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The High Frequency Indian Rhesus Macaque MHC Class I Molecule, Mamu-B*01, Does Not Appear to Be Involved in CD8⁺ T Lymphocyte Responses to SIV₅₃₃₃₉¹

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Although the SIV-infected Indian rhesus macaque (Macaca mulatta) is the animal model most widely used for studying HIV infection, our current understanding of the functional macaque MHC class I molecules is limited. To date, SIV-derived CD8⁺ T lymphocyte epitopes from only three high frequency macaque MHC class I molecules have been extensively characterized. In this study, we defined the peptide-binding properties of the high frequency Indian rhesus macaque class I molecule, Mamu-B*01 (~26%). We first identified a preliminary binding motif by eluting and sequencing endogenously bound Mamu-B*01 ligands. We further characterized the peptide-binding characteristics using panels of single amino acid substitution analogs. Using this detailed motif, 507 peptides derived from SIV₅₃₃₃₉ were identified and tested for their Mamu-B*01 binding capacity. Surprisingly, only 11 (2.2%) of these motif-containing peptides bound with IC₅₀ values ≤500 nM. We assessed the immunogenicity of these peptides using freshly isolated PBMC from ten Mamu-B*01⁺ SIV-infected rhesus macaques in IFN-γ ELISPOT and IFN-γ/TNF-α intracellular cytokine staining assays. Lymphocytes from these SIV-infected macaques responded to none of these peptides. Furthermore, there was no sequence variation indicative of escape in the regions of the virus that encoded these peptides. Additionally, we could not confirm previous reports of SIV-derived Mamu-B*01-restricted epitopes in the Env and Gag proteins. Our results suggest that the high frequency MHC class I molecule, Mamu-B*01, is not involved in SIV-specific CD8⁺ T lymphocyte responses. The Journal of Immunology, 2005, 175: 5986–5997.

Virus-specific CD8⁺ T lymphocytes are implicated in the control of immunodeficiency viral replication. In the initial control of HIV/SIV infection, CD8⁺ T lymphocytes appear concurrently with the reduction in peak viremia (1–3). The transient depletion of CD8⁺ cells in SIV-infected macaques results in dramatically increased viral loads (4–6). Furthermore, CD8⁺ T lymphocytes select for variation in HIV (7–10) and SIV (11–15) infection, suggesting that these lymphocytes play a role in viral containment. Several studies also show an association between certain MHC class I molecules and slow or rapid disease progression in both humans (16–19) and macaques (20–22). With increased interest in AIDS vaccine trials that elicit CD8⁺ T lymphocyte responses (23), a broader understanding of epitope-specific CD8⁺ T lymphocytes and the MHC class I molecules that present these epitopes is critical.

The SIV-infected Indian rhesus macaque is currently the animal model most widely used for the study of immunodeficiency viral pathogenesis and for preclinical development of potential HIV vaccines. The majority of current studies involve Mamu-A*01 selling macaques, making it the best characterized macaque MHC class I molecule (12–15, 24–29). Unfortunately, the focus on Mamu-A*01 macaques has created a bottleneck in HIV vaccine research due to a shortage of animals expressing this allele (30). SIV-specific CD8⁺ T lymphocyte responses restricted by other common MHC class I alleles need to be identified to expand the number of animals available for research. To date, three high frequency macaque MHC class I molecules (Mamu-A*01, -A*02, and -B*17) have been well characterized with respect to their peptide-binding motifs and to the SIV-specific CD8⁺ T lymphocyte epitopes bound by these molecules (24, 25, 31, 32). The study of additional MHC class I alleles will facilitate our understanding of pathogenesis and aid in vaccine development.

Elucidation of detailed peptide-binding motifs for macaque MHC class I molecules has aided in the development of extensive epitope screens that are more rapid and precise than mapping using overlapping 15-mer peptides. Besides immunological assays, virus evolution and escape studies also benefit from the identification of new epitopes as they identify regions of potential viral escape. Only minimal optimal epitopes can be used in the design and synthesis of fluorescent dye-coupled tetrameric peptide-MHC complexes (28, 33). These epitopes can also be evaluated as potential vaccine targets. Increasing the repertoire of known CD8⁺ T lymphocyte epitopes improves the macaque model by enabling more
comprehensive pathogenesis and vaccine studies and facilitating better use of limited animal resources.

PCR-sequence-specific primer (SSP)$^3$ typing demonstrated that Mamu-B*01 is the highest frequency MHC class I allele expressed by Indian rhesus macaques in the Wisconsin National Primate Research Center (WNPRC) colony ($\sim 33\%$). Therefore, we wished to define a detailed peptide-binding motif for Mamu-B*01, identify potential SIV-derived Mamu-B*01 CD8$^+$ T lymphocyte epitopes, and ascertain the breadth of immune responses to these epitopes in SIV-infected macaques. Interestingly, we found that only a small fraction (2.3%) of the peptides that matched the Mamu-B*01 binding motif bound with an affinity (IC$_{50}$) of $\leq 500$ nM. In addition, none of the SIV-derived peptides were found to be immunogenic when tested in SIV-infected Indian rhesus macaques by IFN-γ ELISPOT and IFN-γ-TNF-α intracellular cytokine staining (ICS) assays.

