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Supplementary Materials for

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

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Supplemental Materials List

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    b. Hemagluttinin 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
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    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+}-nitriloacetacetic 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 µl) containing 2,000 pfu of RSV-Luc was added to the dilutions. The mixture was incubated at 20^{o}C for 1 hour and 80 µl was used to inoculate three HAE cultures in Transwell inserts (6.5 µm diameter Corning) at 37°C in 5% CO_2. 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.

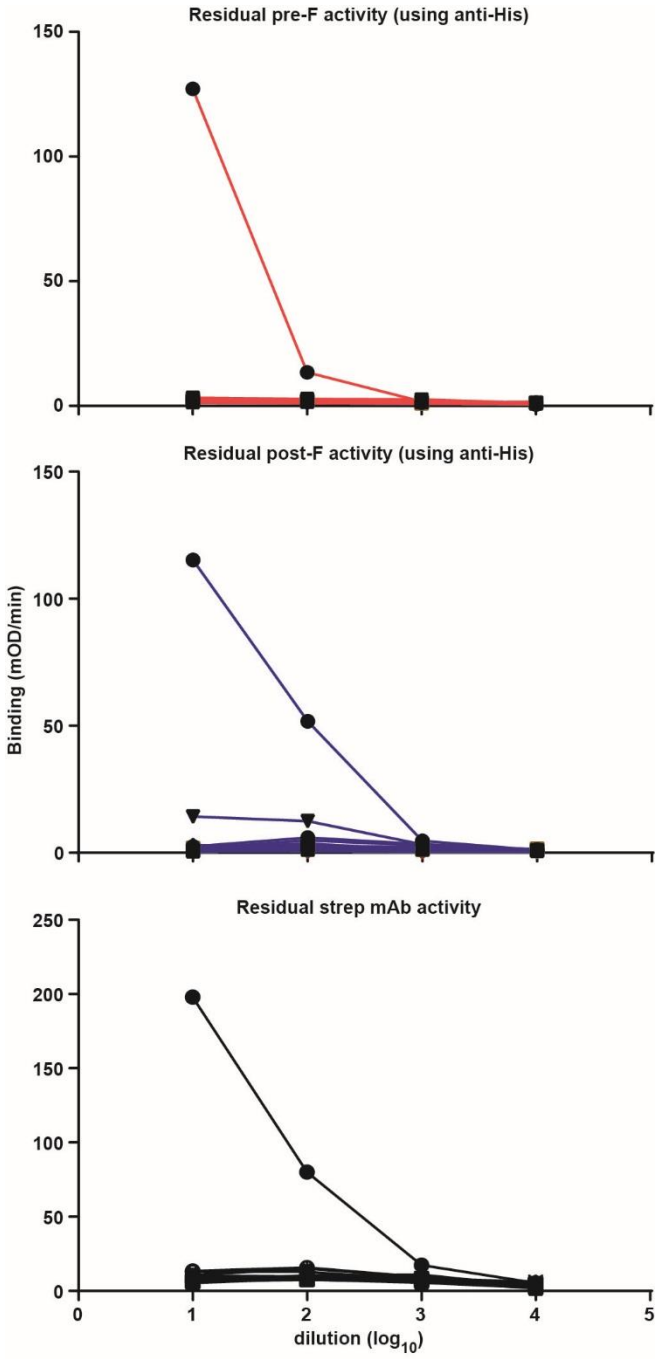

Figure S1. Adsorption assay validation. Following sera adsorption with RSV A2 pre-F and post-F, samples were used in an ELISA to measure residual pre-F (A), post-F (B) and strep mAb (C) for N=8 samples representing different assay batches. Compared to positive control (same amount of F protein/strep 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.

