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VACCINES AND ANTIVIRAL AGENTS

Hemagglutinin Stalk-Reactive Antibodies Interfere with Influenza Virus Neuraminidase Activity by Steric Hindrance

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ABSTRACT Hemagglutinin (HA) stalk-reactive antibodies are the basis of several current "one-shot" universal influenza vaccine efforts because they protect against a wide spectrum of influenza virus strains. The appreciated mechanism of protection by HA stalk-reactive antibodies is to inhibit HA stalk reconfiguration, blocking viral fusion and entry. This study shows that HA stalk-reactive antibodies also inhibit neuraminidase (NA) enzymatic activity, prohibiting viral egress. NA inhibition (NI) was evident for an attached substrate but not for unattached small-molecule cleavage of sialic acid. This finding suggests that the antibodies inhibit NA enzymatic activity through steric hindrance, thus limiting NA access to sialic acids when adjacent to HA on whole virions. Consistently, $F(ab')_2$ fragments that occupied reduced area without loss of avidity or disrupted HA/NA interactions showed significantly reduced NI activity. Notably, HA stalk-binding antibodies lacking NI activity were unable to neutralize viral infection via microneutralization assays. This work suggests that NI activity is an important component of protection mediated by HA stalk-reactive antibodies.

IMPORTANCE This study reports a new mechanism of protection mediated by influenza hemagglutinin stalk-reactive antibodies, i.e., inhibition of neuraminidase activity by steric hindrance, blocking access of neuraminidase to sialic acids when it abuts hemagglutinin on whole virions.

KEYWORDS hemagglutinin, influenza, monoclonal antibodies, neuraminidase, stalk-reactive antibody

Influenza is an acute respiratory illness that causes epidemics and pandemics in the human population, causing up to 640,000 deaths annually worldwide (1). The influenza virus particle contains two major surface glycoproteins, hemagglutinin (HA) and neuraminidase (NA). HA is a trimer protein that contains a globular head and a stalk domain. The head domain mediates binding to host cellular receptor sialic acids, while the stalk domain fuses the virus and host cell membranes to allow the introduction of the influenza virus genes. The NA protein is essential for cleavage of terminal sialic acid residues present on host glycoproteins (Fig. 1A), allowing the ready dispersal of the newly generated virus (2, 3).

Antibodies are a major means of protection from influenza virus infections. Antibody-mediated immunity is the basis of current vaccines and most efforts to improve immunity to influenza. Influenza virus vaccinations induce antibodies that predominantly target the immunodominant globular head of influenza HA, blocking viral attachment to prevent infection (4, 5). However, immunity to the HA head domain is highly susceptible to influenza antigenic drift or viral mutation, which introduces novel amino acids and glycosylation sites that allow the virus to evade existing immunity. The stalk is a more conserved domain, allowing antibodies that target this region to neutralize a wide spectrum of influenza virus subtypes (6–9). The currently

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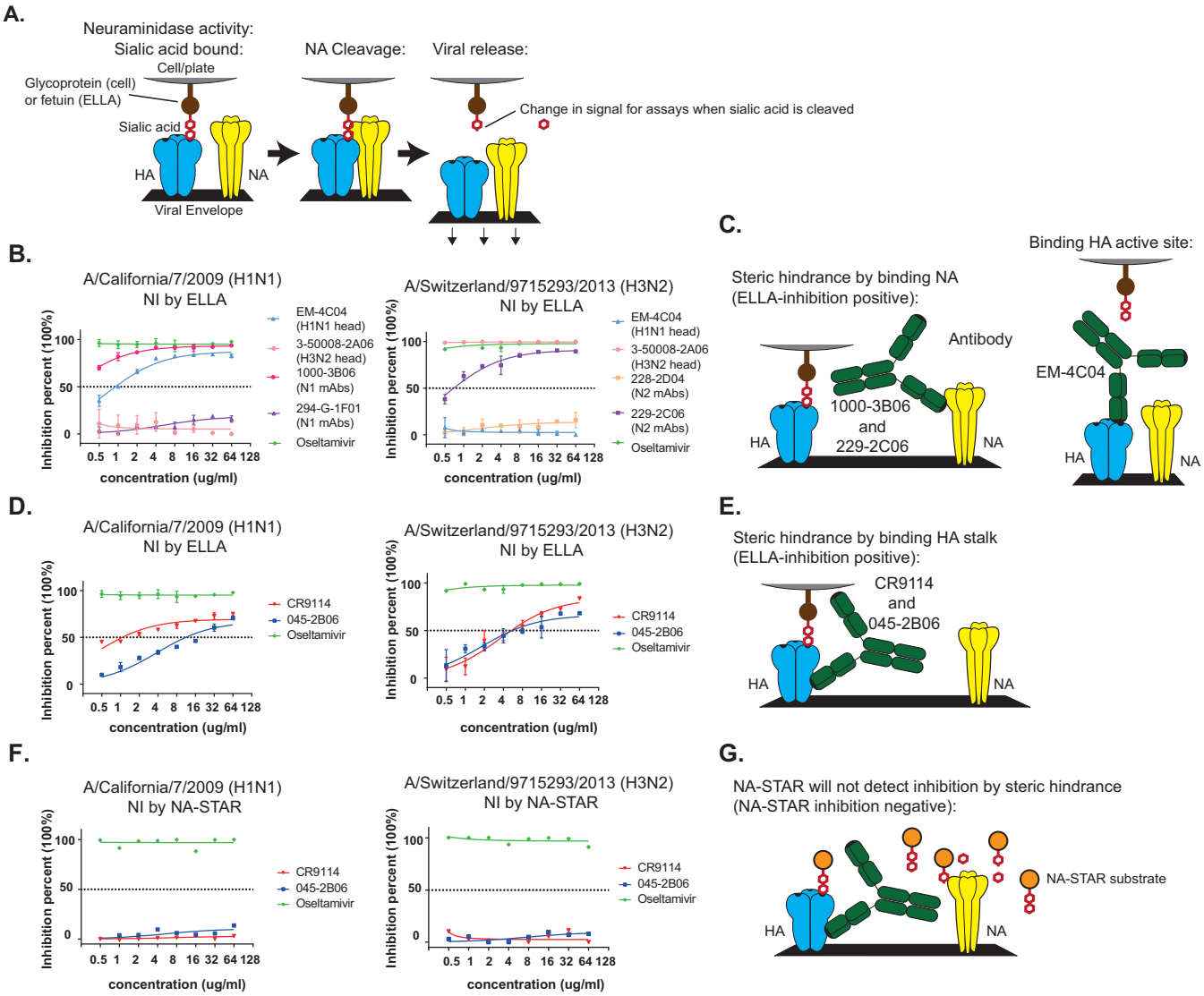

