A Rational Strategy to Design Multiepitope Immunogens Based on Multiple Th Lymphocyte Epitopes

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A Rational Strategy to Design Multiepitope Immunogens Based on Multiple Th Lymphocyte Epitopes

Brian Livingston, Claire Crimi, Mark Newman, Yuichiro Hashimoto, Ettore Appella, John Sidney, and Alessandro Sette

Four HLA-DR-restricted HIV-derived Th lymphocyte (HTL) epitopes cross-reactive with the murine I-A\(^b\) class II molecule were used to evaluate different vaccine design strategies to simultaneously induce multiple HTL responses. All four epitopes were immunogenic in H-2\(^b\) mice, demonstrating the feasibility of murine models to evaluate epitope-based vaccines destined for human use. Immunization with a pool of peptides induced responses against all four epitopes; illustrating immunodominance does not prevent the induction of balanced multispecific responses. When different delivery systems were evaluated, a multiple Ag peptide construct was found to be less efficient than a linear polypeptide encompassing all four epitopes. Further characterization of linear polypeptide revealed that the sequential arrangement of the epitopes created a junctional epitope with high affinity class II binding. Disruption of this junctional epitope through the introduction of a GPGPG spacer restored the immunogenicity against all four epitopes. Finally, we demonstrate that a GPGPG spacer construct can be used to induce HTL responses by either polyepitope or DNA immunization, highlighting the flexibility of the approach.

epitopes. The effect was so marked that recognition of the component epitopes could be completely silenced by the presence of the junctional epitope (17).

The present study defines a rational strategy to design and test HLA-DR-restricted multiepitope vaccine constructs. Epitopes cross-reactive between murine I-A^d and human HLA-DR class II molecules were used to evaluate different delivery systems. These studies revealed significant heterogeneity and suggested that the presence of junctional epitopes can be overcome by the introduction of spacer amino acids designed to retard the binding of the junctional epitope. These findings have broad practical applications in the design of epitope-based vaccines.

Materials and Methods

Peptide and DNA synthesis

Peptides and MAP constructs were synthesized using FMOC chemistry, as described previously (18). After the synthesis was completed, the peptide was cleaved from the resin, the protecting groups were removed, and the peptides were then purified by reverse-phase HPLC. The purity of the peptides was substantiated by mass spectrometry and/or composition analysis and found to be routinely greater than 95%.

DNA expression constructs were constructed using standard PCR techniques. The multiepitope minigenes were cloned into the CMV-driven expression vector pcDNA3.1 (Invitrogen, San Diego, CA); DNA for immunization was purified using endotoxin-free Qiagen kits (Valencia, CA).

MHC purification and peptide-binding assays

Human and murine class II molecules were purified by affinity chromatography from EBV-transformed homozygous cell lines, as previously described (19). In short, cell lysates were filtered twice through two precolumns of inactive Sepharose CL-4B and protein A-Sepharose, and then passed over a column of Sepharose CL-4B beads coupled with the appropriate anti-class II mAbs. Class II molecules were eluted with 50 mM diethylamine in 0.15 M NaCl containing 0.4% N-octylglucoside, pH 11.5. The eluate was then concentrated by centrifugation in Centriprep 30 concentrators at 2000 rpm (Amicon, Beverly, MA).

Peptide-binding affinity was measured by incubating a dose range of the unlabeled test peptides and 1–10 nM 125I-labeled probe peptides with purified class II molecules (5–500 nM) for 48 h. In preliminary experiments, the class II preparation was titrated in the presence of fixed amounts of radiolabeled peptides to determine the concentration necessary to bind 10–20% of the total radioactivity. All subsequent inhibition and direct binding assays were then performed using these concentrations. The percentage of HLA-MHC-bound radioactivity was determined by capturing MHC/peptide complexes on LB1.31 Ab (anti-HLA-DR; American Type Culture Collection, Manassas, VA)-coated Optiplates (Packard Instrument, Meriden, CT) and measuring bound cpm using the TopCount (Packard Instrument) microscintillation counter. In appropriate stoichiometric conditions, the IC_{50} of an unlabeled test peptide to the purified class II molecule is a reasonable approximation of the affinity of interaction (K_a).