Analysis of time-of-death viral genome sequence from eight SIV-infected Mamu-B*01$^+$ macaques showed no indication of viral escape in the regions of the SIV-derived Mamu-B*01 binding peptides. We also discovered that one Env (34) and five Gag (35) SIV-derived peptides previously described as Mamu-B*01-restricted epitopes did not bind to Mamu-B*01. Furthermore, when ELISPOT and ICS assays were performed on these six peptides, we were unable to verify their immunogenicity in ten SIV-infected Mamu-B*01$^+$ macaques. Overall, our results suggested that despite its high frequency, Mamu-B*01 does not appear to bind any immunogenic SIV-derived peptides. Interestingly, Mamu-B*01$^+$ macaques follow a typical SIV disease progression in terms of chronic phase viral loads, suggesting that this allele does not have a protective effect. This is an unusual case of a high frequency MHC class I molecule in Indian rhesus macaques in which no SIV-specific CD8$^+$ T lymphocyte epitopes have been identified despite previous findings that indicated otherwise (34, 35).

Materials and Methods

MHC class I typing of macaques

Indian rhesus macaques (Macaca mulatta) were genotyped for the MHC class I Mamu-A*01, -A*02, -A*08, -A*11, -B*01, -B*03, -B*04, and -B*17 alleles using PCR amplification with sequence-specific DNA Priming (PCR-SSP). The PCR-SSP methodology involves designing at least one primer that will permit amplification based on the 3’-mismatch principle, which theoretically prevents enzymatic primer extension by DNA polymerase due to a single mismatch at the 3’ terminus (36). Whenever possible, each of the 3’-terminal regions target unique MHC class I nucleotide polymorphisms, primarily in exons 2 and/or 3.

PCR conditions were optimized for magnesium ion concentration and pH using a PCR optimizer kit (Invitrogen Life Technologies). Genomic DNA was isolated from 500 μl of EDTA-treated whole blood or buffy coat using the MagNA Pure LC Instrument (Roche Molecular Biochemicals) according to manufacturer’s guidelines. Approximately 75 ng of genomic DNA was amplified for each sample in a total reaction volume of 25 μl. Final reaction mixtures contained 1× PCR buffer (Invitrogen Life Technologies) composed of 60 mM Tris-HCl (pH 9.5), 2 mM MgCl$_2$, and 15 mM ammonium sulfate, 410 μM of each dNTP (Promega), 0.25–0.5 μM of each PCR-SSP primer, 0.3 μM of each internal control primers amplifying a conserved DRB sequence, and 0.961 U of platinum Taq polymerase (Invitrogen Life Technologies). The nucleotide sequence of the primers used for typing Mamu-B*01 are as follows: (forward, 5’-CAG CGA CGC GGA GAG TCG-3’; reverse, 5’-CCG CCG CCG TCC AGG AGT-3’).

Thermal cycling conditions were as follows: a 1 min denaturation at 96°C, followed by five cycles of 96°C denaturation for 25 s, 70°C annealing for 50 s, and 45 s of elongation at 72°C; 21 cycles of 96°C denaturation for 25 s, 67°C annealing for 50 s and a 45 s extension at 72°C; and finally, four cycles of 96°C denaturation for 25 s, 55°C annealing for 60 s and a 120 s extension at 72°C. Subsequently, PCR products were electrophoresed on 2% agarose gels at a constant voltage (0.5× TBE) and analyzed for the presence of a required internal control product and the Mamu-A*01, -A*02, -A*08, -A*11, -B*01, -B*03, -B*04, and -B*17 allele-specific amplicons relative to a 100-bp DNA ladder (Invitrogen Life Technologies).

Virus infections

All macaques used in this study were infected with a molecularly cloned virus, SIVmac239. SIV-infected animals were maintained at the WNPRC, cared for in accordance with the National Research Council Guide for the Care and Use of Laboratory Animals, and under the approval of the University of Wisconsin Research Animal Resources Center review committee.

One-dimensional isoelectric focusing (1D-IEF)

1D-IEF was performed to separate the expressed MHC class I alleles on the basis of differential endpoint migration of individual heavy chains in a discontinuous pH gradient within a polyacrylamide gel as previously described (37). Briefly, $\sim 2.0 \times 10^7$ Indian rhesus macaque PBMC were separated from whole heparin- or EDTA-treated blood by Ficoll-Paque PLUS (GE Health Sciences) density centrifugation and stimulated with 5 μg/ml Con A (Sigma-Aldrich) at a concentration of $2.0 \times 10^6$ cells/ml. After 4 days, the activated PBMC and 721.221 cell lines were metabolically labeled with [35S]methionine. The labeled cells were lysed and immunoprecipitated with W6/32 Ab. The immunoprecipitates were split into two fractions; one fraction was treated with neuraminidase type VIII (Sigma-Aldrich) to remove sialic acid moieties on MHC molecules. The immunoprecipitates were loaded on a pH gradient acrylamide gel for isoelectric focusing. The gel was dried and MHC class I signal was detected by autoradiography following a 7-day exposure to film. The Mamu-B*01 transfectant was created as previously described (38, 39) and was used as the positive control in the 1D-IEF gel. As a negative control, the HLA class I-deficient human B cell line 721.221 and lymphocytes from a Mamu-B*01$^+$ macaque were used.

