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Design and Characterization of Epitope-Scaffold Immunogens that Present the Motavizumab Epitope from Respiratory Syncytial Virus

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Abstract

Respiratory syncytial virus (RSV) is a major cause of respiratory tract infections in infants, but an effective vaccine has not yet been developed. An ideal vaccine would elicit protective antibodies while avoiding virus-specific T-cell responses, which have been implicated in vaccine-enhanced disease with previous RSV vaccines. We propose that heterologous proteins designed to present RSV neutralizing-antibody epitopes and to elicit cognate antibodies have the potential to fulfill these vaccine requirements, as they can be fashioned to be free of viral T-cell epitopes. Here we present the design and characterization of three epitope-scaffolds that present the epitope of motavizumab, a potent neutralizing antibody that binds to a helix-loop-helix motif in the RSV fusion (F) glycoprotein. Two of the epitope-scaffolds could be purified, and one based on a *S. aureus* Protein A domain bound motavizumab with kinetic and thermodynamic properties consistent with the free epitope-scaffold being stabilized in a conformation that closely resembled the motavizumab-bound state. This epitope-scaffold was well-folded as assessed by circular dichroism and isothermal titration calorimetry, and its crystal structure – determined in complex with motavizumab to 1.9 Å resolution – was similar to the computationally-designed model, with all hydrogen-bond interactions critical for binding to motavizumab preserved. Immunization of mice with this epitope-scaffold failed to elicit neutralizing antibodies, but did elicit sera with F-binding activity. The elicitation of F-binding antibodies suggests that some of the design criteria to elicit protective antibodies without virus-specific T-cell responses are being met, but additional optimization of these novel immunogens is required.

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Author contributions

JSM expressed and purified epitope-scaffolds, performed surface-plasmon resonance and isothermal titration calorimetry experiments, and crystallized and solved the structure of MES1 bound to motavizumab. BEC computationally designed the epitope-scaffolds and performed circular dichroism experiments. MC performed mouse immunizations and analyzed sera by ELISA and RSV neutralization assays. YY expressed and purified epitope-scaffolds. All authors designed experiments and analyzed data. JSM wrote the initial draft of the paper, on which all authors commented.

Accession numbers

Coordinates and structure factors have been deposited in the Protein Data Bank with accession number 3QWO

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Keywords

Palivizumab; Synagis; Structure-based vaccine design; X-ray structure

Introduction

Respiratory syncytial virus is a major cause of pneumonia and bronchiolitis in infants, resulting in more than 2 million children under the age of five who require medical attention each year in the United States¹. Worldwide, RSV is estimated to cause more than 30 million lower respiratory tract infections and cause more than 60,000 deaths annually². An effective vaccine has eluded researchers since the 1960 s, when a candidate vaccine composed of formalin-inactivated virus increased disease severity in infants upon natural infection with RSV³. Animal models suggest vaccine-enhanced disease and pathology was associated with an imbalanced T_{H2} response^{4,5} and elicitation of low-affinity antibodies⁶. Passive immunization studies and the subsequent clinical use of the monoclonal antibody palivizumab (Synagis®), have demonstrated that neutralizing antibodies against a single antigenic site on the F glycoprotein can prevent severe disease caused by RSV⁷. Thus, an effective RSV vaccine should elicit potent neutralizing antibodies while avoiding an imbalanced T-cell response. Current vaccines have been designed to promote CD8+ and T_{H1} T-cell responses in addition to neutralizing antibody responses. However, a vaccine that could mimic passive antibody and induce neutralizing antibodies without RSV-specific T-cell responses would be an attractive option, particularly for the neonate.

We hypothesized that immunogens designed to elicit antibodies that target specific neutralizing epitopes would fulfill these vaccine requirements. RSV-neutralizing antibodies target two proteins, the G (attachment) and F (fusion) glycoproteins⁸. Palivizumab targets a major antigenic site on the F glycoprotein⁹, referred to as antigenic site II or site A, which encompasses residues 255–275^{9,10}. Peptides corresponding to this region bind to palivizumab-like antibodies but fail to elicit neutralizing antibodies when injected in mice¹¹, suggesting that the free peptide fails to mimic the conformation of the epitope. In aqueous solution, the free peptide adopts a random coil conformation, but transitions to a helix-loop-helix conformation in the presence of 30% trifluoroethanol¹². It is this helical conformation that is recognized by neutralizing antibodies, as evidenced in the crystal structure of a potent palivizumab derivative, motavizumab, in complex with the peptide¹³.

The elicitation of structure-specific antibodies has recently been achieved by stabilizing the neutralizing antibody-bound conformation of an epitope by using heterologous proteins as scaffolds to support the three-dimensional epitope structure^{14,15}. These “epitope-scaffolds” mimicked the HIV-1 gp41 epitopes of the broadly neutralizing antibodies 2F5 and 4E10, and when used as immunogens elicited polyclonal serum responses that recognized the structure of the epitope in a manner similar to 2F5 and 4E10. Here we apply and extend this methodology to the motavizumab epitope, which can be considered a complex epitope consisting of residues from two separate alpha-helices. A new computational method was derived for identifying scaffold proteins capable of supporting such a discontinuous epitope structure. Three motavizumab epitope-scaffolds were designed, and their biochemical, biophysical, and immunological properties were characterized. The results have implications for the structure of the motavizumab epitope on the F glycoprotein, RSV-vaccine development, and antibody-mediated RSV neutralization.

