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Neutralizing antibodies against the preactive form of respiratory syncytial virus fusion protein offer unique possibilities for clinical intervention

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Human respiratory syncytial virus (hRSV) is the most important viral agent of pediatric respiratory infections worldwide. The only specific treatment available today is a humanized monoclonal antibody (Palivizumab) directed against the F glycoprotein, administered prophylactically to children at very high risk of severe hRSV infections. Palivizumab, as most anti-F antibodies so far described, recognizes an epitope that is shared by the two conformations in which hRSV_F can fold, the metastable prefusion form and the highly stable postfusion conformation. We now describe a unique class of antibodies specific for the prefusion form of this protein that account for most of the neutralizing activity of either a rabbit serum raised against a vaccinia virus recombinant expressing hRSV_F or a human Ig preparation (Respigam), which was used for prophylaxis before Palivizumab. These antibodies therefore offer unique possibilities for immune intervention against hRSV, and their production should be assessed in trials of hRSV vaccines.

Human respiratory syncytial virus (hRSV) is the most important viral cause of severe respiratory tract disease in the pediatric population worldwide (1) and is also of considerable importance in the elderly and immunocompromised adults (2). There is no vaccine available against this virus. A trial conducted with formalin-inactivated hRSV in the 1960s did not confer protection and was associated with enhanced disease in infants upon natural infection with the virus (3).

hRSV belongs to the *Pneumovirus* genus of the *Paramyxoviridae* family. The viral genome consists of a single-stranded RNA molecule of negative polarity that encodes 11 proteins (4). Two of these proteins are the major surface glycoproteins of the virion, namely: (*i*) the attachment protein (G) that mediates binding of the virus to the cell surface and (*ii*) the fusion protein (F) that promotes fusion of the virus and cell membranes during virus entry and also the fusion of the membranes of infected cells with surrounding cells to form syncytia.

Neutralizing antibodies play a major role in protection against hRSV infections. F and G proteins are the only viral antigens able to induce both neutralizing antibodies and relatively long-lived protection in animal models (5, 6). Passive transfer of these antibodies protects mice and cotton rats against a hRSV challenge (7, 8). In human, high titers of serum neutralizing antibodies correlate with protection of adult volunteers against a hRSV challenge (9), and lower risk of hRSV infection in children (10) and the elderly (11). These observations prompted the prophylactic use of an Ig preparation (Respigam) containing high titers of neutralizing antibodies to prevent hRSV infections in high risk infants (12). Respigam has been replaced by a humanized monoclonal antibody (Palivizumab) directed against the hRSV_F glycoprotein (13), which is the only available medicinal product specific for hRSV.

hRSV_F is a type I glycoprotein that assembles as a homotrimer. Each monomer is synthesized as an inactive precursor (F0) that needs to be cleaved at two polybasic sites (I and II) to become fusion competent (14). Site II is equivalent to the single cleavage site of other paramyxovirus F proteins, which precedes a hydrophobic fusion peptide that is inserted into the target membrane during fusion. It has been postulated that the paramyxovirus F proteins remain in a metastable prefusion conformation in the virus particle until the virus binds to the target membrane. The F protein is then activated to initiate a series of conformational changes so that fusion occurs "at the right time and in the right place" (15). After fusion, F acquires a highly stable postfusion conformation determined mainly by the formation of a six-helix bundle (6HB), composed of sequences of two heptad repeats (HRA and HRB) from each monomer (16). The free energy released during the transition of the F protein from the prefusion to the postfusion structure drives the process of membrane fusion.

Palivizumab and most α-F monoclonal antibodies (MAbs) described so far recognizes the postfusion conformation of hRSV_F, represented in an anchorless form of the F protein, referred to as F_{TM-} (17). This artificial protein, engineered to remove the transmembrane region and the cytoplasmic tail of F, is secreted to the culture medium and it folds spontaneously into the postfusion conformation. However, neutralizing MAbs that recognize F_{TM-} inhibit infectivity if preincubated with the virus before being used to infect cells (18); i.e., before the F protein is activated for fusion. It is likely therefore that most α-F MAbs hitherto described recognize epitopes shared by both the pre- and postfusion forms of hRSV_F.

We have published that human Ig (Ig) preparations contain neutralizing α-F antibodies that could be purified by binding to F_{TM-} covalently linked to Sepharose beads (19). However, the antibodies that did not bind to F_{TM-} retained most of the neutralizing activity present in the Ig preparations (even after depletion of α-G antibodies) and were able to bind to cells infected with either hRSV or a recombinant vaccinia virus that expressed hRSV_F. It appears therefore that certain human neutralizing antibodies recognize structures of the F protein, other than those represented in F_{TM-}. We now report that this type of antibodies can be obtained from rabbits immunized with recombinant vaccinia viruses expressing a full-length hRSV_F. In addition, both rabbit and human neutralizing α-F antibodies, depleted of those reacting with F_{TM-}, bind to a prefusion form of hRSV_F,

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stabilized with intermonomeric disulfide bonds. These results therefore offer possibilities to develop unique α-hRSV neutralizing antibodies with potential applications in the clinic.

Results

Induction of Neutralizing Antibodies in Rabbits Inoculated with Recombinant Vaccinia Viruses Expressing Either hRSV_F or hRSV_F_{TM}-. New Zealand White rabbits were inoculated intramuscularly (i.m.) with recombinant vaccinia viruses expressing either a full-length copy of hRSV_F (Vac/Fc) or an anchorless mutant of the same protein (Vac/F_{TM}-). Representative results of each group of rabbits, shown in Fig. 1, demonstrated that both sera contained antibodies able to bind to purified F_{TM}- in an ELISA (Fig. 1*A*) and to inhibit hRSV infectivity in a microneutralization test (Fig. 1*B*). However, the sera of rabbits inoculated with Vac/Fc (named α-Fc, see *Methods* for the system used to label the antibodies) neutralized hRSV significantly better than the sera of rabbits inoculated with Vac/F_{TM}- (α-F_{TM}-).

