Elicitation of structure-specific antibodies by epitope scaffolds

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Elicitation of antibodies against targets that are immunorecessive, cryptic, or transient in their native context has been a challenge for vaccine design. Here we demonstrate the elicitation of structure-specific antibodies against the HIV-1 gp41 epitope of the broadly neutralizing antibody 2F5. This conformationally flexible region of gp41 assumes mostly helical conformations but adopts a kinked, extended structure when bound by antibody 2F5. Computational techniques were employed to transplant the 2F5 epitope into select acceptor scaffolds. The resultant “2F5-epitope scaffolds” possessed nanomolar affinity for antibody 2FS and a range of epitope flexibilities and antigenic specificities. Crystallographic characterization of the epitope scaffold with highest affinity and antigenic discrimination confirmed good to near perfect attainment of the target conformation for the gp41 molecular graft in free and 2FS-bound states, respectively. Animals immunized with 2FS-epitope scaffolds showed levels of graft-specific immune responses that correlated with graft flexibility (p < 0.04), while antibody responses against the graft—as dissected residue-by-residue with alanine substitutions—resembled more closely those of 2FS than sera elicited with flexible or cyclized peptides, a resemblance heightened by heterologous prime-boost. Lastly, crystal structures of a gp41 peptide in complex with monoclonal antibodies elicited by the 2FS-epitope scaffolds revealed that the elicited antibodies induce gp41 to assume its 2FS-recognized shape. Epitope scaffolds thus provide a means to elicit antibodies that recognize a predetermined target shape and sequence, even if that shape is transient in nature, and a means by which to dissect factors influencing such elicitation.

Monoclonal antibodies of enormous utility have been identified, revolutionizing treatments for autoimmune disorders, infectious disease, and different types of cancers (reviewed in ref. 1). Requirements for nonoral means of delivery and in some contexts prolonged treatment regimens, however, have limited their use. While vaccine modalities have potential for improvements, no clear path exists from a clinically useful monoclonal antibody toward elicitation of similar antibodies in a vaccine context. One potential solution is precise immunogen design. The ability of structural biology to provide atomic-level definition of antibody-antigen interactions and of computational biology to manipulate protein structure has raised the possibility—at least for protein antigens—of precisely replicating the antigenic surface recognized by a target antibody. We hypothesized that appropriate immunization with such an antigenic mimic might succeed in eliciting replicas of the original target antibody.

As a first step toward solving the vaccine problem of “reelicitation,” we undertook the challenge of structure-specific elicitation—the elicitation of antibodies capable of binding the sequence and of inducing the structure of a predetermined target epitope. Various protein–scaffold platforms have been described in which structural elements of scaffold proteins act as acceptors of functional or antigenic regions from other proteins (2–4). Here we describe a platform for the elicitation of structure-specific antibodies—the epitope-scaffold platform—in which structural mimics of viral neutralizing determinants are grafted into heterologous protein scaffolds using techniques of computational protein design. As a test system, we chose the 2F5 antibody (5, 6), which recognizes an epitope in the membrane-proximal external region (MPER) of the HIV-1 gp41 transmembrane glycoprotein, for which we and others have determined a number of atomic-level structures (7–14). Although recognition by 2F5 involves not only the structure-specific binding of a gp41 epitope but also nonspecific interactions with membrane (13, 15–17), the system was nonetheless attractive because of the conformational diversity of the MPER, its extensive structural characterization, and the linear nature of the epitope. We show that immunization of animals with epitope–scaffold mimics of the target 2F5 epitope leads to the elicitation of polyclonal serum responses that mimic those of antibody 2F5. Moreover, we confirm crystallographically that monoclonal antibodies elicited by 2F5-epitope scaffolds are capable of binding the sequence and of inducing the conformation of the 2F5 epitope in a flexible gp41 peptide, a conformation that would otherwise only rarely be assumed.

Results

Computational Design of Epitope Scaffolds. To translate structural information into immunogen design, we devised a semiautomated procedure involving the following steps: First, the entire Protein Data Bank was searched for appropriate acceptor proteins (scaffolds) with backbone structural similarity to segments of the 2FS-bound epitope on gp41. Second, a filtering step was applied in which initial structural matches were only retained if the scaffolds could be bound by antibody without significant clashes. Third, epitope side chains were transplanted at appropriate positions. Fourth, additional mutations were introduced into each of the scaffolds to optimize stability, to enhance epitope exposure, and to minimize nonepitope interactions with antibody (Fig. 1).


The authors declare no conflict of interest.

This article is a PNAS Direct Submission.

Freely available online through the PNAS open access option.

Data deposition: Atomic coordinates and structure factors have been deposited in the Protein Data Bank, www.pdb.org (accession codes 3LES and 3LEY for free and 2FS-bound structures of ES2 scaffold, respectively, and accession codes 3LEX and 3LEY for elicited antibodies 11F10 and 6a7, respectively, in complex with a gp41 peptide corresponding to the 2F5 epitope).

See Commentary on page 17859.


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This article contains supporting information online at www.pnas.org/lookup/suppl/doi:10.1073/pnas.1004728107/-/DCSupplemental.

17880–17887 | PNAS | October 19, 2010 | vol. 107 | no. 42
www.pnas.org/cgi/doi/10.1073/pnas.1004728107
Fig. 1. Target epitope and transplantation to select acceptor scaffolds. (A) Epitope for antibody 2F5. The HIV-1 virion (schematic based on ref. 40) employs numerous mechanisms of immune evasion to avoid recognition by neutralizing antibody. A potential site of vulnerability is recognized by antibody 2F5, depicted as an antigen-binding fragment (Fab) with heavy chain in blue and light chain in gray, which binds to residues 659–669 of gp41, depicted in red. The sequence of the HIV-1 MPER is from strain HxB2. (B) Shown are diverse structures of gp41 that have been previously determined (7–11, 14, 41), with residues 659–669 highlighted in red. (C) The 2F5-recognized conformation of the epitope shows essentially the same conformation in two different crystal lattices (12, 13). (D) Computational methods were used to transplant the 2F5 epitope into acceptor scaffolds, which were selected and further modified to present the gp41 epitope (red) in its 2F5-recognized conformation. Epitope scaffolds are drawn in gray as Cα-ribbons, with scaffold residues altered to accept transplantation highlighted in stick representation, and colored orange for ES1, yellow for ES2, green for ES3, blue for ES4, and purple for ES5. (E) Two potential measures of graft flexibility, recognition by sera generated by flexible representations of the epitope (vertical axis) and entropy of 2F5 recognition (horizontal axis), were found to correlate. Data points are colored according to epitope scaffold as in D, with gray for MPER peptide. (F) X-ray crystal structure of unbound ES2. The left image shows a Cα-ribbon of the unbound ES2 structure. The right graph shows rmsds for different residue ranges of the 2F5 epitope in the unbound ES2 structure (yellow) or in the initial ES2-computational model (gray), with both compared to the target 2F5-bound conformation of gp41. (G) Crystal structure of ES2 in complex with antibody 2F5. The left image shows a Cα-ribbon of the ES2:2F5 complex, with the 2F5 Fab colored blue and gray and ES2 yellow. Coloring for the right graph is the same as in F.
This procedure resulted in the design of five epitope scaffolds from parent coordinates 1LGYa, 1KU2a, 2MATa, 1IWLa, and 1D3Bb (refs. 18–22), which we named ES1-ES5, respectively (Fig. 1D and Table 1). On average, eight epitope residues were transplanted and eight additional mutations were made to each of these scaffolds (Fig. S1). In two of the scaffolds (ES2 and ES5), the region encompassing the epitope graft was occluded on the native scaffold oligomer, but in both cases we judged that mutations associated with epitope transplantation would interfere with oligomerization and result in stable monomeric proteins with exposed 2F5 epitopes. Model properties of the resultant 2F5-epitope scaffolds showed main-chain rmsds ranging from 0.7 to 1.3 Å (Table 1 and Fig. S1), indicating reasonable replication of the epitope shape. The nonbound face of the epitope graft, meanwhile, showed up to 70% less solvent-accessible surface area than equivalent residues on the 2F5-bound peptide (Table 1), indicating substantial occlusion of the nonbound face.

Biochemical, Biophysical, and Antigenic Characterization of 2F5-Epitope Scaffolds. Epitope scaffolds were first tested for expression in a mammalian system, which succeeded for scaffolds ES2 and ES4. The remaining scaffolds were expressed bacterially, which, following a refolding step, yielded soluble ES1, ES3, and ES5 scaffolds, although these tended to aggregate. To assess potential utility in elicitation, we tested the scaffolds for binding to antibody 2F5 with surface-plasmon resonance, because high affinity for 2F5 was likely a required property of an immunogen capable of eliciting antibodies that induce the 2F5-recognized shape in gp41. When the epitope scaffolds were directly coupled to surface-plasmon resonance chips, experimental affinities to 2F5 Fab ranged from 0.600 ± 0.004 to 18.80 ± 0.03 nM, which were comparable to 2F5 affinities for free and cyclized peptides of 6.44 ± 0.03 and 1.93 ± 0.02 nM, respectively (Fig. S24 and Table 1).

To evaluate the degree of conformational stabilization of the epitope in the scaffolds, we analyzed the thermodynamics of their interaction with 2F5. Although in general the contributions of configurational entropy are difficult to separate from those of solvation in calorimetry experiments (23, 24), as a first approximation in the cases described here, the latter should be similar for peptides and epitope scaffolds because their interfaces should be nearly identical, assuming contacts made outside the epitope are negligible. Thus, in interaction with antibody 2F5, a more favorable binding entropy for the epitope scaffolds relative to the free peptide is likely to indicate conformational fixation of the epitope. We obtained isothermal titration calorimetry measurements for ES2, ES4, and ES5 as well as for both wild-type and cyclized MPER peptides (Fig. S2B and Table 1); we were unable to obtain accurate measurements for ES1 and ES3, however, likely because of problems with aggregation. For the ES2 scaffold, a −ΔTS change at 37 °C of −10.1 ± 2.3 kcal/mol was observed, indicating an overall increase in entropy upon binding and suggesting the graft in ES2 to be rigid. For the ES4 and ES5 scaffolds, −ΔTS values of −0.9 ± 0.2 and 6.8 ± 0.7 kcal/mol were observed, respectively; the latter value was close to that observed for the cyclized epitope peptide, suggesting the grafts in these scaffolds were more flexible than that in ES2. In contrast, the wild-type epitope peptide had a −ΔTS change of 15.0 ± 0.5 kcal/mol, indicating substantial loss of entropy upon binding, consistent with the expected loss of conformational diversity for an unbound- to bound-peptide transition.

