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DM Peptide-Editing Function Leads to Immunodominance in CD4 T Cell Responses In Vivo

Navreet K. Nanda and Elizabeth K. Bikoff

DM functions as a peptide editor for MHC class II-bound peptides. We examined the hypothesis that DM peptide editing plays a key role in focusing the in vivo CD4 T cell responses against complex pathogens and protein Ags to only one, or at most a few, immunodominant peptides. Most CD4 T cells elicited in the wild-type BALB/c (H-2d) mice infected with Leishmania major predominantly recognize a single epitope 158–173 within Leishmania homologue of activated receptor for c-kinase (LACK), as is the case when these mice are immunized with rLACK. Using DM-deficient (DM−/−) H-2d mice, we now show that in the absence of DM, the in vivo CD4 T cell responses to rLACK are skewed away from the immunodominant epitopes and are diversified to include two novel epitopes (LACK 33–48 and 261–276). DM−/− B10.BR (H-2b) mice showed similar results. These results constitute the first demonstration of the role of DM peptide editing in sculpting the specificity and immunodominance in vivo.

Maj or histocompatibility complex class II molecules on the surface of specialized APC present peptides to CD4 T lymphocytes, and thus select a useful CD4 T cell repertoire in the thymus and guide CD4 T cell responses to pathogens in the periphery. DM, a nonclassical MHC heterodimer, functions as a chaperone to promote capture of diverse peptides by MHC class II molecules inside the endocytic compartments of APC. In addition to releasing CLIP from newly synthesized MHC class II and stabilizing empty MHC class II (1–5), DM edits the repertoire of non-CLIP peptides loaded onto the MHC class II-binding groove (6–9). Peptide editing by DM is thus consequential for recognition of exogenous and endogenous Ags because it introduces prejudice toward display of DM-resistant and/or DM-dependent peptides by MHC class II molecules. Features of the peptide-MHC complex that determine whether DM would release the peptide from the MHC II-peptide complex or enhance its binding remain highly controversial. Although initial studies suggested that DM susceptibility of a peptide is a correlate of low intrinsic stability of its binding to MHC class II (8–10), many recent studies have concluded to the contrary that the intrinsic kinetic instability of a peptide-MHC complex does not predict DM susceptibility of the peptide and vice versa (11–14). Nevertheless, while rules governing DM susceptibility of a peptide-MHC complex are yet to be elucidated, the current evidence indicates that DM diminishes the overall selectivity of the class II-binding groove (12).

DM peptide-editing activity has been extensively described in transfected cell lines and in vitro assays with soluble DM and soluble MHC-peptide complexes (4, 6–14). The significance of peptide-editing function on Ag presentation has been analyzed using mutant/transfected cell lines (7, 15, 16) and APC from DM mutant mice (17–21). However, the consequence of DM peptide editing on in vivo pathogen or protein Ag-specific CD4 T cell responses still remains unknown. A foremost reason for a lack of such analyses is that, until recently, the DM mutant (DM−/−) mice had been constructed only in the H-2b haplotype (17–19). Allele-specific attributes allow formation of extremely tightly bound, long-lived, and SDS-stable Aβ-CLIP complexes, strictly dependent upon DM for CLIP release, resulting in a predominant expression of Aβ-CLIP complexes on the cell surface in the H-2b haplotype DM-deficient mice (17–20, 22, 23). Loading of non-CLIP peptides onto Aβ and Ag and peptide presentation by APC in these mice are severely disrupted as are events in thymic selection (17–20, 22, 23). Due to the severity of defects in the H-2b haplotype, a prevalent in vivo study using DM-deficient H-2b mice infected with Leishmania major provided limited insight into DM function as a peptide editor (24). More recently, BALB/c and B10.BR DM mutant mice have been constructed (22, 23), and in sharp contrast to their H-2b counterparts, APC function and loading of non-CLIP peptides onto MHC class II in these strains show a significantly less acute phenotype. The differences in H-2d and H-2b DM mutant mice (compared with H-2b mutants) are due to allelic polymorphism in MHC class II in these strains that allow formation of only loosely bound and short-lived MHC class II-CLIP complexes (Aβ, CLIP, Eδ-CLIP; Aβ-CLIP, Eδ-CLIP), resulting in a relaxation of DM requirements for peptide loading (22, 23). BALB/c and B10.BR DM mutant mice are thus amenable to examination of the consequence of in vivo peptide editing on CD4 T cell responses against a protein Ag or a pathogen.

