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Computational design of protein antigens that interact with the CDR H3 loop of HIV broadly neutralizing antibody 2F5

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Abstract

Rational design of proteins with novel binding specificities and increased affinity is one of the major goals of computational protein design. Epitope-scaffolds are a new class of antigens engineered by transplanting viral epitopes of pre-defined structure to protein scaffolds, or by building protein scaffolds around such epitopes. Epitope-scaffolds are of interest as vaccine components to attempt to elicit neutralizing antibodies targeting the specified epitope. In this study we developed a new computational protocol, MultiGraft Interface, that transplants epitopes but also designs additional scaffold features outside the epitope to enhance antibody-binding specificity and potentially influence the specificity of elicited antibodies. We employed MultiGraft Interface to engineer novel epitope-scaffolds that display the known epitope of HIV-1 neutralizing antibody 2F5 and that also interact with the functionally important CDR H3 antibody loop. MultiGraft Interface generated an epitope-scaffold that bound 2F5 with sub-nanomolar affinity ($K_D = 400$ pM) and that interacted with the antibody CDR H3 loop through computationally designed contacts. Substantial structural modifications were necessary to engineer this antigen, with the 2F5 epitope replacing a helix in the native scaffold and with 15% of the native scaffold sequence being modified in the design stage. This epitope-scaffold represents a successful example of rational protein backbone engineering and protein-protein interface design and could prove useful in the field of HIV vaccine design. MultiGraft Interface can be generally applied to engineer novel binding partners with altered specificity and optimized affinity.

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INTRODUCTION

The development of rational approaches to efficiently control protein-protein interactions is a major goal of protein engineering, with applications in the fields of protein therapeutics, biosensor engineering and vaccine development. Multiple platforms have been developed and successfully used to design novel or modify existing protein-protein interactions. Numerous studies describe experimental *in vitro* evolution approaches to engineer novel binding partners or to optimize existing interactions¹⁻³. More recently, computational methods have been successfully applied to design novel protein inhibitors⁴ and antigens^{5,6,7}.

MultiGraft^{6,8} is a computational protocol developed within the framework of the Rosetta molecular modeling platform^{9,10} that designs novel binding partners by transferring binding motifs from structurally characterized protein-protein interfaces to heterologous proteins. For a given binding motif, MultiGraft automatically identifies suitable “scaffold” proteins in the Protein Data Bank¹¹ “grafts” the motif onto the scaffolds and subsequently optimizes the interactions both between the epitope and the scaffold and between the scaffold and the desired binding partner. We previously used MultiGraft to design novel antigens, called epitope-scaffolds, by transferring the epitopes of broadly neutralizing antibodies (bnAbs) against Human Immunodeficiency Virus 1 (HIV-1)^{5,6,8,12} and Respiratory Syncytial Virus (RSV)¹³ to suitable scaffold proteins. Epitope-scaffolds are of interest as potential vaccine components to attempt to induce neutralizing antibodies specific for the specified epitope, and present potential advantages over traditional viral-derived immunogens, such as the presentation of the epitope in its antibody-bound state and in an environment devoid of any immune evasion mechanisms that are encoded in natural viral proteins. Recently, epitope-scaffolds displaying a neutralization epitope from RSV elicited neutralizing responses from macaques⁷ demonstrating that epitope-scaffolds can be viable immunogens and encouraging the development of similar antigens for other broadly neutralizing antibodies.

Epitope-scaffolds for three bnAbs against HIV-1 (4E10, b12 and 2F5) were previously described^{5,6,8,12}. Despite their high affinity for the respective antibodies, to date none of these epitope-scaffolds has elicited neutralizing responses against HIV-1 when tested as immunogens in animal studies. Multiple factors potentially contribute to their failure to induce detectable neutralizing activity¹⁴. Recently, auto-antigens have been identified for the 2F5 and 4E10 antibodies, indicating that literal “re-elicitation” of 2F5 or 4E10 might be blocked by tolerance mechanisms¹⁵. However, the odds of re-creating the recombination events and mutational pathways that led to 2F5 or 4E10 are extremely low, and in general we expect to induce a polyclonal response against either of these epitopes. Indeed, 2F5 epitope-scaffolds succeeded to induce mouse antibodies that are genetically unrelated to 2F5 but that bind to a nearly identical conformation of the 2F5 peptide epitope¹². Furthermore, a highly potent HIV bnAb called 10E8 has been shown to lack the autoreactive characteristics of 4E10 while binding to essentially the same epitope¹⁶. Thus we do not believe that

tolerance mechanisms are sufficient to explain the failure to induce neutralizing antibodies with epitope-scaffolds for the 2F5 or 4E10 epitopes. An alternate potential explanation is that these epitope-scaffolds do not fully recapitulate the viral epitopes required for neutralization. In that case, these epitope-scaffolds may be unable to stimulate and drive the maturation of B cell populations capable of secreting such broadly neutralizing antibodies.

Previously designed 2F5 epitope-scaffolds incorporated sub-ranges of the linear 2F5 epitope on the gp41 subunit of the HIV envelope protein^{8,12} and bound the antibody with nanomolar affinity. Recent studies however demonstrated that 2F5 also interacts with the virus outside this well-characterized region. These additional contacts are mediated by the long, hydrophobic CDR H3 loop of the antibody and may involve non-specific hydrophobic contacts with the viral membrane^{17,18} or interactions with other viral protein regions especially within gp41¹⁹. Interactions between the CDR H3 loop of 2F5 and HIV-1 are essential for viral neutralization as changes in either the length or the hydrophobic character of the loop significantly lower the neutralization potency of 2F5^{20,21}. As noted above, one structurally characterized antibody elicited by existing 2F5 epitope-scaffolds lacked a long CDR H3 loop and showed no neutralization ability, despite fully recapitulating the interactions between 2F5 and its gp41 peptide epitope¹². This further suggests that long CDR H3 loops are critical for viral neutralization via mechanisms similar to the one employed by 2F5. Given that none of the existing epitope-scaffolds interact with the CDR H3 loop of 2F5, here we developed novel 2F5 epitope-scaffolds that not only display the gp41 peptide epitope on their surface, but that also interact with this critical antibody loop. By presenting a *de novo* designed binding surface for the 2F5 CDR H3 loop, it was hypothesized that these new epitope-scaffolds would make additional contacts to 2F5 through its CDR H3. It was also hoped that, as immunogens, these new scaffolds might induce neutralizing antibodies that bind not only to the 2F5 peptide epitope but also utilize long CDR H3 loops to engage the CDR H3-complementarity patch.

