Supporting Online Material for

Induction of Broadly Neutralizing H1N1 Influenza Antibodies by Vaccination

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SUPPORTING ONLINE MATERIAL

MATERIALS AND METHODS

Immunogen and plasmid construction. Plasmids encoding different versions of HA (A/PR/8/1934 (1934 PR8), GenBank P03452; A/New Caledonia/20/1999 (1999 NC), GenBank AY289929; A/Singapore/6/1986 (1986 Sing), GenBank ABO38395; A/Beijing/262/1995 (1995 Bei), GenBank AAP34323; A/Solomon Islands/3/2006 (2006 SI), GenBank ABU99109; A/Brisbane/59/2007 (2007 Bris), GenBank ACA28844; A/Wisconsin/67/2005 (2005 WI), GenBank ACF54576; A/Wyoming/3/2003 (2003 Wy), GenBank AAT08000; A/Brisbane/10/2007 (2007 Bris), GenBank ABW23353; A/Vietnam/1203/2004 (2004 VN1203), GenBank AAW80717; A/Singapore/1/1957 (1957 Sing), GenBank AAA64366) and corresponding NA proteins and monoclonal antibodies CR6261 and F10 (S1,S2) were synthesized using human preferred codons as described (S3) by GeneArt (Regensburg, Germany) and cloned into a CMV/R expression vector for efficient expression in mammalian cells (S3). The stem mutations were introduced using the QuikChange Site-Directed Mutagenesis kit (Agilent Technologies).

Production of pseudotyped lentiviral vectors and recombinant adenovirus 5. The recombinant lentiviral vectors expressing a luciferase reporter gene were produced as previously described (S4). For the production of H1N1 and H3N2 pseudovirus, a human type II transmembrane serine protease TMPRSS2 gene was included in transfection for the proteolytic activation of HA (S5). Two replication-defective rAd serotype 5 vectors expressing 1999 NC or 1934 PR8 HA genes were produced as previously described (S6).
Protein expression and purification. Plasmids expressing secreted wild-type or stem mutant 1999 NC HA were expressed in the human embryonic kidney cell line 293F. The stem mutant has an Ile545Asn and a Gly547Thr (H3 numbering) change to introduce an N-linked glycosylation site into helix A in the middle of the stem. The addition of a glycan at this site is unlikely to block regions beyond the stem as it would have to stretch more than 40 Å to block any of the head region of HA (using the PR8/34 HA structure as a reference (PDB entry 1RU7). A complex N-linked glycosylation without terminal sialic acid was modeled to this site with the GlyProt Server (S7). The longest dimensions in this modeled glycosylation are only 32 Å. Even this distance would not be likely for antibody blocking given the uneven surface of the HA protein, the presence of several other nearby glycosylations and the fact that glycans generally project out from the protein surface rather than following the protein contour. Both WT and ΔStem HAs share a similar trimer elution profile (fig. S3A, left panel). Since post-fusion forms of HA are known to aggregate (S8), the presence of soluble trimers indicates that the stem mutant is in the pre-fusion trimeric form. The addition of the extra glycan was evidenced by the increased size of the ΔStem HA on both SDS-PAGE and non-denaturing native Blue gel (fig. S3A). Moreover, both WT and ΔStem HAs have similar binding affinity to a 1999 NC-specific antibody, as indicated by surface plasmon resonance (SPR) analysis, further documenting its structural integrity (fig. S3B).

Monoclonal antibodies CR6261 and F10, as well as the wild type and mutant stem trimer plasmid expression vectors, were transfected into the human embryonic kidney cell line 293F using 293fectin (Invitrogen) according to the manufacturer’s instructions. 293F cells were cultured in Freestyle 293 Expression Medium (Invitrogen) and
supernatant was collected 72-96 hrs post-transfection and cleared by centrifugation and filtration. HA proteins were purified as previously described (S9). Expression of HA proteins was confirmed by Western blotting using an anti-His tag antibody (Qiagen). Antibodies were purified using a Protein G affinity column (GE Healthcare).