The tendency of the immune system to focus T cell responses against a select number of potential epitopes of a complex pathogen or a protein Ag is termed immunodominance (15, 25, 26). A well-studied example is BALB/c (H-2b) mice infected with L. major in which Leishmania-specific T cells predominantly recognize a single epitope within the Leishmania homologue of activated receptor for c-kinase (LACK) (27–30). Thus, CD4 T cells elicited in...
DM PEPTIDE-EDITING FUNCTION LEADS TO IMMUNODOMINANCE

wild-type BALB/c mice infected with L. major or immunized with rLACK recognize a single immunodominant epitope 158–173 (27–30). B10.BR mice challenged with rLACK similarly show an immunodominant CD4 T cell response to a single epitope, LACK 81–96 (this study). In the current study, we tested the hypothesis that in vivo DM peptide editing plays a pivotal role in targeting the CD4 T cell response exclusively to the immunodominant epitopes. This hypothesis was strengthened by our previous in vitro studies using transfected cell lines as APC indicating that: 1) DM was required for presentation of an immunodominant epitope of hen egg-white lysozyme (HEL), and 2) DM antagonized the presentation of recessive epitopes of HEL (15). Using a set of 75 overlapping, 16-mer peptides spanning the 312-aa-long LACK protein, we now show that the phenotype of CD4 T cell response focused on a single immunodominant epitope requires in vivo expression of DM in both the BALB/c and B10.BR strains of mice. DM mutant mice: 1) fail to respond to the immunodominant epitopes in both haplotypes and 2) show a response to two novel epitopes in each strain.

Materials and Methods

Mice

BALB/c (H-2b) DM mutant (DM−/−) and B10.BR (H-2b) DM−/− mice have been previously described (22, 23). The DM mutant mice were bred in specific pathogen-free animal facility of Harvard University. Six- to 8-wk-old female homozygous DM−/− mice in the two haplotypes were shipped to the specific pathogen-free facility of Georgetown University Medical Center, where the mice were housed until immunization 1–3 wk later. Wild-type, female, age-matched, BALB/c/J and B10.BR mice were either littermate controls or the mutant mice bred at Harvard University and shipped to Georgetown University Medical Center or were obtained from The Jackson Laboratory. The animal protocols describing the use of mice were approved by Institutional Animal Care Committees.

Ags and peptides

rLACK Ag was purified, as described before (29). Briefly, Escherichia coli transfected with an expression plasmid (a gift from N. Ghiaichnais, Institut National de la Santé et de la Recherche Medicale, Valbonne, France) encoding LACK protein linked to a 6-histidine tag (29) were grown in large quantities and lysed, and the soluble fraction from E. coli lysates was passed over nickel-agarose affinity (Ni-NTA) columns (Qiagen). Fractions with the highest protein concentration were passed over Ni-agarose columns a second time to get a single band in SDS-PAGE electrophoresis (>95% pure protein) (data not shown). Protein concentration was determined using Bio-Rad protein assay reagent before freezing aliquots of protein at −20°C. Twenty-five overlapping, 16-mer peptides offset by 4 aa (e.g., aa 1-16 (peptide 1), 5-20 (peptide 2), 9–24 (peptide 3)), etc., were custom synthesized according to the published sequence of LACK (30) by Mimotopes (Clayton South). The stock peptides were dissolved in a solvent containing 45% acetonitrile (Sigma-Aldrich) and 55% distilled water containing penicillin-streptomycin (Invitrogen Life Technologies) to give 1 mM peptide solution, and each peptide solution was used at a working dilution of 1/100 in culture wells. Bulk peptides LACK 158–173, LACK 33–48, and LACK 261–276 were custom synthesized by Macromolecular Resources and were dissolved at 400 μM in sterile PBS as stock solutions.

T hybridomas and Abs

LMR 16.2 and LMR 17.1 were LACK 158–173/Aα-reactive CD4 T hybridomas (31) and were a gift of N. Ghiaichnais. Purified anti-Aα Ab (clone MKD6) was obtained from BD Pharmingen. Anti-Eβ (14.4.4S) and some batches of anti-Aα (MKD6) and Abs were purified in the laboratory (32).

Immunizations

Mice were immunized, as described before (32, 33). Briefly, rLACK or LACK 158–173 or LACK 33–48 was emulsified in CFA (1:1 mixture of CFA obtained from Invitrogen Life Technologies and Difco/BD Biosciences) and immunized s.c. in one hind foot pad (between 1 and 7 μM rLACK; 7 μM LACK 158–173, or LACK 33–48, or LACK 261–276 peptide per foot pad in each mouse).

In vitro lymphocyte proliferation assays

Lymphocyte proliferation assays were done as described before (32, 33). Briefly, lymphocytes from the draining lymph nodes were obtained on day 9 or 10 after immunization, and cells were cultured in serum-free medium. HL-1 (Cambrex), in microwell plates without or with purified protein derivative (PPD) of Mycobacterium tuberculosis as a control for immunization, or various concentrations of rLACK Ag or LACK peptides. The wells containing pepscan peptides (2 μl) dissolved in acetonitrile and water mixture (see above) and those with the solvent alone (2 μl) were left without a lid in the sterile flow hood for 60 min to evaporate the solvent before addition of fresh medium and cells. Cells were pulsed with [3H]thymidine, 96 h after initiating cultures, and harvested 20 h later in an automatic cell harvester (Tomtec), and incorporation was measured by liquid scintillation in a Betaplate counter (Wallac).

Ag-specific CD4 T cell lines

Long-term, 33–48-reactive T cell lines were derived from lymph node T cells (LNC) obtained from BALB/c DM−/− mice immunized with 33–48, as described before (32).

