



Structure-based design of a quadrivalent fusion glycoprotein vaccine for human parainfluenza virus types 1–4

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Parainfluenza virus types 1–4 (PIV1–4) are highly infectious human pathogens, of which PIV3 is most commonly responsible for severe respiratory illness in newborns, elderly, and immunocompromised individuals. To obtain a vaccine effective against all four PIV types, we engineered mutations in each of the four PIV fusion (F) glycoproteins to stabilize their metastable prefusion states, as such stabilization had previously enabled the elicitation of high-titer neutralizing antibodies against the related respiratory syncytial virus. A cryoelectron microscopy structure of an engineered PIV3 F prefusion-stabilized trimer, bound to the prefusion-specific antibody PIA174, revealed atomic-level details for how introduced mutations improved stability as well as how a single PIA174 antibody recognized the trimeric apex of prefusion PIV3 F. Nine combinations of six newly identified disulfides and two cavity-filling mutations stabilized the prefusion PIV3 F immunogens and induced 200- to 500-fold higher neutralizing titers in mice than were elicited by PIV3 F in the postfusion conformation. For PIV1, PIV2, and PIV4, we also obtained stabilized prefusion Fs, for which prefusion versus postfusion titers were 2- to 20-fold higher. Elicited murine responses were PIV type-specific, with little cross-neutralization of other PIVs. In nonhuman primates (NHPs), quadrivalent immunization with prefusion-stabilized Fs from PIV1–4 consistently induced potent neutralizing responses against all four PIVs. For PIV3, the average elicited NHP titer from the quadrivalent immunization was more than fivefold higher than any titer observed in a cohort of over 100 human adults, highlighting the ability of a prefusion-stabilized immunogen to elicit especially potent neutralization.

antibody | conformational change | structure | vaccine design | virus

Human paramyxoviruses and pneumoviruses are widespread pathogens (1–3), cause considerable disease burden (4), and include measles virus (MeV) (5), mumps virus (MuV) (6), respiratory syncytial virus (RSV), metapneumovirus (MPV) (7), and parainfluenza virus types 1–4 (PIV1–4) (Fig. 1A). The paramyxovirus family members PIV1 and PIV3 (genus Respirovirus) are important pediatric pathogens, with lower incidence or disease severity caused by the paramyxovirus family members PIV2 and PIV4 (genus Rublavirus) (8). While effective responses to measles and mumps can be induced by live attenuated viral vaccines (9), licensed vaccines for RSV, MPV, and PIV1–4 have not been obtained using the same approach (10–13). Entry by these viruses utilizes the viral fusion (F) glycoprotein (14, 15), a type 1 fusion machine that transitions between a metastable prefusion conformation and a stable postfusion conformation (Fig. 1B) to merge virus and cell membranes. Structure-based stabilization of RSV F in its prefusion conformation has been shown to

induce high levels of RSV-neutralizing activity in vaccine animal models (16–20) and in a recent clinical trial.* While prefusion F stabilization has been proposed as a general means of inducing high titer protective responses, such stabilization with the MPV F glycoprotein does not induce improved neutralization titers (21), thus raising questions about the general utility of prefusion F vaccine stabilization for paramyxoviruses. To investigate the utility of prefusion PIV1–4 F vaccination, we utilized available structural information to engineer soluble prefusion-stabilized

Significance

In the United States alone, over 20,000 children are hospitalized each year from parainfluenza virus (PIV)-related illness. By age 10, most children are seropositive to several PIVs, including PIV1 and PIV2, which cause croup, and PIV3, which causes severe lower-respiratory infections. Here we assess whether the prefusion conformation of the F glycoproteins from PIV types 1–4 could induce potent neutralizing responses. We engineered mutations in the F glycoprotein that stabilized its prefusion conformation and immunized mice and nonhuman primates. The prefusion-stabilized PIV F immunogens elicited significantly higher neutralizing titers than the same F immunogens in the postfusion state, both individually and as a quadrivalent PIV1–4 F vaccine, indicating these prefusion-stabilized PIV F glycoproteins to be promising vaccine candidates.

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Data deposition: The data have been deposited in the Electron Microscopy Data Bank at Protein Data Bank in Europe, www.ebi.ac.uk/pdbe/emdb/ (EMD-9135), and in the Protein Data Bank, www.wwpdb.org (PDB ID 6MJZ).

