

Prefusion F-specific antibodies determine the magnitude of RSV neutralizing activity in human sera

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Respiratory syncytial virus (RSV) is estimated to claim more lives among infants <1 year old than any other single pathogen, except malaria, and poses a substantial global health burden. Viral entry is mediated by a type I fusion glycoprotein (F) that transitions from a metastable prefusion (pre-F) to a stable postfusion (post-F) trimer. A highly neutralization-sensitive epitope, antigenic site Ø, is found only on pre-F. We determined what fraction of neutralizing (NT) activity in human sera is dependent on antibodies specific for antigenic site Ø or other antigenic sites on F in healthy subjects from ages 7 to 93 years. Adsorption of individual sera with stabilized pre-F protein removed >90% of NT activity and depleted binding antibodies to both F conformations. In contrast, adsorption with post-F removed ~30% of NT activity, and binding antibodies to pre-F were retained. These findings were consistent across all age groups. Protein competition neutralization assays with pre-F mutants in which sites Ø or II were altered to knock out binding of antibodies to the corresponding sites showed that these sites accounted for ~35 and <10% of NT activity, respectively. Binding competition assays with monoclonal antibodies (mAbs) indicated that the amount of site Ø-specific antibodies correlated with NT activity, whereas the magnitude of binding competed by site II mAbs did not correlate with neutralization. Our results indicate that RSV NT activity in human sera is primarily derived from pre-F-specific antibodies, and therefore, inducing or boosting NT activity by vaccination will be facilitated by using pre-F antigens that preserve site Ø.

INTRODUCTION

Human respiratory syncytial virus (RSV) infects virtually every child by 2 years of age (1) and annually accounts for an estimated 33 million lower respiratory tract infections in children less than 5 years of age (2). Of 11 proteins expressed by this paramyxovirus, the F and G glycoproteins are known to generate protective neutralizing (NT) antibody responses (3). However, F displays more NT epitopes, is highly conserved, is required for fusion and entry of RSV into host cells, and therefore is a primary target for vaccine-induced protection (4). Currently, at least four described antigenic sites on F are associated with virus neutralization. Site I is a target for monoclonal antibodies (mAbs) such as 2F, 44F, or 45F (5) with weak or negligible NT activity and is defined by a P389 escape mutation. Site II comprises the epitope for palivizumab, a licensed mAb administered prophylactically to infants at high risk of severe disease (6). Site IV is recognized by mAbs such as mAb19 (7) or 101F (8) with moderate NT activity. All the mAbs that recognize these three sites can bind the stable postfusion (post-F) conformation (9). The recent structural definition of the prefusion (pre-F) trimer revealed a new antigenic site (site Ø), which is targeted by mAbs such as D25, AM22, and 5C4 that have NT potency 10- to 100-fold greater than palivizumab (10). Another epitope on F is recognized by the mAb MPE8 (11), which has been mapped to a region adjacent to antigenic site II but

binds almost exclusively to the pre-F conformation of the molecule. Other pre-F-specific antibodies such as AM14 (12), which binds to a quaternary epitope only present in stable trimers (13), have been recently identified.

Immunization with a stabilized version of the pre-F trimer induces significantly higher NT responses than immunization with a post-F immunogen (14), suggesting that pre-F-specific antibodies are more readily elicited and potent than antibodies targeting sites shared by post-F. Therefore, despite the success achieved by passive immunoprophylaxis with palivizumab, which targets the shared antigenic site II, other pre-F-specific surfaces are likely to induce antibody responses with more potent RSV neutralization. Also, there are some limitations in the use of palivizumab. For example, treatment is only recommended for premature infants, those with congenital heart disease, and other select populations at high risk of severe disease (6). Because most hospitalizations occur in infants without identified risk factors (15) and there is a continuing high burden of disease in older children and the frail elderly (16), there remains a need to understand the basis for RSV immunity to develop approaches for preventing RSV disease in the entire birth cohort.

A previous study by Melero and colleagues demonstrated that depletion of antibodies to the post-F conformation does not remove NT activity from the sera of rabbits immunized with RSV. In the same study, pooled polyclonal human sera screened for high levels of NT activity (RSVIG) was shown to retain most of NT activity after adsorption with post-F (17), suggesting the importance of unique NT epitopes present on alternative conformations of F (17). The ability to stabilize pre-F using a structure-guided atomic-level design (14) has enabled the generation of reagents that can detect differential NT activity against the two major conformations of F. We characterized human serum responses to pre-F and post-F and further defined serum NT antibody

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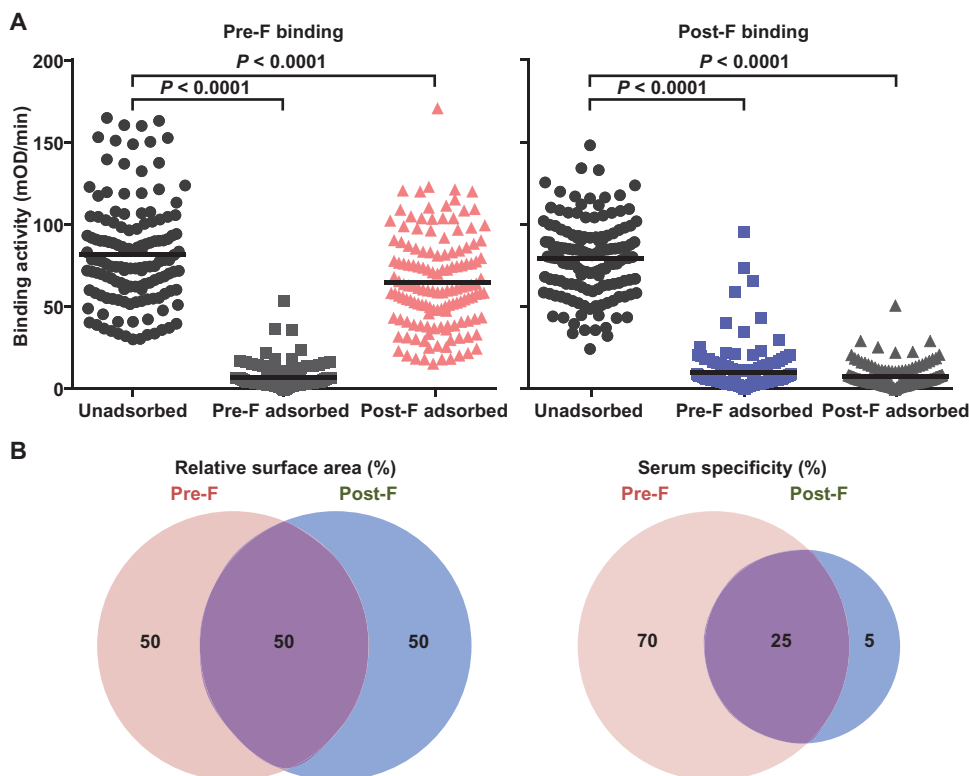


Fig. 1. Serum binding activity to the pre-F and post-F conformations of the RSV F glycoprotein. (A) Human sera were analyzed for binding to pre-F and post-F conformations by kinetic ELISA (black circles, left and right panels, respectively). Binding of unadsorbed sera to pre-F and post-F binding was similar (75.8 and 75.4 mOD/min, respectively). Sera were also analyzed for binding to either F conformation after adsorption with pre-F or post-F subunit proteins. As expected, pre-F adsorption removed antibodies that could bind to pre-F, and post-F adsorption removed antibodies that could bind post-F. Post-F adsorption did not remove a substantial amount of pre-F binding antibodies (pink triangles, left panel), but pre-F adsorption removed nearly all post-F binding antibodies (light blue squares, right panel). (B) Pre-F and post-F conformations share about 50% of their surface, and about 50% of the surface is unique to each. On the basis of the binding data, about 70% of antibodies in human sera bind pre-F-specific surfaces, about 25% bind the shared surfaces, and about 5% bind post-F-specific surfaces, which is disproportionate to the available surface area.

responses that target antigenic sites Ø and II in a population spanning ages 7 to 93 years.

RESULTS

RSV F-specific binding antibodies in human sera are primarily specific for pre-F

To evaluate the level of antibody response to pre-F and post-F (expressed from RSV A2 constructs) in human sera, we used both proteins in a kinetic enzyme-linked immunosorbent assay (ELISA) analysis. Untreated human serum samples bound pre-F and post-F with similar magnitude [geometric mean titers (GMTs) of 75.8 and 75.4 milli-optical density (mOD)/min, respectively; $n = 140$] (Fig. 1A). Serum adsorption with pre-F removed nearly all binding activity to both F conformations (7.6% residual binding to pre-F and 10% residual binding to post-F after pre-F adsorption; $P < 0.0001$ for both cases compared to unadsorbed sera, $n = 140$, Wilcoxon signed-rank test) (Fig. 1A). In contrast, adsorption with post-F did not substantially affect serum binding to pre-F (77% residual binding) but, as

expected, removed binding to post-F (8.7% residual binding, $P < 0.0001$, $n = 140$, Wilcoxon signed-rank test), which suggests that about 90% of antibodies in human sera that bind to RSV F are able to recognize pre-F, whereas <10% can exclusively bind post-F. ELISA confirmed negligible residual pre-F or post-F following adsorption (table S1 and fig. S1), and a hemagglutination inhibition assay (HAI) showed that F proteins did not non-specifically remove influenza-specific antibodies in sera (table S3). The differential antibody binding activity of sera to pre-F and post-F is not proportional to the exposed surfaces of each F conformation: ~50% is shared between pre-F and post-F and ~50% is unique (Fig. 1B) (14). Therefore, either the unique surfaces on pre-F are more immunogenic than those on post-F, or post-F is less abundant or less exposed during natural infection. Whereas there was variation in the binding ratio of pre-F to post-F among individuals, age stratification by decade revealed no major differences in relative binding specificity across age groups (fig. S2).

Adsorption of antibodies directed against pre-F removes NT activity from human sera

We assessed sera for their ability to block HEp-2 infection by RSV A2. Serum adsorption with pre-F removed nearly all serum NT activity, reducing the reciprocal median effective concentration (EC_{50}) GMT from 511 to 9 (Fig. 2A, $P < 0.0001$, $n = 121$, Wilcoxon signed-rank test). Adsorption with post-F reduced serum NT activity by 29% (EC_{50} GMT from 511 to 362; $P < 0.0001$, Wilcoxon signed-rank test) (Fig. 2A). Therefore, these results

suggest that pre-F-specific antigenic sites account for most NT antibody targets, and shared sites (I, II, and IV) on both F conformations must account for most of what is removed by the post-F adsorption. The pattern of pre-F and post-F adsorption on NT activity was consistent across all age groups (Fig. 2, B and C, and fig. S4). Despite the dependence of neutralization on pre-F-specific antibodies, we could not establish a correlation between overall pre-F binding and NT activity, and we therefore assessed binding to individual antigenic sites (Ø and II).

