Molecular modeling predicts novel antibody escape mutations in respiratory syncytial virus fusion glycoprotein

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Abstract

Monoclonal antibodies are increasingly used for the prevention and/or treatment of viral infections. One caveat of their use is the ability of viruses to evolve resistance to antibody binding and neutralization. Computational strategies to predict which mutations will result in antibody resistance would be invaluable because current methods for identifying potential escape mutations are labor intensive and system-biased. Respiratory syncytial virus is an important pathogen for which monoclonal antibodies against the fusion (F) protein are used to prevent severe disease in high-risk infants. In this study, we used an approach that combines molecular dynamics simulations with FoldX to estimate changes in free energy in F protein folding and binding to the motavizumab antibody upon each possible amino acid change. We systematically selected 8 predicted escape mutations and tested them in an infectious clone. Consistent with our F protein stability predictions, replication-effective viruses were observed for each selected mutation. Six of the eight variants showed increased resistance to neutralization by motavizumab. Flow cytometry was used to validate the estimated (model-predicted) effects on antibody binding to F. Using surface plasmon resonance, we determined that changes in the on-rate of motavizumab binding were responsible for the reduced affinity for two novel escape mutations. Our study empirically validates the accuracy of our molecular modeling approach and emphasizes the role of biophysical protein modeling in predicting viral resistance to antibody-based therapeutics that can be used to monitor the emergence of resistant viruses and to design improved therapeutic antibodies.

Importance

Respiratory syncytial virus (RSV) causes severe disease in young infants, particularly those with heart or lung diseases or born prematurely. As no vaccine is currently available, monoclonal antibodies are used to prevent severe RSV disease in high-risk infants. While it is known that RSV evolves to avoid recognition by antibodies, screening tools that can predict which changes to the virus will lead to antibody resistance are greatly needed.

Keywords: Respiratory syncytial virus, fusion glycoprotein, monoclonal antibodies, molecular modeling, monoclonal antibody resistant mutation

Introduction

The use of monoclonal antibodies (mAb) for the treatment or prevention of viral infections has accelerated over the past few years. mAb are a powerful tool against viral infections because of the ability to target specific epitopes to neutralize viral pathogens. The first mAb approved by the FDA for the prophylaxis of viral infection was Synagis® (palivizumab), which is used to prevent respiratory syncytial virus (RSV) infection in high-risk infants (1–3). One of the most notable recent uses of mAb has been the emergency use authorization of three mAb treatments for severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) the causative agent for coronavirus disease 2019 (COVID-19) (4–7). The FDA has also approved the mAb cocktail, Inmazeb™, for the treatment of Ebola virus infection (8) and Trogarzo®, a mAb treatment for drug-resistant human immunodeficiency virus (HIV-1) infection (9). There are also mAb therapies in development for other viruses including influenza virus (10) and herpes simplex virus 1 (11, 12). The use of mAb for the prevention and/or treatment of viral infections has become an important strategy for the medical community.

One caveat of using mAb against viral infections is the increased selective pressure for antibody escape mutations to occur. The current methodology for monoclonal antibody-resistant mutants (MARMs) discovery involves sequencing samples taken from patients or serially passaging virus with the antibody of interest to select for evolved mutations. This presents challenges in that the escape mutation is already circulating in the population and passaging experiments can be time consuming and are biased in only detecting mutations that can arise over limited replication time in cell culture. Given that it is now common to monitor the genetic variation in a population, an aspirational goal in the fight against infectious disease is the ability to predict when viral evolution is about to outpace the effectiveness of protective mAb.

Paramount to this goal is the ability to predict amino acid changes that will significantly disrupt binding of antibodies to viral proteins; a collection of these predicted MARMs would constitute a watch list. An increase in the frequency of MARMs on the watch list in an infected population could signal reduced effectiveness of antibody-based therapies and the possible adaptation to these therapies.

Protein biophysical models can be used to predict protein stability and the disruption of interactions with other biomolecules due to mutations. In our previous studies, we used FoldX software combined with molecular dynamics (MD) simulations (MD+FoldX) to estimate folding and binding stabilities of Ebola virus (EBOV) envelope glycoprotein (GP) and mAbs KZ52, Antibody 100 (Ab100), Antibody 114 (Ab114) and 13F6-1-2. Using this approach, we generated a watch list comprised of 127 mutations that were predicted to disrupt binding between GP and four mAbs but would not disrupt the ability of the glycoprotein to fold and form a trimer (13–16). Three mutations from our watch list have already been seen in humans or are experimentally known to reduce efficacy of the antibody treatment (17, 18). While these previous studies showed the predictive capability of protein biophysical modeling to help guide empirical science in examining phenotypes in viral evolution, these results were not empirically validated to test the accuracy in predicting MARMs.

MARMs for palivizumab have been identified from cell culture, animal models, and clinical samples (19–27). RSV is an important pathogen for infants, elderly, and immune compromised that causes severe lower respiratory infections and is the second leading cause of infant death in the world (2, 3). The only targeted treatment for RSV is the prophylactic mAb palivizumab and additional mAbs are in development, including nirsevimab, which is currently in Phase 3 clinical trials (28–32). Palivizumab and its derivative, motavizumab, target the site II

epitope on the fusion glycoprotein (F protein), the protein responsible for the fusion of viral and host cell membranes during viral entry (28, 33–38). F protein is a desirable target for anti-viral treatments given the function of the protein for viral entry, the response to the antigen by the host immune system, and the conservation of antigenic sites including site II among RSV strains (38–43). F protein is also advantageous to use to test molecular modeling given the multiple co-crystal structures with mAbs (44–47). For this study we used the co-crystal structure of motavizumab-F protein because there is no crystal structure with palivizumab. Motavizumab is also of interest as only one MARM, K272E, has ever been derived from clinical and empirical lab studies (21).

In this study we describe the application and empirical validation of our MD+FoldX approach to RSV F protein and motavizumab complex to predict MARMs by analyzing changes in relative binding affinities due to all possible amino acid changes in the F protein. We empirically tested the fitness, mAb neutralization, and binding characteristics of eight predicted MARMs using an infectious clone. Our modeling approach was able to predict the already known MARM, K272E, and five additional novel variants with decreased neutralization by motavizumab.

Results

Molecular modeling identified amino acid residues of interest in F monomer and motavizumab interaction.