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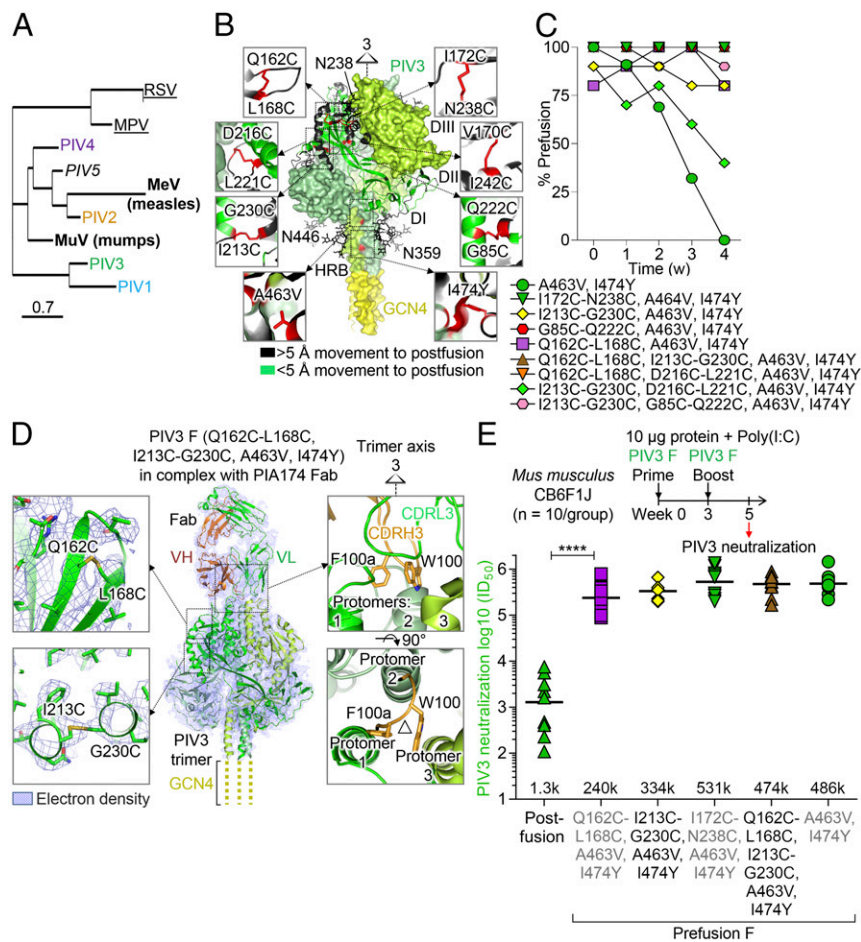


Fig. 1. Structure-based design of a PIV3 F glycoprotein vaccine that elicits high-titer neutralizing responses. (A) Phylogenetic tree of pathogenic human paramyxovirus F glycoproteins, with human parainfluenzas colored blue, orange, green, and purple for types 1, 2, 3, and 4, respectively, throughout all figures. Viruses for which vaccines have been licensed are shown in bold font, viruses for which prefusion F candidate vaccines have been created are underlined, and the simian PIV5 is shown in italics. (B) Structure-based design of prefusion-stabilizing mutations in PIV3 F showing disulfide and cavity-filling changes in the head and stem, respectively. Model was based on PIV5 prefusion structure (PDB ID 4WSG). Additional PIV F variants are shown in *SI Appendix, Fig. S5 B–F*. (C) Temporal stability of prefusion F as determined by negative-stain EM during a 4-wk incubation time course in PBS at 37 °C. (D) Cryo-EM structure at 4.3-Å resolution of prefusion PIV3 F stabilized by Q162C-L168C, I213C-G230C, A463V, I474Y mutations and bound to antibody PIA174. The complex was most ordered at the core of the PIV3 F trimer and at its interface with the PIA174 antibody, where the cryo-EM map showed a local resolution of 4.0 Å, calculated using RELION (38). *Insets* show disulfide bonds (*Left*) and antibody-apex interactions (*Right*). (E) Immunogenicity of various PIV3 F variants in CB6F1J mice (10 per group) showing immunization schema (*Top*), neutralization data (*Bottom*), and statistical comparison of prefusion-versus-postfusion F immunogens by two-tailed Mann–Whitney *U* test (*****P* < 0.0001).

versions of the F glycoprotein from PIV1, 2, 3, and 4, which we characterized structurally and tested for immunogenicity in mice and rhesus macaques.