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<th>PDB code of parent</th>
<th>ES1</th>
<th>ES2</th>
<th>ES3</th>
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<th>ES5</th>
<th>MPER WT</th>
<th>MPER cyc</th>
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<td></td>
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<td>1KU2a</td>
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<td>0.7</td>
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<td>0.8</td>
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<td>Nonbound surface (Å&lt;sup&gt;2&lt;/sup&gt;)</td>
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<td>176</td>
<td>108</td>
<td>160</td>
<td>79</td>
<td>241</td>
<td>193*</td>
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Expression Binding to antibody 2F5 Fab

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<th>ES1</th>
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<th>ES4</th>
<th>ES5</th>
<th>MPER WT</th>
<th>MPER cyc</th>
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<tr>
<td>Bacterial</td>
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<td>Synthetic</td>
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<td>on-rate (1/Ms) (x10&lt;sup&gt;3&lt;/sup&gt;)</td>
<td>6.52 ± 0.04</td>
<td>16.68 ± 0.04</td>
<td>1.56 ± 0.01</td>
<td>7.43 ± 0.02</td>
<td>9.70 ± 0.03</td>
<td>7.17 ± 0.04</td>
<td>8.48 ± 0.05</td>
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<td>off-rate (s&lt;sup&gt;−1&lt;/sup&gt;) (x10&lt;sup&gt;3&lt;/sup&gt;)</td>
<td>3.50 ± 0.02</td>
<td>1.00 ± 0.00</td>
<td>0.547 ± 0.004</td>
<td>13.90 ± 0.02</td>
<td>2.92 ± 0.01</td>
<td>4.62 ± 0.03</td>
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<td>K&lt;sub&gt;D&lt;/sub&gt; (nM)</td>
<td>5.38 ± 0.04</td>
<td>0.600 ± 0.004</td>
<td>3.50 ± 0.02</td>
<td>18.80 ± 0.03</td>
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<td>1.93 ± 0.02</td>
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<td>ND</td>
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<td>ND</td>
<td>−10.0 ± 0.2</td>
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<td>−25.6 ± 0.5</td>
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<td>Flexible MPER A (serum dilution)</td>
<td>45.6 ± 13.8</td>
<td>19.6 ± 16.4</td>
<td>56.0 ± 11.4</td>
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<td>Flexible MPER B (serum dilution)</td>
<td>20.0 ± 31.1</td>
<td>2.1 ± 0.6</td>
<td>19.0 ± 1.9</td>
<td>8.2 ± 8.6</td>
<td>85.2 ± 33.8</td>
<td>357.4 ± 22.3</td>
<td>ND</td>
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<td>MPER flexible loop (serum dilution)</td>
<td>59.2 ± 30.9</td>
<td>38.6 ± 0.9</td>
<td>38.4 ± 16.1</td>
<td>27.9 ± 0.9</td>
<td>723.3 ± 611.4</td>
<td>9227.0 ± 1668.8</td>
<td>ND</td>
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Table 1. Computational design and experimental characterization of 2F5-epitope scaffolds

ES1-ES5 are 2F5-epitope scaffolds; MPER WT is a peptide composed of residues 656–670 of gp41 linked to a C9 tag; MPER cyc is a cyclized version of MPER WT. NA, not applicable; ND, not determined.

*Nonbound surface of MPER cyc is based on a structural model.
design, the ES2 scaffold, which showed the highest affinity to 2F5, the most entropically favorable interaction with 2F5, and one of the most restrictive recognition profiles by flexible MPER-elicited sera, was chosen as a representative scaffold for structural characterization. Crystals of the free form of ES2 were obtained and diffracted to 2.8 Å, with two molecules of ES2 present in the asymmetric unit (Table S1). The structure of ES2 is shown in Fig. 1F, with a close-up view superimposed with the parent gp41 peptide in Fig. S2D. Least squares superposition of gp41 residues 660-667 in the two asymmetric-unit copies of crystalline ES2 against the target 2F5-bound epitope showed Cα rmsds of 0.7 Å and difference-distance matrix (26) averages of 0.3 Å (Table S2). More comprehensive superpositions were performed for various subranges of the epitope graft, and, as shown in Fig. 1F, the crystal structure of free ES2 displayed a pattern of mimicry to gp41 very similar to that predicted by the computational model. The crystal structure of the ES2 scaffold was also determined in complex with the 2F5 antibody at ~2.5 Å resolution (Fig. 1G and Table S1). Binding by antibody 2F5 induced the epitope graft to adopt even more closely the 2F5-bound conformation of gp41. Superposition of the epitope graft showed a Cα rmsd against residues Leu660–Ala667 of the parent gp41 peptide of only 0.2 Å, an almost fourfold decrease from the same superposition in the free ES2 structure (Fig. 1G, Fig. S2D, and Table S2). Extending the alignment subrange by one residue upstream to Gln659, increased the superposition rmsd for Cα atoms to 0.7 Å, while extending it to Leu669 increased the rmsd to 1.3 Å (Table S2). These results indicate that in the ES2 context, the epitope most closely resembled residues Leu660–Ala667 of gp41, and that over these residues, 2F5 induces the graft in the ES2 scaffold to adopt near perfect resemblance to the target conformation of the 2F5-bound gp41 epitope.

**Polyclonal Sera Elicited by Epitope Scaffolds Mimic 2F5 Binding to the gp41 MPER.** We next investigated epitope-scaffold immunogenicity. Guinea pigs were immunized with the 2F5-epitope scaffolds, either using a single scaffold (homologous immunizations) or in combination (heterologous immunizations) (Fig. 2A). ELISAs were performed on prebleeds as well as after the second, fourth, and sixth immunizations. In all cases, robust immune responses were seen against the entire inoculated scaffold (Fig. 2B and Fig. S3A). To assess the degree to which these robust responses were directed against the engrafted gp41 epitope, homologous post-2 and post-4 immunization-elicited sera were tested for binding to the gp41-peptide epitope by ELISA. As shown in Fig. 2C and Fig. S3B, the responses against the grafts depended on the inoculated scaffold, with ES5-immunized animals showing the highest graft-specific responses, ES3- and ES4-immunized animals displaying intermediate responses, and ES1- and ES2-immunized animals displaying lower graft-specific responses. Graft-specific responses did not correlate with the presence of a T-helper epitope (PADRE) (27), the use of Alum/CpG versus AS01B adjuvant, the degree of graft similarity to the 2F5-bound conformation, nor the degree of occlusion of the nonbound surface. The magnitude of the graft-specific response did, however, correlate with the rigidity of the scaffold graft (p < 0.04), as assessed either by recognition with sera elicited with scaffold and flexible versions of the MPER (Fig. 2D) or by entropy of 2F5 recognition (Fig. 2E). These results suggest that epitope flexibility enhances immunogenicity, and conversely, that graft rigidity reduces immunogenicity.

To provide insight into graft-specific immune recognition, elicited responses were interrogated with alanine mutants spanning residues 658–670 of gp41 (Fig. 2F). The 2F5 antibody interacted with alanine mutants in a manner inversely proportional to the contact surface area normally observed with each unaltered residue, with alanine mutants to the central Asp-Lys-Trp tripeptide ablating binding. Sera elicited by both free and cyclized peptide showed alanine-interrogated profiles that were significantly different from 2F5, especially for Asp and Lys in the central tripeptide. Sera elicited by epitope scaffolds, meanwhile, showed a variety of responses (Fig. 2F). When elicited responses were sorted by similarity to 2F5 based on a reliability factor (R value) which quantifies ELISA responses (Fig. 2G and SI Materials and Methods), top responses were derived either from heterologous immunizations or from homologous immunizations with ES5, and these were remarkably similar to antibody 2F5.

**Monoclonal Antibodies Elicited by Epitope Scaffolds Replicate 2F5 Structure-Specific Recognition of the gp41 MPER.** Having thus shown that with select epitope scaffolds it was possible to elicit polyclonal responses that closely mimicked 2F5 in terms of binding across the gp41 MPER, we next sought to determine the biophysical and structural characteristics of specific monoclonal antibodies present in the mimicked responses. Two groups of five mice were immunized with the 2F5-epitope scaffolds: either with ES5 (five times) or with an ES5 prime (two times) followed by an ES1 boost (three times). Mice that had high titers to the ES2 scaffold and the gp41-peptide epitope were chosen for fusion, and B cell-hybridoma clones were selected on the basis of binding to heterologous scaffolds and free peptide. A total of six monoclonal antibodies were isolated in this manner, three from each mouse group. Surface–plasmon resonance analysis (Fig. S4A) revealed that antibodies from the ES5-ES1 prime-boost group had the highest affinity for peptide and scaffolds, and the two tightest binders from this group, 11f10 and 6a7, which were determined to be isogenic variants (Fig. S4D), were selected for structural characterization. The antigen-binding fragments of each of these antibodies was produced and crystallized in complex with a peptide corresponding to gp41 residues 660–667, yielding crystals that diffracted to 2 Å resolution (Fig. 3 and Table S1).

The electron density for the peptide was clearly defined (Fig. 3B), with the exception of Leu660 at the peptide N terminus. Superposition of the 11f10- and 6a7-bound gp41 peptide against 2F5-bound gp41 revealed that the epitope-scaffold-elicited antibodies induced a peptide conformation remarkably similar to that induced by 2F5 (Fig. 3C and Fig. S4B), with rmsds for backbone α atoms of 1.1 and 1.0 Å for 11f10 and 6a7, respectively. Side-chain orientations of gp41 in the 11f10 and 6a7 bound structures were also similar, with the same chi-1 and chi-2 side-chain rotamers observed in roughly half of the peptide residues. When electrostatic potentials were mapped onto antibody surfaces, a high degree of similarity was also observed between antibodies 11f10 and 6a7 and antibody 2F5 (Fig. 3D and E). Although the similarities in the electrostatic surfaces were striking, they did not necessarily correspond to similar residue contacts between the antibodies and gp41 (Fig. 3 F and G and Tables S3 and S4). For instance, Trp 96 of the light chain of 11f10 (and 6a7) packed perpendicular to Trp 666 of gp41, with its Nε atom situated for hydrogen bonding to the π-electrons of the indole ring of Trp 666 of gp41, while in the case of 2F5, interactions with gp41 Trp 666 are mediated by non-Trp residues. Another notable difference between the antibodies is that the tip of the 2F5 CDR H3 loop—likely involved in membrane interactions (15)—had no corresponding partner in 11f10 or 6a7. Nonetheless, similarities were observed between the angles of approach and the spatial orientations of the 11f10 and 6a7 antibody relatives to gp41 as compared to those of 2F5 (Fig. 3H and Fig. S4 B and C). Superposition of the gp41 peptide in the epitope-scaffold-elicited antibodies against the gp41 peptide in the template-2F5 antibody overlays approximately 80% of the variable regions of these antibodies, but with a mode of binding that effectively switches the heavy and light chain positions of the elicited and 2F5 antibodies.
Fig. 2. Immunogenicity of 2F5-epitope scaffolds. (A) Immunization scheme. A priori, it was unclear what factors would influence immunogenicity. We therefore utilized a highly redundant sparse matrix with three primary variables: type of epitope scaffold (ES1, orange; ES2, yellow; ES3, green; ES4, blue; ES5, purple); type of adjuvant (Alum/CpG, circles; ASO1B, squares); and the presence (closed symbols) or absence (open symbols) of linked T-help ("TH"; PADRE) (27). Twelve different immunization schemes were evaluated in guinea pigs, four animals per group, with sera sampled prior to immunizations (Pre) and after two (Post 2), four (Post 4), and six (Post 6) immunization cycles. (B) Overall titers. ELISA EC₅₀ values of polyclonal antibody responses against the entire scaffolds are shown, as assessed by binding of Pre, Post 2, and Post 4 serum time points to the whole scaffold. (C) Graft-specific titers. ELISA EC₅₀ values of polyclonal antibody responses against the 2F5-epitope portion of the epitope scaffolds are shown, as evaluated by binding of Pre, Post 2, and Post 4 serum time points to a 2F5-epitope peptide (individual responses are shown in Fig. S3). (D) Graft-specific titers (Post 2, circles and dashed black line; Post 4, triangles and solid black line) elicited by the epitope scaffolds (vertical axis) are compared with recognition of the epitope scaffolds by sera generated by the epitope when immunized in a flexible context (e.g., as free peptide or placed into a flexible loop) (horizontal axis). The overall fit is shown as a red line. (E) Residue-by-residue interrogation of the elicited responses. Single alanine mutants were introduced into a collection of 2F5-epitope peptides spanning residues 658–670 (top left). The effects of these alanine mutants on antibody 2F5 binding were evaluated by ELISA, with changes to the central Asp-Lys-Trp tripeptide ablating binding and other residues displaying more muted responses (middle left, black line). The alanine mutants were also used to interrogate sera elicited by flexible and cyclized peptides (bottom left, dark brown and light brown, respectively), as well as against all Post 2, Post 4, and Post 6 sera (right panels; lines and symbols are colored based on scaffold coloring depicted in A; for Post 6, symbols are colored based on scaffolds used in the final two immunizations). The 2F5 alanine scan profile (black) is shown in all panels for comparison. (F) Optimal responses. Responses to the alanine-mutant epitope peptides were ranked by R-value of the response, as defined by the expression $R = \frac{\sum_i (EC_{50}^{sera} - EC_{50}^{2F5})}{\sum_i EC_{50}^{sera} - EC_{50}^{2F5}}$, where i is the residue position at which the MPER was mutated to alanine. Alum/CpG, linked T help, increasing number of immunizations, heterologous immunizations, and use of ES5 all biased toward reduced R-values (Fig. S3C and Table S5). Shown here are results from alanine-scanning for the top three responses along with corresponding R-values and p-values of the immunization schemes (p-values were obtained as described in SI Materials and Methods; because 58 different sera or grouped sera were analyzed, Bonferroni adjustments were calculated to account for multiple comparisons, with individual p-values from each serum comparison to 2F5 multiplied by a factor of 58). The 2F5 alanine scan profile (black) is shown in all panels for comparison.
Discussion