MD+FoldX approach was used to predict RSV F protein escape mutations that would allow the F protein to fold correctly but disrupt its recognition by motavizumab. We estimated the effect of all possible mutations of RSV F protein for all 448 amino acids and 19 possible

substitutions at each site for both F protein monomer folding ($\Delta\Delta G_{\text{Fold}}$) and its binding with motavizumab ($\Delta\Delta G_{\text{Bind}}$) (Figure 1 and Appendix). Mutations with $\Delta\Delta G_{\text{Fold}}$ value less than 2 kcal/mol are not predicted to affect the stability of F protein and therefore have the potential to arise under selective pressure (15). To identify MARMs and test the accuracy of the MD+FoldX approach, we selected eight mutations with $\Delta\Delta G_{\text{Bind}}$ values ranging from 0.5 to 5.5 kcal/mol (low to high disruption of binding to motavizumab) and $\Delta\Delta G_{\text{Fold}}$ values less than 2 kcal/mol (Figure 1, Table 1). K272E, which had a $\Delta\Delta G_{\text{Bind}}$ value of approximately 2 kcal/mol and a $\Delta\Delta G_{\text{Fold}}$ value less than 0 kcal/mol, was included as a positive control as the only known escape variant for motavizumab (21).

The eight selected variants were viable and demonstrated different growth kinetics.

To confirm that $\Delta\Delta G_{\text{Fold}}$ values less than 2 kcal/mol did not disrupt F protein function, we first assessed the ability of the eight variants to replicate. To test the growth kinetics of the variants, site directed mutagenesis was used to create the variants, which were subcloned into a recombinant mKate2-expressing RSV infectious clone (48). HEp-2 cells were infected at a multiplicity of infection (MOI) of 0.1 and supernatant media samples were collected over 48 hours then titrated by TCID₅₀ assay. We were able to generate replicating virus from each mutated clone (Figure 2). L258K and L258E both demonstrated delayed growth with extracellular virus remaining undetectable until 15 and 18 hours, respectively. These variants also exhibited significantly reduced overall viral titers when compared to wild-type (WT) over the 48-hour period. N262D and N262Y also displayed significantly reduced viral titers when compared to WT. S275R and S275H were similar to WT virus in growth with small differences in titers at a few of the time points. K272E had reduced viral titers at each time point and N276G

had few differences from WT. All eight mutations were able to replicate but displayed at least some reduced propagation in viral titer when compared to the WT virus.

Six variants demonstrated decreased neutralization by motavizumab compared to WT.

After establishing that all eight variants were able to replicate the next step was to quantify neutralization of the variants by motavizumab. Virus variants were incubated with a two-fold serial dilution of motavizumab for 1 hour prior to infection of HEp-2 cells and mKate2-expressing cells were quantified by flow cytometry after 18 hours. Six of the eight mutations demonstrated reduced neutralization by motavizumab compared to WT (Figure 3). The only known MARM, K272E, was not neutralized at the highest dose of antibody used (10 µg/mL). Previously published data determined that the IC₅₀ for K272E was 30.04 ± 611.35 µg/mL (21). S275H, L258K, and S275R demonstrated a greater than 5-fold increase of IC₅₀ when compared to WT and L258E and N262D had a greater than 3-fold increase in IC₅₀ (Figure 3, Table 2). Variants N262Y and N276G had similar neutralization kinetics to WT (Figure 3, Table 2). In addition to confirming K272E as a motavizumab-resistant mutation, we identified 5 novel MARMs that have not been previously identified by traditional approaches.

MARMs had reduced motavizumab binding to F protein.

Flow cytometry was used to assess motavizumab/F binding in a high-throughput format. HEK 293A cells were transiently transfected with plasmids that express variant F proteins. A subset of the variants that we had tested in the infectious clone for neutralization (L258E, L258K, K272E, S275H, S275R, and N276G) were tested as well as additional variants at the same residues of interest (L258V, K272G, K272R, and S275A). All of the additional variants

tested, except K272G, had $\Delta\Delta G_{\text{Bind}}$ values near 0 kcal/mol (Appendix) and thus would not be expected to have reduced binding by motavizumab. Expression of F protein was analyzed using mAb 101F that binds a distinct antigenic site from motavizumab (24). Motavizumab was conjugated with Alexa Fluor 488 (motavizumab-488) and 101F was conjugated with Alexa Fluor 594 (101F-594). Binding by 101F was used to gate on the F protein positive cell population and the median fluorescent intensity (MFI) of Alexa Fluor 488 was used as an indicator of motavizumab binding (Figure 4A). A reduction in binding was observed in the peak shifts and reduction of motavizumab positive cell percentages for L258E, L258K, K272E, K272G, S275H and S275R (Figure 4A). A significant reduction of MFI was observed in cells expressing L258E, L258K, K272E, S275H, and S275R when compared to WT (Figure 4B). L258V, N276G, and K272G had some reduction in MFI when compared to WT but were not statistically significant. K272R and S275A had MFI values similar to WT indicating that the binding of motavizumab is similar to WT, as expected because they had predicted $\Delta\Delta G_{\text{Bind}}$ values of less than zero. All the MARMs that demonstrated reduced neutralization (Figure 3) also had reduced binding of motavizumab (Figure 4). There was a reasonable correlation ($R^2 = 0.52$) when comparing binding of motavizumab to predicted $\Delta\Delta G_{\text{Bind}}$ values, as $\Delta\Delta G_{\text{Bind}}$ increases there is a decrease in the binding of motavizumab (Figure 4C), suggesting that an increase in $\Delta\Delta G_{\text{Bind}}$ value may be more disruptive to antibody binding.

Escape from neutralization corresponds with altered on rate of motavizumab binding to F protein.

To further evaluate motavizumab binding to MARMs we analyzed binding kinetics of the variants we identified to have the highest increase in IC_{50} compared to WT: K272E, S275H, and

L258K. Surface plasmon resonance (SPR) was used to quantify binding kinetics of purified virions to motavizumab. A decrease in the binding on-rate (k_a) was observed for all three variants, notably a >6-fold reduction of binding for S275H (Figure 5A, Table 3). There was no significant increase in the off-rate (k_d) of antibody binding to the three variants (Figure 5B, Table 3). The overall equilibrium disassociation constant (K_D) for the variants demonstrates a lower affinity between the MARMs and motavizumab when compared to WT (Figure 5C, Table 3). The low K_D for WT is expected as once the antibody binds it is unlikely to be disrupted. There was a correlation between $\Delta\Delta G_{\text{Bind}}$ and on-rate, as $\Delta\Delta G_{\text{Bind}}$ increased the on-rate decreased (Figure 5D). No correlation was observed between $\Delta\Delta G_{\text{Bind}}$ and off-rate, which is expected as there were no significant differences between the off-rate values (Figure 5E). A strong linear correlation was observed between $\Delta\Delta G_{\text{Bind}}$ and overall affinity of motavizumab binding, as $\Delta\Delta G_{\text{Bind}}$ increased, K_D increased (Figure 5F). The escape mechanism for all three variants appears to be a decrease in association with motavizumab rather than an increase of disassociation from motavizumab.

Discussion

The MD+FoldX approach provides rapid and testable predictions of MARMs. In this study, we applied this modeling approach to identify MARMs of RSV F protein and motavizumab antibody complex. This also enabled us to empirically validate the accuracy of our modeling approach. We selected eight variants that were predicted to allow the F protein to fold correctly but disrupt its ability to bind to motavizumab. All the selected variants were found to infect cells and propagate new virus. Six of the eight mutations demonstrated reduced neutralization by motavizumab when compared to WT and flow cytometry confirmed reduced

binding of motavizumab to the mutants. SPR analysis of the variants that had the highest resistance to neutralization suggested that a reduced on-rate of antibody binding to virions was responsible for resistance and correlated with modeling estimations.

