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The Kinetic Stability of MHC Class II:Peptide Complexes Is a Key Parameter that Dictates Immunodominance

Christopher A. Lazarski,[1,2] Francisco A. Chaves,[1] Scott A. Jenks,[1] Shenhong Wu,[2] Katherine A. Richards,[1] J.M. Weaver,[1] and Andrea J. Sant[1,]*
[1]David H. Smith Center for Vaccine Biology and Immunology
Aab Institute of Biomedical Sciences
Department of Microbiology and Immunology
University of Rochester
Rochester, New York 14642
[2]Committee on Immunology
Division of Biological Sciences
University of Chicago
Chicago, Illinois 60637

Summary

T cell priming to exogenous antigens reflects regulated antigen processing in dendritic cells, subsequent homing to lymph nodes, sustained interactions between T cells and antigen-bearing dendritic cells, and, ultimately, selective T cell activation and differentiation. In this study, we test the hypothesis that an intrinsic property of the class II:peptide complex is a key determinant that dictates the specificity of an emerging CD4 T cell response. We found that immunodominant peptides possess extremely long half-lives with class II molecules ($t_{1/2}$ > 150 hr), whereas cryptic peptides displayed half-lives of less than 10 hr. Furthermore, and most importantly, by using a peptide shuttle vector and four independent antigens, we demonstrate a direct, causative relationship between the half-life of peptide epitopes and their immunogenicity in vivo. Taken collectively, our results suggest the half-life of class II:peptide complexes is the primary parameter that dictates the ultimate hierarchy of the elicited T cell response.

Introduction

The evolution of a primary CD4 T cell response reflects sequential events in antigen presentation, dendritic cell homing, and T cell activation and differentiation. Ultimately in this process, CD4 and CD8 T cells respond to a very limited subset of peptides within complex protein antigens. These peptides have been called immunodominant. The focused response to a limited set of peptides within complex proteins reveals a considerable selective pressure on an emerging T cell response.

Previous studies investigating the selectivity of CD4 T cell responses have uncovered several factors that can influence the specificity of T cells including antigen processing and presentation, T cell precursor frequency, and T cell competition (reviewed in Blum et al., 1997; Kedl et al., 2003; Manoury et al., 2002; Medd and Chain, 2000; Sant et al., 2005; Sercarz et al., 1993). Historically, processing of native antigen and subsequent presentation by APCs has been thought to be one of the major factors influencing the specificity of T cells. Endosomal proteolytic processing has the potential to either positively or negatively affect immunogenicity. The assembly of class II:peptide complexes is another potential site of regulation. Assembly can be influenced by interpeptide competition for binding class II molecules, modulation by DM, or "epitope capture" by peptides adjacent to the test peptide. The frequency of peptide-specific T cells can also influence immunodominance and, in particular, negative selection can delete CD4 T cells specific for immunodominant peptides within self-antigens. Finally, competition between T cells for interaction with APCs is a well-documented phenomenon in the CD8 T cell response and has been proposed to extend to CD4 T cell responses.

The preceding studies suggested that many complex events converge to influence the selective specificity of CD4 T cells during primary immune responses, and the relative contribution of any of these parameters could influence immunogenicity. However, our earlier studies demonstrated that DM expression singularly enhanced presentation of immunodominant epitopes while antagonizing presentation of cryptic peptides (Nanda and Sant, 2000). This finding suggested that an intrinsic property of the class II:peptide complex itself might be the most important parameter in determining immunodominance. In the present study, we test the hypothesis that in general, an intrinsic feature of class II:peptide complexes primarily determines the differential priming and expansion of epitope-specific CD4 T cells. Collectively, our studies revealed that the spontaneous kinetic stability of class II:peptide molecules is the principal determinant of the immunogenicity of peptide specificities within the CD4 T cell response to antigen.

Results

Kinetic Stability Correlates with Immunodominance

To begin these studies, we assembled a set of previously identified cryptic and immunodominant epitopes and characterized their relative affinity for class II molecules. We utilized I-A^d-restricted epitopes from divergent origins, including sperm whale myoglobin (SWM), hen-egg lysozyme (HEL), chicken ovalbumin (OVA), and *L. major* (LACK) (Mougneau et al., 1995; Sercarz et al., 1993). The diversity of these epitopes with regard to processing and structure provided the opportunity to isolate a biochemical characteristic that determined in vivo immunodominance. We evaluated the potential of both peptide competition and peptide dissociation assays to distinguish these epitopes. Both assays have been used to determine the relative strength of class II:peptide interactions (Kasson et al., 2000; McFarland et al., 1999a; Sette et al., 1989). Peptide competition assays judge the ability of the test peptide to inhibit formation of complexes between a labeled, standardized peptide with class II molecules,

*Correspondence: andrea_sant@urmc.rochester.edu

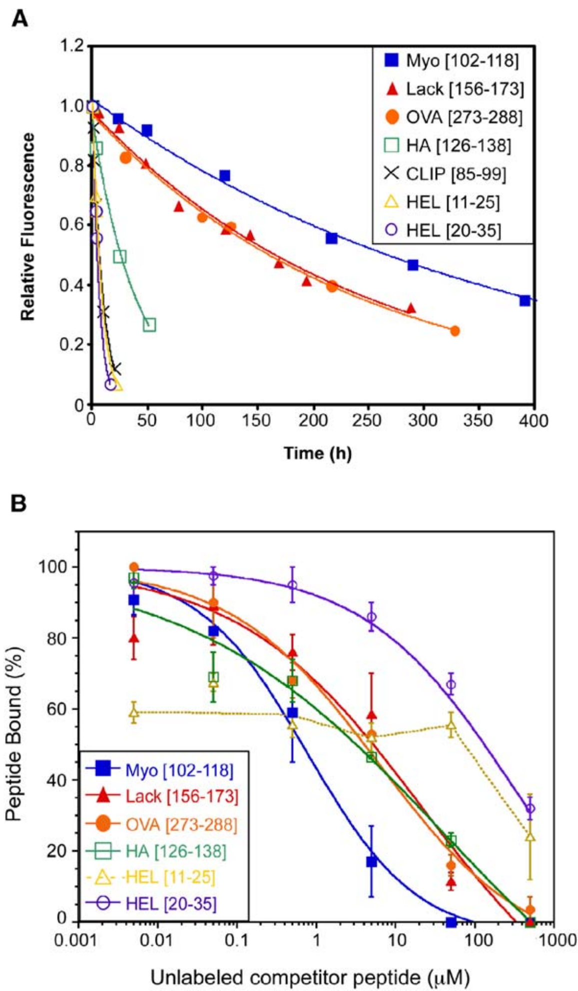

Figure 1. Comparison between Peptide Dissociation and Peptide Competition

(A) The half-life was calculated from the exponential equation fitted to the fluorescence decay curve as a function of the incubation time and was described as the time required to dissociate the 50% of the FITC peptide initially bound to sI-A^d.

(B) The percentage of inhibition of binding of 1 μM N-terminal FITC-HA(126–138) to sI-A^d by the unlabeled competitor peptide was plotted against the concentration of unlabeled inhibitory peptide. Data are represented as a Hill Plot (Hill, 1910) and are the average of two independent experiments. Error bars represent the range obtained in independent experiments.

whereas dissociation assays directly measure the kinetic stability of interaction between the test peptide and class II molecules after the complexes have been assembled.