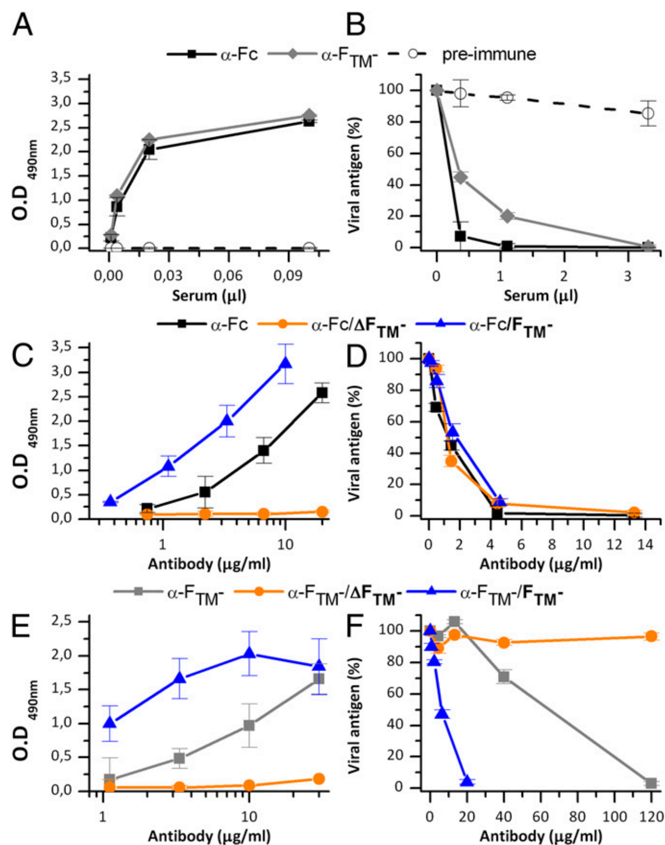

Fig. 1. Induction of binding and neutralizing antibodies in rabbits immunized with recombinant vaccinia viruses expressing different forms of hRSV_F. Serial dilutions of sera from rabbits inoculated with either Vac/Fc (α-Fc) or Vac/F_{TM}- (α-F_{TM}-) were tested for binding to purified F_{TM}- adsorbed to ELISA plates (*A*) or hRSV neutralization (*B*). Control serum from a preimmune rabbit was included as control. Total α-Fc antibodies were purified by protein A-Sepharose chromatography from the sera of rabbits immunized with Vac/Fc. The α-Fc antibodies were loaded onto a column of F_{TM}-Sepharose, and the unbound antibodies (α-Fc/**ΔF_{TM}-**) were collected and saved. After washing, the antibodies bound to the column (α-Fc/F_{TM}-) were eluted. The α-Fc, α-Fc/**ΔF_{TM}-**, and α-Fc/**F_{TM}-** antibodies were then tested for binding to F_{TM}- (*C*) and for hRSV neutralization (*D*). Total α-F_{TM}- antibodies from rabbits inoculated with Vac/F_{TM}- were processed similarly to yield the α-F_{TM}-/**ΔF_{TM}-** antibodies that did not bind to the F_{TM}-Sepharose column and the α-F_{TM}-/**F_{TM}-** antibodies eluted from this column. α-F_{TM}-, α-F_{TM}-/**ΔF_{TM}-**, and α-F_{TM}-/**F_{TM}-** antibodies were tested for binding to F_{TM}- (*E*) and hRSV neutralization (*F*). Results are presented as means ± SEM of three independent experiments.

The α-Fc antibodies were purified with protein A-Sepharose and subsequently loaded onto a column of F_{TM}- protein covalently linked to Sepharose beads. As expected, the antibodies that did not bind to the column (α-Fc/**ΔF_{TM}-**) failed to react with the F_{TM}- protein in an ELISA (Fig. 1*C*), whereas the α-Fc/**F_{TM}-** antibodies eluted from the column showed a higher level of binding to F_{TM}- than the starting antibodies (α-Fc). Remarkably, although the α-Fc/**F_{TM}-** antibodies inhibited hRSV infectivity in a neutralization test, the unbound α-Fc/**ΔF_{TM}-** antibodies retained most of the neutralizing activity of the original α-Fc antibodies (Fig. 1*D*). Considering that the antibodies eluted from the affinity column (α-Fc/**F_{TM}-**) should be specific only for the F protein whereas α-Fc and α-Fc/**ΔF_{TM}-** antibodies should contain multiple specificities present in a rabbit serum, the results of Fig. 1*D* underscore the neutralizing capacity of α-Fc/**ΔF_{TM}-**, although direct comparison of specific activities between α-Fc/**F_{TM}-** and α-Fc/**ΔF_{TM}-** would require estimation of the percentage of F-specific antibodies in each antibody preparation.

The antibodies from rabbits inoculated with Vac/F_{TM}- (referred to as α-F_{TM}-) were processed similarly to the α-Fc antibodies. Again, the antibodies not retained in the column of F_{TM}- (α-F_{TM}-/**ΔF_{TM}-**) were unable to bind to this protein in an ELISA, whereas the antibodies eluted from the column (α-F_{TM}-/**F_{TM}-**) showed a higher level of binding to F_{TM}- than the starting material (Fig. 1*E*). However, in this case, the antibodies unbound to the column of F_{TM}- were unable to neutralize hRSV infectivity (Fig. 1*F*), in clear distinction with the equivalent antibodies obtained from rabbits inoculated with Vac/Fc (compare Fig. 1 *D* and *F*). In addition, both α-F_{TM}- and α-F_{TM}-/**F_{TM}-** antibodies were significantly less neutralizing than the corresponding antibodies obtained from rabbits inoculated with Vac/Fc (compare the *x* axis of Fig. 1 *D* and *F*).

Characterization of the Neutralizing Activity of the α-Fc/ΔF_{TM}- Antibodies. Several tests were performed to confirm that the neutralizing activity of the α-Fc/**ΔF_{TM}-** antibodies was due to antibodies specific against hRSV_F and not to spurious cross-reactions.

First, the reactivity of α-Fc, α-Fc/**ΔF_{TM}-**, and α-Fc/**F_{TM}-** antibodies was tested by flow cytometry with HEp-2 cells infected with hRSV. As shown in Fig. 2*A*, the α-Fc/**ΔF_{TM}-** antibodies could bind specifically to the surface of hRSV-infected cells, although the level of fluorescence was lower than that achieved with α-Fc and α-Fc/**F_{TM}-**.

To exclude illegitimate cross-reactions of α-vaccinia virus antibodies, the α-Fc, α-Fc/**ΔF_{TM}-**, and α-Fc/**F_{TM}-** preparations were adsorbed to HEp-2 cells infected with a vaccinia recombinant expressing the phosphoprotein (P) of hRSV (Vac/P). Before adsorption, the three antibody preparations reacted positively by flow cytometry with cells infected with either Vac/Fc or Vac/P (Fig. 2*B*). However, after adsorption to HEp-2, cells infected with Vac/P the reactivity with cells infected with this virus was lost while the reactivity with cells infected with Vac/Fc remained essentially unchanged (Fig. 2*C*). Finally, the three antibody preparations were adsorbed to HEp-2 cells infected with Vac/Fc. In this case, the three adsorbed antibodies lost the reactivity with cells infected with either Vac/P or Vac/Fc (Fig. 2*D*).