Results

Structure-Based Design of Disulfide Bonds and Cavity-Filling Mutations That Robustly Stabilize the Prefusion PIV3 F Trimer. As PIV3 causes the most hospitalizations (8), we made it our top priority for vaccine design. We used the crystal structures of the simian prefusion PIV5 F glycoprotein (PDB ID 4GIP, 4WSG) (22) to construct a homology model for the prefusion PIV3 F protein, which consisted of three intertwined monomers contributing to the quaternary assembly of four domains—DI, DII, DIII, and the heptad repeat B (HRB) region—enclosing a large internal cavity. We compared this model of the prefusion PIV3 F to the crystal structure of uncleaved postfusion PIV3 F (PDB ID 1ZTM) (23) to predict regions of the F protein that undergo conformational rearrangements between prefusion and postfusion forms. These regions were the focus of design efforts, which resulted in over 100 prefusion-stabilized PIV3 F variants (Fig. 1B and *SI Appendix, Table S1*) and which all had the GCN4-trimerization domain appended to the C terminus at residue 481 (*SI Appendix, Table S1*). These engineered variants were predicted to stabilize the prefusion conformation of the PIV3 F protein and to arrest its transition to the postfusion state. To enable antigenic discrimination of the designed immunogens, we screened B cells for F-directed antibodies capable of neutralizing PIV3 and identified two, antibodies PIA56 and PIA75, which bound postfusion PIV3 F, and two others, antibodies PIA3 and PIA174, which were prefusion F-specific. Antigenic

screening of the F designs for binding to PIV3 prefusion and postfusion F-specific antibodies identified variants that expressed as stabilized prefusion trimers. The antigenic analysis showed that introduction of various combinations of six non-natural disulfide bonds, 172C-238C, V170C-I242C, I213C-G230C, D216C-L221C, Q162C-L168C, and G85C-Q222C, and two cavity-filling mutations, A463V and I474Y, resulted in PIV F glycoproteins that preferentially bound the prefusion-specific neutralizing antibodies PIA3 and PIA174 and not the postfusion F-specific antibody PIA56. (*SI Appendix, Fig. S1 A–E*). Cavity-filling mutations, A463V and I474Y, were both located within the HRB helical coiled-coil region, whereas the engineered disulfide bonds linked regions that were predicted to be close together in the prefusion-homology model, but to separate in the postfusion state.

Numerous disulfide and cavity-filling prefusion F variants (e.g., I172C-N238C/I474Y-GCN4) showed complete prefusion-conformational fixation over 4 weeks at 37 °C, in contrast to the variant stabilized only with GCN4 (22) (Fig. 1C), with other prefusion-stabilized variants displaying temporal stability, enhanced yield, and/or increased prefusion F physical stability (*SI Appendix, Fig. S2 and Table S1*).

Structure of a Prefusion-Stabilized PIV3 F Trimer Bound to an Apex-Binding Antibody Reveals Details of Trimer Organization and Stabilizing Mutations. To provide atomic-level information on the prefusion-stabilized PIV F, we determined the structure of the PIV3 F Q162C-L168C, I213C-G230C, A463V, I474Y variant in complex with the prefusion-specific antibody PIA174 by cryo-EM to an overall resolution of 4.3 Å, as reported according to

the conformational malleability of the prefusion trimer, along with its ability to undergo conformational adjustments while still maintaining a prefusion topology and fold.

Prefusion PIV3 F Trimers Stabilized with Various Mutations Elicit High-Titer Neutralizing Antibodies in Mice. To assess the ability of PIV3 prefusion F-stabilized variants to elicit neutralizing antibodies, we immunized CB6F1/J mice with 10- μ g doses of postfusion or prefusion PIV3 F glycoprotein variants combined with 10 μ g polyinosinic–polycytidylic acid (poly-I:C) adjuvant at weeks 0 and 3 and measured the ability of week 5 sera to prevent PIV3 infection of HEp-2 cells. The PIV3 I213C/G230C/A463V/I474Y-GCN4 prefusion variant elicited PIV3 neutralization titers 250-fold higher than postfusion F (Fig. 1E), and the A463V/I474Y cavity-filling variant elicited neutralizing titers 375-fold higher than postfusion F [geometric mean effective concentrations (EC₅₀ values) of 334,000 and 1,300, respectively]. The double-disulfide variant I213C-G230C/Q162C-L168C/A463V/I474Y (SI Appendix, Fig. S4) elicited neutralizing titers 370-fold higher than postfusion F with an EC₅₀ value of 428,000. Additional PIV3 preF variants showed EC₅₀ values up to 593,000 and similar fold differences with postfusion F (SI Appendix, Fig. S5 A and B). Overall, prefusion PIV3 F stabilized in multiple ways yielded ratios of prefusion F to postfusion F neutralization titers that were substantially higher than observed for RSV, MPV, and other paramyxoviruses.

