Increasing the breadth and potency of response to the seasonal influenza virus vaccine by immune complex immunization

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The main barrier to reduction of morbidity caused by influenza is the absence of a vaccine that elicits broad protection against different virus strains. Studies in preclinical models of influenza virus infections have shown that antibodies alone are sufficient to provide broad protection against divergent virus strains in vivo. Here, we address the challenge of identifying an immunogen that can elicit potent, broadly protective, antiinfluenza antibodies by demonstrating that immune complexes composed of sialylated antihemagglutinin antibodies and seasonal inactivated flu vaccine (TIV) can elicit broadly protective antihemagglutinin antibodies. Further, we found that an Fc-modified, bispecific monoclonal antibody against conserved epitopes of the hemagglutinin can be combined with TIV to elicit broad protection, thus setting the stage for a universal influenza virus vaccine.

TIV | immune complex | universal influenza vaccine | CD23 | sialylated Fc

The influenza A virus (IAV) hemagglutinin (HA) glycoprotein is a major target of both strain-specific and broadly protective influenza virus antibodies. HA is composed of two distinct antigenic domains: the stalk domain, which is relatively conserved, and the globular head, which is characterized by antigenic drift over time. Because of continuous antigenic drift, the composition of current influenza vaccines must be updated annually to reflect strains predicted to circulate in the upcoming season. There are 18 known subtypes of IAV HA, which can be further classified into two major phylogenetic groups; this division correlates with two basic structures taken by the HA stalk domain (1): group 1 subtypes (H1, H2, H5, H6, H8, H9, H11, H12, H13, H16, HA-like H17, and HA-like H18) and group 2 subtypes (H3, H4, H7, H10, H14, and H15). H1 (group 1) and H3 (group 2) subtype IAV viruses have been cocirculating in humans since 1977 and thus are included in all seasonal influenza vaccines.

In recent years, seasonal influenza has caused an estimated 140,000–710,000 annual hospitalizations in the United States (2). In addition to seasonal disease, avian influenza viruses with H5, H7, or H9 subtype hemagglutinins regularly infect people who are exposed to infected birds. Since these viruses are not adapted to transmission between humans, their spread is limited, but H5N1 seroprevalence data show that 1–2% of individuals in rural areas are seropositive for H5N1 at any time, highlighting a substantial rate of exposure and avian-to-human IAV transmission (3). While a majority of H5N1 infections are subacute, several hundred people experience symptomatic and severe avian influenza each year (4). Pandemic influenza would likely result from adaptation of an avian influenza strain to transmissibility between people; it is therefore extremely desirable for next generation influenza vaccines to provide protection against a broad array of both seasonal and avian influenza strains.

The breadth and potency of anti-HA antibody responses are known to be affected by several factors, including: vaccine delivery platform (live attenuated virus vs. protein vs. DNA, etc.) (5–9), the specific HA or chimeric HA structures used in prime-boost vaccination protocols (10–12), timing between prime-boost immunizations (13), and choice of adjuvant, or its absence (14, 15). The common mechanism by which all of these factors affect the final anti-HA antibody repertoire is by impacting the maturation and selection of B cells in the weeks following vaccination. Broadly protective anti-HA antibody responses evolve from the selection of B cells based on their affinity for conserved HA domains (1, 16–18), antibody isotype, and IgG subclass (19–21). This latter requirement determines interactions with activating Fc γ receptors (FcγRs), which are required for potency of broadly protective antibodies in vivo (20, 21).

We have recently shown that anti-HA antibody responses can be enhanced for breadth and potency by direct selection of higher-affinity anti-HA B cells using immune complexes (ICs) with specific FcR-binding properties. We defined a specific pathway by which ICs promote affinity maturation after observing that production of anti-HA Fc domains enriched for sialylated glycoforms correlates with higher-affinity responses to seasonal influenza vaccination (22). ICs comprising the seasonal influenza vaccine (TIV) and sialylated Fc anti-HA IgGs were found to elicit higher-affinity antibody responses that were enhanced for breadth and potency of activity against H1 influenza viruses. The mechanism underlying this enhanced antibody response involved coengagement of CD23 by sialylated Fc domains

Significance

Influenza viruses remain a source of substantial morbidity and mortality worldwide. This is, in part, because current approaches to vaccination elicit strain-specific immune responses. Here, we report a method for targeting the Fc receptor, CD23, during vaccination with existing influenza vaccines (TIV) to increase the breadth and potency of the antibody response. Immunization with the TIV in complex with a monoclonal antibody that is broadly reactive against the hemagglutinin glycoprotein and engineered at the Fc domain to engage CD23 elicited antibodies that were 10-fold increased in potency and that protected against the potential pandemic influenza virus subtype H5N1 in vivo. This work demonstrates that broadly protective influenza immunity can be achieved using existing seasonal vaccines.