FIG 1 HA stalk-reactive MAbs inhibit influenza virus NA activity. (A) Model of NA activity to cleave bound sialic acids to release newly made virus particles. Note that the cleaved sialic acid substrates provide the change in signal (reduced) for the ELLA and NA-Star assay readouts. (B) Examples of HA head-reactive MAbs and NA-binding MAbs with NI activity, illustrating inhibition of NA enzymatic activity via ELLAs against A/California/7/2009 (H1N1) virus and A/Switzerland/9715293/2013 (H3N2) virus. (C) Model for how HA head-reactive MAbs and NA-reactive MAbs inhibit NA from cleaving the sialic acid from the host cell receptors. (D) HA stalk-reactive MAbs tested for inhibiting NA enzymatic activity via ELLAs against A/California/7/2009 (H1N1) virus and A/Switzerland/9715293/2013 (H3N2) virus. (E) Model for how HA stalk-reactive MAbs inhibit NA from cleaving the sialic acid from the host cell receptors via steric hindrance. (F) HA stalk-reactive MAbs tested for inhibiting NA enzymatic activity via NA-Star assays against A/California/7/2009 (H1N1) virus and A/Switzerland/9715293/2013 (H3N2) virus. (G) Model for why NA-reactive but not HA stalk-reactive MAbs can inhibit NA from cleaving the sialic acid in the NA-Star assays. A dilution of virus that resulted in 90 to 95% maximum signal was used in the following NI assays. Oseltamivir was used as a positive control. Results are shown as means ± SDs of three independent experiments.

appreciated mechanism of protection by HA stalk-reactive antibodies is to lock the HA trimer in a prefusion conformation, preventing pH-triggered conformational changes upon viral uptake into endocytic compartments. This conformational change exposes the fusion peptide that mediates fusion of the viral membrane to the host cell membrane, with subsequent introduction of the viral genome (10). Because of the highly conserved epitopes within the HA stalk domain, induction of antibodies targeting this domain is the basis of several universal influenza vaccine concepts and ongoing clinical trials. Herein, we describe an additional mechanism of protection that is mediated by HA stalk-binding antibodies, i.e., the inhibition of NA activity through steric hindrance blocking access to HA-bound sialic acid.

We used a panel of well-characterized HA stalk-reactive monoclonal antibodies

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(MAbs) to explore how the MAbs interfere with NA enzymatic activity, as measured by an enzyme-linked lectin assay (ELLA) or the NA-Star assay (11). The ELLA uses the sialylated glycoprotein fetuin, which is immobilized as a substrate to measure NA inhibition (NI); there is no obvious relationship between the NI ability of an antibody and its virus-binding range (12). This immobilized substrate identifies antibodies that either directly inhibit NA by binding close to the enzymatic active site or sterically inhibit NA from abutting close enough to HA to cleave the sialic acid (13). In contrast, the NA-Star assay uses a small soluble chemiluminescent substrate and so more explicitly distinguishes antibodies that directly inhibit the enzymatic activity of NA by binding close to the enzymatic site (12, 14). The ELLA detects a larger functional area, compared to the NA-star assay. When NA antigenic drift occurs outside the NA enzymatic active domain, it may affect only the ELLA and not the NA-Star assay. For all the ELLA and NA-Star assays, Tamiflu was used as a positive control. Herein, we show that HA stalk-reactive MAbs inhibit the enzymatic activation of influenza A viruses in the ELLA but not in the NA-Star assay, supporting a steric inhibition model. Moreover, we detected reduced NI activity for $F(ab')_2$ fragments from the stalk-reactive MAbs, compared to the same whole antibodies, providing direct evidence that anti-HA stalk antibodies sterically limit NA access to sialic acids. The NI activity of HA stalk-binding but not NA-binding antibodies is disrupted by dissociating HA and NA from virions, further supporting a steric hindrance-based mechanism of NI. Notably, only antibodies against the HA stalk with NI activity, including two with high affinity binding, were able to mediate microneutralization (MN). These findings suggest that an important component of protection by anti-HA stalk antibodies is steric inhibition of NI activity.

(This article was submitted to an online preprint archive [15].)

RESULTS

Stalk-reactive MAbs interfere with influenza A virus NA activity. It has been shown that a subset of NA-reactive antibodies bind NA outside the enzymatic site and inhibit sialic acid cleavage only in ELLAs and not NA-Star assays (12). Two examples of these antibodies are indicated in Fig. 1B (MAb 1000-3B06 against H1N1 influenza and MAb 229-2C06 against H3N2 influenza). The mechanism of action of these antibodies is likely steric inhibition of NA accessing sialic acid molecules bound by HA (Fig. 1C, left). Similarly, antibodies to epitopes on the outside periphery of the globular head of HA that do not inhibit hemagglutination, and so do not bind the HA receptor binding domain, can nonetheless mediate protection by inhibiting viral egress, similar to NI (16). Some HA stalk-reactive antibodies have also been shown to have NI activity (34, 35). Thus, we hypothesized that antibodies to the stalk region of HA, which would occupy space on the side of the molecule, could similarly mediate NI activity, expanding the mechanism of protection to include both inhibition of fusion and inhibition of egress. As expected (17), antibodies binding the HA head region of H1N1 and H3N2 influenza virus strains robustly inhibited NA activity in ELLAs (Fig. 1B, MAb EM-4C04 against H1N1 influenza and MAb 3-50008-2A06 against H3N2 influenza), because HA cannot bind the sialic acid on fetuin and so NA is not close enough for enzymatic activity (Fig. 1C, right). To determine whether anti-HA stalk antibodies elicited NI activity, we tested the well-characterized MAb 045-2B06 (18), which binds the HA stalk regions of group 1 and group 2 influenza A HA molecules, and MAb CR9114, which binds the HA stalk regions of both A and B influenza strain HAs (16). Both of these antibodies neutralize influenza *in vitro* and protect mice *in vivo* upon lethal challenge with influenza, and they can competitively inhibit the binding of each other (18), demonstrating that they bind similar protective HA stalk epitopes. We found that both 045-2B06 and CR9114 provided NI activity against both A/California/7/2009 (H1N1) and A/Switzerland/9715293/2013 (H3N2) in the ELLA (Fig. 1D). The likely mechanism for this inhibition is steric hindrance, in which the antibodies block the access of NA to the sialic acids bound by HA (Fig. 1E). In contrast, neither 045-2B06 nor CR9114 could inhibit the H1N1 and H3N2 viruses cleaving the small unattached substrate in the NA-Star assay (Fig. 1F), suggesting a mechanism of steric hindrance (compare Fig. 1E and G). Our results demonstrate