RSV F knockout proteins serve as a platform to define antibody specificity

To evaluate the contribution of serum antibodies to specific antigenic sites on pre-F to the overall NT activity in sera, we designed knockout mutations that abolished mAb binding to antigenic sites Ø and II (Fig. 3 and table S2). These sites were chosen for analysis primarily because site Ø is pre-F-specific and known to induce potent NT antibodies, whereas site II is present on both F conformations and the target of the currently licensed mAb for prophylactic treatment, palivizumab. RSV F molecules that did not bind to antigenic site Ø-specific antibodies (D25, AM22, and

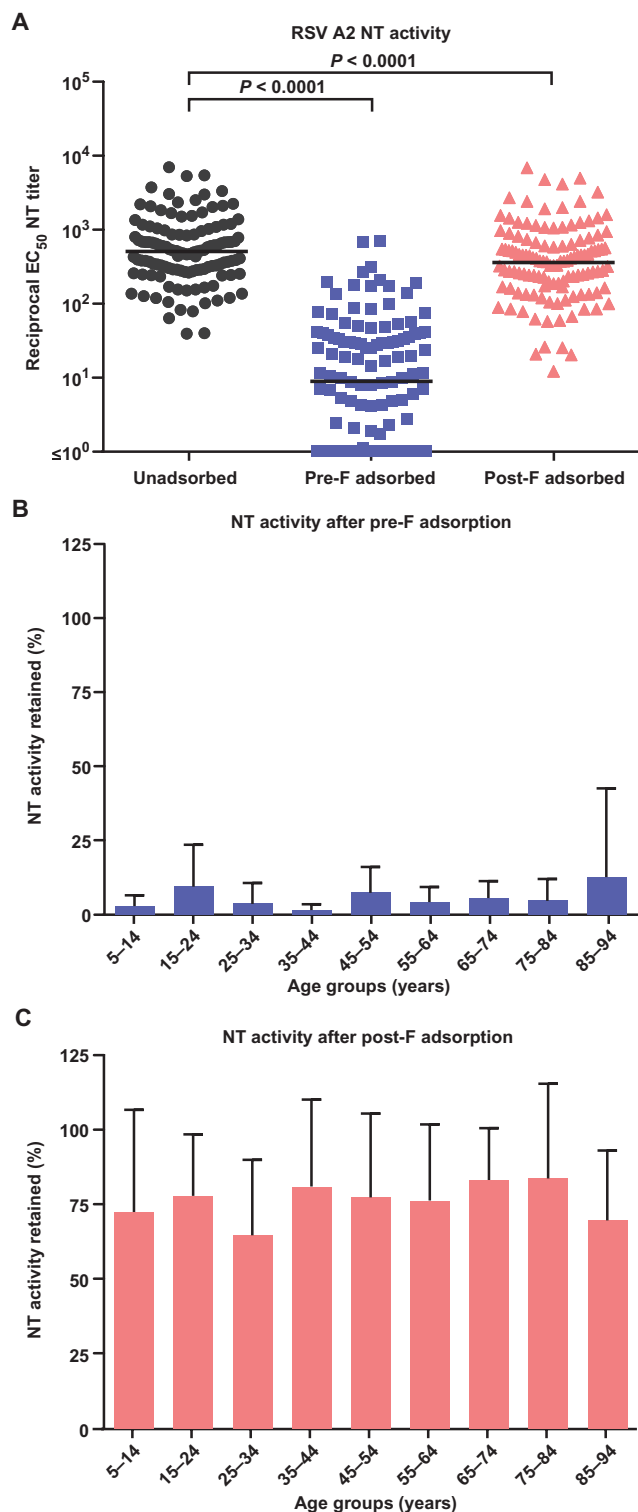


Fig. 2. Serum NT activity against RSV after adsorption with pre-F and post-F. (A) Geometric mean reciprocal EC_{50} NT titers for unadsorbed sera and sera adsorbed with pre-F or post-F were 511, 9, and 362, respectively. (B) Evaluation of samples by age groups showed that less than 5% of NT activity was retained after adsorption with pre-F in all age groups. (C) Adsorption with post-F removed 20 to 45% of the original NT activity, and there were no trends according to age group.

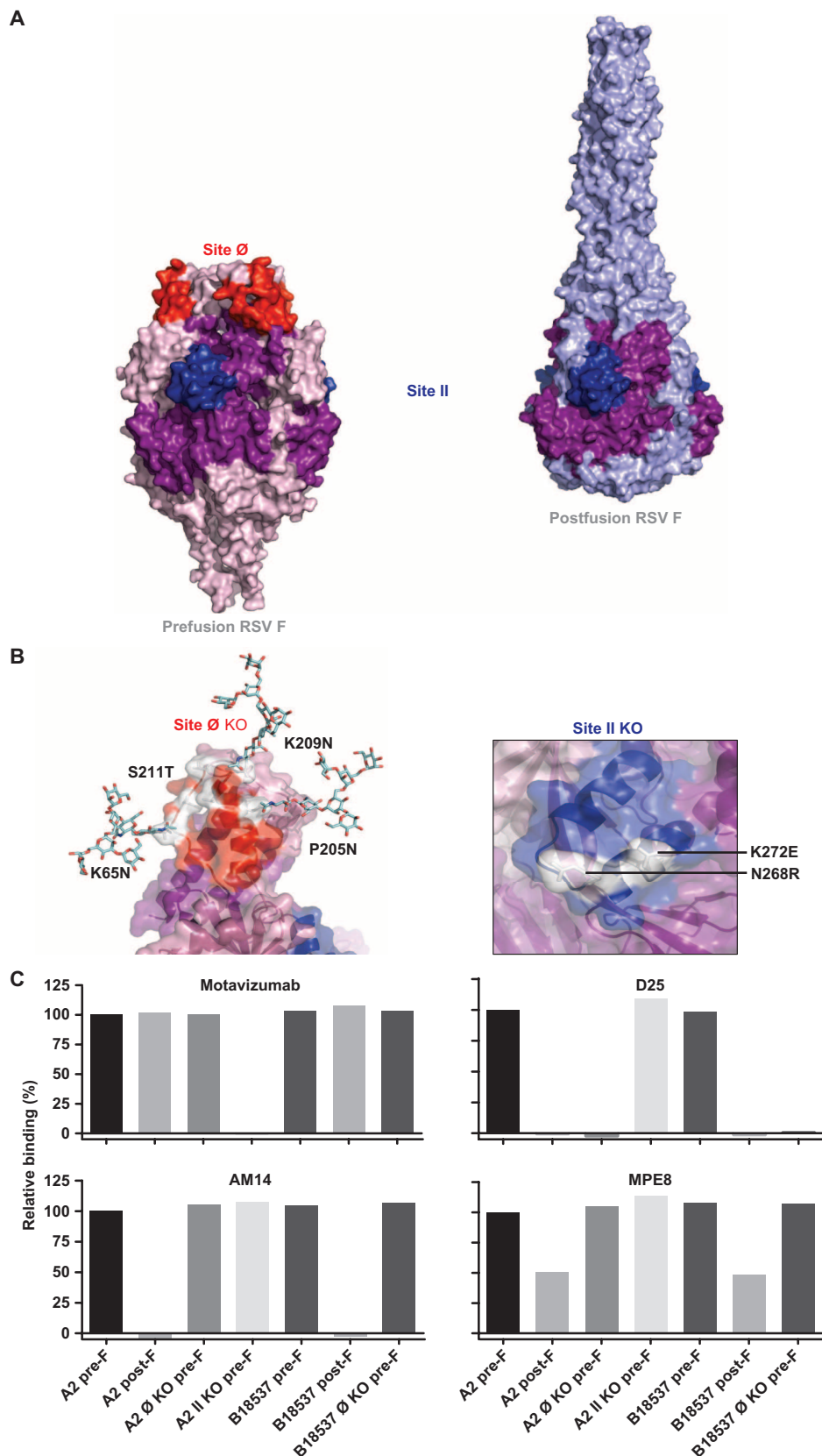
5C4) or site II-specific antibodies (motavizumab and palivizumab) were designed using two approaches: (i) addition of *N*-glycan sequons and (ii) mutation of critical contact residues. For *N*-glycan sequon design, surface-exposed residues were identified on the RSV F molecule that were in close proximity to the D25 binding site (10) or the motavizumab binding site (18). *N*-Glycan sequon (Asn-X-Ser/Thr) mutations were modeled at these sites, and the resulting models were assessed. Three residue positions in RSV F antigenic site Ø and in antigenic site II were chosen on the basis of the prediction that they will allow *N*-glycan introduction with minimal clashes, minimal disruption of hydrogen bonding patterns, and a high likelihood of *N*-glycan addition as assessed by NGlycPred (19). In addition, four residues in antigenic site Ø and three residues in antigenic site II that displayed extensive interactions with either D25 or motavizumab and various mutations to these residues were modeled. Mutants that were chosen exhibited maximal disruption of antibody binding as judged by clash scores and charge incompatibility, and designated site Ø or site II knockout (KO). Octet biolayer interferometry (BLI) was used to assess binding of mAbs to the pre-F variants, and two mutants were chosen that fully disrupted site Ø or site II antibody binding while preserving the binding of mAbs to other pre-F-specific antigenic sites associated with NT activity (Fig. 3C). These mutations were also introduced to RSV F glycoproteins from a B-subtype strain (18537) to knock out binding of the site Ø-specific antibodies (Fig. 3C and table S2). Successful knockout of these sites enabled the analysis of site-specific serum neutralizing activity.

Antigenic site Ø is a major but not the sole target for NT antibodies exclusive to pre-F

The selected pre-F variants with mutations to sites Ø and II, along with wild-type pre-F and post-F, were used in neutralization competition assays to determine what fraction of the pre-F-specific NT activity adsorbed by pre-F could be attributed to sites Ø and II. Assays were performed on samples that exhibited NT titers at the 25th, 50th, 75th, and 95th percentiles within each 10-year age group. Stabilized RSV A2 pre-F, post-F, or pre-F with either site Ø or site II KO were incubated with heat-inactivated serum samples ($n = 32$) before treatment with virus. As observed with the adsorption assay, competition with stabilized wild-type pre-F inhibited >90% of serum NT activity, whereas competition with wild-type post-F removed <30% of NT activity (Fig. 4A). This difference between pre-F- and post-F-specific NT activity was consistent across all subjects. Competition with a site Ø KO pre-F restored 35% (GMT) of NT activity (Fig. 4A; median, 47%; $n = 32$), indicating that antibodies targeting the membrane-distal antigenic site Ø account for a substantial fraction of the overall NT activity in human sera (Fig. 4A). Consistent with these results, incubation of sera ($n = 24$) with pre-F and post-F of the B18537 strain before treatment with virus removed >85 and ~30% of NT activity, respectively. Competition with the site Ø KO pre-F of the B18537 strain restored ~47% of NT activity (Fig. 4B), suggesting the importance of unique pre-F antigenic sites across RSV subtypes in the generation of functional antibody response. In some individuals, nearly all the NT activity was restored when sera were competed with site Ø KO pre-F (Fig. 4C, red bars at the right end). However, in others, the NT activity was largely determined by pre-F-specific antigenic sites other than site Ø (Fig. 4C, extreme left end). These would be sites recognized by MPE8- or AM14-like antibodies or other as yet unidentified pre-F-specific antibodies. Sera competed with the RSV A2 site II KO pre-F had ~8% restoration of NT activity compared to wild-type pre-F-competed sera, suggesting that site II-specific antibodies account for <10% of RSV NT activity in

Fig. 3. Design and characterization of F constructs with mutated antigenic sites Ø and II.

