Respiratory Syncytial Virus Prefusion F Subunit Vaccine Elicits Durable Neutralizing Activity in a Phase 1 Randomized, Open-label Clinical Trial

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Supplementary Appendix

Methods

Assessment of RSV Neutralizing Activity

Secondary endpoints included the evaluation of antigen-specific antibody responses by neutralization assays by neutralization assays 2-4 weeks after the first and second doses; exploratory analyses summarize these responses to week 44. Neutralizing activity was assessed at the Vaccine Immunology Program (VIP) in Gaithersburg, MD with a qualified automated RSV neutralization assay on a Beckman Coulter Biomek integrated automation system. Assay qualification included evaluation of specificity, precision, linearity, accuracy, and range analysis according to ICH guidelines. Briefly, sera were serially diluted four-fold from 1:10 to 1:163840 in Dulbecco's modified Eagle's media (Invitrogen) containing 10% FBS (VWR) (DMEM-10) and mixed 1:1 with recombinant mKate reporter-RSV expressing prototypic F genes from subtype A (strain A2, RSV A) or subtype B (strain 18537, RSV B) for one hour at 37ºC. Next, 50 µl of each serum dilution/virus mixture were added to H28 cells (ATCC) that had been seeded for two hours at a density of $2.5x10^4$ in 25 µl of DMEM-10 in 384-well plates prior to incubation at 37ºC. One day later, the assay media was removed and the plate was washed three times with PBS, followed by a 20-40 minute lysis with 50 µL Glo Lysis buffer (Promega E2661). Next, 25 µL of cell lysate was transferred to a black low volume 384-well plate (Corning 89511-312) for fluorescence intensity analysis at 588 nm excitation and 635 nm emission (SpectraMax Paradigm, Molecular Devices, CA). For post-F inhibition assays, sera were diluted in DMEM-10 containing 25 µg/mL of soluble post-F protein from RSV A2 and incubated for 45 minutes prior to mixing with RSV A reporter virus. For analysis, the concentration that inhibited neutralization by 50% (IC50) for each sample was calculated by fitting a four-parameter logistic curve in GraphPad Prism. Conversion of reciprocal IC50 dilutions to International Units IU/mL was determined by testing the First International Standard for Antiserum to RSV (NIBSC code: 16/284) with RSV standards from BEI (Biodefense and Emerging Infections Research Resources Repository) four times each for both RSV A and B reporter viruses. Using the formula provided with the International Standard, the BEI NR-4020 was determined to be 1235 IU/mL for RSV A and 1754 for RSV B. BEI standard NR-4020 was included in quadruplicate in all runs, and the geometric mean reciprocal IC50 dilution of the BEI NR-4020 was 1146 ± 351 for RSV A across all runs. Thus, a factor of 0·994 was used to convert IC50 to international units/mL. For RSV B, the geometric mean reciprocal IC50 dilution of the BEI NR-4020 was 582 ± 149 and a conversion factor of 4·402 was used to convert IC50 to IU/mL.