Previous studies have identified palivizumab and motavizumab MARMs from patient samples and passage experiments (19–27) and K272E was previously the only known MARM for motavizumab (21). This study examined mutations for motavizumab given the availability of the co-crystal structure and revealed novel escape variants. The binding kinetics of K272E have been previously studied using purified F protein and found that binding on rate rather than off rate appeared to be the mechanism of escape (27). In our study we were able to replicate those findings with purified virions as well as establish the same mechanism for escape for two additional MARMs that we identified. Previous studies have also found that antibody association rates correlate well with viral neutralization (37).

A limitation of the MD+FoldX approach and for molecular modeling in general is the reliance on having a co-crystal structure of the mAb and viral protein. Motavizumab is not used clinically but there are no co-crystal structures available for palivizumab and F protein. However, most mAb in the pipeline for medical use have co-crystal structure with proteins of interest available (49, 50). Another drawback is that many of the viral proteins used in the crystal structures are lab strains of virus and may not reflect circulating viral strains. The RSV-A2 strain used in our study is based on a clinical isolate from 1961(51) and is the prototypic RSV strain for lab use. The F protein used for crystallography, DS-Cav1, is based off the A2 strain with modifications for expression and trimerization (52). However, the predominant circulating RSV A strain, ON1, was first identified in 2006 in Ontario and was classified as a novel genotype based off both F gene and G gene sequencing (53). The use of strains that reflect current

circulating strains will help produce data that are more clinically relevant and can be done in the same reverse genetics system used for these experiments (54). Molecular modeling is also limited to predictions at individual amino acid residues. Studies of MARMs derived from a different F protein mAb, nirsevimab, have demonstrated that some MARMs require two separate residue changes for mAb escape (55). As more advances are made in fast protein-protein binding affinity estimation methods, we may be able to predict the effects of epistatic mutations.

In our previous study, comparing performances of different protein-protein binding affinity prediction methods, MD+FoldX approach showed the best performance for antigen-mAb complexes (56). It achieved an r value of 0.39 for 253 mutations from eight different antigen-mAb complexes with known experimental binding affinity data. We observed a slightly better performance of MD+FoldX approach for our smaller set of mutations tested in this study, where we obtained R^2 of 0.52 between predicted $\Delta\Delta G_{\text{Bind}}$ values and binding measured using flow cytometry (Figure 4C). There were strong correlations between the binding kinetics values and estimated $\Delta\Delta G_{\text{Bind}}$, with R^2 values for on-rate and affinity of 0.86 and 0.84, respectively. However, this data set has only four data points and should be investigated further with more variants. Although this modeling approach remarkably identified six MARMs, there is still a need for better modeling strategies to improve correlation between experimental and predicted $\Delta\Delta G_{\text{Bind}}$ values for antigen-mAb complexes. Use of MD simulation to provide conformation sampling is the key reason behind the performance of the MD+FoldX approach (56). However, empirical validation of the MD+FoldX approach carried out in this study further emphasizes the need for improved conformational strategies for large protein-protein complexes.

K272E was the only previously known MARM for motavizumab and no other study with passage experiments has been able to evolve other mutations. Future studies may investigate the

evolutionary pathway that results in a poorly replicating MARM such as K272E becoming the dominant variant over more fit MARM. We can predict and generate MARMs, but further understanding of viral evolution is essential to refining our predictions and variant watch lists. Expansion of MD simulation and testing for other mAb such as nirsevimab and other viruses such SARS-CoV-2 and the respective mAbs would bolster our proof of concept. This would also be of interest and significance as MARMs have been identified for nirsevimab and both Delta and Omicron variants of SARS-CoV-2 have demonstrated resistance to therapeutic mAb treatments (55, 57, 58). In addition, mutations outside of the receptor binding domain of the SARS-CoV-2 spike protein have demonstrated increased instability in binding of mAb to MARM variants and would be of future interest for modeling studies (59).

In summary, biophysical protein modeling is a powerful tool for predicting MARMs. The ability to predict MARMs for current and future mAb treatments expedites variant discovery and has the potential to help design and optimize mAb. Given the rapid evolution of some viruses, such as SARS-CoV-2 and HIV-1, having a list of potential escape mutations is not only important for public health, but also for the scientific community to develop improved treatments for MARMs.

Materials and Methods

***In silico* predictions of MARMs using MD+FoldX approach.** To predict RSV F protein escape mutations against motavizumab mAb, we applied our approach from previous studies that combines classical molecular dynamics (MD) and FoldX software (MD+FoldX) (15, 16). To designate a mutation as an escape mutation it requires: 1) disrupt binding to a mAb, and 2) leave the F protein monomer stable thus allowing it to fold and assemble. It is thus necessary to

determine how amino acid mutations alter stabilities ($\Delta\Delta G$ values) for F protein monomer folding ($\Delta\Delta G_{\text{Fold}}$) and binding to Motavizumab ($\Delta\Delta G_{\text{Bind}}$). Therefore, we used our MD+FoldX approach to estimate the folding stability of F protein monomer and F protein trimer/Motavizumab complex binding affinities due to all possible single mutations.

Structure preparation. The X-ray crystal structure of RSV F glycoprotein bound to motavizumab was downloaded from Protein Data Bank. (PDB ID:4ZYP) 3D coordinates file was first modified to remove all but F protein trimer and three copies of heavy and light chains of motavizumab bound to each F protein monomer (44). The MODELLER software was then used to alter engineered residues and build the missing residues in all the chains (60). Missing amino acid residues 96 to 137 in F protein monomer represent liberated glycopeptide because of proteolysis by furin like proteases. These residues were ignored in our simulations.

Molecular dynamics simulations. F protein monomer and F protein trimer/motavizumab complex structures were used as starting structures for MD simulations. Similar MD simulation the protocol was applied as reported in our previous studies for both the structures (15, 16). Briefly, the AMBER99SB*-ILDNP forcefield and the GROMACS 5.1.2 software package were used for generating topology files and performing simulation (61, 62). The final production simulation was run for 50 ns and snapshots were saved every 1 ns resulting in 50 snapshots for the F protein monomer and F protein trimer/motavizumab complex.