To perform binding studies with I-A^d, we produced and purified soluble class II molecules (Chaves et al., 2005). For dissociation assays, we monitored the half-life of preloaded class II I-A^d peptide complexes bound with fluorescently labeled peptide in vitro at endosomal pH 5.3 and 37°C. Dissociation was found to fit a single exponential curve with a square correlation coefficient $r^2 > 0.99$ from which the $t_{1/2}$ could be determined. Strikingly, the dissociation curves from Figure 1A and the $t_{1/2}$ values (Table 1) revealed that immunodominant and cryptic epitopes segregated at opposite ends of a very broad kinetic stability spectrum. The immunodominant epitopes LACK (156–173) ($t_{1/2}$ = 170 hr), SWM (102–118); ($t_{1/2}$ = 260 hr), and OVA (273–288); ($t_{1/2}$ = 160 hr) all displayed long half-lives of 150 hr or greater. In contrast, the cryptic epitopes HEL (11–25); ($t_{1/2}$ = 6 hr) and HEL (20–35); ($t_{1/2}$ = 4 hr) both displayed very short half-lives. Collectively, our results suggest that kinetic stability in class II:peptide complexes may be a critical characteristic that determines immunogenicity of a peptide during an immune response.

In contrast to the results involving kinetic stability measurements, cryptic and immunodominant peptides displayed no consistent groupings when assayed by competition (Figure 1B). For example, the classically defined cryptic HEL (11–25) peptide competed well with HA (126–138) for binding to I-A^d, whereas the similarly cryptic HEL (20–35) peptide competed poorly with HA (126–138) for binding to I-A^d, with a predicted IC_{50} > 100 μM. Also, HA (126–138), LACK (156–173), and OVA (273–288) clustered together, even though their potency in immunodominance assays are clearly distinct. Collectively, the studies with these unrelated antigens revealed that under the conditions used in our experiments, dissociation rates of peptides from class II molecules dramatically segregated immunodominant from cryptic peptides. We therefore used this parameter for the remainder of our experiments. In analyses of many peptides whose dissociation rates differ by seven orders of magnitude, McConnell's group (Kasson et al., 2000) reported that the association rates among these peptides were essentially the same. This indicates that measurements of dissociation rates of peptides from class II molecules provide a good measure of relative affinities. Therefore, in general, values for "affinity" and "dissociation rates" will agree with each other. We do, however, continue to generally use the terms "kinetic stability" and "off-rates" in this paper because that is the parameter that we vary and measure in our studies.

Derivation of Peptide Kinetic Stability Variants

We wished to extend the correlative findings between class II:peptide half-lives and immunogenicity to test whether we could show a causative relationship between these two parameters. To address this, we sought peptide variants that possessed increased or decreased kinetic stability with I-A^d and then investigated whether changing the kinetic stability of a given class II:peptide complex caused a corresponding change in its immunogenicity in vivo.

To arrive at generalizable conclusions, three unrelated peptides were chosen: the influenza HA (126–138) peptide, the LACK (156–173) peptide from *L. major*, and HEL (11–25), each of which offered unique biological or biochemical properties. The HA (126–138) peptide was chosen because the crystal structure of HA (126–138):I-A^d has been solved (Scott et al., 1998), providing the register for the peptide bound to I-A^d. A second advantage of the HA (126–138) peptide is its intermediate dissociation rate ($t_{1/2}$ = 26 hr), which provided an opportunity to investigate the biological properties of both higher and lower stability variants with I-A^d. The LACK (156–173) peptide from *L. major* was selected because it is a prototypical immunodominant epitope from a

Table 1. Kinetic Stability of Wild-Type and Variant Peptide Epitopes in Association with I-A^d

Peptide	SEQUENCE (Putative Register)[a] 1 4 6 9	$t_{1/2}$ (h) pH 5.3 I-A^d	Relative Position
Myo (102–118) wt	K Y L E F I S E A I I H V L H S R	260	na
Lack (156–173) wt	I C F S P S L E H P I V V S G S W D	170	na
OVA (273–288) wt	M E E R K I K V Y L P R M K M E	160	na
HA (126–138) wt	H N T N G V T A A C S H E	26	na
CLIP (85–99) wt	K P V S Q M R M A T P L L M R	7	na
HEL (11–25) wt	A M K R H G L D N Y R G Y S L	6	na
HEL (20–35) wt*	Y R G Y S L G N W V C A A K F E	4	na
HA (126–138) T128M	H N *M* N G V T A A S S H E	165	P1
HA (126–138) T128V	H N *V* N G V T A A S S H E	85	P1
HA (126–138) T128Q	H N *Q* N G V T A A S S H E	63 ± 3	P1
HA (126–138) C135S	H N T N G V T A A *S* S H E	26	P8
HA (126–138) wt	H N T N G V T A A C S H E	26	na
HA (126–138) TV,ST	H N *V* N G V T A A S *T* H E	9	P1, P9
HA (126–138) T128G	H N *G* N G V T A A S S H E	1	P1
HA (126–138) V131A	H N T N G *A* T A A S S H E	0.9	P4
HEL(11–25) RQ,NA,GS	A M K *Q* H G L D *A* Y R *S* Y S L	33	P1, P6, P9
HEL (11–25) RQ,GS	A M K *Q* H G L D N Y R *S* Y S L	11	P1, P9
HEL (11–25) wt	A M K R H G L D N Y R G Y S L	6	na
Lack (161–173) wt	S L E H P I V V S G S W D	200	na
Lack (161–173) I166A	S L E H P *A* V V S G S W D	2	P4
OVA (327–339)	K H A A H A E I N E A G R	0.3	na
OVA (327–339) AI,IV	K H A A H *I* E *V* N E A G R	87	P4, P6

Half-lives of peptide:I-A^d complexes were 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 the 50% of the FITC peptide initially bound to sI-A^d. Putative pocket residues for peptides are indicated in bold, and the mutations at pocket residues that modulate kinetic stability are indicated by italics.
[a] Indicates the binding register for HEL (20–35) is unknown. Cys 135 in HA (126–138) is necessary for T cell activation (not shown), but does not affect stability in association with I-A^d. Lys 327 in OVA (327–339) was substituted for Val to eliminate alternate register binding. Data is representative of at least two independent experiments.

model protozoan infection (Mougneau et al., 1995; Reiner et al., 1993). This epitope has been found to have a high number of T cell precursors (Milon et al., 1986; Stetson et al., 2002), a property that offered the opportunity to determine whether reducing kinetic stability of class II:peptide complexes would be sufficient to overcome precursor frequency advantages. The HEL (11–25) peptide is a prototypic cryptic peptide (Moudgil et al., 1997) and thus provided an opportunity to reverse apparent sequestration of a peptide from an immune response solely by stabilizing the interaction of the peptide with class II molecules.

Initial experiments tested the ability of candidate variant peptides to maintain T cell stimulatory capacity when tested with antigen-specific T cell hybridomas. Peptide variants that passed this initial screen were evaluated for dissociation kinetics. HA (126–138) variants included substitutions at P1, P4, or P9 pocket residues. T cells responded to most HA (126–138) variant peptides in vitro when presented by I-A^d-expressing cells (Figure 2A and data not shown). Compared to wild-type (wt) HA (126–138) that displayed a kinetic stability of approximately 26 hr with I-A^d, three P1 mutants displayed increased stability with I-A^d molecules ranging from 63 to 165 hr (Table 1 and Figure 2B). Lower stability variants showed half-lives of 1 and 0.9 hr, respectively (Table 1 and Figure 2B). The variants summarized in Table 1 provided a range of kinetic stabilities to study and also highlighted the importance of single amino-acid interactions with the class II pockets upon the overall stability of the class II:peptide complex.