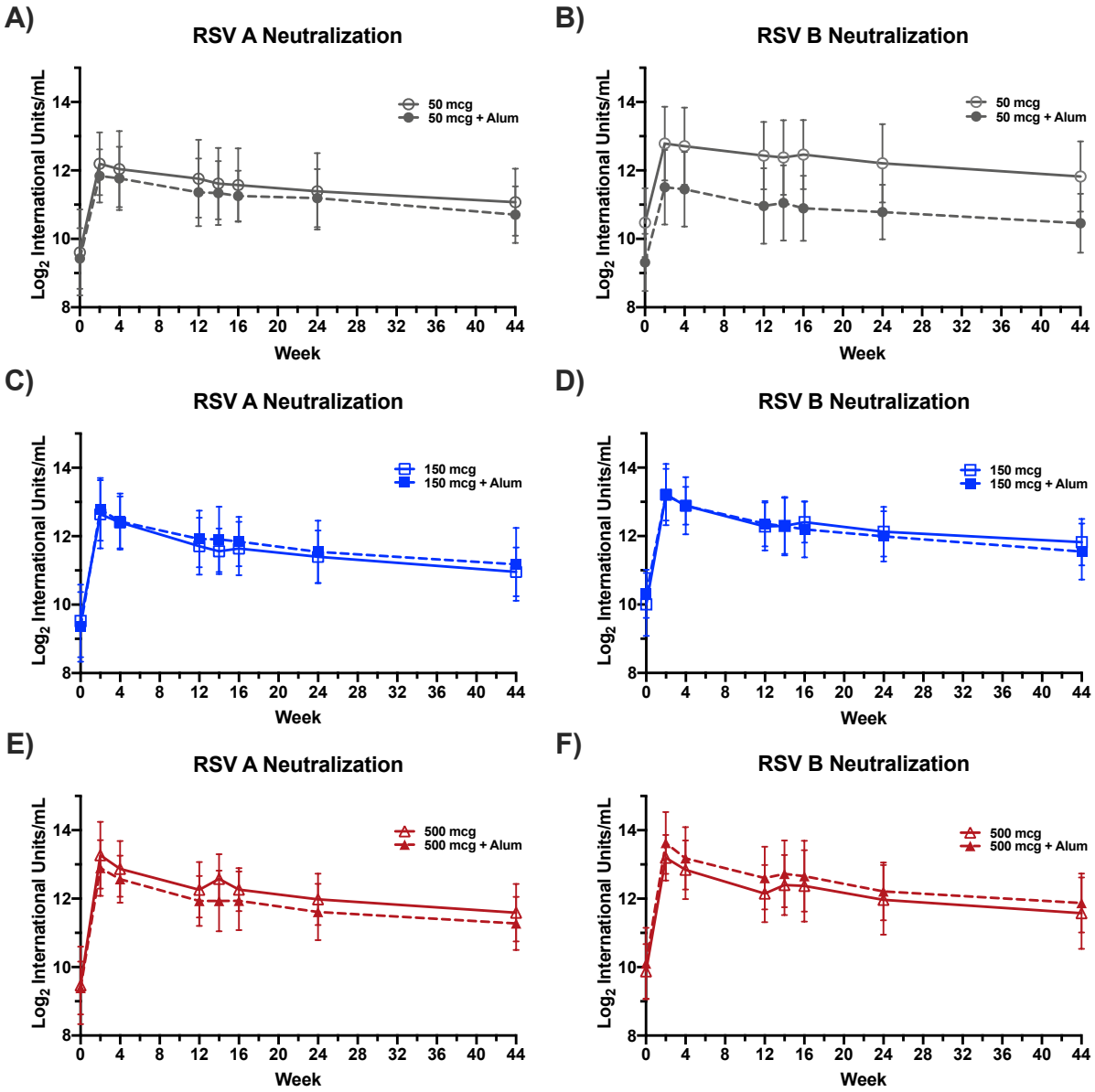
Evaluation of RSV F-specific Antibody Responses

The frequency and magnitude of RSV F-specific antibody responses following DS-Cav1 vaccination were an exploratory endpoint. Pre-F and post-F binding antibodies were quantified using endpoint enzyme-linked immunosorbent assay (ELISA), as previously described.[1] An ELISA with excess post-F was used to measure antibodies binding to pre-F exclusive surfaces.[1] Levels of antibodies competing with D25 binding to pre-F or palivizumab binding to post-F (DCA and PCA, respectively) were quantified using previously described methods.[1,2] The lower limit of quantitation (LLOQ) was 8·4 µg/mL for the pre-F DCA assay and 8·9 µg/mL for the post-F PCA assay. Sera with undetectable levels of competing antibodies were assigned a value half the lower LLOQ.