FoldX. FoldX software was used to analyze MD snapshots of F protein monomer to estimate $\Delta\Delta G_{\text{Fold}}$ and F protein trimer/Motavizumab snapshots were used to estimate $\Delta\Delta G_{\text{Bind}}$ for all possible mutations at each site in F protein. Our FoldX analysis protocol involved processing each

snapshot six times in succession using RepairPDB command to energy minimize the snapshot, BuildModel command to generate all possible 19 single mutations at each site in F protein, and then the folding (ΔG_{Fold}) and binding affinity (ΔG_{Bind}) were estimated using Stability and AnalyseComplex commands, respectively. Both folding and binding $\Delta\Delta G$ values for each mutation was calculated by taking a difference between mutated and WT ΔG_{Bind} and ΔG_{Fold} values. For each mutation, we then averaged $\Delta\Delta G_{\text{Bind}}$ and $\Delta\Delta G_{\text{Fold}}$ values across all individual snapshot estimates. To estimate $\Delta\Delta G_{\text{Bind}}$ values for all possible 19 mutations at each amino acid site of F protein, we performed 1,276,800 FoldX calculations (448 F protein residues $\times$ 19 possible mutations at each site $\times$ 50 MD snapshots $\times$ 3 copies of F protein/Motavizumab). Similarly, to estimate $\Delta\Delta G_{\text{Fold}}$ values for all possible 19 mutations at each amino acid site of F protein monomer we performed 425,600 FoldX calculations (448 F protein residues $\times$ 19 possible mutations at each site $\times$ 50 MD snapshots). Averaging estimates across all individual snapshots ultimately resulted in 8512 $\Delta\Delta G_{\text{Bind}}$ and $\Delta\Delta G_{\text{Fold}}$ values for all possible mutations of F protein (see Appendix).

Cell Lines. HEp-2 cells (ATCC CCL 23) were maintained in minimal essential media (MEM) with Earle's salts, 10% fetal bovine serum (FBS), 1% MEM non-essential amino acids, 1% penicillin-streptomycin-amphotericin (PSF), and 5mM L-glutamine. BHK-21 BSR-T7/5 (63) were supplied by Dr. Ursula Buchhoz (NIH) and maintained in Glasgow's MEM supplemented with 10% FBS, 2% MEM amino acids, 2mM L-glutamine, and 1% PSF. BSR-T7/5 were passaged every other passage with 1mg/mL geneticin to maintain the T7 polymerase-expressing plasmid. HEK 293 A cells were supplied by Dr. Lee Fortunato (University of Idaho) and were maintained in Dulbecco's Modified Eagle's media with 10% FBS, 1% penicillin and streptomycin, and 2 mM L-glutamine All cells were incubated at 37°C and 5% CO₂. FreeStyle™

CHO-STM Cells (Invitrogen) were maintained in FreeStyleTM CHOTM Expression Medium with 8 mM L-glutamine and incubated shaking at 37°C and 8% CO₂

Plasmid Preparation and Viral Propagation. Bacterial artificial chromosome (BAC) containing the antigenomic cDNA of RSV-A2 mKate2 and four RSV helper plasmids (human codon bias optimized N,P,L, M2-1) were provided by Dr. Martin Moore (Emory University). RSV BAC and helper plasmids (WT) were transfected into BSRT7 BHK-21 cells (48). Media and cells were harvested, sonicated in an ice water bath 3 times at 30%, and centrifuged at 2000 rpm for 5 minutes at 4°C. Supernatant media was harvested, flash frozen and stored at -80°C. A working stock of the WT virus was generated by infecting HEp-2 cells for 1-hour rocking at 37°C and 5% CO₂, adding complete media, monitoring cells for fluorescence and cytopathic effect (CPE), and was harvested as described above when fluorescence was detected throughout the flask. The F gene was subcloned from RSV BAC into pBluescript SK +. Site directed mutagenesis to generate the variants of interest was performed by Bioinnovatise Inc. Variant F genes were cloned back into genomic RSV BAC and transfected into BSRT7 BHK-21 cells as described above. Each mutant was plaque purified from HEp-2 cells (64) and confirmed with sequencing. Working stocks of the mutant F gene viruses were propagated as described above. All viruses were titrated by 50% tissue culture infectious dose (TCID₅₀) on HEp-2 cells.

Antibodies. Motavizumab and 101F antibodies and plasmids to produce these antibodies were provided by Dr. Jason McLellan (University of Texas, Austin) (41, 52). Plasmids expressing the heavy and light chains were co-transfected into FreeStyleTM CHO-STM cells (Invitrogen) in serum free FreeStyleTM CHOTM Expression medium. Cell medium was harvested 6-8 days post-transfection and concentrated using a Vivaflow 200. The concentrated medium was purified

using a HiTrap protein A column (GE Healthcare) as previously described (41, 52) and dialyzed into PBS.

Growth Curves. HEp-2 cells were infected at a multiplicity of infection (MOI) of 0.1 in MEM. Cells were rocked for 1 hour at 37°C and 5% CO₂. Cells were washed twice with PBS and the second wash was saved as the zero-hour time point. Complete media was added after aspiration of the PBS. Cell media was sampled at 6, 9, 12, 15, 18, 24, 36, and 48 hours. Viral titers were performed by TCID₅₀ assay on HEp-2 cells.

Microneutralization Assay. Assay was performed as previously described (65). Briefly, motavizumab was serially diluted two-fold starting at 10000 ng/mL and ending at 156.5 ng /mL. Variants were incubated with antibody for 1 hour prior to infecting HEp-2 cells at an MOI of 1. Cells were harvested 18 hours post-infection and counted via flow cytometry. Inhibition curves and IC₅₀ values were calculated using GraphPad Prism v.9.

F gene and Sequencing. RNA was isolated from virus stocks using Quick-RNA Viral Kit (Zymo Research). Viral cDNA was generated using SuperScriptTM IV VILOTM (Thermo Fisher Scientific). The F gene was PCR amplified using primers RSV F 5' (5'GCAAGGATTCCTTCGTGAC3') and RSV F 3' (5'CACACCACGCCAGTAG3') and Phusion High-Fidelity DNA Polymerase (Thermo Fisher Scientific). Sanger sequencing was performed by Elim Biosciences.

F Gene Library and Expression Vector. The F gene variant library was generated by Twist Bioscience, based on the codon-optimized F expressing plasmid, pHRSVFOptA2, provided by Dr. Mark Peeples (The Ohio State University) (66, 67). Each amino acid change at 17 residues

within 5 Angstroms of the motavizumab binding site, based on the co-crystal structure (44), were incorporated into the variant library. F gene variants from the library were cloned into mammalian expression vector pcDNA3.1. Bacterial stocks were plated on LB-ampicillin plates and colonies were picked and sequence confirmed to isolate each variant. Plasmids for transfection were purified using PureLink™ HiPure Plasmid Midiprep Kit (Invitrogen).

Flow Cytometry. HEK 293A cells were transiently transfected with pcDNA3.1-F plasmid variants or pcDNA3.1 as an empty vector control using Lipofectamine 3000™ reagents (Invitrogen). After 6 hours cell media was added to variants L258E, L258K, L258V, K272E, K272G, and K272R to increase protein expression. After 24 hours cells were lifted using Accutase and washed in PBS. Motavizumab and 101F were conjugated using Alexa Fluor™488 and 594 Antibody Labeling Kits (Invitrogen). Cells were stained with 1 µg/mL of Alexa Fluor 488 conjugated motavizumab and 0.7 µg/mL of Alexa Fluor 594 conjugated 101F antibody. Flow cytometry was performed using a Beckman Coulter Cytoflex-S and data were analyzed using CytExpert software.

