

# Broadly Neutralizing Anti-Influenza Virus Antibodies: Enhancement of Neutralizing Potency in Polyclonal Mixtures and IgA Backbones

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## ABSTRACT

Current influenza virus vaccines rely upon the accurate prediction of circulating virus strains months in advance of the actual influenza season in order to allow time for vaccine manufacture. Unfortunately, mismatches occur frequently, and even when perfect matches are achieved, suboptimal vaccine efficacy leaves several high-risk populations vulnerable to infection. However, the recent discovery of broadly neutralizing antibodies that target the hemagglutinin (HA) stalk domain has renewed hope that the development of “universal” influenza virus vaccines may be within reach. Here, we examine the functions of influenza A virus hemagglutinin stalk-binding antibodies in an endogenous setting, i.e., as polyclonal preparations isolated from human sera. Relative to monoclonal antibodies that bind to the HA head domain, the neutralization potency of monoclonal stalk-binding antibodies was vastly inferior *in vitro* but was enhanced by several orders of magnitude in the polyclonal context. Furthermore, we demonstrated a surprising enhancement in IgA-mediated HA stalk neutralization relative to that achieved by antibodies of IgG isotypes. Mechanistically, this could be explained in two ways. Identical variable regions consistently neutralized virus more potently when in an IgA backbone compared to an IgG backbone. In addition, HA-specific memory B cells isolated from human peripheral blood were more likely to be stalk specific when secreting antibodies of IgA isotypes compared to those secreting IgG. Taken together, our data provide strong evidence that HA stalk-binding antibodies perform optimally when in a polyclonal context and that the targeted elicitation of HA stalk-specific IgA should be an important consideration during “universal” influenza virus vaccine design.

## IMPORTANCE

Influenza viruses remain one of the most worrisome global public health threats due to their capacity to cause pandemics. While seasonal vaccines fail to protect against the emergence of pandemic strains, a new class of broadly neutralizing antibodies has been recently discovered and may be the key to developing a “universal” influenza virus vaccine. While much has been learned about the biology of these antibodies, most studies have focused only on monoclonal antibodies of IgG subtypes. However, the study of monoclonal antibodies often fails to capture the complexity of antibody functions that occur during natural polyclonal responses. Here, we provide the first detailed analyses of the biological activity of these antibodies in polyclonal contexts, comparing both IgG and IgA isotypes isolated from human donors. The striking differences observed in the functional properties of broadly neutralizing antibodies in polyclonal contexts will be essential for guiding design of “universal” influenza virus vaccines and therapeutics.

Influenza A viruses (IAVs) remain one of the most pressing global public health concerns due to their widespread distribution, rapid evolution, and potential for reassortment (1). These traits contribute to the ability of IAVs to cause serious pandemics, four of which have occurred in the last 100 years. The potential severity of IAV pandemics is exacerbated by the striking paucity of effective antivirals, and inherent limitations in the speed of vaccine production and distribution when pandemics arise. Importantly, even the annual circulation of seasonal IAV strains carries substantial human and economic tolls.

It has been recognized that the most effective way to alleviate the human and economic impacts of IAV would be through the generation of a more broadly protective, or “universal” influenza virus vaccine. While several strategies have been proposed (2), one of the most promising to date involves the recent discovery of a subset of antibodies that are capable of neutralizing a wide range of IAVs through binding to the highly conserved stalk domain of the hemagglutinin (HA) protein (3). These antibodies seem to be boosted with the greatest magnitude upon sequential exposure to

highly dissimilar HA subtypes, as happens during IAV pandemics (4–8). Studies in mice and ferrets have demonstrated that elicitation of these antibodies via vaccination with chimeric HA molecules can provide broad protection from challenge with a diverse array of IAV subtypes (9–11). However, one of the major concerns that has arisen in the development of vaccines designed to elicit

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TABLE 1 Cal/09 HAI<sup>+</sup> donor characteristics

Donor	Age (yr)	Gender	Race	Collection date
5-1	59	F	Black	June 2012
2-2	54	M	Caucasian	June 2012
4-2	19	M	Black	June 2012
5-2	19	M	Black	June 2012
6-2	49	M	Caucasian	June 2012
7-2	51	M	Black	March 2012
10-3	46	M	Caucasian	April 2013
12-3	20	F	Latino	April 2013
15-3	27	F	Black	April 2013
16-3	35	F	Latino	April 2013

broadly neutralizing antibodies (bnAbs) that target the HA stalk domain is that, in addition to their reduced prevalence relative to antibodies that bind to the HA head, they also appear to be less potently neutralizing *in vitro* (12–14). Yet, current understanding of the mechanisms governing the activity of bnAbs is largely reliant upon studies wherein monoclonal antibodies (primarily of IgG isotypes) have been analyzed in isolation (15). Although informative, this approach fails to recapitulate the complex interactions between differing antibody isotypes and clonotypes that occur naturally in humans as a consequence of the polyclonal response to antigens.

While cooperativity and synergism of monoclonal antibody mixtures have been reported in the past (16–19), emerging data have illustrated striking examples of situations wherein the activity of a monoclonal antibody can be drastically altered when assessed in the context of other antibodies (20, 21). Indeed, the benefits of using polyclonal antibody preparations to treat disease in therapeutic settings are just beginning to be realized (22). In addition, recent discovery of the remarkable effects of heavy-chain constant regions (C<sub>H</sub>) in allosterically modulating the affinity and specificity of identical variable (V) regions is fueling intensive new investigation into isotype-specific effects of antibodies (23–30).

Here, we address these issues by examining the differential properties of HA head and stalk-binding antibodies, as well as the effects of antibody isotypes in mediating neutralization of IAV *in vitro* using polyclonal preparations of antibodies isolated from human donors. We find that the deficiency in the neutralization potency of monoclonal HA stalk-binding antibodies relative to strain-specific head binding antibodies is greatly reduced in polyclonal contexts. Our results also demonstrate a surprising enhancement in both the quality and magnitude of HA stalk-binding antibodies of IgA subtype. Taken together, our findings highlight important differences in the activities of monoclonal versus polyclonal antibodies that bind to the HA glycoprotein of IAV and additionally reveal isotype-specific effects of these antibodies in both monoclonal and polyclonal contexts.