RSV F-specific Antibody in Nasopharyngeal Samples

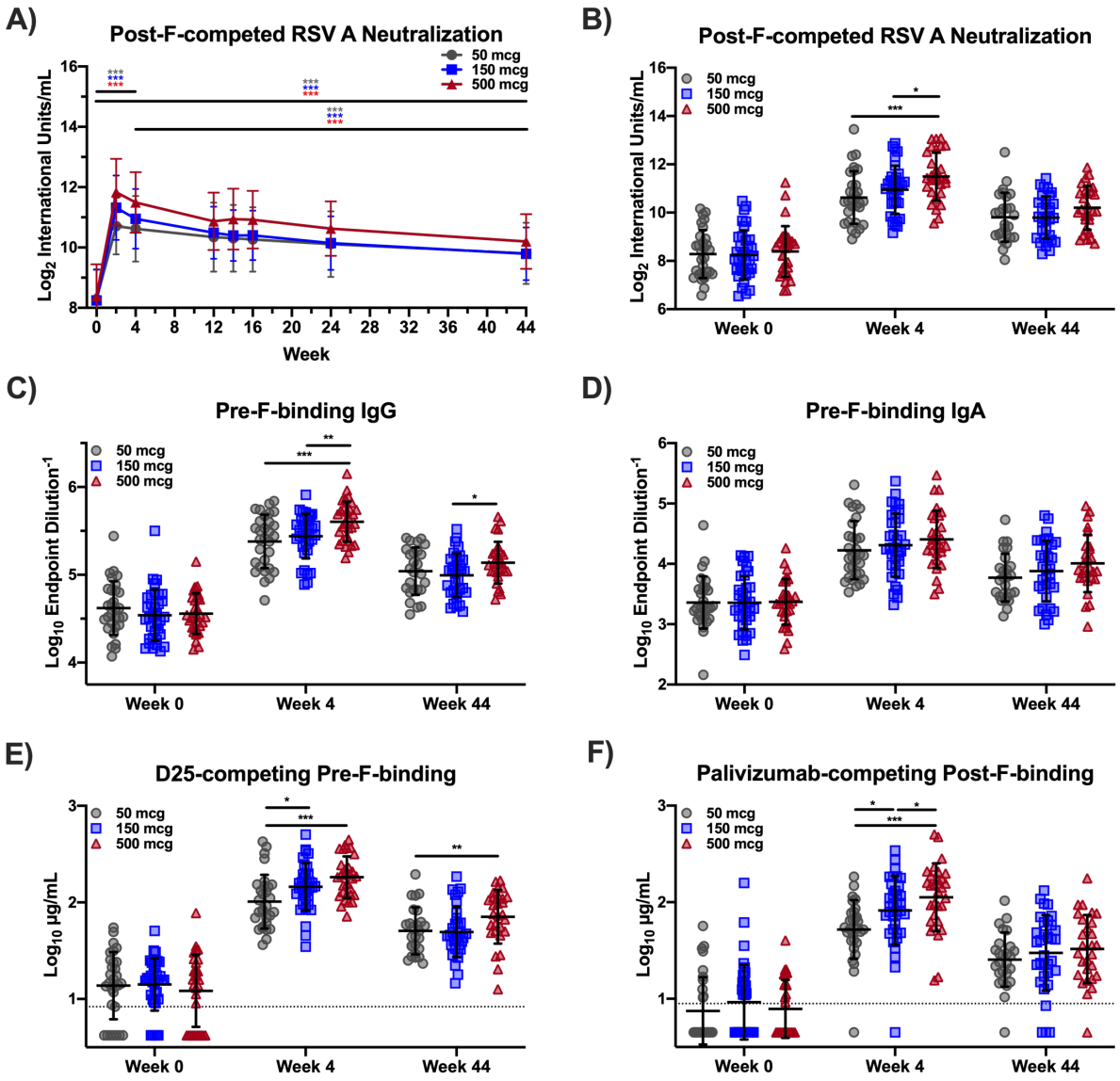
RSC-specific neutralizing antibodies in nasal secretions were assessed in an exploratory endpoint. Flocked swabs (COPAN Diagnostics Inc.) were used to collect nasopharyngeal (NP) samples at study weeks 0 and 14. Due to the variability in acquisition volumes, total IgG and IgA in the nasal fluid was measured with Human IgG and IgA ELISA Kits (Invitrogen). Pre-F-specific IgG and IgA were measured by endpoint ELISA (described above), and units/µg of pre-F-specific IgG and IgA were calculated by dividing 1 µg of total IgG or IgA by the respective endpoint dilution to control for differences in the amount of antibody obtained from the nasal swab.

Figure S1. Alum adjuvant has no effect on neutralizing activity.



Longitudinal serum neutralizing activity measured at study weeks 0, 2, 4, 12, 14, 16, 24 and 44 separated into subjects who received 50 mcg, 150 mcg, or 500 mcg against reporter RSV A2 (RSV A) virus (Panels A, C, and E, respectively), and against the reporter B18537 (RSV B) virus (Panels B, D, and F, respectively). Subjects who received alum are shown in dashed lines, and subjects who did not receive alum adjuvant are shown in solid lines.