To design 2F5 epitope-scaffolds that interact with the CDR H3 loop, we developed a novel algorithm, named MultiGraft Interface, that allows the targeted design of *de novo* interactions outside a grafted motif. For a given binding motif, MultiGraft Interface: i) identifies suitable protein scaffolds that can accommodate the motif and that can be engineered to make additional interactions with the binding partner; ii) structurally alters the identified scaffold to integrate the bound conformation of the motif and iii) builds additional interactions to a user-defined region of the binding partner outside the native interface (Fig. 1). This protocol led to the development of an epitope-scaffold that binds 2F5 with higher affinity ($K_D=400$ pM) than any gp41-derived peptides or previously described epitope-scaffolds. Mutagenesis analysis showed that this antigen interacts specifically with the antibody CDR H3 loop through computationally designed contacts. Aggressive structural remodeling was necessary to engineer this molecule: the 2F5 epitope replaced a native helix in the original scaffold structure and the identity of 15% of the native residues was modified. The successful design of this epitope-scaffold underscores the ability of MultiGraft Interface to rationally engineer protein backbone structure and to design *de novo* protein-protein interactions, two areas of great interest in the field of computational protein design. Outside antigen engineering, Multigraft Interface is generally capable of designing binding partners

with higher affinity or altered specificity and could be applied to develop novel protein inhibitors or biological probes.

MATERIALS AND METHODS

Scaffold identification

MultiGraft Interface was developed within the framework of Rosetta software⁹. The procedure to identify proteins that could both accommodate a given binding site and interact with a region of their binding partner outside the native interface consists of two steps: 1) the PDB is first queried to find scaffolds that can display the motif on their surface, as was previously described for the design of epitope-scaffolds by side-chain grafting⁵ or backbone grafting^{6,8}; 2) once a potential scaffold passes the imposed criteria, a newly implemented spatial filter is employed to filter candidates by the calculated the number of scaffold residues located within a user-defined distance to the targeted interaction region on the binding partner. To speed up the calculations, the identification stage was done using a version of the candidate scaffold where the identity of all non-glycine residues was changed to alanine. The binding motif (epitope) is positioned on the scaffold such that it replaces the corresponding native scaffold region as defined by the alignment at the matching stage. An all-alanine version of the binding partner (antibody) is represented at this stage and the potential complex between scaffold and partner is modeled. Rough energetic filters are used to assess the potential interaction between scaffold and binding partner *in-silico*. For a modeled binding complex at this stage, the spatial filter implemented in MultiGraft Interface counts the number of C β atoms on a potential scaffold located within a user-specified distance from the target interaction region on the binding partner. This number is subsequently used as an exclusion criterion for candidate scaffold selected for the design round.

Scaffold design

In the first stage of the MultiGraft Interface design process, the binding motif is transplanted to the candidate scaffolds as described previously^{5,6,8}. Following the successful transplantation of the binding motif, an additional sequence design stage was implemented that builds *de novo* contacts between the scaffold and the targeted interaction region on the binding partner. Scaffold residues located within a user-defined distance from the interaction region are designed by iterating three times through the following automatic protocol: i) the identity of the residues is allowed to change to any other amino acid except cysteine; ii) the side chains at the binding interface and the rigid body orientation of the binding partner are simultaneously minimized²². Once the scaffold sequence is optimized according to Rosetta energy calculations, the side chains of the residues in the interaction region and its immediate vicinity are repacked one more time before a final Rosetta-based ΔG is calculated and used to evaluate the designed protein complexes.

Screening for 2F5 binding using yeast surface display

The full-length genes encoding the libraries of designed proteins based on the different native scaffolds were assembled by PCR from 20–40 short overlapping oligos (IDT). Degenerate codons were used at different positions to cover the amino acids diversity in the

computational designs. PCR assembly of the full-length genes was done in a two-step process using Phusion DNA polymerase (Thermo Scientific). In the first step, the full-length product was generated for each construct by optimizing the following PCR parameters: the DMSO concentration (0%, 3%, 5%); the oligonucleotide concentration (50nM, 25nM, 12.5nM, 6.75 nM); the reaction buffer (GCTM or HFTM buffer provided by the manufacturer); or the annealing temperature (50°-60°). The resulting full-length product was amplified in a subsequent PCR reaction with regular extension primers. Twenty times molar excess of the gel purified assembled full-length DNA library was mixed with 1µg of digested pCTCON2 vector and used to transform *S. cerevisiae* cells by electroporation²³. Typical transformation efficiency ranged from 10⁶ to 10⁷ and ≈60% of the recovered sequences contained full-length in-frame gene variants. The resulting libraries were screened for cell-surface display and binding to 2F5 mAb using yeast surface display and fluorescence activated cell sorting (BD Influx, BD Biosciences) as previously described^{3,23}. Decreasing 2F5 IgG concentrations were used for labeling at different selection rounds to reduce library diversity and select high affinity binders. The DNA of individual clones was isolated after each selection round using the Zymoprep II kit (Zymo Research) and subsequently sequenced.

Recombinant protein expression and purification

Genes encoding the selected proteins were synthesized with a 6xHis-terminal tag in pet29b+ vector (GenScript) and were subsequently transformed into Arctic ExpressTM *E. coli* cells (Stratagene). Protein expression and purification by immobilized metal affinity chromatography and gel filtration was done as previously described⁸.