## MATERIALS AND METHODS

**Human serum samples.** Human serum samples were obtained from Innovative Research (Novi, Michigan) and were collected between March 2012 and April 2013. Samples were prescreened using HAI assay against wild-type Cal/09 virus. Donor information is shown in Table 1 and 2.

**Expression and purification of recombinant influenza virus proteins.** HA proteins of cH6/1<sub>PR8</sub> (H6 HA globular head domain from A/mallard/Sweden/81/02 and H1 HA stalk domain from A/PR/8/1934), cH9/1<sub>PR8</sub> (H9 HA globular head domain from A/guinea fowl/Hong Kong/WF10/1999 and H1 HA stalk domain from A/PR/8/1934), Cal/09

TABLE 2 Pre-2009 donor characteristics<sup>a</sup>

Donor	Race	Collection date
1	Black	November 2006
2	Black	November 2006
3	Black	November 2006
4	Black	July 2004
5	Black	August 2003
6	Latino	November 2006
7	Black	November 2006
8	Black	November 2006
9	Black	November 2006
10	Black	November 2006

<sup>a</sup> All donors were male. Donor ages are not known.

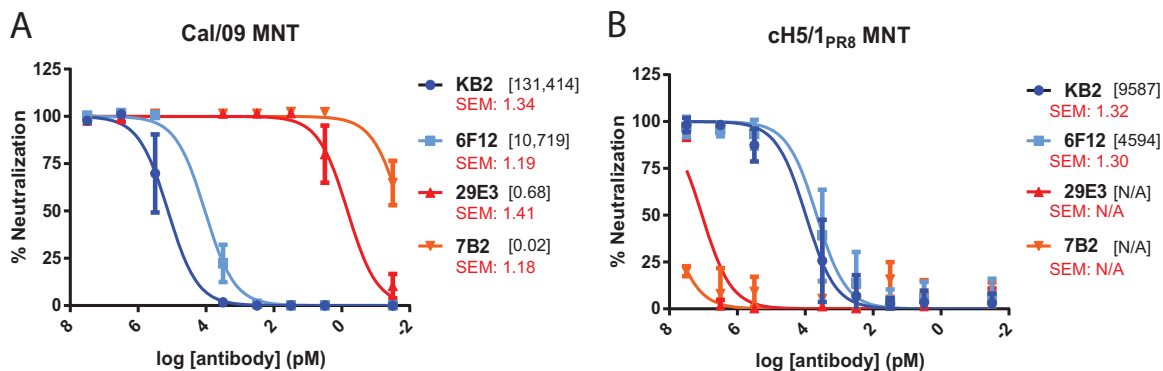
and NC/99 were expressed in BTI-TN5B1-4 (High Five) cells as described previously (4, 9, 31). Briefly, coding sequences of each protein were cloned into a modified pFastBac vector (Invitrogen) with a C-terminal hexahistidine tag and a T4 trimerization domain. Recombinant baculovirus (rBV) expressing each HA was generated using the FastBac system (Invitrogen) according to the manufacturer's recommendations. High Five cells were infected with the rBVs at a multiplicity of infection (MOI) of 10. Cells were harvested 72 to 96 h after infection. Proteins were purified with Ni-nitrilotriacetic acid resin (Qiagen).

**Cells and viruses.** MDCK cells (American Type Culture Collection) were grown in Dulbecco modified Eagle medium (Gibco) supplemented with 10% fetal bovine serum (HyClone) and 100 U of penicillin and streptomycin (Gibco)/ml. The cH5/1 N3 virus (H5 HA globular head domain from A/Vietnam/1204/2004 and H1 HA stalk domain from A/PR/8/1934) and the recombinant Cal/09 N3 virus (H1 HA from A/California/04/09) were rescued as described previously (32). The N3 NA segment of these two viruses was derived from A/swine/Missouri/4296424/2006, and the remaining segments were derived from A/PR/8/1934.

**HAI assays.** HA inhibition (HAI) assays were performed as described previously (4). Briefly, sera were inactivated with TPCK (tolylsulfonyl phenylalanyl chloromethyl ketone)-trypsin at 56°C for 30 min. Viruses and sera were mixed and incubated at room temperature for 30 min. Chicken red blood cells (0.5%) were then added to each well, and the plates were incubated at 4°C for 30 min before reading. Plates were scored for the number of wells exhibiting HAI activity.

**IgG and IgA purification.** Human serum was heat inactivated by incubation at 56°C for 30 min and was then diluted 1:4 in phosphate-buffered saline (PBS) for filtration through a 0.22- $\mu$ m-pore-size filter unit. IgG was purified using a protein G/Sepharose resin (InvivoGen) on a gravity flow column (Qiagen). IgA was purified by a similar process over peptide M/Sepharose resin (InvivoGen). After sera were passed over the columns, the resins were washed with four column volumes of PBS. IgG/IgA was eluted using a 0.1 M glycine-HCl buffer at pH 2.7. The eluate was immediately neutralized in 2 M Tris-HCl buffer (pH 10). Purified IgG/IgA was then concentrated and dialyzed against PBS using Amicon Ultra centrifugal filter units (Millipore) with a 30-kDa molecular mass cutoff in a swinging-bucket rotor.