Prefusion-Stabilized PIV1, 2, and 4 F Trimer Immunogens Obtained Through Structure-Based Design Utilizing EM. Vaccines against PIV1, 2, and 4 are also highly desired, particularly PIV1 and PIV2, which are the primary causes of croup, infect most individuals during infancy, and cause an estimated 14,000 infant hospitalizations (ages 0–4) each year in the United States alone (8). In light of the high neutralization titers induced by PIV3 prefusion F-stabilized immunizations, we used homology design of prefusion F-stabilized variants of PIV1, 2, and 4 (Fig. 2 A–C and SI Appendix, Fig. S6). A strategy similar to that used as for the prefusion PIV3 F design identified regions of high conformational mobility between prefusion and postfusion conformations, which enabled the design of prefusion variants for PIV1, PIV2, and PIV4 (Fig. 2 A–D) that locked the structures in the prefusion state. Since no PIV1, 2, or 4 F-specific antibodies were available to identify antigenically prefusion or postfusion conformation, we used negative-stain EM to assess directly the proportion of prefusion F. The combination of a glycine-rich

linker at the F2-F1 cleavage site and stem cavity-filling mutations yielded variants with high prefusion–postfusion ratios as observed with EM micrographs with over 60% prefusion conformation for PIV1 and PIV2 F, while a soluble linker at the F2-F1 cleavage site and a 172C-238C disulfide bond yielded a variant with over 95% of prefusion conformation for PIV4 F (Fig. 2C). To determine the stability of prefusion F PIV1, 2, and 4 designs, we assessed the proportion of prefusion-to-postfusion conversion over time. Prefusion F-stabilized PIV1, 2, and 4 conformations were retained above 85% over the course of a week at physiological conditions and 37 °C (Fig. 2D).

Prefusion-Stabilized PIV1, 2, and 4 F Elicit High-Titer Virus-Neutralizing Responses. To understand how PIV1, 2, and 4 F conformation impacted elicitation of neutralizing antibodies, we immunized groups of 10 CB6F1/J mice with 10 μ g/dose postF or preF PIV 1, 2, or 4 with 50 μ g poly-I:C at weeks 0 and 3. Mice elicited a statistically significant improved neutralization titer to all three PIV types when immunized with the prefusion form compared with the postfusion form, with fold improvements for PIVs 1, 2, and 4 of 23, 10, and 2.6, respectively (Fig. 2E).

Multivalent Immunization in Mice with Prefusion-Stabilized PIV1–4 F Immunogens Induces Neutralizing Titers Against PIV1–4. To investigate the ability of combinations of the prefusion-stabilized immunogens to elicit pan-PIV neutralizing antibodies, we immunized mice with monovalent, trivalent, or quadrivalent prefusion F (multivalent components were equimolar) and compared this with quadrivalent postfusion F (Fig. 3 A and B). Since PIV1–4 are related, we assessed whether omitting one of the four prefusion F types in a multivalent immunogen might induce cross-neutralization to the omitted virus type. In mice, using the same procedure as for the monovalent immunization, quadrivalent PIV1–4 prefusion F immunization yielded robust neutralization to all four types with prefusion-to-postfusion F-fold differences of 9.3, 17, 450, and 9.7 for PIV1, 2, 3, and 4, respectively. When one component was omitted from the prefusion F immunogen, titers to the omitted type equivalent to the quadrivalent postfusion F were achieved, while titers to the remaining three viral types were equivalent to that achieved by the quadrivalent postfusion F immunogen (Fig. 3C and SI Appendix, Fig. S7). Last, monovalent immunization with PIV1 prefusion F yielded statistically similar titers in both trivalent or quadrivalent immunizations, despite the three- to fourfold reduced amount of immunized PIV1 F. Thus, for PIV1 prefusion F, there