Antibody preparations were further analyzed for binding to F_{TM}- and hRSV neutralization before and after the adsorptions of the previous paragraph. Adsorption to cells infected with Vac/P (ΔVac) had no impact on either antibody binding to purified F_{TM}- (Fig. 3 *A*–*C*) or hRSV neutralization (Fig. 3 *D*–*F*). In contrast, adsorption of the three antibody preparations to cells infected with Vac/Fc reduced the reactivity of α-Fc (Fig. 3*A*) and α-Fc/**F_{TM}-** (Fig. 3*C*) to background levels, whereas the reactivity of α-Fc/**ΔF_{TM}-** remained negligible (Fig. 3*B*). The most significant result was that the neutralizing activity of the three antibody preparations was abrogated after adsorption to cells infected with Vac/Fc (Fig. 3 *D*–*F*).

In summary, the neutralizing activity of α-Fc antibodies depleted of those binding to F_{TM}- (α-Fc/**ΔF_{TM}-**) was removed by adsorption to cells infected with Vac/Fc (Fig. 3*E*). At this point,

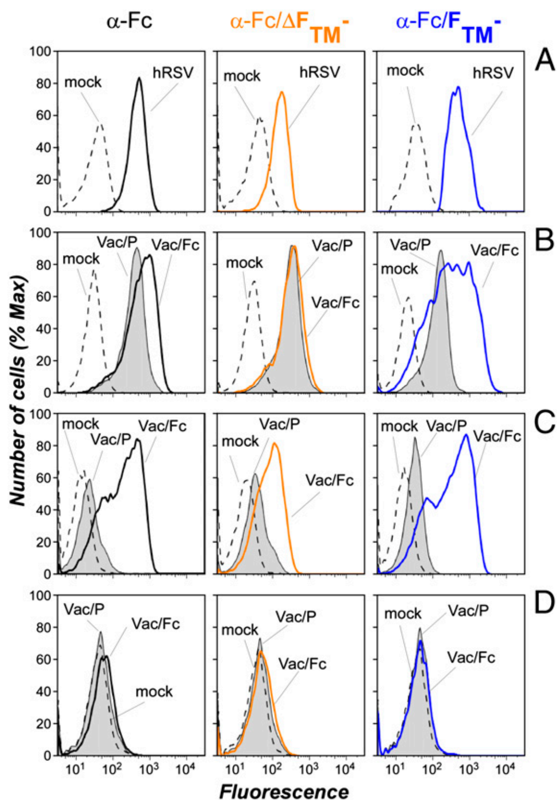

Fig. 2. Rabbit α-Fc antibodies recognize HRSV_F expressed at the cell surface even if depleted of antibodies able to bind to F_{TM}- (α-Fc/$\mathbf{\Delta F_{TM}}$-). HEp2 cells were infected with hRSV (Long strain, multiplicity of infection 5 pfu per cell) and tested 48 h later by flow cytometry for cell surface labeling with α-Fc, α-Fc/$\mathbf{\Delta F_{TM}}$-, and α-Fc/$\mathbf{F_{TM}}$- antibodies (*A*). Alternatively, HEp-2 cells were infected with recombinant vaccinia virus, either hRSV_P (Vac/P) or hRSV_Fc (Vac/Fc), and tested 24 h later by flow cytometry for cell surface labeling with α-Fc, α-Fc/$\mathbf{F_{TM}}$-, and α-Fc/$\mathbf{\Delta F_{TM}}$- antibodies before (*B*) or after adsorption of the antibodies to cells infected with Vac/P (*C*) or Vac/Fc (*D*). Mock infected cells (dashed lines) were included always as control. Note the reactivity of α-Fc/$\mathbf{F_{TM}}$- antibodies with cells infected with Vac/P (*B Right*), although lower than the reactivity of α-Fc and α-Fc/$\mathbf{\Delta F_{TM}}$- probably because anti-vaccinia antibodies that bound unspecifically to the F_{TM}- column. Results are representative of at least three independent experiments.

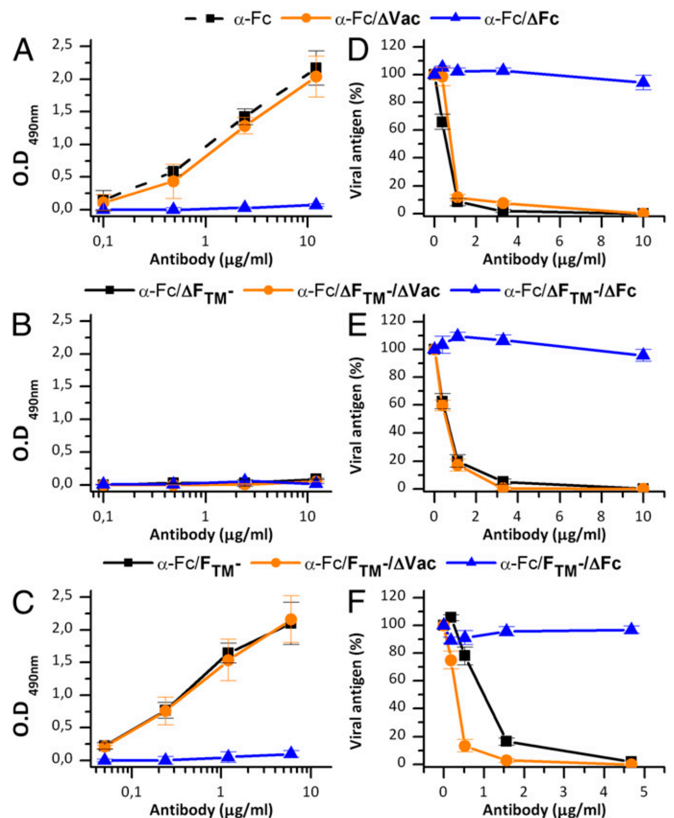

Fig. 3. α-Fc, α-Fc/$\mathbf{\Delta F_{TM}}$-, and α-Fc/$\mathbf{F_{TM}}$- antibodies lost their neutralizing activity after incubation with cells infected with Vac/Fc. The three antibody preparations of Fig. 2 were depleted of antibodies binding to cells infected with either Vac/P (ΔVac) or Vac/Fc (ΔFc) and then tested either before or after the different adsorptions for binding to F_{TM}- (*A*–*C*) and for hRSV neutralization (*D*–*F*). Although the antibodies incubated with cells infected with Vac/Fc were also depleted of anti-vaccinia antibodies (i.e., they were also ΔVac), they were labeled ΔFc to distinguish them of those incubated with cells infected with Vac/P (labeled ΔVac in this figure). Results are presented as means ± SEM of three independent experiments.

we postulated that the α-Fc/$\mathbf{\Delta F_{TM}}$- antibodies recognize a conformation of hRSV_F, which the protein adopts when inserted into membranes but not when expressed as a soluble anchorless molecule. The possibility that this conformation may correspond to the prefusion form of hRSV_F is supported by the results of Fig. S1. Thus, when hRSV was preincubated with saturating amounts of α-Fc, α-Fc/$\mathbf{\Delta F_{TM}}$-, or α-Fc/$\mathbf{F_{TM}}$- and then pelleted to eliminate any free remaining antibodies before being used to infect new cells, virus infectivity was already inhibited. Therefore, the three antibody preparations (and, in particular, α-Fc/$\mathbf{\Delta F_{TM}}$-) could bind and neutralize hRSV before the F protein was activated for fusion. The results of Fig. S1 also suggest that neutralizing α-Fc/$\mathbf{F_{TM}}$- antibodies may recognize epitopes shared by the pre- and postfusion forms of hRSV_F (see below under *Discussion*).