**ELISA.** Enzyme-linked immunosorbent assays (ELISAs) were performed on 96-well plates as described previously (1). Briefly, plates were coated overnight with 2  $\mu$ g of purified recombinant HA (Cal/09 and cH9/1<sub>PR8</sub>)/ml in bicarbonate-carbonate coating buffer (100 mM, pH 9.6). The plates were blocked with 5% nonfat milk for 1 h. Serum was diluted serially in 5% nonfat milk and then incubated on plates for 1 h at room temperature. Plates were washed with PBS–0.1% Tween 20 (PBS-T). Secondary goat anti-human IgG-horseradish peroxidase (HRP; Meridian Life Science, Inc.) or goat anti-human IgA-HRP (Millipore) was incubated on plates at 1:5,000 dilution in 5% nonfat milk for 1 h at room temperature, followed by extensive washing with PBS-T prior to addition of HRP substrate (Sigmafast OPD; Sigma-Aldrich). Reactions were stopped after ~8 min by addition of 3 M HCl, and the optical densities



**FIG 1** HA stalk-binding monoclonal antibodies neutralize virus *in vitro* with much less potency than monoclonal antibodies that recognize the HA head domain. A total of 100 PFU of Cal/09 N3 virus (A) or cH5/1<sub>PR8</sub> N3 virus (B) were preincubated with the indicated concentration of 6F12, KB2, 29E3, or 7B2 prior to infection of MDCK cells. At 20 h postinfection, the cells were fixed, and infectivity was assessed by anti-NP staining. The data representative the means and standard errors of three independent experiments. The *x*-axis scale numbers indicate MNT<sub>50</sub> antibody concentrations in pM. Standard error measurements of MNT<sub>50</sub> values are reported in red.

were read at 490 nm on a Synergy 4 (Bio-Tek) plate reader. Curve fitting was performed with GraphPad Prism 6 software using a “log (agonist) versus normalized response minus variable slope (four-parameter) analysis” with a HillSlope set equal to 1.

**Microneutralization assays.** For multicycle assays, antibodies and viruses (100 PFU/50  $\mu$ l) were preincubated for 30 min at room temperature prior to inoculation of 96-well plates of confluent MDCK cells. The plates were incubated for 1 h at 37°C under 5% CO<sub>2</sub> to allow for adsorption. After a washing step with PBS, the plates were reincubated with infection medium containing equivalent concentrations of diluted antibody supplemented with 1  $\mu$ g of TPCK-trypsin (Sigma)/ml. After 20 h, the cells were fixed with 80% acetone and then blocked with 5% nonfat milk, followed by 3% hydrogen peroxide. The cells were incubated with a 1:2,000 dilution of biotin-conjugated mouse anti-NP (Millipore), followed by a 1:5,000 dilution of secondary HRP-conjugated streptavidin (Millipore). Peroxidase substrate (Sigmafast OPD) was added to the wells, and reactions were stopped with 3 M HCl. Curve fitting was performed with GraphPad Prism 6 software using a “log (inhibitor) versus normalized response minus variable slope (four-parameter) algorithm analysis” with a HillSlope set equal to 1. The 50% microneutralization (MNT<sub>50</sub>) titers were defined as the dilution of antibody that resulted in at least 50% inhibition of infectivity. For single-cycle assays, 1,000 PFU of each virus/50  $\mu$ l was used as the inoculum, and incubations were performed in trypsin-free media. Cells were fixed at 12 h postinfection and stained as described above.

**Plaque reduction (PR) neutralization assay.** cH5/1<sub>PR8</sub> N3 virus or Cal/09 N3 virus (100 PFU) was incubated in minimal essential medium (MEM) with serially diluted purified IgG or IgA (initial concentration of 0.5 mg/ml, followed by 2-fold serial dilutions) for 30 min at room temperature. The virus-antibody mixture was then transferred onto 12-well plates of confluent MDCK cells, followed by incubation at 37°C for 1 h. The virus-antibody mixture was removed, and the cells were overlaid with an agar-MEM mixture containing equivalent concentrations of diluted antibody, as found in the initial inoculum, as well as TPCK-trypsin (1  $\mu$ g/ml) and DEAE-dextran (0.1%). After incubation at 37°C for 48 h, the cells were fixed with 3.7% paraformaldehyde and blocked with 3% bovine serum albumin. The cells were then incubated with a 1:2,000 dilution of biotin-conjugated mouse anti-NP (Millipore), followed by a 1:5,000 dilution of secondary HRP-conjugated streptavidin (Millipore). TrueBlue HRP substrate (KPL) was added to wells to visualize plaques.

**B cell enzyme-linked immunosorbent spot assays (ELISpots).** Human peripheral blood mononuclear cells (PBMCs) were isolated from buffy coats by centrifugation through Lymphoprep (Stemcell Technologies) according to the manufacturer’s recommendations. To stimulate

antibody production by memory B cells (MBCs), PBMCs were cultured for 6 days in B cell stimulation media consisting of RPMI (Gibco) supplemented with 10% fetal bovine serum (FBS; HyClone), penicillin-streptomycin (Gibco), fixed *Staphylococcus aureus* Cowan strain (1:10,000), pokeweed mitogen (1:1,000; Sigma), 50  $\mu$ M  $\beta$ -mercaptoethanol, and 1  $\mu$ g of R848 (Invivogen)/ml. Multiscreen 96-well filtration plates were coated with 2  $\mu$ g/ml of either goat anti-human IgG (Sigma), goat anti-human IgA (Millipore), recombinant NC/99 HA, recombinant Cal/09 HA, or recombinant cH6/1<sub>PR8</sub> HA overnight in PBS at 4°C. The next day, plates were blocked with RPMI supplemented with 10% FBS for 2 h at 37°C. After extensive washing in RPMI, stimulated PBMCs were plated and serially diluted prior to overnight incubation at 37°C. The spots were read by using an immunospot analyzer (CTL, Shaker Heights, OH).