Biophysical characterization

2F5 binding was measured by Surface Plasmon Resonance on a Biacore 2000 (GE Healthcare) using the α-hIgG antibody capture kit (GE Healthcare) and following the manufacturer's instruction. 7000–9000 response units (RUs) of mouse anti-human IgG were immobilized on a CM5 chip via amine coupling. 200–500 RUs of 2F5 IgG were captured and after 1 minute of surface stabilization increasing concentrations of epitope-scaffolds were injected in duplicates at 50–100 µL/min for 1–5 minutes. Between protein runs, two injections of 10 µL 3M MgCl₂ at a flow rate of 10 µL/min were used to dissociate 2F5 IgG from the surface. Experiments were conducted in HBS-EP buffer (GE Healthcare, 0.01 M HEPES pH 7.4, 0.15 M NaCl, 3 mM EDTA and 0.005% (v/v) Surfactant P20). One flow cell with no 2F5 IgG captured was used as a reference cell, and buffer only injections were used for blank subtraction. Data analysis was done using Scrubber 2.0 (BioLogic Software) and for kinetic analysis biosensor data were globally fit to a mass transport limited 1:1 Langmuir binding model. Standard error values were reported for the association, dissociation and binding constants based on the curve fit. The standard error for all the SPR binding constants reported is ±1 of the last significant figure. The solution oligomeric state of the proteins was assessed by static light scattering (miniDAWN TREOS, Wyatt) coupled in-line to HPLC (Agilent, 1200 series) and the stability of the proteins was measured by circular dichroism as described before⁸.

Interface analysis

Interface analysis to determine the buried surface area and the binding interactions in the 1dwu_CM-2F5 complex was done using the web-accessible version of PISA²⁴ (http://www.ebi.ac.uk/msd-srv/prot_int/pistart.html).

Animal immunizations

Adult female New Zealand rabbits received four inoculations intramuscularly with 20 µg of protein formulated in adjuvax adjuvant at one month intervals. Pre-bleeds prior to the first inoculation and bleeds 7- 10 days after each inoculation were collected. Serum was incubated at 55C for 1 hour to heat-inactivate complement and stored at -80C until subjected to analysis.

ELISA

200 ng/well of antigen peptide were incubated overnight at 4C in Maxisorp high binding plate (Nunc) in PBS, pH 7.4. The next day plates were washed five times with PBS, 0.05% Tween 20 and blocked with 300 µL per well of PBS, pH 7.4, supplemented with 2% dry-mild powder and 5% heat inactivated Fetal Bovine Serum for 2 hours at RT and then washed five times. Serum was serially diluted five-fold (1:50 to 1:781,250) in PBS and incubated in antigen coated plates for 1 hour. Plates were washed five times and a goat secondary anti-rabbit immunoglobulin G (H+L) was incubated at a 1:10,000 dilution in PBS and incubated for 1 hour at RT. After washing again, 100 µL of colorimetric TMB (3,3', 5, 5'-tetramethylbenzidine) peroxidase enzyme immunoassay substrate was added to each well, and the reaction was stopped by adding 100 µL of 0.1N H₂SO₄ to each well. The optical density was read on a microplate reader at 450nm using Softmax software.

RESULTS

Computational design of 2F5 epitope-scaffolds that interact with the CDR H3 loop

MultiGraft Interface extends the two-stage protocol of the previously described^{5,6,8} MultiGraft algorithm: in the identification or "matching" stage, candidate scaffolds are selected from the Protein Data Bank based on their ability to accommodate both the 2F5 epitope and an additional potential interaction surface for the CDR H3 loop; in the design stage, the epitope is transplanted to the epitope-scaffold and additional interactions are engineered between the scaffold and the CDR H3 loop (Fig. 1).

2F5 binds to a linear epitope (⁶⁵⁷EQELLELDKWASLW⁶⁷⁰) on the HIV gp41 protein, and mutagenesis studies showed that the core ⁶⁶⁴DKW⁶⁶⁶ residues are essential for binding. Our previous work demonstrated that it is possible to obtain high-affinity 2F5 epitope-scaffolds by transplanting a 6–8 residue epitope sub-range centered around the ⁶⁶⁴DKW⁶⁶⁶ core⁸. Therefore, during the identification stage, epitope segments of lengths 4–10 were aligned for end-point compatibility against a subset of scaffolds in the Protein Data Bank as described before⁸. Once one end of the epitope was aligned on the backbone of the scaffold, several parameters were calculated to select potential scaffolds. An RMSD value was computed between the non-matching end of the epitope and the potential scaffold. Matches with a RMSD value greater than 3 Å were discarded. Two additional filters, to evaluate potential

clashes between the epitope backbone and the scaffold (intraclash<100 Rosetta energy units) or between the antibody and the scaffold region outside the epitope (interclash<100 Rosetta energy units), were used to further reduce the number of candidate scaffolds. To identify scaffolds that could provide additional contacts to the CDR H3 loop of the 2F5 mAb, a spatial filter was then used to select only those scaffolds that had between 2 and 10 C_β atoms within 5.5 Å of the 2F5 mAb CDR H3 loop as defined by the sequence ^{100A}LFGVPI^{100F} (PDBid: 1tji numbering) of the 2F5 heavy chain. This filter was important to identify regions of potential scaffolds where additional contacts to the CDR H3 could be engineered at subsequent stages of the protocol. From over 10,000 unique protein structures initially queried, 323 potential scaffolds based on 202 unique structures were selected for the design stage.