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with the B-cell receptor (BCR), leading to up-regulation of the inhibitory type I FcγR, FcγRIIB. Increased expression of FcγRIIB suppresses the activation/proliferation signal triggered by HA interaction with low-affinity, but not high-affinity, BCR (Fig. 1) (23, 24). The net result was selection for higher-affinity antibody-producing cells that were enhanced for breadth and potency of activity against H1 influenza viruses (22). Overall, CD23 targeting can mediate higher-affinity responses to TIV vaccination, with the abundance of sialylated Fc anti-HA IgGs produced during the early vaccine response impacting the breadth of the ultimate anti-TIV antibody repertoire.

Here, we test the hypothesis that existing influenza virus vaccines can be combined with a broadly reactive anti-HA IgG that engages CD23 through the Fc to elicit a broadly protective response against potential pandemic IAV. Specifically, we demonstrate that TIV can be enhanced to protect against H5N1 by combination with a broadly reactive anti-HA monoclonal antibody that is engineered to engage CD23. Thus, increasing the affinity of the antibody response generated during seasonal vaccination is a mechanism for providing broad and heterologous immunity against influenza viruses.

Results

Our previous studies demonstrated that immunization with sialylated ICs composed of purified HA and sialylated Fc (sFc) anti-HA IgG resulted in anti-HA IgG responses with increased breadth and potency of protective activity (22). To demonstrate the generality of this observation and its application to a standard seasonal influenza vaccine formulation we evaluated whether the antibody response elicited by the seasonal influenza vaccine could be modulated by CD23 targeting during vaccination. To do this, we combined the 2014–15 TIV with purified polyclonal human IgGs from an individual with relatively high sFc abundance [27% sFc abundance on anti-H1 (A/California/7/2009) hemagglutinin IgGs]. A control IgG preparation was prepared by neuraminidase treatment of the purified IgG to remove sialic acids and thus prevent CD23 targeting. The TIV immune complex immunogens, or TIV alone, were administered to wild-type C57BL/6 mice followed by boost immunization 2 wk later with TIV alone. Ten days post boost, serum IgGs were evaluated for breadth of binding by ELISA, which is a measurement that reflects antibody quantity and affinity. All immunogens elicited IgGs with equivalent binding to the homologous TIV preparation and the A/California/7/2009 H1 HA, which was a component of the 2014–15 TIV. In contrast, distinctions between groups were evident when we evaluated breadth of binding of serum IgG against HAs that were not present in the 2014–15 TIV. Serum IgG obtained from animals vaccinated with TIV complexed with sFc IgG showed significantly increased binding to the H1 from A/Brisbane/59/2007, the H3 from A/Brisbane/10/2007, and to the H5 from the avian influenza virus, A/duck/Hunan/795/2002 (Fig. 2 *A*–*E*).

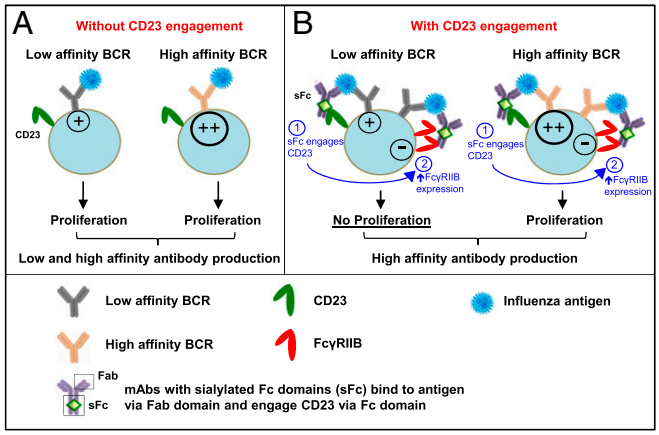


Fig. 1. Model for high-affinity antibody production through B-cell CD23 engagement. (*A*) Signaling through B-cell receptor (BCR) triggers proliferation of low and high-affinity B cells. (*B*) Coengagement of CD23 with BCR induces increased expression of the inhibitory FcγRIIB, which, in turn, elevates the threshold of BCR affinity that is required for cell activation and results in selection of higher-affinity B cells.