## RESULTS

**Monoclonal HA stalk-binding antibodies exhibit reduced *in vitro* neutralization potency compared to monoclonal antibodies that bind to the HA globular head.** In order to compare the potency with which antibodies that bind to the head or stalk domain of the HA molecule neutralize virus, MNT assays were performed using four murine monoclonal antibodies previously described by our group (14, 32–34). 6F12 and KB2 bind to the HA stalk domain of H1 viruses, whereas 7B2 and 29E3 have specificity for the head domain of A/California/04/2009 (Cal/09) HA. Antibodies were preincubated with a recombinant Cal/09 N3 virus prior to infection of Madin-Darby canine kidney (MDCK) cells and maintained in the media during multicycle virus replication. The concentration at which each antibody achieved 50% microneutralization (i.e., MNT<sub>50</sub>) relative to control wells containing no antibody was then calculated. Indeed, striking differences were observed in the potency of neutralization when comparing the head-binding and stalk-binding antibodies. HA head-binding antibodies neutralized Cal/09 N3 virus with an MNT<sub>50</sub> of 0.02 to 0.68 pM, while the stalk-binding antibodies had MNT<sub>50</sub> values of 10,719 to 131,414 pM (Fig. 1A). To control for specificity, the MNT assay was repeated with a recombinant cH5/1<sub>PR8</sub> N3 virus (Fig. 1B). The MNT<sub>50</sub> values of 6F12 and KB2 against cH5/1<sub>PR8</sub> N3 virus were similar to those observed against Cal/09 N3 virus, indicating that the antibodies are not highly sensitive to small differences within H1 stalks. As expected, neither 7B2 nor 29E3 neutralized the cH5/1<sub>PR8</sub> N3 virus effectively, since they are specific