In the first part of the Multigraft Interface design procedure the epitope backbone was transplanted to the scaffold as previously described^{6,6,8}. Briefly, i) the epitope replaced the native scaffold backbone as determined in the identification or "matching" stage; ii) *de novo* backbone regions were built and scaffold polypeptide regions adjacent to the epitope were altered to connect the epitope to the rest of the scaffold; iii) sequence changes were introduced to accommodate the novel backbone conformation around the epitope region and to add interaction between the epitope and the scaffold; iv) the 2F5-scaffold complex was modeled and additional sequence changes were introduced in the scaffold to remove any potential clashes. In the second part of the design stage, MultiGraft Interface introduced an additional sequence optimization routine focused on engineering scaffold contacts to the CDR H3 loop of the antibody. The identities of scaffold residues with sidechain atoms located within 4 Å of the CDR H3 loop were computationally redesigned to identify potential CDR H3-scaffold contacts. Resulting epitope-scaffolds were evaluated based on a Rosetta-calculated binding energy between 2F5 and the epitope-scaffold, the length of the displayed epitope range, the dihedral angles of the remodeled scaffold backbone, the interactions of the epitope with the rest of the scaffold, and the interactions between the epitope-scaffold and the CDR H3 loop. Finally, after visual inspection of the selected models, one additional round of human-guided computational sequence design was performed to correct any obvious defects in the computational models (such as solvent exposed hydrophobic groups, buried hydrogen donor or acceptor groups, undesired non-epitope mediated contacts to the antibody) and to further optimize the interactions between the grafted epitope and the 2F5 CDR H3 loop with the scaffold.

Designed epitope-scaffolds based on four native scaffolds (PDBids: 1dwu²⁵ 1flz²⁶ 1fcu²⁷ 2gas²⁸) passed all the quality control filters and were selected for experimental characterization (Fig. S1). For each of the native proteins, the computational protocol generated 5–15 computational models that were similar based on computational parameters but differed from each other by a few mutations either in the epitope stabilizing or the CDR H3 interacting regions.

Identification of high affinity 2F5 epitope-scaffolds

Rather than characterizing each epitope-scaffold individually, small directed libraries were generated for the models based on the same parent protein and were screened for 2F5

binding on the surface of yeast²³. These libraries encompassed all the sequence diversity present at different positions in the computational models, combined in an all-against-all fashion. This strategy allowed simultaneous examination and optimization of 10^3 - 10^4 constructs based on each of the four parent proteins. The directed libraries attempted to optimize both the interaction of the epitope on the scaffold and of the engineered contacts to the CDR H3 loop. This concurrent optimization is important given that the position of the epitope relative to the scaffold affects the binding orientation of 2F5, which in turn affects the interaction of the CDR H3 loop with the scaffold. Furthermore, previous studies showed that the CDR H3 loop of 2F5 is flexible in solution²⁹ and the scaffolds themselves will sample scaffold-specific conformational variations in solution, therefore the CDR H3 conformation and interaction mode with the scaffolds might differ from the design models. Therefore, this experimental screening procedure attempted to account for the intrinsic flexibility of the CDR H3 loop and for imperfections present in the designed models.

Three of the four libraries tested based on the parent proteins with PDBids: 1f1z, 1fcu, 2gas did not contain any clones that showed epitope-scaffold display on the surface of yeast, which implies that the respective epitope-scaffolds were either not expressed or not stable when produced in yeast. Interestingly, two of the three parent molecules for these designs (PDBids: 1f1z, 2gas) were successfully produced in *E. coli* for crystal structure determination^{26,28}. In order to determine whether the lack of yeast surface display was due to any expression system artifacts or was caused by changes introduced in the parent proteins to engineer the desired epitope-scaffolds, four purely computationally-designed epitope-scaffolds based on the parent proteins PDBid:2gas and PDBid:1f1z were tested for expression and solubility in *E. coli*. None of the resulting proteins were expressed and it was thus concluded that the native proteins could not tolerate the sequence changes introduced during the computational design stage. The protein with the crystal structure described in PDB:1fcu was originally expressed in *Baculovirus*-infected insect cells, therefore the lack of yeast-surface display of epitope-scaffold based on this protein may have been due to an inadequate expression system.

The library of epitope-scaffolds based on PDBid:1dwu scaffold (Fig. 2A, B) contained clones that both displayed on the surface of yeast and bound 2F5 IgG with high affinity. After four rounds of affinity selection, four clones expressing different epitope-scaffolds dominated the library (Fig 2B). Sequence alignment of the four epitope-scaffolds isolated from the library showed good sequence convergence, with the same amino acid selected in every epitope-scaffold at 5 out of the 8 variable library positions.

The 1dwu variants (L1-L4) isolated from the library were expressed recombinantly in *E. coli*, purified by metal affinity and size exclusion chromatography, and biophysically characterized to assess protein stability, solution oligomeric state and 2F5 affinity. One additional epitope-scaffold, 1dwu_CM, with the sequence of the computational model predicted to have the highest 2F5 affinity (Table S1) was also experimentally tested, although its sequence was not isolated from the initial yeast display library. 1dwu_CM was included in the analysis to compare the epitope-scaffolds isolated from the directed library with the highest-scoring computational model.

All 1dwu epitope-scaffolds were expressed recombinantly and purified from *E. coli* successfully. The dissociation constants of the 2F5-epitope-scaffold interactions ranged from 0.4 nM to 3 nM as measured by Surface Plasmon Resonance (Fig. 2B). Remarkably, 1dwu_CM, the epitope-scaffold predicted to have the highest 2F5 affinity by the computational algorithm, showed the tightest 2F5 binding experimentally with a K_D of 400 pM (Fig. 2B, C). 1dwu_CM was determined to be thermodynamically stable ($T_M = 67^\circ \text{C}$) and monomeric in solution (>90% monomeric at 78 μM ; Fig. S2). The affinity of 1dwu_CM for 2F5 is 10-fold higher than that of the gp41-derived full-length 2F5 epitope peptide⁸ ($K_D = 4.1 \text{ nM}$; $k_{\text{on}} = 1.45 \times 10^6 \text{ M}^{-1}\text{s}^{-1}$; $k_{\text{off}} = 6.06 \times 10^{-3} \text{ s}^{-1}$) and higher than any previously designed 2F5 epitope-scaffolds. All 1dwu variants display slower k_{on} and k_{off} constants compared to the gp41 epitope-peptide. The improved binding of 1dwu_CM over the gp41 peptide is primarily due to a k_{off} constant that is almost two orders of magnitude slower.