(A) The RSV fusion glycoprotein is shown in the stabilized pre-F (left) and post-F (right) forms in surface representation with antigenic sites Ø and II highlighted in red and dark blue, respectively. The purple surface shown on the post-F and the dark blue motavizumab epitope are common to the surface on pre-F as mentioned in Fig. 1B. **(B)** Close-up views of the antigenic sites Ø and II with the mutated residues are shown. The three nonnative glycans at residues 65, 205, and 209 introduced to block site Ø binding are shown in stick representation. The surface representation is set to 60% transparency with mutated residues shown in stick representation, with a single protomer shown for clarity. **(C)** BLI was used to measure binding by mAbs that bind pre-F: D25, motavizumab, AM14, and MPE8. Antigenic site Ø KO variant Ø-C (A2 strain) and Ø KO of the B18537 strain showed typical binding to all of the pre-F-targeting antibodies except D25, whereas antigenic site II KO variant II-E bound to all antibodies except motavizumab.



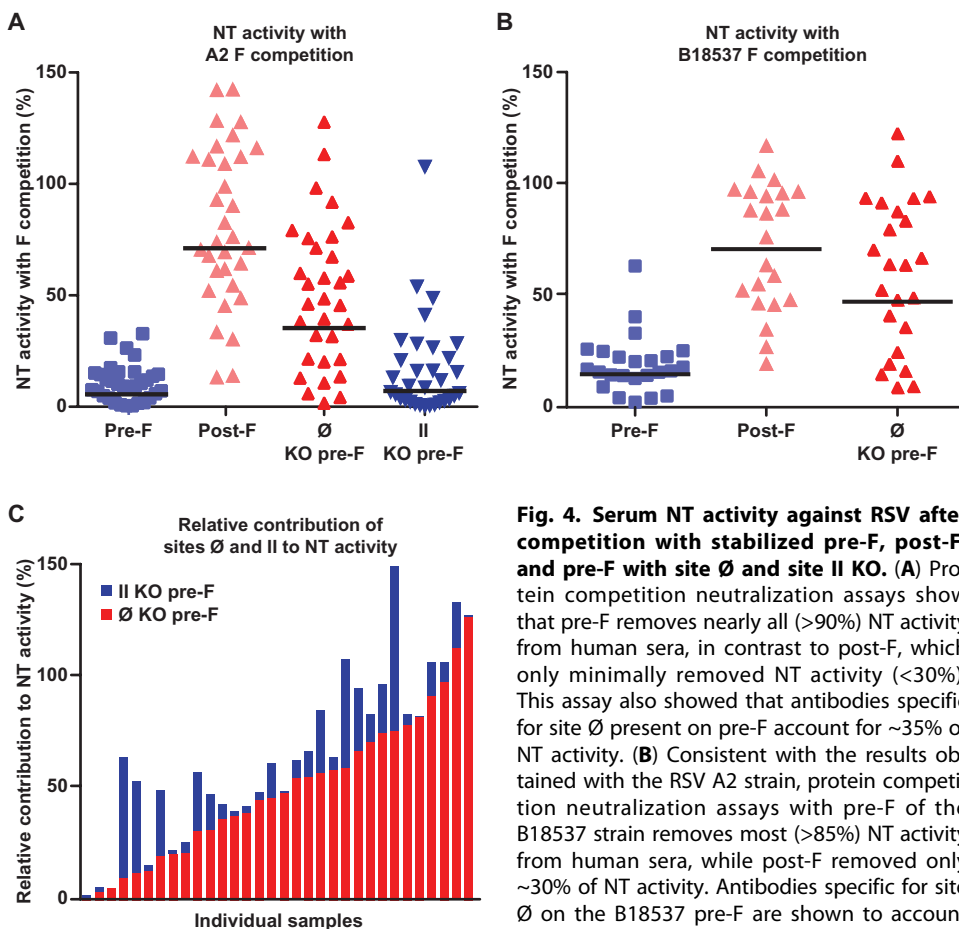


Fig. 4. Serum NT activity against RSV after competition with stabilized pre-F, post-F, and pre-F with site Ø and site II KO. (A) Protein competition neutralization assays show that pre-F removes nearly all (>90%) NT activity from human sera, in contrast to post-F, which only minimally removed NT activity (<30%). This assay also showed that antibodies specific for site Ø present on pre-F account for ~35% of NT activity. (B) Consistent with the results obtained with the RSV A2 strain, protein competition neutralization assays with pre-F of the B18537 strain removes most (>85%) NT activity from human sera, while post-F removed only ~30% of NT activity. Antibodies specific for site Ø on the B18537 pre-F are shown to account for ~45% of NT activity. (C) Results from individual

samples are arranged according to increasing site Ø activity in a stacked bar graph to show the relative amount of NT activity attributed to site Ø or site II. Site Ø-specific antibodies accounted for majority of the NT activity in most subjects (red bars). However, there was significant variation between individuals, and in some subjects, most of NT activity could not be attributed to either site Ø or II (bars on the extreme left) but was clearly pre-F-specific. Although in most individuals, site II antibodies account for some NT activity (dark blue bars), it was a small fraction of the overall activity.

human sera. Similar to site Ø, the relative contribution of site II-specific antibodies varied between individuals, but they contributed a minor fraction of NT activity in most individuals (Fig. 4C, dark blue bars).

Competitive binding assays confirm the presence of unique antigenic sites other than site Ø on pre-F

We next evaluated the fraction of pre-F-specific antibodies binding to individual epitopes by using selected mAbs (Fig. 5A and fig. S6) (D25 and motavizumab) to compete with serum binding to wild-type pre-F by the BLI technique. The assay was validated by six mAbs to RSV, and a cross-competition table was generated (Fig. 5A). We selected the mAbs D25 (site Ø) and motavizumab (site II) for serum antibody competition. The median level of serum antibody blocked by D25 alone was 40% (Fig. 5B). D25 blocked serum binding to a greater extent in higher-NT groups (that is, 75th and 95th percentile samples) than in lower-NT groups (25th and 50th percentiles). The fraction of inhibition by D25 was directly correlated to reciprocal \log_{10} EC_{50} NT titers ($r = 0.45$, $P = 0.0234$, $n = 25$, Pearson's correlation) (Fig. 5C). Motavizumab inhibited about 60% of serum antibody binding, even though antigenic site II antibodies account for

<10% of NT activity. In this assay, pre-F-bound motavizumab prevented serum antibodies from binding not only to its epitope but also to proximal sites such as the MPE8 binding site. This explains the higher overall inhibition of serum antibody binding by motavizumab despite its lack of correlation with NT activity. There was an inverse correlation that did not reach statistical significance between the percent of serum antibody binding inhibited by motavizumab and reciprocal \log_{10} EC_{50} NT titers ($r = -0.26$, $P = 0.20$, $n = 25$, Pearson's correlation) (Fig. 5C), suggesting that there are non-NT antibodies blocked by motavizumab that would also be potentially inhibitory to other more potent MPE8-like NT antibodies in sera. Non-NT antibodies that compete with motavizumab binding to site II were also found in animals immunized with scaffolded motavizumab epitope immunogens (20).

Adsorption of sera with pre-F permits RSV infection of human airway epithelium

Testing neutralization in an immortalized cell line such as HEp-2 is required for large-scale screening but may not completely reflect neutralization in the in vivo target cell. Primary well-differentiated human airway epithelial (HAE) cultures may be better for this purpose (21). RSV uses heparan sulfate as its receptor in immortalized cells (22), but HAE cultures, which display tight junctions, do not display detectable amounts of heparan sulfate on their apical surface where infection is initiated (23). To examine RSV neutralization in HAE cultures,

we chose two sera with high levels of antibody to the RSV A2 G glycoprotein, matching the virus stock used for neutralization assays. Unadsorbed and pre-F adsorbed versions of both samples were evaluated for the presence of antibodies targeting the G glycoprotein associated with preventing RSV infection of HAE cells. The NT curves for unadsorbed and adsorbed sera were similar to the NT curves in HEp-2 cells, suggesting that antibodies to F neutralize at a similar level in both cell types (fig. S7). However, the shapes of the NT curves differed, with a steeper Hill slope for pre-F adsorbed than for unadsorbed sera, a difference particularly obvious in the HAE cultures. Overall, the NT activity that was not removed by pre-F adsorption and therefore potentially attributable to G was 2 to 3% in serum 1 and 14 to 20% in serum 11, further suggesting that antibodies to F are the primary determinants of NT activity, even in primary HAE cells.

DISCUSSION

Understanding the human immune response to natural infection can inform vaccine development because this enables an insight into what

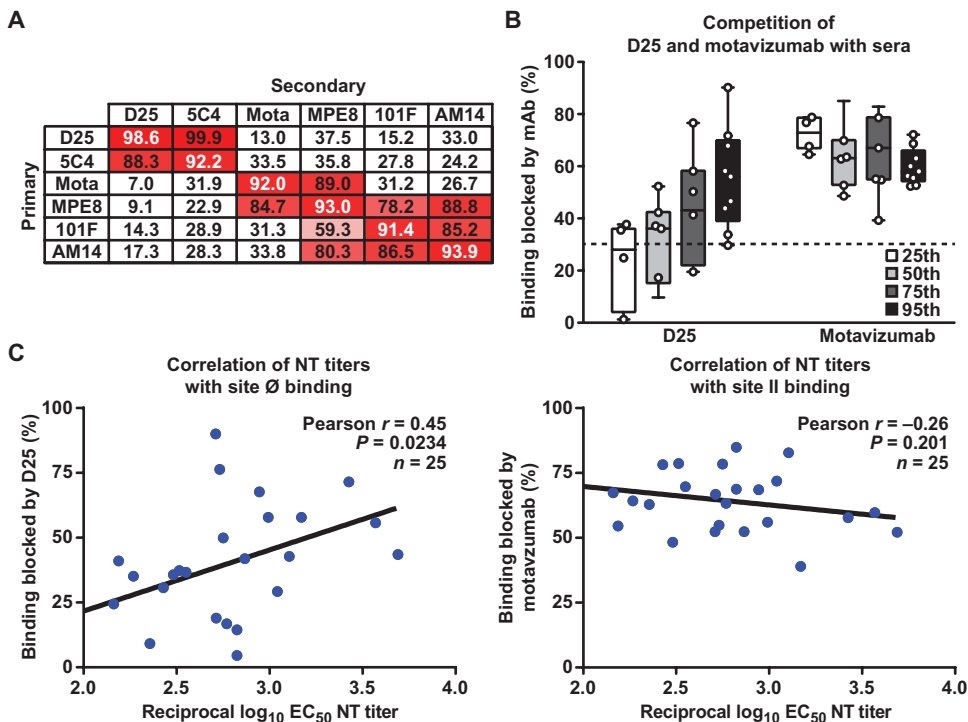


Fig. 5. Octet BLI competition assay using D25 and motavizumab. (A) In a BLI assay, biosensor tips were dipped in pre-F, then into analyte, and then into competing antibody. D25 does not inhibit motavizumab binding to pre-F and vice versa, making these mAbs eligible for use in serum analysis. (B) Biosensor tips were dipped into pre-F, then into D25 or motavizumab, and then into sera. The mean percent inhibition of serum antibody binding by D25 is ~40%, and that by motavizumab is ~60%. Unlike motavizumab, there is increasing inhibition by D25 with increasing NT titers. The dashed line represents background binding inhibition. (C) A moderate correlation is found between percent binding inhibited by D25 and reciprocal EC_{50} NT titers, and a weak inverse correlation was found between percent binding blocked by motavizumab and NT titers.