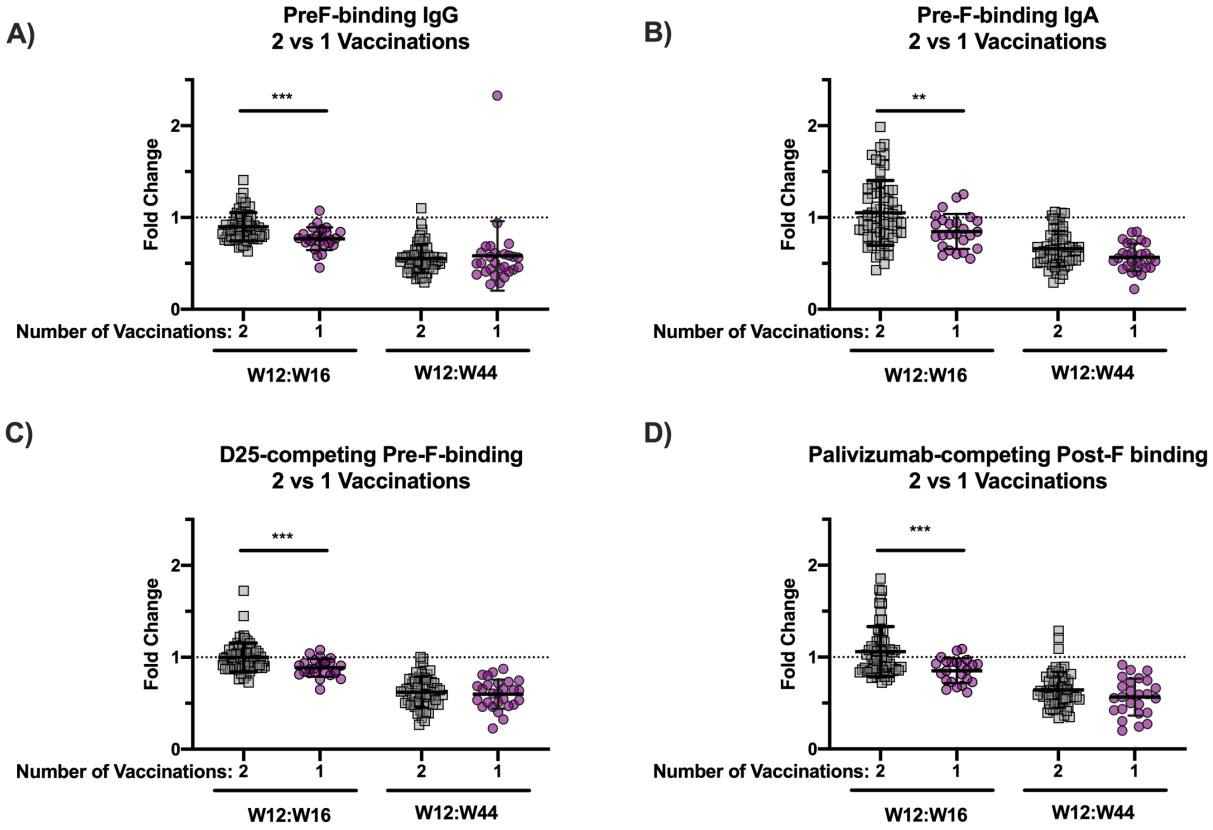
Figure S2. Higher doses of DS-Cav1 have a modest impact on the elicitation of neutralizing activity and binding antibodies at week 4 that is minimized by week 44.



Pre-F conformation-specific serum neutralizing activity against RSV A (Panels A and B) in the presence of excess post-F which competes for binding to post-F exclusive and dual-binding antibodies, demonstrating neutralizing antibodies are directed to the pre-F-exclusive and shared surfaces of pre-F and post-F. RSV A pre-F-binding IgG (Panel C) and IgA (Panel D) measured by ELISA are shown at W0, W4, and W44 for individuals in each dose group. Serum concentrations of apex-binding antibodies to pre-F measured by a D25 competition assay at W0, W4, and W44 (Panel E) and side-binding antibodies to post-F measured by a palivizumab competition assay (Panel F) demonstrate a comparable, dose-dependent increase in antibodies that bind the apex and side of the F protein. Dotted lines indicate the lower limit of quantitation (LLOQ). Any value less than the LLOQ was assigned a value of 1/2 the LLOQ. In Panel D, W0 antibody levels were undetectable in 7 out of 30 subjects (50 mcg), 5 out of 35 subjects (150 mcg), and 10 out of 30 subjects (500 mcg). In Panel E, W0 antibody levels were undetectable in 18 out of 30 subjects (50 mcg), 17 out of 35 subjects (150 mcg), and 17 out of 30 subjects (500 mcg). W44 titers were undetectable in 1 out of 30 subjects in the 50 mcg dose group, but could be quantified in all remaining subjects.