Viral Purification. WT, L258K, K272E, and S275H clonal virus stocks were used to infect HEp-2 cells. Uninfected HEp-2 cells were included as a control. Cells and supernatant media were harvested when mKate2 fluorescence was observed throughout cell culture flasks. Harvested cells and media were flash frozen in liquid nitrogen and rapidly thawed in 37°C water bath. The cells and media were then sonicated in ice water bath at 30% for 30 seconds three times. Cell debris was pelleted at 2000 rpm, supernatant medium was removed and centrifuged again. The supernatant medium was loaded into Ultra-Clear 1 x 3.5 in. ultracentrifuge tubes (Beckman Coulter) with a 15% sucrose cushion in 10 mM Tris-HCl, pH 7.5, 100 mM MgSO₄

and 0.25 M sucrose. Samples were spun at 20000g for 2 hours at 4°C and pellets were resuspended in PBS. F protein concentration was determined using RSV-F ELISA Kit (SinoBiological).

Surface Plasmon Resonance. All SPR experiments were performed on a Nicoya OpenSPR™ Rev 3 one channel and Protein A Sensors Kit (Nicoya) was used to attach 30 µg/mL of motavizumab in PBS to the chip. Purified virions at concentrations 40 nM, 20 nM, 10 nM, and 5 nM were allowed to associate for 5 minutes at 20 µL/minute and disassociate for 5 minutes at 20 µL/minute. Chips were regenerated between runs with 10 mM glycine pH 3 and motavizumab was reattached. All kinetics analysis was performed in TraceDrawer 1.9.

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References

1. A joint statement with the Fetus and Newborn Committee. 1999. Palivizumab and respiratory syncytial virus immune globulin intravenous for the prophylaxis of respiratory syncytial virus infection in high risk infants. *Paediatrics & Child Health* 4:474–480.
2. Griffiths C, Drews SJ, Marchant DJ. 2017. Respiratory Syncytial Virus: Infection, Detection, and New Options for Prevention and Treatment. *Clin Microbiol Rev* 30:277–319.
3. Simões EAF, Bont L, Manzoni P, Fauroux B, Paes B, Figueras-Aloy J, Checchia PA, Carbonell-Estrany X. 2018. Past, Present and Future Approaches to the Prevention and Treatment of Respiratory Syncytial Virus Infection in Children. *Infect Dis Ther* 7:87–120.
4. Baum A, Fulton BO, Wloga E, Copin R, Pascal KE, Russo V, Giordano S, Lanza K, Negron N, Ni M, Wei Y, Atwal GS, Murphy AJ, Stahl N, Yancopoulos GD, Kyratsous CA. 2020. Antibody cocktail to SARS-CoV-2 spike protein prevents rapid mutational escape seen with individual antibodies. *Science* eabd0831.
5. Dougan M, Nirula A, Azizad M, Mocherla B, Gottlieb RL, Chen P, Hebert C, Perry R, Boscia J, Heller B, Morris J, Crystal C, Igbinadolor A, Huhn G, Cardona J, Shawa I, Kumar P, Adams AC, Van Naarden J, Custer KL, Durante M, Oakley G, Schade AE, Holzer TR, Ebert PJ, Higgs RE, Kallewaard NL, Sabo J, Patel DR, Dabora MC, Klekotka P, Shen L, Skovronsky DM. 2021. Bamlanivimab plus Etesevimab in Mild or Moderate Covid-19. *New England Journal of Medicine* 385:1382–1392.
6. Gupta A, Gonzalez-Rojas Y, Juarez E, Crespo Casal M, Moya J, Falci DR, Sarkis E, Solis J, Zheng H, Scott N, Cathcart AL, Hebner CM, Sager J, Mogalian E, Tipple C, Peppercorn A, Alexander E, Pang PS, Free A, Brinson C, Aldinger M, Shapiro AE. 2021. Early Treatment for Covid-19 with SARS-CoV-2 Neutralizing Antibody Sotrovimab. *New England Journal of Medicine* 385:1941–1950.
7. Anti-SARS-CoV-2 Monoclonal Antibodies. COVID-19 Treatment Guidelines. <https://www.covid19treatmentguidelines.nih.gov/therapies/anti-sars-cov-2-antibody-products/anti-sars-cov-2-monoclonal-antibodies/>. Retrieved 5 January 2022.
8. Markham A. 2021. REGN-EB3: First Approval. *Drugs* 1–4.
9. Markham A. 2018. Ibalizumab: First Global Approval. *Drugs* 78:781–785.
10. Hershberger E, Sloan S, Narayan K, Hay CA, Smith P, Engler F, Jeeninga R, Smits S, Trevejo J, Shriver Z, Oldach D. 2019. Safety and efficacy of monoclonal antibody VIS410 in adults with uncomplicated influenza A infection: Results from a randomized, double-blind, phase-2, placebo-controlled study. *EBioMedicine* 40:574–582.
11. Fresnais M, Longuespée R, Sauter M, Schaller T, Arndt M, Krauss J, Blank A, Haefeli WE, Burhenne J. 2020. Development and Validation of an LC–MS-Based Quantification Assay for New Therapeutic Antibodies: Application to a Novel Therapy against Herpes Simplex Virus. *ACS Omega* 5:24329–24339.