Next, we determined whether IgGs elicited by the sialylated TIV ICs could be discriminated based on potency of protective activity in vivo. Purified IgGs from mice immunized with either sialylated or asialylated TIV IC were incubated at 75 μg/mL or 7.5 μg/mL with A/Netherlands/602/2009 virus, a virus expressing an HA that is homologous with the H1 component of the 2014–15 TIV. Virus and IgG were then administered to mice, intranasally (22). At the 75 μg/mL dose, all mice were equally protected from virus challenge. In contrast, only the IgGs elicited by sialylated TIV ICs protected at a 10-fold lower concentration (Fig. 3). Together, this experiment demonstrated that sialylated TIV ICs could elicit IgGs with enhanced breadth of ELISA binding (Fig. 2) and potency of in vivo activity (Fig. 3).

Since our goal is to develop a general method for eliciting broad, neutralizing activity against influenza viruses we sought to develop an immunization approach that would be independent of polyclonal antisera. We recognized that a formulation of immune complexes containing the seasonal influenza vaccine and a broadly reactive monoclonal antibody would be highly desirable, allowing for standardization of the composition and potency of the immunogen. This mAb could be engineered to bind to CD23 either by sialylation of the Fc domain or use of the Fc variant, F241A, previously shown to recapitulate the effect of Fc sialylation on CD23 binding and in vivo activity (25). Development of this mAb proceeded in several steps. First, we needed to determine if the F241A mutation could elicit a CD23 dependent enhancement of an anti-HA response, comparable to that observed for sialylated, polyclonal anti-HA antisera. This was evaluated by generating H1 ICs from A/PR8/1934 (PR8) hemagglutinin with PY102, a monoclonal anti-PR8 IgG, that was expressed as a human IgG1, sialylated Fc, or F241A mutation (26). IgGs elicited by either the sialylated or F241A ICs were of higher affinity for PR8 HA than an unmodified PY102 IgG1 (Fig. 4*A*). This demonstrated that a monoclonal anti-HA IgG with the engineered F241A Fc domain could be used to enhance the affinity of an antihemagglutinin antibody response.

Having defined an Fc domain capable of enhancing the affinity of IC-elicited antibodies, we next evaluated IgGs with broadly reactive Fab domains for their ability to drive the production of broad, potent antibody responses when combined with HA protein. We attempted a series of immunizations with ICs composed of H1 hemagglutinins (PR8 or CAL09) and the H1-reactive mAbs, C05 (27), 2B06, and 2G02 (28) that were generated with the F241A Fc domain. Immune sera elicited by these immune complexes were screened for binding to the H1 HA stalk domain; however, for reasons that have not been clarified in this study, we did not observe enhanced anti-HA antibody responses using these mAbs (Fig. S1). This indicated that specific Fab and Fc domains were required to elicit the enhanced response that we observed with polyclonal anti-HA IgG (Figs. 1 and 2).

We next immunized mice with immune complexes of HA and a bispecific mAb comprised of the broadly reactive 19–4G05 (4G05) (28) and FI6 (29), with the F241A Fc. This 4G05/FI6 bispecific mAb engages H1 HAs within the globular head (4G05) and group 1 and 2 HAs through the stalk domain (FI6) (Fig. S2). The 4G05/FI6 mAb was expressed as a wild-type, human IgG1 or with the F241A Fc domain modification. These mAbs were combined, individually, with PR8 (H1) HA protein, and the resulting immune complex mixtures were used to