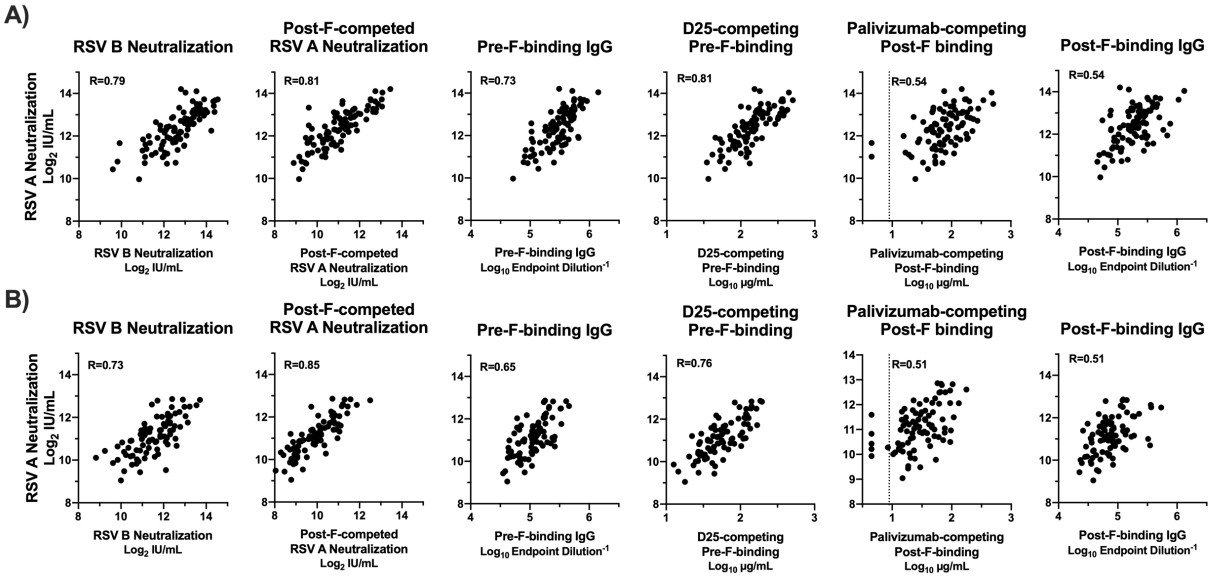
Statistical differences between doses were determined by linear regression and differences between timepoints by linear regression without an adjustment for multiple comparisons. Significance indicated as ***p<0·001, **p<0·01, *p<0·05.

Figure S3. A second dose of DS-Cav1 has a marginal effect on pre-F-binding antibodies between week 12 and week 16 but no significant long-term impact.



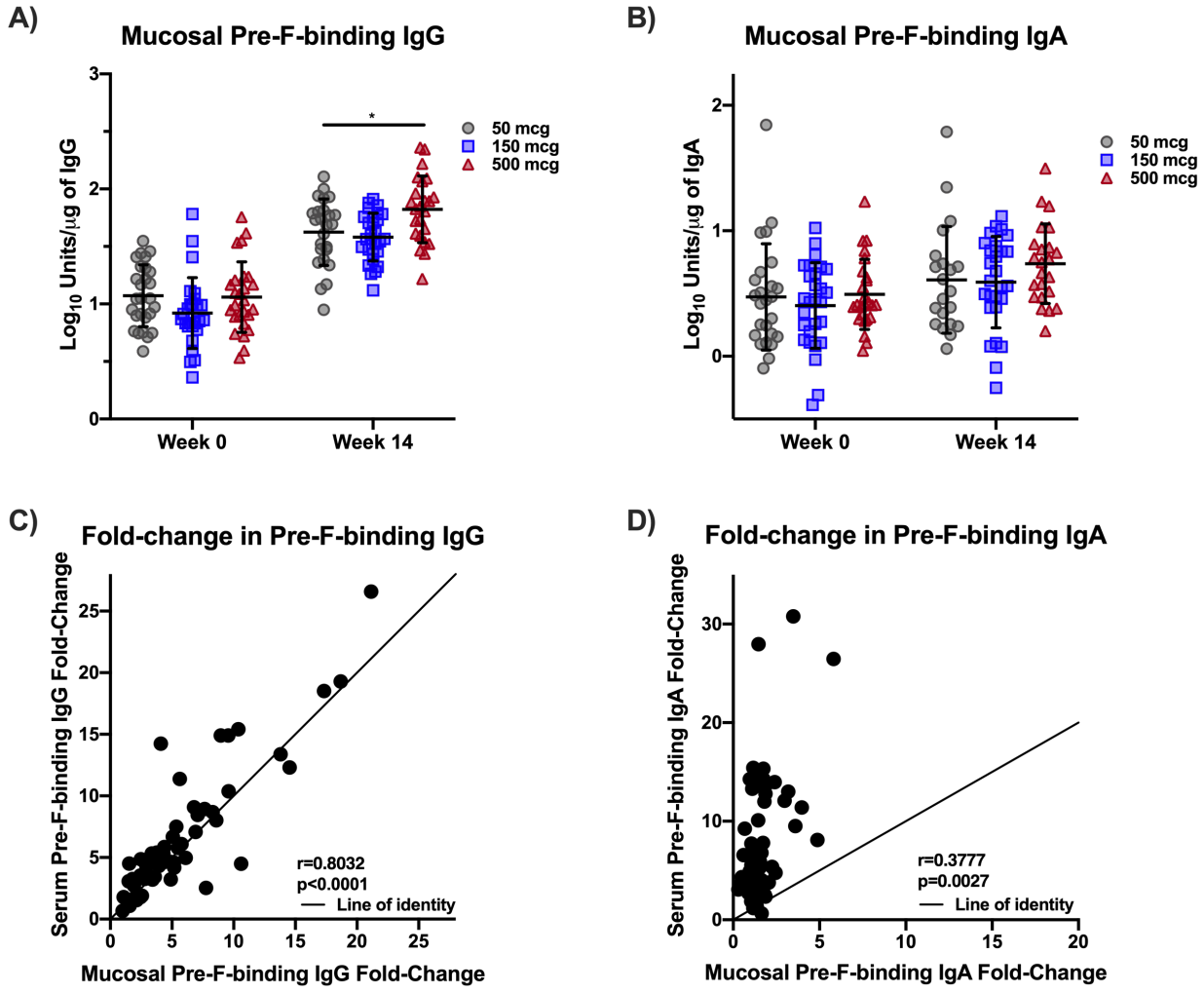
Panels A-D show the fold-change in pre-F-binding IgG and IgA, and D25- and palivizumab-competing antibodies, respectively, between W12 and W16 in subjects that received 2 or 1 vaccinations and between W12 and W44 in these groups. Dotted line represents a fold-change of 1. Significance determined by linear regression without adjustment for multiple comparisons. Significance indicated as ***p<0·001, **p<0·01, *p<0·05.