Results

Computational motavizumab-epitope transplantation

Analysis of the previously determined crystal structure of motavizumab bound to its RSV F peptide epitope¹³ identified 13 discontinuous RSV F residues that were contacted by motavizumab (Fig. 1a). We hypothesized that these 13 residues would be sufficient for eliciting motavizumab-like antibodies provided that their three-dimensional arrangement was preserved. Since all 13 residues were located on two alpha-helices (and none on the connecting loop), the motavizumab epitope could be considered a two-segment epitope. Thus, potential scaffolds did not need to have two helices near each other in the amino acid sequence or in the same order as that found in the F protein. This necessitated an extension of the original side-chain grafting protocol, which was designed for transplantation of single-segment epitopes, such as a loop or alpha helix^{14,15}. In the new protocol, scaffolds were scanned for structural similarity to each of the two epitope segments individually, and whenever a match was found to one of the segments, that scaffold was searched a second time for structural similarity to the other epitope segment while maintaining the rigid-body orientations determined by the first superposition. After an automated search of protein structures in the Protein Data Bank (PDB)¹⁶, three proteins were selected to serve as scaffolds for the 13 residues shown to interact directly with motavizumab¹³. These were Protein Z, which is a domain of Protein A from *S. aureus* (PDB ID: 1LP1, chain B), Cag-Z from *H. pylori* (PDB ID: 1S2X), and the p26 capsid protein from equine infectious anemia virus (PDB ID: 2EIA). These proteins were then taken to the semi-automated design stage, wherein amino acids outside of the motavizumab epitope were modified or removed to optimize epitope-scaffold properties, such as stability, solubility, and binding energetics. This optimization created several variants for each of the three scaffolds, and the variant of each scaffold with the highest motavizumab affinity is shown in Figure 1b, and referred to as MES1, MES2, or MES3. A list of all epitope-scaffolds and derivatives tested is presented in Table 1.

Characterization of motavizumab epitope-scaffolds

The three epitope-scaffolds were initially expressed in HEK293 cells as secreted proteins. Though MES1 expressed well and was purified to homogeneity, MES2 and MES3 expression was poor in mammalian cells. Expression of MES2 and MES3 was then tested in *E. coli*, and MES2 was expressed and purified to homogeneity. MES3 was never sufficiently expressed.

To determine whether MES1 and MES2 were able to bind motavizumab, surface-plasmon resonance (SPR) experiments were performed by flowing the epitope-scaffolds over motavizumab Fab that had been coupled to the sensor chip. Size-exclusion chromatography indicated that MES1 and MES2 were monomeric in solution so the chosen SPR format should not be complicated by avidity effects. MES1 bound motavizumab with a $K_d = 87$ nM, which was ~27-fold tighter than MES2 ($K_d = 2370$ nM). The binding of both epitope-scaffolds to motavizumab displayed fast on/off kinetics (Fig 2a,b). These results were similar to those obtained for the RSV F peptide, which bound motavizumab with a $K_d = 241$ nM and displayed fast binding kinetics (Fig. 2c). The 3-fold tighter motavizumab-binding of MES1 in comparison to the peptide is due to a 3-fold increase in the on-rate, suggesting that free MES1 more closely resembles the bound state than does the free peptide. This is the expected result since the epitope-scaffolds were designed to mimic the motavizumab-bound state. Though motavizumab bound to MES1 tighter than it bound to the peptide, the affinity was several orders of magnitude weaker than that observed for motavizumab binding to the F glycoprotein ($K_d = 0.035$ nM)¹⁷.

In addition to the motavizumab-binding kinetics, we also determined the motavizumab-binding thermodynamics for the two epitope-scaffolds and the peptide using isothermal titration calorimetry (ITC). The binding of MES1 to motavizumab was entropically driven, with little change in enthalpy (Fig. 2d). In contrast, the binding of MES2 to motavizumab was enthalpically driven and entropically unfavorable, which was similar to the thermodynamics of peptide binding to motavizumab (Fig. 2e,f). The K_d determined by ITC was similar to that determined by SPR for MES1 and peptide, but was ~10-fold tighter for MES2. This was likely due in part to the poorer fit of the SPR data for MES2 (Fig. 2b), though a similar K_d was obtained by plotting the response at equilibrium against the protein concentration (data not shown). The favorable change in entropy upon MES1 binding to motavizumab is consistent with free MES1 having a conformation that closely resembles the motavizumab-bound state. This would produce only a small decrease in conformational entropy, with solvation entropy being the driving force. If one were to assume that the solvation entropies are similar for the binding of the epitope-scaffolds and peptide to motavizumab (since the interfaces were designed to be the same), then the unfavorable binding entropy of MES2 and peptide to motavizumab would be due to a large, unfavorable reduction in conformational entropy. This would suggest that the portion of MES2 containing the transplanted epitope is flexible, and not as fixed as in MES1.