Immunizations

Ag, peptides, and polypeptides were suspended in PBS and emulsified in 50% CFA (Sigma-Aldrich, St. Louis, MO). H-2^b mice were immunized with 100 μl emulsion at the base of the tail. For DNA immunizations, mice were pretreated by injecting 50 μl 10 μM cardiotxin bilaterally into the tibialis anterior muscle; 4 days later, the same muscle received 100 μg of plasmid DNA diluted in PBS. Eleven to fourteen days after immunization, 1 × 10^6 syngenic splenocytes (5 × 10^6 cells/well) were added as a source of APCs. Cells were stimulated with various concentrations of peptide ranging from 0.05 to 20 μg/ml. Background proliferation was measured in the absence of peptide. Cultures were incubated 3 days at 37°C and pulsed with 1 μCi [3H]thymidine. Cultures were incubated an additional 16–18 h. Cellular DNA was harvested on glass fiber mats and analyzed for 3H incorporation. Stimulation indexes were calculated using T cell responses observed from cells stimulated with 1 μg/ml peptide.

ELISPOT assay

Membrane-backed 96-well ELISA plates (Millipore, Bedford, MA) were coated with anti-IFN-γ mAb (BD Pharmingen, San Diego, CA) overnight at 4°C and then blocked with medium containing 10% FCS. Purified CD4^+ cells (4 × 10^5/well) were added to the microplate wells and cultured with 1 μg/ml peptide and irradiated splenocyte cells (10^5 cells/well) for 20 h at 37°C. The number of IFN-γ-secreting cells was detected by incubation with biotinylated anti-mouse IFN-γ Ab (BD Pharmingen), followed by incubation with avidin-peroxidase complex (Vectorstain). Finally, the plates are developed using AEC (3-amino-9-ethylcarbazole; Sigma-Aldrich), washed, and dried. Spots are counted using the Zeiss KS ELISPOT reader (Oberkochen, Germany).

Results

Immunogenicity of four HIV-derived HLA-DR epitopes in H-2^d mice

We have previously reported a set of highly conserved HIV-1-derived HLA-DR-restricted HTL epitopes suitable for development of a multiepitope vaccine (20). In the present study, we used four of these epitopes (Pol 711, Gag 171, Pol 303, and Pol 335) to address basic issues related to the design of such multiepitope vaccines. These epitopes were selected because in addition to binding several different HLA-DR molecules, they also bound the murine class II molecule-bound I-A^d with affinities in the 100–600 nM range (Table I). This range of binding affinities is comparable with the affinity of a known I-A^d-restricted epitope, the universal HTL epitope PADRE (21).

The immunogenicity of these epitopes in mice expressing the I-A^d molecule was evaluated by their capacity to prime for in vitro recall proliferative responses. The Pol 758 epitope, which binds I-A^d only weakly (Table I), was included as a negative control. Specifically, H-2^d mice were immunized s.c. with 140 μg of the various epitopes emulsified in CFA. Eleven to fourteen days postimmunization, CD4^+ T cells were purified from the draining lymph nodes, and epitope-specific recall proliferative responses were quantitated based on [3H]thymidine incorporation. As expected, all four I-A^d-binding HIV-derived epitopes induced significant proliferative responses. The Gag 171, Pol 303, and Pol 335 epitopes induced responses similar in magnitude to the response induced by the PADRE epitope (Fig. 1). The epitope Pol 711 was the most immunogenic and induced an ~10-fold stronger response. As anticipated, Pol 758, which binds I-A^d weakly, also failed to induce an HTL response. Collectively, these data identified four HIV-derived HLA-DR-restricted epitopes that are also immunogenic in H-2^d mice. Accordingly, these data defined a murine model system suitable to evaluate strategies for effective delivery of multiple HTL epitopes in vivo.