Stabilization of the Prefusion Form of hRSV_F and Reactivity with Rabbit and Human Antibodies. To test whether α-Fc/$\mathbf{\Delta F_{TM}}$- antibodies could bind to the prefusion form of hRSV_F removed from a membrane environment, we attempted to purify this protein in the prefusion conformation. Previous efforts to solubilize the prefusion form of hRSV_F have failed, most likely because of the metastable state of this conformation and its spontaneous refolding to the postfusion form upon manipulation. Therefore, stabilization of the prefusion form of hRSV_F was attempted via the introduction of intermonomeric disulfide bridges that would prevent refolding after solubilization (Fig. 4*A*). Several artificial disulfide bonds were designed, based on a model of the prefusion hRSV_F (Fig. 4*B*), built with the atomic coordinates of the structure solved for the homologous parainfluenza virus 5 (PIV5) F protein (20).The best results were obtained with a protein referred to as $FcN_{2C\text{-}C}$, in which four amino acids were mutated to cysteines (shown schematically in Fig. 4 *A* and *B*). The basic residues at the two polybasic cleavage sites of $FcN_{2C\text{-}C}$ and the wild-type hRSV_F (referred as FcN) were replaced by asparagine residues to avoid cleavage, as done with PIV5 (20). Finally, a His-tag was added to the C terminus of each protein to facilitate purification in Ni(2+) columns, and both constructs were incorporated into vaccinia virus recombinants. The location of introduced cysteine residues is shown in homology models of both the pre- and postfusion structures in Fig. 4 *B* and *C*. It is worth stressing that the extra disulfide bonds can only be formed intermonomerically and only if the F protein is in the prefusion conformation (Fig. 4*B*), but not if refolded to the postfusion structure (Fig. 4*C*).

SDS/PAGE of boiled samples under nonreducing conditions showed the presence after Coomassie blue staining of a high molecular band (>250 kDa) in purified $FcN_{2C\text{-}C}$—compatible with a F protein trimer—which was absent in FcN (Fig. 4*D*). Under these conditions, the FcN lane contained a major band of ≈80 kDa, corresponding to the F monomer (F0) and a minor band of ≈190 kDa, which might be an F protein dimer, although

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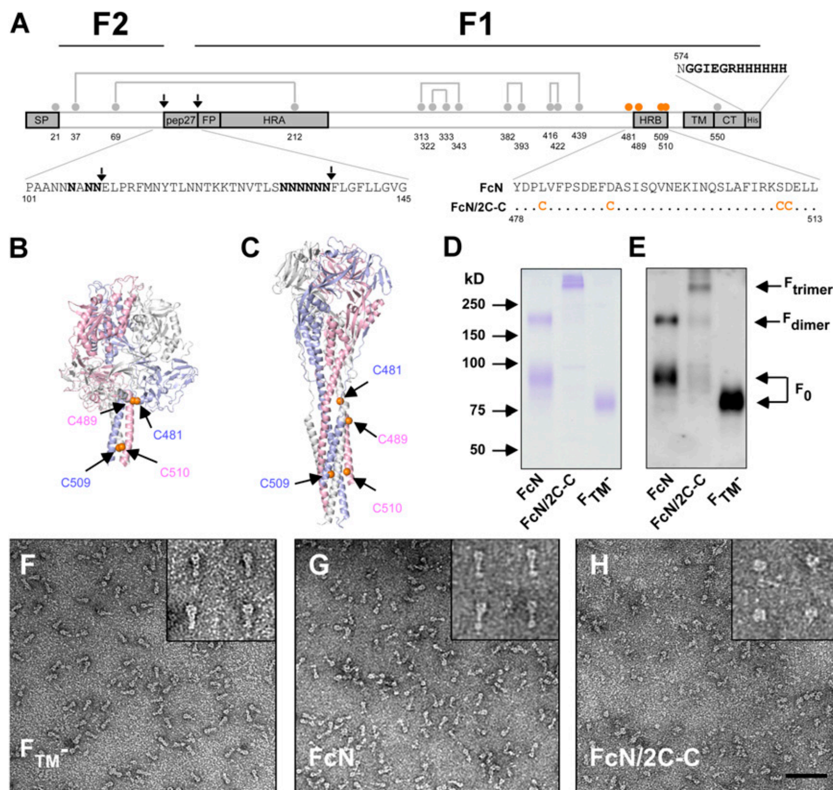

Fig. 4. Stabilization of the prefusion form of hRSV_F with intermonomeric disulfide bonds. (*A*) Scheme of the hRSV_F primary structure, denoting the three main hydrophobic regions: signal peptide (SP), fusion peptide (FP), and transmembrane region (TM). Also indicated are the two furin cleavage sites (arrows) that generate the F2 and F1 chains and the cysteines (gray dots and numbers) that participate in the intramonomeric disulfide bonds observed in the pos-fusion structure of hRSV. HRA and HRB represent the heptad repeat sequences of the F1 chain. Partial sequences, shown below the scheme, highlight the asparagines (N, boldface) introduced to ablate furin cleavage in FcN and FcN_{2C-C} constructs and the cysteines introduced in the latter construct (shown in orange) to make new intermonomeric disulfide bonds. The His-tag sequence added at the C terminus is shown above the scheme. (*B*) 3D model of the prefusion conformation of hRSV_F built with the coordinates of the PIV5_F protein (20) (PDB ID code 2B9B). Each monomer is colored differently. Cysteine residues (C) replacing residues 481, 489, 509, and 510 in FcN_{2C-C} are represented by golden balls alternating in two of the monomers (magenta and blue). (*C*) Postfusion structure of hRSV_F, reported by MacLellan et al. (26) (PDB ID code 3RRR). Colors and locations of engineered cysteine residues are presented as in *B*. Purified FcN, FcN_{2C-C}, and F_{TM}- proteins (*Methods*) were resolved by SDS/PAGE and either stained with Coomassie blue (*D*) or electrotransferred to nylon membranes and stained by Western blot with α-F_{TM}- antibodies (*E*). Electron microscopy of the three purified proteins (*F*–*H*) negatively stained with uranyl acetate. (Scale bar: 50 nm.) Two-times-magnified selected molecules are shown in *Insets*.

the nature of this band has not been investigated further. These two bands were seen as minor components of the unreduced FcN_{2C-C}, indicating that formation of the extra disulfide bonds was not 100% efficient. Purified F_{TM}- (also modified to change the cleavage sites as in FcN and FcN_{2C-C}; *SI Methods*) yielded a single band migrating slightly faster than F0, in agreement with its lower molecular mass. The relatedness of all these bands with hRSV_F was demonstrated by Western blotting with α-F_{TM}- antibodies (Fig. 4*E*).