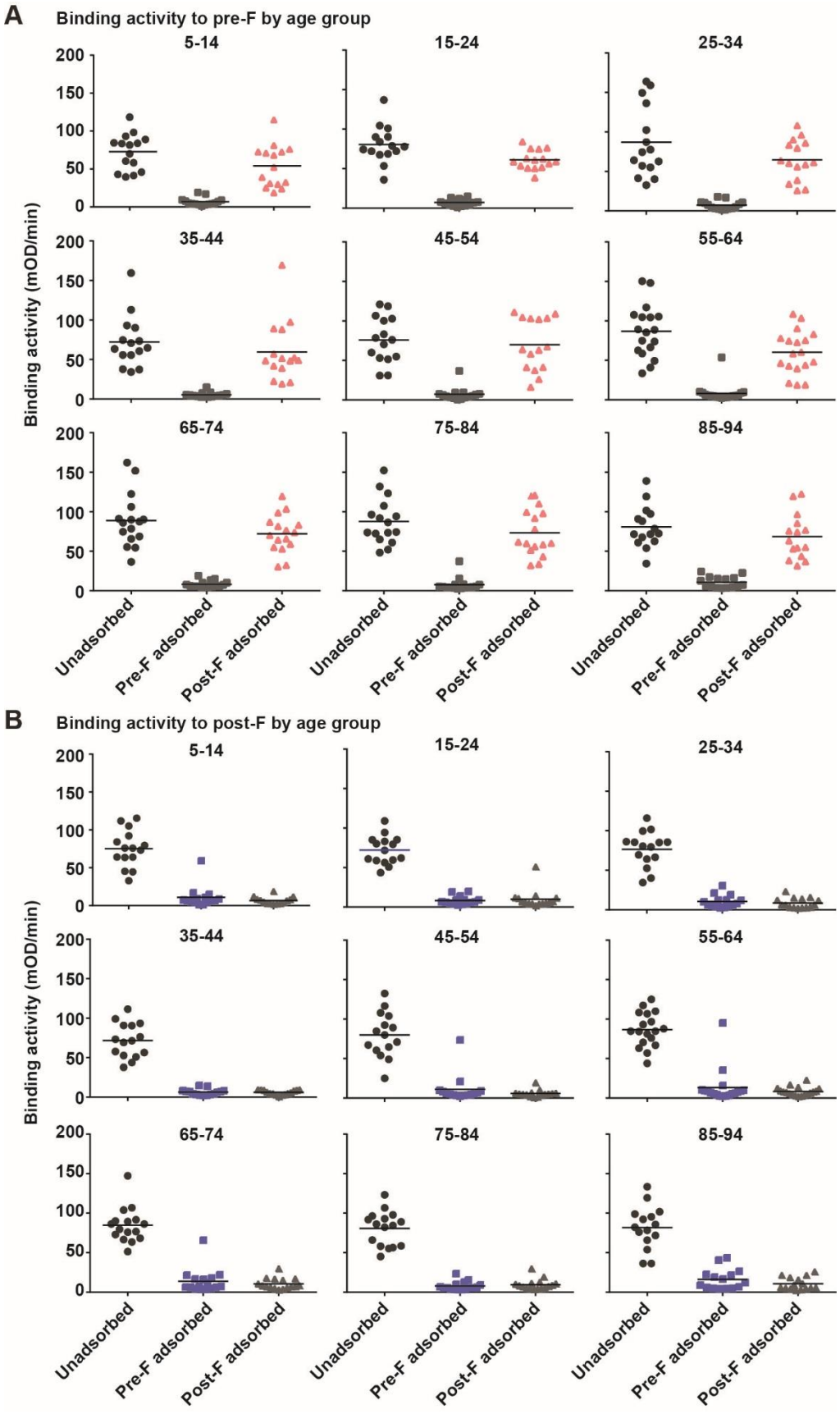
A
Binding activity to pre-F by age group
5-14
15-24
25-34
35-44
45-54
55-64
65-74
75-84
85-94
Binding activity (mOD/min)
200
150
100
50
0
Unadsorbed
Pre-F adsorbed
Post-F adsorbed
B
Binding activity to post-F by age group
5-14
15-24
25-34
35-44
45-54
55-64
65-74
75-84
85-94
Binding activity (mOD/min)
Unadsorbed
Pre-F adsorbed
Post-F adsorbed

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.

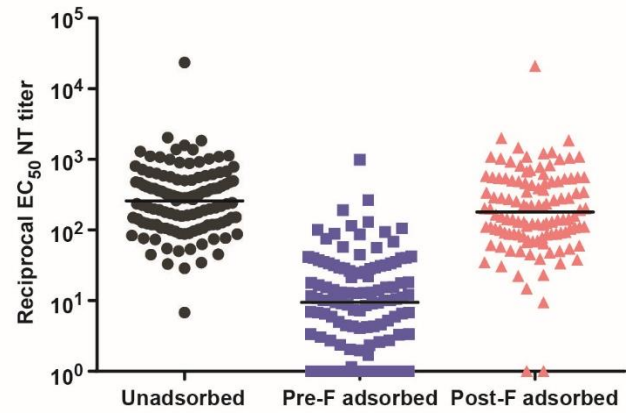

Figure S3. **Neutralization activity with heat inactivation of sera.** (A) Heat-inactivation (HI) before adsorption with pre-F leads to a decrease in overall GMT, however NT activity is still substantially lost with pre-F adsorption but not with post-F adsorption (GMT= 257.5, 9.0 and 174.5 for unadsorbed,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