In vitro Ag presentation assays

In assays using T hybridomas, activation of T hybrids LMR 16.2 and LMR 17.1 was assayed, as described before (15). Briefly, spleen cells from BALB/c DM−/− and wild-type mice were used as APC and incubated with T hybridoma cells with varying concentrations of rLACK or peptides. IL-2 was measured after 24 h by ELISA, as described earlier (15). In assays using LACK 33–48-reactive T cell lines, T cells (105) were cultured with BALB/c (DM−/− or DM−/−) splenic APC and without or with varying concentrations of LACK or 33–48. T cell activation was measured 48 h later by proliferation using [3H]thymidine incorporation, as described before (32). To determine whether 33–48-reactive T cells were restricted by Aα or Eβ molecules, anti-Aα or anti-Eβ Abs (25–50 μg/ml) were added at the start of cultures with or without peptides (data not shown).

Results

DM expression is required for the in vivo CD4 T cell response to be focused to the immunodominant epitope 158–173 of LACK in BALB/c mice

We examined LNC proliferative responses of wild-type (DM+/-) and DM-deficient (DM−/−) BALB/c mice immunized with rLACK to 75 overlapping, 16-mer peptides (pepscan) synthesized according to the sequence of LACK protein (see Materials and Methods). rLACK-immunized DM−/− mice clearly show a phenotype of an immunodominant CD4 T cell immune response (Fig. 1a). Thus, the focus of the entire CD4 T cell response is on overlapping peptides 40 (LACK 157–172) and 41 (LACK 161–176), which represent a previously published immunodominant epitope (27–30), LACK 158–173. The sequences of peptides 40 and 41 are shown in Table I. LACK 158–173 has been previously defined to be restricted to Aα molecules (28, 29).

In sharp contrast, BALB/c DM−/− mice challenged with rLACK revealed divergent results shown in Fig. 1b. The data demonstrate three key observations: 1) the thrust of T cell response, in DM−/− mice, is skewed away from the immunodominant epitope, 158–173/Aα (represented by overlapping peptides 40 and 41), DM−/− mice show no response to peptide 40 (LACK 157–172), but express a modest proliferative response to peptide 41 (LACK 161–176). Because DM−/− LNC consistently showed no reactivity to 157–172, we anticipate that the response observed in DM−/− mice may be directed to a different, but an overlapping determinant within this region of LACK (34). 2) DM−/− mice express a strong T cell response to a novel epitope represented by overlapping peptides 9 (LACK 33–48) and 10 (LACK 37–52) (this epitope is referred to as LACK 33–48 in the remaining text). It should be noted that the DM+/- LNC show no reactivity toward LACK 33–48 or LACK 37–52 peptides. 3) DM−/− mice display a subdominant CD4 T cell response to a second novel epitope represented by peptide 65 (LACK 257–272) and peptide 66 (LACK 261–276) (Fig. 1b). Again, DM−/− mice fail to show any response to this novel epitope.

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LNC cultures from DM

The horizontal line with dashes and dots indicates the 3-fold value for a positive control were as follows: 175,409/H11006

Each peptide was used at 7 μM concentration, and LNC cultures were done as described before (32, 33). The sequence of selected peptides is included in Table I. The results represent mean cpm ± SE of each triplicate culture of LNC. The data show lymphocyte proliferative responses of the LNC obtained from the draining lymph nodes of BALB/c DM

The data show lymphocyte proliferative responses of LNC obtained from the draining lymph nodes of BALB/c DM

FIGURE 2. BALB/c DM+/+ and DM−/− mice show equivalent in vivo responses to LACK Ag. The figure shows LNC proliferative responses of BALB/c DM+/+ (■) and DM−/− (□) mice immunized with rLACK. LNC were cultured with or without various concentrations of LACK protein, as described in Fig. 1. The data represent mean cpm ± SE of triplicate cultures.

indicate that the LNC from DM−/− mice show a strong and dose-dependent T cell response to rLACK Ag that is comparable to that from DM+/+ mice. The half-maximal response in DM−/− mice, however, requires 8-fold more Ag than that in DM+/+ mice. Nevertheless, these results confirm that APC in DM−/− mice are able to process and present LACK Ag to CD4 T cells in vitro, even though the specificity of T cells induced in vivo in these mice is not skewed toward the immunodominant epitope (Fig. 1b).

DM expression is required for the in vivo CD4 T cell response to be focused to the immunodominant epitope LACK 81–96 of LACK in B10.BR mice

FIGURE 3. DM is required for an in vivo CD4 T cell response to be focused to the immunodominant epitope 81–96 of LACK Ag in B10.BR mice.