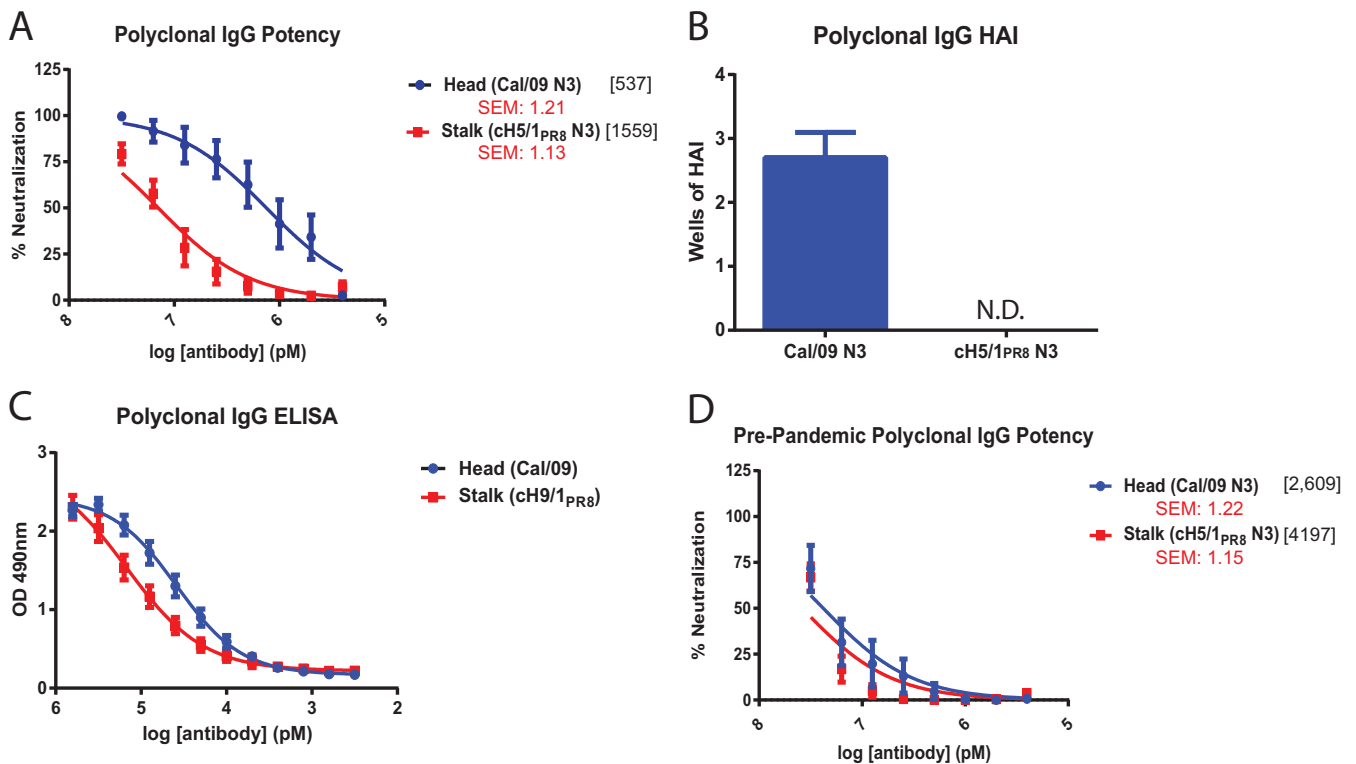


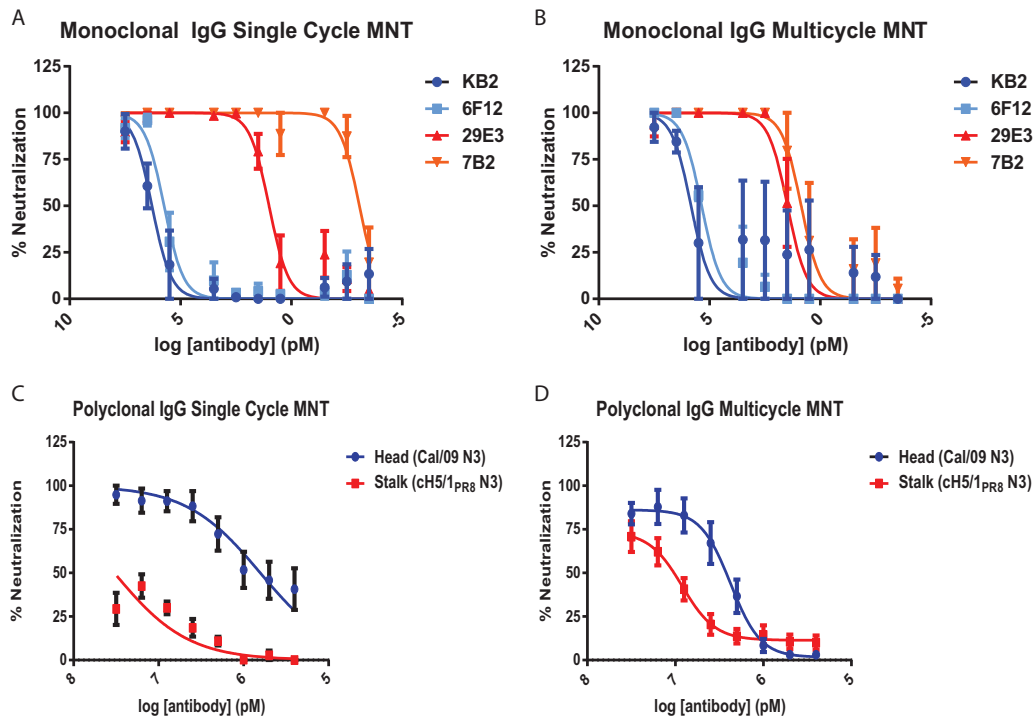
FIG 2 The potency of HA stalk antibody-mediated neutralization is enhanced by the polyclonal response. (A to C) Polyclonal IgG was purified from the sera of ten Cal/09 HAI-positive individuals (Table 1). (B) HAI assays were performed according to standard procedures starting with the maximum dilution of antibodies found in MNT assays (7.5 pM). The number of wells containing sufficient amounts of antibody to retain HAI activity was then assessed. N.D., not detected. (C) Whole HA- and HA stalk-directed reactivity of polyclonal IgG isolated from Cal/09 HAI-positive individuals were assessed by ELISA on plates coated with recombinant Cal/09 HA or cH9/1<sub>PR8</sub> HA, respectively. (A and D) A total of 100 PFU of the indicated viruses was incubated with corresponding dilutions of antibodies prior to infection of MDCK cells. At 20 h postinfection, the cells were fixed, and the infectivity was assessed by anti-NP staining. (D) Polyclonal IgG was also purified from the sera of 10 Cal/09 HAI-negative individuals (Table 2) as a control for nonspecific Cal/09 neutralizing antibody titers. The x-axis scale numbers indicate MNT<sub>50</sub> antibody concentrations in pM. Standard error measurements of MNT<sub>50</sub> values are reported in red.

for the head domain of Cal/09 HA. To further confirm the magnitude of the potency differences observed by MNT assay, plaque reduction (PR) assays were performed with Cal/09 N3 virus. As expected, the striking differences in neutralization potency between HA head and stalk-binding antibodies were maintained (data not shown).

**Potency of HA stalk-mediated neutralization is enhanced in polyclonal contexts.** To determine whether relative differences in the *in vitro* neutralization potency of HA head and stalk-mediated monoclonal antibodies would be recapitulated in the more natural setting of a polyclonal response, IgG was purified from the sera of individuals who were seropositive for Cal/09 (Table 1). Previous work has demonstrated that exposure to Cal/09 virus is capable of robustly boosting the titers of HA stalk-reactive antibodies in individuals who have previously been exposed to other group 1 HA IAVs (4–6, 35, 36). MNT assays were thus performed using the Cal/09 N3 virus to assess HA head-mediated neutralization, and the cH5/1<sub>PR8</sub> N3 virus to assess HA stalk-mediated neutralization. Unlike the multilog potency differences observed using monoclonal antibodies, the human polyclonal IgG preparations neutralized Cal/09 N3 virus and cH5/1<sub>PR8</sub> N3 virus with differences of only 3-fold (Fig. 2A). Again, similar results were obtained by PR assay (data not shown). To confirm that the contraction in potency observed in polyclonal sera was not due to the presence of HAI-positive antibodies binding to the cH5/1<sub>PR8</sub> HA head do-

main, HAI assays were performed using the Cal/09 N3 virus and the cH5/1<sub>PR8</sub> N3 virus. Viruses were mixed with equivalent dilutions of purified IgG, as were used in the MNT assays. These antibodies produced, on average, 2.7 wells of HAI activity against the Cal/09 N3 virus, while no HAI activity was observed against cH5/1<sub>PR8</sub> N3 (Fig. 2B). Differences in reactivity of the IgG preparations to recombinant Cal/09 HA or cH9/1<sub>PR8</sub> HA (used to measure HA stalk binding) as measured by ELISA were even more modest, suggesting that binding alone does not account for the observed differences in neutralization potency and that differences in mechanisms of neutralization are likely to have an influence (Fig. 2C). Consistent with previous findings (4, 35), polyclonal IgG purified from the sera of Cal/09 HAI-negative individuals neutralized Cal/09 N3 and cH5/1<sub>PR8</sub> N3 virus poorly and with similar potency (Fig. 2D).

**HA stalk-mediated neutralization is most potent during multicycle replication.** Previous studies have reported unique mechanisms through which HA head and stalk domain-binding antibodies mediate virus neutralization. Although HA head-binding antibodies primarily block viral attachment and release, stalk-binding antibodies function intracellularly to block fusion of the viral and endosomal membranes, and extracellularly to inhibit proteolytic cleavage of HA (37). To determine whether the mechanism of neutralization exerted by HA head- and stalk-binding antibodies differentially affected neutraliza-