Figure S4. Neutralization assays and D25-competing antibodies are highly correlated with RSV A neutralization.



The correlation of RSV A neutralization with RSV B neutralization, post-F-competed RSV A neutralization, pre-F-binding IgG, post-F-binding IgG, D25-competing pre-F-binding, and palivizumab-competing post-F binding at week 4 (Panel A) and week 44 (Panel B).

Figure S5. Intramuscular immunization with DS-Cav1 elicited mucosal pre-F IgG and IgA antibodies.



Relative pre-F-specific IgG (Panel A) and IgA (Panel B) in the mucosal fluid of subjects immunized with 50, 150, and 500 mcg of DS-Cav1 at W0 and W14, and the Spearman correlation between the fold-change in the serum and mucosal fluid between W0 and W14 (Panel C and D). Significance determined by linear regression without adjustments for multiple comparisons and are indicated as *p<0·05.

Table S1. Local Solicited Reactogenicity

Local Parameters Number of Subjects (%)													
	Group 1 50 mcg		Group 2 50 mcg + Alum		Group 3 150 mcg		Group 4 150 mcg + Alum		Group 5 500 mcg		Group 6 500 mcg + Alum		All
Severity	1 Admin (N=15)	2 Admin (N=10)	1 Admin (N=15)	2 Admin (N=11)	1 Admin (N=20)	2 Admin (N=11)	1 Admin (N=15)	2 Admin (N=9)	1 Admin (N=15)	2 Admin (N=11)	1 Admin (N=15)	2 Admin (N=10)	All Groups (N=95)
Pain/Tenderness													
None	6 (40)	3 (30)	5 (33)	6 (54·5)	11 (55)	6 (54·5)	8 (53·3)	4 (44·4)	7 (46·7)	2 (18·2)	6 (40)	3 (30)	27 (28·4)
Mild	9 (60)	7 (70)	10 (66·7)	5 (45·5)	9 (45)	5 (45·5)	7 (46·7)	5 (55·6)	8 (53·3)	8 (72·7)	9 (60)	7 (70)	67 (70·5)
Moderate	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	1 (9·1)	0 (0)	0 (0)	1 (1·1)
Swelling													
None	15 (100)	10 (100)	15 (100)	11 (100)	20 (100)	11 (100)	15 (100)	9 (100)	15 (100)	11 (100)	14 (93·3)	10 (100)	94 (98·9)
Mild	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	1 (6·7)	0 (0)	1 (1·1)
Redness													
None	15 (100)	10 (100)	15 (100)	11 (100)	20 (100)	11 (100)	15 (100)	9 (100)	15 (100)	11 (100)	15 (100)	10 (100)	95 (100)
Any Local Symptom													
None	6 (40)	3 (30)	5 (33·3)	6 (54·5)	11 (55)	6 (54·5)	8 (53·3)	4 (44·4)	7 (46·7)	2 (18·2)	6 (40)	3 (30)	27 (28·4)
Mild	9 (60)	7 (70)	10 (66·7)	5 (45·5)	9 (45)	5 (45·5)	7 (46·7)	5 (55·6)	8 (53·3)	8 (72·7)	9 (60)	7 (70)	67 (70·5)
Moderate	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	1 (9·1)	0 (0)	0 (0)	1 (1·1)