Limitations of two different multiepitope delivery systems

Although peptide pools are a straightforward means of delivering multiple epitopes, clinical development of peptide pools may be

| Table I. I-A^d binding affinity of HTL epitopes |
|---|---|---|
| Epitope | Sequence | I-A^d Binding Affinity (IC_{50}  μM) |
| Pol 711 | EKVYLAWVPAHKGIG | 101.8 |
| Gag 171 | GQGQMVHQAISPRTLN | 153.1 |
| Pol 335 | SPAIFQSSMTKILEP | 587.8 |
| Pol 303 | FRKYTAFTIPSINNE | 101.4 |
| PADRE | AKFVAAWTLKAAA | 42 |
| Pol 758 | HSNWRAMASDFNLP | 4179 |
HTL responses were pooled and then emulsified in CFA. As in the case of immunization,

![Image](image_url)

**FIGURE 1.** Immunogenicity of four HIV-derived HLA-DR epitopes in H-2^{b} mice. Animals were immunized s.c. at the base of the tail with 140 \( \mu g \) peptide emulsified in CFA. Eleven days postimmunization, CD4^{+} T cells were purified from the draining lymph nodes. T cells were stimulated with various concentrations of peptide in the presence of syngenic splenocytes. After 3 days in culture, cells were pulsed with \(^{3}H\)thyminidine. Proliferative responses, expressed as SI (defined as incorporation in the presence of Ag/incorporation in the absence of Ag), are shown.

associated with significant manufacturing and formulation concerns, particularly if delivery of a large number of epitopes is required. For these reasons, the development of a single vaccine construct capable of delivering multiple epitopes would be desirable. Accordingly, we examined the immunogenicity of two different single component multiepitope Ags. Specifically, a MAP containing the four selected HIV HTL epitopes (Fig. 2A) was synthesized (18). The MAP is a branched structure in which the epitopes are linked together through a C-terminal lysine residue. In addition, we also characterized the immunogenicity of a polypeptide consisting of a sequential arrangement of the Pol 711, Gag 171, Pol 335, and Pol 303 epitopes all colinearly synthesized without intervening flanking residues.

To determine whether HTL responses could be simultaneously induced against Pol 711, Gag 171, Pol 335, and Pol 303, H-2^{b} mice were immunized with equal amounts (28 \( \mu g \)) of each peptide pooled and then emulsified in CFA. As in the case of immunization with the individual peptides, epitope-specific HTL responses were measured using the recall proliferation assay. As shown in Fig. 3, the peptide pool primed significant HTL responses to all four epitopes. Stimulation indexes were typically in the 4–10 range, and the response magnitudes were relatively comparable for the various epitopes. Thus, at least under the conditions tested, little or no interepitope competition was occurring, and no particular epitope appeared to be immunodominant over the other epitopes.

The MAP construct was not very effective in terms of priming for responses to all the component HTL epitopes (Fig. 3). Although a strong response was measured against the Pol 303 epitope, the response to the Pol 711 epitope was relatively weak (stimulation index (SI) = 3.3), and virtually no recall response was detectable against Gag 171 and Pol 335. By comparison, the sequential polypeptide induced stronger HTL responses to three of the four component epitopes. Responses to the Pol 711 and Gag 171 epitopes were similar in magnitude to the responses induced by the peptide pool. Significant recall responses to Pol 303 were also induced, although of lower magnitude than those induced by the peptide pool. Finally, the linear polypeptide failed to prime for responses directed against the Pol 335 epitope.

In conclusion, priming for balanced HTL responses against all four epitopes was not achieved with either the MAP or linear polypeptide. Because a pool of individual peptides could prime for HTL responses to all four epitopes, these data suggest that factors inherent in the design of the MAP and the linear polypeptide hindered their capacity to induce responses to all epitopes.

**Juxtaposition of defined epitopes created a junctional epitope overlapping Pol 335**

Because of the higher intrinsic activity demonstrated by the multiepitope polypeptide, we concentrated on optimizing this construct. We hypothesized that a junctional epitope might have been created by the juxtaposition of the component HIV-derived epitopes and that this might interfere with effective priming of responses to Pol 335. To test this hypothesis, six different peptides spanning the epitope junctions were synthesized. Each of the junctional peptides encompasses 10 aa of one HIV epitope and 5 aa of the adjacent HIV epitope.

These junctional peptides were first characterized by measuring their binding affinity to I-A^{B} molecules. The junctional peptides, 3 and 4, which correspond to the junction between Gag 171 and Pol

![Image](image_url)

**FIGURE 2.** Schematics of two single component multiepitope Ags. A, MAP is a branched structure in which the epitopes are linked through a C-terminal lysine residue. B, Polypeptide consisting of a sequential arrangement of four class II epitopes.