To determine if MES1 and MES2 were properly folded, circular dichroism (CD) spectroscopy was performed. The CD spectrum of each epitope-scaffold showed minima around 208 nm and 221 nm, consistent with alpha-helical proteins (Fig. 3a,c). To determine the melting temperature (T_m) of the epitope-scaffolds and assess their stability, the mean residue ellipticity at 210 nm was measured as the temperature was increased from 5°C to 99°C. MES1 had a $T_m = 57.3^\circ\text{C}$ and showed a sigmoidal melting profile (Fig. 3b), consistent with the cooperative unfolding of a single-domain protein that exists in two states (folded and unfolded). In contrast, MES2 showed a more linear melting profile that could not be fit to a two-state, cooperative unfolding model (Fig. 3d). Collectively, the SPR, ITC, and CD data indicated that MES1 was a rigid, well-folded protein that mimicked the bound conformation of the motavizumab epitope. Therefore, MES1 was chosen for crystallographic studies.

Crystal structure of MES1 in complex with motavizumab

Crystals of MES1 bound by the motavizumab Fab were obtained in space group $P2_1$ and diffracted X-rays to 1.9 Å. A molecular replacement solution was obtained containing two complexes per asymmetric unit, and the structure was refined to an $R_{\text{crys}}/R_{\text{free}} = 18.8\%/23.1\%$ (Fig. 4a). Data collection and refinement statistics are presented in Table 2. The interface between MES1 and motavizumab buries 1,405 Å², and has a high degree of shape complementarity¹⁸, as indicated by its shape correlation (S_c) value of 0.75. This is similar to the interface between the peptide and motavizumab¹³, which buries 1,304 Å² and has an S_c value of 0.76. Thus, based on these statistics, motavizumab binds MES1 and peptide in a similar manner.

Superposition of MES1 coordinates from the crystal structure with RSV peptide coordinates from the motavizumab-peptide structure (PDB ID: 3IXT) shows that MES1 precisely mimicked the desired epitope backbone conformation with a root-mean-square deviation (rmsd) of 0.30 Å for 13 Cα atoms (Fig. 4b). Superposition of MES1 coordinates from the crystal structure and the computational design model shows overall structural similarity, with an rmsd of 1.16 Å for 53 Cα residues (Fig. 4c), but reveals significant differences in the orientation of the N-terminal helix (Fig. 4d). These results indicate that the actual structure of the epitope-scaffold when bound by motavizumab agrees well with the desired structure of the epitope, and also with the predicted structure of the epitope-scaffold, except at the N-terminus.

There are, however, differences in the rigid-body orientation of these ligands relative to motavizumab. When the orientation of the predicted and actual epitope-scaffold structures is compared after superposition of the Fab variable domains, the rmsd jumps to 3.16 Å for 53 Cα residues (Fig. 5a). Further, when comparing the crystal structures of MES1 and peptide bound by motavizumab by superimposing the Fab variable domains (Fig. 5b), the Cα and all-atom rmsds of the 13 transplanted amino acids are 1.91 Å and 2.14 Å, respectively. The difference in rigid-body orientation between MES1 and peptide relative to Fab can be approximated as a 10° rotation of the epitope-scaffold about an axis that is perpendicular to the interface plane and passing through the Cβ atom of Asp269. This rotation of MES1 relative to peptide leads to changes in the atomic interactions with motavizumab but only for residues with significant displacement because they are located furthest from the center of rotation. A detailed comparison of peptide-motavizumab and MES1-motavizumab atomic interactions is given in Table 3. Despite the rotation of MES1 compared to peptide, the locations of three peptide residues (Asn262, Asn268, Lys272) that make important hydrogen bond and salt bridge interactions with motavizumab are conserved, having an all-atom rmsd of 0.34 Å. These three amino acids are critical for antibody-mediated neutralization of RSV, and antibody-escape mutations have been mapped to each residue^{11,19}. Thus, motavizumab interactions that require precise structural mimicry were preserved at the expense of less specific interactions. One final point on comparing peptide and MES1 contacts to motavizumab – three residues on MES1 outside the epitope (absent on the peptide) make contacts to motavizumab but their contribution to the binding affinity is likely modest because they bury only a small amount of additional surface area.

Immunogenicity of epitope-scaffolds

Mice were immunized with MES1 to determine if it was able to elicit motavizumab-like antibodies. We were concerned that the small size of MES1 (55 amino acids) would be insufficient for eliciting a strong humoral immune response due to a lack of T-helper epitopes. Thus we added to the C-terminus of MES1 a pan HLA DR-binding epitope (PADRE) sequence that has been shown to elicit a strong B-cell response in C57BL/6 mice²⁰. MES1 (10 µg) with or without the PADRE sequence were combined with 25 µg of CpG adjuvant and injected into C57BL/6 mice at 0, 3 and 7 weeks, and sera were withdrawn at weeks 5 and 9. The sera were initially tested by ELISA for binding to MES1 to determine if the epitope-scaffolds were able to elicit an immunogen-specific antibody response. Sera from mice immunized with MES1 lacking the PADRE sequences had barely detectable levels of MES1-binding activity (Fig. 6a). In contrast, sera from mice immunized with MES1 fused to PADRE showed substantial MES1-binding activity after three doses (Fig. 6a). To approximate the fraction of MES1-binding antibodies that recognized the transplanted motavizumab epitope, the sera were tested for binding activity to a MES1 mutant that had glutamate substituted for the lysine residue homologous to Lys272 in the RSV F protein. This substitution in the context of the F glycoprotein eliminates motavizumab binding and prevents motavizumab-mediated neutralization²¹. After three doses of MES1, there was a 22% decrease in binding to the mutant compared to the wild-type epitope-scaffold, suggesting some of the elicited antibodies targeted the transplanted epitope (Fig 6a). Thus, MES1 is capable of eliciting antibodies that target the transplanted epitope, but only when a PADRE sequence is present. We therefore used the PADRE sequence for all subsequent immunizations.

