Epitope specificity plays a critical role in regulating antibody-dependent cell-mediated cytotoxicity against influenza A virus

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The generation of strain-specific neutralizing antibodies against influenza A virus is known to confer potent protection against homologous infections. The majority of these antibodies bind to the hemagglutinin (HA) head domain and function by blocking the receptor binding site, preventing infection of host cells. Recently, elicitation of broadly neutralizing antibodies which target the conserved HA stalk domain has become a promising “universal” influenza virus vaccine strategy. The ability of these antibodies to elicit Fc-dependent effector functions has emerged as an important mechanism through which protection is achieved in vivo. However, the way in which Fc-dependent effector functions are regulated by polyclonal influenza virus-binding antibody mixtures in vivo has never been defined. Here, we demonstrate that interactions among viral glycoprotein-binding antibodies of varying specificities regulate the magnitude of antibody-dependent cell-mediated cytotoxicity induction. We show that the mechanism responsible for this phenotype relies upon competition for binding to HA on the surface of infected cells and virus particles. Nonneutralizing antibodies were poor inducers and did not inhibit antibody-dependent cell-mediated cytotoxicity. Interestingly, anti-neuraminidase antibodies weakly induced antibody-dependent cell-mediated cytotoxicity and enhanced induction in the presence of HA stalk-binding antibodies in an additive manner. Our data demonstrate that antibody specificity plays an important role in the regulation of ADCC, and that cross-talk among antibodies of varying specificities determines the magnitude of Fc receptor-mediated effector functions.

Significance

In addition to neutralizing antigens, antibodies are also capable of stimulating cellular responses through Fc–Fc receptor interactions. The type of response stimulated by these interactions is influenced by both the Fc receptor type expressed on the effector cell and the isotype of antibody to which it is bound. However, how antibody specificity influences Fc receptor functions, and how antibodies of different specificities interact to modulate these functions, remain unknown. Using influenza A virus as a model, we demonstrate that antibody specificity profoundly influences the induction of antibody-dependent cell-mediated cytotoxicity by effector cells. In addition, we show that interactions among antibodies that bind to discrete epitopes on the same antigen can influence the induction of Fc-dependent effector functions.
ADCC induction by stalk-binding bnAbs. Inhibition of ADCC by HAI+ antibodies is likely the result of competition for binding to HA in the context of infected cells or virus particles. This competition depends on both the relative affinity of each antibody and the accessibility of the epitope to which it binds. Interestingly, non-neutralizing HA-binding antibodies were not capable of potentially eliciting ADCC on their own, despite being of the appropriate isotype. These antibodies also had no effect on the ability of HA stalk-binding bnAbs to induce ADCC. In contrast, neuraminidase (NA)-binding antibodies could weakly induce ADCC, and also could cooperate with HA stalk-binding bnAbs.

Taken together, the foregoing findings demonstrate that antibody specificity plays a critically important role in the regulation of Fc-dependent effector functions such as ADCC. Because induction of ADCC is known to play an important role in the protection against many viruses, including IAV and HIV (21, 22), as well as in cancer immunotherapies (23), it will be important to consider the effects not only of antibody isotype, but also of specificity in the development of both novel vaccines and therapeutics.

Results

HAI+ Antibodies Inhibit Induction of ADCC by bnAbs That Bind to the HA Stalk Domain. Because monoclonal bnAbs that bind to the HA stalk domain have been reported to potently induce ADCC, whereas mAbs to the HA head domain do not, we sought to investigate how ADCC would be regulated in the context of a polyclonal response, to better recapitulate in vivo conditions (20). To test this, we first made mAbs to the HA head domain do not, we sought to investigate how ADCC would be regulated in the context of a polyclonal response, to better recapitulate in vivo conditions (20). To test this, we first made use of primary human natural killer (NK) cells freshly isolated from the peripheral blood of healthy donors. NK cells were incubated with antigen-presenting cells were incubated with identical antibodies on the cell surface in its natural context. After antibodies were added to the HA-expressing cells, a luciferase reporter cell line expressing murine FcγRIV was added to wells to allow for the quantification of ADCC induction. Cells infected with IAV strain X-31 (H3N2) were incubated with the murine HA stalk-binding bnAb 9H10 (IgG2a) (24) or the H3 head domain neutralizing antibody XY102 (IgG2a) (25) at concentrations ranging from 0.0008 to 5 μg/mL. As expected, 9H10 potently induced ADCC, whereas XY102 did not.