Electron microscopy of negatively stained FcN revealed cone-shaped molecules with a uniform contour and size (Fig. 4*G*) that resembled the structure of F_{TM}- in the postfusion conformation (Fig. 4*F*), except for a smaller globular density at one end of FcN that may represent detergent bound to the transmembrane region of this molecule and that is absent in F_{TM}-. In contrast, FcN_{2C-C} molecules were morphologically more heterogeneous but with a significant proportion of rounded shape structures (Fig. 4*H*), resembling the heterogeneity and shape reported for the prefusion form of PIV5_F (21). Recently, Chaiwatpongsakorn et al. (22) have published electron micrographs of hRSV_F in the prefusion conformation (obtained by alternative methods to those used here) that also resemble the images of FcN_{2C-C} molecules shown in Fig. 4*H*.

The preparations of rabbit antibodies described in previous sections were tested by ELISA for binding to purified F_{TM}-, FcN, and FcN_{2C-C} proteins. The results of Fig. 5*A* demonstrate that α-Fc and α-F_{TM}- antibodies were able to bind similarly to the three proteins, whereas α-Fc/**ΔF_{TM}-** antibodies were able to bind to FcN_{2C-C} but failed to bind to F_{TM}- and FcN. These results strongly support the conclusion that α-Fc/**ΔF_{TM}-** antibodies are specific for the prefusion form of hRSV_F, represented in the FcN_{2C-C} and, therefore, do not require a membrane environment for binding. The conclusion that the FcN_{2C-C} protein is in the prefusion conformation is further supported by the lack of binding of antibodies specific for the 6-helix bundle (a structure unique of the postfusion form), whereas these antibodies bound efficiently to the F_{TM}- and FcN proteins (Fig. 5*A, Bottom Right*).

We have reported that antibodies present in a human Ig preparation (Respigam, RG) were capable of neutralizing hRSV infectivity, even if depleted of those binding to F_{TM}-(19). To clarify the specificities of these antibodies, they were tested in a sandwich ELISA with the F_{TM}-, FcN, and FcN_{2C-C} proteins. As shown in Fig. 5*A, Bottom Left*, RG antibodies bound to the three proteins; however, after depletion of the antibodies binding to F_{TM}-Sepharose (RG/**ΔF_{TM}-**; Fig. 5*A, Bottom Right*), they failed to bind to F_{TM}- and FcN although continued to react with FcN_{2C-C}, mimicking the reactivity of rabbit α-Fc/**ΔF_{TM}-** antibodies. Furthermore, as reported (19), RG and RG/**ΔF_{TM}-** antibodies neutralized efficiently hRSV even if the later antibodies were depleted of α-G antibodies (RG/**ΔF_{TM}-/ΔG**) but not if depleted additionally of antibodies that bound to cells infected with Vac/Fc (RG/**ΔF_{TM}-/ΔG/ΔFc**) or Vac/FcN_{2C-C} (RG/**ΔF_{TM}-/ΔG/ΔFcN_{2C-C}**) (Fig. 5*B*). Thus, a large proportion of the RG neutralizing activity, raised presumably after natural infections with hRSV, is afforded by antibodies able to bind specifically to the prefusion form of hRSV_F and present at the surface of cells infected with either Vac/Fc or Vac/FcN_{2C-C}.

Remarkably, despite significant structural differences between FcN_{2C-C} and FcN (i.e., pre- and postfusion forms of hRSV_F; Fig. 4 *B* and *C*), both proteins (and F_{TM}-) bound similarly four α-F MAbs specific to different epitopes, including palivizumab used in the clinic (Fig. 5*C*).

Discussion

Most α-hRSV_F antibodies described so far in the literature recognize epitopes present in the postfusion form of the F protein. Commonly, reactivity with purified F_{TM}- or Fc or immunoprecipitation of hRSV-infected cell extracts have been used to assign antibody specificities. It is expected that under all of those conditions the F protein will be folded in the postfusion conformation, such as for the FcN protein of Fig. 4. Even virus preparations may contain a sizeable amount of F in the postfusion form, as shown for PIV5_F (23). It is also known that preparations of purified hRSV contain a large proportion of noninfectious particles in which the F protein could be in the postfusion form.