Sera from mice immunized with MES1 were also tested for RSV neutralization, but no neutralizing activity was observed. Though we estimate that ~20% of the antibodies recognized the transplanted epitope, it is likely that only a small percentage of these antibodies bound the epitope in an orientation that was compatible with binding to the trimeric F glycoprotein. To increase this response, a set of resurfaced variants of MES1 were

designed that had increasing numbers of mutations on the non-epitope surface. The goal was to use one of these proteins in prime-boost combination with wild-type MES1 to focus the immune response on the transplanted epitope, which would be the only conserved surface between both immunogens²². The resurfaced MES1 variant “Surf1” had 6 amino acids altered on the non-epitope surface (Fig. 7a,b), expressed well, and had motavizumab-binding characteristics similar to wild-type MES1 (Fig. 7c).

Surf1, along with wild-type MES1 and MES2, were used to immunize C57BL/6 mice in various combinations in an attempt to elicit sera that neutralized RSV. All vaccine regimens elicited sera having MES1-binding activity, with four doses of MES1 producing the highest titers, and two doses of MES1 plus two doses of Surf1 producing the second highest (Fig. 6b). The use of Surf1, however, produced only a modest increase in the fraction of MES1-binding antibodies that target the transplanted epitope, based upon binding to the K272E mutant scaffold (Fig. 6b). While none of the sera displayed RSV-neutralizing activity, MES1-vaccine regimens did elicit sera having RSV F-binding activity, with four doses of MES1 producing the highest titers and immunizations with MES2 producing the lowest titers (Fig. 6c). To determine whether the F-binding activity was specific for the motavizumab epitope, the ELISAs were repeated after pre-incubating the coated antigens with motavizumab IgG. Approximately 24% of the F-binding activity and 17% of the MES1-binding activity was blocked by motavizumab (Fig. 6d). Both values are statistically significant when compared to the 2% decrease in MES1 K272E-binding activity, which served as a negative control. Thus, the low titers of epitope-specific antibodies likely explain the lack of neutralization, though we cannot exclude other factors, such as the precision of epitope recognition.

Discussion

The difficulty in creating an RSV vaccine was increased by the demonstration of vaccine-enhanced illness in infants immunized with formalin-inactivated RSV³, which was due in part to elicitation of a deleterious T-cell response⁵ and low affinity antibodies⁶. Newer vaccine candidates using either full-length sequences or peptides as antigens and presented as a purified polypeptide, a gene-based vector, virus-like particle, or attenuated virus, will elicit both neutralizing antibodies and RSV-specific T-cell responses. Our approach uses atomic-level information obtained from protein crystal structures to design immunogens that replicate neutralizing epitope structures on RSV surface proteins. Uniquely, these epitope-scaffolds do not include stretches of more than three consecutive amino acids from the native viral protein, and thus contain no RSV-specific T-cell epitopes.

Peptides corresponding to the palivizumab epitope on the F glycoprotein fail to elicit neutralizing antibodies when used as immunogens¹¹, likely because the conformation of the peptides in solution differs from that in the context of the full-length F protein. The MES1 epitope-scaffold improves upon peptide immunogens because it maintains the epitope in a conformation approximating its antibody-bound state, as indicated by the SPR (Fig. 2), ITC (Fig. 2) and crystallographic data (Fig. 4). Despite this conformational stabilization, MES1 and its derivatives failed to elicit detectable RSV-neutralizing activity in sera. Although the affinity of MES1 for motavizumab is 3-fold tighter than the interaction between the peptide and motavizumab (87 vs 241 nM, Fig. 2), it is still 4 orders of magnitude weaker than the affinity of motavizumab for the F glycoprotein (0.035 nM)¹⁷. One possibility for this large difference in affinity is that the motavizumab epitope may be more complex than originally described and includes residues located outside of the helix-loop-helix. Another possibility is that MES1 does not sufficiently mimic the motavizumab epitope, despite its high structural similarity to the motavizumab-bound epitope peptide¹³. Additional structural information of the RSV F glycoprotein and motavizumab-like antibodies will greatly aid

these efforts, and may lead to elicitation of protective antibodies and a novel vaccine modality.

Materials and Methods

Multi-segment side-chain grafting

We previously devised a computational method for the transplantation of continuous epitopes, called side-chain grafting^{14,15}. To allow identification of side-chain grafting scaffolds for the discontinuous motavizumab epitope, we extended the Rosetta-implemented matching stage¹⁴ to allow searches for backbone superposition over multiple discontinuous segments. This method, called “Multi-segment side-chain grafting”, conducts the matching stage in a similar manner as the original (single-segment) side-chain grafting – by evaluating backbone RMSDs of the epitope to matched width segments of the scaffold and evaluating steric clashes between antibody and scaffold. However, to identify matches for an epitope with N segments, separate searches are conducted using each of the segments as the “primary”, and for each primary backbone superposition match to one epitope segment, the candidate scaffold is scanned again for superposition matches to the remaining epitope segments with the rigid-body orientation of the remaining segments held fixed relative to the primary matching segment. Further, two candidate rigid-body orientations of the epitope relative to the scaffold are passed on to the design stage – one assigned by the initial single-segment match and another assigned by a subsequent backbone superposition over all the segments – the determination of which orientation is superior is made during design. The design stage is carried out as previously described for (single-segment) side-chain grafting^{14,15}.