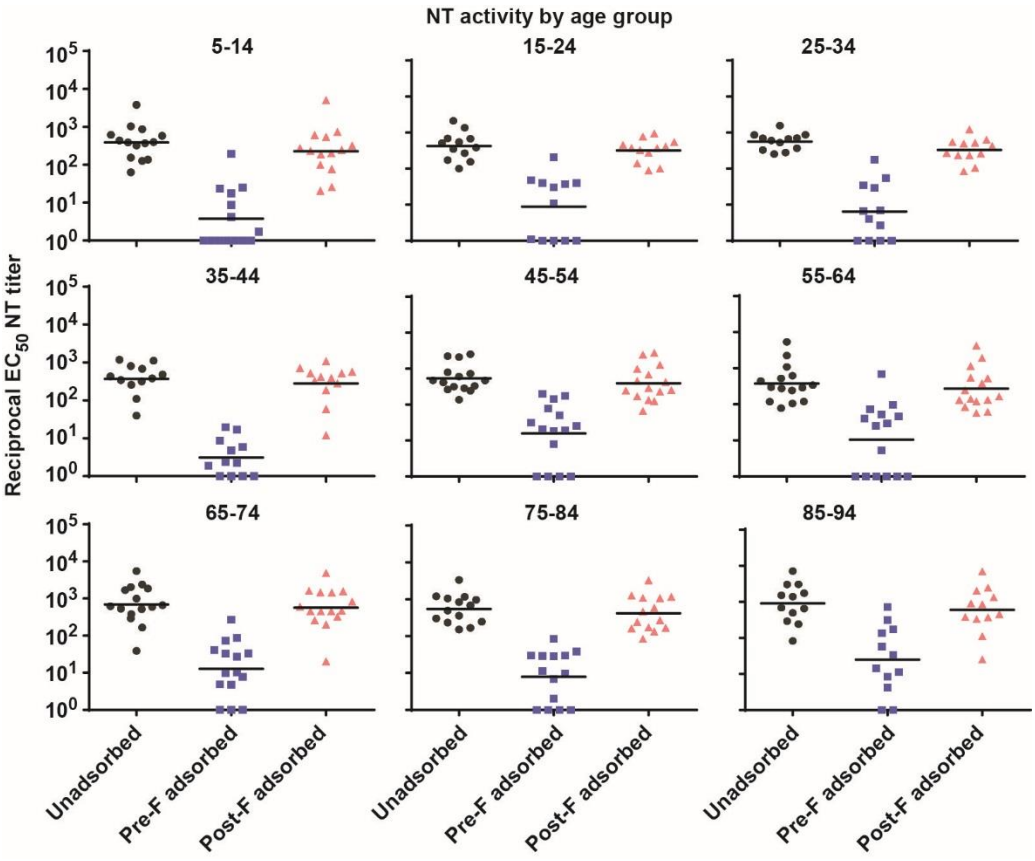

Figure S4. **Neutralization activity in human sera by decase.** Across nine ten-year age groups, pre-F accounts for majority of RSV A2 neutralizing activity in sera.