Flow cytometry

W6/32 Ab surface staining was performed on the 721.221 (negative control), an immortalized macaque B cell line (positive control), and the Mamu-B*01$^+$ transfectant to detect surface expression of the MHC class I protein. Events were acquired on a FACSCalibur (BD Biosciences) and analyzed using FlowJo software 6.1.1 (Tree Star).

Peptide synthesis

Peptides for screening were purchased from either Pepscan Systems, Mi- motopes, A & A Labs, or the Biotechnology Center at the University of Wisconsin-Madison. All were synthesized using standard tertiary butyloxycarbonyl or fluoronylethoxy carbonyl solid-phase methods (40). Peptides were reconstituted and radiolabeled as previously described (32, 41). SIV peptides were derived from the SIVmac239 sequence, GenBank accession number M33262 (42).

MHC purification and peptide-binding assay

Two different stable transfectants expressing either membrane bound or secreted versions of the rhesus macaque MHC class I molecule Mamu- B*01 were created in the HLA class I-deficient human B cell line 721.221, as described previously (38, 39). MHC class I molecules were purified from culture supernatants or cell lysates, respectively, using affinity chromatography with the anti-HLA class I (A-, B-, C) Ab W6/32 (24, 41). Protein purity, concentration, and depletion efficiency steps were monitored by SDS-PAGE.

Quantitative assays for peptide binding to detergent-solubilized Mamu class I molecules were based on the inhibition of binding of a radiolabeled standard probe peptide as detailed elsewhere (32, 41). The radiolabeled peptide used for Mamu-B*01 binding assays was the macaque tumor rejection Ag gp96 256–264 peptide (sequence SDYLELDTI). For each peptide, the concentration of peptide yielding 50% inhibition of the binding of the radiolabeled probe peptide (IC$_{50}$) was calculated in three or more independent experiments. Under the conditions used, where [MHC] and IC$_{50}$ $\leq$ [MHC], the measured IC$_{50}$ values are reasonable approximations of the true $K_d$ values (43). In each experiment, a titration of the unlabeled version of the radiolabeled probe was tested as a positive control for inhibition. The average IC$_{50}$ of the macaque tumor rejection Ag gp96 256–265 peptide was 2.7 nM.
**IFN-γ ELISPOT assay**

PBMC were separated from whole heparin- or EDTA-treated blood by Ficoll-Paque PLUS (GE Health Sciences) density centrifugation. The PBMC were used directly in IFN-γ ELISPOT assays as previously described (25, 32). Briefly, 1.0 × 10^6 PBMC were used per well and incubated 16–18 h at 37°C in 5% CO₂. Peptides were used at 10 μg/ml. In addition, Mamu-B*01+ SIV-infected macaques that also expressed either Mamu-A*01, Mamu-A*02, or Mamu-B*17 were tested for responses against a minimal optimal epitope restricted by the appropriate allele (25, 31, 32). These peptides were used as positive controls to ascertain the immunocompetence of the animal. All tests were performed in triplicate. Wells were imaged with an AID ELISPOT reader (AID), counted by AID ELISPOT reader version 3.1.1 (AID), and analyzed as previously described (32). Spots were counted by an automated system with set parameters for size, intensity, and gradient. Background (mean of wells without peptide) levels were subtracted from each well on the plate. A response was considered positive if the mean number of spot-forming cells (SFC) of triplicate sample wells exceeded background plus two SDs. Assay results are shown as SFC per 1 × 10^6 cells. Responses <50 SFC per 1 × 10^5 cells were not considered positive because these counts are not significantly above background. Wells containing Con A (positive control) were always >1000 SFC per 1 × 10^6 PBMC.

**ICS assays**

ICS assays used fresh PBMC separated from whole heparin- or EDTA-treated blood by Ficoll-Paque PLUS (GE Health Sciences) density centrifugation. The PBMC were used directly in IFN-γ and TNF-α ICS assays as previously described (31, 32). In cases where TNF-α produced a high background in the no peptide (negative) control, IFN-γ and IL-2 production was tested in a subsequent ICS assay. In addition to positive and negative controls, Mamu-B*01+ SIV-infected macaques that also expressed either Mamu-A*01, Mamu-A*02, or Mamu-B*17 were tested for immunocompetence using a minimal optimal epitope restricted by the appropriate allele (25, 31, 32). Between 1 × 10^5 and 2 × 10^5 lymphocyte-gated events were acquired on a FACScalibur (BD Biosciences) and analyzed using FlowJo software 6.1.1 (Tree Star) as previously described (32).

**Amplification of viral RNA (vRNA) from plasma and nucleotide sequencing**

SIVmac239 nucleotide sequencing data was obtained from another study (32). Amplification of viral RNA (vRNA) from plasma and nucleotide sequences were edited with Sequencher 4.1 (Genecodes), and mixed-base sequences had isoleucine (I), leucine (L), or phenylalanine (F) at the C terminus. This finding suggests that the C terminus also plays an important role in binding to Mamu-B*01 and has a specificity for hydrophobic residues. The nine individual eluted ligands were then synthesized and tested for their capacity to bind purified Mamu-B*01 molecules and subjected to pooled Edman sequencing (accession number M33262) (42) in MacVector 7.2 trial version (Accelrys). Amino acid replacements were derived from these nucleotide alignments by use of a script written by Dr. D. H. O’Connor in Lasso dynamic markup language (Blueworld).