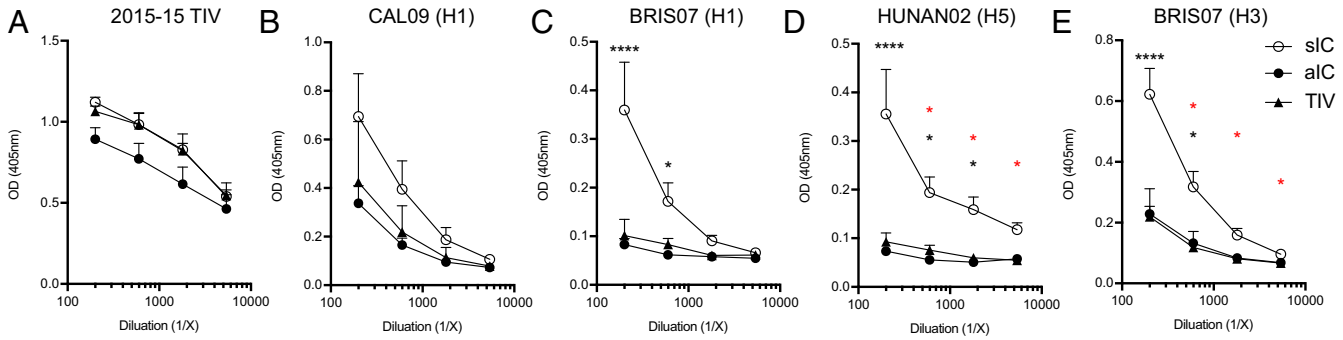


Fig. 2. Increased breadth of anti-TIV antibodies by administration of sialylated TIV immune complexes. Breadth of the anti-HA response was enhanced by TIV+sIgG (sIC) administration over TIV alone or TIV+aIgG (aIC). (*A–E*) Binding of serum IgGs was measured by ELISA to homologous antigen (TIV) or the homologous H1 HA component (Cal09 H1) and was not significantly different between groups. Binding to a heterologous H1 (Bris07), H3 (Bris07), or H5 (Hunan02) was enhanced by administration of sIC over aIC or TIV alone. Stars indicate significance level of difference in binding between the serum IgGs of mice in the sIC and aIC (black) or sIC and TIV (red) immunization groups (five mice per group). Significance at each dilution was determined by unpaired *T* test (Prism 7). $*P \leq 0.05$, $****P \leq 0.00005$.

immunize mice, followed by boost immunizations with PR8 HA 3 wk later. Immune serum taken 10 d post boost showed that the PR8 HA/F241A immune complexes elicited IgGs with higher binding to the H1 stalk domain and higher-affinity IgGs against the conserved stalk domain of the H1 hemagglutinin (Fig. 4 *B* and *C*). The higher-affinity IgGs were not elicited by F241A immune complexes in mice that do not express CD23 (Fig. 4*C*) (30). Affinity measurements were performed by ELISA using a 7M urea incubation that causes dissociation of lower-affinity IgG–antigen interactions (31). Though not significant, there may have been a trend toward higher-affinity IgGs elicited by both of the IgG1/TIV and F241A/TIV immune complexes in CD23 knockout mice. This could be due to enhanced antigen processing and presentation to T cells that occurs when antigen is present in the form of immune complexes (32).

Purified IgGs from the HA/bispecific mAb IC immunizations were then used to evaluate the in vivo potency of the elicited antibodies in passive transfer influenza challenge experiments. Mice that received IgG elicited by the bispecific F241A PR8 IC were protected from lethal challenge with a mouse-adapted virus expressing a heterologous H1 protein, A/Netherlands/602/2009 (Fig. 5 *A* and *B*). In addition, the higher-affinity, F241A IC-elicited IgG protected mice against challenge with a reassortant H5 virus, A/Vietnam/1203/2004 (Fig. 5 *C* and *D*).

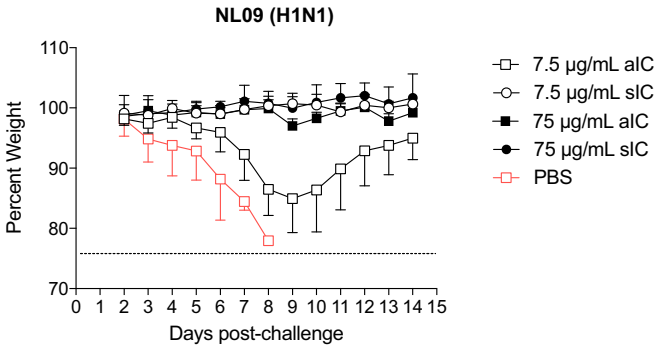


Fig. 3. Increased potency of anti-TIV antibodies by administration of sialylated TIV immune complexes. Purified serum IgGs (at 7.5 or 75 μg/mL) from mice vaccinated with TIV sIC or aIC were incubated with 5 mLD_{50} of maNL09 (H1N1) for 30 min at 37 °C. The virus and IgG mixture was then used to challenge BALB/c mice, intranasally (four mice per group). Mice receiving virus incubated with sIC or aIC IgGs at 75 μg/mL IgG were protected from weight loss. At the 7.5 μg/mL dose, only sIC-elicited IgGs protected from weight loss. Negative control mice received PBS.

Finally, immunizations with the 4G05/FI6 bispecific mAb combined with the 2014–15 TIV or TIV alone were performed. IgGs elicited by the TIV with CD23 targeting (F241A Fc) showed increased binding to the homologous CAL09 HA and enhanced breadth of binding against a heterologous H1 HA, A/Brisbane/59/2007 and an H5 subtype, HA, A/duck/Hunan/795/2002 (Fig. 6 *A–C*). CD23 targeting also increased the potency of antistalk IgGs elicited by TIV immunization, measured by in vitro neutralization of a chimeric cH6/1 virus. This virus expresses the globular head of an H6 subtype virus and an H1 subtype stalk domain (Fig. 6*D*). The enhanced response was also evident in vivo, as IgGs elicited by the F241A/TIV ICs had increased protective activity against lethal A/Vietnam/1203/2004 H5N1 when compared with IgGs elicited by wild-type IgG1/TIV ICs or by TIV alone (Fig. 6 *E* and *F*).

