

The impact of DM on MHC class II-restricted antigen presentation can be altered by manipulation of MHC-peptide kinetic stability

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DM edits the peptide repertoire presented by major histocompatibility complex class II molecules by professional antigen-presenting cells (APCs), favoring presentation of some peptides over others. Despite considerable research by many laboratories, there is still significant uncertainty regarding the biochemical attributes of class II-peptide complexes that govern their susceptibility to DM editing. Here, using APCs that either do or do not express DM and a set of unrelated antigens, we found that the intrinsic kinetic stability of class II-peptide complexes is tightly correlated with the effects of DM editing within APCs. Furthermore, through the use of kinetic stability variants of three independent peptides, we demonstrate that increasing or decreasing the kinetic stability of class II-peptide complexes causes a corresponding alteration in DM editing. Finally, we show that the spontaneous kinetic stability of class II complexes correlates directly with the efficiency of presentation by DM⁺ APCs and the immunodominance of that class II-peptide complex during an immune response. Collectively, these results suggest that the pattern of DM editing in APCs can be intentionally changed by modifying class II-peptide interactions, leading to the desired hierarchy of presentation on APCs, thereby promoting recruitment of CD4 T cells specific for the preferred peptides during an immune response.

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Abbreviations used: HEL, hen egg lysozyme; SWM, sperm whale myoglobin.

Stimulation of antigen-specific CD4 T cells requires the presentation of immunogenic peptide epitopes in association with MHC class II molecules on the surface of professional APCs. The assembly and export of these class II-peptide complexes is a regulated multistep process, culminating in the exchange of CLIP for peptides derived from exogenous or endogenous sources. The class II-like protein DM plays a critical role in facilitating the endosomal exchange reaction. Because DM is a vital component in the peptide exchange reaction, a significant effort has been put forth to understand the nature of the interaction between DM and class II and the consequences of this association upon peptide exchange. Initial studies indicated that DM does not bind peptides itself, probably due to a closed peptide binding groove (1, 2), but rather associates with class II molecules and promotes the release of peptides,

such as CLIP, from the class II binding groove (3–5). DM associates with the class II molecule along the lateral face that accommodates the amino terminus of the antigenic peptide (6, 7). This interaction between class II and DM can prevent inactivation of the class II molecules (8) and sustains their capacity to bind peptides at a low pH (9). Further experiments demonstrated that DM enhances peptide dissociation from class II molecules in a pH-dependent manner (3, 10, 11). Although the physical association of DM with class II molecules is well documented, the underlying mechanisms that govern this interaction are still unknown, as are the factors influencing the subsequent peptide exchange reaction.

“DM editing” refers to the process by which the peptide repertoire presented by MHC class II molecules is modified or “edited” by the DM protein. This biological activity of DM has been revealed by several independent assays. These assays include elution

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and sequencing of peptides bound to class II molecules isolated from APCs that do or do not express DM (12, 13) through functional studies of the peptide repertoire presented by class II using peptide-specific T cells (14–19), the analysis of peptides that bind to class II *in vitro* in the presence or absence of DM (20), or class II–peptide dissociation assays *in vitro* showing differential susceptibility of alternative complexes to DM-promoted dissociation (10, 11, 21–24). That DM editing has important immunological implications was made clear by antigen presentation studies showing that presentation of immunodominant epitopes (those class II–peptide complexes that elicit a robust CD4 T cell response) is enhanced by DM whereas presentation of cryptic peptides (those that do not elicit a response when they are contained in an intact complex protein) is antagonized by DM expression within APCs (25). Collectively, these studies have all suggested that DM editing is selective and have stimulated considerable research toward identifying the characteristics of the class II–peptide complex that can influence the susceptibility to DM editing. Structural features, including peptide length (26), destabilizing amino acid residues (20), the rigidity of the P1 pocket of the class II molecule (27), T cell contact residues (23), and the peptide main chain hydrogen bond network (24), have all been shown to influence DM editing. Although the potential complexity of these overlapping interactions suggests that algorithms to predict the effect of DM may be beyond experimental reach, early studies (10, 13, 21) suggested that the most salient feature that predicted susceptibility to DM editing was the kinetic stability of the class II–peptide complex, where stable peptides were resistant and unstable peptides were susceptible to removal by DM. Subsequent work by Weber et al. (11) suggested that DM was acting as a catalyst for all complexes by accelerating dissociation of all complexes tested to a constant degree, termed the J factor. This direct relationship between kinetic stability and DM activity was challenged, however, in an extensive analysis of nearly 40 independent class II–peptide complexes, representing a diverse range of kinetic stabilities (22). These studies identified a wide range in susceptibility to DM editing, even for class II–peptide complexes of similar off-rates. Furthermore, in analyzing all of their data, these authors found that the modest trend that was observed showed paradoxically that the least stable class II–peptide complexes were the most resistant to negative editing, unlike earlier findings by other groups (10, 13, 21).

The contradictory conclusions drawn from biochemical studies performed thus far made it seemingly difficult to predict qualitative and quantitative relationships between class II–peptide complexes and DM editing. Moreover, within APCs, the complexities of the interaction between DM and class II molecules might additionally confound predictions derived from *in vitro* studies with purified class II–peptide complexes. DM may participate at multiple stages during the transit time of class II through the endosomal loading compartment. Also, DM may interact both during formation and subsequent dissociation of the peptide from the class II com-

plex, and additionally, this interaction may be restricted temporally or spatially. Despite the difficulty in deriving a clear relationship between the kinetic stability of class II–peptide complexes and DM editing from currently published *in vitro* studies using purified class II and DM proteins, our laboratory recently uncovered a striking relationship between the kinetic stability of class II–peptide complexes and immunodominance of CD4 T cell responses (28). Our data demonstrated that a high kinetic stability conferred immunodominance, while conversely, low stability interactions with class II molecules led to crypticity. These recent results, together with our previous investigations discussed above showing that immunodominant and cryptic epitopes were readily distinguishable based on their susceptibility to DM editing within APCs *in vitro* (29), suggested the possibility that highly stable class II–peptide complexes are immunodominant due to the activity of DM within the endosomal loading compartments of APCs.