**Results**

Mamu-B*01 is a high frequency allele in Indian rhesus macaques

When Mamu-B*01 was initially reported to bind an epitope in Env of SIVmac239 (34), the frequency of this MHC allele was unknown. We designed a PCR-SSP-based method to detect this allele in the WNPRC macaque colony (data not shown). We determined the frequency of Mamu-B*01 and seven other MHC class I alleles in Indian rhesus macaques from the WNPRC colony as well as six additional research institutions. PCR-SSP typing of the WNPRC colony revealed that Mamu-B*01 was the most common MHC class I allele, present in approximately one-third (32.8%) of the 1293 MHC-typed animals (Table I). The previously studied high frequency alleles Mamu-A*01 (24.7%), Mamu-A*02 (21.3%), and Mamu-B*17 (16.2%) were present at frequencies comparable to previous studies (27, 31, 44, 45). In addition, 17.0% of our Mamu-B*01+ macaques expressed at least one of the three afore-mentioned MHC class I alleles (data not shown). When typing results were compiled from the six other Indian macaque colonies, the frequency of Mamu-B*01 was greater than 20% in all cases except the Caribbean Primate Research Center, which had a frequency of 13.6% from 771 animals tested (Table I). The overall frequency of Mamu-B*01 (3145 typed animals) was 26.3%.

To confirm that Mamu-B*01 encodes a protein product, we performed 1D-IEF analysis. The Mamu-B*01 transfectant produced a neuraminidase-sensitive MHC class I molecule. This banding pattern corresponded to a protein expressed by lymphocytes from the Mamu-B*01+ macaque, AV59, verifying Mamu-B*01 expression (Fig. 1A). Next, we surface-stained proteins produced by our Mamu-B*01+ transfectant with W6/32 Ab to demonstrate that Mamu-B*01 was indeed expressed on the surface (Fig. 1B). Together, these two methods confirmed that Mamu-B*01 is expressed and is available on the cell surface for Ag presentation (Fig. 1).

Sequencing of endogenously bound Mamu-B*01 ligands indicates a strong preference for aspartic acid at position 2

To determine a putative Mamu-B*01 peptide-binding motif, we transfected the MHC class I-deficient 721.221 cell line with Mamu-B*01. Endogenously loaded ligands were eluted from purified Mamu-B*01 molecules and subjected to pooled Edman sequencing. Pooled sequencing identified a dominant signal at position 2, where aspartic acid (D) was preferred. Dominant residues were not noted at any other position.

The amino acid sequences of nine Mamu-B*01 endogenous peptides was obtained by mass spectrometric analysis (Table II). Notably, eight of the nine endogenously bound ligands possessed a D at the second position. We observed that all of the individual sequences had isoleucine (I), leucine (L), or phenylalanine (F) at the C terminus. This finding suggests that the C terminus also plays an important role in binding to Mamu-B*01 and has a specificity for hydrophobic residues. The nine individual eluted ligands were then synthesized and tested for their capacity to bind purified Mamu-B*01. As shown in Table II, all of the endogenous sequences bound Mamu-B*01 with an IC<sub>50</sub> <75 nM.

### Table I. Frequency of Mamu-B*01 by PCR-SSP in different Indian rhesus macaque colonies

<table>
<thead>
<tr>
<th>Colony</th>
<th>Mamu-B*01&lt;sup&gt;+&lt;/sup&gt;</th>
<th>Total Animals</th>
<th>Frequency (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wisconsin NPRC</td>
<td>424</td>
<td>1293</td>
<td>32.8</td>
</tr>
<tr>
<td>Caribbean Primate Research Center</td>
<td>105</td>
<td>771</td>
<td>13.6</td>
</tr>
<tr>
<td>Oregon NPRC</td>
<td>81</td>
<td>324</td>
<td>25.0</td>
</tr>
<tr>
<td>Various NIH/NCI-funded projects</td>
<td>110</td>
<td>306</td>
<td>35.9</td>
</tr>
<tr>
<td>California NPRC</td>
<td>43</td>
<td>213</td>
<td>20.2</td>
</tr>
<tr>
<td>Southern Research Institute (Birmingham, AL)</td>
<td>41</td>
<td>130</td>
<td>31.5</td>
</tr>
<tr>
<td>University of Pittsburgh</td>
<td>23</td>
<td>108</td>
<td>21.3</td>
</tr>
<tr>
<td>Overall frequency</td>
<td>827</td>
<td>3145</td>
<td>26.3</td>
</tr>
</tbody>
</table>

<sup>*</sup>NPRC, National Primate Research Center; NIH/NCI, National Institutes of Health and National Cancer Institute.
To examine the Mamu-B*01 binding motif in more detail, we synthesized and tested various single amino acid substitution analogs of the high affinity Mamu-B*01 binding peptide tumor rejection Ag gp96 256–264 (sequence SDYLELDTI; see Table III).