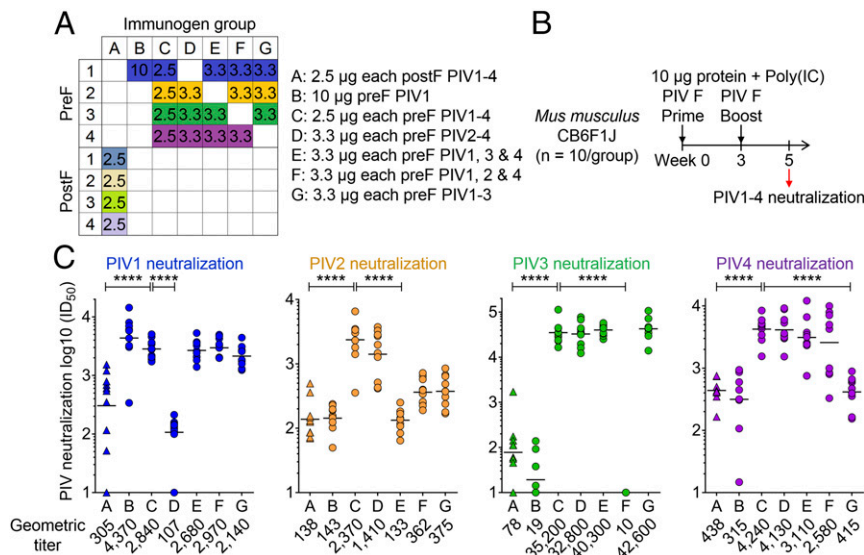


Fig. 3. Multivalent PIV1–4 F immunizations reveal high-titer neutralizing but serotype-specific responses in mice. (A) Immunization matrix of prefusion or postfusion PIV1–4 F glycoprotein mixtures, showing the amounts of each F glycoprotein antigen in the immunogen groups (A–G) with micrograms per immunization shown in the boxes in for each group. (B) Immunization plan for CB6F1J mice ($n = 10$ per group) with mixed immunogens A–G. (C) Week 5 sera neutralization readout with PIV1–4 virus infectivity inhibition assay showing statistically significant differences between quadrivalent prefusion F from quadrivalent postfusion F in all four viral neutralization assays and statistical significance between the quadrivalent prefusion F group and the trivalent prefusion F groups where F glycoproteins corresponding to the neutralization assay virus were omitted from the immunogen. A two-tailed Mann–Whitney U test was used ($****P < 0.0001$).

appeared to be little difference in elicited titer in the multivalent context; however, for PIV3 prefusion F, there did appear to be a substantial reduction in the multivalent context (Figs. 1E and 3C). Overall, the prefusion F responses appeared to be type-specific, the level of titers induced by PIV prefusion F immunization in mono- and quadrivalent formats appeared to also be type-specific, and to achieve pan-PIV immunity, the results indicate a quadrivalent combination of the four PIV1–4 prefusion F immunogens to be required.

Quadrivalent Prefusion-Stabilized PIV1–4 F Immunization of NHPs Yields Pan-PIV1–4 Neutralizing Responses. To determine the ability of the quadrivalent PIV prefusion or postfusion F immunization in nonhuman primates (NHPs) to elicit pan-PIV1–4 neutralizing responses, we immunized two groups of five rhesus macaques with 100 μ g of either quadrivalent prefusion or postfusion F in combination with poly(IC:LC) (31) three times over 16 wk (Fig. 4A). Statistically significant superior neutralization titers to all four PIV types were achieved by immunizing NHPs with quadrivalent prefusion F compared with the quadrivalent postfusion F (Fig. 4B). Prefusion-to-postfusion F-fold differences were 3.3, 6.9, 13, and 32 for PIV1, 2, 3, and 4, respectively. Thus, immunization with a quadrivalent PIV vaccine elicited neutralizing antibody titers of all four major serotypes of PIV.

Prefusion-Stabilized PIV3 F Vaccine Elicits Higher Neutralizing Titers in NHP than Observed in a Cohort of over 100 Healthy Adults. To provide context for the prefusion F-induced titers by the PIV1–4 quadrivalent vaccine, we assessed the naturally occurring level of titers in a cohort of 127 healthy adults. The average PIV3 titer in the cohort was less than 100, with a highest observed titer of \sim 1,600 (Fig. 4D). By contrast, the PIV3 titers after quadrivalent prefusion F immunization were substantially higher, averaging 35,200 in mice and 11,400 in rhesus macaques. As the PIV3 titers were higher in the monovalent context, we tested whether subsequent

monovalent immunization could boost titers in the rhesus macaques. Two additional 100- μ g immunizations with PIV3 prefusion F resulted in further increases in neutralization titers with the prefusion F PIV3 immunized group, reaching an ID₅₀ value of 60,500 (Fig. 4C).