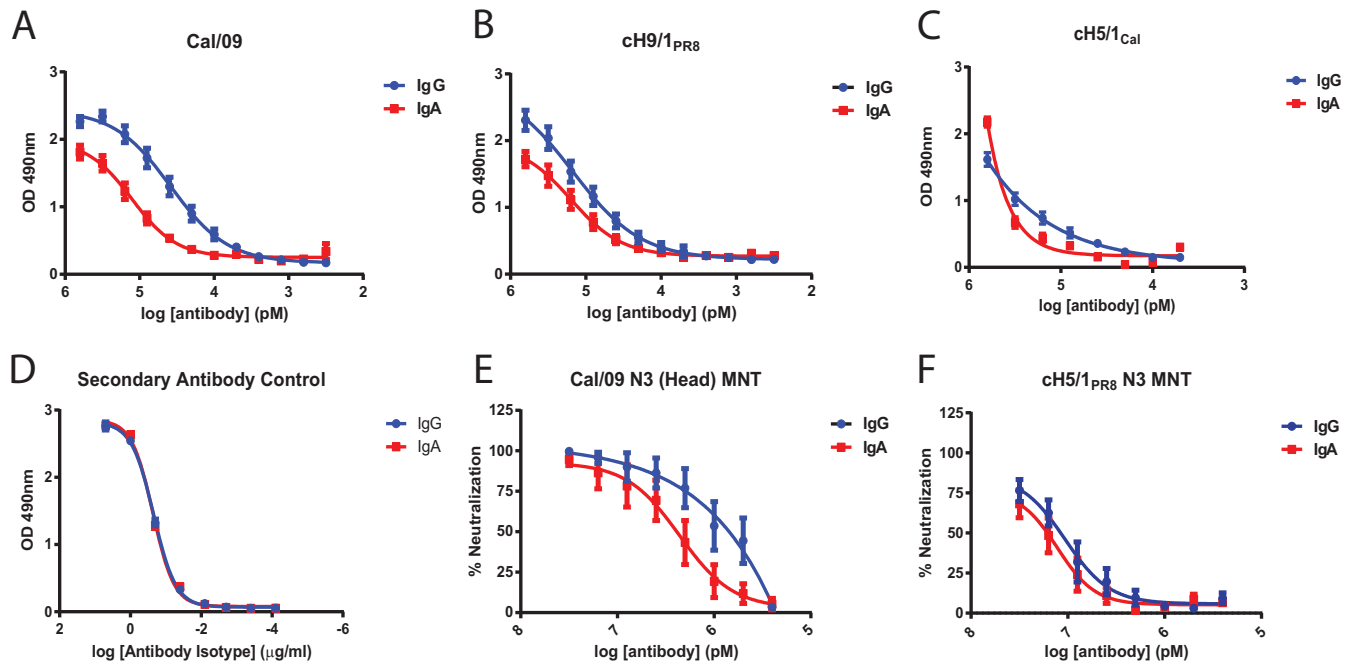
**FIG 3** Polyclonal HA stalk-mediated neutralization is most potent during multicycle replication. MNT assays were performed against Cal/09 N3 virus with monoclonal HA stalk-binding antibodies (6F12 and KB2) and monoclonal HA head-binding antibodies (A and B) or using polyclonal IgG and Cal/09 N3 virus (to assess primarily HA head-mediated neutralization) and cH5/1<sub>PR8</sub> N3 virus (to assess HA stalk-mediated neutralization) (C and D). (A and C) For single-cycle assays, antibody dilutions were preincubated with 1,000 PFU of virus before inoculation of MDCK cells. After adsorption, the cells were incubated in antibody-free and trypsin-free media for 12 h prior to fixation and staining. (B and D) Multicycle assays were performed by inoculating MDCK cells with 100 PFU of virus for 1 h at 37°C. After adsorption, the cells were washed and reincubated in medium containing trypsin and antibody for 20 h prior to fixation and staining. In panels A and B, the data represent the means and standard errors of three independent experiments. For panels C and D,  $n = 10$  (see Table 1).

tion potency at specific stages in the viral life cycle, we performed MNT assays in the context of single-cycle and multicycle infections. For single cycle assays, virus was preincubated with antibody prior to adsorption and cells were infected at high MOIs (Fig. 3A and C). Multicycle assays were performed by adding antibody to media after adsorption of virus at low MOI (Fig. 3B and D). HA stalk-binding monoclonal antibodies KB2 and 6F12 neutralized virus at nearly equivalent potency during both single-cycle and multicycle infections (Fig. 3A and B). In contrast, differences were observed in the neutralizing properties of the head binding monoclonal antibodies 29E3 and 7B2. Although 29E3 was equally potent in neutralizing virus during single-cycle and multicycle growth, 7B2 exhibited markedly less potent neutralization during multicycle replication than during single-cycle replication (Fig. 3A and B). This is likely related to subtle differences in the head domain epitopes recognized by each antibody. For example, the binding site of 7B2 may allow the antibody to efficiently block binding of viral particles to the cell surface during single-cycle replication but may be less efficient in preventing egress/budding during multicycle replication. Similarly, although substantial potency differences were observed in the ability of polyclonal IgGs to neutralize virus via primarily the head (Cal/09 N3) or stalk (cH5/1<sub>PR8</sub> N3) domains during single cycle replication (Fig. 3C), this potency difference was substantially contracted in the context of multicycle replication (Fig. 3D). This is likely a reflection of the unique mechanisms of neutralization medi-

ated by HA head and stalk binding antibodies, which have been reported previously (37, 38).

**Preferential stalk-specificity exhibited by human IgA.** Despite the known importance of IgA in maintenance of mucosal immunity, little is known about the biology of HA stalk-reactive antibodies of isotypes other than IgG. Therefore, polyclonal serum-derived IgG and IgA were purified from 10 individuals as matched samples. As expected, the serum-derived IgA was mainly monomeric (39). Reactivity of the donor-matched polyclonal IgG and IgA preparations were first compared by ELISA against recombinant Cal/09 HA (Fig. 4A) and against recombinant cH9/1<sub>PR8</sub> and cH5/1<sub>Cal</sub> HA to measure stalk-specific reactivity (Fig. 4B and C). Although the EC<sub>50</sub> of IgA binding to Cal/09 HA was ~4-fold lower than that of IgG (Fig. 4A), the difference in binding to the HA stalk was minimal (Fig. 4B and C). These differences were not due to differences in detection by secondary antibodies, since the antibodies produced equivalent signals when incubated with equal concentrations of IgG or IgA (Fig. 4D). To evaluate whether these differences in binding corresponded to functional differences in neutralization, MNT and PR assays were performed using Cal/09 N3 and cH5/1<sub>PR8</sub> N3 viruses (Fig. 4E and F and data not shown). In agreement with the ELISA data, IgG preparations neutralized Cal/09 N3 virus 2- to 4-fold more potently than IgA preparations (Fig. 4E), whereas neutralization of cH5/1<sub>PR8</sub> N3 was essentially equivalent for both isotypes (Fig. 4F).