Fig. 3a shows the LNC proliferative responses of wild-type B10.BR (DM+/+) mice immunized with rLACK Ag to 75 overlapping, pepscan peptides of LACK protein. As is the case for the

Table I. Sequences of selected lack pepscan peptides

<table>
<thead>
<tr>
<th>Mice</th>
<th>Epitopes</th>
<th>Pepscan No.</th>
<th>LACK aa</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>DM+/+</td>
<td>40</td>
<td>157–172</td>
<td>CFSFSLHEPIVVSGSWV*</td>
<td></td>
</tr>
<tr>
<td></td>
<td>41</td>
<td>161–176</td>
<td>SLEHP1VVSGSW0NTI</td>
<td></td>
</tr>
<tr>
<td></td>
<td>9</td>
<td>33–48</td>
<td>SRDGTIVSWKANPDRH</td>
<td></td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>37–52</td>
<td>TVIWSRNPDHPSVDS</td>
<td></td>
</tr>
<tr>
<td></td>
<td>65</td>
<td>257–272</td>
<td>SSLSYDLESKAVIARL</td>
<td></td>
</tr>
<tr>
<td></td>
<td>66</td>
<td>261–276</td>
<td>YDLESKAVIAELTPDG</td>
<td></td>
</tr>
<tr>
<td></td>
<td>21</td>
<td>81–96</td>
<td>LTAGWDRSIRMDLRN</td>
<td></td>
</tr>
<tr>
<td></td>
<td>22</td>
<td>85–100</td>
<td>WDRSIRMDLRNQCCQ</td>
<td></td>
</tr>
<tr>
<td>H-2K</td>
<td>55</td>
<td>217–232</td>
<td>AALLWDLSTGEQLKFRI</td>
<td></td>
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<tr>
<td></td>
<td>56</td>
<td>221–236</td>
<td>WDLSTGEQLKFIRNEVS</td>
<td></td>
</tr>
<tr>
<td></td>
<td>37</td>
<td>145–160</td>
<td>LRDGHEDVYSGICFSP</td>
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* Bold letters represent the common amino acid residues in overlapping peptides; the sequence of bold residues is expected to include contact residues required for binding to the MHC and for interacting with TCR.

** No overlapping peptide in the region LACK 145–160 was stimulatory.

BALB/c DM+/+ and DM−/− mice, overall, show comparable in vivo CD4 T cell responses to rLACK Ag

Fig. 2 depicts the LNC proliferative responses to LACK Ag by DM+/+ and DM−/− mice immunized with rLACK Ag. The results

indicate that the LNC from DM−/− mice show a strong and dose-dependent T cell response to rLACK Ag that is comparable to that from DM+/+ mice. The half-maximal response in DM−/− mice, however, requires 8-fold more Ag than that in DM+/+ mice. Nevertheless, these results confirm that APC in DM−/− mice are able to process and present LACK Ag to CD4 T cells in vitro, even though the specificity of T cells induced in vivo in these mice is not skewed toward the immunodominant epitope (Fig. 1b).

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<td>221–236</td>
<td>WDLSTGEQLKFIRNEVS</td>
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<td></td>
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<td>145–160</td>
<td>LRDGHEDVYSGICFSP</td>
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DM pepsin. The sequences of these peptides are shown in Table I.

The entire CD4 T cell response is on a single epitope, represented by peptide 21 (LACK 81–96) and peptide 22 (LACK 85–100) of the CD4 T cell immune response (Fig. 3a). Thus, the focus of the entire CD4 T cell response is on a single epitope, represented by peptide 21 (LACK 81–96) and peptide 22 (LACK 85–100) of the pepsin. The sequences of these peptides are shown in Table I.

Fig. 3b depicts the LNC proliferative responses in B10.BR DM−/− mice immunized with rLACK. Three key observations revealed by these data are as follows: 1) DM−/− mice fail to elicit a T cell response targeted to the immunodominant epitope 81–96 (as represented by peptides 21 and 22) (see Fig. 3a). 2) DM−/− mice express a strong T cell response to a novel epitope represented by peptide 55 (LACK 217–232), and to a smaller extent to overlapping peptide 56 (LACK 221–236) and perhaps 54 (LACK 213–228). It should be noted that the DM+/+ LNC exhibit no reactivity toward these peptides. 3) DM−/− mice display a CD4 T cell response to a second novel epitope represented by peptide 37 (LACK 145–160) (Fig. 3b). Once again, the wild-type B10.BR mice fail to show any response to this novel epitope.

H-2d mice (Fig. 1a), it is clear that the DM+/+ H-2b haplotype mice immunized with rLACK Ag also show an immunodominant phenotype in their CD4 T cell immune response (Fig. 3a). Thus, the focus of the entire CD4 T cell response is on a single epitope, represented by peptide 21 (LACK 81–96) and peptide 22 (LACK 85–100) of the pepsin. The sequences of these peptides are shown in Table I.

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DM+/+ and DM−/− mice, overall, show comparable in vivo CD4 T cell responses to rLACK Ag

Data in Fig. 4 demonstrate that LNC from DM+/+ and DM−/− B10.BR mice immunized with rLACK protein show equivalent T cell proliferative responses to LACK Ag. These results also confirm that APC in DM−/− mice are able to process and present LACK Ag to CD4 T cells in vitro; however, the specificity of T cells induced in vivo in these mice is not skewed toward the immunodominant epitope.

