VACCINES

A proof of concept for structure-based vaccine design targeting RSV in humans

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Technologies that define the atomic-level structure of neutralization-sensitive epitopes on viral surface proteins are transforming vaccinology and guiding new vaccine development approaches. Previously, iterative rounds of protein engineering were performed to preserve the prefusion conformation of the respiratory syncytial virus (RSV) fusion (F) glycoprotein, resulting in a stabilized subunit vaccine candidate (DS-Cav1), which showed promising results in mice and macaques. Here, phase I human immunogenicity data reveal a more than 10-fold boost in neutralizing activity in serum from antibodies targeting prefusion-specific surfaces of RSV F. These findings represent a clinical proof of concept for structure-based vaccine design, suggest that development of a successful RSV vaccine will be feasible, and portend an era of precision vaccinology.

Respiratory syncytial virus (RSV) is a leading cause of severe respiratory disease in young infants and the elderly. Since its discovery in 1956, multiple vaccine development programs have been initiated, but none have resulted in a licensed vaccine. Protein-based RSV vaccines have had a particularly complicated history, especially those in which the primary immunogen has been the fusion (F) glycoprotein, which exists in two major conformational states: prefusion (pre-F) and postfusion (post-F). During the 1960s, clinical trials were performed in young children and infants by using a whole-inactivated virus formulated with aluminum salts (FI-RSV), in which immunized children experienced vaccine-associated disease enhancement after natural infection in the subsequent RSV season (*1*–*4*). Since then, clinical trials of F subunit vaccine candidates, which contain a post-F or structurally undefined F protein (*5*), have induced an approximately two- to fourfold increase in neutralizing activity and a ~10- to 30-fold increase in F-protein binding antibody levels (*6*, *7*). Although the products are immunogenic, a substantial proportion of antibodies elicited are non- or poorly neutralizing, and field trials have shown no or minimal efficacy. In retrospect, many of these failures can be explained by the atomic-level understanding of F conformational states, antigenic sites, and the specificity of the human B cell repertoire and serum antibody response to infection (*8*–*20*). A substantial boost in neutralizing activity will be necessary for a vaccine to protect infants beyond 3 months of age through maternal immunization or to substantially protect the elderly (*21*, *22*).

RSV F mediates membrane fusion and is required for infection. The structures of pre-F and post-F have been solved, and major antigenic sites have been defined on the basis of structural domains, antibody competition, and sequencing of neutralizing antibody-escape mutants (Fig. 1) (*9*–*11*, *17*, *23*, *24*). Most of the sites on the side and membrane-proximal regions of the pre-F head domain are retained on the post-F molecule after rearrangement (sites II, III, and IV). The apex of pre-F contains sites Ø and V, which are highly neutralization-sensitive and exclusive to the pre-F conformation (*9*, *11*, *25*). The majority of neutralizing activity in human sera can be adsorbed by using pre-F, whereas post-F removes substantially less (*15*, *16*). Monoclonal antibodies (mAbs) to the shared surfaces of pre-F and post-F have variable levels of RSV-neutralizing potency but are generally less potent than mAbs to pre-F–exclusive sites Ø and V (*11*). Mutations have been introduced to stabilize a pre-F trimeric subunit protein, DS-Cav1, and to eliminate triggering and rearrangement into the post-F conformation (*9*). DS-Cav1 was produced in Chinese hamster ovary cells for clinical evaluation as a vaccine candidate that maintained structural integrity (Fig. 1 and fig. S1) (*9*).

We evaluated whether the preservation of pre-F–exclusive antigenic sites on DS-Cav1 improved the quality of elicited antibody in healthy adult volunteers. A randomized, open-label phase I dose-escalation clinical trial [Vaccine Research Center (VRC) 317] was initiated to evaluate two administrations of DS-Cav1 with and without aluminum hydroxide (alum) formulation at three different dose levels in healthy adults between 18 and 50 years of age (table S1). The vaccine was well tolerated without any serious adverse events reported (tables S2 to S4). Here, we report the prospectively planned interim analysis, evaluating immune responses to the first administration of 50 or 150 μg of DS-Cav1 with or without alum (n = 10 subjects per group) (fig. S2).