Whereas the successful crystallization of I-A^d:HA (126–138) facilitated the design of variant peptides, the core binding sequence of LACK (156–173) was not known. Use of truncated peptides (see Figures S1A and S1B in the Supplemental Data available with this article online) indicated the binding core to be residues (163–171) and suggested the following amino acid register from P1 to P9: **EHPIVVSGS**. This register fits with some of the known binding preferences for I-A^d, particularly Ile at P4 and Val at P6 (Figure S1C). To derive lower kinetic stability variants for LACK, we substituted Ala for Ile at P4 (I166A). This variant sustained T cell stimulation to wt LACK (161–173) (Figure 2C) and displayed a half-life of only 2 hr, nearly 100-fold less than wt LACK (Figure 2D). The changes in stability observed with I166A demonstrated that this residue interacted with I-A^d pockets as hypothesized and verified this register for LACK (156–173):I-A^d.

Determination of the register of the HEL (11–25) peptide presented us with unique challenges. Because of its extremely weak interaction with I-A^d, this peptide was not expected to possess even the poorly defined "motif" for I-A^d (Sette et al., 1988). As shown (Figure S1D), we used functional studies to determine its binding register with I-A^d. These analyses suggested that the likely register for the HEL (11–25) peptide with I-A^d was AMK**R**HG**L**D**N**YR**G**YSL, with the bold residues indicating P1, P4, P6, and P9. Higher stability variants of HEL (11–25) displayed half-lives of 11 or 35 hr, respectively (Figure 2F).

Implementation of a Peptide Shuttle Vector for CD4 T Cell Epitopes

To study the relationship between peptide off-rates and immunogenicity, we required a protein shuttle vector that could accept heterologous peptide inserts. To prevent self-reactive T cells from interfering with re-

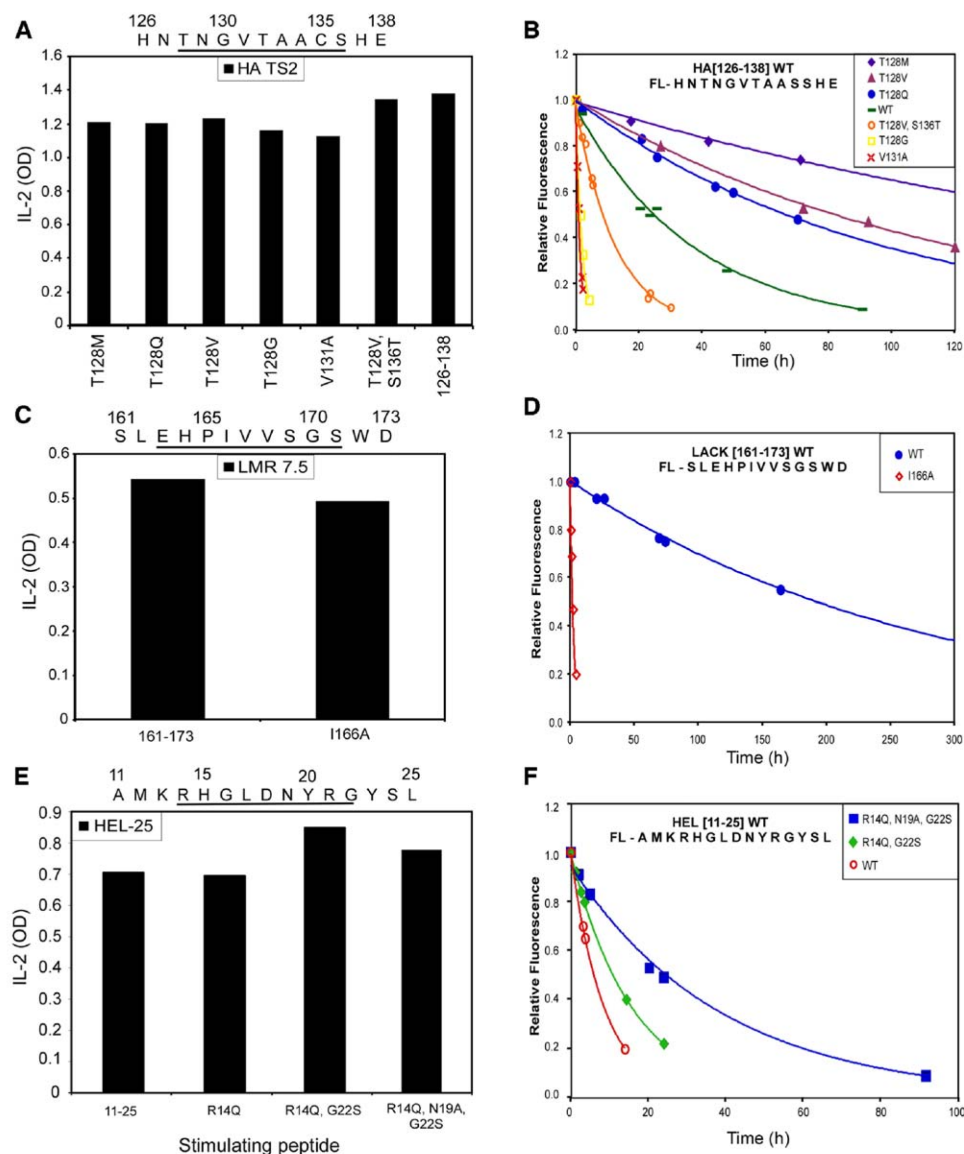

Figure 2. Identifying Kinetic Stability Variants of HA (126–138), LACK (156–173), and HEL (11–25)

(A, C, and E) Candidate peptide variants were identified by in vitro stimulation of 5×10^4 specific hybridomas with soluble peptide presented by 4×10^4 I-A^d-expressing L cells. IL-2 production by HA TS2 (A), LMR 7.5 (C), and HEL 25 (E) hybridomas in response to 667 nM (A and E) or 300 nM (C) peptide was measured as described in the Experimental Procedures. Data are representative of three independent experiments. (B, D, and F) Dissociation of peptides from I-A^d was found to fit a single exponential curve with a square correlation coefficient, $r^2 > 0.99$, from which $t_{1/2}$ could be determined. Data are representative of at least two independent experiments.

sponses to inserted epitopes, we used a vector that had no murine homolog. The protein vector chosen, MalE, encodes a subunit of the *E. coli* maltose binding protein and can accept inserts of greater than twenty amino acids (Martineau et al., 1992). The use of the same protein vector for all of the test peptides has the advantage of providing the same set of competing peptides, thus controlling for T cell competition events and allowing us to track responses to these MalE peptides in all the immunization studies. To take advantage of this, we characterized the immunodominant epitopes within MalE and found that in BALB/c mice, MalE (69–82) was consistently dominant, whereas MalE (103–118) and MalE (269–285) were subdominant (S.A.J. and A.J.S., unpublished data).