Structural specificity is a defining characteristic of mature antibody recognition. While antibodies may use other specialized mechanisms of recognition, such as posttranslational mimicry (antibody 412d, ref. 28) or co-membrane binding (antibody 2F5 investigated here, refs. 13, 15, 16), they all bind with high affinity to a specific protein-epitope structure. Here we utilize epitope scaffolds to teach structure-specific recognition of a target epitope. Antibodies elicited with the epitope scaffolds bound to the 2F5 epitope with high affinity, induced a conformation similar to that induced by 2F5, and showed similar angles of epitope approach (Fig. 3C and H and Fig. S4). Nonetheless, we do not expect these variables to recreate fully the antibody properties of 2F5, because the membrane-binding component of 2F5 recognition was not addressed in the design procedure. This component is responsible for a substantial portion of the free energy of 2F5 recognition, without which neutralization is difficult to attain (15, 16, 29, 30).

A number of other groups have also studied the expression of the nominal 2F5-epitope sequence (ELDKWAS) in scaffold systems: a variable loop of the HIV-1 gp120 envelope glycoprotein; a surface loop of human rhinovirus; and a surface loop of bovine papilloma virus (25, 31–33). All of these studies resulted in the elicitation of antibodies targeted to the ELDKWAS epitope, and two of these studies reported the induction of weak neutralizing antibodies. However, none of the studies demonstrated similarity between the elicited polyclonal response and 2F5 binding to gp41, nor the elicitation of monoclonal antibodies able to induce the 2F5-bound conformation in gp41, both of which we demonstrate with the 2F5-epitope scaffolds to provide proof of concept for the “reelicitation” of structure-specific antibodies.

The modular nature of the epitope scaffolds—with the same epitope placed into different acceptor–scaffold backgrounds—provides a means to examine factors that influence elicitation. We observed a range of flexibilities for the engrafted epitope, with flexibility inferred from two different measures: antigenic recognition by sera generated with the epitope in a highly flexible context and thermodynamic measurements of the entropy of 2F5 recognition. While equating binding entropies with conformational entropies is generally not permissible (23, 24), the similarity of the recognition by 2F5 appears to permit such analysis in this particular case. The results with respect to immunogenicity were striking: Notably, the flexibility of the engrafted epitope, as judged by thermodynamics and antigenic recognition, correlated with immunogenicity, with a flexible but otherwise equivalent epitope generating significantly higher immune responses than a rigid one. This finding provides an explanation for prior observations that elicited responses against viral antigens are often focused on flexible loops—as has been classically observed with influenza virus (34) and more recently with adenovirus chimeras (35). One possible mechanism for this finding may relate to the ability of flexible epitopes to utilize more fully mechanisms of induced fit, thereby engaging nascent immunoglobulins on a larger percentage of B cells and initiating their maturation (as has been proposed for the inverse problem of antibody recognition) (36). Perhaps relevant to this, the finding that heterologous immunizations more often elicit graft-specific responses that closely resemble those of the target antibody suggests that successive populations of B cells can be appropriately expanded and affinity matured by successive epitope-scaffold boosts, as we have done with ES5 and ES1 to elicit antibodies highly similar to the 2F5 antibody. Lastly, the epitope-scaffold-elicited antibodies th
selves provide insights into the diversity of molecular recognition enabled by the adaptive immune response. Antibodies 1F10 and 2FS show related recognition chemistries—with similarities in electrostatics (Fig. 3 D and E) and comparable combining site-amino acid chemistries (Fig. 3 F and G)—even though their orientations of heavy and light chains are dramatically different (Fig. 3 H) as are their progenitor immunoglobulin genes (Fig. S4D). Thus, in addition to the potential utility of epitope scaffolds in vaccine modalities, the antibodies they elicit provide a means to delineate the diversity of molecular recognition by the adaptive immune response as it fulfills requirements of structure-specific elicitation.

Materials and Methods
A summary of experimental techniques is given here, with full methods and associated references presented in SI Materials and Methods.

Creation of 2FS-Epitope Scaffolds. The central concept was to employ, as acceptor scaffolds, proteins with preexisting structural similarity to the antibody-bound conformation of the target epitope. The gp41-2FS structure revealed significant peptide-antibody contacts over the range of gp41 residues from E659 to L669 (refs. 12, 13), so we designed epitope scaffolds to mimic this bound conformation of the target epitope. The gp41-2F5 structure revealed tor scaffolds, proteins with preexisting structural similarity to the antibody-specific elicitation.

The adaptive immune response as it fulfills requirements of structural and functional consequences of changing a sulfur atom to a methylene group in the M31Ne mutation in ribonuclease B. EMBO J. 33(28):8578–8593.


Generation and Structural Characterization of Monoclonal Antibodies Elicted with 2FS-Epitope Scaffolds. Mice were immunized in two week intervals with 20 ug of epitope scaffold in Alum/CpG. Those with the highest titers of graft-specific responses, as assessed by ELISA against peptide or heterologous epitope scaffold, were chosen for production of monoclonal antibody with standard fusion and selection techniques (ProSci). Monoclonal antibodies obtained were characterized by surface-plasmon resonance for affinity to gp41-peptide epitope and heterologous scaffolds, and those with highest affinity were sequenced, crystallized, and analyzed by X-ray crystallography as antigen-binding fragments in complex with the 2FS-epitope peptide (residues 660–667).

ACKNOWLEDGMENTS. We thank B.K. Chakrabarti and G.J. Nabel for sera generated from placing the 2FS epitope into the flexible V3 loop of gp120; Y. Geng and V. Savich (ProSci) for assistance for monoclonal antibody elicitation; K. Kattinger for antibody 2F5; J.R. Mascola and K. McKee for neutralization analysis; G.J. Nabel, J.R. Mascola, L. Shapiro, I. Wilson, and members of the Structural Bioinformatics and Biology Sections, Vaccine Research Center, for discussions and comments on the manuscript; J. Stuckey for assistance with figures; and GlaxoSmithKline for AS01B adjuvant. Support for this work was provided by the Intramural Research Program of the National Institutes of Health and by the International AIDS Vaccine Initiative and its Neutralizing Antibody Consortium. Use of SER-CAT at the Advanced Photon Source was supported by the U.S. Department of Energy, Basic Energy Sciences, Office of Science.


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Supporting Information

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SI Text


The overall design procedure consisted broadly of “matching” and “design” stages. The computational work flow was carried out by custom shell scripts (1). In the “matching” stage, the Protein Data Bank (PDB) (2) was searched for scaffolds with backbone-structural similarity to any segment of the 2F5-bound gp41 peptide spanning the range 659–669, and the initial structural matches were filtered based on antibody clash. PISCES (3) was used to cull the PDB of November 11, 2004, down to 14,383 chains of native than 50 residues with resolution better than 3.0 Å. MAMMOTH (4) was used to search the culled PDB for sequence-independent structural matches based on Ca coordinates. Structural matches were ranked by the ratio of rmsd to the number of superimposed residues (rmsd/nsup). RosettaDesign (5, 6) was then used for clash-checking. The best 5% of matches by rmsd/nsup (700 matches with rmsd/nsup < 0.132) were evaluated for steric clash (Rosetta score “E.rep”) between the scaffold backbone (side-chains mutated to glycine) and the antibody (all atoms retained), with the relative orientation of scaffold and antibody determined by the structural superposition of the epitope segment onto the scaffold. The 25 scaffolds with the least clash were retained for further analysis; these scaffolds had E_rep (bind) < 0.5 score units above the E_rep (bind) measured for the 2F5-gp41 crystal structure with gp41 mutated to glycine, where E_rep (bind) = E_rep (complex)- E_rep (partner1)-E_rep (partner2). Scaffolds with higher clash values were judged to require too many severe atomic overlaps with antibody to allow binding. Five scaffolds with co-factors were then eliminated. Many of the remaining 20 scaffolds were oligomeric in their native state, so the clash analysis was repeated with the native oligomers, resulting in the final selection of five scaffolds—ILGYa (ES1), 1KU2a (ES2), 2MA1a (ES3), 1WLa (ES4), 1D3Bb (ES5). In two of the five chosen scaffolds, the epitope was occluded on the native oligomeric ES2 homodimer (7) and ES5 hetero-dimer (8), but in both cases we judged that oligomerization was not essential to maintain the scaffold structure and that the mutations associated with epitope transplantation could result in monomeric proteins with an exposed 2F5 epitope.

