconservative (V$\rightarrow$K) substitution was made in the P(-1) position of MBP 84-99 (Table I). These findings are consistent with previous reports (40) and our own observations (M. P. Belmares and R. Busch, unpublished) that the N-terminal peptide overhang does not substantially affect DM activity.

**Comparisons of structurally related complexes**

To obtain more information about structural factors influencing DM susceptibility, we compared other closely related complexes.
In considering differences in DM susceptibility ($k_{\text{obs}}/k_{\text{in}}$), the overall correlation between $k_{\text{in}}$ and $k_{\text{obs}}$ (Fig. 3A) is a possible confounding factor, since a structural change may affect DM susceptibility simply by virtue of its effect on intrinsic stability. Specifically, from the correlation in Fig. 3A, a 10-fold (1 log) change in $k_{\text{in}}$ predicts a $10^{0.47}$ or 3.0-fold increase in $k_{\text{obs}}$ and a $10^{0.53}$ or 3.4-fold decrease in $k_{\text{obs}}$. Thus, to identify effects on DM susceptibility that are independent of intrinsic stability, we examined the deviations of observed data points from the best-fit line for pairs of complexes. One special case occurs when two structurally related complexes are equally DM-susceptible despite differing substantially in intrinsic stability: in this case, the lack of a net change in DM susceptibility may reflect compensating effects. Examples of these different scenarios follow.

Roles of the P1 anchor and C-terminal anchor residues

Based on individual comparisons, we found one example suggesting that the peptide P1 anchor may influence DM susceptibility. A mutation from Val to Ile in the P1 anchor of the peptide f-MFBI84–99, which stabilizes a complex with HLA-DR*1501 by 6-fold in the presence and the absence of DM, leaves the dissociation enhancement by DM ($k_{\text{obs}}/k_{\text{in}}$) unchanged. The change at P1 thus reduces DM susceptibility 2.6-fold from the value predicted by its effect on intrinsic stability (Fig. 3A). P1 anchor effects do not appear dominant, however: a wide range of dissociation enhancement by DM is seen among peptide/DR*0401 complexes where the peptide P1 anchor is Phe (Table I). These results suggest that at least some of the variations in DM susceptibility are due to peptide/MHCII interactions outside the P1 pocket.

Consistent with this possibility, comparisons between closely related complexes suggest that C-terminal anchor residues (P4–P10) can influence DM susceptibility. One informative comparison involves the following complexes: f-KPVSQMRMATPLLLR/DR4*0402 (t1/2,ms = 1.4 h; $k_{\text{obs}}/k_{\text{in}}$ = 19.4), f-KPVSQMRMATPLL/DR4*0402 (t1/2,ms = 11.4 h; $k_{\text{obs}}/k_{\text{in}}$ = 21.5), and f-KPVSQMRMATQAPMR/DR4*0402 (t1/2,ms = 340 h; $k_{\text{obs}}/k_{\text{in}}$ = 73.1). These peptides are murine f-Ii 85–99 variants with a mutation(s) in the C-terminal sequence (P5–P10 positions, shown in bold) but with identical N-termini. The Met P1 anchor of each peptide is underlined. In qualitative agreement with the trend shown in Fig. 3A, the DM susceptibility is 3.8-fold larger for the most stable peptide than for the least stable one. However, the difference is ~5-fold less than might have been expected from the correlation in Fig. 3A. Similarly, the change in DM susceptibility due to the Leu228Glu change in P9 is less than expected from the change in intrinsic stability, which increases 8-fold and predicts a 2.8-fold difference in DM susceptibility (as shown in Fig. 3A). These results suggest that DM susceptibilities are influenced (in these cases) both by differences in the intrinsic stability of the complexes and by compensatory effects of the C-terminal substitutions.

In a second example, the DM susceptibilities of complexes between DR*0401 and the peptides f-KMRMATPLLMQALPM (t1/2,ms = 6.0 h; $k_{\text{obs}}/k_{\text{in}}$ = 13) and f-QMRMATPLLMR (t1/2,ms = 7.8 h; $k_{\text{obs}}/k_{\text{in}}$ = 42) are measurably different, even though their intrinsic stability is essentially the same. The core sequences in both peptides are the same, with differences in bold. As discussed above, the Gln/Lys difference in the P(-1) position is not likely to account for the dissociation enhancement differences, leaving the C-terminal difference as a more likely explanation. Taken together, these examples argue that C-terminal anchor residues can affect the DM susceptibility of peptide/MHCII complexes independently of their effects on intrinsic stability.