Surprisingly, when the concentration of 9H10 was held constant at 5 μg/mL and XY102 was titrated into the system, ADCC was inhibited in a dose-dependent manner (Fig. 1E). To ensure that this phenomenon was not subtype-specific, we repeated the experiment by infecting cells with A/California/04/09 (Cal/09) H1N1, followed by incubation with the H1 stalk-binding bnAb 6F12 (IgG2b) or the Cal/09-specific head domain neutralizing antibody 7B2 (IgG2a). Consistent with our previous results, 6F12 potently induced ADCC, whereas 7B2 failed to activate the reporter cell line; however, when the two antibodies were mixed, 7B2 antagonized ADCC induction by 6F12 in a dose-dependent manner (Fig. 1F).

Fig. 1. HAI+ antibodies inhibit induction of ADCC by bnAbs that bind to the HA stalk domain. (A and B) The ADCC assay was performed using primary NK cells. ADCC was measured as a percentage of activation above HK/68 H3-expressing target cells without antibody. The data shown represent the mean of technical replicates from each independent donor. (C and D) ADCC activation of NK cells grown in continuous culture was also assessed after incubation on infected A549 target cells incubated with the indicated antibodies. Data represent the mean and SEM of four independent experiments performed in duplicate. *P < 0.05, Student’s t test. (E–G) ADCC reporter assays were performed on X-31 (H3N2) cells (E and F) and on Cal/09-infected A549 cells (F) using combinations of murine mAbs (E and F) or human mAbs (G). The mean and SEM values for biological experiments performed in triplicate are shown.

**References**

1. He et al. (2016) PNAS 113 (10), 2828–2833.

2. S. Bowdle, J. E. Grabowski, M. G. Karp, J. T. McCutchan, P. J. M. Heijne, D. A. H. Westra, et al. (2016) Nature 531 (7593), 442–445.

3. E. C. S. Bowdle, J. E. Grabowski, M. G. Karp, J. T. McCutchan, P. J. M. Heijne, D. A. H. Westra, et al. (2016) Nature 531 (7593), 442–445.
To confirm that our findings are indeed relevant in the context of human IAV infection, we repeated the aforementioned experiments using human antibodies and a reporter cell line expressing human FcRIIIa. This served the dual purpose of ensuring that our observations were not artifacts of the murine FcRyIV cell line or of hybridoma-derived antibodies. CR8020 (IgG1) is a human H3 stalk-binding bnAb, whereas C05 binds to the H3 head domain and exhibits classical HAI activity. Both antibodies are capable of binding to HA or NA but lack neutralization activity in vitro. Nonneutralizing HA-binding antibodies neither induce nor inhibit ADCC by the bnAb 9H10 (Table S1). Therefore, to determine whether the lack of potent ADCC induction by nonneutralizing antibodies is related to affinity, we analyzed three additional human nonneutralizing antibodies—IG05 (stalk-binding), 3B01 (stalk-binding), and 5E02 (head-binding)—that bound to HA with affinity equivalent to the human stalk-binding bnAb CR8020 (Fig. S24), yet did not neutralize virus in vitro (Fig. S28). These antibodies were capable of only modest ADCC induction and did not affect the induction of ADCC by CR8020 (Fig. S2 C-E).