Multi-segment side-chain grafting was employed to design epitope-scaffolds for the motavizumab helical hairpin epitope. A filtered version of the PDB¹⁶ was used for matching, that included 13337 protein chains assigned as monomers and 39621 protein chains assigned as multimers. The assignment of the oligomeric state of protein structures was performed according to information available in the PDB and in the PQS (<http://www.ebi.ac.uk/pdbe/pqs/>) databases. The thresholds for matching were 1 Å for backbone RMSD and 30000 Rosetta energy units for the clash check. MES1 (1LP1) and MES2 (1S2X) were selected from the non-monomeric set and MES3 (2EIA) was selected from the monomeric set. Of the three matches obtained, two (MES2 and MES3) could have been obtained with standard side-chain grafting matching for continuous epitopes (backbone rmsds to the epitope were 1.0 and 0.92 Å over 19 residues for MES2 and MES3, respectively), but identification of the highest affinity epitope-scaffold (MES1) required the new multi-segment matching method.

In the design stage, epitope side chains responsible for the key interactions were transplanted to all-glycine versions of the matched scaffolds. The side chains transferred were: S255, L258, S259, I261, N262, D263, N268, D269, K271, K272, L273, S275, N276 (RSV F residue numbering as in PDB ID:3IXT). As previously described for side-chain grafting¹⁴, native scaffold side-chain rotamers outside the epitope were recovered, and residues near (heavy atom distance <4 Å) the epitope or the antibody were designed, categorized as intra and inter positions, respectively. Inter residues were allowed to be ALA, GLY, SER, and THR, and intra positions were allowed to be all amino acids except CYS. Epitope-scaffolds were ranked by antibody binding energy and a final step of human-guided design was performed to revert unnecessary or potentially destabilizing mutations, eliminate unpaired cysteines and undesired functional sites, and trim scaffold termini to avoid clash with antibody.

Computational resurfacing

To generate the Surf1 variant of the original MES1, multiple positions at the surface of the protein were designed using RosettaDesign²³. The residue positions allowed to mutate were: 2, 5, 9, 12, 15, 21, 24, 17, 37, 39, 40, 43, 44, 46, 47, 50, 53 and 54 in the residue numbering of the MES1-motavizumab crystal structure. Different resurfaced MES1 constructs vary in the number of surface mutations. To achieve this mutational gradient, subsets of the enumerated residues were allowed to change in different design simulations, and in the most distinct resurfaced variants all of the residues were allowed to change simultaneously. The amino-acid identities allowed in the designed residues were ALRNDEQKST. To ensure greater sequence diversity, in some of the designed molecules amino-acid identities were restricted to subsets of the initial amino-acids allowed.

MES1 cloning, expression and purification

Mammalian codon-optimized genes encoding MES1 and its variants were synthesized by GeneArt with an N-terminal secretion signal (MGSLQPLATLYLLGMLVASVLA) and a C-terminal HRV3C cleavage site, PADRE epitope (AKFVAAWTLKAAA), Caspase-3 cleavage site, 6x His-tag and Strep-tag II. The genes were cloned into the mammalian expression vector pαH, which is a modified version of pLEXm²⁴. Proteins were expressed from the plasmids by transient transfection using the Free Style 293 expression system (Invitrogen). MES1 proteins were purified from the media using Ni²⁺-NTA resin (Qiagen) and then Strep-Tactin resin (IBA) as per manufacturer's instructions, followed by passage over a 16/60 Superdex 75 column (GE Healthcare). For SPR, ITC and CD measurements, all tags were retained. For immunization experiments, Procaspase-3 D9A, D28A (a kind gift from A. Clay Clark²⁵) was added to remove the 6x His-tag and Strep-tag II. The tags and protease were removed from cleaved MES1 by passage over Ni²⁺-NTA resin. MES1 F2Y/H15N used for crystallization was similarly prepared, but it lacked the HRV3C site and PADRE epitope on the C-terminus.

MES2 cloning, expression and purification

E. coli codon-optimized genes encoding MES2 and its variants were synthesized by GeneArt and cloned into a custom vector based on pMAL-c2X (New England Biolabs). The expression vectors were transformed into BL21(DE3) cells, and the cells were grown in Terrific Broth at 37°C until OD₆₀₀ = 2.0. The temperature was then reduced to 22°C, and isopropyl β-D-thiogalactoside (IPTG) was added to 1 mM. After overnight incubation at 22°C, the cells were harvested and lysed with Bug Buster (Novagen), and MES2 proteins were purified using Ni²⁺-NTA resin (Qiagen). Fusion tags were removed by incubation with Procaspase-3 D9A, D28A and passage over Ni²⁺-NTA resin. MES2 proteins were further purified by passage over a 16/60 Superdex 75 column (GE Healthcare), and anion exchange chromatography using a MonoQ column (GE Healthcare).