The primary immunogenicity endpoint was vaccine-induced neutralizing activity. We normalized to international units (IU) for RSV subtype A by using a panel of serum standards and the First International Standard for Antiserum to Respiratory Syncytial Virus [National Institute for Biological Standards and Control (NIBSC) code 16/284] (fig. S3 and table S5). RSV A neutralization was measured at baseline [week 0 (W0)], W4, and W12. At W4, neutralizing activity was increased sevenfold after immunization with 50 μg of DS-Cav1 without or with alum and 12- and 15-fold after 150 μg without and with alum, respectively (all $P < 0.001$) (Fig. 2, A and C, and Table 1). These increases in neutralizing activity were higher than those previously reported for F protein subunit vaccines (*5*, *26*–*28*) and exceeded the threefold increase in neutralization reported after experimental human challenge with RSV (*29*). Neutralization remained 5- to 10-fold above baseline at W12 ($P < 0.001$) (Fig. 2C and Table 1). We used a reporter RSV expressing the F protein of subtype B strain 18537 (RSV B) to assess neutralizing activity against a virus with a maximally divergent F protein (fig. S4). Neutralization of RSV B increased by four- and sixfold in the groups receiving 50 μg DS-Cav1 without and with alum, respectively, and ninefold in groups receiving 150 μg DS-Cav1 without or with alum (all $P < 0.001$) (Fig. 2, B and C, and Table 1) between W0 and W4. Neutralization of RSV B also remained significantly higher at W12 than at baseline in groups administered 50 μg with alum and 150 μg without alum ($P < 0.001$) (Fig. 2C and Table 1). The boost in neutralizing activity to subtype B after a single immunization with a subtype A–based F vaccine reflected the high conservation of F between subtypes and suggested that multiple prior infections by both RSV A and B subtypes establishes a broad pre-existing B cell repertoire.

Statistical analysis identified no significant effect of adjuvant. There was a marginal vaccine dose-effect on neutralization of RSV A and RSV B at the W4 time point (P = 0.012 and P = 0.004,

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respectively) when controlling for adjuvant and baseline neutralization. This effect was not seen without adjustment for baseline neutralization, or at W12. Therefore, we performed subsequent analyses on compiled data from all four groups. We used competition neutralization assays to assess the contribution of antibodies specific for post-F and pre-F to neutralization. In the presence of excess post-F protein, which adsorbs post-F–exclusive antibodies and antibodies that bind the shared surfaces on post-F and pre-F, neutralization activity was substantially dampened (depleted by 75% at W0, 81% at W4, and 80% at W12). Nevertheless, post-F–competed neutralization was significantly higher at W4 and W12 than at baseline ($P < 0.001$) (Fig. 3A), demonstrating that pre-F–exclusive antibodies were successfully elicited through DS-Cav1 vaccination. Conversely, competition with excess pre-F protein, which adsorbs pre-F–exclusive antibodies and antibodies that bind both pre-F and post-F, nearly abrogated neutralizing activity at all time points (depleted by 97% at W0 and 100% at W4 and W12) (Fig. 3A). Thus, the antibodies responsible for vaccine-induced increase in neutralizing activity bound surfaces on the pre-F conformation. Similarly, excess post-F diminished immunoglobulin G (IgG) binding to pre-F in an enzyme-linked immunosorbent assay, but a significant increase in pre-F–exclusive binding was retained between W0 and the W4 and W12 time points ($P < 0.001$) (Fig. 3B). Uncompeted IgG binding to post-F was similarly increased sixfold and fourfold over baseline at W4 and W12, respectively, indicating that binding antibody was directed toward both pre-F–exclusive surfaces and shared surfaces on pre-F and post-F (fig. S5A). IgA binding to pre-F was also significantly increased at W4 ($P < 0.001$) (fig. S5B). The fold-change ratio of binding to neutralization—calculated as the fold-change in post-F binding IgG at W4 divided by the fold-change in RSV A neutralization at W4—was determined to compare our results with those from prior clinical trials. This ratio averaged 0.65, indicating that DS-Cav1 immunization increased the average neutralizing potency of F-specific antibodies compared with preexisting F-specific antibodies (fig. S5C). Because non-neutralizing antibodies have been associated with the FI-RSV vaccine–enhanced respiratory disease (*7*), the relative increase in neutralizing potency of antibodies elicited by DS-Cav1 may both improve vaccine efficacy and reduce the risk of vaccine-associated adverse events. Additionally, vaccination with DS-Cav1 induced $CD4^+$ T cells with a T helper 1 (T_H1)–biased cytokine profile while not generating significant T_H2 or T_H17 $CD4^+$ T cell responses (figs. S6 and S7).