In the “design” stage, epitope side chains were transplanted to the appropriate positions on the remaining scaffolds, and further mutations were designed on each scaffold to minimize nonepitope interactions with antibody and to optimize stability. RosettaDesign was used to pack side chains on the scaffold backbone, with side-chain conformations restricted to those contained in the backbone-dependent rotamer library of Dunbrack and Cohen (9) or to native scaffold rotamer conformations, and an energy function dominated by a 12-6 Lennard–Jones potential, and an implicit solvation model as detailed in the supplementary information for RosettaDesign (5, 6). Antibody-contacting epitope residues (E659, L661, E662, D664, K665, W666, A667, L669) were transplanted to corresponding scaffold positions according to the MAMMOTH structural alignment where possible, while scaffold positions adjacent to the epitope or to the antibody were designed to accommodate epitope side chains and avoid interactions with antibody, and all other scaffold positions were assigned native-scaffold amino-acid rotamers. On average, eight epitope residues were transplanted and eight additional mutations were made to each of these scaffolds.

Subsequent to the original design of the epitope scaffolds, the protocol was improved to include transplantation of native epitope rotamers to each scaffold, followed by energy minimization (10) of rigid-body and rotameric torsional degrees of freedom at the scaffold-antibody interface, prior to design of scaffold positions outside the epitope. Scaffold models analyzed in this manuscript were produced by the updated protocol using the originally designed scaffold sequences.

Cloning, Expression, and Purification of Epitope Scaffolds.

Cloning.

Plasmids for the expression of all scaffolds were derived by de novo gene synthesis, followed by subcloning into the CMV/R expression plasmid (11) (GenScript, Bionexus). All constructs were appended with an N-terminal CD5 leader sequence for secretion from mammalian cells, as well as with a C-terminal Hisx6 and C9 (TETSQVAPA) tags, the latter of which reacts with the ID4 mouse antibody (12). Given that several 2F5-epitope scaffolds (ES1, ES3, and ES5) did not express or secrete in the mammalian expression system, the genes were subcloned into the bacterial expression plasmid pET-17b (Novagen). PCR reactions were carried out with primers that introduced two unique restriction sites (Nde I and BamH I) to facilitate the ligation of the PCR products into the pET-17b expression vector. Ligation of the PCR products and vector pET-17b was carried out after digestion. The plasmids were transformed into Rosetta BL21 E. coli cells. Addition of a universal heterologous T-cell helper epitope (AfKvAATLkAa) to the scaffolds was undertaken either by QuickChange site-directed mutagenesis (Stratagene) for ES3, or by de novo gene synthesis for ES2 and ES5. A plasmid for a shorter fragment of ES2 was cloned using QuickChange site-directed mutagenesis (Stratagene) to add a stop codon after residue 271.

Expression.

Transient transfection of epitope-scaffold mammalian expression plasmids into adherent HEK 293 cells or Freestyle 293F cells (Invitrogen) was undertaken using Lipofectamine or 293fectin transfection reagents, respectively (Invitrogen). For 293F Freestyle expression, cells were grown in 2L flasks to a density of 1.2 x 10^6 cells per ml and transfected with 250-500 µg of plasmid DNA per liter of 293 Freestyle growth medium (Invitrogen). Cell culture supernatants were collected 4–5 days after transfection, centrifuged at 3,500 x g to remove cell debris and filtered using a 0.22 micron filter unit. Of the five scaffolds, ES2 and ES4 were successfully secreted from mammalian cells.

For the remaining scaffolds, ES1, ES3, and ES5, bacterial expression was undertaken. A 50 ml culture of Rosetta BL21 E.coli transformed with the expression vector was grown overnight at 37 °C. The next morning a 1L culture was grown from the 20 ml overnight culture to an OD600 of 0.6–1.0 and induced with IPTG to a final concentration of 1 mM. After growth for 6 h, the cells were harvested and pelleted by centrifugation at 5,000 x g.

Purification.

Mammalian expressed scaffolds (ES2 and ES4) were purified from the supernatants through a combination of a chelating (nickel), affinity (2F5-antibody), and size-exclusion chromatography (Superdex 75 or 200). In brief, 1L supernatants were either applied directly to a nickel column or first buffer-exchanged into PBS, pH 7.4. Binding was undertaken in 500 mM NaCl, 20 mM Tris-Cl, pH 8.0, 20 mM imidazole, followed by washes and elution with binding buffer supplemented with 40 and 500 mM imidazole, respectively. Eluates were concentrated, buffer-exchanged into PBS (Amicon Ultra centrifugal filter devices, Millipore), and
then loaded onto a 2F5 antibody affinity column. Samples were eluted using Pierce IgG elution Buffer, pH 2.8, followed by rapid pH adjustment with Tris-Cl, pH 8.5-9. Eluates were pooled, concentrated, and then loaded onto a size-exclusion column (Superdex 75 or 200).

All bacterially expressed scaffolds—ES1, ES3, and ES5—seg- regated into inclusion bodies, and purification was undertaken first by lysing the bacterial cell pellet with Bugbuster reagent con taining lysozyme and nucleases (Novagen), followed by centrifugation at 10,000 g to pellet the inclusion bodies. Inclusion bodies were resuspended overnight under denaturing conditions of 8 M urea, 20 mM Tris-Cl, pH 8.0, 1 mM beta-mercaptoethanol or DTT, and then centrifuged and filtered, and loaded onto a nickel column equilibrated with binding buffer comprised of 8 M urea, 500 mM NaCl, Tris-Cl, pH 8.0, imidazole 10 mM. Loaded samples were then washed with binding buffer supplemented with 40 mM imidazole, and eluted with binding buffer supplemented with 300–500 mM imidazole. Eluates were concentrated to 1 ml using an Amicon Ultra concentrator (Millipore). Refolding of concentrated eluates was performed overnight at 4 °C by quick dilution (1:100) into refolding buffer comprised of 50 mM Tris-Cl, pH 8.0, 250 mM NaCl, 500 mM L-Arginine, 0.1 mM glutathione reduced, 0.01 mM glutathione oxidized, 0.03% N-lauryl sarcosine and 0.1 mM ZnCl2. Refolded samples were then concentrated to 5–7 ml using Centricon plus-80 (Millipore) and dialyzed into PBS supplemented with 125 mM NaCl. For ES3 and ES5, a further round of purification was undertaken by applying the samples to a 2F5 antibody affinity column. The ES1 scaffold did not remain soluble after elution from the affinity column, and was therefore only subjected to nickel column purification.

**Surface-Plasmon Resonance.** For the binding of 2F5 to the epitope scaffolds, the scaffolds were directly immobilized onto Biacore CM5 sensor chips (GE Healthcare) to final surface densities of ∼500 RU. 2F5 antigen-binding fragment (Fab) was then used analyte at concentrations ranging from 0.5 nM to 500 nM, at 2-fold serial dilutions, with association and dissociation phases of up to 5 min, at a flow rate of 30 pl/min. To determine binding affinities of antibodies 2F5, 11F10 and 6a7 to gp41 peptides, the antibody IgGs were immobilized directly to CM5 sensor chips at surface densities of ∼4,000–5,000 RU, and the gp41 MERR-C9 peptide (EQELLEDKWASLGTTGVTSSQA) or the MERR-C9-cyclized peptide (EQELLE-Dap-DKWDDSLWGVC, respectively) (Washington Biotechnology). Guinea pig sera generated previously by insertion of the gp41 MERR sequence LLELDKWA into a flexible surface loop on HIV-1 gp140 were also used, and were a kind gift from B. Chakrabarti and G. Nabel (13).

**Generation of Animal Sera.** Sera generated from immunogens which had the gp41 MERR inserted into flexible contexts were generated by injecting rabbits with free or cyclized MERR peptides linked to KLH through thiol linkage (NEQELLEDKWASLG GCC or EQELLE-Dap-DKWDSSLWG GCC, respectively) (Washington Biotechnology). Guinea pig sera generated previously by insertion of the gp41 MERR sequence LLELDKWA into a flexible surface loop on HIV-1 gp140 were also used, and were a kind gift from B. Chakrabarti and G. Nabel (13). Sera elicited by epitope scaffolds were generated by intramuscular injection of Hartley guinea pigs (females, ∼10 weeks of age) with 20 μg of affinity purified protein formulated in either a GlaxoSmithKline Adjuvant System AS01B or a combination of Alum (2% Aluminum Hydroxide gel, 50 μl per animal) with CpG (250 μg per animal) at 4 week intervals. The protein-adjuvant emulsion was always prepared within 1 h of inoculation into animals. The first four inoculations were all homologous protein. Subsequent inoculations were either homologous or heterolo- gous protein. Bleeds were collected 7–10 days after each inoculation. Serum was collected and incubated at 55 °C for 1 h to heat-inactivate complement and stored at −80 °C until subjected to analysis.

**Analysis of Animal Sera.** ELISA profiles.

100 ng/well of mouse monoclonal antibody 1D4 (12) (with specificity to C9 tags) was adsorbed overnight at 4 °C onto a Maxisorp plate (Nunc) in PBS. The next day plates were washed five times with wash buffer composed of PBS supplemented with 0.2% Tween 20, and then blocked for 2 h at 25 °C in PBS supplemented with 5% dry-milk powder (Difco) and 5% heat-inactivated Fetal Bovine Serum (Sigma or Gibco). Following a wash step, scaffolds or peptides in PBS, all of which had a covalently linked C9 tag at the C terminus, were loaded onto the plates at 10X molar excess with respect to the adsorbed 1D4 antibody and incubated for 1 h at 25 °C. Following a wash step, serum was added at fivefold serial dilutions (1:50 to 1:781250) in PBS, 0.2% Tween 20 and incubated for 1 h at 25 °C. Pooled preimmune sera per animal group were also tested (at the same serum dilutions), as was antibody 2F5, at fivefold serial dilutions from 5 μg/ml to 6.4 × 10⁻⁶ μg/ml. Plates were washed five times with wash buffer followed by incubation with a donkey secondary anti- guinea pig immunoglobulin (H + L) (Jackson Labs) at a 1:10000 dilution in PBS, 0.2% Tween 20 for 1 h at 25 °C. Plates were washed again, followed by addition of 100 uL of the colorimetric TMB (3,3′,5,5′-tetramethylbenzidine) peroxidase enzyme immuno- noassay substrate (Bio-Rad) to each well. The reactions were performed using Microcal VP-ITC or ITC-200 at 37 °C. All pair- wise 2F5/ligand samples were dialyzed extensively into identical buffer for 0.5X of the solution 350 mM NaCl, 2.5 mM Tris-Cl, pH 7.1 or PBS supplemented with 125 mM NaCl. 2F5 IgG was placed into the calorimeter cell at a concentration of 1–2 μM, and the scaffolds or peptides were injected as titrants. Between 25–30 injections were performed per run, at 5–10 μl per injection. Heats of dilution of the titrants were assessed based on control experiments of titrator into buffer and buffer into macromolecule, or by analysis of the heats observed for the last set of injections after saturation. Concentrations of all ligands were determined using Aں absorbance and known extinction coefficients for each of the proteins. If measured concentrations yielded fits of the ITC data that were inconsistent with expected stoichiometries of n = 2 for an IgG interaction, effective active concentrations of the epitope scaffolds were assessed based on achieving stoichiometries of n = 2. These effective concentrations were then used in obtaining the final thermodynamic parameters. With the exception of the ES5 scaffold, the effective concentrations of all titrants were within ∼90% of the concentrations measured using Aں absorbance. For ES5, based on expected stoichiometry of binding, came to 38% that measured by Aں absorbance to within 10% the effective concentration predicted in the first format (ES5 as titrant). The estimated effective concentrations of the scaffolds were therefore as follows: ES2, 40 μM; ES4, 24 μM; ES5, 135 μM; MPER WT peptide, 25 μM; MPER cyclized peptide, 30 μM. Profiles were fit using Origin Ver. 7.0 (Microcal).
stopped with 100 μL of 0.1N H$_2$SO$_4$ per well. Optical density was read on a microplate reader at 450 nm using Softmax software (Molecular Devices). All samples were performed in duplicate. GraphPad Prism Version 5.0 was used to fit four parameter logistic curves to all ELISA profiles.