FIGURE 4. DM susceptibility of full-length and soluble MHCII/peptide complexes. The susceptibility of various soluble MHCII/peptide complexes to soluble DM ($k_{\text{obs}}/k_{\text{in}}$) was plotted against the susceptibility of the corresponding full-length MHCII/peptide complexes to full-length DM (y-axis). In order of increasing susceptibility to soluble DM (x-axis), the complexes tested were HCGp-39281–311/DR*0401, HCGp-39322–337/DR*0402, HCGp-39298–331/DR*0402, HCGp-39262–278/DR*0402, and HCGp-39298–331/DR*0402. Note the similar rank order for full-length and soluble complexes.

Differential susceptibility of full-length MHCII/peptide complexes to full-length DM

Removing the membrane anchors of either DM or DR decreases the efficiency of interaction by at least 2 orders of magnitude (45, 58). This is the main functional difference between soluble recombinant and native DM. To address whether the membrane anchors reduce the effect of peptide structure on DM susceptibility, we assayed peptide dissociation using full-length recombinant DM and DR molecules. Fig. 4 compares the dissociation enhancements for full-length DM and full-length DR/peptide complexes with values observed using soluble molecules. Even though under the conditions used, DM effects generally are less pronounced in the full-length system, we found a clear correlation between the DM susceptibility of different complexes in both systems. Enhancement of dissociation by full-length DM ranged between 2.2- and 16.3-fold (a 7.5-fold variation), compared with a 14.7-fold variation in DM enhancement (range, 7- to 103-fold) for the same complexes using soluble molecules. Thus, substantial variation in DM susceptibility exists for full-length molecules. Quantitative differences could be due to a genuine effect of the membrane anchors or to assay differences, including the use of detergent to solubilize full-length molecules.

Effect of HLA-DM on the relative association rates of two peptides to MHCII

To test whether differences in DM susceptibility between complexes are also detectable during association, we measured the relative association rates of two peptides to MHCII with and without HLA-DM. Upon dissociation of a pre-bound peptide, MHCII molecules are released in a peptide-reactive state, allowing rapid binding of added peptides (48). However, the peptide-reactive state inactivates rapidly ($t_{1/2} \leq 10$ s; M. P. Belmares, unpublished observations), making measurements of absolute association rates difficult. Even the best methods currently available for estimating absolute on-rates make critical simplifying assumptions regarding both the mechanism of peptide association to the peptide-reactive state and the mechanism of MHCII inactivation (48, 59). However, deviations from these assumptions have been noted previously (48, 59) (M. P. Belmares et al., unpublished observations). It is unclear to what extent such complications might interfere with the detection of small differences in on-rates. However, we reasoned that these difficulties might be circumvented by measuring the relative association rates of two different peptides. To this end we decided...
Discussion

Whether all peptide/MHCII complexes are equally susceptible to DM-catalyzed dissociation has been controversial (30, 38–42). Our survey of a wider range of peptides and MHC class II alleles clearly established that there are large variations in DM susceptibility, ranging over several orders of magnitude. Large differences in DM susceptibility were seen for different peptides binding to the same MHCII allele as well as for one peptide binding to different MHCII alleles. Our results thus establish a role for both peptide sequence and MHC polymorphism in controlling DM susceptibility.

The correlation between DM susceptibility and intrinsic stability (Fig. 3A) has interesting implications for the mechanism by which DM causes peptide release. It was surprising that, on the average, less stable peptides tended to be less susceptible to DM than stable ones. Since differences in intrinsic stability between complexes are controlled by peptide side chain/MHCII specificity pocket interactions, the correlation suggests that DM disrupts side chain/pocket interactions. However, the disruption is not complete; otherwise, dissociation rates in the presence of DM would not differ between peptides and would be much faster than those observed here. In addition, the fact that the correlation between \( k_{\text{obs}} \) and \( k_{s} \) is modest suggests that DM not only disrupts side chain/pocket interactions, but also distorts the Ag binding groove, probably including disruption of conserved hydrogen bonds.