In an effort to equalize three-dimensional context and protease sensitivity among the variant or wt peptides, we chose a single insertion site for the peptides. Also, because insertions that perturb structure diminish affinity to maltose, the purification strategy chosen was based on the functional association of peptide-inserted MalE with crosslinked amylose (Martineau et al., 1992). DNA encoding each peptide of interest was inserted into amino acid 133 flanked by five to seven carboxyl-terminal and amino-terminal residues (Figure 3A) of the native peptide to preserve potential T cell receptor

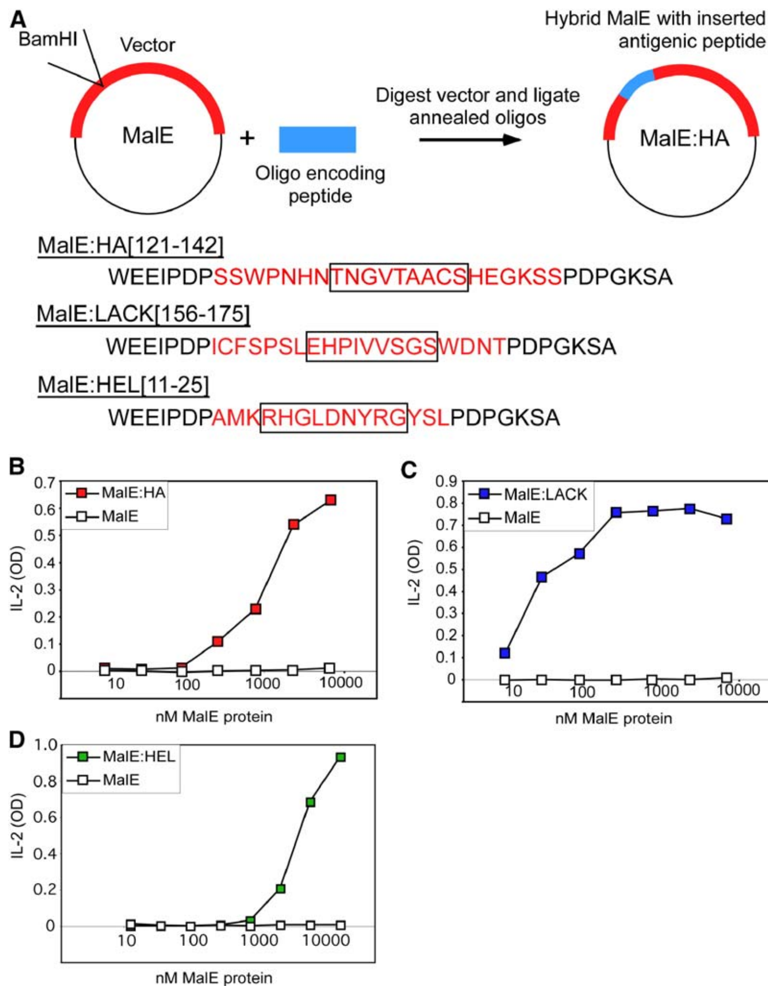

Figure 3. Construction of Hybrid MalE Used to Incorporate Antigenic Peptides

(A) DNA encoding antigenic peptides with flanking residues was inserted in-frame into MalE at amino acid 133 via BamHI ligation.

(B) MalE:HA, (C) MalE:LACK, or (D) MalE:HEL purified from sequenced clones was tested for the ability to activate 5×10^4 peptide-specific hybridomas in vitro by using 5×10^5 BALB/c spleen as APCs. As a measure of T cell stimulation, IL-2 production was assayed by CTLL proliferation with an MTT assay.

(TCR) contacts (Arnold et al., 2002). With insertion of the HA (126–138), LACK (156–173), and HEL (11–15) peptide epitopes into MalE, the respective T cell hybridomas gained reactivity (Figures 3B–3D), indicating that the inserted peptides were liberated during processing of the protein.

Immunogenicity of Peptide Variants In Vivo

To examine the immunogenicity of the heterologous test peptide variants within MalE, IL-2 ELISPOT assays were used to quantify the number of CD4 T cells specifically responding to peptides ex vivo. To compare data collected from independent experiments, we normalized spot counts for all the tested peptides relative to the total number of T cells that responded in vitro to the original MalE:insert protein used for immunization. Data shown represent the average of at least three independent experiments. We found HA (126–138) to be cryptic in BALB/c mice when inserted into MalE. Very few T cells specific for HA (126–138) could be detected in immunized mice. The occasional single spot above background corresponded to less than one in 500,000 lymph node cells. Thus, a kinetic stability of 26 hr is insufficient for recognition of HA (126–138). We next investigated whether variants of HA (126–138), which displayed increased kinetic stability, could overcome the crypticity of wt HA (126–138). BALB/c mice were immunized with the high-stability variant HA T128V ($t_{1/2}$ = 86 hr) encoded in MalE (MalE:T128V). Strikingly, this assay revealed that the higher-stability HA peptide successfully recruited T cells in vivo. Approximately 20% of the specific response was dedicated to the HA variant peptide, similar to the magnitude of MalE (103–118)- and MalE (269–285)- specific responses (Figure 4A), demonstrating that a half-life of 86 hr is sufficient for recognition of HA (126–138) in vivo. We also found that the two other high-stability variants of HA gained immunogenicity. The number of CD4 T cells responding to HA T128M ($t_{1/2}$ = 165 hr) was also equivalent to the two subdominant backbone MalE peptides previously identified. When inserted into MalE, the HA:T128Q variant that displayed a kinetic stability of 63 hr was more immunogenic than the wt peptide, although not as potent as the higher-stability variant. In contrast, when low-stability variants of HA were inserted into MalE and tested for immunogenicity, these peptides were not immunogenic. Responses to HA V131A (P4) and T128G (P1), both of which displayed quite low kinetic stability interactions with I-A^d ($t_{1/2}$ of 3 and 1 hr, respectively; Figure 4B), represented less than 4% of the overall re-