**Analysis of ELISA profiles of flexible MPER sera.**

Means of replicate EC$_{50}$S and standard deviations of the means are reported. In cases where the ELISA curves were too flat to fit, resulting in lack of convergence or ambiguity, constraints were added to limit the tops of the fits so that an EC$_{50}$ could be interpolated. This was the case for curves of recognition of ES2 by Flexible MPER B (2 replicates); for recognition of ES4 by Flexible MPER B (1 replicate); for recognition of ES1 by Flexible MPER B (2 replicates); and for recognition of the negative control 1D4 antibody by Flexible MPER A (1 replicate) and Flexible MPER B (2 replicates). In the case of recognition of ES2 by Flexible MPER A, the curve was too flat to fit even in the presence of constraints, and the EC$_{50}$ was set to the highest dilution of 7.6.

**Analysis of ELISA profiles of epitope scaffold elicited sera.**

Mean EC$_{50}$S of the individual sera were obtained by applying the fits to all replicates per scaffold per bleed. EC$_{50}$S calculated per scaffold (Fig. 2C) entailed 2 replicates each for scaffolds ES1, ES3, ES4, and 8 replicates each for scaffolds ES2 and ES5. A constraint on the top of the fits to be less than 5 was applied in all cases. In cases where the curves of individual replicates were too flat to obtain a proper fit of the data, the replicates were excluded from the analysis. This was the case for profiles ES3,AS01B.Post 2 (1 replicate), ES1,AS01B2.Post 2 (1 replicate), ES2,Alum/CpG.Post 4 (1 replicate), ES2,AS01B1.Post 2 (1 replicate), ES2,AS01B3.Post 4 (1 replicate), ES2,TH.Alum/CpG.Post 4 (1 replicate), ES2.TH.AS01B.Post 2 (1 replicate), ES2.TH.AS01B.Post 4 (2 replicates), and ES4,Alum/CpG.Post 2 (1 replicate).

**Analysis of alanine scan ELISA profiles.**

EC$_{50}$S of alanine-interrogated ELISA profiles were determined as described above (per replicate). In cases where the responses were too flat to accurately obtain a fit, replicates were excluded from the analysis. All EC$_{50}$S were normalized relative to the EC$_{50}$ of wild-type MPER peptide, as defined by the peptide for which position 667 of gp41 is an alanine. To determine similarity of profiles to 2F5, an R-value calculation was used, as defined by the expression $R = (\frac{\sum_{i=658}^{670} |EC50_{ES Sera} - EC50_{2F5}|^2}{\sum_{i=658}^{670} |EC50_{2F5}|^2})$, where $i$ is the residue at which the peptide is mutated to alanine. Unique R-values were computed from the normalized EC$_{50}$ data for each technical replicate of each serum, then mean R-values and standard errors were computed from the two technical replicate R-values. Resampling tests were computed in R 2.10 (http://www.R-project.org) to determine if any of the test sera were significantly similar to the 2FS-MAB profile. Bootstrap samples of the normalized EC$_{50}$ measurements were collected from the pool of 41 test sera (all sera except for MAB 2F5) at each of the 13 residues, then mean R-values were computed from the resampled normalized EC$_{50}$ data to simulate a distribution of all possible R-values. Mean R-values estimates from the individual sera and from select groups of sera were compared to the histogram and p-values were computed as the proportion of the area under the histogram to the left of the mean R-factors. A Bonferroni adjustment was used to account for multiple comparisons (30); p-values were only considered statistically significant if $p < 0.05/58 = 0.0008621$, yielding two profiles that were statistically significant in their similarity to 2F5: ES2.TH.AS01B.Post 6 and ES2.TH.Alum/CpG.Post 6. A less conservative False Discovery Rate (FDR) step-up adjustment was also applied (31), yielding six profiles that were statistically significant in their similarity to 2F5 with a 3.75% false discovery rate: ES2.TH.AS01B.Post 6, ES2.TH.Alum.CpG.Post 6, ES5.Alum.CpG.Post 6, ES1.AS01B2.Post 6, ES5.TH.AS01B.Post 4, ES5.Alum.CpG.Post 6.

Because the distribution of the resampled R-factors was approximately normally distributed, Student’s t-tests were used to compare the mean R-values from several groups of sera. A Bonferroni adjustment was used to account for multiple comparisons (30); p-values were only considered statistically significant if $p < 0.05/18 = 0.0027778$. Only the comparison of R-values of groups that did or did not possess a T-helper epitope, was statistically significant, with $p = 0.0154$ after Bonferroni multiplication by 18. The results of the Student’s t-test were confirmed by Mann-Whitney and Kruskal–Wallis tests.

**Generation of Graft-Specific Monoclonal Antibodies Using Epitope Scaffolds.**

Balb/c mice were inoculated subcutaneously with 20 μg of affinity purified protein (ES5, ES1) formulated in a combination of Alum (2% Aluminum Hydroxide gel, 50 μL per animal) with CpG (250 μg per animal) at 2 week intervals (ProSci). Serum was collected 7–10 days after each inoculation. The protein-adjuvant emulsion was always prepared within 1 h of inoculation into animals. ELISA IgG titers measured using selection antigens ES2 (no tags) and MPER peptide (EOELLELDK-WASLWNWFDDTTKWY1K8K8K8) were used to determine mice that were to be sacrificed to proceed with fusion of spleen. Secondary selections of positive clones were carried out using selection antigens ES2, ES3, and ES4.

The 11f10 and 6a7 antibodies were sequenced by a combination of N-terminal Edman sequencing followed by reverse transcriptase polymerase chain reaction (RT-PCR). For N-terminal sequencing, the antibodies were first run on SDS/PAGE gels and then transferred to PVDF membranes (Invitrogen). The heavy and light chains were cut out of the blots and sent for sequencing (Columbia University Protein Core). For the 11f10 and 6a7 antibodies, the N-terminal sequences of the light chains were determined to be DVPVMTQTPLS and DVPVMTQTPET, respectively, and those of the heavy chains were DVLQLESGLGLVK and DVQLQESGP, respectively. The N-terminal sequences were input into the International Immunogenetics Information System (IMGT, http://imgt.cines.fr/) and possible germline precursors determined. Light chains were suggested to be IGKV1-131, IGKV1-132, IGKV1-133, or IGKV1-135, and heavy chains suggested to be IGHV3-2, IGHV3-5, or IGHV3-6. N-terminal DNA primers were ordered based on the N-termini of these genomic precursors, as were constant region C-terminal primers based on isotyping of the antibodies, which suggested both antibodies had k light chains and IgG2a heavy chains. The sequences of the N-terminal primers were IgVK1-132, 5'-gatgttggtgtgacctccag-3', IgVH3-a, 5'-gatgtgctacgctagtagtggtc-3', IgVH3-d, 5'-gatgtgctacgctagtagtggggtagtggagc-3'. The sequences of the C-terminal primers were IGKC rev, 5'-acatccagctgctgctgccac-3', and IgG2a rev, 5'-ttgattgtgggccctctg-3'. Total cellular RNA was then prepared from the respective murine hybridoma cells using RNAeasy (Qiagen) and was used in one step RT-PCR reactions (Qiagen) with the above primers. Reactions which yielded DNA PCR products were sequenced to give the final sequences of the light and heavy chains, which were then read into the IMGT database and determined to be isogenic descendents of genomic precursors IGKV1-135-01 and IGHV3-2-02, respectively, and putatively of IGKV1-135-01 and IGKV1-135-01.

**Preparation of Antibody-Binding Fragments of Antibodies 2F5, 11f10, 6a7.** Antibody Fab's of 2F5, 11f10, and 6a7 were prepared as previously described (14, 15). Briefly, antibody IgGs were first reduced with 100 mM DTT for 1 h at 37 °C, followed by 1 h of dialysis in PBS or Hepes, pH 7.6, to reduce the DTT concent-
tration to 1 mM. Antibodies were then dialyzed against 2 mM Iodoacetamide for 48 h at 4°C, and subjected to a final dialysis against PBS or Hapes, pH 7.6, for 2 h. After reduction and alkylation, antibodies were cleaved with Lys-C (Roche), run over a Protein A column to segregate away the Fc fragment, and then subjected to ion exchange (Mono S, 2F5; Mono Q, 11f10 and 6a7) and size-exclusion chromatography.

Crystallizations. ES2 scaffold.

We observed that the full length ES2 scaffold was prone to proteolysis at Arg271, and since the resulting N-terminal proteolytic fragment was stable, a stop codon was introduced after Arg271 and the shorter fragment was expressed and purified as described above. Crystallizations using 576 conditions adapted from the commercially available Hampton (Hampton Research), Precipitant Synergy (Emerald Biosystems), and Wizard (Emerald Biosystems) crystallization screens were set up robotically (Honeybee) using vapor diffusion sitting drops. Robotic crystal hits were hand optimized and crystals that were grown in 23% PEG 4000, 120 mM (NH₄)₂SO₄, 20 mM ATP at 20°C diffracted to 2.8 Å resolution.

ES2: 2F5 Fab complex.

Purified 2F5 Fab was incubated with excess ES2 (Arg271) scaffold for 0.5 h at 25°C and the complex was then loaded onto a gel filtration column (Superdex 200). A clear peak could be discerned for the complex and crystallizations were set up robotically as described for free ES2. Crystal hits were hand optimized, and crystals grown in 16% PEG 400, 2.8% PEG 3350, 100 mM sodium acetate (C₄H₆NaO₂), pH 5.5, 20 mM ATP, diffracted to 2.5 Å, though were only 33% complete in the highest resolution shell.

11f10: gp41-peptide and 6a7: gp41-peptide complexes.

Threefold molar excess of gp41 peptide LLELDKWA corresponding to residues 660–667 of gp41 (HxB2 numbering) was added to purified 11f10 and 6a7 Fab's and the complexes set up for robotic crystallization, as described above. For the 11f10: gp41 complex, crystal hits were hand optimized and those grown from 16% PEG 4000, 80 mM sodium acetate (C₄H₆NaO₂), 40 mM Tris-Cl, pH 8.5 diffracted to better than 2 Å. For the 6a7:gp41 complex, crystals grown in 31.5% PEG 1000, 200 mM zinc acetate (C₄H₆O₂Zn), 100 mM sodium acetate (C₄H₆NaO₂), pH 4.5 diffracted to better than 2 Å, in a space group that was distinct from the 11f10:gp41 crystals.