**NA-Inhibiting Antibodies Cooperate with HA Stalk-Binding bnAbs to Enhance Induction of ADCC in an Additive Manner.** In addition to HA, a substantial antibody response is also generated against the second major IAV surface glycoprotein, NA. Although present in antigenically lower quantities than HA-binding antibodies, NA-binding antibodies can inhibit NA activity, which has been shown to confer protection from infection in vivo (28–30). Certain HA-binding antibodies have been shown to exhibit NA-inhibiting (NAI) activity through steric hindrance of the NA enzymatic site (28). Therefore, we examined whether NA-binding antibodies by themselves could induce ADCC or influence ADCC induced by HA stalk-binding bnAbs. We studied three NAI+ mAbs: 3C2, 8C10, and 4D6 (all IgG2a) (Fig. 3A). Alone, these antibodies were capable of only modest ADCC induction; however, when combined with the bnAb 9H10, these NAI+ antibodies exhibited cooperative activity and enhanced induction of ADCC in an additive manner (Fig. 3B–D). These data demonstrate that although NAI+ antibodies do not potently induce ADCC on their own, they are capable of enhancing bnAb-mediated ADCC.

**Induction of ADCC by Human Polyclonal IgG Is Influenced by the Ratio of ADCC-Inducing and -Inhibiting Antibodies.** The foregoing results clearly demonstrate that antibody specificity (i.e., the HA epitope to which a particular antibody binds) profoundly influences both the ability to induce ADCC and the induction of ADCC by antibodies of other specificities in a polyclonal response. Thus, we turned our attention to investigating whether our model would hold true in the natural context of human polyclonal serum. To do this, we purified polyclonal IgG from the serum of three healthy adult donors (Table S2), and assessed the ability of these antibodies to induce ADCC in X-31-infected cells. We first performed ELISAs to quantify the extent of H3 stalk-binding antibodies using recombinant cH5/3 HA (Fig. 4A), total HK/68 HA-binding antibodies (Fig. 4B), and total HK/68 NA-binding antibodies (Fig. 4C), as described previously (2, 3, 18). As expected, IgG antibodies from each donor were capable of

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**Fig. 2.** Nonneutralizing HA-binding antibodies neither induce nor inhibit ADCC induced by HA stalk-binding bnAbs. (A) Western blot analysis was performed against 293T cells transfected with plasmids expressing His-tagged HK/68 H3 or N2 (negative control) using anti-His, FEE8, or FEB9. (B) The neutralization activities of 9H10, FEE8, and FBE9 were tested against X-31 by a microneutralization assay on MDCK cells. (C and D) The ability of FEE8 (Neut+, HA head-binding) and (C) FBE9 (Neut+, HA stalk-binding) (D) to induce ADCC or inhibit ADCC induced by 9H10 was evaluated on X-31–infected A549 cells. Because these assays were performed in parallel, the curves for 9H10, XY102 + 9H10, and control IgG + 9H10 are duplicated in C and D for clarity and ease of comparison. Data represent the mean and SEM values of biological experiments performed in triplicate.

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**Fig. 3.** NAI+ antibodies are capable of cooperating with HA stalk-binding bnAbs to boost ADCC induction. (A) ELISAs were performed using recombinant HK/68 N2 protein to test the binding affinities of 3C2, 4D6, and 8C10. (B–D) In vitro ADCC assays were performed on X-31–infected A549 cells using the HA stalk-binding antibody 9H10 either alone or in combination with the NAI+ antibodies 3C2 (B), 8C10 (C), or 4D6 (D). Data represent the mean and SEM values of biological experiments performed in triplicate.
inducing ADCC when added to the infected monolayer. Notably, antibodies purified from donor 3, who had the strongest ch53/5 reactivity (which specifically measures stalk-binding antibodies) and the strongest NA reactivity, also most potently induced ADCC (compare Fig. 4 A–C and F). Consistent with our earlier results, the extent of ADCC induction was influenced by the proportion of ADCC-activating antibodies (stalk and NA binding) and ADCC-inhibiting antibodies, given that spiking the polyclonal IgG samples with C05 (HA1+) could inhibit ADCC induction in a dose-dependent manner (Fig. 4 D–F). Thus, during infection of influenza-exposed humans, the extent of ADCC induction seems to be regulated by the relative proportion of ADCC-activating and inhibiting antibodies. Owing to the complex nature of antibody specificities in polyclonal mixtures, it is possible that other species of ADCC-activating and -inhibiting antibodies (i.e., ADCC-inhibiting antibodies that bind to NA) may exist that influence the magnitude of response observed for each donor.