![Image](image_url)

**FIGURE 3.** Induction of immune responses to multiple HTL epitopes using different delivery systems. H-2^{b} mice were immunized s.c. at the base of the tail with either a pool of peptides, MAP, or a linear polypeptide emulsified in the CFA. Eleven days postimmunization, CD4^{+} T cells were purified from the draining lymph nodes. T cells were stimulated with various concentrations of peptide in the presence of syngenic splenocytes. After 3 days in culture, cells were pulsed with \(^{3}H\)thyminidine. Naive responses were measured in animals immunized with CFA alone. Proliferative responses, expressed as SI (defined as incorporation in the presence of Ag/incorporation in the absence of Ag), are shown. Responses with a SI > 2 were considered significant. Error bars represent variation observed between multiple replicate experiments.
335, bound I-A\textsuperscript{b} with affinities of 14 and 75 nM, respectively. These affinities are on average 25-fold higher than the binding affinity of the Pol 335 epitope. (Table II). By contrast, the junctional peptides 1, 2 and 5, 6 bound purified I-A\textsuperscript{b} poorly (1474 nM for peptide 6) or not at all (peptides 1, 2, and 5).

The six junctional peptides were also tested for their capacity to recall proliferative responses from mice immunized with the linear polypeptide. HTL responses to junctional peptide 3, which corresponds to the junction of Gag 171 and Pol 335, were readily detected (Fig. 4). The magnitude of this response (SI = 5.7) was comparable with those observed for Pol 711, Gag 171, and Pol 303, which stimulated mean responses of SI = 5.1 (see Fig. 2 for comparison). In conclusion, the binding and immunogenicity data demonstrate the presence of a junctional epitope created by the juxtaposition of the Gag 171 and Pol 335. The presence of this junctional epitope may be associated with the inability of the linear polypeptide to effectively prime Pol 335-specific responses.

The design of an amino acid spacer to disrupt junctional epitopes

Peptide binding to class II molecules is predicated on specific sequence motifs based on the occurrence of certain amino acid residues with defined spacing. Disrupting these specific binding motifs, through either the alteration of primary anchor residues or their relative spacing, is predicted to deleteriously affect peptide binding. Both these approaches could be used to disrupt the junctional epitope in the polypeptide; however, changing the anchor residues responsible for junctional peptide binding would require changing the sequence of the epitopes themselves, and thereby may be associated with altering the specificity of the HTL response. Consequently, we sought to disrupt the junctional epitope through the introduction of spacer amino acids between the HIV epitopes.

Although MHC class II molecules typically bind peptides of 12–20 residues in length, a nine-residue core region is usually the main determinant of the binding energy. In the case of the binding motifs associated with most human class II molecules, positions 1 and 6 (referred to as P1 and P6, respectively) are generally the most important anchor residues (19). The I-A\textsuperscript{b} class II molecule also appears to share a similar motif and anchor spacing (12). Accordingly, we reasoned that a spacer with a minimum of five residues would be required to disrupt potential peptide-binding motifs occurring as the result of sequentially linking two different epitopes. We also reasoned that to be effective, the spacer should be made of residues not commonly allowed at either P1 or P6.

A survey of the side chain specificities for main anchor residues of prevalent MHC class II molecules (Table III) highlighted that in most instances, Y, F, I, L, V, or W is preferred at P1. Small or hydrophobic residues such as S, A, T, or C at P6 are also important for most peptide-DR interactions. As G and P are residues not typically found at main anchor positions, we predicted that insertion of a GPGPG spacer between epitopes would have a high likelihood of preventing the formation of most junctional epitopes.

Validation of the GPGPG spacer as a tool to disrupt junctional epitopes

To test this approach directly, a peptide was synthesized in which a GPGPG spacer was inserted into the peptide spanning the Gag 171-Pol 335 junction. The binding of the spacer peptide relative to that of the junctional peptide was measured for several common HLA-DR molecules (Table IV). Binding to HLA-DR molecules was measured because our goal is to develop a strategy applicable to multiepitope vaccines for human use.