In the studies described here, we sought to rigorously evaluate whether the kinetic stability of the class II–peptide complex controlled DM editing in the context of APCs during processing and presentation of exogenous antigen. To explore the activities of DM upon peptide presentation on class II molecules within APCs, we examined a diverse set of peptides and encoded them or their variants within a protein vector, thus allowing us to manipulate their kinetic stability with class II. These recombinant proteins were then used in antigen presentation experiments to sensitize APCs that did or did not express DM. The efficiency of antigen presentation was monitored via stimulation of antigen-specific T cell hybridomas. Our experiments demonstrate that the intrinsic kinetic stability of class II–peptide complexes indeed correlates with their presentation in the presence of DM. The contribution of kinetic stability to survival and export of the class II–peptide complex occurs independently of processing requirements or sequence origin. Most importantly, we demonstrate that the magnitude of DM enhancement or antagonism can be rationally modulated through conservative changes in the peptide, leading to the desired changes in the kinetic stability of class II–peptide complexes.

RESULTS

A peptide's susceptibility to DM editing in APCs depends upon its spontaneous kinetic stability with MHC class II molecules

In our initial study on the consequence of DM expression upon the display of class II–peptide complexes, we found a consistent correlation between DM's effect on presentation of epitopes from the model antigens hen egg lysozyme (HEL) and sperm whale myoglobin (SWM) and the immunodominance and crypticity *in vivo* (29). The efficiency of antigen presentation and surface display of class II–peptide complexes was estimated through comparative dose response curves using DM⁺ or DM⁻ APCs and assessment of activation of epitope-specific T cell hybridomas via IL-2 production. Presentation of the cryptic epitopes HEL [11–25] and HEL

Table I. Presentation of immunodominant and cryptic epitopes by APCs expressing DM

Epitope	$t_{1/2}$ (h)	Response in vivo	Presentation by APCs expressing DM
H-2L ^d [69-81]	0.2	cryptic	antagonized
OVA [327-339]	0.3	cryptic	antagonized
HEL [20-35]	4	cryptic	antagonized
HEL [11-25]	6	cryptic	antagonized
HEL [102-116]	ND	immunodominant	enhanced
OVA [273-288]	160	immunodominant	enhanced
LACK [156-173]	170	immunodominant	enhanced
SWM [102-118]	260	immunodominant	enhanced

The patterns of presentation of protein antigens encoding the listed epitopes was determined by comparative IL-2 production of antigen-specific T cells stimulated by APCs expressing DM and graded doses of native protein antigen. The half-life was calculated from the exponential equation fitted to the fluorescence decay curve as a function of the incubation time and described as the time required to dissociate 50% of the FITC peptide initially bound to I-A^d. Data are representative of at least three independent experiments.

[20–35] was antagonized by DM expression, whereas presentation of the immunodominant HEL [102–116] and immunodominant SWM [102–118] epitopes was enhanced by DM expression. We have since expanded on these earlier published findings and examined the effects of DM expression in APCs on the efficiency of presentation of a larger set of independent, structurally unrelated epitopes. Table I shows a summary of these results demonstrating that presentation of the immunodominant epitopes HEL [102–116], OVA [273–288], SWM [102–118], and LACK [156–173] derived from exogenous native protein was enhanced by DM expression in APCs. When measured for their spontaneous off-rate from class II molecules using purified I-A^d molecules (30), we found that each of these complexes also displayed exceedingly long half-lives ($t_{1/2} > 150$ h) at endosomal pH. Conversely, the presentation of the cryptic epitopes HEL [11–25], HEL [20–35], and OVA [327–339] from native exogenous antigen, as well as the endogenous H-2L^d [69–81], was inhibited by DM expression in APCs. Measurements of the kinetic stability of these DM-antagonized epitopes revealed extremely short half-lives of association with I-A^d ($t_{1/2} < 10$ h). These results suggested that the half-life of class II–peptide complexes is a critical parameter that dictates the effect of DM-regulated class II–restricted antigen presentation.

The efficiency of presentation by DM-expressing APCs can be up- or down-modulated by altering the kinetic stability of class II–peptide complexes

To more rigorously examine the linkage between kinetic stability and DM editing in APCs, we selected the MalE protein vector to encode peptides that could be used for presentation by APCs that either did or did not express DM. The MalE protein vector was previously demonstrated to accommodate long peptide inserts without perturbation of its structure and could maintain the pattern of immunodominance or crypticity of the peptide that was previously observed within the native protein (28, 31). As a protein antigen, epitopes encoded within MalE will require endosomal processing to be liberated for presentation. Importantly, potential differences

in context between independent peptides, such as proteolysis and competing peptides within the antigen, will be minimized, as all peptides are placed within the same insertion site of the same protein. Thus, the MalE system unifies the delivery of the encoded peptide epitopes that are being evaluated, making it an efficient tool for identifying the influence that kinetic stability has upon presentation.