MES3 cloning, expression and purification

A mammalian codon-optimized gene encoding MES3 was synthesized and cloned as described for MES1. Protein expression and purification were also performed as described for MES1.

Surface plasmon resonance

All experiments were carried out on a Biacore 3000 instrument (GE Healthcare). For the detection of motavizumab binding to MES1 and MES2, motavizumab antigen-binding fragments (Fabs) were covalently coupled to a CM5 chip at 530 RU, and a blank surface with no antigen was created under identical coupling conditions for use as a reference. Initially, epitope-scaffolds were serially diluted 2-fold, starting at 10 μM, into 10 mM

HEPES pH 7.4, 150 mM NaCl, 3 mM EDTA and 0.005% polysorbate 20 (HBS-EP) and injected over the immobilized Fab and reference cell at 40 μ l/min. MES1 measurements were repeated using lower protein concentrations, with the 2-fold dilutions starting at 500 nM. The data were processed with SCRUBBER-2 and double referenced by subtraction of the blank surface and a blank injection (no analyte). Binding curves were globally fit to a 1:1 binding model.

For the detection of motavizumab binding to peptide, motavizumab Fab was covalently coupled to a CM5 chip at high density (1,950 RU) and a blank surface with no antigen was created for use as a reference. An N-terminally acetylated peptide with the sequence NSELLSLINDMPITNDQKKLMSNNGYSGTETSQVAPA and a C-terminal biotinylated lysine residue was serially diluted 2-fold, starting at 500 nM, into HBS-EP and injected over the immobilized motavizumab Fab and reference cell at 40 μ l/min. Data were processed with BIAevaluation software and double referenced by subtraction of the blank surface and a blank injection. Binding curves were globally fit to a 1:1 binding model with drifting baseline and no Bulk RI.

Isothermal titration calorimetry

Experiments were carried out on an iTC₂₀₀ calorimeter (MicroCal Inc) at 25°C. Samples were dialyzed into phosphate-buffered saline (PBS) and degassed prior to titrations. MES1 or peptide at 350 μ M was titrated into 14 μ M motavizumab IgG in 2 μ l aliquots with stirring at 1,000 rpm. MES2 at 170 μ M was titrated into 7 μ M motavizumab IgG in 2 μ l aliquots with stirring at 1,000 rpm. Data were processed with Origin software and best fit by a single binding-site model.

Circular dichroism

To evaluate the secondary structures and thermostabilities of the epitope-scaffolds in solution, circular dichroism experiments were performed with an Aviv 62A DS spectrometer. Far-UV wavelength scans (190 nm –260 nm) at the concentration of 20 μ M were collected in a 1 mm path length cuvette. Temperature-induced protein denaturation was followed by change in ellipticity at 210 nm.

Protein crystallization and data collection

A five-fold molar excess of MES1 F2Y/H15N was incubated with motavizumab Fab for 1 hour at 22°C, and the complex was concentrated to 11.2 mg/ml in an Amicon Ultra centrifugal filter with a 30 kDa molecular weight cut-off (Millipore). 192 crystallization conditions were screened using a Cartesian Honeybee crystallization robot, and initial crystals were grown by the vapor diffusion method in sitting drops at 20°C by mixing 0.2 μ l of protein complex with 0.2 μ l of reservoir solution (20.5% (w/v) PEG 4000, 0.2 M lithium sulfate monohydrate, 0.1 M Tris-HCl pH 8.5, 100 mM NaCl). These crystals were manually reproduced in hanging drops by mixing 0.8 or 1.6 μ l protein complex with 0.8 μ l of the initial reservoir solution containing a range of PEG 4000 concentrations. Larger, single crystals were obtained by streak seeding with clusters of crystals that had been pulverized with a PTFE bead, and these crystals were flash frozen in liquid nitrogen in 24% (w/v) PEG 4000, 30% (v/v) ethylene glycol, 0.2 M lithium sulfate monohydrate, 0.1 M Tris-HCl pH 8.5. Data to 1.90 Å were collected at a wavelength of 1.00 Å at the SER-CAT beamline ID-22 (Advanced Photon Source, Argonne National Laboratory).

Structure determination, model building and refinement

Diffraction data were processed with the HKL2000 suite²⁶ and a molecular replacement solution consisting of two motavizumab Fab molecules¹³ and two Protein Z molecules²⁷ per

asymmetric unit was obtained using PHASER²⁸. Model building was carried out using COOT²⁹, and refinement was performed with PHENIX³⁰. Final data collection and refinement statistics are presented in Table 2. The Ramachandran plot as determined by MOLPROBITY³¹ shows 97.7% of all residues in favored regions and 99.8% of all residues in allowed regions. All structural images were created using PyMol (The PyMOL Molecular Graphics System, Version 1.1, Schrödinger, LLC).

Mice and immunizations

6–8 week old female C57BL/6 mice (Jackson Laboratory, Bar Harbor, ME) were used for all experiments. Mice were immunized with scaffold proteins and 25 µg CpG per mouse intramuscularly. Mice were boosted with either the same protein or an alternative scaffold protein, and the sera were tested by kinetic ELISA for binding to RSV F protein or scaffolds and for neutralization activity.