**ADCC Inhibition by HAI+ Antibodies Is Achieved Through Competitive Binding to HA on Virus Particles and on the Surface of Infected Cells.** We next sought to define the mechanism responsible for HAI+ antibody-mediated inhibition of ADCC induced by HA stalk domain-binding bnAbs. To establish whether binding of HAI+ antibodies to HA was required for their inhibitory activity, we performed an ADCC assay on cells infected with a recombinant A/Puerto/Rico/8/1934 (PR8) virus expressing a chimeric 4/3 HA protein (ch14/3). This protein contains the stalk domain of A/Panama/10/16/1903 H3 and the head domain of an H4 avian virus. As expected, the HA stalk-binding bnAb 9H10 was capable of inducing ADCC in a dose-dependent manner. However, XY102, which is specific for the H3 head domain of A/Hong Kong/1/1968 (HK/68) and thus unable to bind to ch4/3 HA, lost its ability to inhibit ADCC activation (Fig. 5A).

Because binding of XY102 was clearly required to mediate inhibition of ADCC by 9H10, we examined the possibility that XY102 might interfere with 9H10 binding to HA. To do this, we first performed a competitive binding assay using biolayer interferometry. Recombinant HK/68 H3 was immobilized on probes, and binding to XY102 alone, to 9H10 followed by XY102, or to 9H10 followed by 9H10 again (as a positive control for competition), was measured. As expected, XY102 alone bound to the probe efficiently. Furthermore, 9H10 and XY102 were able to bind to the probe at the same time without any interference/competition (Fig. S3A).

Despite the fact that no competition in binding to immobilized recombinant HA was observed between 9H10 and XY102, we recognized that this does not accurately recapitulate the natural arrangement of HA on the surface of infected cells. Thus, we also performed ELISA-based binding competition assays against HA on the surface of infected cells, in the same context in which it is found naturally. (This is also the context in which our ADCC assays were performed.) Indeed, when the assay was performed under these conditions, XY102 was able to outcompete 9H10 for binding to HA in a dose-dependent manner (Fig. 5B). The same phenomenon could be recapitulated by assessing competition using purified virus particles (Fig. 5C). If competition for binding to HA can explain the ADCC inhibition observed previously, then it stands to reason that antibodies that are unable to inhibit ADCC also should not compete with HA stalk-binding bnAbs.

Consistent with this line of reasoning, we observed no competition for binding to purified virus particles between the nonneutralizing antibody FE8 and 9H10 (Fig. 5D). Thus, direct competition for binding to HA expressed on virus particles and the surface of infected cells is sufficient to explain the inhibition of stalk antibody-mediated ADCC by HAI+ antibodies. This competition is influenced by both antibody affinity and epitope accessibility (Fig. S3 and SI Results).

**Discussion**

Owing to the exquisite neutralization potency exhibited by HAI+ IAV strain-specific antibodies, the role of Fc-dependent effector functions in contributing to protection from homologous infection is thought to be negligible (20). However, extensive work by the Ravetch, Kent, and Ennis laboratories has demonstrated that cross-reactive antibodies are capable of eliciting ADCC against nonhomologous IAVs, and that the induction of ADCC by these antibodies might contribute to protection in vivo (22, 31–37). Furthermore, work by DiLillo et al. (20) has demonstrated that ADCC induction by HA stalk-binding bnAbs is required for optimal protection on a lethal challenge of mice that received a passive transfer of a monoclonal HA stalk-binding bnAb.

The most prominent targets of ADCC-inducing antibodies are the two major surface glycoproteins of IAV, HA and NA.