BALB/c DM−/− mice have an intact T cell repertoire able to recognize LACK 158–173/A4α epitope

Underlying mechanisms of differential T cell responses in DM+/+ and DM−/− mice were further examined in BALB/c mice. Thus, to pursue the argument as to whether an absence of LACK 158–173/A4α-specific CD4 T cell responses in BALB/c DM−/− mice was

FIGURE 5. LACK 158–173/A4α-specific T cell repertoire exists in BALB/c DM−/− mice; however, DM−/− APC are unable to display this epitope after processing. a and b, BALB/c DM−/− (a) and DM+/+ (b) mice were immunized with peptide LACK 158–173, as described before (32). The data show LNC proliferative responses of four individual DM−/− (a) and DM+/+ (b) mice. LNC were cultured without or with various concentrations of LACK 158–173 (top panels) or rLACK Ag (bottom panels). Each symbol represents data from an individual mouse. Data in the top and bottom panels in a were obtained from the same mice, as represented by each symbol, as is the case in b. The LNC cultures were done as described in Fig. 1. The horizontal lines (dashed or otherwise) represent medium control values for each mouse, which were as follows: DM−/−, 7726 ± 97, 5537 ± 739, 3197 ± 238, and 4096 ± 113; DM+/+, 2655 ± 229, 1825 ± 159, 1935 ± 326, and 1411 ± 77. c, Activation of T hybridoma LMR 16.2 specific for LACK 158–173 is shown at different concentrations of rLACK Ag using either BALB/c DM−/− (■) or DM+/+ (○) spleen cells as APC. The activation of the T hybrid is measured by release of IL-2, analyzed by ELISA, as described before (15). Inset, Shows stimulation of the same T hybrid with LACK peptide 158–173 using DM−/− (□) or DM+/+ (○) splenic APC. The data are shown as mean ± SE of triplicate cultures. Identical results were obtained with hybridoma LMR 17.1 (data not shown).
due to a deficient T cell repertoire specific for this peptide, we immunized the DM\textsuperscript{+/+} and DM\textsuperscript{--/} mice with the peptide LACK 158–173. Because peptide 158–173 can be presented by fixed APC to T cell hybrids specific for this epitope (data not shown), the peptide 158–173 does not need Ag processing in endocytic compartments. The data presented in Fig. 5, a and b, show the LNC response of individual mice immunized with peptide LACK 158–173. As expected, DM\textsuperscript{+/+} mice show a strong LNC response against peptide LACK 158–173 (Fig. 5a, top panel). Furthermore, it is clear from Fig. 5a (bottom panel) that peptide LACK 158–173-elicited CD4 T cells are able to recognize rLACK Ag in in vitro recall assays, APC in DM\textsuperscript{+/+}/H11002, and presented by DM\textsuperscript{+/+}/H11002 APC and DM\textsuperscript{+/+}/H11002 APC. In contrast, there is no activation of this T hybrid when LACK is processed and presented by DM\textsuperscript{--/} splenic APC. A strong stimulation of this T hybrid is seen at concentrations of LACK Ag as low as 2 nM when presented by DM\textsuperscript{--/} APC. The possibility that APC in LACK 158–173-immunized DM\textsuperscript{--/} mice (Fig. 5a) were unable to express the same conformation of the peptide LACK 158–173, as expressed by DM\textsuperscript{+/+} APC also signifies the expression of comparable levels of A\textsuperscript{b} MHC molecules by both APC. Similar results were obtained using LMR 17.1 T hybrid (data not shown). The data in Fig. 5c thus suggest that the absence of a response of LNC from the 158–173-primed DM-deficient mice (shown in Fig. 5b, lower panel) to rLACK could be due to a deficient display of the 158–173/A\textsuperscript{b} epitope after processing of LACK protein by DM\textsuperscript{--/} APC.