We hypothesized that the improved increase in neutralizing activity relative to the increase in binding antibodies reflected the elicitation of antibodies specific to the neutralization-sensitive apex of the pre-F molecule (*11*). Antibodies that recognized the apex of pre-F were assessed through competition with biotinylated, site Ø–directed mAb D25 and were significantly increased over baseline at W4 and W12 ($P < 0.001$) (Fig. 3C) (*30*). Similarly, antibodies that bound the side of pre-F, measured through competition with the site II–directed mAb palivizumab, were significantly boosted by vaccination whether a pre-F antigen (Fig. 3D) or a post-F antigen (fig. S5D) was used as a substrate ($P < 0.001$). Thus, DS-Cav1 vaccination elicited antibody responses to pre-F–exclusive surfaces on the apex and shared pre-F and post-F surfaces on the side of pre-F. Analysis of experimental endpoints at W4 and W0 demonstrated that elicitation of pre-F–specific antibody correlated with neutralization (figs. S8 and S9 and tables S6 and S7).

Pre-F and post-F probes were developed to analyze B cell responses (fig. S10A) from before (W0) and 2 weeks after vaccination (W2) in three subjects per group (12 total). Human IgG^+ and IgA^+ B cells were partitioned into three categories: cells that preferentially bound the pre-F probe, cells that bound both the pre-F and post-F probes (dual-binding cells), and cells that preferentially bound the post-F probe (Fig. 3E and fig. S10B). The number of IgG^+ and IgA^+ B cells that preferentially bound the pre-F probe increased as well as B cells that bound both the pre-F