Discussion

As humans can generate immunity to infection by PIV3 (32), with titers in our cohort averaging \sim 100, it seems likely that the high-PIV3 neutralizing titers induced by the quadrivalent vaccine in NHPs would be protective against PIV3 infection. The superior titers induced by prefusion-stabilized F versus postfusion F for PIV1–4 indicate that these viruses are similar to RSV F, for which prefusion F-induced titers were superior to those induced by postfusion F, and, unlike MPV F, for which prefusion and postfusion F immunization generated similar titers (21). Notably, the PIV3 neutralization titers elicited by prefusion-stabilized PIV3 F were much higher than those observed for prefusion-stabilized RSV (16), MPV (21), PIV1, PIV2, or PIV4 F glycoproteins, suggesting the prefusion-stabilized PIV3 F immunogens described here may be highly suitable PIV3 vaccine candidates. It is not clear why prefusion PIV3 F elicits such high neutralization titers; one possibility is that highly neutralization-sensitive epitopes are exposed on the protein immunogen structure that can engage effective counterpart neutralizing antibodies, such as the apex-binding epitope for PIA174 and epitopes similar to site \emptyset of prefusion RSV F (16). Despite identifying numerous prefusion-stabilizing designs for PIV3 F, all nine combinations of stabilized immunogens elicited similar neutralizing titers, suggesting that stabilization into the prefusion state was the primary criterion for the improved titers and contrasts with prefusion-stabilized RSV F titers, where more graded immunogenicity was observed depending on the prefusion-stabilizing mutations (16).

It may be possible to develop a multivalent vaccine, which combines prefusion-stabilized F immunogens from RSV and

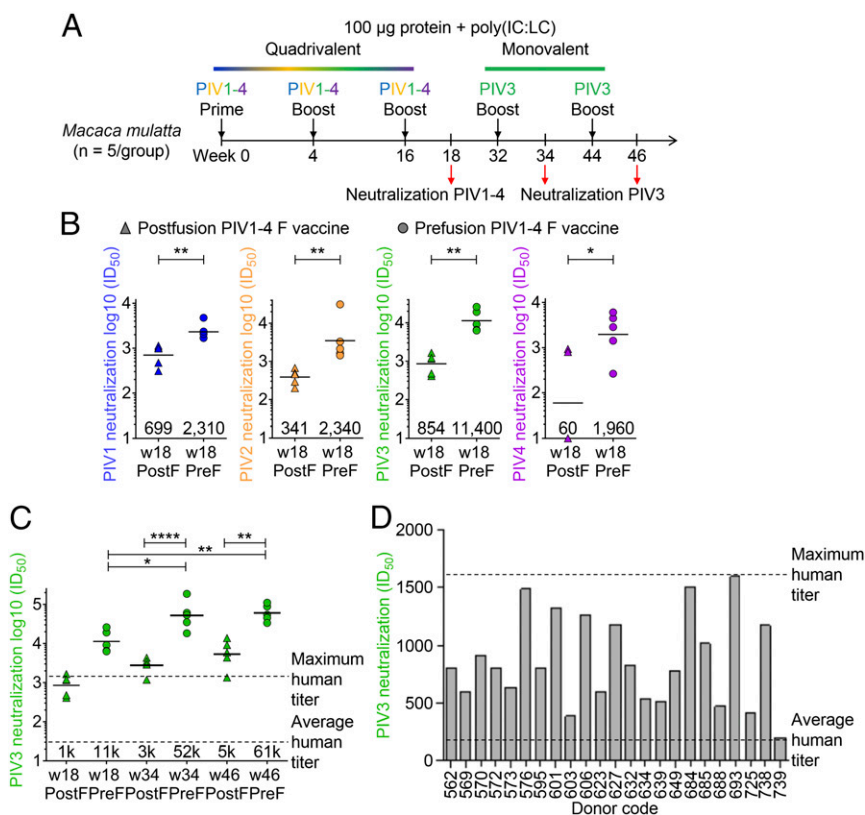


Fig. 4. Quadrivalent immunization with prefusion PIV1–4 F glycoproteins in NHP elicits high-titer neutralizing responses. (A) NHP immunization scheme for quadrivalent prefusion or postfusion PIV1–4 F whereby three immunizations were administered containing the four viral F glycoproteins, followed by two immunizations with only PIV3 F glycoproteins. (B) Neutralization titers for NHP sera using PIV1, 2, 3, or 4 virus following three quadrivalent immunizations at week 18. Statistical significance is observed when comparing prefusion and postfusion groups for all four PIV virus types using a two-tailed Mann–Whitney U test ($^{**}P < 0.01$, $^{*}P < 0.05$). Geometric titers are shown above the x axis. (C) Neutralization titers in NHPs at weeks 34 and 46 following two PIV3 F-only boosts. A fivefold increase in prefusion-specific titers and statistically significant differences between prefusion and postfusion groups were observed ($^{****}P < 0.0001$, $^{**}P < 0.01$, $^{*}P < 0.05$). (D) Analysis of 127 human sera tested for PIV3 neutralization showing the top 23 neutralizers for which the highest recorded titer was 1,600.