protein(s) must be included in a vaccine to generate protective immunity. An earlier study showed that in high-titer pooled human immunoglobulin, NT activity could not be removed with post-F, suggesting that pre-F-specific antibodies may be important in RSV neutralization (17). Here, we sought to determine the specificity of antibodies in individual human sera for the two major conformations of the RSV F glycoprotein. Therefore, a cross-sectional serological analysis was performed to evaluate the neutralization potency and specificity of sera from healthy volunteers ranging in age from 7 to 93 years. We found that unadsorbed sera had similar binding activity to both conformations of RSV F, which was not surprising because ~50% of the surface area of pre-F and post-F conformations is shared, including well-characterized antigenic sites II and IV to which the prototypic NT mAbs palivizumab/motavizumab and 101F bind, respectively (10). In contrast, the NT activity in human sera is nearly all removed by adsorption with pre-F, whereas only a small fraction is removed by adsorption with post-F. As noted, the moderately neutralization-sensitive antigenic sites II and IV are present on both conformations of F, whereas pre-F also displays the more neutralization-sensitive sites such as antigenic site Ø and the MPE8 and AM14 epitopes. Given the overlap of epitopes, post-F presentation—in the context of vaccination or natural infection—will induce a significant amount of antibodies that bind pre-F. This is consistent with our finding that there is no correlation between pre-F or post-F binding (or their ratio) and overall NT activity or NT activity within a particular age

group. It is likely that the level of NT activity in human serum is a more complex function of epitope access, binding affinity and specificity for epitopes associated with different levels of neutralization, non-NT antibodies that may interfere with binding to more functionally relevant sites, and synergy or interference of different antibodies when multiple sites are targeted simultaneously. Therefore, a simple ELISA assay using pre-F or post-F will not be sufficient to predict the NT activity in serum.

The analysis of sera by competition with site Ø and site II KO probes demonstrated that there are diverse patterns of binding to the various sites associated with NT activity. Whereas a substantial amount of NT activity in most human sera can be accounted for by antigenic site Ø-specific antibodies, there are some individuals whose predominant response is to other pre-F-specific sites (Fig. 4C), because some samples that lost NT activity after competition with wild-type pre-F did not regain this activity when site Ø KO pre-F was used in competition. As the complete antigenic topology of pre-F and the antibody content of human serum become more fully defined, it may be possible to predict NT activity on the basis of the magnitude and composition of epitope-specific antibodies. About 10% of samples had an increase in NT activity after adsorption with post-F.

One potential explanation for this phenomenon is that non-NT antibodies that recognize post-F surfaces may also bind pre-F and block the access of antibodies with more potent NT activity. For example, the abundance of antibodies binding to site II (Fig. 5B) or site IV with relatively little NT activity could potentially interfere with the access of potent pre-F-specific antibodies in sera with similar characteristics as MPE8 or AM14, which are both potent pre-F-specific mAbs. This may explain our finding that site II-specific antibodies in human sera had a 60% inhibition of pre-F binding when competed with site II-specific mAbs and yet accounted for <10% of NT activity.

A limitation of our analysis was the use of immortalized human epithelial cells (HEp-2) for the neutralization assay cellular substrate. Immortalized cells have a large amount of heparan sulfate on surface proteoglycans that act as a receptor for both the F and G proteins and diminish the need for attachment via G proteins. However, heparan sulfate is not present on the apical surface of primary well-differentiated HAE cultures, strongly suggesting that a different receptor is used by RSV to infect these cultures, and therefore, antibodies against the G protein may neutralize differently in HEp-2 and HAE cultures. We partially addressed this by selecting two subjects with high levels of G protein-specific binding (fig. S7). One subject had a low (2 to 3%) and the other a moderate (16 to 20%) NT activity remaining after adsorption with pre-F, which was likely attributable to G-specific antibodies. However, the overall pattern of neutralization was similar in both cell systems, indicating that antibodies against pre-F are the dominant NT antibodies in

human sera. More individual sera will need to be tested on primary and immortalized cell lines to fully define the contribution of G protein-specific antibodies to RSV neutralization.

Addition of guinea pig complement to sera has been shown to increase RSV NT titers in sera of naturally infected adults (24). The adsorption and neutralization assays in this study were performed without and with heat inactivation (Fig. 2A and fig. S3, respectively). The level of NT activity in heat-inactivated samples ($n = 117$) was lower than that of serum not heat-inactivated ($n = 121$) (Fig. 2A and fig. S3). With or without heat inactivation of sera, the effect of removing antibodies specific to pre-F through adsorption and competition assays remained the same (Figs. 2A, 4, A and B, and fig. S3). In all age groups, including the 65- to 84-year-old cohort, NT activity was retained after sera were adsorbed with post-F, and a previous study showed that children, young adults, and the elderly had comparable levels of NT activity to RSV (25). Therefore, the cumulative impact of repeated RSV infections with multiple subtypes over the course of a lifetime could promote the maintenance of pre-F-specific NT antibodies. It is not known whether the susceptibility of humans to recurrent infection with RSV throughout life is related to the overall magnitude of NT activity, the specificity of antibodies mediating neutralization, or other factors involving innate or adaptive cellular responses.

Although natural infection with RSV occurs throughout life, young infants and children <5 years old are much more likely than healthy individuals >5 years old to require hospitalizations due to lower respiratory tract infections. We speculate that antibodies specific for pre-F account for most of the NT activity that limits infection to the upper airway in older children and adults. We therefore suggest that the pre-F protein may be better suited than post-F as a vaccine antigen on the basis of the observation that most NT activity induced by natural infection is pre-F-specific. This is of particular importance for target populations that have had prior natural infection where the intent is to boost serum NT activity, such as pregnant women or the elderly. In addition, there are neutralization-sensitive antigenic sites unique to pre-F, whereas post-F-binding antibodies may be distracted from the functional pre-F trimers in lieu of binding post-F that has no functional value to the virus except as a decoy, supported by the observation that adsorption with post-F resulted in an increase in serum NT activity in about 10% of individuals. These observations suggest that induction of pre-F-specific responses may be more favorable for antigen-naïve populations to prime for more functionally relevant antibody responses. As antibody epitopes on RSV F are further defined, it will be important to characterize the pattern of antibody specificities induced in children under 5 years of age experiencing primary or secondary RSV infections.

MATERIALS AND METHODS

Study design

With the stabilization of the pre-F conformation of RSV F and the availability of human serum samples, we sought to understand the antibody responses to RSV. All samples for this study were collected with informed consent of volunteers, and approval for this study was obtained under protocol number VRC 700 (Clinicaltrials.gov NCT01262079). All volunteers were at least 6 years of age, healthy, and ranged in age from 7 to 93 years. Of all samples collected, 140 were systematically selected to fill up nine 10-year age groups with at least 15 per age group, first through blinded selection from stock and later by randomization. Actual

ages were concealed until results were obtained and analyzed for each individual subject.

Viruses and cells

Virus stocks were generated in HEP-2 cells cultured with 10% minimum essential medium (MEM) according to previously described methods (26). Briefly, T175 flasks of cells at 80% confluence were infected with 1 ml of RSV A2 or B18537 master stock and allowed to incubate on a shaker for 1 hour at room temperature (RT). Fifty milliliters of 10% MEM was then added to the adherent cultures, which were incubated at 37°C for an additional 4 days. When substantial syncytia had formed, cells were scraped loose from the flask and transferred to 50-ml tubes for sonication (six 1-s bursts) and centrifugation at 4°C and 1000 rpm for 15 min. The supernatant containing virus was aliquoted into dram vials, quick-frozen in an alcohol-dry ice slurry, and stored at -80°C.

Serum adsorption assay

Each serum sample (300 μ l) was diluted 1:9.7 in 1 \times phosphate-buffered saline (PBS) and split into three parts. Twenty microliters of pre-F or post-F (0.5 mg/ml) expressed from an RSV A2 construct using previously described methods (9, 14) was added to 970 μ l of diluted samples, whereas the positive control, 970 μ l of diluted unadsorbed serum, was further diluted 1:10 with 1 \times PBS. All samples were then incubated at RT for 2 hours, and positive control samples were stored at 4°C until use. To F-treated samples, 10 μ l of reconstituted Strep-tag II mAb at 0.5 mg/ml (GenScript) was added, and the mixture was placed on a 360° rotator at 4°C for 2 hours. Eighty microliters of sheep anti-mouse immunoglobulin G Dynabeads (2 mg/ml) (Life Technologies) was washed twice with wash buffer [1% bovine serum albumin (BSA), 2 mM EDTA in 1 \times PBS (pH 7.4)]. The wash buffer was separated from the beads using a DynaMag-2 magnet (Life Technologies). The Sera-RSV F-strep tag mAb mixture was added to corresponding labeled tubes containing Dynabeads and incubated for 1 hour at 4°C on a rotator. The beads were separated from supernatant using DynaMag-2; the supernatant was stored at 4°C.

Kinetic ELISA

Plates (96-well) were coated with 100 μ l of stabilized pre-F (1 μ g/ml) or post-F (1 μ g/ml) diluted in 1 \times PBS (pH 7.4) and incubated overnight at 4°C. The plates were washed with wash buffer (0.2% Tween 20 in 1 \times PBS) and had 200 μ l of 2% BSA (in 1 \times PBS) added per well as a blocking buffer. Plates were then incubated for 1 hour at RT. Three tubes containing 100 μ l (1:10 dilution) of unadsorbed serum, serum adsorbed with pre-F, and serum adsorbed with post-F were added to pre-F- and post-F-coated wells. The plates were incubated for 1 hour at RT, washed, and coated with 100 μ l of goat anti-human IgG (1:5000, Santa Cruz Biotechnology). After 1 hour of incubation, the plates were washed, and 100 μ l of Super AquaBlue substrate (eBioscience Inc.) was added to each well. An ELISA plate reader (Molecular Devices LLC) was used to read signals at 538-nm wavelength.