To identify likely main anchor positions, the first panel represented single, nonconservative substitutions to lysine (K) at each position of the peptide. Substitution to K in positions 2, 3, and 5, and the C terminus resulted in greater than 100-fold decreases in binding capacity when compared with the wild-type sequence, suggesting these as the most crucial positions influencing Mamu-B*01 binding capacity. At positions 4 and 7, K substitutions were associated
with 15- to 20-fold reductions in binding capacity. Overall, 6 of 9 positions were associated with >10-fold decreases in Mamu-B*01 binding capacity (Table III).

Additional single substitution panels were synthesized and tested to further probe the binding specificity at each position. At all positions, we designed a set of substitutions to provide at least one example of a conservative, semi-conservative, and non-conservative substitution (Table III). In the present analyses, as in previous studies of HLA class I molecules (26, 46, 47), preferred residues at each position were defined as those whose average relative binding capacity (ARB) was ≥0.1, when compared with the binding capacity of the optimal residue at the same position. We defined residues with an ARB between 0.01 and 0.1 as tolerated. The aromatic residue tyrosine (Y) was tolerated, and tryptophan (W) were also preferred with ARB values in the 0.03–0.08 range (Table III). Only the four of the five other substitutions tested, representing small, aromatic, aliphatic, and polar chemical specificities were also tolerated with ARB values in the 0.011–0.054 range. These data implied that position 2 plays an important role in binding and is associated with a dominant preference for acidic residues, although it appeared to tolerate a reasonably broad range of residues.

At position 5, only the acidic residue E was preferred. However, four of the five other substitutions tested, representing small, aromatic, aliphatic, and polar chemical specificities were also tolerated with ARB values in the 0.03–0.08 range (Table III). Only the basic residue K was associated with an abrogation of binding. This pattern, where the majority of substitutions were tolerated and only rare residues were deleterious, suggested that position 5 was a secondary anchor position.

Only hydrophobic residues were preferred or tolerated at the C terminus. Specifically, the aliphatic residue I was found to be optimal, but aliphatic residues L and V and the aromatic residues F and tryptophan (W) were also preferred with ARB values in the 0.15–0.6 range. The aromatic residue tyrosine (Y) was tolerated, yet it was associated with a 90-fold decrease in binding capacity. None of the other substitutions tested, including small, polar, basic, and acidic residues, were tolerated.

Taken together, these data indicated that Mamu-B*01 binds its ligands using the residues in position 2 and at the C terminus as main anchors (Fig. 2). Although acidic residues were preferred at position 2, a broad range of chemical specificities was tolerated. Aliphatic, hydrophobic, or aromatic residues were distinctly preferred at the C terminus. Position 5, where a preference for acidic residues (D and E) was noted, may also be an important secondary anchor position.

### Identification of SIV-derived Mamu-B*01 binding peptides

To identify SIV-derived Mamu-B*01 binders, we first scanned the SIV<sub>mac239</sub> proteome for the presence of peptides 8–11 residues in length bearing the stringent main anchor motif derived from the pool sequencing described above. Specifically, each peptide was required to have D in position 2 and F, L, or I at the C terminus. A total of 97 peptides were identified and tested for their capacity to bind Mamu-B*01. As in previous studies, we used a threshold of IC<sub>50</sub> ≤ 500 nM to define binders. This threshold had previously been shown to be associated with T cell recognition in vivo in human, macaque, and murine systems (25, 43, 48, 49). Surprisingly, it was found that only three (3.1%) of these peptides had binding affinities of ≤500 nM.

To identify additional binders, we expanded our search criteria to include the expanded motif identified in the single amino acid substitution analyses (see Fig. 2). In this case, we scanned for peptides with E, A, S, N, G, I, Q, L, T, or V in position 2, and I, F, L, V, W, or Y at the C terminus. Based on chemical similarity, methionine (M) was also allowed at either main anchor position. Congruent with previous definitions of an expanded motif, at least one of the residues occupying a main anchor position was required to be preferred. Using these criteria, 331 additional peptides were identified and synthesized. When these peptides were tested for their capacity to bind Mamu-B*01, it was found that only 2.1% (seven of 331) bound with an affinity of ≤500 nM (Supplemental Table I).

Taken together, only 10 of 428 (2.3%) motif containing peptides were Mamu-B*01 binders (Supplemental Table I). Given this low incidence of binding, we further relaxed the search criteria by eliminating the requirement that one of the anchor residues must be preferred. We also included several peptides with T, N, or A at the C terminus, as these residues were weakly identified in the pool and/or single amino acid substitution analyses. As expected, these peptides also bound poorly. Overall, only 1 of 79 (1.3%) additional “non-motif” peptides tested was a binder (Supplemental Table I). In total, the binding capacity of only 11 of the 507 SIV-derived peptides had an affinity of ≤500 nM (Table IV).