**Isotype-intrinsic differences in the potency of HA stalk binding and neutralization.** To explore the possibility that antibody iso-



**FIG 4** Preferential targeting of the HA stalk domain by human IgA. Matched preparations of polyclonal human IgG and IgA were isolated from the sera of 10 donors (Table 1). (A to C) ELISAs were performed to compare the binding of polyclonal IgG and IgA to whole recombinant Cal/09 HA (A), recombinant cH9/1<sub>PR8</sub> HA (B), or recombinant cH5/1<sub>Cal</sub> HA (C) to evaluate stalk binding. (D) Control for secondary antibody reactivity showing that when equivalent concentrations of IgG and IgA are present, no differences are observed in the optical density (OD) values. (E and F) MNT assays were performed to assess neutralization potency using Cal/09 N3 virus (E) and cH5/1<sub>PR8</sub> N3 virus (F) to measure stalk-mediated neutralization, as described in Fig. 2A. IgG data from Fig. 2A have been reproduced here to facilitate comparison. The data points represent the mean and standard error of three replicates. The x-axis scale numbers indicate MNT<sub>50</sub> antibody concentrations in pM. Standard error measurements of MNT<sub>50</sub> values are reported in red.

type might influence the binding and neutralizing properties of HA stalk-binding antibodies, chimeric IgG and IgA antibodies were generated by cloning the variable regions of the group 1 HA stalk-binding monoclonal antibodies, 6F12 and KB2, into human IgG and IgA backbones (chKB2 and ch6F12). Both monomeric and dimeric IgA molecules were expressed for each variable (V) region. Comparable binding of mIgA and dIgA to recombinant PR8 HA was observed when the antibodies were added at equal molarity. However, in the case of both ch6F12 and chKB2, the IgG antibodies bound substantially weaker (Fig. 5A and B). MNT assays were then performed to determine whether isotype would influence the *in vitro* neutralization potency of antibodies with identical V regions (Fig. 5C and D). For both ch6F12 and chKB2, the most potent neutralization was exerted by the IgA molecules. In agreement with the ELISA data, IgG molecules consistently exhibited the weakest neutralization potency (Fig. 5C and D). Thus, structural features of the IgA backbone appear to increase the binding and neutralization potency of HA stalk-specific V regions.

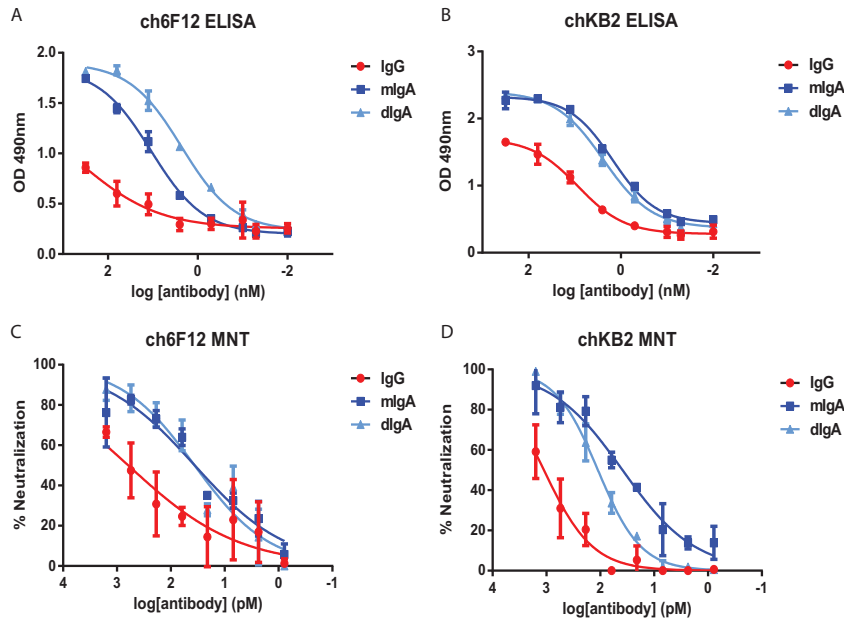
**The IgA-secreting memory B cell compartment is biased toward HA stalk specificity.** Finally, B cell ELISpots were performed using PBMCs isolated from human donors and stimulated *in vitro* to determine whether differences existed in the frequency of stalk-specific MBCs belonging to IgG and IgA pools. Although no significant differences were observed in the frequency of total IgG-secreting cells versus total IgA-secreting cells with specificity for Cal/09 HA (Fig. 6A) or A/New Caledonia/20/99 (NC/99) HA (Fig. 6B), a substantially greater proportion of total IgA-secreting cells were stalk specific relative to the pool of IgG-secreting cells (Fig. 6C). These findings suggest a previously unappreciated epitope-

specific bias for antibodies of unique isotypes. Whether the basis for this skewing is genetic in nature or a result of exposure route is the source of ongoing investigation.

## DISCUSSION

The discovery of bnAbs that bind to the HA stalk domain has opened new frontiers in both “universal” influenza virus vaccine design, and in biological anti-influenza virus therapeutics. Although much has been learned from studies of monoclonal stalk-binding IgGs derived from both mice and humans, the activities of HA stalk binding antibodies in polyclonal contexts and in non-IgG isotypes has gone largely unstudied. However, these issues are nontrivial. Indeed, the activities of monoclonal antibodies can change drastically in polyclonal mixtures (20, 21), and vaccine regimens invariably elicit polyclonal responses. In addition, IgA isotype antibodies play an important role in the maintenance of mucosal immunity against respiratory pathogens and may be of particular relevance in terms of protection afforded to individuals immunized with live-attenuated influenza virus vaccines (40–42).