An additional factor to consider is the rate of bimolecular association of DM and peptide/MHCII complexes. The dissociation rate of the least stable complexes in the presence of DM (mur. flIi 89–99/DR*1501; Table I) establishes a lower limit for the bimolecular DM/DR association rate as \( \geq 2 \times 10^{3} \text{M}^{-1}\text{s}^{-1} \). This is based on the assumption that the DM-catalyzed peptide release cannot be faster than the rate at which DM and DR/peptide complexes collide. Based on this estimate, we conclude that under the conditions used, DM/DR association may become rate limiting at dissociation half-times of 2.5 min or less.

The length of the N-terminal overhang did not correlate significantly with DM susceptibility. Furthermore, although there were hints that interactions involving the P1 pocket might affect DM susceptibility, this pocket was not unique in that regard, with C-terminal interactions making contributions of similar or greater magnitude. These findings are surprising, because recent mutagenesis studies have mapped the DM interaction site on HLA-DR to a lateral face near the peptide N terminus (37), and there is evidence that the P1 pocket, near this N terminus, controls DM resistance (42). Interestingly, a conformational change involving DR \( \beta_{38–60} \), near the C terminus of bound peptides, has been detected upon peptide binding to the MHCII groove using a mAb specific for this region of the molecule (60). Interaction between DR and DM may, among other changes, perturb this region, which is modulated by C-terminal peptide-MHCII interactions. Overall, our results are most consistent with a model in which DM causes a global conformational change in the Ag binding groove.

Recently, it was speculated that DM facilitates peptide dissociation by selectively destabilizing one or two hydrogen bonds near the peptide N terminus (35). Indeed, disruption of the peptide backbone, greatly enhances peptide release (18–20). Hydrogen bond disruption in this system leads to a greater dissociation enhancement for less kinetically stable complexes. If DM promoted peptide release by a similar mechanism, we would predict a slope of >1 in plots of \( \log k_{\text{obs}} \) vs \( \log k_{s} \). In contrast, we observed that the dissociation enhancement by DM tended to be greater for complexes with higher kinetic stability, with a best-fit slope of 0.47 in Fig. 3A, although significant variation occurred. We conclude that while the mechanism of DM action may include disruption of conserved hydrogen bonds, this is unlikely to be its only mechanism.

Single substitutions often had only slight effects on DM susceptibility. If DM susceptibility is controlled cumulatively by spatially distributed interactions, this might explain why such effects were
misused by Weber et al. (40), who employed hybrid peptides combining sequences from a small number of parent peptides, each of which had a similar degree of DM susceptibility to begin with. DM has been reported to act as a peptide editor in APC, enhancing presentation of some peptide/class II complexes while extinguishing others (23–25). It has been proposed that DM performs a kinetic proofreading function by accelerating peptide dissociation. Thus, complexes that dissociate rapidly in the presence of DM (compared with the endosomal residence time of MHCII molecules) would be at a disadvantage relative to complexes that survive in the presence of DM. Our finding that DM differentially enhances the dissociation of different complexes is compatible with a kinetic proofreading model. However, we show that the effects of kinetic proofreading by DM cannot be predicted accurately from the intrinsic stability of peptide/MHCII complexes in the absence of DM. In the future, experimental strategies and computational algorithms to predict immunodominant epitopes from protein sequences might be improved by explicitly considering differences in DM susceptibility. However, achieving this will require extensive follow-up studies.

Our observation that peptide association rates can also be differentially affected by DM adds another layer of complexity, whose impact on Ag presentation is difficult to assess. Critical unknown parameters include both the effective concentration and unknown parameters include both the effective concentration and the time of exposure to DM in vivo. One intriguing possibility is that both highly DM-susceptible and highly DM-resistant complexes might be removed during DM editing; the resistant ones would be at a disadvantage during peptide association, whereas the susceptible ones would be removed during dissociation. The net outcome might be that most immunodominant peptides that have undergone DM editing in vivo would be selected to possess an average degree of DM susceptibility, as originally proposed by Weber et al. (40), based on studies of several immunodominant peptides and CLIP. Our study may have revealed a larger range of DM susceptibility by including non-natural peptide/MHCII combinations.

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References