Kinetic ELISAs

Proteins were diluted in PBS to a concentration of 1 µg/ml and coated onto 96-well flat bottom ELISA plates overnight at 4°C. Nonspecific adsorption was prevented with 200 µL/well of blocking buffer (2% BSA in PBS) for 1 h at room temperature. Plates were washed four times on an automated plate washer (Bio-Tek Instruments, Winooski, VT) with wash buffer (0.02% Tween-20 in PBS). 100 µL of diluted test sera (1:100 in blocking buffer) and positive serum control were added to each well. Plates were incubated for one hour at room temperature, washed four times, and incubated for 1 hour at room temperature with HRP-conjugated goat anti-mouse IgG antibody (Jackson ImmunoResearch Laboratories, West Grove, PA). Plates were washed with wash buffer four times followed by distilled water. 100 µL of Super AquaBlue ELISA substrate (eBioscience, San Diego CA) was added to each well and plates were read immediately using a Dynex Technologies microplate reader (Chantilly, VA). The rate of color change in mOD/min was read at a wavelength of 405 nm every 9 s for 5 min with the plates shaken before each measurement. The mean mOD/min reading of duplicate wells was calculated, and the background mOD/min was subtracted from the corresponding control well.

Competition Kinetic ELISA

Competitive ELISA was used to determine the binding specificity of MES1-induced antibodies for the motavizumab epitope. Motavizumab IgG was used as a competitor for the binding of serum antibody from MES1-immunized mice to RSV F, MES1, or MES1 K272E. Motavizumab (50 µL, 1 µg/ml) was added to ELISA plates coated with each antigen and incubated 30 minutes before adding serum samples. Each sample was tested with and without motavizumab. The data are normalized for each sample pair as the percent of binding compared to the untreated well by dividing mOD/min of the motavizumab-treated well by the mOD/min of the control untreated well. This experiment was performed with sera from a group of 5 immunized mice. The P value was determined by Student's two-tailed T-test.

RSV neutralization assay

Antibody-mediated RSV neutralization was measured by a flow cytometry neutralization assay³². Briefly, HEp-2 cells were infected with RSV-GFP and infection was monitored as a function of GFP expression at 18 hours post-infection by flow cytometry. Data were analyzed by curve fitting and non-linear regression (GraphPad Prism, GraphPad Software Inc., San Diego CA).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Fig. 1. Motavizumab epitope-scaffolds

Three proteins were computationally designed to accept and maintain the motavizumab epitope in the conformation observed in the complex between motavizumab and its epitope peptide. (a) Ribbon diagram of a pre-fusion RSV F model, and the structure of the motavizumab epitope peptide bound to motavizumab Fab¹³. The motavizumab heavy chain is green, and the light chain is blue. The epitope peptide is grey, with the 13 residues that were transplanted into the acceptor-scaffolds colored red. (b) Models of the three motavizumab epitope-scaffolds, in an orientation similar to that of the peptide in (a). The transplanted residues are colored red, and the PDB ID and chain ID of the original structures are listed.

Fig. 2. Kinetics and thermodynamics of epitope-scaffolds binding to motavizumab
 MES1 has a faster on-rate than the peptide, and MES1 binding is entropically driven, suggesting that the motavizumab epitope present on MES1 is fixed in the bound-conformation. (a–c) SPR sensorgrams of MES1, MES2 and F peptide binding to immobilized motavizumab Fab. Red lines represent best fit of kinetic data to 1:1 binding models. (d–f) ITC data for MES1, MES2 and F peptide binding to motavizumab IgG. Experiments were performed at 25°C in PBS, and red lines represent best fit of data to a single binding-site model.

Fig. 3. Circular dichroism data for MES1 and MES2

Both epitope-scaffolds have spectra indicative of alpha-helical proteins, but only MES1 displays cooperative-unfolding. (a) MES1 CD spectrum. (b) MES1 melting profile obtained by measuring mean residue ellipticity at 210 nm while increasing the temperature from 5°C to 99°C. Red line represents best fit of the data to a two-state model. (c) MES2 CD spectrum. (d) MES2 melting profile obtained by measuring mean residue ellipticity at 210 nm while increasing the temperature from 5°C to 99°C. The non-sigmoidal plot indicates a lack of cooperative unfolding, and the data could not be fit to a two-state model.

Fig. 4. Crystal structure of MES1 bound to motavizumab

The conformation of motavizumab-bound MES1 is similar to the motavizumab-bound peptide and the computationally-designed model. (a) Structure of MES1 in complex with motavizumab Fab. A semi-transparent molecular surface is overlaid on a ribbon representation of the complex. The motavizumab heavy chain is green, the light chain is blue, and MES1 is magenta. (b) Ca superposition of the 13 shared residues between MES1 (magenta) and the F peptide (yellow) in ribbon representation. The rmsd is 0.30 Å for the 13 Ca residues. (c) Ca superposition of MES1 from the crystal structure (magenta) and the model (grey) in ribbon representation. The rmsd is 1.16 Å for 53 Ca residues. (d) Plot of the Ca distances of the superposition shown in (c). The 20 helical residues which contain the 13 transplanted residues are colored red.