**FIGURE 6.** DM\textsuperscript{+/+} BALB/c mice are deficient in LACK 33–48-reactive T cell repertoire, although DM\textsuperscript{+/+} APC can efficiently display this epitope. a and b, BALB/c DM\textsuperscript{+/+} and DM\textsuperscript{--/} mice were immunized with peptide LACK 33–48, as described in Fig. 5. The data show proliferative responses of the LNC obtained from the draining lymph nodes of three individual DM\textsuperscript{+/+} (a) and DM\textsuperscript{--/} (b) mice. LNC from each mouse were cultured without or with various concentrations of LACK 33–48 (top panels) or rLACK Ag (bottom panels). The assay was done, as described in Fig. 1, and the results represent mean cpm ± SE of triplicate culture. The data are presented as in Fig. 5, with each symbol representing an individual mouse. The horizontal lines (dashed or solid) represent medium control values for each mouse in each panel, which were in the same range as in Fig. 5. The responses at the highest concentration of rLACK in a (bottom panel) reflect a contaminant in the batch of rLACK used despite observation of a single band of protein on SDS-PAGE. Mean cpm obtained using PPD as a positive control for DM\textsuperscript{+/+} and DM\textsuperscript{--/} mice ranged from 77,716 ± 36,350 to 102,026 ± 3,787 and 65,126 ± 2,192 to 86,693 ± 4,856, respectively. c and d, Activation of 33–48-specific T cell line 3 is shown at different concentrations of rLACK Ag or peptide 33–48 using BALB/c (DM\textsuperscript{+/+}) splenic cells as APC. LACK concentrations 1 and 2 and in c represent 45.8, 183.5, and 735 nM, respectively, and LACK 33–48 concentrations 1, 2, and 3 represent 3.125, 12.5, and 50 μM, respectively (c). LACK concentrations 1 and 2 in d represent 70.8 and 283 nM, respectively. LACK 33–48 concentrations 1 and 2 represent 12.5 and 50 μM, respectively (d). Activation of the T cell line is measured by [\textsuperscript{3}H]thymidine incorporation after 48 h of culture with either medium alone or Ag or peptide 33–48, as described before (32) and in Fig. 1. The data are shown as mean cpm ± SE of triplicate cultures.
To pursue why DM⁺⁻⁺/- mice fail to show CD4 T cell response to peptide LACK 33–48, we immunized BALB/c DM⁺⁻⁺ and DM⁺⁻⁻ mice with the peptide LACK 33–48. Because peptide 33–48 can be presented by fixed APC to T cell lines specific for this epitope (data not shown; see below), the peptide 33–48 does not need Ag processing in endocytic compartments. The data presented in Fig. 6a show in vitro LNC responses of individual mice to the peptide LACK 33–48 (top panels, Fig. 6, a and b) and to rLACK Ag (bottom panels, Fig. 6, a and b). It is obvious that DM⁺⁻⁻/- mice are unable to respond to this epitope even though mice were immunized with the peptide 33–48 (Fig. 6a). The DM⁺⁻⁻/- mice, as expected, show a strong CD4 T cell response to this epitope when immunized with the same peptide (Fig. 6b). Fig. 6b (bottom panel) also indicates that T cells elicited with the peptide LACK 33–48 can be stimulated by rLACK Ag. To further examine the ability of LACK-pulsed DM⁺⁻⁻⁻/⁻ APC to display epitope 33–48, we used long-term LACK 33–48-reactive T cell lines (derived from DM⁺⁻⁻/- mice) as probes to analyze display of this peptide by Ag-pulsed APC. In Fig. 6, c and d, our results show that DM⁺⁻⁻⁻/⁻ BALB/c APC pulsed with LACK or peptide 33–48 are strongly able to activate 33–48-reactive CD4 T cell line. Fixed DM⁺⁻⁻⁻/⁻ APC pulsed with LACK in this experiment were unable to present the 33–48 epitope (data not shown). In Fig. 6d, we show that there is an equivalent stimulation of the 33–48-reactive CD4 T cell line when either DM⁺⁻⁻⁻/⁻ or DM⁺⁻⁻/- splenic cells were used as APC to present LACK protein. Thus, DM⁺⁻⁻⁻/⁻ BALB/c APC are efficiently able to process LACK to display 33–48 epitope, indicating that an absence of LNC T cell responsiveness to LACK 33–48 in DM⁺⁻⁻⁻/⁻ mice, as shown in Fig. 6a, is consistent with a deficient LACK 33–48-reactive T cell repertoire in these mice.

Results presented in this study show that DM is required for skewing the CD4 T cell immune response toward immunodominant epitopes in vivo. Although multiple elements, such as differential peptide affinity for MHC and differential enzymatic processing (25, 26, 35, 36), within APC could be responsible for anointing a single peptide in a 312-aa protein to be the immunodominant Ag, we envision to translate into a prejudiced display of some peptides (DM dependent/DM resistant) and an exclusion from presentation of other peptides (DM susceptible) by APC. The peptide-editing properties of DM could thus sculpt the outcome of T cell immune responses, however, remain unexamined until now. Results presented in this study show that DM is required for skewing the CD4 T cell immune response toward immunodominant epitopes in vivo. Although multiple elements, such as differential peptide affinity for MHC and differential enzymatic processing (25, 26, 35, 36), within APC could be responsible for anointing a single peptide in a 312-aa protein to be the immunodominant epitope, the current study shows that in the case of LACK Ag, DM peptide editing is a major determining factor leading to the expression of the immunodominant phenotype in both the BALB/c and B10.BR strains of mice. DM-deficient mice were unable to elicit T cells reactive with either the immunodominant LACK 158–173 in H-2b mice or the immunodominant LACK 81–96 in H-2k mice, indicating that these epitopes were DM dependent (rather than simply DM resistant) for their display by MHC class II molecules. Whether these results reflect the ability of DM to impact the association or dissociation rates of these peptide-MHC.

T cell response to LACK 33–48 is restricted by A⁺⁻ molecules

In earlier studies, a divergent pattern of DM dependence for display of epitopes restricted by MHC II isotypes A⁺⁻ and E⁺⁻ expressed in BALB/c mice was shown (22, 23). To explore our concerns that disparate responses to epitopes 158–173 and LACK 33–48 in DM⁺⁻⁺ and DM⁺⁻⁻/- BALB/c mice might be simply due to different restricting molecules, we analyzed the restriction element for the LACK 33–48 epitope. In proliferation assays using LNC from LACK 33–48-immunized BALB/c DM⁺⁻⁻/- mice cultured with LACK 33–48 in the absence or presence of anti-A⁺⁻ and anti-E⁺⁻ Abs, we found that LACK 33–48-specific T cell response is restricted by A⁺⁻ MHC molecules (data not shown) (see Materials and Methods).