Discussion

In a study of TIV vaccination in healthy adults, we previously observed that the abundance of sFc on anti-HA IgGs produced during the early plasmablast response correlated with vaccine efficacy (22). This suggested the involvement of type II FcR signaling in the ontogeny of protective TIV responses. Subsequent experiments demonstrated that sFc within HA immune complexes engaged germinal center B-cell CD23, triggering upregulation of FcγRIIb, which modulates the selection of B cells in favor of those expressing higher-affinity B-cell receptors (Fig. 1). IgGs elicited by sFc H1 ICs were of higher affinity for the H1 stalk domain and demonstrated increased breadth and potency of protective activity in vivo against antigenically drifted H1 IAV strains (22).

Further studies will be needed to dissect the biochemical mechanisms involved in enhancing the breadth of in vivo protection against influenza viruses through increased affinity of the anti-HA response. The mechanisms involved are likely multiple, including: improving the function of IgGs that act by restricting the low-pH–triggered conformation change required for fusion of the host and viral membranes (1), enhancing binding of antistalk IgGs and the recruitment of innate effector cells (21, 33), and/or increasing virus opsonization and clearance. In addition, while it is known that Fab specificity plays a critical role in the in vivo activity of broadly protective anti-HA mAbs, we are not aware of prior reports of selectivity of the response to IC immunizations based on Fab specificity, and additional studies will be required to uncover the basis for this observation (20, 21, 34).

The present work expands on prior universal influenza vaccine studies in three key ways. First, we have demonstrated that IgGs elicited with CD23 targeting against a seasonal H1 vaccine

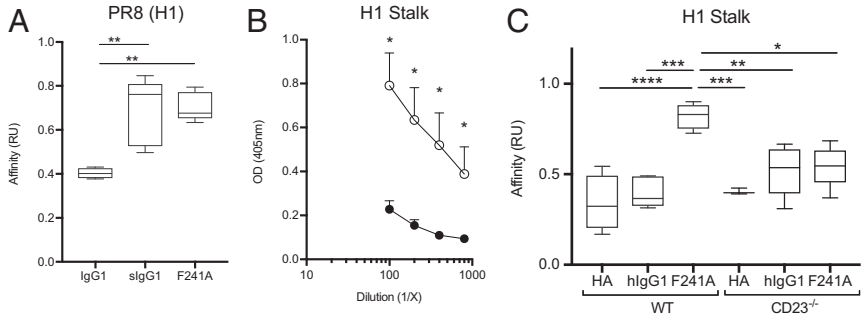


Fig. 4. F241A Fc domain and FI6 and 4G05 Fab domains combine to elicit enhanced anti-HA IgGs. (*A*) F241A mAb+H1 HA (PR8) enhances the affinity of anti-H1 response to a level that is comparable with the sialylated Fc IgG. Thus, the F241A Fc domain was selected for use in subsequent experiments. (*B*) The anti-H1 stalk titer, a measurement of antibody quantity and affinity, was not increased by every mAb that was tested (Fig. S1), but was increased by immunization with a FI6/4G05 bispecific mAb+H1 HA. (*C*) A F241A bispecific mAb comprised of 4G05 (28) and FI6 (29) enhances the affinity of the antistalk response when delivered with HA protein. The increase in affinity was not observed in CD23 knockout mice. Affinity was determined by 7M urea ELISA (31). Data are expressed as serum IgG bound to HA following 7M urea treatment/IgG bound without 7M urea treatment. Significance at each dilution was determined by unpaired *T* test (Prism 7). $*P \leq 0.05$, $**P \leq 0.005$, $***P \leq 0.0005$, $****P \leq 0.00005$.

component have increased potency against the seasonal H1 virus and can also provide protection against an H5N1 IAV. Though other universal influenza vaccination strategies have shown impressive breadth of heterologous protection in animal models, the strategy shown here is unique in that it provides both enhanced potency against the homologous seasonal strain and protection against a heterologous virus of potential pandemic HA subtype.

The second important way that this work expands on prior studies is by the identification of a single, broadly reactive anti-HA mAb that could be used to adjuvant seasonal influenza virus vaccines or in combination with other HA-based vaccines. This mAb is engineered with an F241A Fc domain that engages CD23 upon IC formation. F241A ICs directly select for higher-affinity B cells; this, in turn, enhances the potency and breadth of the anti-HA antibody response elicited by seasonal influenza vaccination.