We next wanted to investigate why the 1dwu_CM sequence was not selected from the directed library, given that its sequence was in theory part of the library and that the experimental library of 10^6 transformants oversampled the theoretical sequence space (576 sequences) by 2000-fold. To this end, we transformed a vector encoding 1dwu_CM into the same *S. cerevisiae* strain used for screening and tested it for surface display and 2F5 binding in the same experimental conditions used to screen the initial directed library. No surface display was observed for 1dwu_CM, thus explaining our inability to identify it from the directed library. Compared to the variants isolated from the library, 1dwu_CM has a phenylalanine as opposed to a valine at position 27 and a leucine as opposed to a valine at position 162 (Fig. 2A, B). Both of these residues are predicted to interact with the CDR H3 loop, with Phe27 modeled to be at the core of interface. We hypothesized that the increased hydrophobic character of the 1dwu_CM surface may affect its expression and display on the surface of yeast.

Significant structural and sequence changes were introduced in the native 1dwu protein to engineer the 1dwu_CM epitope-scaffold (Fig. 3). Based on the computational model, the epitope conformation transplanted on the scaffold is significantly different from the native 1dwu backbone replaced during the design process (RMSD=3.7 Å over the remodeled epitope region; Fig. 3A). Of the 213 total residues in the native 1dwu protein, 15 amino acids were mutated and the backbone conformation of a 17 residue stretch was modified, in the computational model, to accommodate the 2F5 epitope (Fig. 3B).

1dwu_CM interacts with the CDR H3 loop of 2F5

Based on the computational model of the 1dwu_CM-2F5 mAb complex, nine scaffold residues make hydrophobic interactions with the CDR H3 loop of the antibody (Phe22, Lys25, Phe27, Ile29, Ala160, Leu162, Ser201, Ala203, P207). Forty-five percent (234 Å^2) of the total surface area (517 Å^2) of these residues was predicted to become buried upon 2F5 binding. Residues Phe27 (78 Å^2 91% buried) and Ala203 (41 Å^2 82% buried) account for half of the predicted interface contacts with the CDR H3 loop. On the antibody side, 48% (283 Å^2) of the total CDR H3 loop surface is buried in the modeled complex and all residues outside Gly100^C are predicted to contribute significantly to the binding interface (Leu100^A:

94 Å² 99% buried; Phe100^B: 77 Å² 36% buried; Ile100^F: 49 Å² 46% buried; Val100^D: 46 Å² 52% buried; Pro100^E: 17.5 Å² 24% buried).

Next, we carried out mutagenesis studies to experimentally dissect the binding contributions of the epitope to the overall 2F5 affinity. Scaffold residue Lys95, corresponding to residue 665 of the core ⁶⁶⁴DKW⁶⁶⁶ gp41 native epitope, was mutated to alanine and the 2F5 binding to the resulting construct was measured. This mutation completely abrogated 2F5 binding when introduced into the HIV gp41 epitope peptide or in other 2F5 epitope-scaffolds that we previously engineered (Table S1 and not shown). 1dwu_CM_K95A showed 1000-fold decreased 2F5 binding ($K_D = 300$ nM), indicating that the epitope is the major interaction site of the antibody with 1dwu_CM (Fig. 4). However, the fact that 2F5 1dwu_CM_K95A still maintained considerable affinity for 2F5 suggested that additional interactions were made between the scaffold and the antibody outside the epitope region.

To test whether scaffold interactions to the CDR H3 loop contribute to the affinity, we measured the binding of 1dwu_CM and 1dwu_CM_K95A to two 2F5 variants previously described¹⁸ that had the length of the CDR H3 loop either extended by two residues (2F5_e2, ⁹⁸PTTGLFGVGP¹⁰⁰IAA¹⁰⁰G) or reduced by two residues (2F5_r2, ⁹⁸PT-LFGV-IAA¹⁰⁰G) relative to WT 2F5 (⁹⁸PTTLFGVPIAA¹⁰⁰G) (Fig. 4). As reported by Guenaga et al.³¹ these antibody variants had the same affinity as WT 2F5 for ES2, a previously described epitope-scaffold¹² that stabilizes the 2F5-bound conformation of the gp41 peptide epitope on its surface. However, 2F5_e2 and 2F5_r2 had more than 10-fold reduced affinity compared to WT 2F5 towards a gp41 peptide fragment that adopts a helical conformation in solution. The CDR H3 loop is not thought to interact directly with the epitope peptide, based on lack of contacts between the CDR H3 and the peptide in co-crystal structures, however the peptide binding differences in the mutated 2F5 constructs were attributed to a potential role for the CDR H3 to induce an extended-loop conformation of the gp41 epitope¹⁸. Given that 1dwu_CM was engineered to display the bound conformation of gp41 on its surface, minimal structural reorganization of the epitope region should be necessary upon binding to 2F5, 2F5_e2 or 2F5_r2. Therefore, we anticipated that the antibody interactions through the epitope would be similar for all three 2F5 variants and that any measured binding differences would be primarily due to different interaction modes of the CDR H3 loops with 1dwu_CM. 2F5_e2 and 2F5_r2 bound 1dwu_CM with 8-fold ($K_D = 6$ nM) and 16-fold ($K_D = 13$ nM) lower affinities, respectively, than wild-type 2F5, indicating that the binding interactions were sensitive to the length of the CDR H3 loop. The binding affinities of 1dwu_CM_K95A for 2F5_e2 and 2F5_r2 ($K_D > 40$ μM in both cases) were lower than the affinity of 1dwu_CM for 2F5 by a factor of $>10^5$ demonstrating that both the native 2F5 paratope and the CDR H3 loop represent the major sites of scaffold interactions with the antibody.