4 The online version of this article contains supplemental material.
The low binding incidence within this peptide library precluded a detailed analysis of binding specificity at secondary anchors, except in the case of 8-mer and 9-mer ligands. The 8-mer and 9-mer analyses, which have been used to generate an algorithm for predicting the binding of 8-mer and 9-mer ligands to Mamu-B*01 (50), confirmed that position 5 was a dominant secondary anchor with a preference for the acidic residue E (data not shown). It was also noted that an acidic residue was present in position 5 of 6 of the 11 SIV binders, and 3 of the 9 endogenously bound ligands. In total, 9 of the 20 (45%) Mamu-B*01 binders described herein have an acidic residue in position 5. Significant influences at other secondary positions, however, could not be identified.

In terms of size preferences, it was noted that 8 of the 11 binders (73%) were 9-mers. Similarly, among peptides with the preferred or extended motif, 9-mers (6.9%) were about three times more likely to bind than 8-mers (2.0%), while 10- and 11-mer binders were virtually nonexistent (0.4%). These observations suggest that 9-mers are the optimal ligand size for Mamu-B*01 (Supplemental Table I).

Finally, we analyzed the binding capacity of a previously described Mamu-B*01 Env epitope (34) and five recently published Mamu-B*01 Gag epitopes (35). Three of these six epitopes were identified by the motif scan described above. Unexpectedly, we observed no binding to Mamu-B*01 with any of these epitopes at any dose tested up to 50,000 nM (Table IV). Because of this surprising finding, we tested nine additional permutations (truncation, extensions, and frame shifts) of the Env epitope. In all cases, no binding to Mamu-B*01 could be detected when we tested the peptides in excess of 50,000 nM (Supplemental Table I).

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Analysis of Mamu-B*01+ SIV-infected rhesus macaques identified no immunogenic SIV-derived peptides that bind to Mamu-B*01

We then analyzed whether the 11 selected peptides (IC_{50} ≤ 500 nM) were recognized in vivo by fresh PBMC derived from SIV-infected Mamu-B*01+ macaques. Two uninfected Mamu-B*01+ animals were initially tested in ELISPOT assays. None of the 11 peptides induced significant responses in either of our control animals (data not shown). Using IFN-γ ELISPOT and IFN-γ and
TNF-α ICS assays with fresh PBMC derived from 10 SIV-infected Mamu-B*01+ macaques, we were unable to demonstrate that any of the 11 newly defined Mamu-B*01-binding peptides were immunogenic (Fig. 3). This was surprising when compared with four previous MHC class I characterization studies (25, 31, 32, 51) in which at least 5 binding peptides were immunogenic in multiple rhesus macaques. Therefore, we expanded our analysis to a broader peptide panel, IC50 less than 4000 nM, in IFN-γ ELISPOT assays. The inclusion of these peptides expanded our testing pool of immunogenic (Fig. 3). This was surprising when compared with four of the 11 newly defined Mamu-B*01-binding peptides were immunogenic, but were not required.

FIGURE 2. Summary motif of Mamu-B*01. Summary map of primary and secondary anchor preferences derived from single amino acid substitution analysis. Main and secondary anchor positions are indicated. Preferred and tolerated anchor residues were defined in the text. Preferred residues at primary anchor positions (position 1) were associated with increased binding affinity, but were not required.

Table IV. Binding capacity of SIV-derived peptides to Mamu-B*01

<table>
<thead>
<tr>
<th>Protein</th>
<th>Amino Acid Positions</th>
<th>Length</th>
<th>Sequence</th>
<th>Mamu-B<em>01 binding (IC50 nM)</em></th>
</tr>
</thead>
<tbody>
<tr>
<td>Pol</td>
<td>389–397</td>
<td>9</td>
<td>VQYMDILI</td>
<td>5.3</td>
</tr>
<tr>
<td>Env</td>
<td>644–651</td>
<td>8</td>
<td>VDFLEHNI</td>
<td>19</td>
</tr>
<tr>
<td>Pol</td>
<td>848–856</td>
<td>9</td>
<td>SDFEEAVI</td>
<td>63</td>
</tr>
<tr>
<td>Env</td>
<td>652–660</td>
<td>9</td>
<td>TALLEEAQI</td>
<td>137</td>
</tr>
<tr>
<td>Pol</td>
<td>610–618</td>
<td>9</td>
<td>TDYQWVTWI</td>
<td>168</td>
</tr>
<tr>
<td>Vpr</td>
<td>59–69</td>
<td>11</td>
<td>GELRILQRAL</td>
<td>374</td>
</tr>
<tr>
<td>Env</td>
<td>644–652</td>
<td>9</td>
<td>VDFLEHNI</td>
<td>387</td>
</tr>
<tr>
<td>Pol</td>
<td>320–328</td>
<td>9</td>
<td>GDAFPSIPL</td>
<td>388</td>
</tr>
<tr>
<td>Pol</td>
<td>508–516</td>
<td>9</td>
<td>EAEEVEIKI</td>
<td>389</td>
</tr>
<tr>
<td>Env</td>
<td>75–82</td>
<td>8</td>
<td>FDANNTVT</td>
<td>398</td>
</tr>
<tr>
<td>Vpx</td>
<td>37–45</td>
<td>9</td>
<td>VNHLPRELI</td>
<td>499</td>
</tr>
<tr>
<td>Env</td>
<td>501–509b</td>
<td>9</td>
<td>EITPIGLAP</td>
<td>—</td>
</tr>
<tr>
<td>Gag</td>
<td>39–46c</td>
<td>8</td>
<td>NELDRFGL</td>
<td>—</td>
</tr>
<tr>
<td>Gag</td>
<td>169–177</td>
<td>9</td>
<td>EVVPQFQAL</td>
<td>—</td>
</tr>
<tr>
<td>Gag</td>
<td>198–206c</td>
<td>9</td>
<td>ANQIIIRD</td>
<td>—</td>
</tr>
<tr>
<td>Gag</td>
<td>257–265</td>
<td>9</td>
<td>IPVGNLYR</td>
<td>—</td>
</tr>
<tr>
<td>Gag</td>
<td>296–305c</td>
<td>10</td>
<td>SYVDRFKSL</td>
<td>—</td>
</tr>
</tbody>
</table>