Finally, these studies demonstrate that enhanced breadth of anti-HA immunity can be achieved using a single HA antigen (22) or seasonal influenza virus vaccine preparation. This contrasts with other universal influenza vaccine strategies that promote expansion of broadly reactive anti-HA B cells by using modified HA antigens or combinations of HA antigens in various delivery platforms and immunization schedules (5, 35–42). While many of the universal influenza vaccine strategies developed around antigen modification show great promise, none of them is based on a single HA antigen or on existing vaccines.

The approach defined here has potential for use with vaccines involving other IAV proteins or against noninfluenza pathogens. For example, the influenza neuraminidase (NA), like the HA, can mediate immunity against distinct IAV, and affinity is likely to play a significant role in the function of antibodies with neuraminidase-inhibiting activity (43, 44). Thus, a CD23-targeting anti-NA mAb in combination with NA could improve the potency of the anti-NA response. CD23 targeting might also be useful in other systems where high-affinity IgG responses are important for protective immunity such as vaccines against *Streptococcus pneumoniae* and other capsular polysaccharide vaccines (45–47). Overall, this study defines an approach for universal influenza vaccination that targets the endogenous B-cell affinity maturation pathway and demonstrates that existing influenza vaccines can be used to provide broad and potent anti-IAV immunity that extends to the potential pandemic IAV subtype, H5N1.

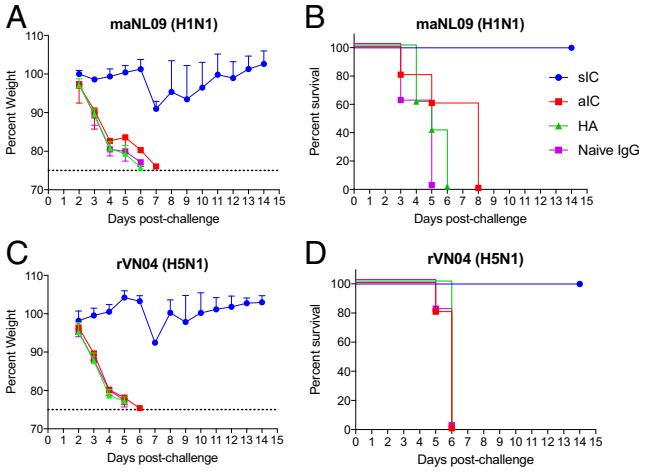
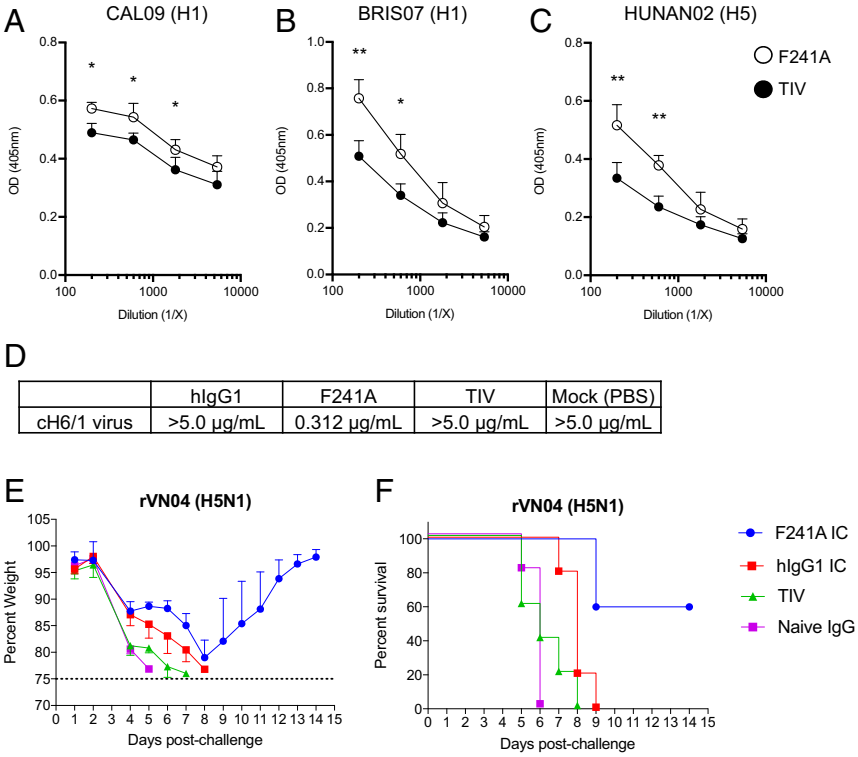


Fig. 5. IgGs elicited by the F241A bispecific mAb/H1 HA ICs protect against an H1 or H5 virus in vivo. (*A* and *B*) IgG elicited by PR8 H1 IC immunization protected against lethal challenge with a heterologous H1 virus [mouse adapted A/Netherlands/602/09 (maNL09) or (*C* and *D*) lethal H5 subtype virus challenge (reassortant virus expression the HA and NA of A/Viet Nam/1203/2004 (rVN04) (H5N1) with the internal segments of PR8 (H1N1) influenza A virus (50)] (five mice per group).