Several independent peptides, including LACK [156–173], HA [126–138], and HEL [11–25], were inserted into MalE at amino acid position 133. LACK [156–173] is the immunodominant peptide derived from the parasite *Leishmania major*. This peptide, which induces a strong CD4 response in infected mice (32, 33), displays a correspondingly high kinetic stability in association with I-A^d ($t_{1/2} = 170$ h; reference 28). HA [126–138] is derived from the influenza hemagglutinin molecule, and the I-A^d–HA [126–138] complex has been crystallized (34). HA [126–138] displays moderate stability in association with I-A^d ($t_{1/2} = 26$ h), and several kinetic stability variants of HA [126–138] have already been characterized (28, 35, 36). HEL [11–25] is a cryptic epitope derived from HEL, which has an unstable interaction with class II molecules ($t_{1/2} = 6$ h), the presentation of which has been demonstrated to be antagonized by DM expression within APCs when it is contained in the native HEL protein (29). The efficiency of antigen presentation and surface display of class II–peptide complexes was estimated through comparative dose response curves using DM⁺ or DM[−] APCs, peptide–MalE constructs, and assessment of activation of the epitope-specific T cell hybridomas via IL-2 production. These studies revealed that MHC class II–restricted presentation of epitopes that displayed a high intrinsic stability with I-A^d was enhanced by DM expression within APCs, whereas the presentation of epitopes that displayed a low intrinsic stability was antagonized by DM expression within APCs. Using LACK-specific T cells, we found a dramatic enhancement in LACK [156–173] presentation by APCs expressing DM compared with DM[−] APCs (Fig. 1 A). DM expression facilitated nearly a 10-fold shift in the efficiency of antigen presentation by APCs. Conversely, presentation of HEL [11–25]

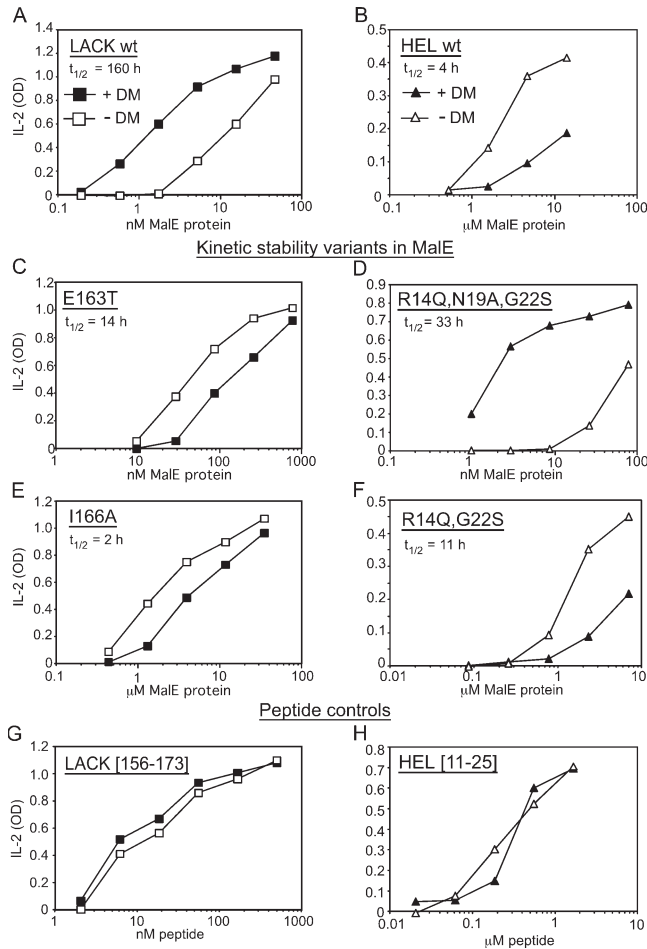


Figure 1. Modulating the kinetic stability correspondingly modifies presentation by DM-expressing APCs. WT and kinetic stability variants of LACK [156–173] and HEL [11–25] were encoded within MalE and tested for their ability to sensitize APCs that did (filled symbols) or did not (open symbols) express DM for stimulation of LMR 7.5 hybridoma cells (left panels) or HEL-25 hybridoma cells (right panels). IL-2 production by the antigen-specific hybridoma cells in response to the indicated dose of the antigen indicated in each panel was assayed as described in Materials and methods. Data are representative of at least three independent experiments.

was antagonized in APCs expressing DM compared with DM⁻ APCs. HEL-specific T cells required nearly 10-fold more protein to stimulate equivalent production of IL-2 on APCs that were expressing DM (Fig. 1 B). Table II summarizes the effects of DM upon presentation seen with the WT LACK and HEL peptides and their respective variants inserted within MalE. We further compared the presentation of HEL and LACK peptides when encoded within native protein or within MalE to determine whether the protein context influenced presentation (Fig. S1, available at <http://www.jem.org/cgi/content/full/jem.20060058/DC1>). These data suggested that the pattern of DM-mediated presentation of a class II-peptide complex appeared to be independent of the protein context that the peptide epitope was derived. Thus,

competing peptides liberated during processing of the protein antigen do not appear to play a significant role in regulating the effect of DM upon antigen presentation.