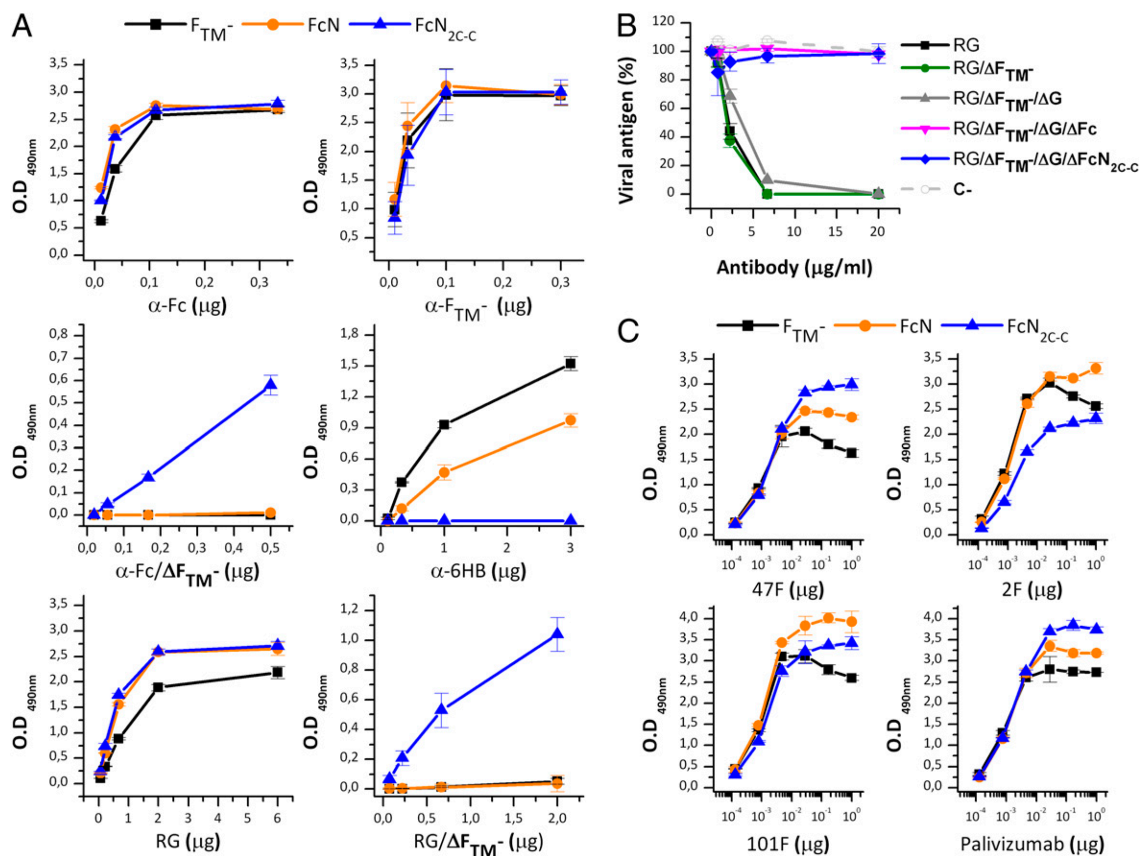

Fig. 5. The stabilized prefusion hRSV_F protein is bound by rabbit and human neutralizing antibodies depleted of those that recognize F_{TM}-. (*A*) The three proteins, F_{TM}-, FcN, and FcN_{2C-C} of Fig. 4 were bound to ELISA plates via a bridge made with MAb 101F and then tested for binding to the antibodies indicated at the bottom of each image. α-Fc, α-F_{TM}-, and α-Fc/$\mathbf{\Delta F_{TM}}$- have been described in Fig. 1. The α-6HB antibodies (*Methods*) were raised in rabbits inoculated with a bacterially expressed construct that mimics the six-helix bundle present in the postfusion form of hRSV_F (28). RG is the Ig preparation (Respigam) used some years ago in the clinic (a generous gift of MedImmune). A sample of this preparation was also depleted of antibodies binding to F_{TM}-Sepharose (RG/$\mathbf{\Delta F_{TM}}$-). (*B*) RG was depleted sequentially of antibodies binding to F_{TM}-Sepharose (RG/$\mathbf{\Delta F_{TM}}$-), G-Sepharose (RG/$\mathbf{\Delta F_{TM}}$-/**ΔG**), and cells infected with either Vac/Fc (RG/$\mathbf{\Delta F_{TM}}$-/**ΔG/ΔFc**) or Vac/FcN_{2C-C} (RG/ΔF_{TM}-/ΔG/ΔFcN_{2C-C}), and all these antibody preparations were used in a microneutralization test with hRSV. C- is a preimmune rabbit antibody. (*C*) F_{TM}-, FcN, and FcN_{2C-C} proteins were adsorbed directly to microtiter plates and used in an ELISA with the four MAbs indicated in each image. 2F, 47F, and 101F are murine MAbs that recognize nonoverlapping epitopes in hRSV_F (29) Palivizumab (a generous gift of Abbott España) is a humanized MAb in use as a prophylactic treatment for hRSV infections. Escape mutants selected with Palivizumab partially overlap those selected with MAb 47F (30), and both antibodies bind to similar peptides of hRSV_F (31, 32).

Therefore, it was only after depletion of antibodies that bound to F_{TM}- that those specific for the prefusion form of hRSV_F could be uncovered. These antibodies account for most of the neutralizing activity found in human Ig preparations (not only Respigam but also another Ig preparation, Flebogamma; ref. 24) and in sera of rabbits inoculated with Vac/Fc. These antibodies, however, were absent from sera of rabbits inoculated with Vac/F_{TM}-, illustrating the importance of the F protein structure presented to the immune system for the antibody outcome.

Previously we have shown that antibody affinity is crucial for protection of mice from hRSV infection (25). It is likely that a major proportion of the F protein present in the formalin or UV inactivated virus or the purified F protein that failed to induce protection in those studies was in the postfusion structure. Taking into consideration our new results showing that antibodies specific for the prefusion form of hRSV_F account for most of the neutralizing activity of an immune response, it is possible that absence of this form may have contributed to a failure to produce a protective immune response.

The structure of hRSV_F in the postfusion conformation has been solved recently by X-ray crystallography (26, 27). Modeling of the prefusion form and comparison with the actual structure of the postfusion form confirmed the preservation of local structures in both conformations that contained epitopes recognized by certain MAbs. These results are in full agreement with previous electron microscopy studies of immune complexes of F_{TM}- with MAbs (17) and with the results of Fig. 5*C*, which showed cross-reactivity of four MAbs with the pre- and postfusion forms of hRSV_F. These results also explain the frequent isolation of MAbs that recognize both conformations and the capacity of MAbs or antibodies such as α-Fc/$\mathbf{F_{TM}}$- (Fig. S1) to inhibit virus infectivity even before activation of the F protein for fusion (18).

At least a proportion of FcN and FcN_{2C-C} proteins should be in the prefusion form when expressed at the cell surface as inferred from their capacity to deplete the α-Fc/$\mathbf{\Delta F_{TM}}$- and RG/$\mathbf{\Delta F_{TM}}$- antibodies of their respective neutralizing activities (Fig. S2 and Fig. 5*B*). However, it is worth pointing out that both proteins were engineered to ablate the two cleavage sites required for activity and, consequently, remain uncleaved. Therefore, subtle structural differences between those two proteins (while inserted in the cell membrane) and the actual prefusion protein may have escaped the antigenicity tests and electron microscopy studies, carried out in the present work. When solubilized, however, FcN apparently refolded into the postfusion form as seen by electron microscopy. In contrast, FcN_{2C-C} (for which the yield is 10–20 times lower than for FcN) was inhomogeneous but contained a proportion of rounded molecules that resemble the reported prefusion shapes of PIV-5 (21) and

hRSV_F proteins (22). $FcN_{2C\text{-}C}$ preparations also contain a small proportion of molecules in which the additional cysteine residues were not disulfide bonded, as seen by SDS/PAGE (Fig. 4 *D* and *E*). This heterogeneity was reproducibly found in several preparations of $FcN_{2C\text{-}C}$ and was maintained at similar levels during storage. Thus, additional efforts are needed to obtain homogeneous preparations of prefusion hRSV_F amenable for structural studies. Furthermore, $FcN_{2C\text{-}C}$ (or other alternative constructs) may require further improvements for efficient immunogenicity, because preliminary results indicate that Vac/$FcN_{2C\text{-}C}$ is able to induce neutralizing antibodies in rabbits but at lower titers than Fc, most likely due to the lower expression level (Fig. S3).