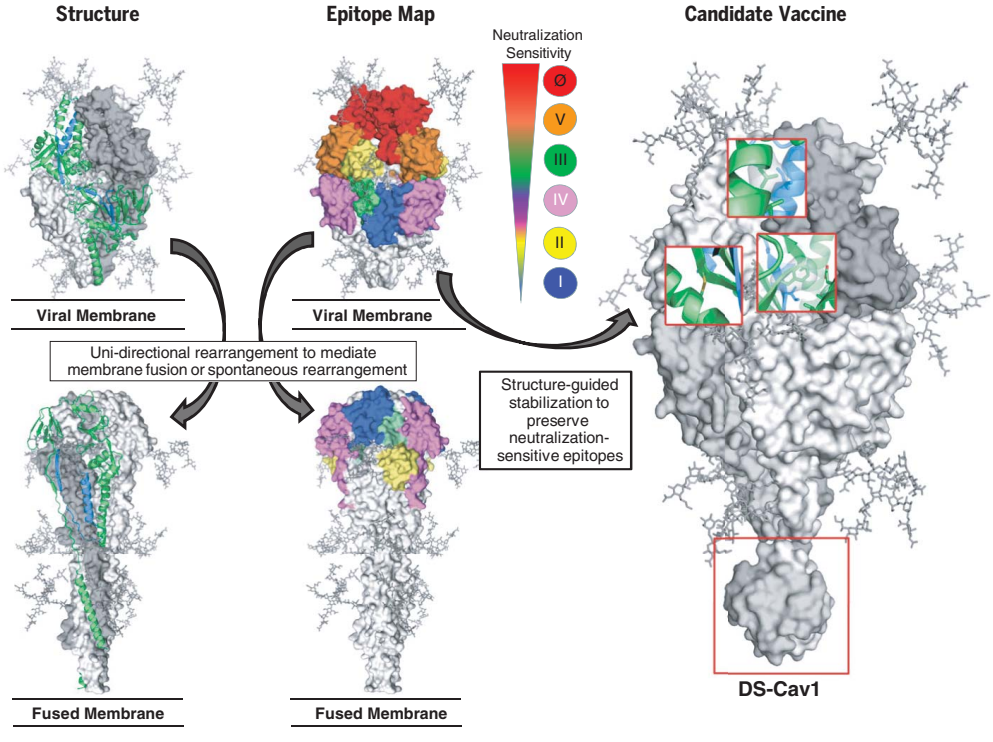

Fig. 1. DS-Cav1, a stabilized prefusion RSV F subunit protein candidate vaccine. RSV F is a class I fusion protein displayed as a trimer of F2-F1 heterodimers on the infected cell or virus membrane. The pre-F conformation is metastable and undergoes an irreversible rearrangement to the nonfunctional post-F conformation when mediating membrane fusion or when spontaneously triggered. Although ~50% of the surface is shared between the pre-F and post-F, the most neutralization-sensitive antigenic sites are exclusively on the pre-F conformation. Antibodies to site Ø (red) and V (orange) have the most potent neutralizing activity. Antibodies to site III (green) have the uncommon property of neutralizing RSV using germline immunoglobulin sequences (*17*). Boxed in red are a foldon trimerization domain at the C terminus of F1, two mutations (S155C and S290C) that form a disulfide bond between fixed and mobile portions of the protein, and two cavity-filling modifications (S190F and V207L) that were used to produce the stabilized pre-F trimer vaccine, DS-Cav1. These modifications prevent the molecule from rearranging into the post-F conformation. (Single-letter abbreviations for the amino acid residues are as follows: C, Cys; F, Phe; L, Leu; S, Ser; and V, Val. In the mutants, amino acids were substituted at certain locations; for example, S155C indicates that serine at position 155 was replaced by cysteine.)

and post-F probes (Fig. 3, E to H). By contrast, there was no detectable increase in B cells that preferentially bound the post-F probe (Fig. 3, F and H). The pre-F probe clearly delineated a boost in the frequency of memory IgA-expressing B cells at W2 after a single administration of DS-Cav1. IgA^+ memory B cells were not identified after experimental human challenge, but the assay used in that analysis used structurally undefined F protein and therefore may have missed pre-F–reactive IgA-expressing cells (*29*). The increase in pre-F–preferring and dual-binding B cells elicited by DS-Cav1 vaccination was consistent with the serological profile of elicited binding and neutralizing antibody.

This interim analysis demonstrates DS-Cav1 immunization elicits antibody responses with a superior functional profile relative to historical RSV subunit vaccines. This indicates that structure-guided design of stabilized RSV pre-F preserves neutralization-sensitive epitopes not present on prior RSV F subunit vaccines and provides the basis for development of an effective RSV vaccine. Further stabilization of the pre-F conformation may demonstrate improved immunogenicity, as shown in preclinical animal models (*31*). Vaccine development has traditionally been an empirical process, and licensed antiviral vaccines have typically used whole-virus or virus-like particles without prior knowledge