Data Collection, Structure Solution, and Refinement. Crystals were transferred into stabilizing solutions containing 5–10% higher concentrations of precipitant and a cryoprotectant (40% glycerol for ES2, 15% 2R:3R butanediol for 11f10 and 6a7 crystals). For the 11f10 and 6a7 crystals, cryosolutions were supplemented with 4 mM gp41 peptide to ensure the peptide did not dissociate from the crystal after dilution with the cryo. After mounting the crystals on a loop, they were flash cooled and data was collected on SER CAT ID-22 or BM-22 beamlines (APS) and processed using HKL-2000 (16).

Structures were solved with molecular replacement using AmoRe (17, 18) or Phaser (19). For the free ES2 scaffold the parental ES2 structure 1KU2a was used as a search model. For the ES2:2F5 complex, both 1KU2a and the 2F5 crystal structure 1TIJ were used as search models. For the 6a7:gp41 complex, 1BB3 was used as a search model, and the solved 6a7 model structure with a few rounds of refinement was subsequently used as a search model for 11f10:gp41.

Refinement of the ES2 and ES2:2F5 crystal structures was undertaken using CNS (20), with iterative model building using O (21). For the 11f10:gp41 and 6a7:gp41 crystal structures, Phe

Structural Analyses. The program Grasp (24) was used to determine electrostatic potential maps of structural surfaces, which were then read into PyMOL (PyMOL Molecular Graphics System, Versions 0.99 and 1.2r3pree). Grasp was also used to determine exposed surface area of the non-antibody-bound face of the core five residues (662–666, HxB2 numbering) of the epitope. This was performed for epitope scaffolds ES1-ES5, and for the free and cyclized peptides. In each case, 2F5-bound and unbound surface areas were calculated for the whole ligand molecule. The exposed surface area of the core five residues was defined as the area contributed by the core residues to the nonbound surface.

To determine spatial overlap of antibody Fv fragments, the antibodies were superimposed using an alignment of their respective complexed gp41 peptides. The total volume enfolding both antibody Fvs was then determined using Grasp, and the percent overlap defined as the mean volume of the individual Fvs divided by the volume of their superposition.

Structural alignments were performed using the program lsqkab (ccp4 Package (25)). Distance-difference matrix calculations were determined using Differences Distance Matrix Plot (DDMP) software, as previously described (26) [Fleming, P. http://www.roselab.jhu.edu/ddmp/ (2004)]. MS, PISA, and HPLUS were used to determine contact surfaces and residues (27–29).

All structural figures were generated with use of PyMOL (Versions 0.99 and 1.2r3pree).


5. Kuhlman B & Baker D (2000) Native protein sequences are close to optimal for their
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All structural figures were generated with use of PyMOL (Versions 0.99 and 1.2r3pree).
Fig. S1. Sequences of 2F5-epitope scaffolds. Shown are alignments of the amino acid sequences of epitope scaffolds ES1, (A), ES2, (B), ES3, (C), ES4, (D), and ES5, (E), with their parental proteins, labeled based on PDB ID code. Residues that were mutated from the parental sequence to build in the gp41 graft or accommodate 2F5-binding are shaded. The sequence of gp41 residues 659–669 (red), upon which the grafting was based, is aligned onto the graft of each scaffold, with residues of gp41 that are present in the graft labeled with asterisks. Root-mean-square deviations (rmsd) of the epitope graft against gp41 residue ranges 660–667, 659–667, and 659–669 from PDB ID 1TJI are shown for main-chain (MC) and Cα atoms.
Experimental characterization of epitope scaffolds. (A) Surface-plasmon resonance analysis of 2F5 Fab binding to epitope scaffolds. To determine binding affinities of epitope scaffolds to antibody 2F5 Fab, epitope scaffolds ES1-ES5 were directly immobilized onto Biacore CM5 sensor chips and 2F5 Fab was flowed over as analyte. For affinities to wild type and cyclized MPER-C9 peptides, 2F5 IgG was directly immobilized onto the sensor surface and the peptides flowed over as analyte. Shown sensograms, black, are twofold serial dilutions of 2F5 Fab at concentrations 0.97–7.8 nM (ES1), 0.49–3.9 nM (ES2), 0.97–15.6 nM (ES3), 1.95–15.6 nM (ES4), and 0.49–3.9 nM (ES5), or peptide analyte concentrations of 0.49–7.8 nM (MPER-C9) and 1.95–7.8 nM (MPER-C9-cyclized). Sensograms were fit with a 1∶1 Langmuir model, shown in red. (B) Thermodynamics of interaction between 2F5 and epitope scaffolds. Shown are isothermal titration calorimetry profiles (VP-ITC, Microcal) of 2F5 IgG with epitope scaffolds ES2, ES4, and ES5, and with wild-type and cyclized MPER-C9 peptides. In all experiments shown, 2F5 IgG was placed into the calorimeter cell at a concentration of 1–2 μM, and the scaffolds or peptides injected as ligands. Effective concentrations of the ligands were 40 μM, ES2, 24 μM, ES4, 135 μM, ES5, 25 μM, MPER-C9 WT peptide, and 30 μM, MPER-C9 cyclized peptide (see SI Materials and Methods). After baseline adjustment, the profiles were fit using Origin Ver. 7.0 (Microcal), and the results are presented in each panel and in Table 1. (C) Recognition of epitope scaffolds by flexible MPER-elicited serum. ELISA mean EC₅₀s of flexible MPER-elicited serum recognition of epitope scaffolds ES1-ES5 and of wild-type and point mutant K665E MPER peptides are shown (flexible MPER A serum, left slanting hatch, flexible MPER B serum, solid bars, MPER flexible loop serum, right slanting hatch). As a negative control, reactivity with 1D4 antibody was also examined (for flexible MPER A and MPER flexible loop sera), and served as a reference baseline. All experiments were performed in duplicate. (D) Shown are superpositions of residues 659–669 of gp41 in its 2F5-bound conformation (red) against corresponding residues (105–115) of the epitope graft in the crystal structure of unliganded ES2 (top, yellow) and 2F5-bound ES2 (bottom, yellow). Root-mean-square deviations (rmsd, Å) of Cα atoms are listed in each case.
Fig. S3. Characterization of epitope-scaffold-elicited sera. (A) Overall responses elicited by epitope scaffolds. Sera elicited by epitope scaffolds were examined for antibody titers against the entire respective scaffolds (including regions outside of the graft). Post 2 and Post 4 bleeds of all animal groups shown in Fig. 2A, with the exception of group 2, were analyzed in ELISA format for reactivity with the respective scaffold immunogen. Shown are EC_{50} values obtained by fitting ELISA profiles with four parameter logistic curves (GraphPad Prism Version 5.0). (B) Graft-specific responses of scaffold-elcited sera. Sera elicited by epitope scaffolds were also examined for graft-specific immune responses, using reactivity to MPER-C9 peptide. ELISAs were performed and analyzed as in A, for Post 2, 4, and 6 bleeds of all animal groups. For Post 6 samples, hatched bars indicate heterologous boosts for the last two immunizations, while solid bars indicate homologous boost. All ELISA experiments were performed in duplicate. (C) Factors influencing the quality of 2FS epitope-scaffold-elicited responses. Alanine scan profiles of all serum samples were grouped based on different parameters used in the immunizations—immunogen, bleed, T-cell help, or adjuvant. Mean R-values per group were then examined for effects of each parameter, with the lower the R-values the closer the mimicry to the alanine scan profile of 2FS (see SI Materials and Methods). Shown, clockwise from upper left, are mean R-values per immunogen (for post 2 and post 4 immunizations), per serum bleed (with post 6 profiles separated based on whether the boosts were homologous or heterologous), per adjuvant, and per presence of a T-cell helper epitope (PADRE). For reference, the R-values for sera elicited by MPER in flexible formats were 1.02 ± 0.01 and 0.87 ± 0.02, for flexible MPER immunized rabbits A and B, respectively, and 0.91 ± 0.03 and 1.19 ± 0.04 for cyclized MPER immunized rabbits 1 and 2, respectively.

Ofek et al. www.pnas.org/cgi/doi/10.1073/pnas.1004728107
C

Comparison of Angles of Approach to gp41.

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<td>6a7₁₁₀</td>
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Volumes determined using the program Grasp (24).

₁%-Overlap defined as the ratio between the mean volume of the individual Fv’s with the volume of their superposition.

na, not applicable.
Characterization of epitope-scaffold-elicited monoclonal antibodies. (A) Surface–plasmon resonance analysis of binding of elicited monoclonal antibodies to gp41 peptide. Monoclonal antibodies elicited by homologous immunization with the ES5 epitope scaffold—9f8, 1d9, and 1c11—and those elicited by heterologous immunization with epitope scaffolds ES5 and ES1—11f10, 6a7, and 6f4 were directly immobilized onto Biacore CM5 sensor chips and the gp41 MPER-C9 peptide was flowed over as analyte. Twofold increasing concentrations of analyte were used, ranging from 3.9–125 nM (11f10, 6f4), 3.9–62.5 nM (6a7), 0.97–125 nM (9f8), and 15.6–500 nM (1d9, 1c11). Sensograms, black, were fit with a 1∶1 Langmuir model, red. Binding constants are reported in each panel, with the exception of 1c1 antibody, for which binding to peptide was too low to obtain proper values at the concentrations tested. (B) Structure of monoclonal antibody 6a7 in complex with gp41. Shown in the left panel is a backbone representation of the crystal structure of the 6a7 Fab (heavy chain, magenta; light chain, green) in complex with the gp41 peptide LLELDKWA (cyan). The crystal structure of the isogenically related antibody 11f10 (heavy chain, orange; light chain, purple) in complex with gp41 peptide (yellow) is superimposed. A closeup view of the alignment of all atoms of the gp41 peptides from the 6a7 and 11f10 crystal structures against corresponding residues of 2F5-bound gp41 (salmon) is shown on the right. (C) Comparison of angles of approach to gp41. To compare angles of approach to gp41 of the elicited and 2F5 antibodies, spatial overlap of the antibody Fvs relative to gp41 was determined. The antibody Fvs were first oriented by superimposing their complexed gp41 peptides, and then the total volume enfolding them both was determined. Percent-overlap was calculated by dividing the mean volume of the two individual Fvs by the volume of their superposition. (D) Sequences, progenitor genes, and affinity maturation of antibodies 11f10 and 6a7. Shown are sequence alignments of the heavy and light chains of monoclonal antibodies 11f10 and 6a7, against their common heavy and light chain precursors. Residues that are shaded (orange, 11f10 heavy chain; magenta, 6a7 heavy chain; purple, 11f10 light chain; green, 6a7 light chain) are different from their genomic precursor at that position. Antibody residues that contact gp41 peptide in the crystal structures are labeled with red circles (open circles, main-chain contacts only; open circles with rays, side-chain contacts only; closed circles, both main-chain and side-chain contacts).
Table S1. Crystallographic data and refinement statistics

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<th>Crystallographic Data</th>
<th>ES2</th>
<th>ES2:2F5-Fab</th>
<th>11f10:gp41\textsubscript{660-667}</th>
<th>6a7:gp41\textsubscript{660-667}</th>
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<td>γ (°)</td>
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<td>90</td>
<td>90.3</td>
<td>90</td>
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<tr>
<td>Resolution (Å)*</td>
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<td>46.1 – 2.502 (2.50)</td>
<td>50.00 – 1.972 (2.04 – 1.97)</td>
<td>50.00 – 1.982 (2.05 – 1.98)</td>
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<td>23675 (862)</td>
<td>49925 (5030)</td>
<td>26922 (2394)</td>
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<tr>
<td>Completeness (%)*</td>
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<td>I/σ*</td>
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<td>R\text{sym} (%)†</td>
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Refinement statistics (F > 0)

Non-hydrogen atoms

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PDB ID

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<th>ES2</th>
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<td>9.0 (47.0)</td>
<td>3.8 (9.2)</td>
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*Data in parentheses are for highest resolution shell.