Neutralization assay

Fourfold dilutions were done on unadsorbed and pre-F- and post-F-adsorbed serum samples (with or without heat inactivation) starting at 1:10 through 1:40,960 with 45 μ l in each well. Each dilution was mixed with 45 μ l of diluted RSV strain A2 carrying the Katushka fluorescent protein mKate (27). The mixture was incubated for 1 hour at 37°C. Fifty microliters of the serum dilution/virus mixture was then added to HEP-2 cells that had been seeded

at a concentration of 1.8×10^4 cells in 30 μ l of 10% MEM in each well of 384-well black clear-bottom plates. The plates were incubated for 22 to 26 hours at 37°C before spectrophotometric reading at 588-nm excitation and 635-nm emission (Molecular Devices LLC).

Production of KO RSV F proteins

RSV F mutations were carried out by site-directed mutagenesis using Quik-Change (Stratagene), as was the addition of the AviTag sequence at the C terminus of the RSV F protein. Expi293F cells were transiently transfected with plasmids expressing RSV F site \emptyset and site II KO pre-F constructs and grown in suspension. The culture supernatants were harvested 5 days after transfection and centrifuged at 10,000 rpm to remove cell debris. The culture supernatants were sterile-filtered, and RSV F glycoproteins were purified by a Ni^{2+} -nitrilotriacetic acid resin (Qiagen) and a Strep-Tactin resin (Novagen). Relevant fractions containing the RSV F variants were pooled, concentrated, and subjected to size-exclusion chromatography. Fractions corresponding to the trimer peak (fig. S5) were concentrated, and frozen at -80°C .

Assessment of KO RSV F proteins

RSV F KO proteins with an introduced N-glycan sequon were initially examined by SDS-polyacrylamide gel electrophoresis to assess whether the novel glycan had been added to the RSV F protein and whether the glycan addition was uniform. RSV F KO proteins that expressed the glycan (9 of 10) were assessed for binding to antigen-binding fragments (Fabs) specific for site \emptyset or site II by BLI with an Octet Red384 (FortéBio Corp.). Assays were performed with agitation at 1000 rpm in PBS (100 μ l per well) supplemented with 1% BSA to minimize nonspecific interactions at 30°C in solid black 96-well plates (Greiner Bio-One). Anti-mouse Fc probes were sequentially incubated for 300 s with Strep-tag II mAb (35 μ g/ml) in PBS buffer, and in RSV F KO proteins that bound via their C terminus (location of the Strep-tag II purification tag). Typical capture levels were between 0.7 and 1 nm, and variability within a row of eight tips did not exceed 0.1 nm for each of these steps. Biosensor tips were equilibrated for 300 s in 1% BSA in 1 \times PBS before measuring association with Fabs in solution (1 μ M) for 300 to 600 s followed by dissociation for 100 to 600 s.

Competition neutralization assay

A total of 32 samples with NT titers closest to the 25th, 50th, 75th, and 95th percentiles for each age group were randomly selected. Fourfold dilutions were done on uncompeted sera, and sera mixed with 6 μ g of different versions of RSV A2 F (starting concentration of sera, 1:10): stabilized pre-F, post-F, site \emptyset KO pre-F, and site II KO pre-F (starting concentration of F constructs, 0.1 μ g/ml). Dilutions were done through 1:40,960 with 45 μ l in each well. Plates were incubated for 1 hour at 37°C, after which 45 μ l of diluted RSV A2 virus was added to each well. This was followed by another hour of incubation at 37°C. Fifty microliters of serum-virus or of serum-F protein-virus mixture was added to 384-well black clear-bottom plates that had been seeded with HEp-2 cells at 1.8×10^4 in 30 μ l of 10% MEM, and plates were incubated for 22 to 26 hours at 37°C, followed by NT assessment as previously described. Competition neutralization assays were also performed with different versions of RSV B18537 F: stabilized pre-F, post-F, and site \emptyset KO pre-F for $n = 24$ samples as described above. Both virus strains used in these assays expressed the Katushka fluorescent protein, mKate.

Biolayer interferometry

Inhibition of serum antibody binding to pre-F-specific antigenic sites on the pre-F trimer was carried out using the Octet Red384 instrument (FortéBio Corp.). The RSV pre-F trimer was loaded onto HIS biosensors

through a polyhistidine tag for 300 s in 1% BSA in 1 \times PBS. Typical capture levels were between 0.9 and 1.2 nm, and variability within a row of eight tips did not exceed 0.2 nm. Biosensor tips were then equilibrated for 60 s in 1% BSA in 1 \times PBS before capturing competing mAbs. Pre-F was diluted to 30 μ g/ml and mAbs D25, motavizumab, and isotype control VRC01 were diluted to 35 μ g/ml in the same buffer. Binding of competing mAbs was assessed for 300 s followed by a baseline equilibration for 120 s in buffer before dipping into serum samples. Sera were diluted 1:50 in 1% BSA in 1 \times PBS and binding was assessed for 300 s. After the pre-F load was normalized, serum antibody binding was calculated by subtracting the binding of competing mAbs. Percent inhibition of serum antibody binding to pre-F by competing mAbs was determined by the following equation: inhibition (%) = $100 - [(\text{serum antibody binding in the presence of competitor mAb}) / (\text{serum antibody binding in the presence of isotope mAb})] \times 100$. All the assays were done twice at 30°C, and the values reported were the average of two independent experiments.

Statistical analysis

Comparisons between unadsorbed and adsorbed serum samples (Figs. 1A and 2A) were done using the Wilcoxon signed-rank test. Thirty-five samples were selected from all 140 for further analysis by protein and antibody competition assays. Four samples were chosen within each age group that represented the 25th, 50th, 75th, and 95th percentiles of the geometric mean for the corresponding group, which allowed a spread-out of EC₅₀. Thirty-two samples were analyzed in the RSV A2 competition neutralization assays, 24 were analyzed in the RSV B18537 competition neutralization assays, and 25 were analyzed in the competition binding assays. Correlations between continuous variables were assessed using Pearson's correlation. All *P* values were compared to a two-sided α level of 0.05. All statistical analyses were done with GraphPad Prism v6.

SUPPLEMENTARY MATERIALS

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Methods

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Fig. S2. RSV F-specific binding activity in human sera by decade.

Fig. S3. Neutralization activity with heat inactivation of sera.

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Fig. S5. Characterization of RSV A2 site \emptyset and site II KO pre-F proteins.

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Table S1. ELISA assay readings to validate adsorption assay and show negligible residual pre-F/post-F and Strep-tag mAb in sera after adsorption.

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Table S3. HAI assay shows that RSV F proteins did not non-specifically remove influenza-specific antibodies in human sera.

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Acknowledgments: We thank all members of the Viral Pathogenesis Laboratory, the Biodefense and Structural Biology Sections, and the Clinical Trials Program of the National Institute of Allergy and Infectious Diseases (NIAID) Vaccine Research Center, NIH, as well as the study sites and participants that provided serum samples for the analysis. The RSV A2 construct used in this study was derived from a recombinering system for producing molecular clones of RSV provided by A. Hotard Lopez-Ona and M. Moore from Emory University. We also thank M. Teng (University of South Florida) for the RSV expressing *Renilla* luciferase (RSV-Luc) used in the neutralization studies in HAE cultures, and B. Hartman of the NIH Vaccine Research Center for preparing the manuscript for submission. **Funding:** Funding for the VRC 700 trial was provided by the American Recovery and Reinvestment Act of 2009 (Recovery Act) through contract #HHSN2722010000491 awarded to the EMMES Corporation by the NIH. **Author contributions:** J.O.N., M.C., K.M., and B.S.G. planned the study. J.O.N. performed adsorption, binding, and neutralization assays and prepared working RSV stocks for this study. A.K. purified and expressed native RSV A2 pre-F and post-F proteins. J.O.N., M.K., and A.M.K. performed BLI experiments. H.M.Y. designed and performed the hemagglutinin assay. S.M.M. engineered and made the reporter RSV A2 master stock. M.C.N. was our consulting statistician, and M. Enama and J.E.L. made human samples available for this study. A.K., K.M., J.S.M., and M.G.J. expressed and purified the mAbs used for the competition binding assays. RSV A2 and B18537 pre-F knockout proteins were designed and characterized by J.S.M., M.G.J., G.B.E.S.-J., G.-Y.C., I.S.G., and P.D.K.; expressed (RSV A2) by Y.Y., B.Z., A.D., and J.O.N.; and purified (RSV A2) by M.G.J., G.B.E.S.-J., M.S., E.J.R., A.K., and J.O.N. J.O.N. and A.K. expressed and purified RSV B18537 pre-F, post-F, and site Ø KO pre-F proteins. C.C. and M.E.P. performed and analyzed neutralization in the HAE cultures and were supported by grants AI095684 and AI093848 from the NIH. J.O.N., M.C., M.K., and B.S.G. analyzed the data; J.O.N., K.M., and B.S.G. wrote the manuscript with contributions from M.G.J., M.K., and M.E.P. All authors reviewed and edited the manuscript. **Competing interests:** M.C., J.S.M., M.G.J., M.K., G.B.E.S.-J., G.-Y.C., I.S.G., M.S., Y.Y., B.Z., P.D.K., and B.S.G. are named as inventors on a patent pending entitled “Prefusion RSV F proteins and their use,” WO 2014160463.

Submitted 24 April 2015
Accepted 12 August 2015
Published 14 October 2015
10.1126/scitranslmed.aac4241

Citation: J. O. Ngwuta, M. Chen, K. Modjarrad, M. G. Joyce, M. Kanekiyo, A. Kumar, H. M. Yassine, S. M. Moin, A. M. Killikelly, G.-Y. Chuang, A. Druz, I. S. Georgiev, E. J. Rundlet, M. Sastry, G. B. E. Stewart-Jones, Y. Yang, B. Zhang, M. C. Nason, C. Capella, M. E. Peebles, J. E. Ledgerwood, J. S. McLellan, P. D. Kwong, B. S. Graham, Prefusion F-specific antibodies determine the magnitude of RSV neutralizing activity in human sera. *Sci. Transl. Med.* **7**, 309ra162 (2015).

Supplementary Materials for
**Prefusion F-specific antibodies determine the magnitude of RSV
neutralizing activity in human sera**

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Published 14 October 2015, *Sci. Transl. Med.* **7**, 309ra162 (2015)
DOI: 10.1126/scitranslmed.aac4241

The PDF file includes:

Methods

Fig. S1. Adsorption assay validation.