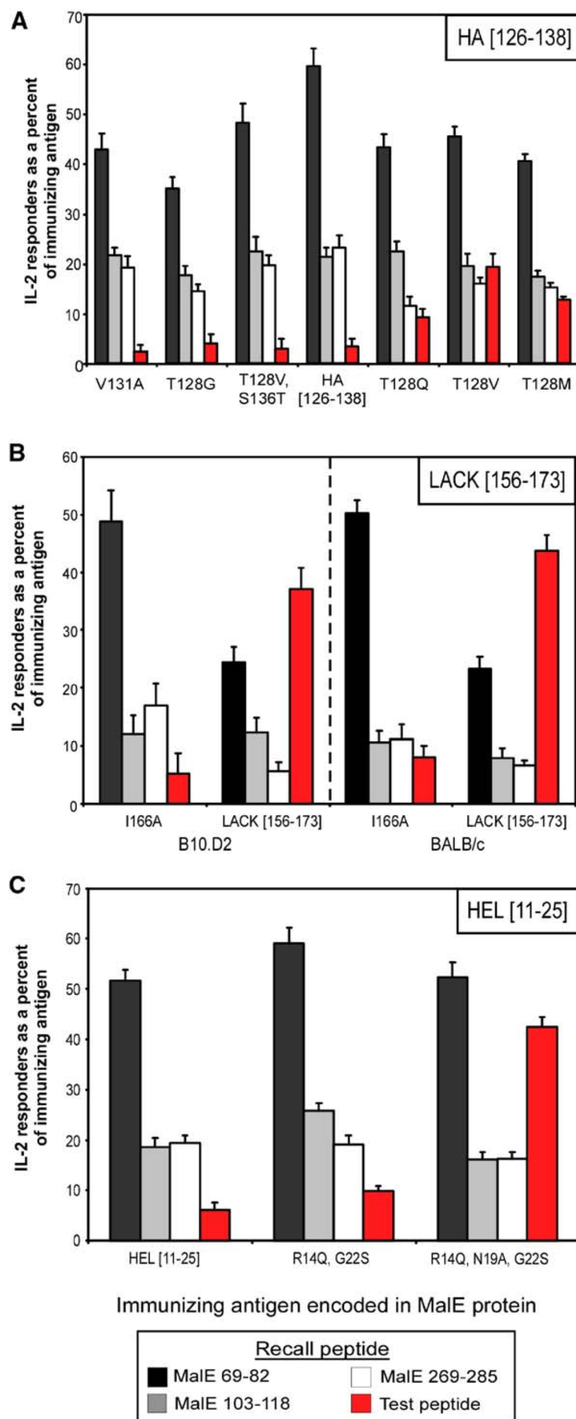

Figure 4. Kinetic Stability of Peptide:MHC Complexes Controls In Vivo Recognition of CD4 T Cell Epitopes

Groups of two mice (A, B, and C, BALB/c; B, B10.D2) were immunized in the footpad with 20 μg/ml (A and B) or 200 μg/ml (C) of the indicated protein emulsified in 50 μl PBS:CFA. The number of IL-2-producing cells at day 10 was determined by 16 hr in vitro stimulation of unpurified (A and B) or CD4 purified (C) cells with syngeneic spleen cells and 20 μg/ml (A and B) or 200 μg/ml (C) MalE protein or 5 μM peptide antigen by using IL-2 ELISPOT assays. Data represent the mean counts of two separate cell dilutions ([A] and [B], 1×10^6 and 5×10^5; [C], 5×10^5 and 2.5×10^5) of triplicate wells normalized as a percent of the response against the immunizing protein with background spot counts subtracted. Data presented represent the mean of at least three independent experiments ± SD.

sponse (Figure 4A). Finally, we were able to reverse the pattern of immunodominance gained with the HA T128V peptide. A new variant of T128V described above was designed to reduce its stability with I-A^d molecules. By substituting the P9 residue Ser in HA with the closely related Thr residue, the kinetic stability of HA T128V was reduced from 85 hr to 9 hr (Figure 2B). As shown in Figure 4A, the T128V, S136T variant of HA became cryptic in the CD4 T cell response when inserted into MalE. These data further support the idea that the immunogenicity of a peptide can be up or down regulated solely by changing its kinetic stability with class II molecules.

We next investigated recognition of the LACK (156–173) peptide epitope inserted within MalE (MalE:LACK) as a protein immunogen for H-2^d mice. The results of this experiment demonstrated that LACK (156–173)-specific T cells dominated the in vivo response, in fact surpassing the response to the MalE epitopes (Figure 4B). Over one in 16,000 lymph-node cells recognized LACK (156–173), which corresponded to an average of 37% of the total response to MalE:LACK protein in B10.D2 mice and 44% in BALB/c mice. These results show that the kinetically stable LACK (156–173) epitope is immunodominant within MalE. To determine whether reducing the kinetic stability of LACK with I-A^d would be sufficient to extinguish its immunodominance, the I166A variant of LACK (156–173) ($t_{1/2}$ = 2 hr) was tested (Figure 2D). The results of this experiment (Figure 4B) showed a lack of priming toward the low stability LACK:I166A epitope when inserted within MalE. T cells from MalE:I166A-immunized mice did not respond detectably to either the I166A variant peptide or the wt LACK (156–173) peptide. The number of lymph node cells specifically responding to LACK dropped precipitously, corresponding to fewer than one in 500,000 lymph-node cells. Responses against the MalE protein and backbone MalE peptides were clearly evident, indicating the MalE:I166A variant protein was effectively processed by APCs and that other MalE peptides within it were successfully presented. These results suggest that by reducing the kinetic stability of the binding of LACK to the I-A^d molecule, we have successfully eliminated the in vivo response to a normally immunodominant peptide.

To address whether the modulation of immunodominance with the MalE shuttle vector is unique to this expression system, we mutated the LACK (156–173) epitope in its normal molecular context. Recombinant LACK protein containing the wt peptide sequence or with a mutation at residue I166A, described above, was used to immunize BALB/c mice. T cells from the draining lymph node were tested for reactivity with the intact LACK protein, the wt LACK peptide, the I166A variant peptide, or PPD as an immunization control (Figure 5). The results of this experiment confirmed the results obtained with the MalE peptide shuttle protein. The

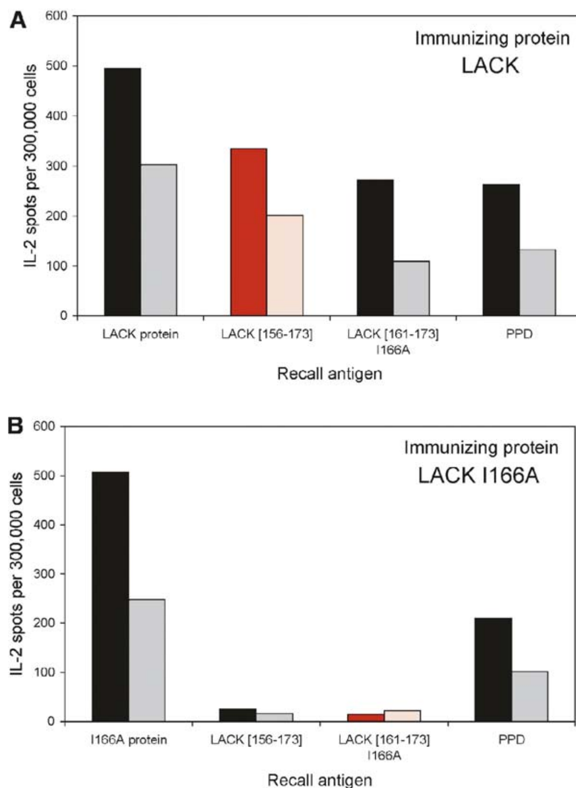

Figure 5. Kinetic Stability of LACK (156–173) with I-A^d Controls the Immunogenicity within Native LACK Protein

Groups of two BALB/c mice were immunized in the footpad with 200 μg/mL of purified LACK protein (upper) or purified LACK:I166A protein (lower) emulsified in 50 μL of PBS:CFA. The number of IL-2-producing cells at day 10 was determined by 16 hr in vitro stimulation of CD4-purified cells with syngeneic spleen cells and a range of protein or peptide concentrations. Shown are: LACK and LACK I66A protein, 200 μg/mL and 8 μg/mL; LACK and LACK:I166A peptide, 50 μM and 2 μM; PPD, 5 μg/mL and 1 μg/mL. Spots were quantified by using triplicate wells of IL-2 ELISPOT assays with background subtracted and are representative of two independent experiments.

strongly immunodominant LACK (156–173) peptide can be rendered cryptic by simply reducing the stability of its interaction with I-A^d. Our results also show that the loss in immunodominance is not due to loss of T cell reactivity, because the LACK:I166A variant peptide effectively stimulates the T cells that were primed against the wt protein.