12. Heidelberg ImmunoTherapeutics GmbH. 2020. A Randomised, Double-Blind Phase II Trial of Topical HDIT101 Versus Placebo in Patients With Chronic Recurrent HSV-1 Infection and Orolabial Lesion. NCT04539483. Clinical trial registration. clinicaltrials.gov.
13. Schymkowitz J, Borg J, Stricher F, Nys R, Rousseau F, Serrano L. 2005. The FoldX web server: an online force field. *Nucleic Acids Research* 33:W382–W388.
14. Hollingsworth SA, Dror RO. 2018. Molecular Dynamics Simulation for All. *Neuron* 99:1129–1143.
15. Miller CR, Johnson EL, Burke AZ, Martin KP, Miura TA, Wichman HA, Brown CJ, Ytreberg FM. 2016. Initiating a watch list for Ebola virus antibody escape mutations. *PeerJ* 4:e1674.
16. Patel JS, Quates CJ, Johnson EL, Ytreberg FM. 2019. Expanding the watch list for potential Ebola virus antibody escape mutations. *PLOS ONE* 14:e0211093.
17. Leroy EM, Rouquet P, Formenty P, Souquière S, Kilbourne A, Froment J-M, Bermejo M, Smit S, Karesh W, Swanepoel R, Zaki SR, Rollin PE. 2004. Multiple Ebola Virus Transmission Events and Rapid Decline of Central African Wildlife. *Science* 303:387–390.
18. Kugelman JR, Kugelman-Tonos J, Ladner JT, Pettit J, Keeton CM, Nagle ER, Garcia KY, Froude JW, Kuehne AI, Kuhn JH, Bavari S, Zeitlin L, Dye JM, Olinger GG, Sanchez-Lockhart M, Palacios GF. 2015. Emergence of Ebola Virus Escape Variants in Infected Nonhuman Primates Treated with the MB-003 Antibody Cocktail. *Cell Reports* 12:2111–2120.
19. Zhao X, Sullender WM. 2005. In Vivo Selection of Respiratory Syncytial Viruses Resistant to Palivizumab. *J Virol* 79:3962–3968.
20. Zhao X, Chen F-P, Sullender WM. 2004. Respiratory syncytial virus escape mutant derived in vitro resists palivizumab prophylaxis in cotton rats. *Virology* 318:608–612.
21. Zhu Q, McAuliffe JM, Patel NK, Palmer-Hill FJ, Yang C, Liang B, Su L, Zhu W, Wachter L, Wilson S, MacGill RS, Krishnan S, McCarthy MP, Losonsky GA, Suzich JA. 2011. Analysis of Respiratory Syncytial Virus Preclinical and Clinical Variants Resistant to Neutralization by Monoclonal Antibodies Palivizumab and/or Motavizumab. *J Infect Dis* 203:674–682.
22. Zhao X, Liu E, Chen F-P, Sullender WM. 2006. In Vitro and In Vivo Fitness of Respiratory Syncytial Virus Monoclonal Antibody Escape Mutants. *J Virol* 80:11651–11657.
23. Papenburg J, Carbonneau J, Hamelin M-È, Isabel S, Bouhy X, Ohoumanne N, Déry P, Paes BA, Corbeil J, Bergeron MG, De Serres G, Boivin G. 2012. Molecular Evolution of Respiratory Syncytial Virus Fusion Gene, Canada, 2006–2010. *Emerg Infect Dis* 18:120–124.

24. Boivin G, Caouette G, Frenette L, Carbonneau J, Ouakki M, De Serres G. 2008. Human respiratory syncytial virus and other viral infections in infants receiving palivizumab. *J Clin Virol* 42:52–57.
25. Zhu Q, Patel NK, McAuliffe JM, Zhu W, Wachter L, McCarthy MP, Suzich JA. 2012. Natural Polymorphisms and Resistance-Associated Mutations in the Fusion Protein of Respiratory Syncytial Virus (RSV): Effects on RSV Susceptibility to Palivizumab. *J Infect Dis* 205:635–638.
26. Adams O, Bonzel L, Kovacevic A, Mayatepek E, Hoehn T, Vogel M. 2010. Palivizumab-Resistant Human Respiratory Syncytial Virus Infection in Infancy. *Clinical Infectious Diseases* 51:185–188.
27. Bates JT, Keefer CJ, Slaughter JC, Kulp DW, Schief WR, Crowe JE. 2014. Escape from neutralization by the respiratory syncytial virus-specific neutralizing monoclonal antibody palivizumab is driven by changes in on-rate of binding to the fusion protein. *Virology* 454–455:139–144.
1998. Palivizumab, a humanized respiratory syncytial virus monoclonal antibody, reduces hospitalization from respiratory syncytial virus infection in high-risk infants. The IMPact-RSV Study Group. *Pediatrics* 102:531–537.
29. MedImmune LLC. 2019. A Phase 2b Randomized, Double-Blind, Placebo-controlled Study to Evaluate the Safety and Efficacy of MEDI8897, a Monoclonal Antibody With an Extended Half-life Against Respiratory Syncytial Virus, in Healthy Preterm Infants. study/NCT02878330. Clinical trial registration. clinicaltrials.gov.
30. MedImmune LLC. 2021. A Phase 2/3 Randomized, Double-blind, Palivizumab-controlled Study to Evaluate the Safety of MEDI8897, a Monoclonal Antibody With an Extended Half-life Against Respiratory Syncytial Virus, in High-risk Children (MEDLEY). NCT03959488. Clinical trial registration. clinicaltrials.gov.
31. MedImmune LLC. 2021. A Phase 3 Randomized, Double-blind, Placebo-controlled Study to Evaluate the Safety and Efficacy of MEDI8897, a Monoclonal Antibody With an Extended Half-life Against Respiratory Syncytial Virus, in Healthy Late Preterm and Term Infants (MELODY). NCT03979313. Clinical trial registration. clinicaltrials.gov.
32. AstraZeneca. 2021. A Phase 2, Open-label, Uncontrolled, Single-dose Study to Evaluate the Safety and Tolerability, Pharmacokinetics, and Occurrence of Antidrug Antibody for Nirsevimab in Immunocompromised Children $\leq$ 24 Months of Age. NCT04484935. Clinical trial registration. clinicaltrials.gov.
33. Johnson S, Oliver C, Prince GA, Hemming VG, Pfarr DS, Wang SC, Dormitzer M, O’Grady J, Koenig S, Tamura JK, Woods R, Bansal G, Couchenour D, Tsao E, Hall WC, Young JF. 1997. Development of a humanized monoclonal antibody (MEDI-493) with potent in vitro and in vivo activity against respiratory syncytial virus. *J Infect Dis* 176:1215–1224.