The peptide spanning the Gag 171-Pol 335 junction binds two DR alleles, DRB1*1201 and DRB4*0101, with good affinity (<1000 nM). The peptide also binds DRB1*0101 and DRB1*1302 with low affinity. The introduction of the GPGPG spacer abolished the binding to all four molecules completely. The peptide spanning the Pol 335-Pol 303 junction was also characterized. This peptide binds seven DR alleles, DRB1*1501, DRB1*1101, DRB1*1201, DRB1*0701, DRB1*0802, DRB1*0901, and DRB5*0101, with affinities well below 1000 nM, the binding threshold predictive of immunogenicity. The introduction of the GPGPG spacer effectively disrupted DR binding. In the case of DRB1*0701, the binding was reduced over 100-fold and the binding to the other alleles was almost completely abrogated. These data demonstrate that inserting spacer residues between epitopes can disrupt the binding of junctional epitopes.

### Table II. I-A\textsuperscript{b} binding affinity of junctional peptides

<table>
<thead>
<tr>
<th>Junctional Peptide No.</th>
<th>Sequence</th>
<th>I-A\textsuperscript{b} Binding Affinity (IC\textsubscript{50} nM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>AWVPAHKIGGQGQMV</td>
<td>—</td>
</tr>
<tr>
<td>2</td>
<td>HKIGGQGOMVHQAI</td>
<td>—</td>
</tr>
<tr>
<td>3</td>
<td>HQAISPRTLNSPAIF</td>
<td>14</td>
</tr>
<tr>
<td>4</td>
<td>PRTLNSPAIFQSSMT</td>
<td>75</td>
</tr>
<tr>
<td>5</td>
<td>QSSMTKILEFPRKYT</td>
<td>—</td>
</tr>
<tr>
<td>6</td>
<td>KILEFPRKYTAAFTIP</td>
<td>1474</td>
</tr>
</tbody>
</table>

* Binding affinity >20 \mu M.
The ability of the GPGPG spacer to disrupt I-A^b binding was also evaluated. As presented in previous sections, the peptide spanning the Gag 171-Pol 335 junction binds I-A^b with high affinity of 14 nM. Insertion of a GPGPG spacer decreased the binding affinity to 313.3 nM, a greater than 20-fold decrease in binding affinity compared with the native peptide without spacers. By comparison with the effects seen on DR binding, the impact on I-A^b binding was relatively modest. We reasoned that the use of the murine model system would represent a stringent functional test of the ability of the GPGPG spacer to disrupt I-A^b binding.

Immunogenicity of a GPGPG spacer containing HTL polypeptide

Based on these results, the sequential polypeptide was redesigned to include GPGPG spacers between all the epitopes. To determine the impact this modification would have on immunogenicity, H-2^d mice were immunized with equimolar doses of either the sequential polypeptide (112 μg/mouse) or the GPGPG spacer polypeptide (140 μg/mouse) emulsified in CFA. The presence of the GPGPG spacer restored the immunogenicity of the Pol 335 epitope that was lost in the context of the sequential polypeptide (Fig. 5). In addition, the GPGPG spacer did not appreciably effect responses to the Pol 711, Gag 171, and Pol 303 epitopes. In each of those cases, the magnitude of the responses was indistinguishable from those obtained from mice immunized with the sequential polypeptide.

The immunogenicity of the GPGPG spacer polypeptide was further characterized using a primary IFN-γ ELISPOT assay. CD4 T cells were purified from animals immunized with the GPGPG spacer polypeptide, and epitope-specific responses were directly measured in the absence of restimulation to expand the T cell population. As shown in Fig. 6A, responses against all four epitopes were detected in these experiments. The frequency of IFN-γ-secreting CD4 cells was in the range of 90–500 spot-forming cells/10^6 cells. These data illustrate that the spacer polypeptide effectively induces multiple HTL responses characterized by both the ability to secrete IFN-γ and proliferate in response to specific epitopes. More importantly, these data demonstrate how junctional epitopes can be disrupted and how a spacer-optimized polypeptide could simultaneously prime responses against all the HTL epitopes incorporated into the construct.

The GPGPG spacer design allows effective induction of HTL responses by DNA immunization

Although DNA immunization has been an effective means to induce CTL responses, the approach has been less effective in stimulating HTL responses. To further examine the applicability of the GPGPG spacer design, we constructed a minigene encoding the GPGPG spacer polypeptide.