To extend our studies to a third antigen, the cryptic HEL peptide (Moudgil et al., 1997) and its variants were analyzed for immunogenicity. When incorporated into the MalE protein vector, the failure in immunogenicity of the HEL (11–25) peptide persisted (Figure 4C). A higher stability variant ($t_{1/2}$ = 11 hr; Figure 2F) with changes at P1 and P9 (R14Q and G22S, respectively) was incorporated into MalE and tested in vivo, and it remained nonimmunogenic. However, when an HEL peptide variant with changes at P1, P6, and P9 (R14Q, N19A, and G22S, respectively; $t_{1/2}$ = 35 hr; Figure 2F) was inserted into MalE, we observed a significant gain in immunogenicity, suggesting a peptide ordinarily sequestered from an immune response can become immunogenic by enhancing the stability of its interaction with class II molecules.

Kinetic Stability Variants Retain Crossreactivity In Vivo

The possibility existed that we had created novel TCR contact profiles with the designed kinetic stability variants that either enhanced or abrogated T cell recognition compared to wt peptides. To evaluate this issue comprehensively, mice were immunized with wt or variant peptides and T cells were tested for recognition of both wt and variant peptide (Figure S2). We observed no change in the number of lymph-node cells that recognized the wt HA (126–138) or T128V variant peptide within each immunization condition (Figure S2A). Additionally, when tested for crossreactivity, we found no significant difference in the number of lymph node cells recognizing the wt or low-stability LACK (156–173) variants (Figure S2B). The vigorous responses to both HA (126–138) and LACK:I166A (156–173) as peptide immunogens also demonstrate that a lack of T cell precursors is not the explanation for the observed crypticity of the epitopes within MalE. The HEL variants were not as straightforward in their phenotype. When mice were immunized with the HEL:R14Q, N19A, G22S peptide, a significant portion of the T cells elicited were nonreactive with the wt HEL peptide (Figure S2C), suggesting that this peptide variant offers additional epitopic residues and can thus recruit T cells with additional specificities. To address the relative contribution of epitopic changes versus kinetic stability in the immunogenicity of the HEL peptides, mice were immunized with MalE containing either the wt HEL or R14Q, N19A, G22S peptide inserts. Although some of the gain in reactivity is attributable to recruitment of T cells of new specificity, a significant portion is due solely to the increased stability of the R14Q, N19A, G22S peptide complexed with I-A^d (Figure S2D).

The Normally Cryptic OVA (327–339) Epitope Recognized by the 3DO11.10 TCR Can Be Made Immunodominant

To provide another example of a cryptic epitope that can gain immunodominance by simply changing its kinetic stability with I-A^d, we studied one of the peptide registers contained within the prototypic I-A^d-restricted peptide OVA (323–339) (Jenkins et al., 2001; Sette et al., 1988). Several studies (McFarland et al., 1999b; Robertson et al., 2000) have shown that this long peptide contains several alternative registers, including the most amino-terminal segment that was cocrystallized with I-A^d (Scott et al., 1998). The register of OVA (323–339) peptide recognized by the 3DO11.10 T cell is the most carboxyterminal segment, with residue 329 constituting the P1 position (Robertson et al., 2000; Buus et al., 1987), which we confirmed through the use of the truncated peptide 327–339 (not shown). When the stability of this peptide with I-A^d was measured (Figure 6A), we found, surprisingly, that it displayed a very low kinetic stability of 0.3 hr. To test whether this low stability peptide was cryptic, we immunized mice with the OVA protein and tested for T cells that recognize OVA (327–339). Less than 1% of T cells raised against OVA recognize

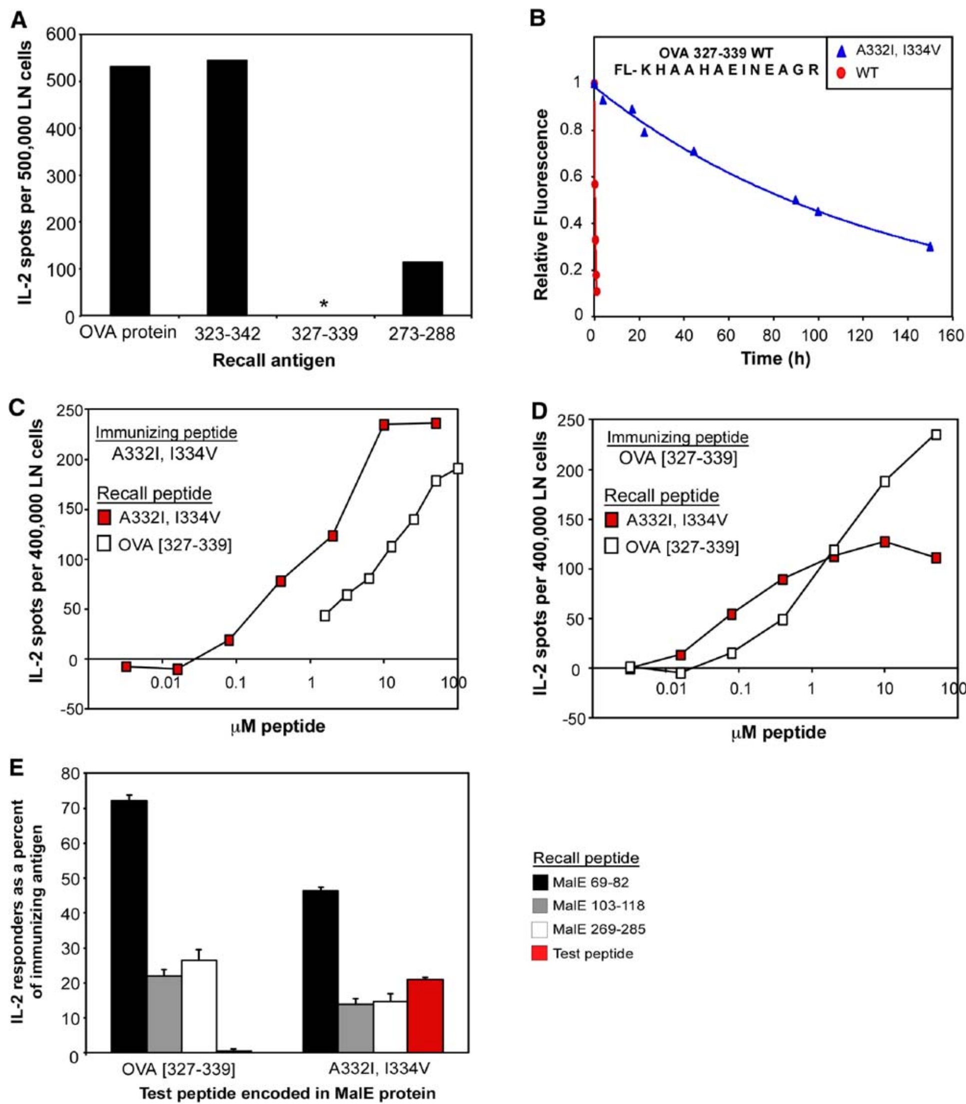

Figure 6. Increasing the Kinetic Stability of OVA (327–339) with I-A^d Enhances immunogenicity

Groups of two BALB/c mice were immunized in the footpad with (A) 200 μg/mL OVA protein, (B) 200 μg/mL of the indicated MalE protein, or (D and E) 5 nmol of the indicated peptide emulsified in 50 μL PBS:CFA. The number of IL-2-producing cells at day 10 was determined by 16 hr in vitro stimulation of CD4-purified cells with syngeneic spleen cells and (A) 200 μg/mL protein or 12.5 μM peptide, (B) 200 μg/mL protein or 5 μM peptide, or (D and E) the indicated amount of peptide and quantified with IL-2 ELISPOT assays with background subtracted. The data presented represent the mean of two independent experiments ± SD.