![Fig. 4.](image-url)

**Fig. 4.** The induction of ADCC by antibodies in human polyclonal serum is regulated by the relative proportion of ADCC activating and inhibiting antibodies. Polyclonal IgG was purified from the serum of human donors over protein G-Sepharose columns. (A) Quantities of H3 stalk-binding antibodies were determined by ELISA against recombinant ch53/5 HA. (B and C) Quantities of total HA-binding antibodies (θ) and N2-binding antibodies (C) were determined by ELISA against recombinant HK/68 HA or NA. (D–F) In vitro ADCC assays were performed on X-31-infected A549 cells using polyclonal IgG isolated from healthy adult donors either alone or in combination with the HAI+ human mAb antibody C05. (D) Donor 1. (E) Donor 2. (F) Donor 3. Data represent the mean and SEM values of biological experiments performed in triplicate.

![Fig. 5.](image-url)

**Fig. 5.** ADCC inhibition by HAI+ antibodies is mediated by competition for binding to HA on the surface of infected cells and virus particles. (A) In vitro ADCC assays were performed on ch4/3 virus-infected A549 cells using XY102 (HA1+), 9H10 (stalk-binding bnAb), or combinations thereof. (B and C) ELISA-based binding competition assays were performed on X-31-infected A549 cells (B) or on purified X-31 (C) using XY102 (HA1+), biotinylated 9H10 (HA stalk-binding bnAb), or both. The limit of detection of these assays is shown as a dashed line (background + 3 SD). (D) Another binding competition assay was performed on purified X-31 using biotinylated 9H10 and FE8 (HA1+, head-binding). Data represent the mean and SEM values of biological experiments performed in triplicate.
Whereas the ability of different antibody isotypes to mediate downstream Fc-effector functions has been well established, how antibody specificity affects the induction of FcR-dependent mechanisms like ADCC has not been addressed. We therefore systematically characterized multiple antibody specificities that bind to the IAV surface glycoproteins for their ability to induce ADCC and for ADCC-modifying interactions among antibodies of distinct specificities (Fig. S4). Although we have limited our studies to ADCC specifically, these findings are likely to extend to other FcR-dependent mechanisms as well, such as antibody-dependent cellular phagocytosis (ADCP).

Consistent with previous studies, we found that HA stalk-binding bnAbs were able to potently induce ADCC, whereas HAI* antibodies were not (20). Nonneutralizing antibodies directed against HA were not capable of potent ADCC induction, despite being of the appropriate isotype (IgG2a). Nevertheless, these antibodies have been reported to have the capability to protect from lethal infection in vivo (37, 38). Interestingly, NA+ antibodies induced ADCC weakly on their own, and cooperated with HA stalk-binding bnAbs to enhance ADCC induction. These results are consistent with recent studies demonstrating that some NA-specific mAbs are capable of protecting against influenza virus challenge in vivo, albeit at much higher concentrations than for HA stalk-binding bnAbs (37). This firmly demonstrates that antibody specificity plays a critical role in the stimulation of Fc-dependent effector functions like ADCC.

The reason why bnAbs targeting the HA stalk domain seem to be the most potent activators of ADCC in the context of IAV infection remains elusive. The fact that HAI* antibodies do not elicit ADCC suggests that binding of HA to the sialic acid residues on the surface of effector cells may potentiate ADCC. These dual interactions might increase receptor clustering or the strength and/or duration of the interaction between target cells and effector cells. This also would explain why NA+ antibodies cooperate with HA stalk-binding bnAbs, because binding of the antibodies to NA instead of their enzymatic activity. This model does not explain why nonneutralizing HA-specific antibodies fail to induce ADCC, however. It has been suggested that in vivo antibodies have unique glycoforms that potentiate activating Fc–Fc receptor interactions (39, 40). It is also possible that binding to the HA stalk domain alters the conformation of the Fc region in a way that promotes the Fc receptor binding and/or clustering required for ADCC activation. Given the requirement for Fc receptor clustering to achieve optimal downstream signaling, it is possible that the relatively weak ADCC induced by NA+ antibodies is related to the relatively weak neutralization of NA relative to HA on the surface of virus particles and infected cells, which may not be sufficient to promote downstream signaling (41).