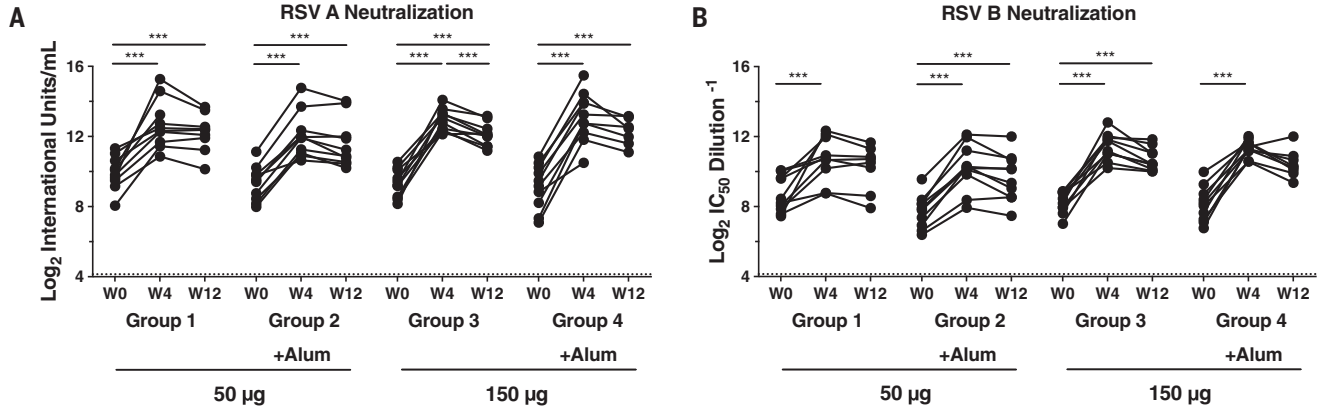

Fig. 2. DS-Cav1 vaccination elicits potent neutralizing antibody responses to RSV subtypes A and B. (**A**) Neutralization of recombinant reporter RSV A2 virus (RSV A) was measured in sera obtained before vaccination (W0), W4, and W12. Measurements were normalized to international units per milliliter. (**B**) Neutralization of reporter RSV B virus (RSV B) at W0, W4, and W12. ***$P < 0.001$. Dotted lines indicate limit of detection (LOD). Data shown are from two technical replicates from a single experiment. For all assays, results for 10 subjects per group were reported for all time points, with the exception of W12, for which results for seven subjects were reported for group 4. All P values were determined by using paired Student's t tests without adjustment for multiple comparisons. The full dataset used to generate these and all other graphs and tables in the manuscript are available in the supplementary materials (Data File S1), and geometric mean titers (GMT) and fold-changes are listed in Table 1.

Table 1. Neutralizing activity against RSV A and RSV B. GMT and fold-change in neutralizing activity between W0, W4, and W12. IC_{50}, median inhibitory concentration.

	Group	GMT (range)			Fold-change (range)		
		W0	W4	W12	W0 to W4	W0 to W12	W4 to W12
RSV A neutralization IU/mL	Group 1, 50 µg	991 (270 to 2585)	6647 (1877 to 39,869)	4779 (1140 to 13,240)	6.7 (2.2 to 49.2)	4.9 (2 to 16.3)	0.8 (0.3 to 1.2)
	Group 2, 50 µg + alum	648 (255 to 2264)	4287 (1608 to 28,235)	3108 (1193 to 16,694)	6.6 (1.8 to 12.5)	4.8 (1.6 to 12.7)	0.7 (0.4 to 1.1)
	Group 3, 150 µg	656 (289 to 1516)	7888 (4526 to 17,635)	4427 (2354 to 9312)	12.0 (4.1 to 42.0)	6.7 (2.9 to 32.2)	0.6 (0.4 to 1.0)
	Group 4, 150 µg + alum	577 (138 to 1863)	8851 (1461 to 46,006)	4988 (2208 to 9203)	15.3 (3.2 to 42.5)	10.2 (4.7 to 37.0)	0.6 (0.3 to 0.9)
RSV B neutralization IC_{50} dilution^{-1}	Group 1, 50 µg	415 (175 to 1083)	1700 (433 to 5200)	1194 (240 to 3226)	4.1 (1.5 to 29.8)	3.4 (1.1 to 18.5)	0.8 (0.6 to 1.3)
	Group 2, 50 µg + alum	212 (84 to 756)	1203 (243 to 4462)	813 (177 to 4100)	5.7 (2.9 to 14.1)	3.8 (2.1 to 9.2)	0.7 (0.4 to 1.1)
	Group 3, 150 µg	304 (129 to 473)	2763 (1182 to 7196)	1661 (1030 to 3676)	9.1 (3.3 to 24.7)	5.5 (2.4 to 15.7)	0.6 (0.3 to 0.9)
	Group 4, 150 µg + alum	283 (109 to 1012)	2607 (1516 to 4186)	1384 (658 to 4108)	9.2 (3.2 to 24.8)	4.8 (1.3 to 37.5)	0.6 (0.3 to 1.5)