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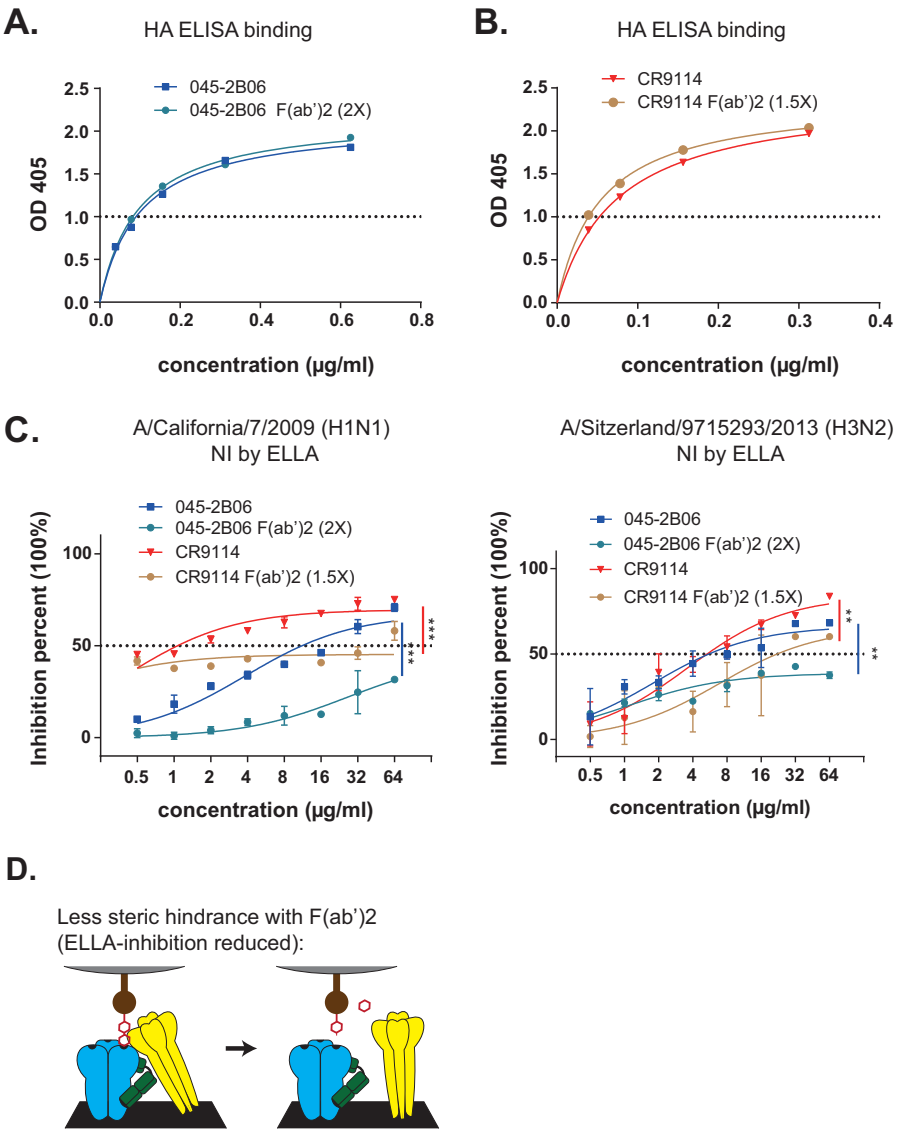

FIG 2 F(ab′)$_2$ molecules from stalk-reactive MAbs have reduced interference of virus NA activity by steric hindrance. (A) Binding avidity of 045-2B06 and its F(ab′)$_2$ fragments against A/California/7/2009 recombinant HA. (B) Binding avidity of CR9114 and its F(ab′)$_2$ fragments against A/California/7/2009 recombinant HA. (C) HA stalk-reactive MAbs and their F(ab′)$_2$ fragments tested for inhibiting NA enzymatic activity via ELLAs against A/California/7/2009 (H1N1) virus and A/Switzerland/9715293/2013 (H3N2) virus. (D) Model for why F(ab′)$_2$ fragments of HA stalk-reactive MAbs have reduced inhibition of NI in ELLAs. Results are shown as means ± SDs of three independent experiments. Statistical significance is indicated as follows: **, $P \leq 0.05$; ***, $P \leq 0.01$.

that human antibodies reactive with the HA stalk inhibit the enzymatic activity of the NA protein on H1N1 and H3N2 influenza virus particles.