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Fig. S3. Neutralization activity with heat inactivation of sera.

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Table S3. HAI assay shows that RSV F proteins did not non-specifically remove influenza-specific antibodies in human sera.

Supplemental Materials List

1. Methods for:
 - a. Production of RSV wild-type pre-F and post-F proteins
 - b. Hemagglutinin assay to test specificity of pre-F for pre-F specific antibodies.
 - c. Description of NT assay to assess G-specific NT activity in sera
2. Supplemental table 1 shows adsorption assay validation assays; Supplemental Table 2 shows antigenic site Ø and II KO variant mutations; and Supplemental Table 3 represents results of the hemagglutination inhibition assay (HAI) which show that RSV F proteins did not non-specifically remove influenza-specific antibodies from human sera.
3. Figures to show:
 - a. Adsorption assay validation experiments
 - b. Binding activity per age group to pre-F and post-F with and without adsorption.
 - c. NT activity of heat-inactivated sera samples following adsorption with pre-/post-F
 - d. NT activity per age group with/without adsorption with pre-F/post-F
 - e. Size-exclusion and gel profiles for site Ø and II KO pre-F
 - f. Analysis of F-specific mAbs by octet biolayer interferometry
 - g. NT activity in human airway epithelial cells with/without adsorption with pre-F

Expression of RSV pre-F and post-F. Proteins were expressed from RSV A2 constructs using previously described methods (1, 2). Briefly, Expi293F cells were transiently transfected with plasmids expressing either construct and grown in suspension for six days. The culture supernatants were harvested and centrifuged at 6,600 rpm for 30 min to remove cell debris. The supernatants were sterile-filtered and purified by Ni^{2+} -nitriloacetic acid (NTA) resin (Qiagen) followed by purification with StrepTactin resin (Novagen). Purified fractions were concentrated and subjected to size-exclusion chromatography.

Hemagglutination inhibition assay (HAI). To measure the specificity of the adsorption assay, ten samples were selected at random (unadsorbed and adsorbed sera) to measure influenza antibody titer using the HAI assay. Briefly, 25 μl of 1X PBS was added to wells of rows B through H of three V bottom 96-well plates representing ten unadsorbed sera, and their corresponding pre-F and post-F adsorbed sera. 50 μl of unadsorbed, pre-F and post-F adsorbed sera was added to wells of row A of plates 1, 2 and 3 and diluted 1:2 down the columns. 25 μl of 8HA units/50 μl California 09 H1N1 influenza virus was added to all wells and plates were gently tapped and incubated for 30 min at room temperature in a tissue culture hood. 50 μl of 0.5% Turkey red blood cells was added to all wells and incubated for another 30 min at room temperature without shaking. Titers were then read by visual observation for hemagglutination.

Neutralizing assay on human airway epithelium (HAE). Quantification of neutralizing antibodies was performed using RSV-Luc. Two sera samples were chosen that had high antibody titers against the subtype A2 G glycoprotein, using a recombinant soluble G in an ELISA assay. This G protein matched the sequence of the G protein in our test virus, RSV-Rluc. Sera (1:10 dilution) that had been adsorbed with pre-F were heat-inactivated (56°C, 30 min). Undiluted, unadsorbed sera were likewise heat-inactivated before diluting 1:10. With a starting dilution of

1:4, all samples were serially diluted 10 times before an equal volume (150 μ l) containing 2,000 pfu of RSV-Luc was added to the dilutions. The mixture was incubated at 20°C for 1 hour and 80 μ l was used to inoculate three HAE cultures in Transwell inserts (6.5 μ m diameter Corning) at 37°C in 5% CO₂. After 4 hours the inoculum was removed and the cultures were incubated for another 48 hours under the same conditions, allowing RSV-Luc to express *Renilla* luciferase. Cultures were lysed and assayed as described by the manufacturer (*Renilla* Luciferase Assay System; Promega). Samples were transferred to 96-well solid black plates and luminescence was quantified using a PE Wallac Victor 2 1420-012 system microplate reader. Assays were performed in triplicates for each dilution and plotted using GraphPad Prism 6. Error bars indicate the standard deviation.

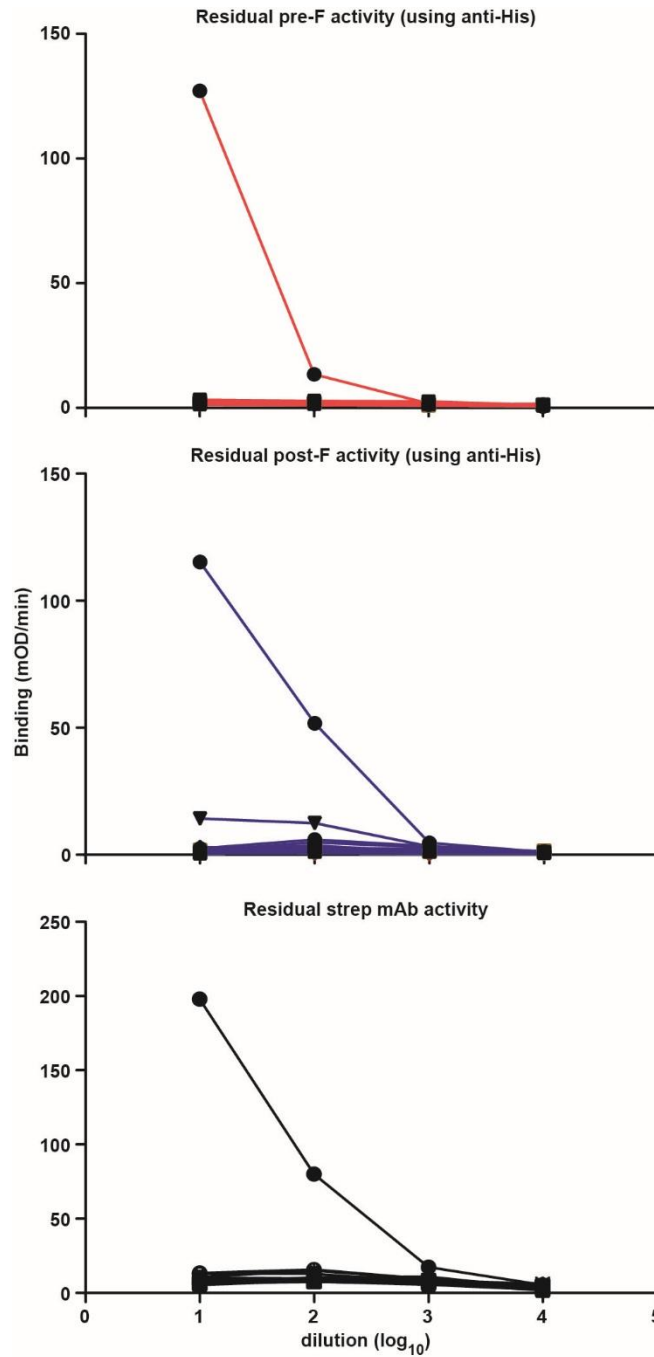
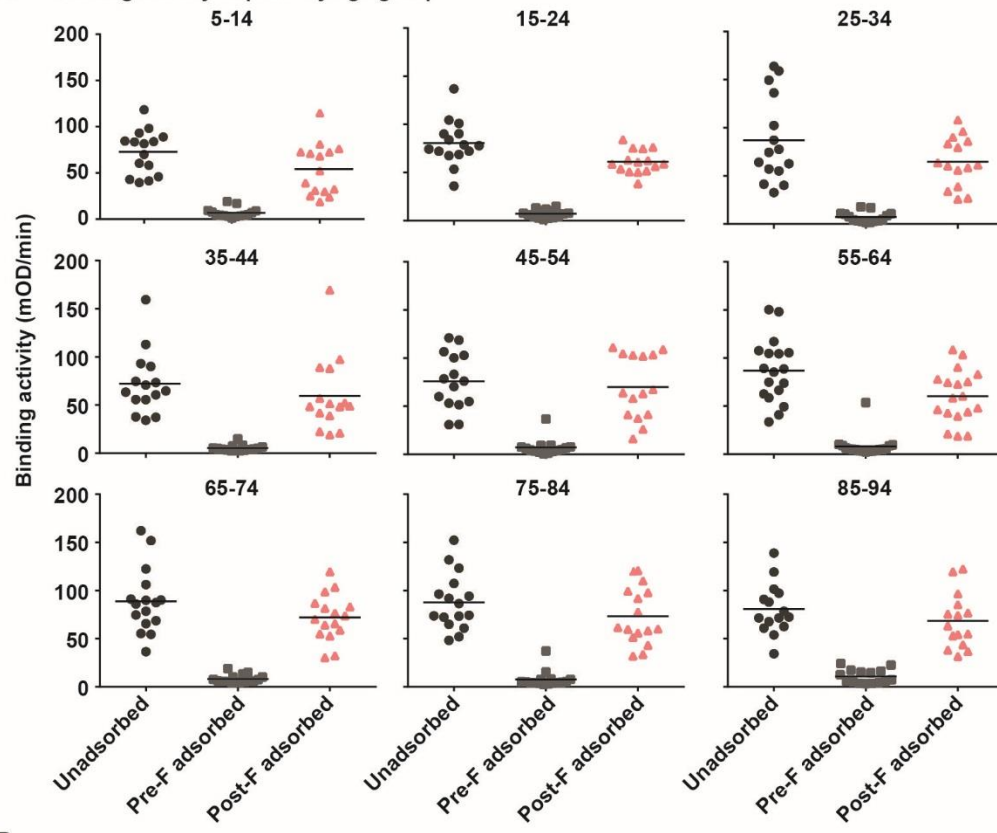


Figure S1. Adsorption assay validation. Following sera adsorption with RSV A2 pre-F and post-F, samples were used in an ELISA to measure residual pre-F (A), post-F (B) and strep mAb (C) for N=8 samples representing different assay batches. Compared to positive control (same amount of F protein/strep mAb used per sample in adsorption in 1X PBS), HRP-conjugated anti-HIS antibodies show negligible residual pre-F (A) and post-F (B), and HRP-conjugated anti-mouse antibodies show negligible residual strep mAb in sera.