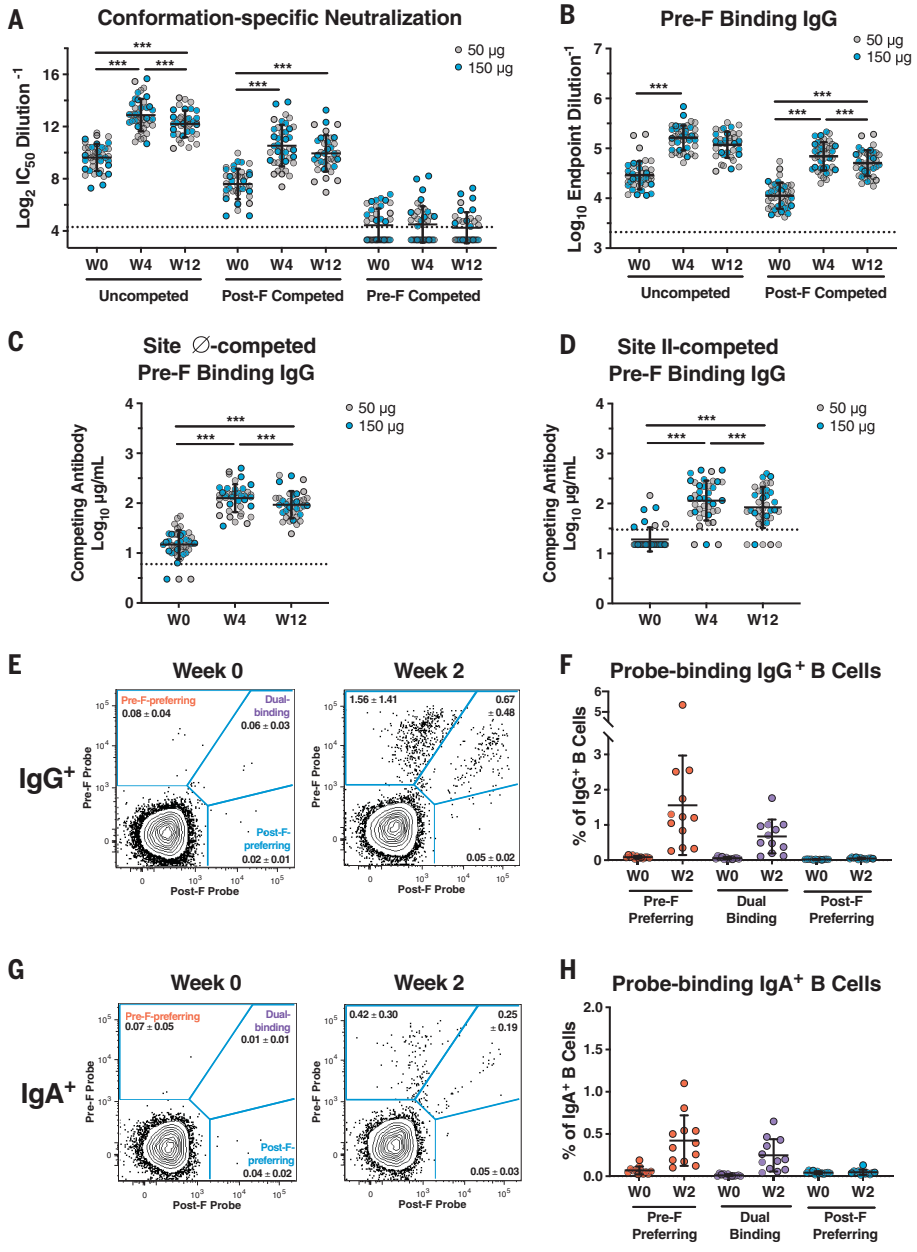

Fig. 3. DS-Cav1 elicits pre-F–preferring and dual-binding antibody and B cell responses. (**A**) Conformation-specific neutralization was measured at W0, W4, and W12 in the presence of excess post-F (competes for post-F–exclusive and dual-binding antibodies) or pre-F (competes for pre-F–exclusive and dual-binding antibodies). Bars represent mean ± SD of log_2 neutralization titer. (**B**) Pre-F–binding IgG endpoint titers were measured in the absence (uncompeted) or presence (post-F competed) of excess post-F protein at W0, W4, and W12. Bars represent mean ± SD of log_{10} endpoint titer. (**C**) Concentrations of serum antibody that compete for pre-F binding with the site Ø antibody D25 were measured in sera obtained at W0, W4, and W12. Bars represent mean ± SD of log_{10} data. (**D**) Concentrations of serum antibody that compete for pre-F binding with the site II antibody palivizumab were measured at W0, W4, and W12. Bars represent mean ± SD of log_{10} data. (**E**) Representative flow cytometry staining from a single subject comparing profiles of pre-F and post-F probe-binding B cells at W0 and W2 after DS-Cav1 immunization. Class-switched B cells were partitioned into pre-F–preferring, dual-binding, and post-F–preferring populations on the basis of binding to fluorescently labeled pre-F and post-F probes. Numbers inside each gate represent the mean ± SD percentage of total IgG^+ B cells binding to each of the F probes of 12 subjects (three per group). (**F**) Compiled percentage of total IgG^+ B cells that bind pre-F, post-F, or both probes from 12 subjects (three per group) at W0 and W2. Bars represent mean ± SD. (**G**) Representative flow cytometry staining from a single subject comparing profiles of pre-F and post-F probe binding B cells at W0 and W2 after DS-Cav1 immunization. Numbers inside each gate represent the mean ± SD percentage of total IgA^+ B cells binding to each of the F probes of 12 subjects (three per group). (**H**) Compiled percentage of total IgA^+ B cells that bind pre-F, post-F, or both probes from the same 12 subjects (three per group) at W0 and W2. Bars represent mean ± SD. For (A) to (D), data are from two technical replicates from a single experiment. For these assays, results for 10 subjects per group were reported for all time points, with the exception of W12, for which results for seven subjects were reported for group 4. For (E) to (H), each experiment was performed once. For all graphs, ***$P < 0.001$, determined by means of paired Student's t test without adjustment for multiple comparisons. Gray symbols indicate groups given 50 µg DS-Cav1, and blue symbols indicate groups given 150 µg DS-Cav1. Dotted lines indicate LOD. Any value less than the LOD was assigned a value of ½ the LOD.