Importantly, we found that antibodies with discrete specificities have the ability to cross-talk to regulate ADCC induction. Here we report that HAI* antibodies are capable of inhibiting ADCC induction by HA stalk-binding bnAbs in a concentration-dependent manner. This has important consequences not only for IAV, but also in many natural scenarios in which ADCC might occur in the context of a polyclonal antibody response. In the case of IAV specifically, these observations imply that when sufficient quantities of HAI*–neutralizing antibodies are present, ADCC is inhibited, and neutralization functions as the primary mode of protection. This scenario would most likely occur in the context of reexposure to a homologous IAV strain, after either infection or vaccination. However, when HAI* antibodies are absent or limiting, bnAbs targeting the HA stalk domain engage effector cells to stimulate ADCC and thereby limit the spread of infection. This is likely to be the primary mechanism through which a “universal” IAV vaccine would confer protection. Thus, it is encouraging that we also observed no inhibition of ADCC by cross-reactive, nonneutralizing antibodies, which are likely to be present in most preexposed individuals.

The ability of HAI* antibodies to inhibit HA stalk antibody-mediated ADCC on both virus particles and on the surface of infected cells suggests that other Fc-dependent effector functions, such as ADCP and antibody-mediated complement-dependent cell lysis, also may be regulated by interactions among antibodies of differing specificities. Indeed, the signaling pathways that regulate these processes are largely conserved (42). Given that in the case of IAV, the inhibition of ADCC relies on competition for binding to HA, both affinity and epitope accessibility play roles in determining the eventual outcome. Despite having lower affinity than 9H10, XY102 is capable of competing for binding, because the HA head domain on virus particles and on the surface of infected cells is substantially more accessible than the HA stalk domain. These data are consistent with recent studies showing that poor accessibility of the stalk domain is partly responsible for the relatively low quantities of HA stalk-specific antibodies produced under normal conditions (43).

Taken together, our findings identify a previously unappreciated role for antibody specificity in regulating Fc-dependent effector functions such as ADCC. We also demonstrate that interactions among antibodies of unique specificities in polyclonal mixtures can modulate the extent of Fc receptor activation. These findings should be carefully considered in the design of novel vaccines and therapeutics in which induction of Fc-dependent effector functions are important for achieving efficacy, including those targeting HIV and cancer.

Materials and Methods

Cells and Viruses. Adenocarcinomic human alveolar basal epithelial cells (A549; American Type Culture Collection) and Madin–Darby canine kidney (MDCK) cells were grown in DMEM (Gibco) supplemented with 10% (vol/vol) FBS (HyClone) and 100 U/mL penicillin-streptomycin (Gibco). X-31 and ch43 viruses are reassortant viruses that have six internal proteins of PR8. X-31 viruses express the HA and NA of HK/68 H3N2 and ch43 viruses express chimeric ch43 HA (H4 globular head domain from A/duck/Czechoslovakia/1956 and H3 stalk domain from A/Perth/16/2009) and N1 of PR8 virus.

IgG Fab. Preparation. XY102 Fab(ab), antibody fragments were created using the Fab(ab)2 Preparation kit (Pierce). XY102 IgG was digested by immobilized papain at acidic pH. Then the digested product was used through an immobilized NAB Protein A Plus Spin Column (Thermo Fisher Scientific) to separate undigested IgG. The column was washed with PBS using Amicon Ultra centrifugal 50-KDa filter units and separated between small peptides of the Fc portion. The final product was XY102 Fab(ab). ELISA. ELISA was performed as described previously (18).

Micronutralization Assays. Micronutralization assays were performed as described previously (19).