**Neutralization, protein competition and cell absorption assays.** The pseudotype neutralization assay has been widely accepted for defining the specificity of neutralizing antibodies targeting influenza HAs (S3, S4, S10). The increased sensitivity of the pseudotype neutralization assay makes it a useful tool to assess the neutralization induced by antibodies targeting the conserved stem region of HA which the MN assay often fails to pick up (S11). Moreover, because the HAI assay relies on antibodies that recognize the HA head, anti-stem antibodies cannot be detected using the HAI assay.

HA NA-pseudotyped lentiviral vectors encoding luciferase were first titrated by serial dilution. Similar amounts of virus (p24 ≈ 6.25 ng/ml) were then incubated with indicated amounts of mouse antisera for 20 minutes at room temperature and added to 293A cells (10,000 cells/well in a 96-well plate) (50 μl/well, in triplicate). Plates were then washed and replaced with fresh media 2 hours later, and luciferase activity was measured after 24 hours. For the protein competition assay, mouse or ferret antisera were pre-absorbed with HIV env (control), wild-type or stem mutant 1999 NC HA trimers (40 µg/ml) for 30 min and the pre-absorbed antisera were then used to measure neutralization activity against the indicated pseudoviruses. Cell absorptions of ferret or monkey sera were performed as previously described (S10). Monkey sera pre-absorbed with cells expressing wild-type or stem mutant 1999 NC HA were then used to perform ELISA assays on plates coated with 1999 NC, 1986 Sing or 2007 Bris HA trimers. For CR6261
antibody competition, ELISA plates coated with 1999 NC or 1986 Sing HA trimers were incubated with CR6261 (10 µg/ml) before the addition of ferret sera pre-absorbed with cells expressing the stem mutant 1999 NC HA. Ferret antibodies were detected with an anti-ferret secondary antibody (Rockland). The ELISA assay was performed as described previously (S12).

Mouse and monkey immunization. Female BALB/c mice (6-8 weeks old; Jackson Laboratories) were immunized intramuscularly with 15 µg of plasmid DNA in 100 µl of PBS (pH 7.4) at weeks 0, 3, and 6. At week 9, mice were boosted with either PBS, 5 µg of 2006-2007 seasonal influenza vaccine (Sanofi Pasteur) containing HA from the following strains: A/New Caledonia/20/1999 (H1N1), A/Wisconsin/67/2005 (H3N2), and B/Malaysia/2506/2004, or 10⁸ PFU of rAd5 vector expressing 1999 NC HA. Mice that received two doses of seasonal influenza vaccine were immunized at a 3-week interval. Blood was collected 14 days after each immunization and serum was isolated. For monkeys, three groups of four 3- to 4-year-old rhesus macaques were immunized with 4 mg of empty vector or 1999 NC HA DNA in 500 µl of PBS (pH 7.4) at weeks 0, 4, and 8. At week 16 the animals were boosted with either 45 µg of 2006-2007 seasonal influenza vaccine or PBS. The vaccine was administered intramuscularly into the anterior quadriceps. Blood was collected 14 days and 28 days after each immunization and serum was isolated. Animal experiments were conducted in full compliance with all relevant federal regulations and NIH guidelines.

Mouse challenge. BALB/c female mice were anesthetized by i.p. injection with 0.0025 mg xylazine and 0.125 mg ketamine per gram body weight. Influenza virus strain A/PR8/1934 was diluted in PBS to obtain the appropriate LD50 and instilled drop-wise
intranasally at 0.025 ml per nostril into each mouse. Mice were weighed daily for up to 21 days starting on the day of infection and monitored for signs of influenza virus infection such as ruffled fur, hunched posture, and listlessness. Any mice that had lost more than 25% body weight were euthanized.