To better map the interactions of the CDR H3 loop with the epitope-scaffold, we mutated 1dwu_CM residues located in the proximity of the CDR H3 loop according to our model and assessed the 2F5 binding of the resulting constructs (Fig. 5). First, we measured the 2F5 binding of a construct in which the 1dwu_CM residues hypothesized to interact with the CDR H3 loop were reverted to their identity in the native 1dwu protein. Five residues were accordingly mutated in 1dwu_CM (K25S, F27D, A160H, S201K, A203T) while another

four residues (F22, I29, L162, P207) were kept unchanged since they had the same identity in 1dwu_CM and the native scaffold. The resulting construct, name 1dwu_CM_native_patch, bound 2F5 with a K_D of 2.3 nM, six times lower than the affinity of 2F5 for 1dwu_CM, showing that novel interactions were engineered between the epitope-scaffold and the CDR H3 loop.

Next, we measured the effect on binding affinity caused by alanine substitutions of scaffold residues predicted to interact with the CDR H3 loop. Phe27 is located at the core of the interaction, and mutating it to alanine or glutamate decreased the binding affinity of 1dwu_CM by 7-fold ($K_D = 2.8$ nM) and 9-fold respectively ($K_D = 3.7$ nM). Ser201 was shown to play a key role in the interaction with the CDR H3 loop. Mutation of Ser201 to alanine reduced the 2F5 affinity of 1dwu_CM by 25-fold ($K_D = 10$ nM). While Ser201 is predicted to be centrally located at the computationally modeled CDR H3-1dwu_CM interface, it is predicted to contribute only a small binding area ($12 \text{ \AA}^2$) and it is not predicted to form any polar interactions with the CDR H3 loop. Nevertheless, the significant effect of the S201A substitution on the 2F5 affinity indicates that Ser201 may make additional polar and/or van der Waals interactions with the CDR H3 loop that are not captured in the computational model. To further confirm the position of S201 relative to the CDR H3 loop, we mutated it to Leu and showed that the 2F5 affinity of the resulting construct was 300-fold ($K_D = 140$ nM) lower than that of 1dwu_CM. This loss of binding is probably due to a clash between the introduced leucine and the CDR H3 loop, although such a clash is not predicted in the computational model. Interestingly, mutating residue Tyr199 to Ala lowered the affinity of 1dwu_CM for 2F5 by 10-fold ($K_D = 4.7$ nM). In the computational model, Tyr199 is located at the periphery of the epitope-scaffold-CDR H3 loop interface, $8 \text{ \AA}$ from residue Phe107^B of the antibody. However, it may be possible for Tyr199 to contact the tip of the CDR H3 if the side chain of Tyr199 adopted a different conformation or if the CDR H3 loop were more extended than in the model.

Single alanine substitutions of residues Phe22, Ile29 and Leu162 result in 2–3-fold reduced 2F5 affinity, indicating that they interact with the CDR H3 loop (Fig. 5C). Replacing residues Lys25 and Pro206 with alanine does not lead to a significant change in 2F5 affinity. These residues are located within 3–5 $\text{\AA}$ of the CDR H3 loop in the computational model, and while they are partially buried in the complex, they not directly contact any CDR H3 loop residues. Similarly, mutating residue Met204 to alanine has no effect on the 2F5 affinity, consistent with the position of this residue outside the binding interface in the model.

We then wanted to assess whether the decrease in binding affinity of the alanine mutations was predicted computationally. Using Rosetta, binding interface residues were independently changed to alanine, and the total binding energy of the resulting 1dwu_CM-2F5 models was calculated. Changes in both CDR H3 and 1dwu_CM interface residues led to a decrease in computed binding energy (Table S1). However, the magnitude of the changes was small for each individual residue at the interface, and did not correlate with the significant binding contributions of residues Phe27 or Ser201 measured experimentally. It is important to note that this may be due to the lack of sensitivity in the Rosetta scoring function in our case, due to either the intrinsic characteristics of the

biological system used or to the computational protocol employed (see Supplementary Computational Methods). It may also be due to the fact that our computational modeling is not attempting to capture an explicit role for waters in the binding interaction. Epitope mutations known to reduce the affinity to 2F5 by a factor of 10000 or more (D664A, K665A, W666A; PDBId: 1tji numbering) result in binding energy decreases of 2.4 to 4.9 Rosetta energy units compared to the binding energy of the wild type epitope to the antibody. Therefore, smaller experimentally measured changes in affinity by factors of only 2 to 10, would not be detectable using our Rosetta protocol.

Interpretation of the experimental analysis of the affinity contributions of the individual scaffold residues was limited by the known flexibility of the CDR H3 loop that made computational prediction of its structure difficult. Furthermore, it is likely that the loop undergoes fine structural rearrangements in response to changes in the epitope-scaffold sequence, thus leading to slightly different binding modes between 1dwu_CM and the tested mutants. Nevertheless, our results indicate that 1dwu_CM makes specific and significant contacts to the CDR H3 loop of 2F5. Although not all the affinity contributions of the different epitope-scaffold residues can be precisely correlated with the proposed computational model of the 2F5-1dwu_CM complex, our model captures the most significant features of the interaction and can explain any imperfections based on structural rearrangement either in the CDR H3 loop or in the loop interacting region on the scaffold. To get a better understanding of the interactions between the epitope-scaffold and the antibody, we attempted to determine the crystal structure of 1dwu_CM in complex with 2F5 Fab. Unfortunately, all our attempts to obtain diffraction quality crystals were unsuccessful.