PIV1–4; together with licensed vaccines for measles and mumps, such a multivalent vaccine would provide broad-spectrum immunity against pathogenic paramyxoviruses to which humans are exposed throughout life. Multivalent vaccines have been found to be useful against a number of pathogens and are currently licensed for flavivirus vaccines (dengue virus serotypes 1–4) (33), rotavirus vaccines (34), and HPV vaccines (35). Although our work here focused on the use of purified recombinant proteins for vaccination, it seems likely that the prefusion designs can be utilized in genetic contexts, as has been shown for prefusion-stabilized RSV F in the context of a PIV1 vector (36) or as a mRNA vaccine (37). Furthermore, prefusion-stabilized PIV F nanoparticles may be particularly effective. Overall, our results show how prefusion stabilization can substantially increase the titer of virus-neutralizing responses against PIV types, especially type 3. It will be interesting to see if prefusion F vaccine strategies utilized here can succeed with other members of the paramyxovirus family that are considered high risk for pandemic outbreak, such as Nipah and Hendra viruses.

Materials and Methods

Prefusion and postfusion PIV3 F-specific monoclonal antibodies were isolated by comparing B-cell culture supernatant neutralization potency and post-fusion PIV3 F ELISA. The design of prefusion-stabilizing PIV3 F mutations was performed based on the prefusion PIV5 F structure and antigenically analyzed using prefusion and postfusion PIV3 F-specific monoclonal antibodies.