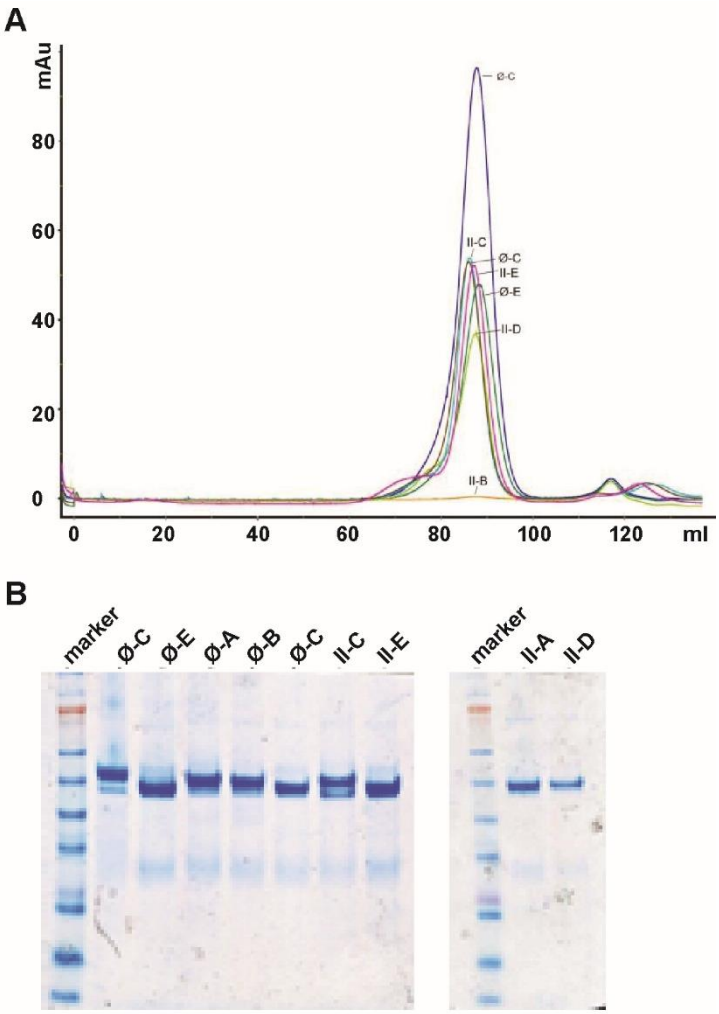

Figure S5. Characterization of RSV A2 site Ø and site II KO pre-F proteins. (A) Site Ø and site II KO molecules were purified by size-exclusion chromatography using a 120 ml Superose-6 column and the elution profiles are shown. (B) Samples in reducing conditions were loaded onto a 4-12% Bis-Tris SDS-PAGE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