The actual mechanism of neutralization by the antibodies specific for the prefusion form of hRSV_F remains to be elucidated. For instance, it is unknown whether the high neutralizing capacity of the α-Fc/$\mathbf{\Delta F_{TM}}$- or RG/$\mathbf{\Delta F_{TM}}$- relies on synergistic effects of antibodies binding simultaneously to several epitopes. In this sense, it would be important to evaluate the neutralizing potency of individual antibodies (e.g., MAbs) specific for the prefusion form of hRSV_F in comparison with antibodies that recognize epitopes shared by the prefusion and postfusion conformations, such as those of Fig. 5*C*. The availability of stabilized prefusion forms of hRSV_F should facilitate the search of MAbs or other type of molecules that bind specifically to this conformation and interfere with its activation. This approach could be used to develop a new generation of more effective prophylactic antibodies for treatment of infants at high risk of hRSV infections or even extended to other paramyxoviruses for development of novel prophylactic and/or therapeutic agents.

Methods

Preparation and Testing of Polyclonal Antibodies Directed Against Different Forms of hRSV_F. New Zealand White rabbits were inoculated intramuscularly (i.m.) with the vaccinia virus recombinants indicated in Fig. 1 legend on days 0 and 21 and bled 3 wk later. Serum antibodies were purified by using a protein A-Sepharose column and further processed by affinity chromatography to F_{TM}- protein covalently linked to Sepharose beads. The unretained fraction (named ΔF_{TM}-; see *SI Methods* for antibody nomenclature) was saved, and the bound antibodies were eluted with acidic buffer. The different antibody preparations were tested for ELISA binding to F_{TM}- and virus neutralization. Depletion of certain specific antibodies was also achieved after incubation with cells infected with either hRSV or vaccinia virus recombinants expressing different forms of the F protein. Human antibodies present in Respigam were processed similarly to rabbit antibodies.

Stabilization of the Prefusion Form of hRSV_F. Vaccinia virus recombinant expressing full-length F (Vac/Fc) has been described (17). This recombinant was modified by changing the basic residues at the two cleavage sites of hRSV_F to Asparagines as indicated in Fig. 4*A* to generate Vac/FcN. Additionally, the residues Leu481, Asp489, Ser509, and Asp510 of hRSV_F were substituted by Cysteines to generate Vac/$FcN_{2C\text{-}C}$. Finally, a His tag was added to the C terminus of FcN and $FcN_{2C\text{-}C}$ for purification purposes. Additional experimental details are provided in *SI Methods*.

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Supporting Information

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SI Methods

Viruses. The Long strain of hRSV was grown in HEp-2 cells with DMEM supplemented with 2.5% FCS (DMEM2.5). Previously synthesized recombinant vaccinia viruses expressing either the phosphoprotein (Vac/P) (1), the G glycoprotein (Vac/G) (2), or the full-length (Vac/Fc) or soluble forms (Vac/F_{TM}-) of the hRSV_F protein (3) were grown in CV-1 monkey as described. Additional recombinant vaccinia viruses were made by the method of Blasco and Moss (4), expressing either an uncleaved version of hRSV_F with the basic amino acids at the two cleavage sites Arg106, Arg108, Arg109, Lys131, Lys132, Arg133, Lys134, Arg135, and Arg136 substituted with asparagines residues (Vac/FcN) or additionally having amino acids Leu481, Asp489, Ser509, and Asp510 substituted with cysteine residues (Vac/FcN_{2C-C}) (Fig. 4*A*). Both FcN and FcN_{2C-C} were additionally modified by the insertion of a 6His tag (Gly-Gly-Ile-Glu-Gly-Arg-**His-His-His-His-His-His**) at the C terminus to facilitate purification in Ni(2+) columns.

Protein Expression and Purification. A soluble, anchorless form of the hRSV_F protein was purified from the supernatants of HEp-2 cells infected with Vac/F_{TM}-, as described (5). This construct was engineered to substitute the basic amino acids of the two cleavage sites by asparagines, as done for FcN and FcN_{2C-C} (see previous section). Four milligrams of purified F_{TM}- were covalently linked to CNBr-activated Sepharose beads to make F_{TM}-Sepharose columns, following manufacturer's recommendations.

FcN and FcN_{2C-C} proteins were solubilized from CV-1 cells infected with the respective recombinant vaccinia viruses in a buffer containing: 50 mM sodium phosphate at pH 8.0, 300 mM NaCl, 10 mM imidazole, and 1% octyl-glucoside. The solubilized proteins were loaded onto columns of His-Select Nickel affinity gel (Sigma) and washed with the same buffer containing 20 mM imidazole. The proteins retained in the columns were eluted by increasing the imidazole concentration to 250 mM. Protein purity was confirmed by SDS/PAGE and Western blot analysis.

Rabbit Immunization, Purification of Antibodies and Depletion of Conformation Specific Antibodies. New Zealand White rabbits were inoculated intramuscularly (i.m.) on days 0 and 21 with 10^7 pfu of vaccinia recombinants encoding the proteins mentioned in each case. Groups of two or three rabbits were inoculated with each virus. Sera were collected 3 wk later and pooled by group. Total antibodies were purified by using protein A-Sepharose columns as recommended by the manufacturer (GE Healthcare).

Depletion of α-F_{TM}- antibodies was achieved by loading purified total antibodies onto a F_{TM}- Sepharose column. The unretained material containing the depleted antibodies was collected and saved, and the antibodies bound to the column were eluted with 0.1 M Glycine-HCl at pH 2.5 and neutralized with saturated Tris.

Depletion of specific antibodies was also achieved by incubating 100–300 μg of total antibodies for 30 min at room temperature with 10^7 HEp-2 cells infected 24 h earlier with recombinant vaccinia viruses at a multiplicity of 5 infectious units per cell. After incubation, cells were pelleted by centrifugation at low speed and the supernatants were collected and saved until used.

Depletion of antibodies present in a human Ig preparation (Respigam) was done as for the rabbit antibodies.

Antibodies specific for the six-helix bundle (6HB) of the post-fusion F protein were generated by inoculation of rabbits with 100 μg of a protein made of GST followed by HRA (aa 160–207) and HRB (aa 479–520) linked by the sequence GSSGG. The protein was purified by standard procedures using glutathione-Sepharose and mixed with an equal volume of Freund's adjuvant for inoculation into rabbits. Animals were bled 3 wk after injection.

Nomenclature of Polyclonal Antibodies. The rabbit antibodies used in this study have been named according to the following system: The first part of the name refers to the protein encoded by the vaccinia recombinant used in the immunization, followed by the manipulations made in vitro, indicated in boldface. For instance, α-Fc/**ΔF_{TM}-** correspond to antibodies raised in rabbits inoculated with Vac/Fc and that were depleted of those binding to F_{TM}-. This system has also been used to label the antibodies derived from the Respigam (RG) preparation.