Materials and Methods

ELISA. Purified hemagglutinin proteins (2 μg/mL in PBS) in PBS were added in volume of 50 μL per well to Costar 96-well enzyme immunoassay/RIA (EIA/RIA) high-binding plates (Corning Inc.) and incubated, overnight, at 4 °C. The next day, plates were blocked with 1% BSA, PBS for 30 min at room temperature, followed by washing, three times, with 0.1% Tween 20, PBS (TPBS). Mouse serum was diluted in 1% BSA, PBS and added to plates followed by incubation for 3 h at 37 °C. After the incubation, plates were washed five times with TPBS. Secondary antibody consisting of goat anti-mouse IgG γ-chain conjugated to alkaline phosphatase (Southern Biotech), at a 1:2,500 dilution in 1% BSA, PBS, was added to plates and incubated at 37 °C for 2 h. Plates were then washed five times with TPBS and developed with PNPP substrate (Sigma–Aldrich) for 5–15 min. The signal was read at an absorbance of 405 nm. Negative control (naïve mouse serum or binding values of human IgGs on the irrelevant protein BSA) values were subtracted from readings given by test samples. HA proteins were obtained from BEI resources [National Institute of Allergy and Infectious Diseases (NIAID)], chimeric cH5/1 protein [head domain derived from 11 H5N1 strain A/Viet Nam/1203/04 and a stalk domain derived from H1N1 strain A/Puerto Rico/8/34 (PR8)] was obtained from Florian Krammer. The affinity ELISA was performed by incubating wells with 7M urea for 10 min, followed by washing three times with TPBS before addition of secondary antibody. Data are expressed as serum IgG bound to HA following 7M urea treatment/IgG bound without 7M urea treatment.



	hIgG1	F241A	TIV	Mock (PBS)
cH6/1 virus	>5.0 μg/mL	0.312 μg/mL	>5.0 μg/mL	>5.0 μg/mL

Fig. 6. Breadth and potency of the anti-HA TIV response was enhanced by administration with the F241A bispecific mAb. (*A–C*) IgGs elicited by TIV+F241A bispecific mAb had increased binding activity to homologous H1 HA (Cal09 H1), heterologous H1 (Bris07), or H5 (Hunan02) subtype hemagglutinins. (*D*) In addition, the F241A/TIV immune complexes elicited more potent antistalk IgGs, measured by in vitro neutralization using a virus that expresses the cH6/1 hemagglutinin (H1 HA stalk domain with an H6 subtype globular head domain) (10). The H1 stalk domain is highly conserved among group 1 influenza virus subtypes, including H5. Endpoint microneutralization titers against cH6/1 virus are shown. (*E* and *F*) IgGs elicited by TIV+F214A bispecific mAb protected mice against lethal H5N1 (rVN04) challenge (five mice per group). Significance at each dilution was determined by unpaired *T* test (Prism 7). $*P \leq 0.05$, $**P \leq 0.005$.

Recombinant Proteins and Generation of Immune Complexes. Recombinant anti-HA bispecific mAb was expressed as a human IgG1 in 293T cells, either wild-type or 293T cells stably expressing human ST6GAL1 and B4GALT1 and purified using protein G chromatography as previously described (48). ICs were formed by incubation of molar ratio 30:1 (purified, polyclonal human IgG) or 3:1 (mAb or bispecific mAb) IgG:HA trimer for 1 h at 4 °C. These IgG:HA ratios were designed to generate 1:1 complexes with IgG and HA monomer; a 30:1 ratio was used for polyclonal IgG:HA based on a predicted anti-HA frequency of 1 in 10 IgGs post vaccination. IgG subclass and Fc glycan composition were determined by mass spectrometry.

In Vivo Studies. All mice were maintained in a specific-pathogen–free facility at the Icahn School of Medicine at Mount Sinai, and all studies were approved by the Icahn School of Medicine at Mount Sinai Animal Care and Use Committee.