1dwu_CM elicits antibodies that cross-react with the gp41 peptide

A preliminary animal study was conducted to assess the immunogenic potential of 1dwu_CM. Two groups of four rabbits each were inoculated four times, at one month intervals, with either 1dwu_CM or ES5/1d3Bb, a previously described 2F5 epitope-scaffold that does not interact with the CDR H3 loop¹². In previous immunization experiments with more than ten different 2F5 epitope-scaffolds, ES5 elicited the strongest antibody responses that cross-reacted with the HIV gp41 peptide, although no viral neutralizing activity was detected (references ^{12,32} and not shown). Here, serum samples were collected 10 days after each inoculation and sera reactivity was measured against the epitope-scaffolds themselves to determine if the scaffolds were immunogenic. Both 1dwu_CM and ES5 generated robust autologous antibody titers (Fig. S3, top). Additionally, serum was tested for binding to two different HIV gp41 peptide variants to measure gp41 epitope specific responses: the full length membrane proximal external region (MPER) and a flexible MPER variant containing an artificial Gly₄SerGly₂ linker inserted immediately after the 2F5 epitope (Fig. S3). The antibody 2F5 binds equally to both peptides¹⁸ and thus 2F5-like antibodies should also display this behavior. 1dwu_CM immunized animals generated gp41 specific antibodies that recognized both peptides to the same degree as the antibody 2F5 would, while the sera from ES5 immunized animals did not. Unfortunately, no HIV neutralizing activity was observed from the 1dwu_CM or ES5 elicited sera (data not shown).

DISCUSSION

Although significant advances were recently reported^{4,33} accurate computational modeling of protein backbone structure and design of *de novo* protein-protein contacts remain great challenges of protein engineering. Here we developed MultiGraft Interface, a new computational protocol that expands MultiGraft, our previously described algorithm for binding motif transplantation. In the first stage of MultiGraft Interface, a particular conformation of a binding motif is transplanted and stabilized on a candidate scaffold identified from all the existing structures in the Protein Data Bank. Subsequently, *de novo* contacts are engineered between the scaffold and a user-defined area of the binding partner outside the grafted interface. MultiGraft Interface is general and can be applied outside antigen development, for example to design novel protein inhibitors or biosensors by engineering novel binding partners for a given target or by altering the specificity and affinity of existing binding interactions. As evidenced by the successful design of 1dwu_CM, MultiGraft Interface is capable of significant structural and sequence modifications. In 1dwu_CM, the 2F5 epitope loop replaced an α -helix in the native protein, resulting in one residue deletion and the structural modification of a 17-residue segment. The resulting RMSD between the design model and the native crystal structure in the epitope region was 3.7 Å, with an RMSD of 6 Å between the core ⁶⁶⁴DKW⁶⁶⁶ epitope segment and the corresponding native structure. Furthermore, the identity of 15% (32/231) of the native residues was altered during the design process.

Aggressive structural modifications of native proteins can adversely affect their thermodynamic stability and may explain why designs based on three out of four parent scaffolds could not be expressed in soluble form. Other computational protein design studies that involved protein backbone modeling or protein interface design similarly reported low rates of soluble proteins and usually required directed evolution to improve protein stability and binding affinity^{4,6}. For example, candidate scaffolds required extensive structural changes to accommodate the two-loop epitope of HIV bnAb b12, and as a result only one out of 62 experimentally characterized pure computational designs bound b12, and in that case the affinity was 10000-times worse than the native antibody-antigen interaction⁶. In contrast, over twenty 2F5 and 4E10 epitope-scaffolds engineered by side-chain grafting, which aims to change the sequence of the scaffold without altering backbone conformation, were produced as stable proteins and showed binding levels comparable to the native antibody epitope^{5,8}. These results underscore the difficulty of correctly predicting and designing protein backbone conformation as opposed to making more conservative changes to the sequence of a given protein that are not intended to alter backbone structure³⁴. In this context, and given the large structural modifications introduced in the native 1dwu scaffold, it is notable that the computationally generated sequence for 1dwu_CM was stable and interacted with 2F5 as predicted without any additional experimental optimization.

Many factors have to be considered for the accurate design of protein-protein interactions, such as the rigid body orientation of the binding partners, the buried area of the interaction, the chemical complementarity of the interface and the conformation of the interface residues in solution³⁵. To reduce this complexity, MultiGraft Interface implements an anchored interface design³⁶ approach to build binding interactions. Given a functional motif and a

suitable scaffold, our protocol first “anchors” the motif (i.e. the 2F5 epitope) on the scaffold and thus defines a rigid body orientation for the binding partners. Additional contacts can then be engineered to target a region (i.e. the antibody CDR H3 loop) outside the native interface (i.e. the antibody paratope). All the designed interactions between the epitope-scaffolds and the CDR H3 loop in this study were hydrophobic. While this is partly due to the nature of the CDR H3, other interface design studies that used Rosetta similarly produced largely non-polar protein-protein interfaces³⁷. In contrast, natural protein-protein interfaces rely both on hydrophobic interactions and on intricate hydrogen bonds and electrostatic interactions to modulate binding affinity and specificity. Further improvements in the computational engineering of polar contacts with Rosetta are necessary to facilitate the design of protein binders with fine specificity for a wide range of targets.

1dwu_CM bound 2F5 with 10-fold higher affinity than both the full-length epitope peptide on HIV gp41 and any previously designed epitope-scaffold. Unlike previous 2F5 epitope-scaffolds, 1dwu_CM made specific contacts to the functionally critical CDR H3 loop of the antibody through computationally designed contacts. As evidenced by the mutagenesis analysis, the CDR H3 loop interaction is sensitive to both the length of the loop and to the epitope-scaffold sequence. While crystallographic analysis of the 2F5-1dwu_CM complex would be definitive in dissecting the molecular details of the interaction, the generation of diffraction-quality crystals remains elusive. In the absence of a crystal structure, the mutagenesis data demonstrated that the computational model of the interaction is in qualitative agreement with the experimental observations. While some residues appear to interact with the CDR H3 loop as expected from the computational model (e.g. Phe27), it is likely that others interact with the loop differently than the model predicted (e.g. Ser201) or that there are residues at the periphery of the modeled interface that may contribute to binding (e.g. Tyr199). Some differences between the computational model and the actual interaction mode were expected given i) the intrinsic flexibility of the 2F5 CDR H3 loop that was not modeled during the design process; ii) the probable existence of modeling errors in the epitope orientation that may lead to slight changes in the antibody binding angle, which would subsequently alter the orientation of the CDR H3 loop relative to the scaffold; iii) the possible structural changes in the scaffold regions that interact with the CDR H3 loop given the large number of introduced mutations. Furthermore, the flexible CDR H3 loop may change conformation between binding to 1dwu_CM and to the tested mutagenized variants, thus limiting the interpretation of the mutagenesis studies.