- Russell CJ, Simões EAF, Hurwitz JL (2018) Vaccines for the paramyxoviruses and pneumoviruses: Successes, candidates, and hurdles. *Viral Immunol* 31:133–141.
- Jain S, et al.; CDC EPIC Study Team (2015) Community-acquired pneumonia requiring hospitalization among U.S. adults. *N Engl J Med* 373:415–427.
- Jain S, et al.; CDC EPIC Study Team (2015) Community-acquired pneumonia requiring hospitalization among U.S. children. *N Engl J Med* 372:835–845.
- Lozano R, et al. (2012) Global and regional mortality from 235 causes of death for 20 age groups in 1990 and 2010: A systematic analysis for the Global Burden of Disease Study 2010. *Lancet* 380:2095–2128, and erratum (2013) 381:628.
- Rota PA, et al. (2016) Measles. *Nat Rev Dis Primers* 2:16049.
- Hviid A, Rubin S, Mühleemann K (2008) Mumps. *Lancet* 371:932–944.
- Panda S, Mohakud NK, Pena L, Kumar S (2014) Human metapneumovirus: Review of an important respiratory pathogen. *Int J Infect Dis* 25:45–52.
- Abedi GR, et al. (2016) Estimates of parainfluenza virus-associated hospitalizations and cost among children aged less than 5 years in the United States, 1998–2010. *J Pediatric Infect Dis Soc* 5:7–13.
- Davidkin I, Jokinen S, Broman M, Leinikki P, Peltola H (2008) Persistence of measles, mumps, and rubella antibodies in an MMR-vaccinated cohort: A 20-year follow-up. *J Infect Dis* 197:950–956.
- Crowe JE, Jr, et al. (1994) Satisfactorily attenuated and protective mutants derived from a partially attenuated cold-passaged respiratory syncytial virus mutant by introduction of additional attenuating mutations during chemical mutagenesis. *Vaccine* 12:691–699.
- Karron RA, et al. (1997) Evaluation of two live, cold-passaged, temperature-sensitive respiratory syncytial virus vaccines in chimpanzees and in human adults, infants, and children. *J Infect Dis* 176:1428–1436.
- Kapikian AZ, Mitchell RH, Chanock RM, Shvedoff RA, Stewart CE (1969) An epidemiologic study of altered clinical reactivity to respiratory syncytial (RS) virus infection in children previously vaccinated with an inactivated RS virus vaccine. *Am J Epidemiol* 89:405–421.
- Weibel RE, et al. (1967) Respiratory virus vaccines. VII. Field evaluation of respiratory syncytial, parainfluenza 1, 2, 3, and Mycoplasma pneumoniae vaccines, 1965 to 1966. *Am Rev Respir Dis* 96:724–739.
- Scheid A, Choppin PW (1974) Identification of biological activities of paramyxovirus glycoproteins. Activation of cell fusion, hemolysis, and infectivity of proteolytic cleavage of an inactive precursor protein of Sendai virus. *Virology* 57:475–490.
- Aguilar HC, Henderson BA, Zamora JL, Johnston GP (2016) Paramyxovirus glycoproteins and the membrane fusion process. *Curr Clin Microbiol Rep* 3:142–154.
- McLellan JS, et al. (2013) Structure-based design of a fusion glycoprotein vaccine for respiratory syncytial virus. *Science* 342:592–598.
- McLellan JS, et al. (2013) Structure of RSV fusion glycoprotein trimer bound to a prefusion-specific neutralizing antibody. *Science* 340:1113–1117.
- Krarup A, et al. (2015) A highly stable prefusion RSV F vaccine derived from structural analysis of the fusion mechanism. *Nat Commun* 6:8143.
- Liang B, et al. (2015) Enhanced neutralizing antibody response induced by respiratory syncytial virus prefusion F protein expressed by a vaccine candidate. *J Virol* 89: 9499–9510.
- Stewart-Jones GB, et al. (2015) A cysteine zipper stabilizes a pre-fusion F glycoprotein vaccine for respiratory syncytial virus. *PLoS One* 10:e0128779.
- Battles MB, et al. (2017) Structure and immunogenicity of pre-fusion-stabilized human metapneumovirus F glycoprotein. *Nat Commun* 8:1528.
- Welch BD, et al. (2012) Structure of the cleavage-activated prefusion form of the parainfluenza virus 5 fusion protein. *Proc Natl Acad Sci USA* 109:16672–16677.
- Yin HS, Paterson RG, Wen X, Lamb RA, Jardetzky TS (2005) Structure of the uncleaved ectodomain of the paramyxovirus (hPIV3) fusion protein. *Proc Natl Acad Sci USA* 102: 9288–9293.
- Liu Q, et al. (2017) Quaternary contact in the initial interaction of CD4 with the HIV-1 envelope trimer. *Nat Struct Mol Biol* 24:370–378.
- Lee JH, et al. (2017) A broadly neutralizing antibody targets the dynamic HIV envelope trimer apex via a long, rigidified, and anionic β -hairpin structure. *Immunity* 46: 690–702.
- Doria-Rose NA, et al.; NISC Comparative Sequencing Program (2014) Developmental pathway for potent V1V2-directed HIV-neutralizing antibodies. *Nature* 509:55–62.
- Walker LM, et al.; Protocol G Principal Investigators (2009) Broad and potent neutralizing antibodies from an African donor reveal a new HIV-1 vaccine target. *Science* 326:285–289.
- Julien JP, et al. (2013) Asymmetric recognition of the HIV-1 trimer by broadly neutralizing antibody PG9. *Proc Natl Acad Sci USA* 110:4351–4356.
- McLellan JS, et al. (2011) Structure of HIV-1 gp120 V1/V2 domain with broadly neutralizing antibody PG9. *Nature* 480:336–343.
- Kabat EA, Wu TT, Perry HM, Gottesman KS, Foeller C (1991) *Sequences of Proteins of Immunological Interest* (US Dept Health Human Serv, Natl Inst Health, Bethesda), 5th Ed.
- Martins KA, Bavari S, Salazar AM (2015) Vaccine adjuvant uses of poly-IC and derivatives. *Expert Rev Vaccines* 14:447–459.
- Lee MS, et al. (2001) Half-life of human parainfluenza virus type 3 (hPIV3) maternal antibody and cumulative proportion of hPIV3 infection in young infants. *J Infect Dis* 183:1281–1284.
- McArthur MA, Szein MB, Edelman R (2013) Dengue vaccines: Recent developments, ongoing challenges and current candidates. *Expert Rev Vaccines* 12:933–953.
- Jonesteller CL, Burnett E, Yen C, Tate JE, Parashar UD (2017) Effectiveness of rotavirus vaccination: A systematic review of the first decade of global postlicensure data, 2006–2016. *Clin Infect Dis* 65:840–850.
- Kash N, et al. (2015) Safety and efficacy data on vaccines and immunization to human papillomavirus. *J Clin Med* 4:614–633.
- Liu X, et al. (2017) Attenuated human parainfluenza virus type 1 expressing the respiratory syncytial virus (RSV) fusion (F) glycoprotein from an added gene: Effects of prefusion stabilization and packaging of RSV F. *J Virol* 91:e01101-17.
- Geall AJ, et al. (2012) Nonviral delivery of self-amplifying RNA vaccines. *Proc Natl Acad Sci USA* 109:14604–14609.
- Nakane T, Kimanius D, Lindahl E, Scheres SH (2018) Characterisation of molecular motions in cryo-EM single-particle data by multi-body refinement in RELION. *eLife* 7: e36861.