Stalk-reactive MAbs interfere with NA activity by steric hindrance. Because the antibodies inhibited NI activity on the attached substrate of the ELLA but not on the free substrate of the NA-Star assay, as depicted in Fig. 1D and F, it appears that the mechanism of inhibition is likely steric hindrance of NA access to the sialic acid when bound by HA. To investigate this possibility directly, we generated F(ab′)$_2$ molecules from the 045-2B06 and CR9114 MAbs to reduce the size of the molecules, and thus the degree of steric hindrance, without affecting the avidity of binding. The F(ab′)$_2$ protein concentrations were increased to ensure binding of the F(ab′)$_2$ molecules equivalent to that of the MAbs (Fig. 2A and B). Comparison via the ELLA showed that indeed the F(ab′)$_2$ molecules had significantly reduced NI activity, in comparison

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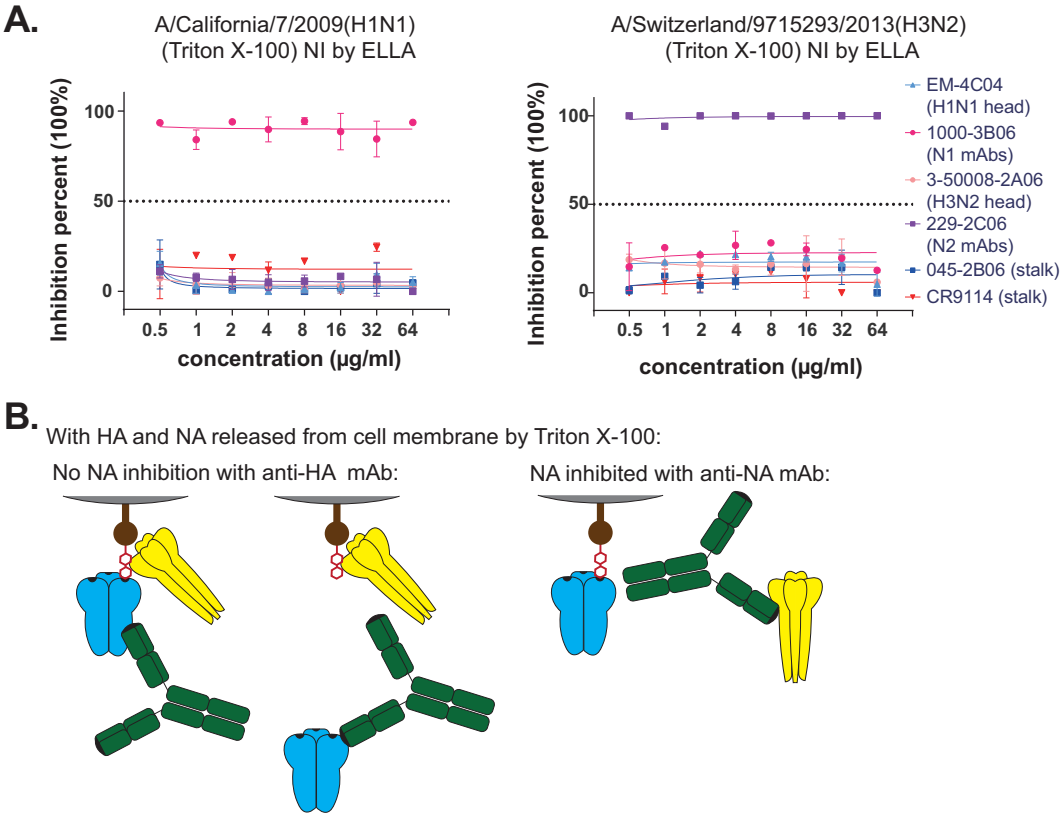

FIG 3 HA stalk-reactive MAbs cannot interfere with influenza virus NA activity if HA and NA are dissociated from the viral envelope. (A) A/California/7/2009 (H1N1) virus and A/Switzerland/9715293/2013 (H3N2) virus were treated with 1% Triton X-100 for 1 h at 37°C, and HA stalk-reactive and HA head-reactive MAbs, as well as NA-reactive MAbs, were tested for NI activity via ELLAs against Triton X-100-treated A/California/7/2009 (H1N1) virus and A/Switzerland/9715293/2013 (H3N2) virus. (B) Model for why HA reactive MAbs cannot disrupt NA activity, whereas NA-reactive MAbs can, when the HA and NA glycoproteins are dissociated from the viral envelope. Results are shown as means ± SDs of two independent experiments.

to the whole MAbs, against both H1N1 and H3N2 influenza viruses (Fig. 2C). Thus, while not completely allowing access of NA to the sialic acid, the smaller antibody fragments allowed increased NA access to the substrate (Fig. 2D), supporting a steric hindrance model of NI by HA stalk-reactive antibodies. As a final verification that antibodies to the HA stalk were inhibiting NA through steric hindrance, we dissociated the HA and NA molecules from the virions, thus allowing NA to access the sialic acid substrates completely independent of HA, regardless of the presence of antibody. For this analysis, we performed the ELLA in the presence of 1% Triton X-100 to disrupt the viral envelope lipid bilayer, releasing HA and NA in a way similar to seasonal split influenza virus vaccine production (19). The detergent treatment completely abrogated the NI activity of all of the HA stalk-reactive and HA head-reactive MAbs against both H1N1 and H3N2 virus strains, further supporting the steric hindrance model for NI activity (Fig. 3A and B). However, the NA-reactive antibodies were able to inhibit NI activity, demonstrating the integrity of the assay after detergent treatment. In total, these various results demonstrate that, in addition to the well-appreciated mechanism of action of disrupting viral entry, antibodies to the HA stalk region inhibit the access of NA to sialic acid through steric hindrance and thus reduce viral egress.

Stalk-reactive MAbs have different capacities for NI activity, which appears important for potency. Various HA stalk-reactive antibodies have been shown to have distinct amino acids involved in binding, various angles of interaction, and differences in specificity, as indicated by the spectrum of influenza strains bound (10). Thus, it was predicted that not all HA stalk-binding antibodies would elicit NI activity to the same degree. We tested a panel of 13 human MAbs that had been verified to bind HA stalk