34. Feltes TF, Cabalka AK, Meissner HC, Piazza FM, Carlin DA, Top FH, Connor EM, Sondheimer HM, Cardiac Synagis Study Group. 2003. Palivizumab prophylaxis reduces hospitalization due to respiratory syncytial virus in young children with hemodynamically significant congenital heart disease. *J Pediatr* 143:532–540.
35. Cingoz O. 2009. Motavizumab. *MAbs* 1:439–442.
36. Wu H, Pfarr DS, Johnson S, Brewah YA, Woods RM, Patel NK, White WI, Young JF, Kiener PA. 2007. Development of Motavizumab, an Ultra-potent Antibody for the Prevention of Respiratory Syncytial Virus Infection in the Upper and Lower Respiratory Tract. *Journal of Molecular Biology* 368:652–665.
37. Wu H, Pfarr DS, Tang Y, An L-L, Patel NK, Watkins JD, Huse WD, Kiener PA, Young JF. 2005. Ultra-potent antibodies against respiratory syncytial virus: effects of binding kinetics and binding valence on viral neutralization. *J Mol Biol* 350:126–144.
38. Beeler JA, van Wyke Coelingh K. 1989. Neutralization epitopes of the F glycoprotein of respiratory syncytial virus: effect of mutation upon fusion function. *J Virol* 63:2941–2950.
39. Melero JA, Moore ML. 2013. Influence of Respiratory Syncytial Virus Strain Differences on Pathogenesis and Immunity. *Curr Top Microbiol Immunol* 372:59–82.
40. McLellan JS, Ray WC, Peeples ME. 2013. Structure and Function of RSV Surface Glycoproteins. *Curr Top Microbiol Immunol* 372:83–104.
41. McLellan JS, Chen M, Kim A, Yang Y, Graham BS, Kwong PD. 2010. Structural basis of respiratory syncytial virus neutralization by motavizumab. *Nat Struct Mol Biol* 17:248–250.
42. McLellan JS. 2015. Neutralizing Epitopes on the Respiratory Syncytial Virus Fusion Glycoprotein. *Curr Opin Virol* 11:70–75.
43. Arbiza J, Taylor G, Lopez JA, Furze J, Wyld S, Whyte P, Stott EJ, Wertz G, Sullender W, Trudel M, Melero JA. 1992. Characterization of two antigenic sites recognized by neutralizing monoclonal antibodies directed against the fusion glycoprotein of human respiratory syncytial virus. *Journal of General Virology* 73:2225–2234.
44. Gilman MSA, Moin SM, Mas V, Chen M, Patel NK, Kramer K, Zhu Q, Kabeche SC, Kumar A, Palomo C, Beaumont T, Baxa U, Ulbrandt ND, Melero JA, Graham BS, McLellan JS. 2015. Characterization of a Prefusion-Specific Antibody That Recognizes a Quaternary, Cleavage-Dependent Epitope on the RSV Fusion Glycoprotein. *PLoS Pathog* 11:e1005035.
45. Zhu Q, McLellan JS, Kallewaard NL, Ulbrandt ND, Palaszynski S, Zhang J, Moldt B, Khan A, Svabek C, McAuliffe JM, Wrapp D, Patel NK, Cook KE, Richter BW, Ryan PC, Yuan AQ, Suzich JA. 2017. A highly potent extended half-life antibody as a potential RSV vaccine surrogate for all infants. *Sci Transl Med* 9:eaaj1928.

46. Gilman MSA, Furmanova-Hollenstein P, Pascual G, B. van 't Wout A, Langedijk JPM, McLellan JS. 2019. Transient opening of trimeric prefusion RSV F proteins. 1. *Nature Communications* 10:2105.
47. Jones HG, Battles MB, Lin C-C, Bianchi S, Corti D, McLellan JS. 2019. Alternative conformations of a major antigenic site on RSV F. *PLoS Pathog* 15:e1007944.
48. Meng J, Lee S, Hotard AL, Moore ML. 2014. Refining the Balance of Attenuation and Immunogenicity of Respiratory Syncytial Virus by Targeted Codon Deoptimization of Virulence Genes. *mBio* 5.
49. McLellan JS, Chen M, Leung S, Graepel KW, Du X, Yang Y, Zhou T, Baxa U, Yasuda E, Beaumont T, Kumar A, Modjarrad K, Zheng Z, Zhao M, Xia N, Kwong PD, Graham BS. 2013. Structure of RSV Fusion Glycoprotein Trimer Bound to a Prefusion-Specific Neutralizing Antibody. *Science* 340:1113–1117.
50. Copin R, Baum A, Wloga E, Pascal KE, Giordano S, Fulton BO, Zhou A, Negron N, Lanza K, Chan N, Coppola A, Chiu J, Ni M, Wei Y, Atwal GS, Hernandez AR, Saotome K, Zhou Y, Franklin MC, Hooper AT, McCarthy S, Hamon S, Hamilton JD, Staples HM, Alfson K, Carrion R, Ali S, Norton T, Somersan-Karakaya S, Sivapalasingam S, Herman GA, Weinreich DM, Lipsich L, Stahl N, Murphy AJ, Yancopoulos GD, Kyratsous CA. 2021. The monoclonal antibody combination REGEN-COV protects against SARS-CoV-2 mutational escape in preclinical and human studies. *Cell* 184:3949–3961.e11.
51. Lewis FA, Rae ML, Lehmann NI, Ferris AA. 1961. A Syncytial Virus Associated with Epidemic Disease of the Lower Respiratory Tract in Infants and Young Children. *Medical Journal of Australia* 2:932–933.
52. McLellan JS, Chen M, Chang J-S, Yang Y, Kim A, Graham BS, Kwong PD. 2010. Structure of a Major Antigenic Site on the Respiratory Syncytial Virus Fusion Glycoprotein in Complex with Neutralizing Antibody 101F. *Journal of Virology* <https://doi.org/10.1128/JVI.01579-10>.
53. Eshaghi A, Duvvuri VR, Lai R, Nadarajah JT, Li A, Patel SN, Low DE, Gubbay JB. 2012. Genetic Variability of Human Respiratory Syncytial Virus A Strains Circulating in Ontario: A Novel Genotype with a 72 Nucleotide G Gene Duplication. *PLoS One* 7:e32807.
54. Jo WK, Schadenhofer A, Habierski A, Kaiser FK, Saletti G, Ganzenmueller T, Hage E, Haid S, Pietschmann T, Hansen G, Schulz TF, Rimmelzwaan GF, Osterhaus ADME, Ludlow M. 2021. Reverse genetics systems for contemporary isolates of respiratory syncytial virus enable rapid evaluation of antibody escape mutants. *PNAS* 118.
55. Zhu Q, Lu B, McTamney P, Palaszynski S, Diallo S, Ren K, Ulbrandt ND, Kallewaard N, Wang W, Fernandes F, Wong S, Svabek C, Moldt B, Esser MT, Jing H, Suzich JA. 2018. Prevalence and Significance of Substitutions in the Fusion Protein of Respiratory Syncytial Virus Resulting in Neutralization Escape From Antibody MEDI8897. *The Journal of Infectious Diseases* 218:572–580.