Polyclonal IC immunization. For mouse immunizations using human serum-derived ICs, A/California/04/2009 HA protein (10 μg per immunization) or 2014–15 Fluvirin TIV (15 μg total HA per immunization), per dose was delivered alone, or in complex with pooled, protein G-purified, human hyperimmune IgG taken 3 wk post TIV administration. The polyclonal IgGs had 27% sFc on anti-HA IgG, measured by mass spectrometry. As control, asialylated IgG was prepared by treatment of IgG with α2–3,6,8 Neuraminidase (New England Biolabs) as described (49). Mice were primed with TIV or HA ICs or TIV or HA alone in PBS. Two to three weeks later, mice were boosted, s.c., with the same dose of HA or TIV as used in the primary immunization, in adjuvant (MF59 or polyIC). Serum IgGs were tested 10 d post boost immunization. For in vivo neutralization studies, anesthetized mice (female C57BL/6J; 6–8 wk old) were infected intranasally with 5 mLD_{50} of a mouse-adapted A/Netherlands/602/09 (maNL09) (H1N1) or 10 mLD_{50} of a reassortant virus expressing the HA and NA of A/Viet Nam/1203/2004 (rVN04) (H5N1) with the internal segments of PR8 (H1N1) influenza A virus (50). The purified IgG preparation from vaccinated mice was from pooled sera using protein G (GE Healthcare) and mixed with virus for 30 min at 37 °C before infections. For the maNL09 challenge, 10 μg/mL IgG was mixed with virus; for the rVN04 challenge, 300 μg/mL IgG was used. Mouse body weight was recorded daily, and death was determined by a 25% body weight loss threshold.

Monoclonal IC immunizations. For mAb immunizations, 10 μg HA and 25 μg mAb or 10 μg PR8 HA alone was delivered i.v. to 6-wk-old wild-type or $CD23^{-/-}$ mice. Two to three weeks later, mice were boosted, intravenously, with 10 μg HA in alum. For challenge studies, mice were anesthetized and infected as described above. A mixture of mouse-adapted virus PR8 (H1N1), maNL09 (H1N1), or rVN04 and purified polyclonal IgG (75 or 7.5 μg/mL) from vaccinated mice was preincubated at 37 °C for 30 min. Six- to eight-week old female BALB/c mice were then infected with 5 mLD_{50} of virus. Mice were weighed daily to monitor morbidity, and animals that exceeded 25% weight loss were euthanized.

Microneutralization Assay. MDCK cells were seeded onto 96-well plates at 2e10^4 cells per well and grown overnight in 37 °C, 5% CO_2. In a separate 96-well plate, purified IgGs were serially diluted by twofold at a starting dilution of 5 μg/mL. Fifty microliters of purified IgGs were preincubated with 50 μL of 100 tissue culture infectious dose, 50%, of cH6/1 [a recombinant influenza A virus expressing a chimeric HA: globular head of H6 and the stalk region of H1 (PR/8 HA)] for 30 min at 37 °C, 5% CO_2. The MDCK monolayer was then washed twice with 1× PBS. One hundred microliters of the IgG:virus mixture was put onto the MDCK monolayer and infected for 1 h at 37 °C, 5% CO_2. Inoculum was aspirated and replace with 100 μL of 1× MEM supplemented with corresponding purified IgG dilutions and TPCK-treated trypsin (1 μg/mL). Plates were then incubated at 37 °C, 5% CO_2. Twenty-four hours post infection, 50 μL of cell culture supernatant was transferred to a V-bottom 96-well plate. Fifty microliters of 0.5% chicken red blood cell were added to each sample for total volume of 100 μL and incubated on ice for 1 h. Samples were observed for presence of agglutination (presence of virus). The endpoint titer is the lowest concentration that inhibited virus growth and prevented agglutination. The experiment was performed in duplicate.

Clinical Studies. Human IgGs used in vaccination studies were purified from serum obtained from a TIV vaccination study conducted at the Rockefeller University Hospital in New York City. Informed consent was obtained from

study participants and experiments were conducted in accordance with a protocol approved by the Institutional Review Board of Rockefeller University (protocol TWA-0804), and in compliance with guidelines of the International Conference on Harmonization Good Clinical Practice guidelines, and was registered on www.clinicaltrials.gov (NCT01967238).

Statistical Analysis. All data were analyzed in Prism 7 (GraphPad). Results from multiple experiments are presented as mean ± SEM. Correlation analysis was used to determine the Pearson correlation coefficient, r. Linear regression was used to determine goodness of fit, r^2. Two-tail unpaired student's or ANOVA tests followed by Tukey post hoc analysis were performed to assess differences in the mean values of quantitative variables. Nonparametric tests of significance were performed if normal distribution could not be assessed or if populations were not normally distributed.

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