If kinetic stability influences DM activity, we reasoned that decreasing the kinetic stability of the highly stable LACK [156–173] epitope might lead to a corresponding change in the effect of DM upon antigen presentation. Similarly, we hypothesized that increasing the kinetic stability of the HEL [11–25] epitope would lead to a corresponding increase in the efficiency of presentation by APCs expressing DM. To test these predictions, two kinetic stability variants of LACK [156–173] and two kinetic stability variants of HEL [11–25] were selected for further investigation. The P1 mutant E163T and P4 mutant I166A of the LACK peptide both display significantly reduced kinetic stability ($t_{1/2} = 14$ h and $t_{1/2} = 2$ h, respectively) in association with I-A^d compared with WT LACK [156–173] ($t_{1/2} = 170$ h). When these antigens were incorporated into MalE and processed and presented by DM-expressing APCs, we found that reducing the kinetic stability of LACK [156–173] to under 15 h completely eliminated the DM enhancement previously observed for the WT LACK [156–173] epitope (Fig. 1, C and E, and Table II). Although single amino acid substitutions were sufficient to reduce the kinetic stability of LACK [156–173], multiple substitutions were found to be necessary to improve the relatively poor fit of the HEL [11–25] peptide in association with I-A^d. Increasing the kinetic stability of HEL [11–25]–I-A^d to 11 h with P1 and P9 substitutions, R14Q and G22S, respectively, revealed no statistically significant change in DM-mediated presentation (Fig. 1 F), although a summary of all experiments with MalE–R14Q, G22S (Table II) indicates a small attenuation in DM-mediated antagonism. However, an additional amino acid substitution (N19A), which increased the stability of R14Q, G22S by a factor of 3 ($t_{1/2} = 33$ h), led to a striking increase in presentation of MalE–HEL RQNAGS by DM-expressing APCs as compared with APCs lacking DM (Fig. 1 D and Table II). Pulsing APCs with peptide indicated no significant difference in the presentation by the two sets of APCs (Fig. 1, G and H). We further examined the stimulation of two additional independently derived HEL [11–25]–specific T cell hybridomas and one additional LACK [156–173]–specific T cell hybridoma to determine whether differential T cell reactivity may have influenced our estimates of DM editing (Fig. S2, available at <http://www.jem.org/cgi/content/full/jem.20060058/DC1>). Our data demonstrate that these additional T cells identify a similar degree of DM-mediated enhancement or antagonism in presentation compared with our original T cells, and thus offer a consistent measure of class II-peptide complex presentation.

The experiments described above indicated that kinetic stability of class II-peptide complexes could profoundly alter the qualitative influence of DM expression upon antigen presentation. We sought to further test this relationship by examining a class II-peptide epitope of moderate stability. Presentation of WT HA [126–138] inserted in MalE was modestly antagonized by the presence of DM in APCs (Fig. 2 A),

Table II. Presentation of kinetic stability variants of LACK [156–173], HEL [11–25], and HA [126–138]

Epitope inserted in MalE	$t_{1/2}$ (h)	DM enhancement	DM antagonism	<i>n</i>
I166A	2		1.8 ± 0.5	7
E163T	14		2.1 ± 0.8	8
LACK WT	170	9.9 ± 2.5		8
HEL WT	4		9.7 ± 3.8	3
R14Q, G22S	11		6.6 ± 1.3	5
R14Q, N19A, G22S	33	29.7 ± 7.6		4
V131A	0.9		14 ± 1.6	3
T128G	1		4 ± 1.8	2
T128V, S136T	9		1.8 ± 0.7	2
HA WT	26		1.2 ± 0.4	5
T128Q	63	3.5 ± 0.7		3
T128V	85	3 ± 0.8		4
T128M	165	6.2 ± 1.4		4

MalE protein, which encoded either WT or kinetic stability variants of the indicated antigen, was tested for its ability to sensitize APCs that did or did not express DM for stimulation of antigen-specific T cells. The fold DM effect was determined by comparing the amount of protein sufficient for equivalent IL-2 production and averaged over the indicated number of experiments ± SD. The half-life was calculated from the exponential equation fit to the fluorescence decay curve as a function of the incubation time and described as the time required to dissociate the 50% of the FITC peptide initially bound to si-A^d.

whereas peptide presentation was equivalent (Fig. 2 B). Substitutions in the peptide that increased the kinetic stability of this class II–peptide complex enhanced the positive effects of DM editing upon presentation (Fig. 2, D, F, and H), whereas substitutions that decreased kinetic stability of the epitope magnified the negative effects of DM editing upon presentation (Fig. 2, C, E, and G). For example, Fig. 2 D shows that the HA [126–138]–specific T cells required only 90 nM MalE–T128M protein for detectable IL-2 production when processed by APCs expressing DM, as compared with 650 nM by APCs lacking DM, representing a sevenfold enhancement in the efficiency of antigen presentation. Collectively, these results with three independent MHC class II–peptide epitopes demonstrate that the effect of DM activity upon the efficiency of presentation by APCs is determined by the spontaneous kinetic stability of the peptide epitope in association with class II molecules.

The relationship between the half-life of class II–peptide complexes and DM editing in APCs

One of the questions that arises from this type of study is how strict the correlation is between kinetic stability and DM editing and the accuracy with which this correlation can predict the effect of DM on presentation of new class II–peptide complexes. To address this question, data from T cell assays involving many different class II–peptide complexes and independent replicate T cell assays were consolidated, and we calculated the impact of DM editing on presentation from the quantity of protein that was required to stimulate comparative IL-2 production from T cells using APCs that did or did not express DM (Table II). As an example, presentation of the high stability HA variant T128M ($t_{1/2}$ = 165 h) was 6.2-fold enhanced by DM expression after averaging four independent experiments. DM's effect upon presentation of the WT and kinetic stability variants of HA [126–138],

LACK [156–173], and HEL [11–25], as well as OVA [273–288], SWM [102–118], and MalE [69–84], was arranged according to the log ($t_{1/2}$ [h]) versus the log (DM effect).

This analysis, shown in Fig. 3, demonstrated that intrinsic stability was a good predictor of presentation by APCs that express DM, as the correlation coefficient was strong (r = 0.921). The slope of the best-fit line through the data (slope = 0.849) predicts that a 10-fold increase in kinetic stability of a class II–peptide complex corresponds to a 7.1-fold increase in presentation of that complex by DM-expressing APCs. The triple substitution HEL [11–25] variant was considered an outlier in these analyses; however, the slope of the best-fit line was not significantly different when this complex was included (slope = 0.864). Thus, although there are occasional class II–peptide complexes that appear to be unusually sensitive to either the positive effects of DM editing (Fig. 1 D) or its negative effects, the consistent trend demonstrates that modifying the kinetic stability of class II–peptide complexes reliably influences the overall susceptibility to DM editing in a qualitative and quantitative manner.