Mice were immunized i.m. with 100 μg plasmid expressing the GPGPG polypeptide. The induction of epitope-specific CD4 T cells was measured using a primary ELISPOT. IFN-γ-secreting T cells were detected for all epitopes (Fig. 6B). The HTL responses were in general comparable with those induced by polypeptide

### Table III. Peptide motifs associated with common human HLA-DR and murine H-2IA^b molecules

<table>
<thead>
<tr>
<th>Ag Allele</th>
<th>Position</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
<th>7</th>
<th>8</th>
<th>9</th>
<th>Reference</th>
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</thead>
<tbody>
<tr>
<td>DR1</td>
<td>DRB1*0101</td>
<td>YFILVWMA</td>
<td>SATGILVPC</td>
<td>AILMY</td>
<td>19, 29, 30, 31</td>
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<tr>
<td>DR2</td>
<td>DRB1*1501</td>
<td>LVM</td>
<td>FTY</td>
<td>ILVMF</td>
<td>32, 31</td>
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<td>DRB1*0301</td>
<td>LIFMY</td>
<td>DENQST</td>
<td>KR</td>
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<td>MLV</td>
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<td>RK</td>
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<td>VYFI</td>
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### Table IV. Binding affinity of junctional peptides to MHC class II molecules

<table>
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<tr>
<th>MHC Class II Molecule</th>
<th>Junctional Gag 171-Pol 335 (HQAISPRTLNSPAIF)</th>
<th>GPGPG No. 3</th>
<th>Junctional Pol 335-Pol 303 (KLEPFKRYTAFTIP)</th>
<th>GPGPG No. 6 (KILEPGPGPGFRKYY)</th>
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<td>313</td>
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* Binding affinity >20 μM.
immunization, thus underlying the flexibility of this approach in the induction of multispecific HTL responses.

Discussion

In the present study, we examine strategies to design and test immunogens containing multiple HLA-DR-restricted HTL epitopes. Using the cross-reactivity between the murine I-`A1 and HLA-DR class II molecules, various multiepitope delivery systems were tested in a murine model. We report the development of a GPGPG spacer that disrupts junctional epitopes and thereby enables the rational design of multiepitope HTL vaccine constructs. This vaccine design is compatible with both polypeptide- and DNA-based immunogens. The breadth and potency of the HTL responses induced by the GPGPG-containing immunogens are superior to other designs such as MAPs or linear epitope arrangements.

The definition of peptides that bind and are immunogenic in the context of different HLA-DR molecules enables the design of epitope-based vaccines capable of being recognized by the vast majority of the human population. In general, human and murine class II molecules are thought to recognize different ligands. This different specificity restricts the experimental testing of these constructs in laboratory animals. To overcome this difficulty, transgenic mice expressing human class II molecules have been derived (10). We describe an alternative strategy based on the observation that peptide-binding motif for the murine class II molecule I-`A1 (12) overlaps with the motif recognized by several common DR molecules (19). Accordingly, we predicted that it should be possible to identify DR-restricted epitopes capable of binding I-`A1 molecules and inducing epitope-specific HTL responses in nontransgenic mice. Our results validate this concept and enable the use of H-2b mice as an experimental model to design HLA-DR-restricted responses.

One fundamental question concerning the development of multiepitope vaccines is whether or not it is possible to simultaneously induce responses to a number of epitopes. In general, immune responses are only directed at a small fraction of the possible peptides derived from a given pathogen are recognized as epitopes. This phenomena of immunodominance can be attributed to a number of factors, including binding affinity, holes in the T cell repertoire, or epitope processing (9). Immunodominance primarily focused on one or a few epitopes is more commonly observed in murine systems, as a number of studies have shown human responses are often directed against multiple epitopes (20, 22). We find in the present study that immunization with a pool of peptides, each of which individually induces potent HTL responses, was nearly as effective at inducing responses as the individual peptides. Some caution should be exercised in interpreting these results, as dose-response experiments were not performed. Nevertheless, these data were encouraging, in that they alleviated possible concerns over immunodominance and demonstrated that it is possible to prime for a balanced multispecific HTL response at least up to a tetravalent epitope construct. Current experiments are examining whether there is a limit to the number of epitopes that can be recognized.