**HAI assay.** A seed stock of the A/PR/8/1934 (H1N1) virus was obtained from ATCC (Cat. #VR-95) and the A/New Caledonia/20/1999 (H1N1) seed stock was obtained from the CDC (Atlanta, GA). Stock virus was expanded in the allantoic cavities of 10-day-old embryonated chicken eggs at 35°C for 48 hr and stored at -80°C. The TCID$_{50}$ of the PR8/34 stock used for the mouse challenge experiment was $10^{7.5}$/ml. Sera were treated with receptor-destroying enzyme (RDE) by diluting one part serum with three parts enzyme and incubated overnight in a 37°C water bath. The enzyme was inactivated by 30 min incubation at 56°C followed by addition of six parts PBS for a final dilution of 1/10. HAI assays were performed in V-bottom 96-well plates using four hemagglutinating units (HAU) of virus and 0.5% turkey RBC.

**Microneutralization (MN) assay.** Neutralizing antibody activity was analyzed in an MN assay based on the methods of the WHO Global Influenza Program (S13). Sera were treated with RDE by diluting one part serum with three parts enzyme and incubated overnight in a 37°C water bath and heat-inactivated as described for the HAI assay.

**Ferret immunizations and challenge.** 6 month old male Fitch ferrets (Triple F Farms, Sayre, PA), seronegative for exposure to currently circulating pandemic H1N1, seasonal H1N1, H3N2, and B flu strains, were housed and cared for at BIOQUAL, Inc. (Rockville, MD). These facilities are accredited by the American Association for the Accreditation of Laboratory Animal Care (AAALAC) International and meet NIH
standards as set forth in the Guidelines for Care and Use of Laboratory Animals (*SI4*).

Prior to study start, a temperature transponder (Biomedic Data Systems, Inc.) was implanted into the neck of each ferret. Ferrets were immunized intramuscularly with 250 µg of 1999 NC HA DNA in 500 µl of PBS (pH 7.4) at weeks 0, 3, and 6. At week 9 the animals were boosted with either 7.5 µg of 2006-2007 seasonal influenza vaccine or an rAd5 vector expressing 1999 NC HA (10^9 PFU). The vaccine was administered via IM injections into the upper thigh muscle. Blood was collected 14 days after each immunization and serum was isolated. Approximately 4 weeks after the last immunization the ferrets were challenged with 10^{6.5} EID_{50} of the seasonal influenza viruses, A/Brisbane/59/2007 (H1N1) or A/PR/8/1934 (H1N1). This virus had been expanded in 10-day-old chicken eggs from a seed stock obtained from the CDC (Atlanta, GA) and has a titer of 10^{6.5} EID/ml. The virus stock was inoculated intranasally into ferrets, which had been anesthetized with Ketamine/Xylazine, in a volume of 0.5 ml per nostril. The ferrets were observed for clinical signs twice daily and weight and temperature measurements recorded daily by technicians blind to the treatment groups.

Nasal washes were obtained on days 1, 2, 3, 5, 7, 9, and 14 post challenge and infectious viral titers determined by a TCID_{50} or EID_{50} assay. For TCID_{50}, 10-fold dilutions of nasal wash were added to a 96-well round-bottomed tissue culture plate. As a positive control, 10-fold dilutions of the challenge virus were included in each experiment. MDCK cells were then added to all wells and the plates were incubated overnight at 37°C, 5% CO_2. The medium was discarded and replaced with TPCK-trypsin-containing medium. The plates were incubated for another 4 days. At this time, 50 µl from each well was transferred to a 96-well V-bottomed microtiter plate, 0.5% turkey RBC were
added to all wells and the presence of virus detected by hemagglutination as a read-out. The infectious titer was calculated by the method of Reed and Muench (S15). For EID$_{50}$, virus titers in clarified nasal washes were determined in eggs from an initial dilution of 1:10 in PBS and expressed as EID$_{50}$/ml. The limit of virus detection was $10^{1.5}$ EID$_{50}$/ml.