Immunoassays. ELISAs were performed in 96-well plates coated with purified recombinant proteins, F_{TM}-, FcN, and FcN_{2C-C} either by direct adsorption to the plastic or by capture with a specific monoclonal antibody MAb 101F. Nonspecific antibody binding was blocked with 2% porcine serum in PBS with 0.05% Tween 20. Antibody preparations were incubated on plates for 1 h at 37 °C, and unbound antibody was removed by washing five times with water. Antibody binding was assessed by incubation with a peroxidase-conjugated anti-rabbit or anti-human secondary antibody (GE Healthcare) and subsequent addition of OPD (Sigma) as substrate.

Western blotting was done after separation of proteins by SDS/PAGE and electrotransfer to nylon membranes that were developed with the antibodies indicated in the figure legends and Lumigen assays (GE Healthcare).

Microneutralization tests were performed in 96-well plates with 90% confluent HEp-2 cell monolayers (6). The Long strain of hRSV was used to infect the monolayers (0.2–0.5 infectious units per cell) either in the presence or absence of antibodies. After 72 h, the medium was removed, and after washing with 0.05% Tween-20 in PBS, cells were fixed with chilled 80% acetone in PBS. Then, the amount of viral antigen in each well was quantified by ELISA, using a pool of MAbs specific for the F and G glycoproteins (6). Neutralization is referred as reduction of viral antigen production normalized to an infected control without antibody.

Cytometry of cells infected with hRSV and recombinant vaccinia viruses was performed by standard procedures, after detaching the cells from the plates with 10 mM EDTA in PBS.

Electron Microscopy. Two to five microliters of each purified protein was applied to glow-discharged carbon-coated grids and negatively stained with 2% aqueous uranyl acetate. Micrographs were recorded with a Tecnai 12 FEI microscope operated at 120 kV at a nominal magnification of 67,000×.

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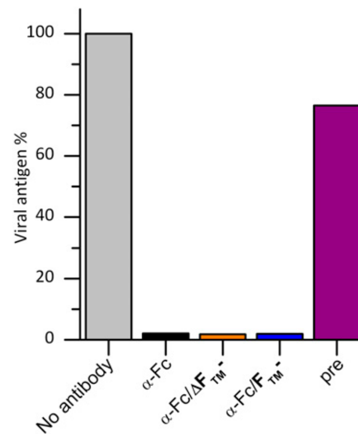

Fig. S1. Virus neutralization by antibodies after ultracentrifugation. The Long strain of hRSV [8×10^5 infectious units (pfu)] were incubated with 10-fold excess of the minimal neutralizing dose of α-Fc, α-Fc/**Δ**$\mathbf{F_{TM}}$**-**, and α-Fc/$\mathbf{F_{TM}}$**-** antibodies (previously titrated; pre is the same amount as α-Fc but of preimmune antibody) for 30 min at 37 °C. Then, samples were loaded on top of a 25% glycerol cushion in PBS and ultracentrifuged at 126,000 × *g* for 2 h. Pellets were resuspended in DMEM2.5 and used to infect HEp-2 cells. Viral antigen produced after 72 h was quantified by ELISA as indicated in *Methods*. Results are representative of two independent experiments.

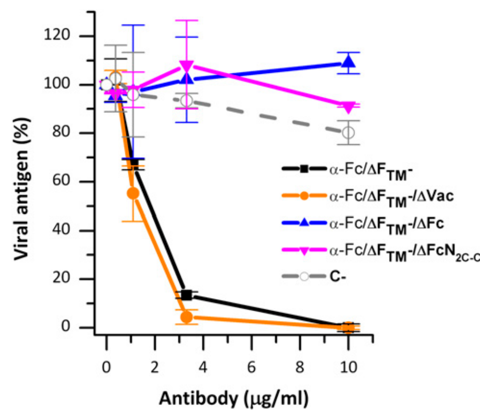

Fig. S2. The neutralizing activity of rabbit α-Fc/**Δ**$\mathbf{F_{TM}}$**-** antibodies is ablated by depletion with either Vac/Fc or Vac/Fc$N_{2C\text{-}C}$ infected cells. The purified α-Fc antibodies (raised in rabbits inoculated with Vac/Fc) that did not bind to the column of F_{TM}-Sepharose (α-Fc/**Δ**$\mathbf{F_{TM}}$**-**) were incubated with HEp-2 cells infected with Vac/P (α-Fc/**Δ**$\mathbf{F_{TM}}$**-/ΔVac**), Vac/Fc (α-Fc/**Δ**$\mathbf{F_{TM}}$**-/ΔFc**), or Vac/Fc$N_{2C\text{-}C}$ (α-Fc/**Δ**$\mathbf{F_{TM}}$**-/ΔFc**$\mathbf{N_{2C\text{-}C}}$). After adsorption, the antibodies were used in a microneutralization test with hRSV. Note that incubation of antibodies with cells infected with Vac/P had no effect on neutralization but incubation with cells infected with either Vac/Fc or Vac/ Fc$N_{2C\text{-}C}$ ablated the neutralizing activity of α-Fc/**Δ**$\mathbf{F_{TM}}$**-** antibodies. C- is a preimmune rabbit antibody. Results are presented as means ± SEM of three independent experiments.

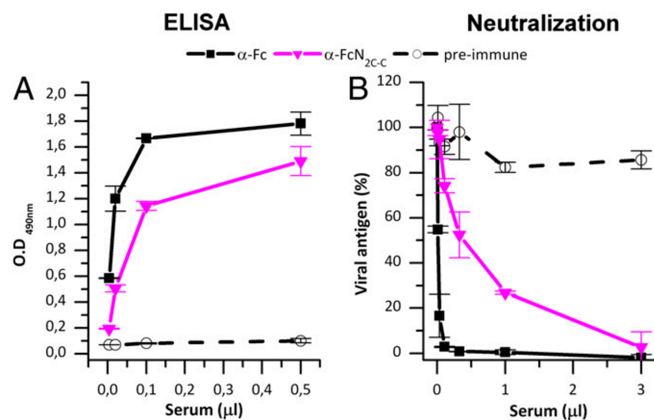

Fig. S3. Immunogenitiy of Vac/Fc$N_{2C\text{-}C}$. New Zealand White rabbits were inoculated with Vac/Fc$N_{2C\text{-}C}$. The antiserum (named α-Fc$N_{2C\text{-}C}$) was tested for binding to F_{TM}- adsorbed to ELISA plates (*Left*) or hRSV neutralization (*Right*). The antiserum raised against Vac/Fc (α-Fc) or preimmune serum were tested in parallel.