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epitopes (11) for NI activity by ELLA. Criteria for being classified as HA stalk reactive included competitive binding with structure-verified HA stalk-reactive antibodies, including CR9114 (16) and SC70-F02 (20), and binding in a pH-dependent fashion, because the HA stalk epitope is disrupted at low pH (11). The majority, but not all, of the anti-HA stalk antibodies tested could inhibit NA activity, including 69% (9 of 13 antibodies) of those that are reactive with an H1N1 influenza virus (Fig. 4A and C) and 3 of 4 that bind an H3N2 influenza virus (Fig. 4B and D). Notably, none of those antibodies had NI activity using the NA-Star assay (Fig. 4E to H), suggesting that, like CR9114 and 045-2B06, they all inhibit NI via steric interference between NA and sialic acid. Importantly, after testing the panel of MAbs using a MN assay, we noted that only the antibodies with NI activity were able neutralize viral infection *in vitro* (Fig. 5A and B). While 2 of the antibodies that lacked MN capacity had low avidity, possibly explaining the lack of both MN and NI activities, the other 2 were of equal avidity, compared to the set of HA stalk-binding antibodies that did inhibit NI and neutralize infection (Fig. 5C). The lack of NI and MN activities of 2 of the HA stalk-binding antibodies with high avidity suggests that the epitope is bound in an atypical fashion. Thus, we considered whether these antibodies were of the stereotypical variety with restricted variable gene repertoires, biased for VH1-69 and VH1-18 immunoglobulin heavy chain gene usage. Notably, while most of the NI-positive antibodies appeared to be stereotypical (Fig. 6A), none of the NI-negative anti-HA stalk antibodies used the most typical anti-HA stalk VH gene, VH1-69 (Fig. 6B). One of the high-avidity NI-negative antibodies used VH3-23, which is indeed atypical, while the other high-avidity NI-negative antibody used VH1-18, which is a common stalk-related gene that is highly similar to VH1-69. There were no other features of the immunoglobulin gene repertoire that were distinct between NI-positive and NI-negative antibodies (Fig. 6C to F). These studies demonstrate that, while the majority of HA stalk-reactive antibodies inhibit NA activity, a subset do not and the capacity for NI appears to be important for potency.

DISCUSSION

The development of a "one-shot" universal influenza vaccine that will provide long-term or improved immunity to influenza epidemics and pandemics is currently a major focus of the biomedical community (21, 22). The HA stalk is an important target for the ongoing design of universal influenza vaccines and for recent and ongoing clinical trials, because the protective epitopes in this portion of HA are highly conserved across many influenza subtypes (10, 23, 24). While the appreciated mechanism of action of HA stalk-binding antibodies is to inhibit viral entry by disrupting the HA conformational change required for fusion of the viral envelope to the host cell membrane, one recent study using a microscopy-based assay suggested that HA stalk-reactive antibodies also block influenza virus particle release (25). Herein, we show that antibodies to the HA stalk region inhibit the activity of NA through steric hindrance on whole virions, adding to the mechanisms of protection mediated by this broadly reactive class of antibodies and providing a mechanism for the previously observed inhibition of viral release.

It is notable that not all antibodies that bind the HA stalk region can inhibit NI activity, although, for the collection of antistalk antibodies tested for this study, NI activity correlated perfectly with MN activity. Thus, NI activity is at least an important correlate of neutralization capacity and at most a critical component of the activity of HA stalk-binding antibodies. It is known that preexisting NA-reactive antibodies can reduce the number of cases of infection and decrease disease severity (26, 27). Moreover, adult influenza virus challenge studies showed that the NI titer is more predictive of protection and reduced disease (28). In future studies, it will be important to distinguish the relative contributions of inhibition of viral entry versus viral egress in protection for HA stalk-reactive antibodies.

A second interesting observation that should be evaluated is that there is a potential correlation between the capacity for NI activity and the type of anti-HA stalk antibody elicited, in that none of the NI-negative antibodies binding the HA stalk was encoded

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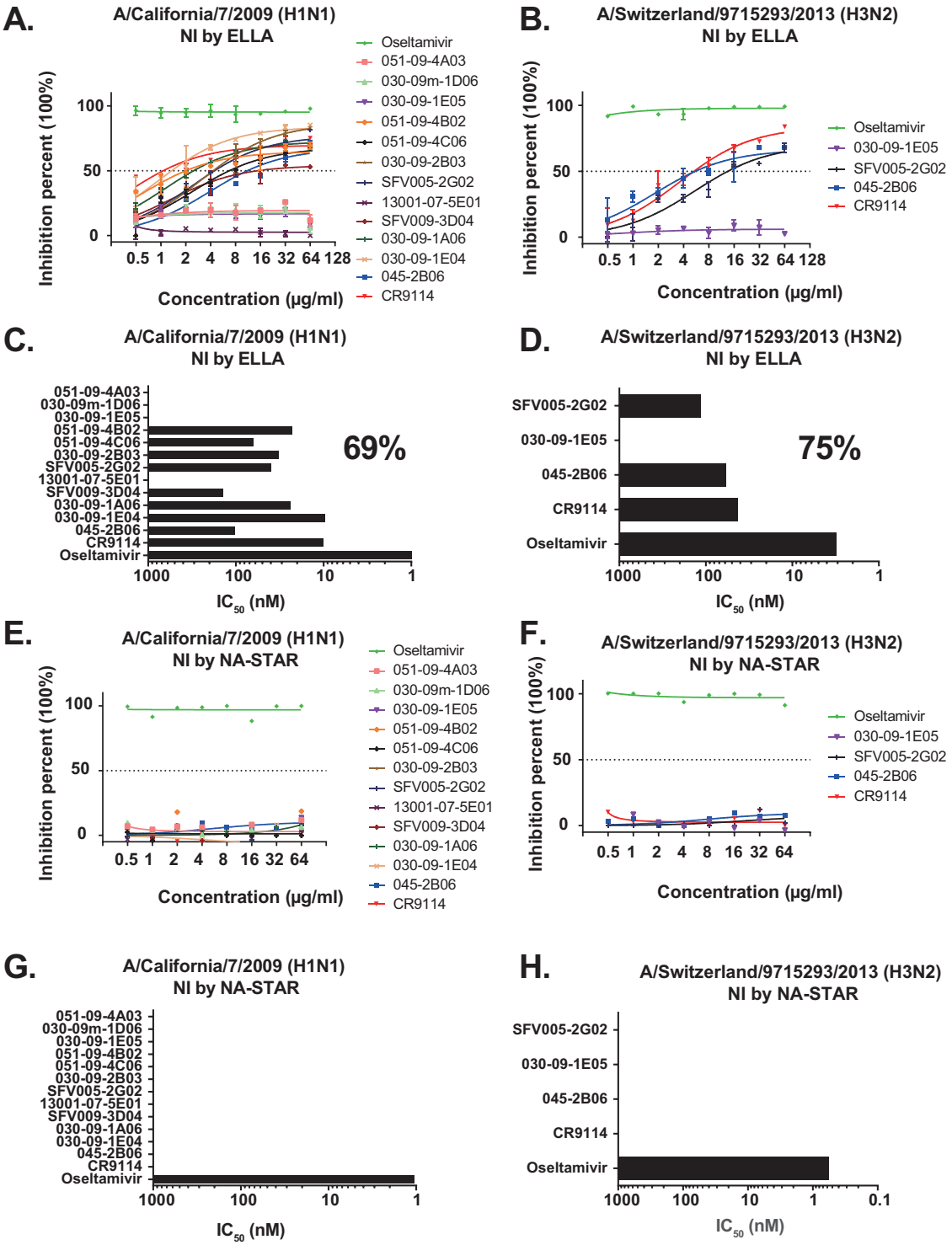