Table S2. Systemic Solicited Reactogenicity

Systemic Parameters Number of Subjects (%)													
	Group 1 50 mcg		Group 2 50 mcg + Alum		Group 3 150 mcg		Group 4 150 mcg + Alum		Group 5 500 mcg		Group 6 500 mcg + Alum		All
Severity	1 Admin (N=15)	2 Admin (N=10)	1 Admin (N=15)	2 Admin (N=11)	1 Admin (N=20)	2 Admin (N=11)	1 Admin (N=15)	2 Admin (N=9)	1 Admin (N=15)	2 Admin (N=11)	1 Admin (N=15)	2 Admin (N=10)	All Groups (N=95)
Malaise													
None	13 (86·7)	9 (90)	13 (86·7)	9 (81·8)	17 (85)	11 (100)	12 (80)	8 (88·9)	11 (73·3)	8 (72·7)	11 (73·3)	9 (90)	72 (75·8)
Mild	2 (13·3)	0 (0)	2 (13·3)	2 (18·2)	3 (15)	0 (0)	3 (20)	1 (11·1)	4 (26·7)	3 (27·3)	4 (26·7)	1 (10)	22 (23·2)
Moderate	0 (0)	1 (10)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	1 (1·1)
Myalgia													
None	15 (100)	9 (90)	13 (86·7)	10 (90·9)	19 (95)	9 (81·8)	9 (60)	8 (88·9)	12 (80)	10 (90·9)	13 (86·7)	7 (70)	76 (80)
Mild	0 (0)	1 (10)	2 (13·3)	1 (9·1)	1 (5)	2 (18·2)	6 (40)	1 (11·1)	3 (20)	1 (9·1)	2 (13·3)	3 (30)	19 (20)
Headache													
None	13 (86·7)	8 (80)	11 (73·3)	10 (90·9)	18 (90)	10 (90·9)	10 (66·7)	8 (88·9)	13 (86·7)	8 (72·7)	10 (66·7)	9 (90)	69 (72·6)
Mild	2 (13·3)	2 (20)	4 (26·7)	1 (9·1)	2 (10)	1 (9·1)	5 (33·3)	1 (11·1)	2 (13·3)	2 (18·2)	5 (33·3)	1 (10)	25 (26·3)
Moderate	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	1 (9·1)	0 (0)	0 (0)	1 (1·1)
Chills													
None	14 (93·3)	10 (100)	15 (100)	11 (100)	20 (100)	11 (100)	14 (93·3)	9 (100)	13 (86·7)	11 (100)	13 (86·7)	9 (90)	89 (93·7)
Mild	1 (6·7)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	1 (6·7)	0 (0)	2 (13·3)	0 (0)	2 (13·3)	1 (10)	6 (6·3)
Nausea													
None	14 (93·3)	10 (100)	14 (93·3)	11 (100)	19 (95)	11 (100)	15 (100)	9 (100)	14 (93·3)	11 (100)	14 (93·3)	9 (90)	89 (93·7)
Mild	1 (6·7)	0 (0)	1 (6·7)	0 (0)	1 (5)	0 (0)	0 (0)	0 (0)	1 (6·7)	0 (0)	1 (6·7)	1 (10)	6 (6·3)
Temperature													
None	15 (100)	10 (100)	15 (100)	11 (100)	20 (100)	11 (100)	15 (100)	9 (100)	14 (93·3)	11 (100)	15 (100)	9 (90)	93 (97·9)
Mild	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	1 (6·7)	0 (0)	0 (0)	1 (10)	2 (2·1)
Any Systemic Symptom													

None	12 (80)	7 (70)	11 (73·3)	9 (81·8)	15 (75)	9 (81·8)	9 (60)	7 (77·8)	10 (66·7)	7 (63)	9 (60)	6 (60)	56 (58·9)
Mild	3 (20)	2 (20)	4 (26·7)	2 (18·2)	5 (25)	2 (18·2)	6 (40)	2 (22.2)	5 (33·3)	3 (27·3)	6 (40)	4 (40)	37 (38·9)
Moderate	0 (0)	1 (10)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	1 (9·1)	0 (0)	0 (0)	2 (2·1)