The efficiency of antigen presentation by APCs expressing DM is directly related to immunodominance in vivo

Once we established that the kinetic stability of class II–peptide complexes determines the consequences of DM activity within APCs, we analyzed our previously published data on immunodominance (28) and compared these data to the studies described here. These analyses revealed a striking agreement between the efficiency of antigen presentation by DM-expressing APCs in vitro and the in vivo patterns of immunodominance (Fig. 4). Within the set of variants for each individual peptide, the presentation of immunodominant high stability epitopes on DM⁺ APCs was significantly more efficient than presentation of cryptic low stability epitopes. We calculated the amount of MalE protein necessary for

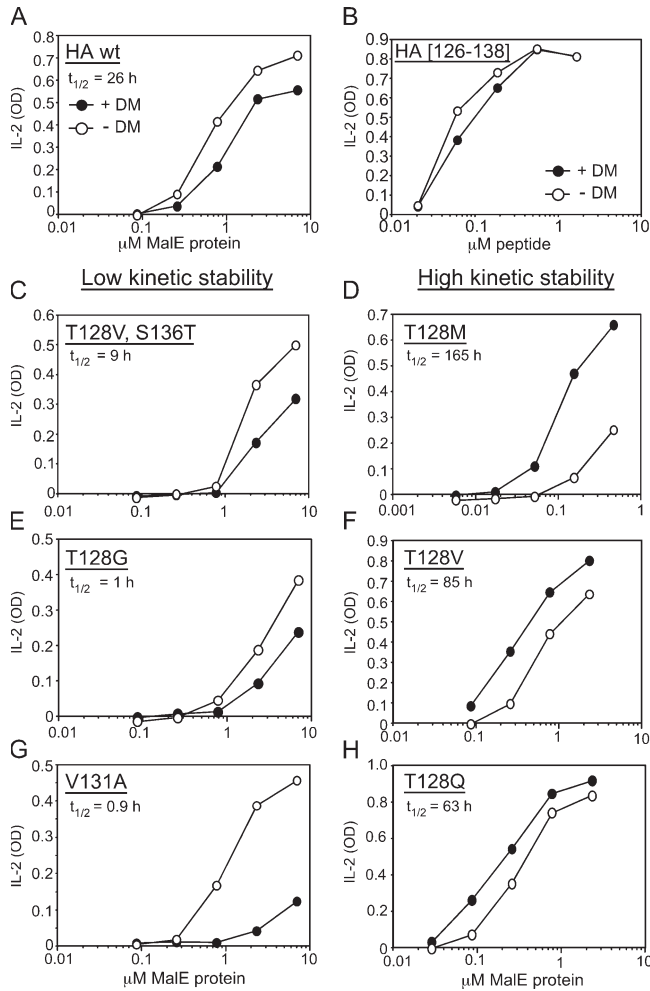


Figure 2. The kinetic stability of HA [126–138] with I-A^d determines the pattern of presentation by APCs that express DM. WT and kinetic stability variants of HA [126–138] cloned into MalE and indicated in each panel were tested for their ability to sensitize APCs that did (filled circles) or did not (open circles) express DM for stimulation of TS2 hybridoma cells. IL-2 production in response to the indicated dose of protein or HA [126–138] peptide was assayed as described in Materials and methods. Data are representative of at least two independent experiments.

detectable IL-2 production on DM-expressing APCs, arranging the epitopes according to their kinetic stability in association with I-A^d, and plotted this versus the relative magnitude of the in vivo response to the peptide variant when it was encoded in the complex antigen MalE. Fig. 4 A shows that the immunogenic variants of HA (T128M, T128V, and T128Q) were up to 10-fold more efficient at sensitizing APCs for recognition in vitro by DM⁺ APCs as compared with cryptic variants (T128G, V131A and T128V, S136T) and WT HA [126–138]. A similar relationship was uncovered comparing presentation of immunogenic and nonimmunogenic variants of LACK [156–173] and HEL [11–25] (Fig. 4, B and C).

Finally, we investigated the presentation of several MalE-encoded kinetic stability variants of HA [126–138] by splenic APCs to determine whether freshly isolated, naturally oc-

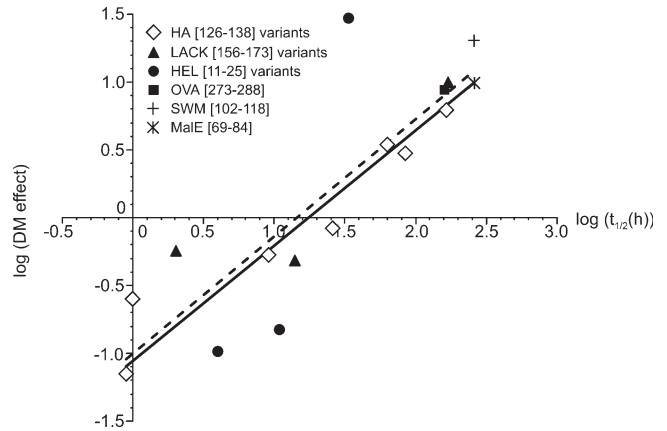


Figure 3. Kinetic stability predicts the activity of DM. The fold enhancement or antagonism of presentation of the indicated class II-peptide complexes was quantified across experiments by comparing the amount of protein necessary for detectable IL-2 production by specific T cell hybridomas on APCs that did or did not express DM. The log (DM effect) was then plotted against the $\log(t_{1/2})$ of the complex in question. The solid best-fit straight line excluding the triple substitution variant of HEL [11–25] was overlaid on the dataset (slope = 0.849), as well as the dashed straight line representing the best-fit line that included the triple substitution variant (slope = 0.864). The DM effect of native SWM [102–118] was derived from our previous publication (reference 29), whereas the DM effect of OVA [273–288] inserted within MalE and the MalE [69–84] epitope was determined from three independent experiments (not depicted).