**Flow Cytometric Analysis of Intracellular Cytokines.** CD4$^+$ and CD8$^+$ T-cell responses were evaluated using intracellular cytokine staining for IFN-$\gamma$ and TNF-$\alpha$ as described (S16) with peptide pools (15 mers overlapping by 11 aa, 2.5 $\mu$g/ml each) covering 1999 NC HA proteins. Cells were then fixed, permeabilized, and stained by using rat monoclonal anti-mouse CD3, CD4, CD8, IFN-$\gamma$, and TNF-$\alpha$ (BD-PharMingen). The IFN-$\gamma$ and TNF-$\alpha$ positive cells in the CD4$^+$ and CD8$^+$ cell populations were analyzed with the program FlowJo (Tree Star).

**Surface plasmon resonance.** Biacore 3000 (GE Healthcare) was used in all experiments. Antibodies were coupled to Biacore CM5 chips using standard amine coupling. HA or control proteins (500 nM) were flowed over the chip at a flow rate of 30 $\mu$L/min for either 5 min or 8 min, followed by injection of Biacore HBS-EP buffer (10 mM HEPES, pH 7.4, 150 mM NaCl, 3mM EDTA, 0.005 % surfactant P-20) for 5 min.
Figure S1. Humoral responses against H3N2 influenza HAs from animals primed with H3 DNA vaccine and boosted with 2006-2007 seasonal flu vaccine. (A)

Neutralizing antibodies against HA from A/Wisconsin/67/05 (2005 WI) after homologous H3 HA DNA or seasonal flu vaccine alone or prime boost immunization.

(B) Cross-reactive antibodies against heterologous H3N2 HAs from A/Wyoming/3/03
(2003 WY) and A/Brisbane/10/07 (2007 Bris) after DNA alone, seasonal flu vaccine alone, or prime/boost vaccination.
Figure S2. Prime/boost regimen elicited cross-reactive neutralization to other group 1 subtypes. Antisera from mice immunized with 1999 NC HA DNA/rAd5 or DNA/seasonal vaccine neutralize HAs from A/Singapore/1/1957 (H2N2) (left, 1957 Sing) and A/Vietnam/1203/2004 (H5N1) (right, 2004 VN1203), respectively. The control groups received an empty vector. The rAd5 expressed HA from the 1999 NC strain.
Figure S3. Biochemical characterization and surface plasmon resonance binding analysis of wild-type and stem mutant 1999NC HA trimers. (A) Gel filtration elution profile of wild-type (WT, black line) and stem mutant (ΔStem, red line) of 1999 NC HA proteins on a Superdex200 16/60 column. The figures present superimposed elution profiles of HA proteins with calibration standards (green dotted line). Trimeric fractions
were collected and further analyzed by SDS-PAGE (left) and native blue gel (right). (B) Representative Biacore binding profiles are shown for 1999 NC WT HA (black line), ΔStem (red line), and an unrelated protein (HIV env, blue line) binding to a 1999 NC-specific antibody or anti-stem monoclonal antibodies C179, F10, and CR6261. Antibodies were coupled to Biacore CM5 chips to final densities ranging from ~700 to 3000 response units (RU). The WT HA protein bound to all four antibodies, while ΔStem lacked observed binding to C179, F10, and CR6261. The HIV Env protein failed to bind to any of the antibodies and served as a negative control.
Figure S4. T-cell responses to 1999 NC HA after DNA priming and seasonal vaccine boosting. Spleens from immunized animals were taken 12 days after the seasonal vaccine boosting. Spleen cells were re-stimulated with 1999 NC HA peptides. Intracellular cytokine staining for IFN-γ and TNF-α in CD4+ and CD8+ T cells was measured by flow cytometry following staining with a mixture of antibodies to the two cytokines. Five animals per group were analyzed individually. The percentage of activated T cells that produced either IFN-γ or TNF-α in response to stimulation is shown. Symbols indicate the response of individual animals.
REFERENCES