Table S3. RSV A Neutralizing activity

Group	**GMT International Units/mL (Range)**							
	W0	**W2**	**W4**	**W12**	**W14**	**W16**	**W24**	**W44**
Group 1 50 mcg	777 (192-3167)	4691 (1652-16058)	4203 (1001-10910)	3465 (991-13102)	3133 (870-11290)	3048 (842-9545)	2648 (715-8430)	2155 (692-7249)
Group 2 50 mcg + Alum	689 (288-2141)	3663 (1927-9037)	3486 (1385-18852)	2631 (1253-19017)	2588 (1264-15130)	2446 (1566-10771)	2338 (1186-9549)	1674 (910-7066)
Group 3 150 mcg	736 (216-4676)	6385 (1458-18052)	5422 (2050-13104)	3345 (1152-8996)	3019 (1305-8000)	3191 (1184-9673)	2699 (729-7079)	1988 (528-4265)
Group 4 150 mcg + Alum	653 (186-2476)	7061 (1627-22575)	5513 (1715-17602)	3874 (1910-10644)	3835 (1328-9072)	3676 (1922-7977)	2980 (1331-7777)	2317 (739-7435)
Group 5 500 mcg	707 (230-5725)	9860 (2555-33081)	7495 (2370-16870)	4904 (1735-11420)	6109 (1991-11601)	4914 (2597-9251)	4030 (1729-8645)	3081 (1081-6238)
Group 6 500 mcg + Alum	669 (321-1840)	7606 (2045-21263)	6083 (2301-13522)	3906 (1750-9882)	3910 (1467-10061)	3921 (1469-10551)	3131 (1199-9674)	2474 (881-7310)

Table S4. RSV B Neutralizing Activity

Group	GMT International Units/mL (Range)							
	W0	W2	W4	W12	W14	W16	W24	W44
Group 1 50 mcg	1421 (540-4442)	7046 (2124-29903)	6685 (1826-20843)	5530 (1913-18374)	5316 (1305-13862)	5646 (1466-13312)	4728 (1262-17045)	3619 (1137-13382)
Group 2 50 mcg + Alum	636 (233-1608)	2913 (985-13998)	2795 (774-10855)	1993 (513-6748)	2118 (494-6088)	1904 (611-6810)	1760 (596-3610)	1407 (454-3363)
Group 3 150 mcg	1273 (535-5001)	9514 (2108-24713)	7562 (2210-21794)	5257 (2632-11855)	5008 (1665-15174)	4693 (1776-14742)	4074 (1862-14689)	2991 (1023-8373)
Group 4 150 mcg + Alum	1025 (342-3145)	9482 (4477-21856)	7568 (3638-15645)	4974 (2583-12039)	5031 (2166-20927)	5436 (2808-12832)	4477 (2216-14258)	3624 (1498-9583)
Group 5 500 mcg	1102 (401-7039)	12652 (4763-30634)	9281 (3129-23744)	6223 (2428-17001)	6783 (2458-18400)	6470 (1981-18506)	4742 (1566-10851)	3751 (1204-11938)
Group 6 500 mcg + Alum	940 (414-2560)	9372 (3980-21068)	7348 (2494-15614)	4546 (1820-9667)	5400 (2229-11802)	5300 (1979-13580)	3995 (1128-10323)	3056 (790-8998)

Table S5. RSV Neutralizing Activity Fold-change

Group	RSV A Neutralization Fold-change (Range)		RSV B Neutralization Fold-change (Range)	
	W0-W4	W0-W44	W0-W4	W0-W44
Group 1 50 mcg	5.4 (0·9-25·4)	2.9 (0·7-12·3)	4.7 (1·2-35·7)	2.6 (1·0-9·4)
Group 2 50 mcg + Alum	5.1 (1·2-24·8)	2.4 (1·2-4·2)	4.4 (1·2-16·1)	2.3 (1·1-4·0)
Group 3 150 mcg	7.4 (0·8-28·0)	2.7 (0·5-13·9)	5.9 (0·7-24·7)	2.3 (0·4-6·2)
Group 4 150 mcg + Alum	8.4 (2·3-29·5)	3.3 (1·3-20·2)	7.4 (1·9-25·1)	3.4 (1·3-15·3)
Group 5 500 mcg	10.6 (1·5-39·1)	3.9 (0·8-14·5)	8.4 (1·8-22·4)	3.3 (0·7-8·6)
Group 6 500 mcg + Alum	9.1 (3·2-26·8)	3.7 (1·7-11·8)	7.8 (3·2-23·8)	3.3 (1·2-8·9)

References

1. Crank MC, Ruckwardt TJ, Chen M, et al. A proof of concept for structure-based vaccine design targeting RSV in humans. *Science* 2019; **365**(6452): 505-9.
2. Phung E, Chang LA, Morabito KM, et al. Epitope-Specific Serological Assays for RSV: Conformation Matters. *Vaccines (Basel)* 2019; **7**(1).