curing DM⁺ APCs would display the same hierarchy of presentation as observed with transfected fibroblasts. Fibroblasts transfected with DM may not fully reconstitute all physiological loading compartments or the stoichiometric relationship between class II and DM within native APCs. HA [126–138]-specific T cell hybridoma cells were incubated with splenic APCs and graded doses of MalE-encoded protein antigen, and IL-2 production by the T cells was measured by CTLL proliferation (Fig. 5). These experiments revealed that the high kinetic stability variants of HA [126–138] (T128V: $t_{1/2} = 86$ h; T128Q: $t_{1/2} = 65$ h) were significantly more efficient at sensitizing the APCs for recognition by the T cells than WT HA [126–138] ($t_{1/2} = 26$ h; Fig. 5). Similarly, the lower kinetic stability variants T128G and V131A were unable to sensitize splenic APCs for T cell recognition at even the highest dose of protein used (Fig. 5). The same pattern was observed with bone marrow-derived dendritic cells (not depicted). These data suggest that the hierarchy of presentation of epitopes by APCs expressing DM correlates with the kinetic stability of class II-peptide complexes, which furthermore is reflective of the immunodominance of these complexes.

DISCUSSION

These experiments were initiated to determine whether it is possible to modulate the qualitative and quantitative effects of DM editing in APCs by altering the kinetic stability of the

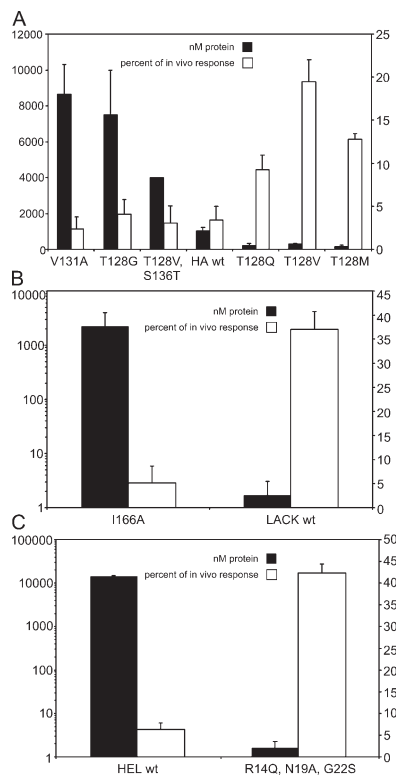


Figure 4. The efficiency of presentation in vitro correlates with immunodominance in vivo. The average amount of protein (left axis, filled bars) sufficient for detectable IL-2 stimulation by WT and kinetic stability variants of (A) HA [126–138], (B) LACK [156–173], and (C) HEL [11–25] was averaged across experiments \pm SD in comparison to the published (reference 28) immunodominance (right axis, open bars) of the protein in question.

presented MHC class II–peptide complex. We used a bacterial protein expression system to incorporate a set of peptides that possesses a range of kinetic stabilities in association with the I-A^d class II molecule. These antigens were tested for their ability to sensitize APCs with or without DM expression for recognition by antigen-specific T cells. Our results presented here provide strong evidence that the kinetic stability of the class II–peptide complex has a direct and causative influence on DM-mediated presentation within APCs. Class II–restricted presentation of low kinetic stability variants is antagonized by DM expression, whereas presentation of high kinetic stability variants is enhanced by DM expression. Finally, we show that the efficiency of class II–peptide presentation from complex antigens by DM⁺ APCs can be modified by altering its spontaneous kinetic stability, and that such a change is directly related to alterations in immunodominance in vivo. Collectively, our data suggest that the efficiency of antigen presentation on DM⁺ APCs is a critical parameter of MHC class II–peptide complexes that determines their relative ability to recruit T cells during an in vivo immune response.

The peptide variants investigated here included substitutions at each of the four major pockets for I-A^d. In each case,

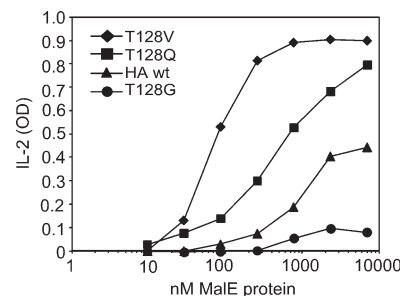


Figure 5. The kinetic stability predicts the hierarchy of presentation by splenic APCs. WT and kinetic stability variants of HA [126–138] encoded within MalE were tested for their ability to sensitize 5×10^5 BALB/c spleen cells for stimulation of 5×10^4 TS2 hybridomas. Cells were incubated with the indicated doses of protein overnight, and IL-2 production was assayed as described in Materials and methods. Data are representative of three independent experiments.

a substitution that increased kinetic stability resulted in enhanced presentation by DM–expressing APCs, and substitutions that decreased kinetic stability caused a corresponding decline in presentation by DM–expressing APCs. This result has several important implications. First, in terms of improving strategies in vaccine design, this finding suggests that for a given class II–peptide complex, there are multiple sites that can be modified in the peptide that can lead to the desired change in DM editing within APCs. This is important because it allows considerable flexibility in designing variants that maintain T cell reactivity while altering interactions with class II molecules. Second, these data argue in favor of the idea that DM recognizes a feature characteristic of the overall structure of the class II binding pocket rather than a specific site on the class II–peptide complex.