* A dash (—) indicates IC50 greater than 50,000 nM.
** Previously described in 1995 (34).
*** Previously described in 2005 (35).
shown). None of these animals expressed the Mamu-B*01 molecule. In addition, Mamu-B*01 appeared to have little influence on disease progression with a mean survivorship of 46 wk post-SIV infection. The geometric mean of the chronic phase viral load of 32 Mamu-B*01 macaques was 1.27 $10^6$ viral RNA copies per milliliter of plasma (data not shown).

**Discussion**

The importance of CD8$^+$ T lymphocyte responses in controlling HIV/SIV replication has become evident in numerous studies. However, our knowledge of the immunogenetics of the SIV-infected Indian rhesus macaque model is sorely limited when compared with humans. The Indian rhesus macaques at the WNPRC and in colonies at other institutions express Mamu-B*01 with relatively high frequency (13.6–35.9%; see Table I). In the WNPRC colony, it was the most prevalent of eight MHC class I alleles tested by PCR-SSP (32.8%).

In this study, we defined a detailed peptide-binding motif of a high frequency MHC class I allele expressed by Indian rhesus macaques, Mamu-B*01. Mamu-B*01 bound its ligands using position 2 and the C terminus as main anchors, a typical finding with MHC class I binding (24–26, 31, 32, 46, 47, 51). D was the dominant residue at position 2, whereas hydrophobic residues were preferred at the C terminus. Position 5, where the acidic residue E was preferred, appeared to be a strong secondary anchor (Fig. 2).

Information regarding the binding specificity of Mamu-B*01 enabled an exhaustive scan of the SIVmac239 proteome for potential CD8$^+$ T lymphocyte epitopes. SIVmac239 is a well-defined viral isolate used in previous SIV epitope identification studies (25, 31, 32, 51). As a molecular clone, it has facilitated our ability to...
screen for candidate peptides and carry out viral escape analysis. Nevertheless, it should be noted that it is one of many different viral isolates used as a model for HIV research. Surprisingly, very few SIVmac239-derived peptides bound Mamu-B*01 with an IC$_{50}$ value of $\leq$500 nM (11 of 507). Furthermore, we showed that CD8$^+$ lymphocytes responded to none of these peptides in ex vivo ELISPOT and ICS assays (Fig. 3). Subsequent viral sequencing analysis provided no evidence of escape (Fig. 4). We expanded our studies to include peptides with IC$_{50}$ values $\leq$4000 nM and obtained similar results, bringing us to the conclusion that Mamu-B*01 does not contribute to the breadth of immune responses directed against SIV.

Despite definition of its preferred motif and ligand sizes, relatively few peptides were found that bound Mamu-B*01 with high affinity. This low incidence of Mamu-B*01 binding was observed whether peptides carried the most preferred (stringent) motif or a more expanded motif. Specifically, 3 of 97 (3.1%) of the preferred motif peptides were binders, compared with 7 of 331 (2.1%) of the peptides associated with the expanded motif (Supplemental Table I). This overall low incidence of binding is on the order of 5- to 25-fold lower than what has been observed with almost all human, macaque, chimpanzee, or mouse MHC class I molecules that have been studied to date. Typically at least 15%, and in some cases as many as 75%, of corresponding motif-bearing peptides are binders (25, 43, 48, 49, 52).

Overall, 7 of 11 (64%) of the peptides that bound Mamu-B*01 had the extended motif, and 3 of 11 (27%) had the preferred motif (Table IV). This ratio of binders identified from the extended versus preferred motifs is nearly identical with that observed for several common HLA class I molecules (53).
After testing 44 SIV-derived peptides with an IC50 <4000 nM in ex vivo IFN-γ ELISPOT assays, no positive responses were detected in any of the 10 SIV-infected Mamu-B*01+ macaques tested (Fig. 3). However, it should be noted that our approach might fail to detect CD8+ T lymphocyte responses with low binding affinities. Additionally, responses detected during chronic SIV infection may not include those that escape during acute infection, such as the Mamu-A*01-restricted epitope Tat21–28SL8 (13), due to reduced CD8+ T lymphocyte frequencies.