Although pools of peptides have been used clinically to elicit multispecific immune responses (23), practical issues relating to the manufacture and formulation limit the usefulness of this approach to the development of a vaccine designed to incorporate multiple HTL epitopes. An alternative approach is the use of MAP conjugates in which distinct epitopes are chemically linked into a
single molecule. Such constructs have been shown to be immunogenic, capable of inducing both humoral and cellular immune responses to *Plasmodium falciparum* epitopes (15). An alternative approach is to produce an Ag representing a string of select epitopes. Using a baculovirus expression system, Shi et al. (14) produced an immunogenic polypeptide composed of 21 *P. falciparum* epitopes. Although both Ags induced immune responses that conferred in vitro protective efficacy, there was no direct comparison of immunogenicity of the Ags, and means of optimizing immunogenicity were not addressed. In fact, the polypeptide was found to contain two junctional epitopes that may contribute to reduced potency (24). This study represents the first direct comparison of these delivery strategies and examines means to improve vaccine design through the use of specialized spacer sequences.

The presence of junctional epitopes could redirect the immune response to irrelevant and possibly even immunodominant epitopes. In the present study, we observed similar effects in a sequential tetravalent polypeptide. This problem was overcome by the introduction of a GPGPG spacer. The use of spacers has been used in constructs consisting of helper and Ab epitopes to preserve conformational dependent immunogenicity. Spacers have also been used between two neighboring CTL epitopes to facilitate epitope processing (25, 26). In this study, the spacers are applied to eliminate junctional epitopes and tailor the specificity of the immune response. The introduction of GPGPG spacers does not preclude the possibility that such linear arrangements of epitopes might contain other cryptic epitopes. Nonetheless, the use of GPGPG spacers should be broadly applicable at minimizing purely junctional epitopes because the approach is not context dependent.

The receptor-binding pockets of MHC class II molecules are best suited to bind amino acids with specific biochemical properties and spacing. The characterization of these parameters has led to the definition of detailed peptide-binding motifs. Although detailed knowledge of these binding motifs has been used for epitope identification, it can also be used to minimize the creation of artificial epitopes, such as junctional epitopes, in the design of specialized epitope-based vaccines. The peptide-binding motifs of common MHC class II molecules are typically based around two primary anchor residues separated by 4 aa. As such, the introduction of a 5-aa spacer between epitopes would preclude the creation of a junctional epitope that uses amino acids from both epitopes as anchor residues. To prevent the formation of junctional epitopes, it becomes a matter of using a spacer based on residues that are infrequently used as primary anchors.

Among various possible spacers, GPGPG was selected for two reasons. First, GPGPG extensions around the core binding region becomes a matter of using a spacer based on residues that are best suited to bind amino acids with specific biochemical properties and spacing of a 5-aa spacer between epitopes would preclude the creation of a junctional epitope that uses amino acids from both epitopes as anchor residues. As such, the introduction of a 5-aa spacer between epitopes would preclude the creation of a junctional epitope that uses amino acids from both epitopes as anchor residues. To prevent the formation of junctional epitopes, it becomes a matter of using a spacer based on residues that are infrequently used as primary anchors.

Among various possible spacers, GPGPG was selected for two reasons. First, GPGPG extensions around the core binding region greatly decrease binding affinity, thus suggesting that GPGPG-containing epitopes would not bind efficiently unless the spacer was removed by Ag processing. Consequently, we felt that this spacer would maximize the likelihood that the relevant epitope would be regenerated with little or no extraneous sequences attached to its N and C terminus. Second, the GPGPG spacer was selected because regions rich in G and P are known to be associated with β (27). The presence of this spacer at ~15–20 residue intervals might help create some secondary and possibly tertiary structure, thereby facilitating Ag expression and potential purification.

Polypeptides are frequently used as a means of delivering HTL epitopes, as such Ags are likely to be endocytosed by APC, where they could directly access the endosomes in which class II molecules are loaded. Recent data suggest that HTL epitopes may also be effectively delivered by DNA immunization (28). Our data demonstrate that the GPGPG spacer can also be applied to DNA-based vaccines, underlying the broad applicability of spacers in optimizing the potency of multi-epitope HTL vaccine constructs.

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**References**