(A and B) Data represent the mean counts of two separate cell dilutions (2.5×10^5 and 1.25×10^5) of triplicate wells normalized as indicated.

(C) Dissociation of peptides from I-A^d was found to fit a single exponential curve with a square correlation coefficient, $r^2 > 0.99$, from which $t_{1/2}$ could be determined. Asterix (*) indicates no detectable stimulation above background. Data are representative of at least two independent experiments.

OVA (327–339) (Figure 6B), indicating that it is cryptic. A variant with substitutions at P4 and P6 (A332I and I334V, respectively) that was anticipated to improve binding to I-A^d, was recognized by the 3DO11.10 T cell hybridoma (not shown) and showed a greatly enhanced stability with I-A^d, with a $t_{1/2}$ greater than 80 hr (Figure 6B). Additionally, T cells raised against the P4/P6 variant crossreacted back onto the wt OVA peptide (Figures 6C and 6D). To evaluate dominance of the higher stability peptide, both the wt and variant OVA peptides were incorporated into MalE and tested for immunogenicity in BALB/c mice (Figure 6E). These studies revealed that improved P4 and P6 pocket interactions converted the cryptic OVA peptide into an immunodominant peptide. Collectively, our results suggest, unlike the situation with the HEL (11–25) peptide, that the gain in immunogenicity of the OVA peptide can be accounted for by its more stable interaction with the class II-presenting molecule.

Discussion

We initiated these experiments to determine whether the magnitude of CD4 T cell priming to a given peptide

in a complex antigen is due to an intrinsic property of the class II:peptide complex. After investigating both peptide competition and peptide dissociation, we found that the kinetic stability of class II:peptide complexes correlated dramatically with the epitopes' observed immunodominance. These findings agree with studies of the murine T cell response to human cartilage glycoprotein 39 (Hall et al., 2002). Dissociation assays provide a more simplified measure of affinity because competition assays more completely reflect the mechanistically complicated process of peptide binding to class II molecules (Beeson and McConnell, 1994, 1995; Joshi et al., 2000; Kasson et al., 2000; Natarajan et al., 1999; Rabinowitz et al., 1998; Sadegh-Nasseri and McConnell, 1989) and are highly subject to experimental variability (Table S1). To investigate a causative relationship between kinetic stability of a peptide with the class II molecule and its in vivo recognition by T cells, kinetic stability variants of four independent test peptides (HA [126–138], LACK [161–173], HEL [11–25], and OVA [327–339]) were incorporated into MalE. These experiments revealed that immunodominance can be conferred by increasing the kinetic stability of a given peptide with MHC class II and conversely can be eliminated by decreasing the kinetic stability of a peptide with MHC class II molecules.

The dramatic linkage between kinetic stability and in vivo recognition demonstrated here raises several critical questions. Most importantly, at what stage during presentation does the kinetic stability of a class II:peptide complex physiologically influence the recognition and expansion of specific T cells? Peptide epitopes must overcome at least three distinct hurdles for recognition by specific T cells in vivo: (1) loading onto class II in endocytic compartments and export to the surface of antigen-presenting cells; (2) survival of class II:peptide molecules during the transit of antigen-bearing dendritic cells to the draining lymph node; and (3) sustained TCR signaling once contact between CD4 T cells and the antigen-bearing dendritic cells is established. The first checkpoint is monitored by the presence of DM (Nanda and Sant, 2000), which exerts a critical regulatory role in editing peptides presented by class II molecules through discrimination among assembled class II peptide complexes within endosomal compartments of APCs (reviewed in Busch et al., 2000; Kropshofer et al., 1997; Sant et al., 2005; Vogt et al., 1999). A mechanism for DM-mediated selection of immunodominant epitopes based on kinetic stability is consistent with evidence that the catalytic effect of DM on class II:peptide dissociation is determined by the intrinsic off-rate of the complexes (reviewed in Jensen et al., 1999; Vogt et al., 1999), although recent data suggest this to be an imperfect correlation (Belmares et al., 2002). In this model, although dissociation of immunodominant class II:peptide complexes may be promoted by DM, the high stability of these complexes will lead to negligible effects during the time frame of endosomal colocalization with DM. Thus, most immunodominant complexes persist in the presence of DM and are displayed at a high initial density on the surface of priming APCs.

High kinetic-stability peptides may possess additional advantages during later events in T cell priming. Once class II:peptide complexes reach the cell surface, they must persist long enough to survive transit of the priming dendritic cell from the site of antigen uptake to the draining lymph nodes (reviewed in Germain and Jenkins, 2004). Studies by Mellman and colleagues have shown that uptake of antigen is lost during maturation of DC (Mellman and Steinman, 2001). Therefore, dendritic cells are essentially pulsed with antigen before transit to the lymph node, and any peptides that dissociate from class II molecules are not replenished. This transit time represents the second kinetic-stability checkpoint for a class II:peptide complex to survive. After relocation to lymph nodes, dendritic cells must maintain surface presentation of class II:peptide complexes in order to recruit antigen-specific T cells and to engage in sustained interactions, estimated to be 10–20 hr (Huppa et al., 2003; Lanzavecchia and Sallusto, 2001). Dissociation of class II:peptide complexes during T cell-APC interactions might lead to an abortive activation signal that does not sustain ultimate expansion and differentiation of T cells (Mirshahidi et al., 2004; Ryan et al., 2004). Finally, kinetically stable complexes may be required to stimulate daughter cells from early cell divisions. These factors, when combined, may lead to a significant competitive advantage for T cells specific for the high-stability complexes.

One question that may arise from our studies is how closely the off-rates we measure relate to off-rates of peptides from class II molecules on live APCs in vivo. We do not expect the measured off-rates here to precisely mimic dissociation rates in live APCs, although these values will likely reflect relative kinetic stabilities in endosomal compartments. We have found reasonably good agreement between off-rates in vitro of fluorescently labeled peptide at pH 7.2 with dissociation rates of peptides from live APCs measured with T cells (Figure S3).