DM⁺⁻⁻⁻/⁻ BALB/c mice do not have a LACK 33–48-reactive T cell repertoire

Results presented in this study show that DM peptide editing, during presentation of a pathogen or a protein Ag in vivo, can be envisioned to translate into a prejudiced display of some peptides (DM dependent/DM resistant) and an exclusion from presentation of other peptides (DM susceptible) by APC. The peptide-editing properties of DM could thus sculpt the outcome of an in vivo T cell immune response in two ways: 1) by expression of a biased spectrum of peptides by thymic APC and a consequent maturation of an altered T cell repertoire in the host (22), and 2) by display of selected peptides by macrophages and dendritic cells (DC) recruited in the host defense against pathogens and proteins. The precise in vivo consequences for DM peptide editing on CD4 T cell immune responses, however, remain unexamined until now.

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Discussion

DM peptide editing, during presentation of a pathogen or a protein Ag in vivo, can be envisioned to translate into a prejudiced display of some peptides (DM dependent/DM resistant) and an exclusion from presentation of other peptides (DM susceptible) by APC. The peptide-editing properties of DM could thus sculpt the outcome of an in vivo T cell immune response in two ways: 1) by expression of a biased spectrum of peptides by thymic APC and a consequent maturation of an altered T cell repertoire in the host (22), and 2) by display of selected peptides by macrophages and dendritic cells (DC) recruited in the host defense against pathogens and proteins. The precise in vivo consequences for DM peptide editing on CD4 T cell immune responses, however, remain unexamined until now.

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Table II. T cell responses to LACK 261–276 in peptide-primed mice

<table>
<thead>
<tr>
<th>Ag</th>
<th>BALB/c DM⁺⁻⁻⁻</th>
<th>BALB/c DM⁺⁻⁻⁻⁻</th>
</tr>
</thead>
<tbody>
<tr>
<td>LACK 261–276</td>
<td>100 µM 13.10 ± 2.4</td>
<td>24.43 ± 13.52</td>
</tr>
<tr>
<td>rLACK</td>
<td>25 µM 8.56 ± 7.07</td>
<td>6.78 ± 3.83</td>
</tr>
<tr>
<td></td>
<td>6.25 µM 3.74 ± 2.89</td>
<td>5.86 ± 3.25</td>
</tr>
<tr>
<td></td>
<td>588 nM 36.17 ± 20.97</td>
<td>16.87 ± 7.78</td>
</tr>
<tr>
<td></td>
<td>147 nM 26.09 ± 18.24</td>
<td>8.84 ± 4.76</td>
</tr>
<tr>
<td></td>
<td>9.2 nM 4.85 ± 3.01</td>
<td>2.04 ± 2.02</td>
</tr>
</tbody>
</table>

*The data represent LNC responses of mice immunized with LACK peptide 261–276. Three mice were included in each group and were individually tested as described in Fig. 5. The range of stimulation indices for each group was as follows: DM⁺⁻⁻⁻⁻: 261–276: (100 µM) 10.41 to 15.02, (25 µM) 2.74 to 16.42 and (6.25 µM) 0.9 to 6.67; rLACK: (588 nM) 15.59 to 59.50, (147 nM) 7.53 to 44.00, and (9.2 nM) 1.69 to 7.69. DM⁺⁻⁻⁻: 261–276: (100 µM) 8.82 to 32.64, (25 µM) 2.36 to 9.03 and (6.25 µM) 2.76 to 9.25; rLACK: (588 nM) 11.19 to 25.85, (147 nM) 3.97 to 13.49 and (9.2 nM) 0.77 to 4.37. The stimulation index was calculated by dividing the mean cpm of triplicate cultures with Ag by the cpm obtained from cultures with medium alone.
complexes is unclear and is a subject of further investigation (13). The requirement of DM for APC display of 158–173/A^d was nonetheless further confirmed by evidence that DM^−/− mice indeed had 158–173/A^d-reactive T cells that could be elicited in DM-deficient mice by immunization with the processed peptide (Fig. 5). Furthermore, the inability of DM^−/− APC to display 158–173/A^a after processing of LACK (Fig. 5c) was shown using 158–173-reactive T cell hybridomas (Fig. 5c). The data in the inset (Fig. 5c) also indicate that the preprocessed peptide 158–173 can be efficiently presented in the same conformation by both DM^−/− and DM^+/+ APC (Fig. 5c).

Remarkably, we found that CD4 T cells elicited in the DM^−/− mice in the two haplotypes responded to two novel epitopes in each case, although the predominant T cell response in DM^−/− BALB/c mice was to a novel epitope, 33–48/A^d (Fig. 1b) and the predominant response in DM^−/− B10.BR mice was toward the novel epitope 217–232 (Fig. 3b). An expression of a response to two novel epitopes in each case is evidence for the ability of the enzymatic machinery in BALB/c and B10.BR APC, respectively, to produce these peptides and the ability of these peptides to bind MHC class II molecules. Note that the differential epitope specificity of T cells in DM^+/+ and DM^−/− mice in our study is a reflection of processing reactions within late endocytic compartments within APC in each case because mice were immunized with the intact protein, LACK, and processing and loading of intact proteins take place in late compartments containing nascent MHC class II and reductive activity (37).