A Binding activity to pre-F by age group



B Binding activity to post-F by age group

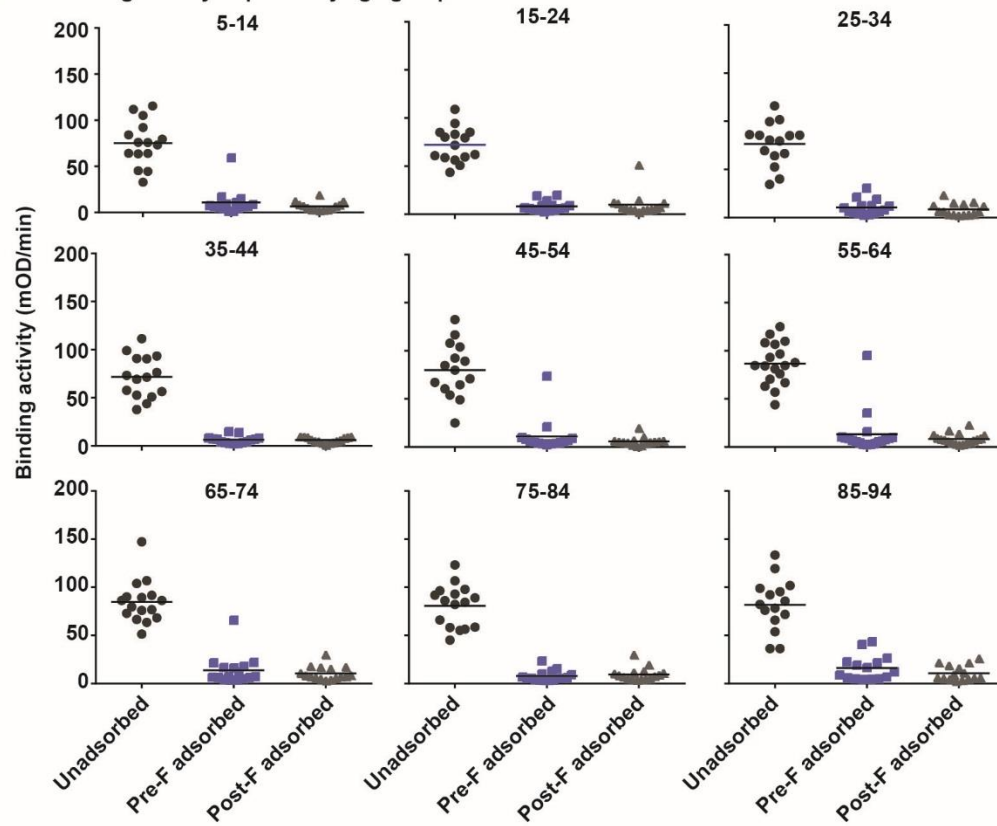


Figure S2. RSV F-specific binding activity in human sera by decade. Following sera adsorption with pre-F and post-F, serum samples were used in an ELISA binding assay to measure the impact of adsorption on binding to pre-F (A) and post-F (B). Across age groups, binding activity to either conformation is lost after adsorption with pre-F, while binding activity to pre-F is substantially retained when samples are adsorbed with post-F.

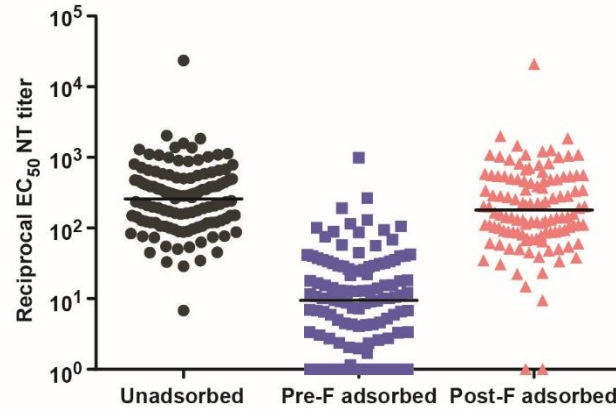


Figure S3. Neutralization activity with heat inactivation of sera. (A) Heat-inactivation (HI) before adsorption with pre-F leads to a decrease in overall GMT, however NT activity is still substantially lost with pre-F adsorption but not with post-F adsorption (GMT= 257.5, 9.0 and 174.5 for unadsorbed, pre- and post-F adsorbed HI sera respectively, N=118).

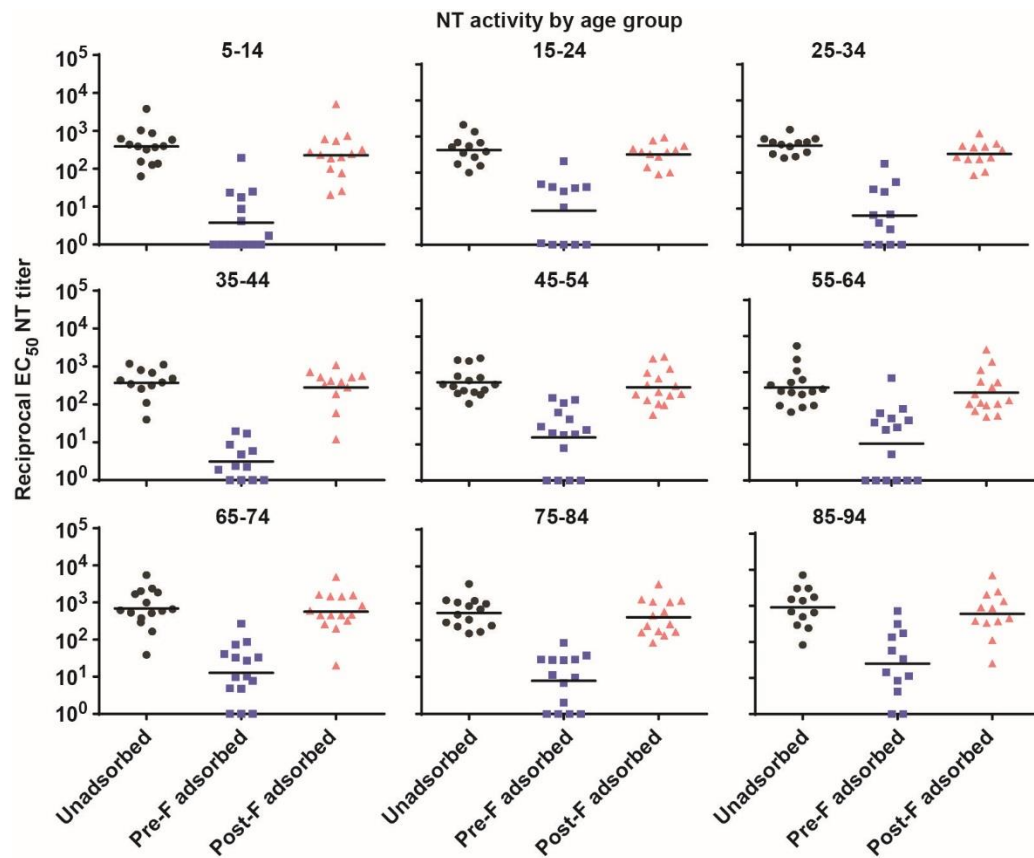


Figure S4. Neutralization activity in human sera by decade. Across nine ten-year age groups, pre-F accounts for majority of RSV A2 neutralizing activity in sera.

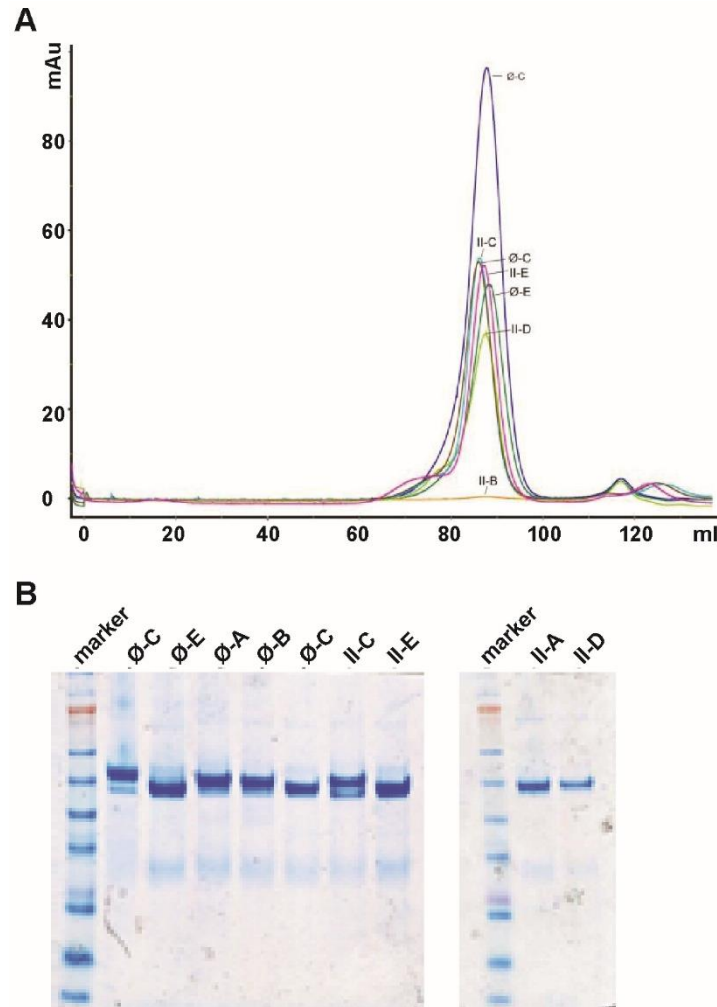


Figure S5. Characterization of RSV A2 site Ø and site II KO pre-F proteins. (A) Site Ø and site II KO molecules were purified by size-exclusion chromatography using a 120 ml Superose-6 column and the elution profiles are shown. (B) Samples in reducing conditions were loaded onto a 4-12% Bis-Tris SDS-PAGE gel in reducing conditions. A See-Blue Plus 2 marker was used to assess molecular weight of the site Ø and site-II KO proteins.