Fig. 5. MES1 binding to motavizumab Fab

MES1 binding to motavizumab is shifted with respect to the model and the epitope peptide, though side-chain hydrogen-bond interactions are preserved. (a) MES1 in complex with motavizumab based on coordinates from the crystal structure (magenta) and the model (grey). Superposition of the Fab molecules was used to orient the complexes. The top image shows the molecular surface of the Fab, and MES1 alpha-helices are depicted as cylinders. The bottom image shows both MES1 and the Fab as ribbons, with the side-chains of the 13 transplanted residues shown as sticks. Oxygen atoms are colored red and nitrogen atoms are blue. (b) Same as (a), except the peptide/motavizumab crystal structure (yellow) replaces the MES1/motavizumab model.

Fig. 6. Epitope-scaffold immunogenicity

MES1 elicits antibodies when fused to a PADRE sequence, and sera with F protein-binding activity are elicited by combinations of MES1, a modified derivative of MES1, or MES2. (a) ELISA analysis of sera from MES1-immunized mice. MES1 or a mutant that ablates motavizumab binding (MES1 K272E), were coated on the plate. (b,c) Sera from mice immunized with epitope-scaffolds were tested for binding to MES1 (b) and recombinant RSV F protein (c). (d) Sera from mice immunized four times with MES1 were tested for binding to proteins that had been pre-incubated with motavizumab IgG. For all panels, plotted data represent the mean of 5 mice $\pm$ SD. As a reference, a result of 100 mOD/min in the kinetic ELISA is equivalent to the F binding activity of ~ 0.5 μ g/ml of palivizumab (Synagis[®], Medimmune, Gaithersburg, MD) and a result of 20 mOD/min ~ 0.01 μ g/ml.

Fig. 7. MES1 derivative for focusing the immune response

Ribbon diagram of MES1 model (a), with residues replaced in Surf1 (b) shown as sticks with semi-transparent surfaces. (c) SPR sensorgram of Surf1 binding to immobilized motavizumab Fab. Five 2-fold dilutions of Surf1 starting at 500 nM were measured. Red lines represent best fit of kinetic data to a 1:1 binding model.

Table 1

Expression yields and motavizumab-binding affinities as determined by SPR for all of the epitope-scaffolds created for this study.

Name	Expression (mg/L)	Motavizumab K_D (nM)
MES1	4.2	87
MES1a	1.6	181
MES1b	2.7	700
MES1c	2.4	296
MES2	12.0	2370
MES2a	2.0	4755
MES2b	7.0	3638
MES2c	12.0	3950
MES3	0.1	N.D.
MES3a	0.0	N.D.
MES3b	0.0	N.D.
Surf1	3.0	114
Surf2	0.0	N.D.
Surf3	1.2	117

N.D. = Not determined

Table 2

Data collection and refinement statistics

Motavizumab/MES1	
PDB ID	3QWO
Data collection	
Space group	$P2_1$
Cell dimensions	
a, b, c (Å)	75.8, 74.4, 86.4
β (°)	101.8
Resolution (Å)	50–1.90 (1.93–1.90)
R_{merge}	7.9 (44.9)
$I/\sigma I$	16.7 (2.0)
Completeness (%)	99.4 (92.0)
Redundancy	3.6 (3.0)
Unique reflections	73,143
Refinement	
Resolution (Å)	1.90
No. reflections	69,917
$R_{\text{work}}/R_{\text{free}}$ (%)	18.8/23.1
No. atoms	
Protein	7362
Ligand/ion	27
Water	509
B -factors	
Motavizumab	43.6
MES1	43.8
Ligand/ion	73.3
Water	40.5
R.m.s. deviations	
Bond lengths (Å)	0.007
Bond angles (°)	1.03
Ramachandran plot	
Outliers (%)	0.2
Favored (%)	97.7

Only one crystal was used for data collection

Values in parentheses are for highest-resolution shell The sigma cutoff on F used in refinement was zero

Table 3

Intermolecular Van der Waals contacts in peptide-motavizumab and MES1-motavizumab crystal structures. The interactions were assessed using the contact application included in the CCP4 software package using a cutoff distance of 3.9 Å.

3IXT		MES1/Motavizumab	
Peptide	Motavizumab	MES1	Motavizumab
Ser255	Ala32H	Ser25	Ala32H
			Trp53H
Leu258	Ala32H	Leu28	Ala32H
	Phe98H		Phe98H
			Trp53H
			Ile97H
Ser259	Trp53H	Ser29	Trp53H
			Asp54H
Asn262	Trp53H	Asn32	Trp53H
	Asp54H		Asp54H
	Lys56H		Lys56H
			Trp52H
		Ser1	Ser92L
		Tyr2	Ser92L
Asn268	Gly91L	Asn3	Gly91L
	Ser92L		Ser92L
	Tyr94L		Tyr94L
	Phe96H		Phe96H
	Phe100H		Phe100H
			Gly93L
Asp269	Gly31L	Asp4	Gly31L
	Ser92L		Ser92L
			Tyr32L
Lys271	Lys56H	Lys6	Phe100H
Lys272	Tyr32L	Lys7	Tyr32L
	Asp50L		Asp50L
	Ile97H		Ile97H
	Phe98H		Phe98H
	Phe100L		Phe100L
Ser275	Ile97H	Ser10	Ile97H
	Phe98H		Phe98H
Asn276	Phe98H	Asn11	Phe98H
		Ala14	Phe98H