The epitope-scaffolds in this study were engineered to stabilize the epitope conformation and interact with the CDR H3 loop structure described in the 2F5-gp41 complex with PDBid:1tji³⁸. More exhaustive crystallographic analysis showed that the CDR H3 loop is flexible and that the conformation observed in PDBid:1tji is influenced by crystal packing interactions²⁹. In the absence of such artefactual interactions no discernable density is observed for the CDR H3 loop, indicative of its conformational flexibility in solution. We used the conformation of the CDR H3 loop described in PDBid:1tji in our computational modeling, on the assumption that it represents a low-energy loop conformation that could be further stabilized energetically through the addition of interactions with the scaffold. Nevertheless, other yet-unidentified CDR H3 conformations may be more physiologically relevant in the context of HIV interaction and viral neutralization. Our modeling and binding

experiments were carried in the absence of a lipid membrane environment, which affects the conformation of the CDR H3 loop³⁹. Given that no atomic-level details exist of the interaction between the CDR H3 loop of 2F5 and HIV, the physiological relevance of the engineered 1dwu_CM-CDR H3 contacts may be limited.

In our preliminary animal study, 1dwu_CM elicited sera that cross-reacted with gp41 but did not neutralize HIV. While multiple factors likely contribute to the lack of neutralization, it is possible that 1dwu_CM elicits antibodies that cannot access the HIV gp41 due to steric clashes with the membrane lipid in the native gp41 environment, given that the modeling, experimental validation and immunizations were done without lipid present. Another possibility may be that the CDR H3 loops of the elicited antibodies, while binding strongly to 1dwu_CM, do not interact with the virus the same way that the CDR H3 loop of 2F5 does, an interaction that has not yet been elucidated in molecular detail. Additional immunogenicity studies will better characterize the specificity of the sera and in particular should attempt to evaluate whether 1dwu_CM can induce antibodies that both bind to the 2F5 peptide epitope and possess CDR H3 loops that contact the 2F5-CDR H3-complementarity patch engineered onto the scaffold. Future studies will assess the potential of 1dwu_CM to elicit 2F5-like antibodies either on its own or in combination with other epitope-scaffolds or MPER peptide derivatives. It may be important to test 1dwu_CM in non-human primates that produce antibodies with a CDR H3 length distribution similar to humans. Notably, for RSV, the epitope-scaffolds that elicited neutralizing antibodies did so only when tested in macaques, but not in mice⁷. 1dwu_CM may also prove useful as a probe to screen for novel 2F5-like antibodies with long hydrophobic CDR H3 loops from the sera of HIV infected patients. If such antibodies could be isolated, they may serve to improve our understanding of responses to the 2F5 epitope during natural infection, and they may serve as templates for further vaccine development.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

Stages of the MultiGraft Interface computational protocol developed to engineer 2F5 epitope-scaffolds that interact with the antibody CDR H3 loop. (epitope: yellow; 2F5 heavy chain: blue; 2F5 light chain: magenta; CDR H3 loop: light blue; scaffold: red; epitope-scaffold contacts to the CDR H3 loop: green).

Figure 2.

Characterization of 1dwu epitope-scaffold variants. (A) The location of variable positions (black spheres) in the 1dwu computation-guided directed library. (B) Sequence comparison and 2F5 binding kinetics of the 1dwu variants selected from the library (1dwu_L1-L4) and 1dwu_CM. The sequence of the native 1dwu protein (1dwu_native) is included for reference. (C) SPR characterization of 2F5 mAb-1dwu_CM interaction. 1dwu_CM was injected in duplicates over chip-captured 2F5 IgG at the following concentrations: 944nM, 189nM, 37.8nM, 7.6nM, and 1.5nM.

Figure 3.

(A) Structural and (B) sequence alignment between 1dwu_CM (red) and the native scaffold it was engineered from, 1dwu_WT. The epitope region is shown in yellow, the corresponding native structure in 1dwu_WT is shown in green and sequence differences outside the epitope region are shown in blue.

Figure 4.

SPR analysis of the 1dwu_CM and 1dwu_CM_K95A interactions with 2F5 mAb and with two 2F5 variants with the CDR H3 loop either extended by two residues (2F5_e2) or shortened by two residues (2F5_r2). In separate experiments for the two epitope-scaffolds, equal amounts of antibodies were captured on different flow cells of the same chip. 1dwu_CM was injected at 500nM, 167.8nM, 18.9nM, 6.3nM and 2.1nM. 1dwu_CM_K95A was injected at 10μM, 2μM, 400nM, 80nM, 16nM, 3.2nM and 0.64nM. The different ranges in the overall response units (y-axis) are due to the different amounts of the epitope-scaffolds bound to the chip surface according to the binding affinities of the constructs.

Figure 5.

1dwu_CM interacts with the CDR H3 loop of 2F5. (A) Computational model of the interaction between the CDR H3 loop (cyan) with epitope-scaffold residues (yellow); (B) 2F5 dissociation constants of 1dwu_CM variants with residues predicted to be at the CDR H3 binding interface mutagenized; (C) Qualitative 2F5 binding analysis of 1dwu_CM alanine mutants of residues located at the CDR H3 interface. 2F5 IgG was captured on the chip and the respective constructs were subsequently injected at a concentration of 500nM; (D) SPR analysis of 2F5 binding interaction with 1dwu_CM_F27A (concentrations: 1 μ M, 339nM, 113nM, 37.7nM, 12.56nM, 4.2nM, 1.4nM), 1dwu_CM_S201A (500nM, 168nM, 56.3nM, 18.9nM, 6.3nM, 2.1nM, 0.7nM) and 1dwu_CM_Y199A (500nM, 168nM, 56.3nM, 18.9nM, 6.34nM, 2.1nM, 0.7nM).