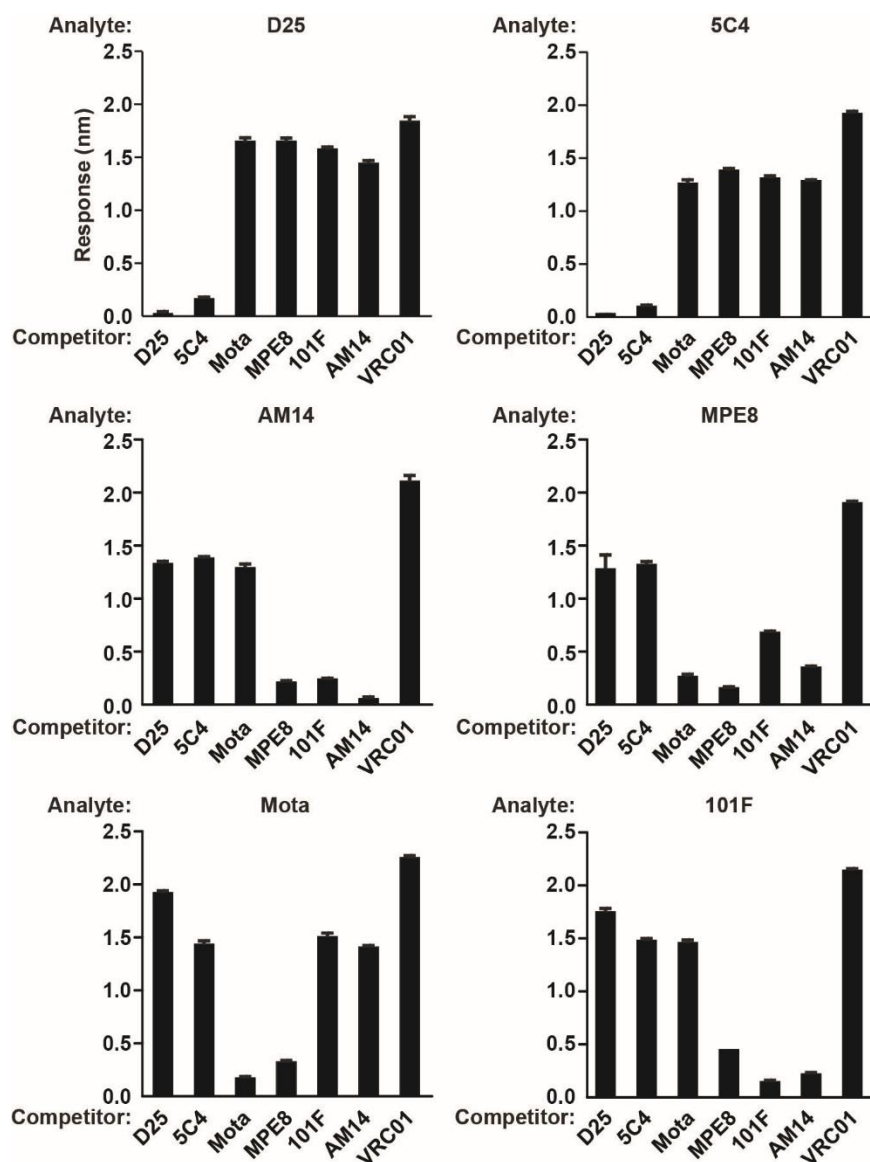


Figure S6. Analysis of RSV F-specific mAbs by binding competition. The data supporting Fig. 5A shows the extent of competition between mAbs using Octet biolayer interferometry. There is no evidence of competition between mAbs specific for sites Ø and II, which is consistent with structural data. MPE8 competes with antibodies to sites II, IV, and the AM14 epitope. 101F (a site IV mAb) and AM14 compete with each other and MPE8, but not site II.

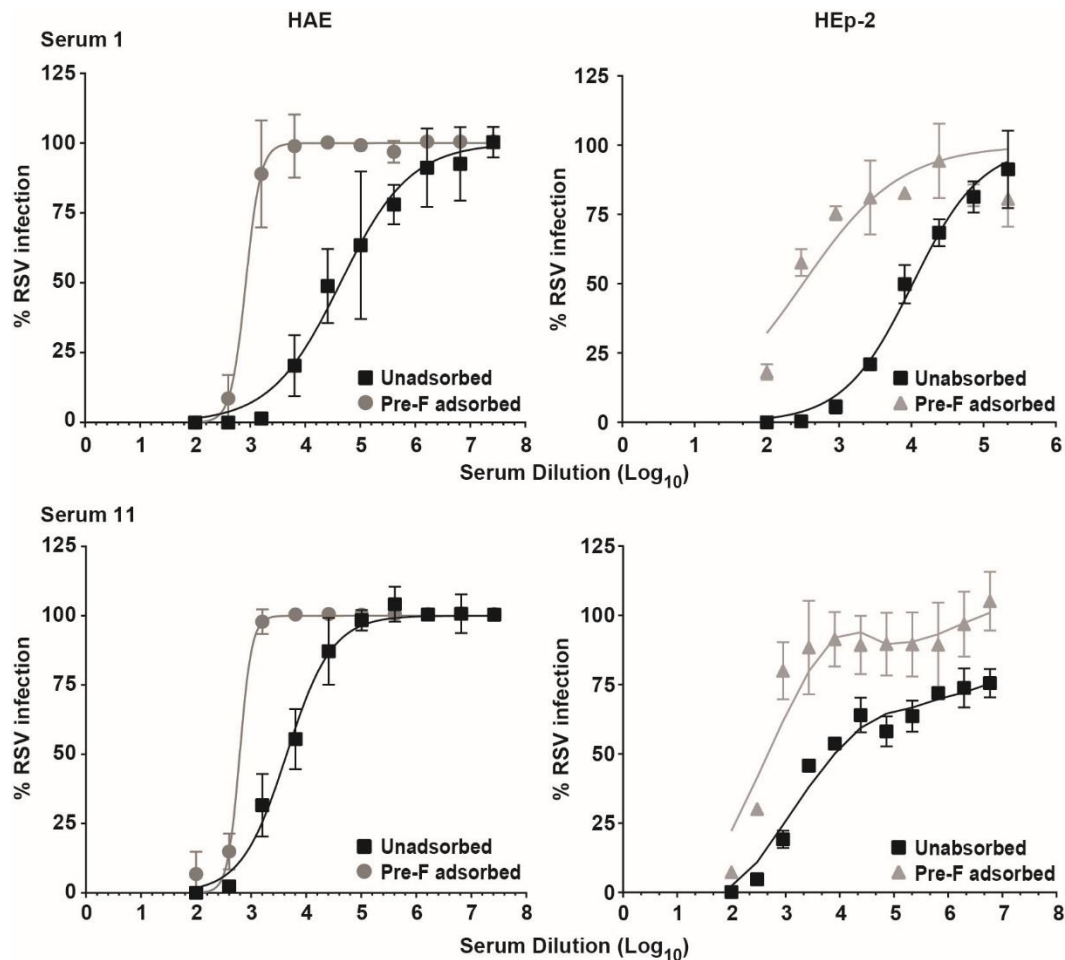


Figure S7. NT activity in HEp-2 versus primary HAE cells. NT activity of two selected serum samples was measured on HEp-2 and HAE cells. Pre-F adsorption of Serum #1 removed all but 2-3% of the NT activity on both HEp-2 and HAE cells, indicating that the NT activity in this serum was largely pre-F specific. In contrast, pre-F adsorption of Serum #11 removed all but 16-20% of the NT activity on both HEp-2 and HAE cells, indicating that a portion of the NT activity was due to antibodies against the only other viral NT antigen, the G protein. Note that the unadsorbed and pre-F adsorbed NT curves particularly those in HAE cultures, display different slopes. The NT slopes of the unadsorbed sera in which antibodies to F predominate are more gradual than those of the pre-F adsorbed sera that likely represent antibodies to the G protein, suggesting a broader diversity of antibodies to the F protein than to the G protein.

Supplemental Table 1. ELISA assay readings to validate adsorption assay and show negligible residual pre-F/post-F and Strep-tag mAb in sera after adsorption.

Dilution	Pre-F	65	66	42	46	1	2	12	13
10	127.2	2.1	1.579412	3.255882	1.411765	2.461765	2.523529	1.658824	1.261765
100	13.46471	1.941176	1.747059	2.779412	1.173529	1.976471	1.994118	1.641176	1.147059
1000	1.773529	1.623529	1.8	2.567647	1.147059	1.217647	1.005882	1.270588	0.855882
10000	0.582353	1.270588	0.979412	1.120588	0.811765	1.358824	1.208824	1.614706	0.635294

Dilution	Post-F	65	66	42	46	1	2	12	13
10	115.2706	0.9	0.6	14.24118	3.008824	2.444118	1.852941	1.5	0.758824
100	51.75882	2.788235	1.288235	12.40588	2.205882	5.911765	3.529412	4.870588	1.932353
1000	4.623529	2.170588	1.305882	3.3	1.252941	3.397059	1.217647	2.841176	1.076471
10000	1.023529	0.935294	0.864706	0.555882	0.714706	1.279412	1.579412	0.847059	0.644118

Dilution	Strep mAb	4 Pre-F	4 Post-F	5 Pre-F	5 Post-F	14 Pre-F	14 Post-F	15 Pre-F	15 Post-F
10	197.9559	9.9	6.105882	8.267647	7.385294	6.855882	6.097059	8.188235	5.497059
100	80.16177	9.220588	9.529412	8.594118	7.702941	8.567647	8.938235	7.773529	8.223529
1000	17.39118	7.402941	9.167647	8.285294	5.541176	7.8	7.905882	6.088235	6.520588
10000	5.444118	4.614706	1.941176	3.970588	2.161765	4.844118	2.364706	3.617647	1.852941

Dilution	Strep mAb	18 Pre-F	18 Post-F	20 Pre-F	20 Post-F	32 Pre-F	32 Post-F	39 Pre-F	39 Post-F
10	197.9559	10.47353	10.85294	7.102941	5.929412	9.105882	13.07647	6.538235	13.57059
100	80.16177	9.679412	15.06177	7.720588	10.59706	7.967647	13.00588	7.588235	15.88235
1000	17.39118	7.35	9.229412	6.273529	11.02059	8.585294	7.411765	6.926471	9.370588
10000	5.444118	4.95	3.361765	6.432353	3.017647	6.167647	2.717647	4.95	3.520588

Supplemental Table 2. RSV F antigenic site Ø and site II KO variant mutations, production, and characterization.

Name	Strategy	Mutations	Yield (mg/L)	Targeted Site	Antibody binding (nm)			
					5C4	AM2 ₂	D25	Motavizumab
A2 Ø –A	Glycan	K209N-S211T	0.94	Ø	0.00	0.01	0.29	0.50
A2 Ø –B	Glycan	P205N-V207T	1.70	Ø	0.00	0.07	0.02	0.46
A2 Ø –C (Ø –KO)	Glycan	K65N-N67T, P205N-V207T, K209N-S211T	2.02	Ø	0.00	0.00	0.00	0.46
A2 Ø –D	Protein	I206E-K209E	1.51	Ø	0.00	0.00	0.29	0.39
A2 Ø –E	Protein	K201E-N208Y-K209E	0.40	Ø	0.00	0.03	0.03	0.49
A2 II-A	Glycan	D206N-K271S	0.11	II	0.29	0.36	0.36	0.06
A2 II-B	Glycan	S275N-V277S	0.01	II	N/A	N/A	N/A	N/A
A2 II-C	Glycan	Q270T	0.95	II	0.37	0.37	0.39	0.14
A2 II-D	Protein	K272E	0.25	II	0.23	0.26	0.35	0.06
A2 II-E (II-KO)	Protein	N268R-K272E	1.52	II	0.38	0.38	0.41	0.00
B18537 Ø KO	Protein	K65N, P205N, V207T, Q209N	4.0	Ø	N/A	N/A	0.00	0.60

Supplemental Table 3. HAI assay shows that RSV F proteins did not non-specifically remove influenza-specific antibodies in human sera.

Sample ID	Serum		
	Unadsorbed	pre-F adsorbed	post-F adsorbed
7	160	160	80
8	80	80	80
9	80	80	80
15	40	40	40
16	40	40	40
17	<20	<20	<20
18	40	40	40
21	160	160	160
26	20	20	20
27	80	40	80

	mAb Control	
	CR6261	CH65
Reactivity	-	+++