†R\text{sym} = \sum|I_{\text{obs}} - I_{\text{avg}}| / \sum I_{\text{avg}}

‡R = \sum(|F_{\text{obs}}| - |F_{\text{calc}}|)/\sum|F_{\text{obs}}|

Table S2. Structural comparison of free and 2F5-bound ES2 grafts to 2F5-bound gp41

Rmsd (Å) of gp41 Residues 660–667/659–667/659–669*

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*In each subection, rmsd of main-chain atom (N, Ca, C) superpositions shown in lower left, and rmsd of Ca atom superpositions shown in upper right. Row and column headings correspond to PDB IDs used in the respective superpositions. Alignments of gp41 residues 660–667 are shaded red, 659–667 blue, and 659–669 yellow.

†Rmsds calculated using the program lsqkab (25).

‡Mean difference of absolute values shown in lower left; Root-mean-square difference shown in upper right. Row and column headings correspond to PDB IDs used in the respective calculations. Residues for gp41 residues 660–667 and 659–667 are shaded red and blue, respectively.

§Difference-distance matrix means were determined using Differences Distance Matrix Plot (DDMP) software, as previously described [(26), Fleming, P. http://www.roslab.jhu.edu/ddmp/].
Table S3. Interactive surface areas on 2F5 and gp41 in the ES2, 11f10, and 6a7 crystal structures

<table>
<thead>
<tr>
<th>2F5 (residue)</th>
<th>gp41&lt;sub&gt;2PM&lt;/sub&gt;</th>
<th>gp41&lt;sub&gt;TJII&lt;/sub&gt;</th>
<th>ES2&lt;sub&gt;LEV&lt;/sub&gt;</th>
<th>2F5 (residue) cont'd</th>
<th>gp41&lt;sub&gt;2PM&lt;/sub&gt;</th>
<th>gp41&lt;sub&gt;TJII&lt;/sub&gt;</th>
<th>ES2&lt;sub&gt;LEV&lt;/sub&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ala&lt;sub&gt;1&lt;/sub&gt;</td>
<td>11</td>
<td>11</td>
<td>12</td>
<td>Asp&lt;sub&gt;154&lt;/sub&gt;</td>
<td>13</td>
<td>17</td>
<td>21</td>
</tr>
<tr>
<td>Leu&lt;sub&gt;2&lt;/sub&gt;</td>
<td>18</td>
<td>25</td>
<td>22</td>
<td>Asp&lt;sub&gt;156&lt;/sub&gt;</td>
<td>15</td>
<td>15</td>
<td>28</td>
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<tr>
<td>Glu&lt;sub&gt;3&lt;/sub&gt;</td>
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<td>4</td>
<td>0</td>
<td>Arg&lt;sub&gt;158&lt;/sub&gt;</td>
<td>19</td>
<td>20</td>
<td>33</td>
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<td>Ser&lt;sub&gt;26&lt;/sub&gt;</td>
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<td>5</td>
<td>0</td>
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<td>0</td>
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<td>4</td>
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<tr>
<td>Glu&lt;sub&gt;27&lt;/sub&gt;</td>
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<td>44</td>
<td>16</td>
<td>Pro&lt;sub&gt;161&lt;/sub&gt;</td>
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<td>0</td>
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<tr>
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<td>0</td>
<td>Arg&lt;sub&gt;165&lt;/sub&gt;</td>
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<td>1</td>
<td>Arg&lt;sub&gt;166&lt;/sub&gt;</td>
<td>5</td>
<td>7</td>
<td>5</td>
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<td>Leu&lt;sub&gt;91&lt;/sub&gt;</td>
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<td>11</td>
<td>11</td>
<td>Gly&lt;sub&gt;177&lt;/sub&gt;</td>
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<td>His&lt;sub&gt;92&lt;/sub&gt;</td>
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<td>41</td>
<td>39</td>
<td>Pro&lt;sub&gt;184&lt;/sub&gt;</td>
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<td>Phe&lt;sub&gt;93&lt;/sub&gt;</td>
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<td>50</td>
<td>53</td>
<td>Val&lt;sub&gt;190&lt;/sub&gt;</td>
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<td>74</td>
<td>84</td>
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<td>10</td>
<td>Ala&lt;sub&gt;190&lt;/sub&gt;</td>
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<td>67</td>
<td>67</td>
<td>67</td>
<td>Gly&lt;sub&gt;190&lt;/sub&gt;</td>
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<td>0</td>
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<tr>
<td>Gly&lt;sub&gt;98&lt;/sub&gt;</td>
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<td>11</td>
<td>9</td>
<td>Gly&lt;sub&gt;190&lt;/sub&gt;</td>
<td>22</td>
<td>26</td>
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<tr>
<td>Ser&lt;sub&gt;99&lt;/sub&gt;</td>
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<td>0</td>
<td>4</td>
<td>Asn&lt;sub&gt;190&lt;/sub&gt;</td>
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<td>4</td>
<td>2</td>
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<td>Subtotal</td>
<td>300</td>
<td>357</td>
<td>335</td>
<td></td>
<td>130</td>
<td>273</td>
<td>291</td>
</tr>
<tr>
<td>Total</td>
<td>430</td>
<td>630</td>
<td>626</td>
<td></td>
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<td></td>
</tr>
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</table>

Table S3. Interactive surface areas on 2F5 and gp41 in the ES2, 11f10, and 6a7 crystal structures

<table>
<thead>
<tr>
<th>gp41 graft Residue</th>
<th>11f10&lt;sub&gt;3LEXa&lt;/sub&gt;</th>
<th>11f10&lt;sub&gt;3LEXb&lt;/sub&gt;</th>
<th>6a7&lt;sub&gt;3LEX&lt;/sub&gt;</th>
<th>2F5&lt;sub&gt;TJII&lt;/sub&gt;</th>
<th>2F5&lt;sub&gt;3LEX&lt;/sub&gt;</th>
<th>2F5&lt;sub&gt;2PM&lt;/sub&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glu&lt;sub&gt;657/103&lt;/sub&gt;</td>
<td>20</td>
<td></td>
<td></td>
<td>13</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Glu&lt;sub&gt;658/104&lt;/sub&gt;</td>
<td></td>
<td></td>
<td></td>
<td>17</td>
<td>8</td>
<td>15</td>
</tr>
<tr>
<td>Glu&lt;sub&gt;659/105&lt;/sub&gt;</td>
<td>17</td>
<td></td>
<td></td>
<td>8</td>
<td>15</td>
<td></td>
</tr>
<tr>
<td>Leu&lt;sub&gt;660/106&lt;/sub&gt;</td>
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<td>1</td>
<td>14</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Leu&lt;sub&gt;661/107&lt;/sub&gt;</td>
<td>80</td>
<td>81</td>
<td>86</td>
<td>67</td>
<td>75</td>
<td>69</td>
</tr>
<tr>
<td>Glu&lt;sub&gt;662/108&lt;/sub&gt;</td>
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<td>48</td>
<td>45</td>
<td>57</td>
<td>57</td>
<td>60</td>
</tr>
<tr>
<td>Leu&lt;sub&gt;663/109&lt;/sub&gt;</td>
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<td>23</td>
<td>26</td>
<td>35</td>
<td>37</td>
<td>45</td>
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<tr>
<td>Asp&lt;sub&gt;664/110&lt;/sub&gt;</td>
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<td>54</td>
<td>54</td>
<td>65</td>
<td>59</td>
<td>66</td>
</tr>
<tr>
<td>Lys&lt;sub&gt;665/111&lt;/sub&gt;</td>
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<td>58</td>
<td>58</td>
<td>63</td>
<td>71</td>
<td>67</td>
</tr>
<tr>
<td>Trp&lt;sub&gt;666/112&lt;/sub&gt;</td>
<td>93</td>
<td>94</td>
<td>88</td>
<td>98</td>
<td>105</td>
<td>85</td>
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<tr>
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<td>29</td>
<td>29</td>
<td>32</td>
<td>29</td>
<td>28</td>
<td>12</td>
</tr>
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<td>Ser&lt;sub&gt;668/114&lt;/sub&gt;</td>
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<td>0</td>
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<td>0</td>
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<td>0</td>
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<tr>
<td>Leu&lt;sub&gt;669/115&lt;/sub&gt;</td>
<td>51</td>
<td>29</td>
<td>0</td>
<td>29</td>
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<td>0</td>
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<tr>
<td>Asp&lt;sub&gt;671/117&lt;/sub&gt;</td>
<td>37</td>
<td>37</td>
<td>17</td>
<td>7</td>
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<td>Total</td>
<td>385</td>
<td>387</td>
<td>402</td>
<td>560</td>
<td>496</td>
<td>419</td>
</tr>
<tr>
<td>Total (gp41&lt;sub&gt;660-667&lt;/sub&gt;)</td>
<td>385</td>
<td>387</td>
<td>402</td>
<td>415</td>
<td>431</td>
<td>404</td>
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<tr>
<td>Acidic (gp41&lt;sub&gt;660-667&lt;/sub&gt;)</td>
<td>103</td>
<td>101</td>
<td>98</td>
<td>123</td>
<td>116</td>
<td>126</td>
</tr>
<tr>
<td>Basic (gp41&lt;sub&gt;660-667&lt;/sub&gt;)</td>
<td>56</td>
<td>58</td>
<td>58</td>
<td>63</td>
<td>71</td>
<td>67</td>
</tr>
<tr>
<td>Hydrophobic (gp41&lt;sub&gt;660-667&lt;/sub&gt;)</td>
<td>226</td>
<td>228</td>
<td>246</td>
<td>229</td>
<td>245</td>
<td>212</td>
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</table>

* Molecular contact surface areas by residue represent sums of main-chain and side-chain contact surfaces, as determined by the program MS (27).
† The residue ranges of the gp41 peptides are: 1TJI (654-670-[N-Ac]), 2P8M (659-671), 3LEX (660-667-[N-Ac]), 3LEY (660-667-[N-Ac]).
Table S4. Hydrogen bonds and salt bridges between gp41 and antibodies 11f10, 6a7, and 2F5