To address this issue, we analyzed the viral genome sequence of SIVmac239 from 35 SIV-infected macaques at time-of-death. Eight of these macaques expressed Mamu-B*01 and none had amino acid replacements suggestive of escape in the 44 Mamu-B*01 SIV-derived peptides (Fig. 4 and data not shown). In addition, analysis of the entire SIV genome did not reveal any additional regions with consistent amino acid substitutions indicative of viral escape from strong, acute phase CD8+ T lymphocyte responses (data not shown). The lack of Mamu-B*01-restricted viral escape is surprising because previously every macaque MHC class I allele studied had at least one identified epitope associated with viral escape (11–15, 22, 29, 32, 45). Interestingly, WNPRC animals expressing Mamu-B*01 appear to progress to simian AIDS in a typical fashion. Therefore, the lack of identifiable Mamu-B*01-restricted CD8+ T lymphocyte epitopes does not seem to influence SIV infection in these animals.

Our findings contradict previously published reports of a Mamu-B*01-restricted Env epitope (34) and five Mamu-B*01 Gag epitopes (35). None of these putative epitopes were found to have the capacity to bind Mamu-B*01 with biologically relevant affinities (Table IV). Similarly, lymphocytes from none of our 10 Mamu-B*01+SIV-infected macaques made a detectable cytokine response to these peptides in either ELISPOT or ICS assays using freshly isolated PBMC, although other SIV-specific CD8+ responses were detected in these animals (Fig. 3). We also vaccinated one Mamu-B*01+ macaque, 97111, before SIVmac239 challenge with a DNA-prime adenovirus-boost regimen that contained whole Gag, Tat, Rev, and Nef protein (data not shown). The vaccine elicited broad immune responses against fourteen unique CD8+ epitopes after vaccination alone in 97111, making it unlikely that the vaccine focused the immune response to only immunodominant epitopes. However, although PBMC from this animal made many robust CD8+ lymphocyte responses against epitopes in several SIV proteins, including Gag, none were detectable against the five previously described epitopes purported to bind to Mamu-B*01 (data not shown). It is therefore unlikely that these six epitopes are indeed Mamu-B*01-restricted. Without Mamu-B*01 transfectant data to confirm MHC restriction, it is possible that these six peptides may be presented by multiple MHC class I molecules or a single MHC class I molecule other than Mamu-B*01. For instance, MHC class I allele cloning and sequencing of 13 Mamu-B*01+ macaques (data not shown) revealed that all 13 animals also possessed the previously published allele Mamu-B*07 (39, 54).

Although only a limited number of peptides bound to Mamu-B*01, it was still surprising to find that none were immunogenic. It is possible that epitope processing may be involved in the lack of detectable responses. Therefore, we analyzed the sequence regions flanking the 11 Mamu-B*01 binders to see whether we could detect anything that would influence the efficiency with which these peptides can be processed. After analyzing a window of five residues on either side of the peptide, we found an increase of the acidic residue glutamic acid in both N-terminal (11/55 = 20%) and C-terminal flanking residues (10/55 = 18%) of the peptide compared with the occurrence of glutamic acid in the SIV sequence as a whole (252/3376 = 7.5%). Due to the small sample size, no definite conclusions could be drawn from this finding. However, it is conceivable that these flanking residues could have a negative impact on the processing efficiency, especially as glutamic acid is known to be a poor residue for cleavage by the human immunoproteasome (55, 56) as well as for transport into the endoplasmic reticulum by TAP (57, 58).

It is also feasible that there could be low frequency memory responses that are below the limits of detection and that these responses may be measurable only after peptide stimulation. However, previous work with Mamu-A*01 showed that in vitro stimulated PBMC yielded comparable results to fresh PBMC when identifying new epitopes (25). CD8+ T cell anergy may play a role in the paucity of Mamu-B*01 epitope-specific T cell responses. However, the large number of macaques tested at various time points with multiple techniques makes this unlikely.

When identifying additional MHC class I alleles from total RNA and synthesizing cDNA by reverse transcriptase for cloning and sequencing from the cohort of 13 Mamu-B*01+ animals used in this study, we identified three variants of Mamu-B*01: Mamu-B*0101 (34), Mamu-B*0102, and Mamu-B*0103. These variants are identical to Mamu-B*0101 except for a single amino acid substitution. At position 161 of the α2 domain, Mamu-B*0101 contains the acidic residue, glutamic acid, while Mamu-B*0102 exhibits the basic amino acid, lysine. In contrast, Mamu-B*0103 possesses serine in the place of histidine at position 9 of the α1 domain. Currently, our PCR-SSP technique for MHC class I typing from genomic DNA cannot distinguish among the three Mamu-B*01 subtypes. We are now in the process of producing MHC class I transfectants of the alternative allelic subtypes to confirm their expression and binding properties. Future research will clarify the importance, if any, of these Mamu-B*01 variants.

In conclusion, we have characterized the peptide binding specificity of the high frequency Indian rhesus macaque molecule, Mamu-B*01. Contrary to previous studies, we detected no Mamu-B*01-restricted CD8+ T lymphocyte responses. In fact, Mamu-B*01 appeared to have little or no effect on SIV disease progression. Despite these findings, the detailed peptide-binding motif described here may be useful for other disease pathogens, allowing better utilization of the limited numbers of available Indian rhesus macaques.

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Disclosures

The authors have no financial conflict of interest.

References