56. Gonzalez TR, Martin KP, Barnes JE, Patel JS, Ytreberg FM. 2020. Assessment of software methods for estimating protein-protein relative binding affinities. *PLOS ONE* 15:e0240573.
57. Planas D, Veyer D, Baidaliuk A, Staropoli I, Guivel-Benhassine F, Rajah MM, Planchais C, Porrot F, Robillard N, Puech J, Prot M, Gallais F, Gantner P, Velay A, Le Guen J, Kassis-Chikhani N, Edriss D, Belec L, Seve A, Courtellemont L, Péré H, Hocqueloux L, Fafi-Kremer S, Prazuck T, Mouquet H, Bruel T, Simon-Lorière E, Rey FA, Schwartz O. 2021. Reduced sensitivity of SARS-CoV-2 variant Delta to antibody neutralization. 7871. *Nature* 596:276–280.
58. VanBlargan LA, Errico JM, Halfmann PJ, Zost SJ, Crowe JE, Purcell LA, Kawaoka Y, Corti D, Fremont DH, Diamond MS. 2022. An infectious SARS-CoV-2 B.1.1.529 Omicron virus escapes neutralization by therapeutic monoclonal antibodies. *Nat Med* 1–6.
59. Lusvarghi S, Wang W, Herrup R, Neerukonda SN, Vassell R, Bentley L, Eakin AE, Erlandson KJ, Weiss CD. Key Substitutions in the Spike Protein of SARS-CoV-2 Variants Can Predict Resistance to Monoclonal Antibodies, but Other Substitutions Can Modify the Effects. *J Virol* 96:e01110-21.
60. Webb B, Sali A. 2016. Comparative Protein Structure Modeling Using MODELLER. *Current Protocols in Bioinformatics* 54:5.6.1–5.6.37.
61. Aliev AE, Kulke M, Khaneja HS, Chudasama V, Sheppard TD, Lanigan RM. 2014. Motional timescale predictions by molecular dynamics simulations: Case study using proline and hydroxyproline sidechain dynamics. *Proteins: Structure, Function, and Bioinformatics* 82:195–215.
62. Van Der Spoel D, Lindahl E, Hess B, Groenhof G, Mark AE, Berendsen HJC. 2005. GROMACS: Fast, flexible, and free. *Journal of Computational Chemistry* 26:1701–1718.
63. Buchholz UJ, Finke S, Conzelmann K-K. 1999. Generation of Bovine Respiratory Syncytial Virus (BRSV) from cDNA: BRSV NS2 Is Not Essential for Virus Replication in Tissue Culture, and the Human RSV Leader Region Acts as a Functional BRSV Genome Promoter. *Journal of Virology* <https://doi.org/10.1128/JVI.73.1.251-259.1999>.
64. McKimm-Breschkin JL. 2004. A simplified plaque assay for respiratory syncytial virus—direct visualization of plaques without immunostaining. *Journal of Virological Methods* 120:113–117.
65. Chen M, Chang JS, Nason M, Rangel D, Gall JG, Graham BS, Ledgerwood JE. 2010. A flow cytometry-based assay to assess RSV-specific neutralizing antibody is reproducible, efficient and accurate. *Journal of Immunological Methods* 362:180–184.
66. Morton CJ, Cameron R, Lawrence LJ, Lin B, Lowe M, Luttick A, Mason A, McKimm-Breschkin J, Parker MW, Ryan J, Smout M, Sullivan J, Tucker SP, Young PR. 2003. Structural characterization of respiratory syncytial virus fusion inhibitor escape mutants: homology model of the F protein and a syncytium formation assay. *Virology* 311:275–288.

67. Branigan PJ, Liu C, Day ND, Gutshall LL, Sarisky RT, Del Vecchio AM. 2005. Use of a novel cell-based fusion reporter assay to explore the host range of human respiratory syncytial virus F protein. *Virology Journal* 2:54.

Figure Legends

Figure 1. Molecular modeling of F protein and motavizumab interaction. (A) F protein trimer (dark gray) monomer (gray ribbon) with motavizumab (gold ribbon) structure with interacting residues of interest: L258 (pink), N262 (teal), K272 (lavender), S275 (purple), and N276 (blue). Based on PDB ID:4ZYP. (B) Plot of MD+FoldX predictions of $\Delta\Delta G_{\text{Fold}}$ and $\Delta\Delta G_{\text{Bind}}$ of all possible mutations in F with eight selected variants.

Figure 2. All eight variants demonstrated ability to replicate and propagate new virus.

HEp-2 cells were infected with 0.1 MOI of variant or WT virus and supernatant media were collected over time. Virus was titrated by TCID₅₀ assay on HEp-2 cells and is shown as the average and SEM of three independent experiments. * $p < 0.05$; ** $p < 0.005$; *** $p < 0.001$.

Figure 3. Microneutralization assay revealed reduced neutralization by motavizumab for six of the eight variants. Motavizumab was serially diluted two-fold (10000 ng/mL-156 ng/mL) and incubated with variants prior to infection of HEp-2 cells at an MOI of 1. mKate2-positive cells were counted by flow cytometry at 18 hours post-infection. Error bars indicate SEM of three independent experiments.

Figure 4. Flow cytometry revealed reduced binding of motavizumab to MARMs. Cells were transiently transfected with variant F protein-expressing plasmids or an empty vector control (pcDNA3.1) and dual stained with motavizumab-488 and 101F-594. Cells were gated on the

101F positive cell population, and the MFI of the motavizumab-Alexa Fluor 488 was quantified. (A) Histograms of motavizumab-488 positive cells gated on 101F-594 positive cell population are representative of three replicate experiments. (B) MFI of motavizumab-488 channel on 101F-594-gated cell population showing averages and SEM from three replicate experiments. $*p < 0.05$. (C) Graph of $\Delta\Delta G_{\text{Bind}}$ vs. average MFI of motavizumab-488.

Figure 5. SPR revealed reduced on-rate as escape mechanism for variants. Purified virions were flowed over motavizumab at 40 nM, 20 nM, 10 nM, and 5 nM and (A) on-rate, (B) off-rate, and (C) affinity measurements were calculated. Three independent experiments were carried out for each variant. $*p < 0.05$; $**p < 0.005$; $***p < 0.001$. Graphs of (D) on-rate, (E) off-rate, and (F) affinity and $\Delta\Delta G_{\text{Bind}}$.

Table 1. $\Delta\Delta G_{\text{Fold}}$ and $\Delta\Delta G_{\text{Bind}}$ values and standard deviations of eight selected variants

Table 2. IC_{50} values and fold change compared to WT of selected variants

Table 3. Binding kinetics of selected variants to motavizumab

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Table 1. $\Delta\Delta G_{\text{Fold}}$ and $\Delta\Delta G_{\text{Bind}}$ values and standard deviations of eight selected variants

Mutation	$\Delta\Delta G_{\text{Bind}}$	SD of Bind	$\Delta\Delta G_{\text{Fold}}$	SD of Fold
N276G	0.50	0.59	0.32	0.43
L258K	1.02	0.52	0.37	0.53
L258E	1.59	0.48	0.96	0.29
K272E	2.09	0.98	-0.28	0.59
N262D	2.56	1.11	0.48	0.67
N262Y	2.90	3.61	-0.58	0.73
S275R	3.79	2.76	-0.87	0.90
S275H	5.56	3.82	1.24	1.45

Table 2. IC₅₀ values and fold change compared to WT of selected variants

Variant	WT	L258E	L258K	N262D	N262Y	K272E	S275H	S275R	N276G
IC ₅₀ (ng/mL)	290.4	1101	1890	1081	223.5	>10000	3966	1633	756.4
Fold change	-	3.8	6.5	3.7	0.8	-	13.7	5.6	2.6

Table 3. Binding kinetics of selected variants to motavizumab

Variant	k_a (1/(M*s))	Fold Change	k_d (1/s)	Fold change	KD (M)	Fold Change
WT	6.01E+05		4.64E-04		9.2E-10	
L258K	2.26E+05	-2.7	2.14E-04	+0.46	9.56E-10	-1.0
K272E	1.70E+05	-3.5	2.32E-04	+0.50	1.35E-09	-1.5
S275H	9.81E+04	-6.1	3.14E-04	+0.68	3.01E-09	-3.3