of atomic-level structure. A notable exception comes from the field of bacterial vaccines, in which structural definition of the pertussis toxin enzymatic site directed the mutagenesis of vaccine antigens to reduce reactogenicity (*32*). We are now entering an era of vaccinology in which new technologies provide avenues to define the structural basis of antigenicity and to rapidly isolate and characterize human monoclonal antibodies. These technologies provide information and reagents for guiding immunogen design and understanding mechanisms of viral neutralization that may be required to achieve calculable outcomes and solve our remaining and future vaccine challenges. The clinical data derived from immunization with RSV F stabilized in its pre-F conformation demonstrate the feasibility of eliciting targeted immune profiles by using structure-based vaccine design and mark a step toward a future of precision vaccines.

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SUPPLEMENTARY MATERIALS

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Materials and Methods
Figs. S1 to S10
Tables S1 to S8
Data File S1

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Science

A proof of concept for structure-based vaccine design targeting RSV in humans

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Building a better RSV vaccine

Respiratory syncytial virus (RSV) causes severe respiratory disease, especially in infants and the elderly. However, attempts to produce effective human vaccines have largely been unsuccessful. Structure-based design has been used to generate an RSV fusion glycoprotein stabilized in its prefusion conformation (DS-Cav1). This immunogen is highly effective in mice and macaques. Crank *et al.* now report the results of a phase I vaccine clinical trial using the stabilized prefusion DS-Cav1 molecule. Four weeks after immunization, these vaccines elicited substantially more high-quality antibody titers than those typically generated using earlier RSV immunogens. The findings provide a proof of concept for how structural biology can contribute to precision vaccine design.

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