In general, there was a strong predictive relationship between the induced changes in the kinetic stability of class II–peptide complexes and changes in DM editing in APCs. For a given peptide, modulation of its interaction with class II caused a predictable change in DM editing. We did not observe any examples of high stability complexes (i.e., $t_{1/2} > 50$ h) that were not enhanced by DM expression in APCs nor any examples of low stability complexes (i.e., $t_{1/2} < 10$ h) that were not antagonized by DM. There were, however, some interesting exceptions to the largely linear nature of the data, where a few peptide complexes seem to fall significantly off the diagonal line one generates when plotting kinetic stability of class II–peptide complexes versus DM editing. The HEL variant with substitutions at P1 (R14Q) and P9 (G22S) possessed a half-life of 11 h with I-A^d. With regard to DM editing, presentation of this class II–peptide complex is antagonized sixfold by DM expression within the APCs. Strikingly, however, when one further modifies this peptide, enhancing interactions via the P6 pocket (N19A) and promoting a modest increase in kinetic stability ($t_{1/2} = 33$ h), presentation of this complex is remarkably (30-fold) enhanced by DM expression in APCs. Therefore, a threefold change in kinetic stability causes almost a 200-fold shift in the effects

of DM. We confirmed this effect on the efficiency of presentation pattern with two additional, independently derived HEL [11–25]–specific hybridomas (not depicted). One possibility to explain dramatic changes in DM editing is that the triple-substituted HEL complex has unique conformational features that have altered its interaction with DM or the consequences of that interaction, perhaps making it completely resistant to DM-mediated dissociation. The possibility that this class II–peptide complex has altered conformational features relative to the complex made with the WT peptide is supported by our previous studies on T cell recognition of the HEL peptide complexes (28). These studies showed that T cells display a strong discrimination between WT HEL and the P1, P6, and P9 variant. Another example of atypical DM effects was observed with a variant of the HA [126–138] peptide complex. In general, substitutions in this peptide that increased kinetic stability enhanced positive DM editing, whereas substitutions that diminished the kinetic stability with I-A^d led to a conversion to negative DM editing. However, two of the low stability variants differed significantly from each other. The P1 substitution (T128G) and the P4 substitution (V131A) each caused a 20-fold drop in kinetic stability compared with the WT peptide complexed to I-A^d, and each showed accentuated sensitivity to negative DM editing compared with the WT or higher stability variants of HA. However, the change at P4 led to significantly more sensitivity to negative DM editing (14-fold) compared with the substitution at P1 (four-fold). Therefore, equivalent destabilization of class II–peptide complexes, but at different sites or through different substitutions in the peptide, can lead to quantitative differences in DM editing within APCs. In general, the quantitative effect of DM editing was less predictable with low stability class II–peptide complexes. Although the presentation of each of these complexes was antagonized by DM, there was considerable scatter in the magnitude of DM effects. We hypothesize that some of the variability of DM's cumulative effects for very low stability complexes (e.g., $t_{1/2} < 5$ h) may be due to the combined effect of DM antagonism during peptide loading and after formation of the complex, which will then be subject to accelerated dissociation due to the continued interaction with DM. Recent publications have proposed that DM may differentially affect loading onto class II molecules, depending on the peptide sequence (22, 37, 38). Resolution of this possibility will require detailed biochemical assessment of DM's effect at these discreet events in vitro with purified DM and class II proteins.

We predict that DM's ability to edit peptides is a major mechanistic link that effectively favors the cell surface presentation of class II–peptide complexes displaying high kinetic stability. This epitope hierarchy expressed on the priming APC likely plays a key role in the establishment of the well-described patterns of immunodominance observed with foreign-derived exogenous antigens (for review see references 39–41). Accumulated evidence suggests that maturation of dendritic cells enhances the accumulation of class II–peptide complexes on the cell surface while concomitantly reducing

endocytosis, effectively presenting a snapshot of the antigens available during exposure to the maturation signal (42, 43). For low stability epitopes, the susceptibility to DM editing is a barrier that must be overcome and may in fact prove insurmountable in such instances of limiting antigen dose or exposure time. Additionally, at stabilities of below 1 h, the inability of a peptide to form any meaningful association with class II may prove the ultimate antagonist for detectable presentation. For high stability epitopes, the activity of DM results in a significant boost to the efficiency of presentation. Recent data suggest that the persistence of antigen presentation is necessary for continued proliferation and differentiation of CD4 cells (44–47). The initial advantage high stability complexes possess in epitope density that should promote the priming of high numbers of CD4 T cells may thus further extend to daughter T cells arising from initial antigen-dependent proliferation. It should now be possible to design peptides that can take advantage of these observed preferences exhibited by DM to elicit a maximal level of immunogenicity toward the desired epitopes at a minimum dose.

MATERIALS AND METHODS

Purification of soluble I-A^d proteins

Production and purification of class II molecules were performed as described previously (28, 30). In brief, transfectants expressing the phosphatidyl-inositol-linked class II molecules were solubilized with 50 mM Tris, 150 mM NaCl, and 6.5 mM CHAPS containing protease inhibitors at pH 7.8 for 1 h at 4°C. I-A^d phosphatidyl-inositol molecules were purified by class II mAb affinity chromatography and treated with 0.1 U/ml of phosphatidylinositol-specific phospholipase C (Sigma-Aldrich) in 50 mM Tris, pH 7.4, containing 1% BSA at room temperature for 1.5 h to remove the phosphatidylinositol linker. The soluble I-A^d protein (sI-A^d) was then eluted from the column at pH 11.0 with a buffer containing 1 mM n-dodecyl maltoside (n-dodec; Sigma-Aldrich), 100 mM sodium carbonate, and 500 mM NaCl at 4°C. After neutralization, dialysis, and concentration, the purified sI-A^d proteins were analyzed by SDS-PAGE, Western blot, and peptide binding.