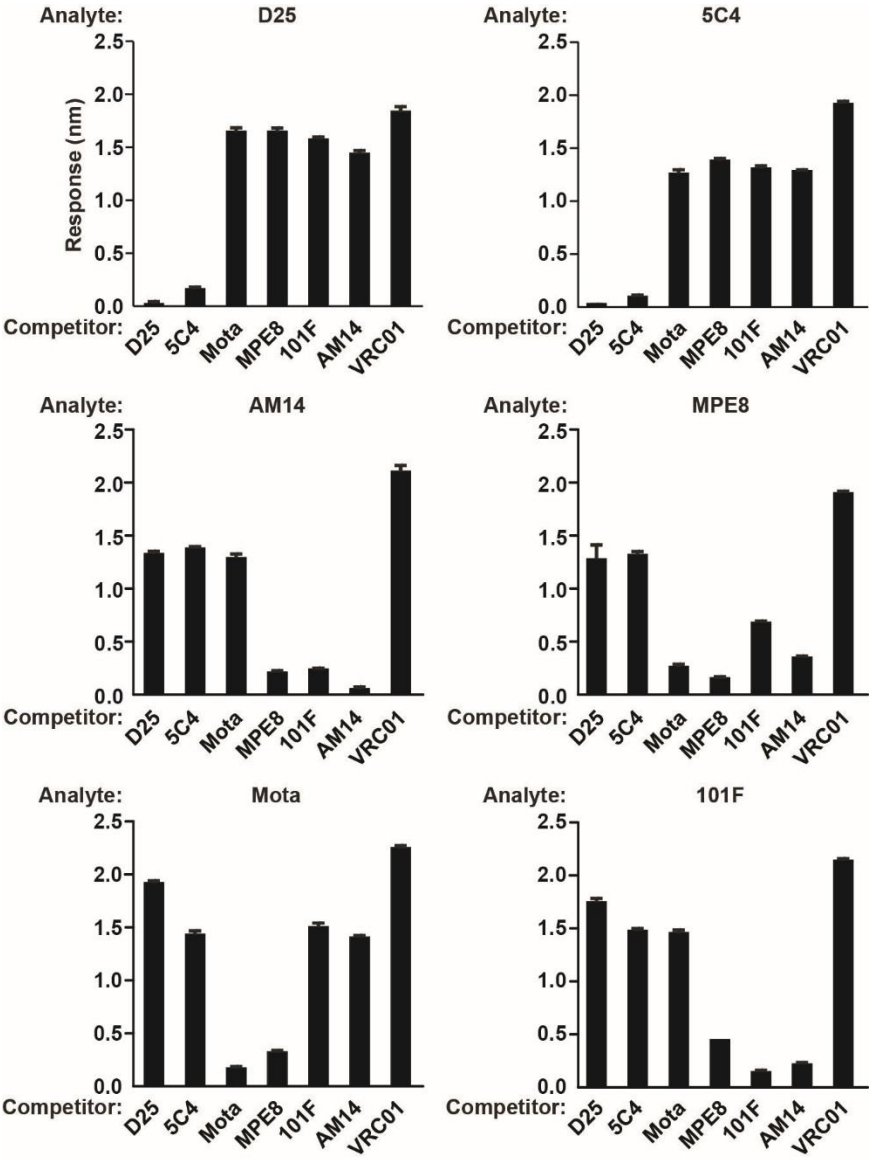

Figure S6. Analysis of RSV F–specific mAbs by binding competition. The data supporting Fig. 5A shows the extent of competition between mAbs using Octet biolayer interferometry. There is no evidence of competition between mAbs specific for sites Ø and II, which is consistent with structural data. MPE8 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.

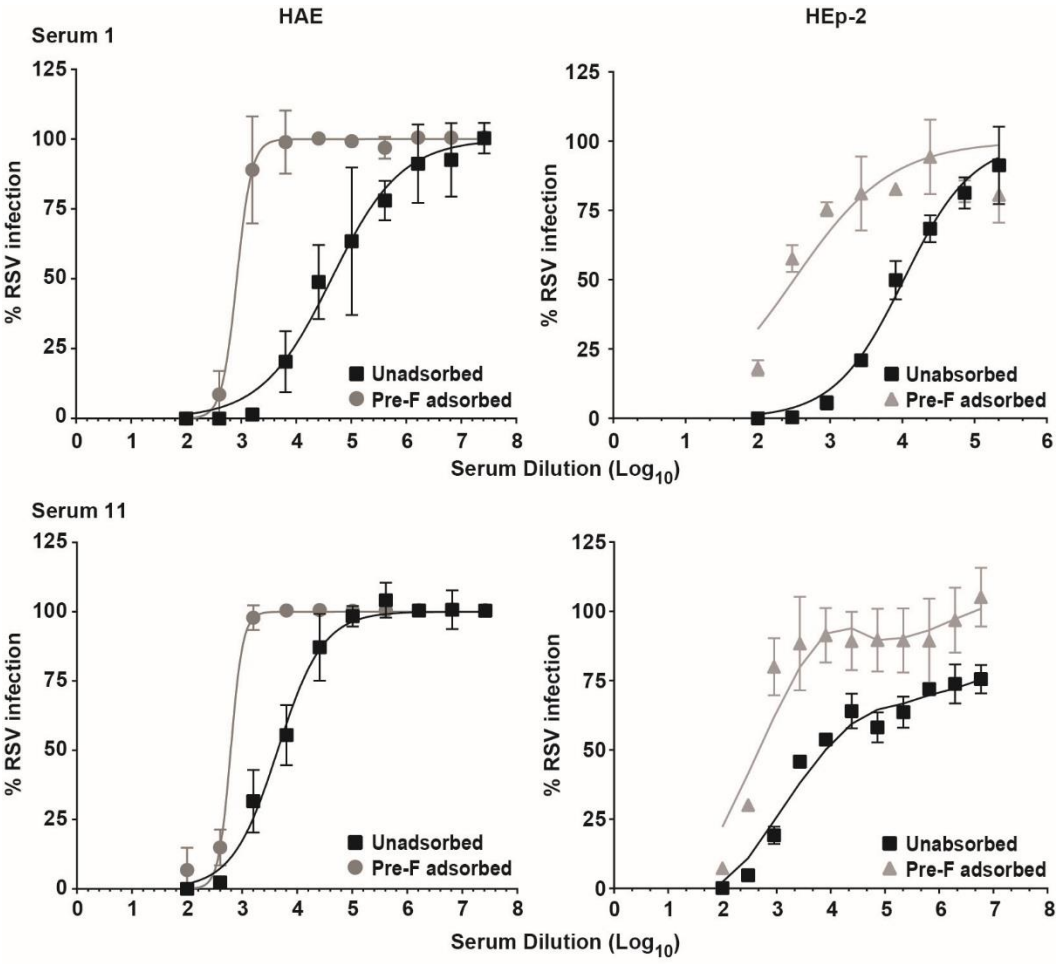

Figure S7. NT activity in HEp-2 versus primary HAE cells. NT activity of two selected serum samples was measured on HEp-2 and HAE cells. Pre-F adsorption of Serum #1 removed all but 2-3% of the NT activity on both HEp-2 and HAE cells, indicating that the NT activity in this serum was largely pre-F specific. 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)	TargetedSite	Antibody binding (nm)			
					5C4	AM22	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	-	+++