FIG 4 Stalk-reactive MAbs affect virus NA activity differently. (A and C) HA stalk-reactive MAbs were tested for NI activity via ELLAs against A/California/7/2009 (H1N1) virus. (B and D) HA stalk-reactive MAbs were tested for NI activity via ELLAs against A/Switzerland/9715293/2013 (H3N2) virus. (E and G) HA stalk-reactive MAbs were tested for NI activity via NA-Star assays against A/California/7/2009 (H1N1) virus. (F and H) HA stalk-reactive MAbs were tested for NI activity via NA-Star assays against A/Switzerland/9715293/2013 (H3N2) virus. Oseltamivir was used as a positive control for all assays. Data are representative of two independent experiments.

by the stereotypical VH1-69 gene. A more substantial evaluation of many anti-HA stalk antibodies for various properties and structural studies to evaluate the particular contact residues and angle of binding for stalk-reactive antibodies with NI activity will provide insight into this issue. Identifying stereotypical features of anti-HA stalk anti-

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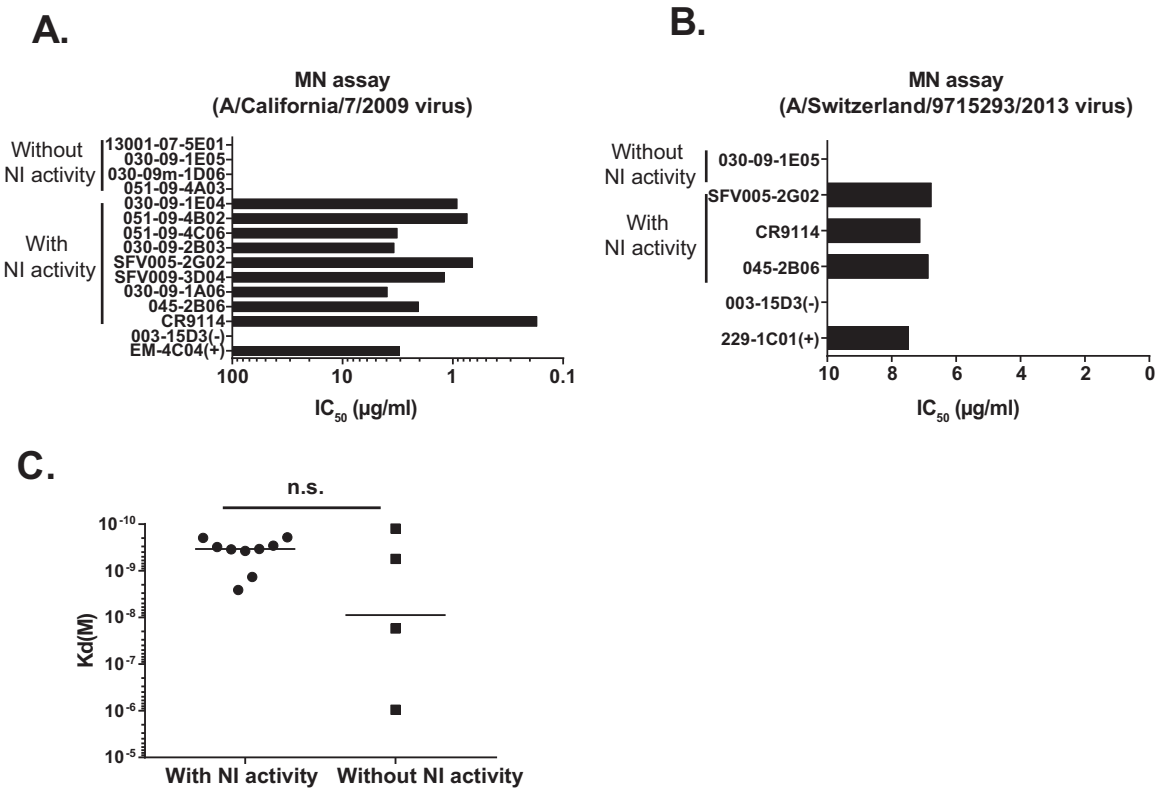

FIG 5 NI activity of influenza HA stalk-reactive antibodies correlates with MN capacity. (A and B) HA stalk-reactive MAbs with or without NI activity were tested for neutralization in *in vitro* MN assays using A/California/7/2009 (H1N1) virus (A) and A/Switzerland/9715293/2013 (H3N2) virus (B). Positive-control HA reactive MAbs EM-4C04 (anti-H1N1) and 229-1C01 (anti-H3N2) bind HA and neutralize these influenza virus strains. Influenza-nonreactive MAb 003-15D3 was used as a negative control in the assays. (C) HA stalk-reactive MAbs were tested for binding affinity against recombinant HA protein. K_d values for antibody binding were determined by Scatchard analysis of ELISA data using nonlinear regression (one-site binding model) with GraphPad Prism software. Bars indicate median values. Data are representative of two independent experiments. n.s., not significant.

bodies with NI activity could inform universal influenza vaccine design, by, for example, providing an impetus to develop germline-boosting prime-boost strategies to elicit a particular class of anti-HA stalk antibodies preferentially. In conclusion, this study identifies NI inhibition as a new and additional mechanism of action for the important class of anti-influenza antibodies that bind highly conserved epitopes on the HA stalk.