<table>
<thead>
<tr>
<th>gp41/ES2 (Atom)</th>
<th>11f10/6a7 (Atom)</th>
<th>gp41: 11f10 (3LEXa)</th>
<th>gp41: 11f10 (3LEXb)</th>
<th>gp41: 6a7 (3LEY)</th>
<th>2F5 (Atom)</th>
<th>gp41: 2F5 (1TJI)</th>
<th>gp41: 2F5 (2P8M)</th>
<th>ES2: 2F5 (3LEV)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gln656 O</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Glu658 O</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Leu661 N</td>
<td>Tyr663 OH</td>
<td>3.4</td>
<td>3.4</td>
<td>3.3</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Leu661 O</td>
<td>Asn665 Nα2</td>
<td>3.1</td>
<td>3.0</td>
<td>3.3</td>
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<td></td>
</tr>
<tr>
<td>Glu658 O/Glu660 O</td>
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<td></td>
<td></td>
<td></td>
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</tr>
<tr>
<td>Glu658 O</td>
<td>Tyr659 OH</td>
<td>3.2</td>
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<td>Arg660 NH2</td>
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<td>2.5</td>
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<tr>
<td>Asp664 O</td>
<td>Leu666 N</td>
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<td>Tyr667 N</td>
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<tr>
<td>Asp664 O</td>
<td>Asp668 N</td>
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<tr>
<td>Lys665 N</td>
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<td>Asp668 O</td>
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<tr>
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<td>Gly671 O</td>
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<td>2.6</td>
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<tr>
<td>Trp666 N</td>
<td>Arg670 N</td>
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<td>2.9</td>
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</tr>
<tr>
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<td>6</td>
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*Hydrogen bonds and salt bridges and corresponding distances determined using HBPLUS (29).
†The residue ranges of the gp41 peptides are: 1TJI (654-670-[N-Ac]), 2P8M (659-671), 3LEX/3LEY (660-667-[N-Ac]).
### Table S5. Individual and grouped R-factors for alanine-interrogated epitope-scaffold-elicited responses

<table>
<thead>
<tr>
<th>Individual sera*</th>
<th>Mean R-value (SE)¹</th>
<th>p-value (Bootstrap)</th>
<th>p-value (Bonferroni) ‡</th>
<th>p-value (FDR, §)</th>
<th>Grouped sera</th>
<th>Mean R-value (SE)¹</th>
<th>p-value (Bootstrap)</th>
<th>p-value (Bonferroni) ‡</th>
<th>p-value (FDR, §)</th>
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</thead>
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<tr>
<td>2F5.MAb</td>
<td>0.12 (0.01)</td>
<td>0.00000</td>
<td>0.00000</td>
<td>0.00000</td>
<td>ES1</td>
<td>2.23 (0.99896)</td>
<td>1.00000</td>
<td>1.00000</td>
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<tr>
<td>ES2.TH.AS01B.Post.6</td>
<td>0.36 (0.04)</td>
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<td>0.00278</td>
<td>0.00070</td>
<td>ES2</td>
<td>1.21 (0.94379)</td>
<td>1.00000</td>
<td>1.00000</td>
<td>0.99994</td>
</tr>
<tr>
<td>ES2.TH.Alum.CpG.Post.6</td>
<td>0.44 (0.03)</td>
<td>0.00057</td>
<td>0.03294</td>
<td>0.00659</td>
<td>ES3</td>
<td>1.40 (0.97995)</td>
<td>1.00000</td>
<td>1.00000</td>
<td>0.99994</td>
</tr>
<tr>
<td>ES5.Alum.CpG.Post.2</td>
<td>0.49 (0.04)</td>
<td>0.00265</td>
<td>0.15358</td>
<td>0.02560</td>
<td>ES4</td>
<td>1.07 (0.86575)</td>
<td>1.00000</td>
<td>1.00000</td>
<td>0.99994</td>
</tr>
<tr>
<td>ES1.AS01B2.Post.6</td>
<td>0.51 (0.02)</td>
<td>0.00401</td>
<td>0.23246</td>
<td>0.03508</td>
<td>ES5</td>
<td>0.64 (0.05532)</td>
<td>1.00000</td>
<td>1.00000</td>
<td>0.20758</td>
</tr>
<tr>
<td>ES5.TH.AS01B.Post.4</td>
<td>0.51 (0.03)</td>
<td>0.00642</td>
<td>0.24638</td>
<td>0.03080</td>
<td>Flexible Peptide*</td>
<td>1.00 (0.78277)</td>
<td>1.00000</td>
<td>1.00000</td>
<td>0.99994</td>
</tr>
<tr>
<td>ES5.Alum.CpG.Post.6</td>
<td>0.53 (0.02)</td>
<td>0.00582</td>
<td>0.33733</td>
<td>0.03748</td>
<td>Post 2</td>
<td>1.71 (0.99511)</td>
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<tr>
<td>ES4.Alum.CpG.Post.4</td>
<td>0.57 (0.01)</td>
<td>0.01591</td>
<td>0.92290</td>
<td>0.08279</td>
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<td>1.00000</td>
<td>1.00000</td>
<td>0.99994</td>
</tr>
<tr>
<td>ES5.TH.AS01B.Post.2</td>
<td>0.57 (0.02)</td>
<td>0.01600</td>
<td>0.92800</td>
<td>0.08279</td>
<td>Post 6</td>
<td>0.93 (0.66051)</td>
<td>1.00000</td>
<td>1.00000</td>
<td>0.99994</td>
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<tr>
<td>ES3.AS01B.Post.4</td>
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<td>0.99342</td>
<td>0.08279</td>
<td>Flexible MPER A</td>
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<td>1.00000</td>
<td>0.28970</td>
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<td>ES5.Alum.CpG.Post.4</td>
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<td>0.19699</td>
<td>T-Helper (+PADRE)</td>
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<td>ES5.Alum.CpG.Post.2</td>
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<td>0.04938</td>
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<td>0.20459</td>
<td>T-Helper (–PADRE)</td>
<td>1.38 (0.97805)</td>
<td>1.00000</td>
<td>1.00000</td>
<td>0.99994</td>
</tr>
<tr>
<td>ES5.TH.AS01B.Post.2</td>
<td>0.65 (0.01)</td>
<td>0.06600</td>
<td>1.00000</td>
<td>0.20758</td>
<td>ES01B</td>
<td>1.45 (0.98441)</td>
<td>1.00000</td>
<td>1.00000</td>
<td>0.99994</td>
</tr>
<tr>
<td>Flexible Peptide*</td>
<td>0.71 (0.11)</td>
<td>0.14207</td>
<td>1.00000</td>
<td>0.39239</td>
<td>Flexible Peptide*</td>
<td>0.73 (0.16)</td>
<td>0.17907</td>
<td>1.00000</td>
<td>0.47210</td>
</tr>
<tr>
<td>ES5.Alum.CpG.Post.2</td>
<td>0.74 (0.02)</td>
<td>0.20521</td>
<td>1.00000</td>
<td>0.51748</td>
<td>Flexible Peptide*</td>
<td>0.79 (0.01)</td>
<td>0.30667</td>
<td>1.00000</td>
<td>0.71458</td>
</tr>
<tr>
<td>ES5.TH.Alum.CpG.Post.4</td>
<td>0.79 (0.01)</td>
<td>0.30801</td>
<td>1.00000</td>
<td>0.71458</td>
<td>Flexible Peptide*</td>
<td>0.79 (0.01)</td>
<td>0.30801</td>
<td>1.00000</td>
<td>0.71458</td>
</tr>
<tr>
<td>Flexible MPER B</td>
<td>0.87 (0.02)</td>
<td>0.51290</td>
<td>1.00000</td>
<td>0.99994</td>
<td>Flexible MPER B</td>
<td>0.88 (0.05)</td>
<td>0.54114</td>
<td>1.00000</td>
<td>0.99994</td>
</tr>
<tr>
<td>Flexible MPER Cyc1</td>
<td>Flexible MPER Cyc1</td>
<td>0.91 (0.03)</td>
<td>0.62683</td>
<td>1.00000</td>
<td>Flexible MPER Cyc1</td>
<td>0.93 (0.04)</td>
<td>0.67272</td>
<td>1.00000</td>
<td>0.99994</td>
</tr>
<tr>
<td>ES5.Alum.CpG.Post.6</td>
<td>Flexible MPER Cyc1</td>
<td>0.97 (0.09)</td>
<td>NA</td>
<td>0.74286</td>
<td>Flexible MPER Cyc1</td>
<td>1.02 (0.01)</td>
<td>0.81178</td>
<td>1.00000</td>
<td>0.99994</td>
</tr>
<tr>
<td>Flexible MPER A</td>
<td>Flexible MPER A</td>
<td>1.06 (0.03)</td>
<td>0.85527</td>
<td>1.00000</td>
<td>Flexible MPER A</td>
<td>1.13 (0.14)</td>
<td>0.90893</td>
<td>1.00000</td>
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</tr>
<tr>
<td>Flexible MPER Cyc2</td>
<td>Flexible MPER Cyc2</td>
<td>1.19 (0.04)</td>
<td>0.93626</td>
<td>1.00000</td>
<td>Flexible MPER Cyc2</td>
<td>1.32 (0.18)</td>
<td>0.97022</td>
<td>1.00000</td>
<td>0.99994</td>
</tr>
<tr>
<td>ES2.Alum.CpG.Post.6</td>
<td>Flexible MPER Cyc2</td>
<td>1.51 (0.10)</td>
<td>0.98843</td>
<td>1.00000</td>
<td>Flexible MPER Cyc2</td>
<td>1.75 (0.59)</td>
<td>0.99575</td>
<td>1.00000</td>
<td>0.99994</td>
</tr>
<tr>
<td>Flexible MPER A</td>
<td>Flexible MPER A</td>
<td>2.22 (0.24)</td>
<td>0.99891</td>
<td>1.00000</td>
<td>Flexible MPER A</td>
<td>2.56 (0.22)</td>
<td>0.99949</td>
<td>1.00000</td>
<td>0.99994</td>
</tr>
<tr>
<td>ES5.AS01B2.Post.4</td>
<td>Flexible MPER A</td>
<td>2.68 (0.68)</td>
<td>NA</td>
<td>0.99999</td>
<td>Flexible MPER A</td>
<td>2.68 (0.68)</td>
<td>NA</td>
<td>1.00000</td>
<td>0.99994</td>
</tr>
<tr>
<td>Flexible MPER Cyc1</td>
<td>Flexible MPER Cyc1</td>
<td>3.46 (1.39)</td>
<td>0.99988</td>
<td>1.00000</td>
<td>Flexible MPER Cyc1</td>
<td>4.52 (1.39)</td>
<td>0.99994</td>
<td>1.00000</td>
<td>0.99994</td>
</tr>
<tr>
<td>Flexible Peptide*</td>
<td>Flexible Peptide*</td>
<td>4.97 (2.06)</td>
<td>NA</td>
<td>0.99994</td>
<td>Flexible Peptide*</td>
<td>4.97 (2.06)</td>
<td>NA</td>
<td>1.00000</td>
<td>0.99994</td>
</tr>
</tbody>
</table>

*For Post 6 samples, scaffold for immunizations 5 and 6 is listed if heterologous (see Fig. 2A).

¹SE, standard error.

²Bootstrap p-values were adjusted due to multiple comparisons, as described in (30).

³p-values computed using a step-up False Discovery Rate procedure, as described in (31). Significant results have a false discovery rate of 3.75%.

⁴Flexible peptide group includes Flexible MPER A, B, Cyc1, and Cyc2 sera.

NA, not applicable.

Red, statistically significant p-values.

Ofek et al. www.pnas.org/cgi/doi/10.1073/pnas.1004728107