Our experiments do not discount the influence of processing upon the selectivity of the in vivo T cell response, and we expect that there will likely be exceptions to the paradigm we have established here. Endopeptidases have been described to either enhance (Schneider et al., 2000) or destroy (Burster et al., 2004; Manoury et al., 2002) epitopes available for class II molecules. A prominent protease cleavage site within the minimal epitope can prevent an immune response if these peptides are destroyed before they can bind and be protected from proteolysis by the class II molecule. Also, antigens that either have a high degree of tertiary structure or tend to aggregate may sequester peptides from proteolytic release and, thus, from availability for binding to class II molecules. Finally, the requirement for high-stability interactions of a peptide with the class II molecule for immunodominance in CD4 T cells may be bypassed during responses to antigens synthesized within the APC or those made by pathogens that replicate within the host APC. Continual synthesis of these peptides may obviate the need for high-stability interactions with class II molecules. This may explain why HA (126–138), which has a modest kinetic stability with I-A^d (25–30 hr), was found to be well represented in the response to influenza virus (Gerhard et al., 1991). Our own studies of self-antigens recognized by T cells (Katz et al., 1996) suggest that these are frequently antagonized by DM, consistent with lower-affinity interactions with class II molecules (Sant et al.,

2005). Finally, we expect that T cell repertoire asymmetries would, in some cases, lead to exceptions to the close predictive relationship between stability and immunodominance, which we have found in our studies. Class II-restricted presentation of self-antigens or those closely related to self in the thymus can skew the developing T cell repertoire, selecting out T cells responsive to immunodominant epitopes. Thus, subsequent antigenic challenge in the periphery would reveal a bias in the T cell response for epitopes with low apparent affinity for class II molecules. Such effects on T cell repertoire are supported by a number of papers in the literature (Harrington et al., 1998; Liu et al., 1995; Phelps et al., 1998; Targoni and Lehmann, 1998; Wong et al., 2001). For this reason, we have restricted our studies primarily to exogenous foreign antigens.

Collectively, the experiments described here show that for many antigens, a single biochemical feature of a given peptide within a complex immunogen directly and quantitatively controls the immunogenicity of that peptide during the CD4 T cell response. Furthermore, this parameter—the off-rate of the peptide from the presenting class II molecule—can be profoundly modulated by single amino-acid changes in the peptide, leaving T cell recognition intact. Thus, varying kinetic stability of MHC class II:peptide complexes represents a unique and powerful method of tuning in vivo recognition towards a preferred outcome in CD4 T cell responses.

Experimental Procedures

Antibodies and Peptides

Purified rat anti-mouse IL-2 (JES6-1A12) antibodies and biotinylated rat anti-mouse IL-2 (JES6-5H4) antibodies were obtained from BD Pharmingen. Synthetic peptides were obtained from commercial sources or were the generous gifts of C. Beeson (Medical University of South Carolina), N. Glaichenhaus (University of Nice), and D. Fowell (University of Rochester).

Purification of Soluble I-A^d Proteins

A chimeric soluble I-A^d protein (sI-A^d) with a small segment of the carboxyterminal domains of I-A replaced with I-E sequences was used for peptide binding studies. We have shown that the modifications improve dimer stability but do not affect peptide binding characteristics of class II molecules (Chaves et al., 2005). Transfectants expressing the PI-linked class II molecules were used as a source of class II molecules, which were obtained from detergent lysates of transfectants by antibody affinity chromatography as described (Chaves et al., 2005).

Dissociation Experiments

sI-A^d (50 nM final concentration) was mixed with FITC peptide (5 μM final concentration) in McIlvaines buffer pH 5.3 (0.2 M citric acid, 0.5 M Na_2HPO_4), 0.2 mM n-dodecyl maltoside, 0.025% NaN_3 in the presence of protease inhibitors for 1–16 hr at 37ºC. sI-A^d-FITC-peptide complexes were separated from free FITC-peptide by passage over a Micro Bio-Spin 30 column, and the complexes were incubated at 37ºC and pH 5.3 for increasing lengths of time in the presence of 5 μM unlabeled Eα (52–68) peptide to avoid rebinding of the fluorescinated peptide. At each time point, a sample of the dissociation mixture was injected into a LC-10AT HPLC (Shimadzu Corporation) equipped with a Bio-Sep-SEC-S 3000 column 300 × 7.8 mm (Phenomenex Inc.) connected to an in-line fluorescence detector (RF-10AXL fluorescence detector; Shimadzu Corporation) as described (Chaves et al., 2005).

Competition Experiments

For competition assays, 20 nM of soluble I-A^d and 1 μM FITC-HA (126–138) were used, which we determined to be in the titratable range of both components. Unlabeled competitor peptides at final concentrations ranging between 0–500 μM were mixed and, after 16–20 hr of incubation at 37ºC, the sI-A^d-FITC-HA(126–138) complex was separated from free peptide and quantified as described above. Inhibition by competitor peptides was calculated with the Hill equation (Hill, 1910), with the IC_{50} value as the concentration of unlabeled competitor peptide required to achieve 50% inhibition of the labeled peptide binding to class II molecules.

T Cell Hybridoma Assays

The LACK-specific hybridoma (4F7) and HA-specific hybridoma (TS2) were created by fusion of peptide-activated LN cells from the ABLE mouse (Reiner et al., 1998) (4F7) or HNT-TCR mouse (Scott et al., 1994) (TS2) with BW5147 lymphoma cells. T cell assays were performed as previously described in overnight cultures (Peterson and Sant, 1998), with peptide or protein at the specified dose in a flat-bottom 96-well dish. IL-2 produced by the T cells was quantified by using CTL.L and MTT assays as previously described (Peterson and Sant, 1998).

Immunizations

BALB/c or B10.D2 mice were immunized in the footpad with 50 μl of 20 μg/ml MalE protein or 5 nmole of peptide emulsified in CFA (Sigma-Aldrich). Ten days later, cells were isolated from draining popliteal lymph nodes. IL-2 production by the unpurified lymphnode cells was measured by ELISPOT assay as described previously (Wang and Mosmann, 2001) by using DMEM media with 10% fetal calf serum (Katz et al., 1996) instead of RPMI and triplicate wells for each condition. Quantification of IL-2-producing cells was accomplished with an Immunospot reader series 2A by using Immunospot software version 2.0 (Cellular Technologies Ltd.).

MalE Protein Purification

Poly acrylamide gel electrophoresis (PAGE)-purified synthetic oligonucleotides encoding the desired peptide were obtained from Integrated DNA Technologies (IDT) and resuspended in 10 mM Tris and 1 mM EDTA at a concentration of 100 μM. Annealed double-stranded DNA was ligated into the MalE133 vector, and sequenced clones were transformed into MalE (–/–) ER2507 *E. coli*. MalE protein was prepared as described (Martineau et al., 1992) with some modifications (see Supplemental Data).

LACK Protein Synthesis

LACK cDNA expression vector (Mougneau et al., 1995) was mutated at position 166 via Quikchange site-directed mutagenesis (Stratagene) and confirmed by sequence analysis. Overnight cultures of BL21(DE3λ) bacteria (Novagen) transfected with LACK or LACK:I166A were inoculated into 500 mL of LB with ampicillin and chloramphenicol and grown at 37ºC until an OD_{600} of 0.5 was reached. 0.25 mL of 1 M IPTG was added to induce protein expression, and bacteria were grown for another 3 hr at 37ºC and subsequently harvested by centrifugation at 5000 × g for 15 min at 4ºC. Pellets were resuspended in 100 mL of 10 mM Imidazole, 50 mM $NaHPO_4$, and 300 mM NaCl (pH 8.0) and sonicated for 1 min. Supernatants were pelleted by centrifugation at 26,000 × g for 25 min at 4ºC. Protein was purified from supernatants via Ni-NTA affinity column and assayed for quantity and purity via sodium dodecylsulfate-PAGE analysis.

Supplemental Data

Supplemental Data including three figures, one table, Supplemental Experimental Procedures, and Supplemental References are available online with this article at http://www.immunity.com/cgi/content/full/23/1/29/DC1/.

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