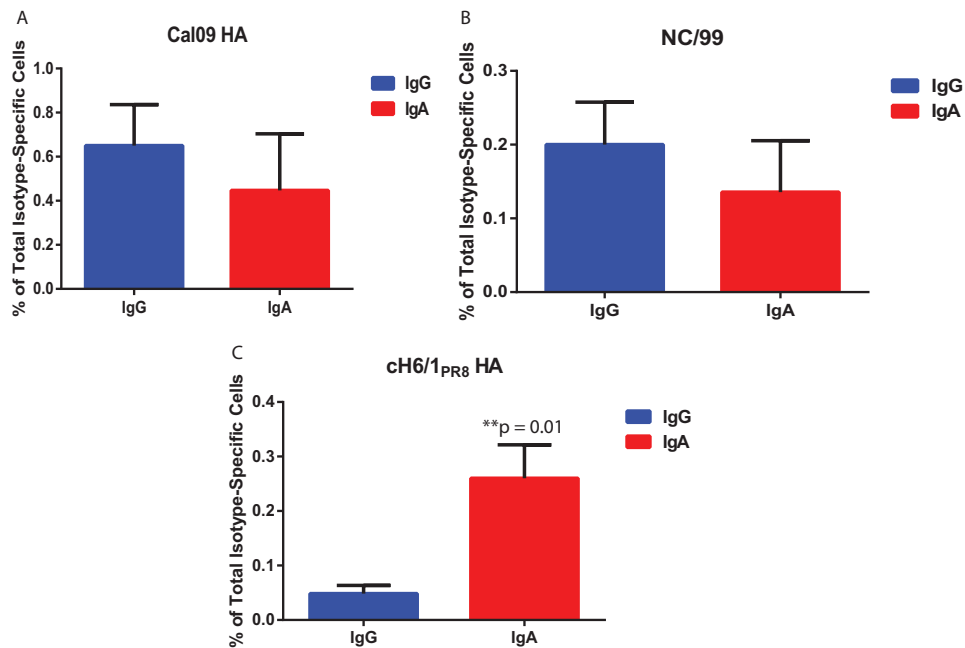
Despite the broad neutralizing capacity of HA stalk-binding antibodies relative to those that bind to the head domain, concerns have been raised about their suboptimal neutralization potency (12, 13). Indeed, when compared directly *in vitro*, the neutralization potencies of HA stalk-binding monoclonal antibodies were several orders of magnitude weaker than HAI-positive monoclonal antibodies that bind to the HA head domain. Previous work has indicated that HA stalk-binding antibodies appear to be capable of accessing their epitope on virions (43), and thus their inferior potency is likely due to their inability to inhibit HA bind-



**FIG 5** IgA exhibits an isotype-intrinsic enhancement in HA-stalk binding and neutralization activity. (A to D) The variable regions of mouse monoclonal HA stalk-binding antibodies, KB2 and 6F12, were cloned into human IgG or IgA backbones to generate chimeric antibodies. Dimeric IgA was generated by coexpression of human J chain. (A and B) ELISA plates were coated with recombinant PR8 protein in order to compare the binding properties of ch6F12 (A) and chKB2 (B) antibodies. (C and D) MNT assays were performed against cH5/1<sub>PR8</sub> N3 virus using the indicated dilutions of ch6F12 (C) or chKB2 (D). The data represent the means and standard errors of three independent experiments.

ing to cell surface receptors: the robust means of neutralization achieved by HA head-binding antibodies (37). A recent study has also suggested that stalk-binding monoclonal antibodies have limited activity against cell-bound viruses, which would further limit their neutralization potency (44). Despite this inferior ca-

capacity to neutralize virus, passive transfer experiments in mice have demonstrated a strong propensity for these antibodies to activate antibody-dependent cellular cytotoxicity (ADCC), which correlates with enhanced protection *in vivo* (13). Importantly, we found that the *in vitro* potency of HA stalk-mediated neutraliza-



**FIG 6** IgA-secreting MBCs exhibit a bias toward HA stalk specificity. (A to C) PBMCs were isolated from the blood of seven healthy donors, and MBCs were stimulated *in vitro* for 6 days to elicit antibody production. ELISpots were performed to evaluate the total number of IgG- and IgA-secreting cells from each donor, as well as the number of IgG- or IgA-secreting cells specific for recombinant Cal/09 HA (A), recombinant NC/99 HA (B), or recombinant cH6/1<sub>PR8</sub> HA (C). *P* values were determined by using a paired Student *t* test.

tion was also enhanced by several orders of magnitude relative to conventional HAI-mediated neutralization when the antibodies were evaluated in a polyclonal setting. Taken together, these findings are particularly encouraging when considering the feasibility of “universal” influenza virus vaccine approaches seeking to exploit HA stalk binding antibodies. Indeed, the inferior *in vitro* neutralization potency of HA stalk-binding monoclonal antibodies can likely be overcome or substantially reduced in the context of polyclonal responses and ADCC, which occur *in vivo*.

Given that influenza viruses initiate infection at the respiratory mucosa, understanding the biology of HA-stalk binding antibodies of IgA isotype is of critical importance. We demonstrate here that serum-derived polyclonal monomeric IgA possesses an equivalent capacity to neutralize virus *in vitro* compared to IgG from matched donors. Using a reductionist approach, we observed that when expressing identical variable regions, IgA molecules bound and neutralized virus via the HA stalk domain with elevated potency compared to corresponding IgG antibodies. This enhancement in the potency of IgA-mediated neutralization was also recently reported for HAI-positive antibodies that bind to the head domain (30) and may therefore reflect a more general structural feature of IgA constant regions that contribute to enhanced binding and neutralization. Stalk-binding V-regions expressed in the context of IgA constant regions may experience subtle conformational differences that promote their binding to the relatively discrete and well-characterized neutralizing epitope found in the HA stalk/stem.

In addition to the antibody-intrinsic enhancement of IgA stalk-mediated neutralization, analysis of MBCs present in human PBMCs highlighted an enrichment in stalk-specific antibody secreting cells of the IgA subtype. Although the source of this repertoire biasing remains under investigation, it could reflect either a genetic predisposition for particular V regions during class-switch recombination or an environmental factor, such as exposure route, which results in more potent elicitation of HA stalk-specific antibodies of IgA subtype during natural infection at respiratory surfaces.

Taken together, our findings provide important new insights regarding the biological activity of HA stalk-specific antibodies in the natural context of polyclonal human sera. The enhanced activity of these antibodies in polyclonal contexts and in the context of IgA should be carefully considered in the design of HA stalk antibody therapeutics and for design of “universal” influenza virus vaccines.

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