MATERIALS AND METHODS

Cells and viruses. Both human embryonic kidney (HEK) 293T cells and Madin-Darby canine kidney (MDCK) cells were obtained from the ATCC. The 293T cells were maintained at 37°C with 5% CO_2 in advanced Dulbecco's modified Eagle's medium (DMEM) with 2% Ultra-Low IgG fetal bovine serum (FBS) (Gibco), 2 mM GlutaMAX (Gibco), and penicillin and streptomycin (100 mg/ml; Gibco). The MDCK cells were maintained at 37°C with 5% CO_2 in DMEM with 10% FBS (Gibco), 1% L-glutamine (Gibco), and 1% antibiotic-antimycotic (Gibco). All influenza virus stocks (A/California/7/2009 H1N1 and A/Switzerland/9715293/2013 H3N2) used for the assays were freshly grown in specific-pathogen-free (SPF) eggs, harvested, purified, tittered, and stored at −80°C.

Recombinant MAb expression and purification. Antibodies were generated as described previously (11, 29–31). Briefly, the VH, Vκ, or Vλ genes amplified from each single cell were cloned into IgG1, Igκ, or Igλ expression vectors as described previously. Nine micrograms of each paired heavy and light chain plasmid DNA was transfected into the 293 cells using polyethylenimine (PEI) 25 K (catalog no. 23966-2; Polysciences), and the cells were incubated overnight. The next day, the transfection medium was aspirated from each plate and replaced with 25 ml of protein-free hybridoma medium (PFHM) II (Gibco). Four to 5 days later, secreted MAbs were purified from the supernatant using protein A beads. The MAbs was further concentrated and the buffer was exchanged with phosphate-buffered saline (PBS) (pH 7.4) using Amicon Ultra centrifugal filter units (30-kDa cutoff; Millipore). The final protein concentrations were determined using a NanoDrop device (Thermo Scientific).

Preparation of F(ab′)$_2$ fragments. The whole antibodies CR9114 and 045-2B06 were reduced to F(ab′)$_2$ fragments using the Pierce F(ab′)$_2$ digestion kit (catalog no. 44988; Thermo Fisher), according to the manufacturer's instructions.

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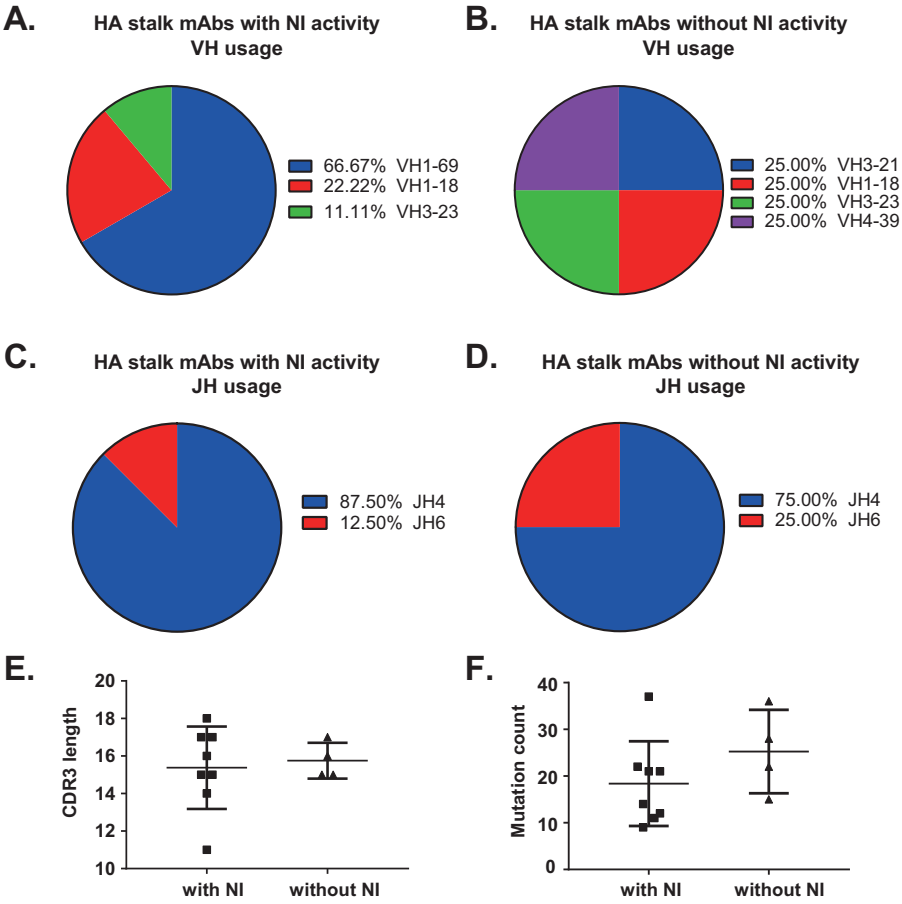

FIG 6 Heavy chain gene features of HA stalk-reactive MAbs with or without NI activity. (A and B) Usage of VH immunoglobulin genes by HA stalk-reactive B cells with NI activity (A) and HA stalk-reactive B cells without NI activity (B). (C and D) Usage of JH immunoglobulin genes by HA stalk-reactive B cells with NI activity (C) and HA stalk-reactive B cells without NI activity (D). (E) Complementarity-determining region 3 (CDR3) length of HA stalk-reactive MAbs with and without NI activity. Data are represented as means ± SDs. (F) Total heavy chain amino acid mutations for HA stalk-reactive MAbs with or without NI activity, based on analysis using the NCBI IgBlast tool (https://www.ncbi.nlm.nih.gov/igblast). Data are represented as means ± SDs. The antibodies represented in this figure were cloned from six different subjects (subjects 13001, 030, 045, 051, SFV005, and SFV009).