There could be two reasons, both related to DM peptide-editing actions, for the inability of the wild-type BALB/c and B10.BR mice to show any response to these novel epitopes: 1) these peptides are edited out from the MHC class II groove in the presence of DM in APC, and 2) DM edits peptide repertoire within the thymus of the wild-type strains such that either T cells reactive to the novel epitopes are not positively selected or are negatively selected. Consistent with this, the results presented in this work show that whereas the LACK 158–173-reactive T cell repertoire remains intact in DM^+/+ and DM^−/− mice (Fig. 5), LACK 33–48/A^d-reactive T cell repertoire appears to be absent in DM^+/+ mice (Fig. 6) as a result of DM-editing events during thymic selection. T cell repertoire directed to epitope 261–276 remains intact in DM^+/+ mice. The data in Table II and Fig. 1a are suggestive of a compromised display of 261–276 epitope by APC in rLACK-primed DM^+/+ mice, leading to a lack of response to this epitope.

The current in vivo results support our previous in vitro studies, using transfected APC lines and HEL and sperm-whale myoglobin as model Ags, indicating that DM has a profound influence in selection of immunodominant epitopes (15). Note that the in vitro results (15) were obtained using fibroblast cell lines transfected with genes encoding appropriate molecules including invariant chain and DM. It is of significance that the current study using intact DM-deficient animals demonstrates that even though the professional APC such as DC act as chief APC during development of an in vivo immune response (38), DM peptide-editing function in professional APC is still a requirement for focusing the CD4 T cell immune response to the immunodominant epitopes in two different haplotypes. DM peptide-editing activity perhaps is significant for DC function, because unlike B cells, DC lack expression of DO (17–20, 22, 23, 39), another chaperone in the MHC class II pathways, described in several recent reports to be inhibitory for DM actions.

In the context of a recent study indicating that the absence of DM in vitro leads to formation of a novel conformer in the case of epitope HEL 48–63 bound to A^d molecules (21), our study demonstrates that LNC and CD4 T cell lines derived from peptide 33–48-immunized DM^−/− mice recognize LACK 33–48 bound to A^d from Ag and peptide-pulsed APC from both the DM^+/+ mice (shown in Fig. 6c) and DM^−/− mice (Fig. 6b). Thus, in the case of LACK 33–48, the same conformer of this epitope can be expressed by APC pulsed with the protein or the peptide and in the absence or presence of DM. These results also indicate that peptide-immunized DM^−/− mice can predominantly generate type A (defined by their ability to be activated both by Ag or peptide-pulsed APC (21)) T cell specific for 33–48/A^d using either wild-type or DM-deficient APC (Fig. 6, b and c). Likewise, priming with peptide 261–276 in BALB/c DM^−/− mice also is able to stimulate 261–276-reactive type A T cells within LNC that are able to recognize both the peptide and the protein (Table II).

The question of the impact of a differential epitope display by APC in DM^+/+ and DM^−/− mice on the outcome of infection with L. major is currently under further investigation. However, because L. major is a complex pathogen, the current study represents the impact of DM on specificity of an in vivo T cell response to rLACK so that it is independent of pathogen-related effects, such as the ability of L. major to internalize both the MHC class II as well as DM molecules from parasitophorous vacuoles (lysosomal compartments) within APC (40). Another issue of great interest is to understand and define the features of peptide MHC complex that determine whether the display of a peptide by an MHC molecule is dependent or independent of DM molecules, and is a focus of further analyses. In this context, our preliminary data suggest that binding of both the 158–173 and 33–48 peptides to A^d molecules is SDS stable (N. Nanda and S. Sadegh-Nasseri, unpublished observations), a reflection of a tight association between the peptide and A^d MHC molecules in both cases. Because LACK 158–173 is dependent on DM (Fig. 5c) and 33–48 is independent of DM (Fig. 6c) for binding to A^d molecules, additional factors, other than rigid peptide-MHC binding, are likely to play a role in DM dependence.

We conclude that DM peptide-editing function sculpts the specificity and immunodominance in the CD4 T cell response to an Ag, and that DM may have evolved to conservatively focus the T cell response to a selected few epitopes of a pathogen. Such a strategy could be protective for the host on one hand because it prevents cross-reactive autoimmune response (41), but detrimental on the other because it allows a pathogen to develop antigenic variation and immune escape (42). We speculate that the evolutionary pressures to focus T cell responses to fewer epitopes may have outweighed the disadvantage to the host due to potential immune escape by the pathogen during an immune response directed to one or only a few epitopes. Nevertheless, our results could reveal critical insights for developing tools to diversify the epitope specificity of a T cell response when an in vivo T cell response targeted to multiple specificities is beneficial to the host.

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Disclosures

The authors have no financial conflict of interest.

References