Antigens

Peptides. Synthetic, unlabeled, and fluorescein-labeled peptides were obtained from commercial sources. Fluoresceinated peptides were purified by HPLC to >90% purity.

MalE protein purification. MalE protein was purified as described previously (28). In brief, PAGE-purified synthetic oligonucleotides encoding the desired peptide were ligated into MalE133 vector, and sequenced clones were transformed into MalE (–/–) ER2507 *Escherichia coli* for overnight culture. Protein was isolated from periplasmic extracts of bacterial cultures expressing the MalE construct. Periplasmic shock fluid was filtered over a 0.45- μ m membrane and added to an amylose column (48) for purification and elution. Collected fractions containing MalE were pooled, dialyzed against 1 \times PBS, and concentrated with a Centricon ym-10 KD cutoff filter to \sim 1 ml final volume. Concentrated MalE in PBS was sterile filtered through a 0.2- μ m syringe filter, quantified by Bradford assay and SDS-PAGE, and stored at 4°C.

LACK protein synthesis. The LACK protein, originally derived by Mougneau et al. (32), and LACK-I166A proteins were purified as described previously (28). In brief, BL21(DE3 λ) bacteria (Novagen) were transfected with LACK or LACK-I166A constructs, and protein was isolated from IPTG-induced cultures via Ni-NTA affinity column and assayed for quantity and purity via SDS-PAGE analysis. Protein was dialyzed in PBS and sterile filtered through a 0.2- μ m syringe filter.

Protein antigens. Purified HEL, SWM, and OVA were purchased from Sigma-Aldrich. Protein was resuspended in PBS and sterilized by passage through a 0.2- μ M syringe filter.

Dissociation experiments

Dissociation assays were performed as described previously (30). In brief, an aliquot of 25 μ l of 100 nM sI-A^d molecule was mixed with 1 μ l of 250 μ M FITC peptide plus 5 μ l of McIlvaines buffer, pH 5.3 (0.2 M citric acid, 0.5 M Na₂HPO₄), 0.2 mM n-dodecyl, and 0.025% NaN₃ in the presence of protease inhibitors. The association mixture was incubated at 37°C and pH 5.3 to allow formation of complexes, and the sI-A^d-FITC peptide complex was separated from free FITC peptide by passage over a Micro Bio-Spin 30 column (Bio-Rad Laboratories). Complexes were incubated at 37°C and pH 5.3 in the presence of 5 μ M unlabeled E α [52–68] peptide to avoid rebinding of the fluorescinated peptide. After increasing periods of time, a sample was injected into a LC-10AT HPLC (SHIMADZU Corporation) equipped with a Bio-Sep-SEC-S 3000 column (300 \times 7.8 mm; Phenomenex Inc.) and passed through the column with PBS containing 0.2 mM n-dodecyl plus 0.025% NaN₃ at room temperature and pH 7.4 at a flow rate of 0.8 ml/min. The complex was detected with an RF-10AXL fluorescence detector (SHIMADZU Corporation) by measuring the emission intensity at 525 nm. The emission intensities were normalized with respect to the intensity of the first sample, taken right before the complex incubation.

Generation and maintenance of transfected fibroblasts

Ltk⁻ cells were transfected by calcium phosphate with genes encoding the I-A^d α and β chains plus the neomycin resistance gene. MHC class II⁺ cells were subcloned and supertransfected either with the murine genomic Ii gene and pSV2gpt for resistance to MXH (49), or the murine genomic Ii gene and DM α and β genes along with the pSV2bsr2 for resistance to blasticidin (14). Transfected cells were maintained in selection media and stained for class II expression every 3–6 wk but were removed from selective drug for 2 d before T cell assays.

Hybridoma stimulation assays

The HEL-specific hybridoma (HEL 25) was created by a fusion of peptide-activated LN cells from a BALB/c mouse immunized 10 d prior in the footpad with 50 μ l of 5 nmol of HEL [11–25] peptide emulsified in PBS-CFA. The HA-specific hybridoma (TS2) was created by the fusion of peptide-activated LN cells from a HNT-TCR mouse (TS2; reference 50) with BW5147 lymphoma cells. For assays, 5 \times 10⁴ specific T cell hybridomas were mixed with 4 \times 10⁴ APCs expressing DM, class II, and invariant chain (DMI), or 4 \times 10⁴ APCs expressing class II and invariant chain (gIi) as described previously (51), and peptide or protein at the specified dose in a flat-bottom 96-well dish. After overnight culture, plates were frozen and thawed, and 50 μ l supernatant was removed and added to 3 \times 10⁴ CTLL cells. After 16–20 h, CTLLs were incubated with 0.4 mg/ml MTT for 6 h, followed by 100 μ l 10% SDS/0.01 N HCl overnight. OD was calculated from measurements at 570–630 nm.

Online supplemental material

To address whether protein context of the peptide epitope and thus competing peptides within the antigen influence the presentation of high versus low affinity peptides, Fig. S1 compares the presentation of the highly stable LACK [156–173] epitope and the unstable LACK-I166A and HEL [11–25] epitopes when presented within either native protein or within MalE. Our data demonstrate that the pattern of DM-mediated presentation of a class II-peptide complex appears to be independent of the protein context from which the peptide epitope is derived. To address whether the use of T cells as a measure of class II-peptide complex presentation introduces bias according to unique aspects of the individual T cells used, Fig. S2 compares the presentation of class II-peptide complexes as detected by the activation of additional T cell hybridomas. These data demonstrate that these additional T cells detect a similar degree of DM-mediated enhancement or antagonism of presentation as compared with our original

T cells. Figs. S1 and S2 are available at <http://www.jem.org/cgi/content/full/jem.20060058/DC1>.

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