Enzyme-linked immunosorbent assay. High-protein-binding microtiter plates (Costar) were coated overnight at 4°C with recombinant HAs or NAs at 1 μg/ml in PBS. After blocking, serially diluted (3-fold) antibodies starting at 10 μg/ml were incubated for 1 h at 37°C. Horseradish peroxidase (HRP)-conjugated goat anti-human IgG antibody diluted 1:1,000 (Jackson Immuno Research) was used to detect binding of MAbs, and the result was developed with Super AquaBlue enzyme-linked immunosorbent assay (ELISA) substrate (eBiosciences). Absorbance was measured at 405 nm on a microplate spectrophotometer (Bio-Rad). To standardize the assays, antibodies with known binding characteristics were included on each plate, and the plates were developed when the absorbance of the control reached an optical density at 405 nm (OD_{405}) of 3.0 (32). To determine the binding of $F(ab')_2$ fragments by ELISA, a HRP-conjugated goat anti-kappa antibody (Southern-Biotech) was used as a secondary antibody. Affinity constant (K_d) values for antibody binding were determined by Scatchard plot analysis of ELISA data using nonlinear regression (one-site binding model) with GraphPad Prism software.

NA ELLA. ELLAs were performed as described previously (13, 33). Flat-bottom 96-well plates (Thermo Scientific) were coated overnight at 4°C with 100 μl of fetuin (Sigma) at 25 μg/ml. To determine the amount of virus to use in the ELLA, serial dilutions (2-fold) of the targeted virus were made in Dulbecco's PBS (DPBS) with 0.05% Tween 20 and 1% bovine serum albumin (BSA) (DPBSTBSA), into fetuin-coated plates containing an equal volume of DPBS. The plates were incubated for 18 h at 37°C and washed six times with PBS with 0.05% Tween 20, and 100 μl/well of HRP-conjugated peanut agglutinin (PNA) lectin (Sigma-Aldrich) in DPBSTBSA was added for 2 h at room temperature in the dark. The plates were washed six times and developed with Super AquaBlue ELISA substrate (eBiosciences). Absorbance was read at 405 nm on a microplate spectrophotometer (Bio-Rad). The dilution of virus that resulted in 90 to 95% of the maximum signal was chosen for use in the subsequent ELLAs. To measure the NI titers of the

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antibodies, the antibodies were serially diluted in DPBSTBSA and then incubated for 18 h at 37°C with an equal volume (50 μl) of the selected virus dilution in duplicate wells of a fetuin-coated plate. Eight wells containing diluent without antibody served as the positive (virus-only) control. Data were analyzed using Prism software, and the 50% inhibitory concentration (IC_{50}) was defined as the concentration at which 50% of the NA activity was inhibited, compared to the negative control.

NA-Star assay. The NA-Star assay was performed according to the manufacturer's instructions for the resistance detection kit (Applied Biosystems, Darmstadt, Germany) (14). In brief, 25 μl of test MAbs in serial 2-fold dilutions in NA-Star assay buffer [26 mM 2-(*N*-morpholino)ethanesulfonic acid [MES], 4 mM calcium chloride [pH 6.0]) were mixed with 25 μl of 1.58×10^3 50% tissue culture infectious doses ($TCID_{50}$)/ml of A/California/7/2009 virus or 2.5×10^4 $TCID_{50}$/ml of A/Switzerland/9715293/2013 virus, and the plates were incubated at 37°C for 30 min. After addition of 10 μl of 1,000-fold diluted NA-Star substrate, the plates were incubated at room temperature for another 30 min. The reaction was stopped by the addition of 60 μl of NA-Star accelerator. The chemiluminescence was determined by using a DTX 880 plate reader (Beckman Coulter). Data points were analyzed using Prism software, and the IC_{50} was defined as the concentration at which 50% of the NA activity was inhibited, compared to the negative control. The final concentration of antibody (IC_{50}) was determined using Prism software (GraphPad).

NI assay in the presence of detergent. To perform the NI assay in the presence of detergent, all steps remained identical to those listed above except as follows. First, a final concentration of 1% Triton X-100 (Fisher Bioreagents) was added directly to the virus particles, and they were shaken gently at 37°C for 1 h. During that time, antibodies were diluted using PBS with 1% Triton X-100. Prior to incubation with MAbs, the virus preparation was diluted in PBS containing 1% BSA and 1% Triton X-100. The NI assay was then performed as detailed above.

MN assay. The MN assay for antibody characterization was carried out as described previously (18). Briefly, MDCK cells were maintained at 37°C with 5% CO_2 in DMEM supplemented with 10% FBS. On the day before the experiment, confluent MDCK cells in a 96-well format were washed twice with PBS and incubated in minimal essential medium (MEM) supplemented with 1 μg/ml tosyl phenylalanyl chloromethyl ketone (TPCK)-treated trypsin. Serial 2-fold dilutions (starting concentration, 64 μg/ml) of MAb were mixed with an equal volume of 100 $TCID_{50}$ of virus, and the mixture was incubated for 1 h at 37°C. The mixture was removed, and cells were cultured for 20 h at 37°C with 1× MEM supplemented with 1 μg/ml TPCK-treated trypsin and an appropriate MAb concentration. Cells were washed twice with PBS, fixed with 80% acetone at −20°C for 1 h or overnight, washed three times with PBS with 0.05% Tween 20, blocked for 30 min with 10% FBS, and then treated for 30 min with 2% H_2O_2 at room temperature. A biotinylated anti-nucleoside phosphorylase antibody (1:3,000) in 3% BSA-PBS was added for 1 h at room temperature. The plates were developed with Super AquaBlue ELISA substrate and read at 405 nm. The signals from uninfected wells were averaged to represent 100% inhibition, and the signals from virus-infected wells without MAb were averaged to represent 0% inhibition. Duplicate wells were used to calculate the mean and standard deviation (SD) of neutralization, and IC_{50} values were determined from a sigmoidal dose-response curve. The inhibition ratio was calculated as follows: [(OD_{405} for positive control − OD_{405} for sample)/(OD_{405} for positive control − OD_{405} for negative control)] × 100%. The final concentration of antibody that reduced infection to 50% (IC_{50}) was determined using Prism software (GraphPad).

Data analysis and statistics. GraphPad Prism 7 was used to perform all statistical analyses. Data are presented as means and SDs of triplicates.

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Y.-Q.C. designed and performed experiments, analyzed data, and wrote the manuscript, L.Y.-L.L., M.H., and C.H. performed experiments and revised the manuscript, and P.C.W. designed and directed the project, analyzed data, and wrote the manuscript.

We declare no financial or commercial conflicts of interest.

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