Biolayer Interferometry. For binding competition assays, biotinylated recombinant HA proteins (20 μg/mL) were immobilized onto streptavidin-coated biosensors (Fortebio) for 5 min. After the baseline signal was measured in kinetics buffer for 3 min, biosensor tips were immersed into the wells containing primary antibody at a concentration of 50 μg/mL for 5 min. Biosensor tips were then immersed into wells containing competing mAbs at a concentration of 50 μg/mL for 5 min. The values of y-axes were recorded in real time. For Kd determination, purified mAbs were loaded onto AMC (anti-mIgG Fc capture) biosensors (Fortebio) in kinetics buffer for 180 s. For the measurement of Kd, the association was measured for 180 s by exposing the sensors to seven concentrations of purified HA of HK/68 in 1× kinetics buffer for 2 min. For the measurement of Kd, the dissociation was measured for 180 s in 1× kinetics buffer. Experiments were performed at 30 °C. Kd values were calculated according to the manufacturer’s guidelines.

Primary NK Cell ADCC Assay. NK cells were either purified from peripheral blood monoclonal cells (PBMCs) isolated from peripheral blood using Lymphoprep density gradient medium (Stem Cell Technologies), followed by a CD56+ magnetic selection kit (Stem Cell Technologies), or were isolated from peripheral blood and put into continuous culture with K562 cells (clone 9) at a 1:2 ratio in RPMI supplemented with 10% (vol/vol) FBS, 1 mM HEPES, 1 mM L-glutamine, 100 μM penicillin-streptomycin, and 100 ng/mL IL-2 (Peprotech) every 2–3 d, as reported previously (44). As targets, HEK 293T cells were transfected with a HK/68 HA plasmid or A549 cells were infected with X-31 (H3N2) for 18 h to allow for HA expression. At 24 h after transfection, the cells were incubated with antibodies for 1 h. Antibodies were then
removed, and 4 x 10^7 CD56+ cells were added to the transfected cells along with anti-human CD107a-APC (HAAS; BD Biosciences). After 1 h of incubation, 5 μg/mL brefeldin A (Sigma-Aldrich) and Golgistop (BD Biosciences) were added. Following 3.75 μM paraformaldehyde and blocked with 3% BSA. A mixture of biotinylated (EZ-link Micro NHS-PEG4-Biotinylation Kit; Thermo Fisher Scientific) stalk and unlabeled head antibodies were added to the cells, followed by incubation at room temperature for 1 h. Plates were washed with PBST. Secondary HRP-conjugated streptavidin (Millipore) was added at 1:5,000 dilution for 1 h. After the addition of HRP substrate (Sigmafast OPD; Sigma-Aldrich), reactions were stopped by the addition of 3 M HCl, and the optical densities were read at 490 nm on a Synergy 4 plate reader (Bio-Tek).

**Virus Particle-Based Competition Assay.** The 96-well plates were coated with 2 μg/mL sucrose gradient-purified X-31 virus particles overnight in bicarbonate-carbonate coating buffer (100 mM, pH 9.6). All other steps were performed as described above.

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1. Kramer F, Palese P (2015) Advances in the development of influenza virus vaccines. *Nat Rev Drug Discov* 14(3):167–182.
2. Miller MS, et al. (2015) Broadly neutralizing antibodies 2009 H1N1 influenza virus vaccine boost anti-hemagglutinin stalk antibodies in humans. *J Infect Dis* 217(1):98–105.
3. Miller MS, et al. (2013) Neutralizing antibodies against previously encountered influenza virus strains increases over time: A longitudinal analysis. *Sci Transl Med* 5(198):198ra107. 106.
4. Eibenboh A, et al. (2014) Induction of broadly cross-reactive antibody responses to the influenza HA stem region following H5N1 vaccination in humans. *Proc Natl Acad Sci USA* 111(36):13133–13138.
5. Li G-M, et al. (2012) Pandemic H1N1 influenza vaccine induces a recall response in humans that favors broadly cross-reactive memory B cells. *Proc Natl Acad Sci USA* 109(23):9047–9052.
6. Wrammert J, et al. (2011) Broadly cross-reactive antibodies dominate the human B cell response against 2009 pandemic H1N1 influenza virus infection. *J Exp Med* 208(1):181–193.
7. Halliley JL, et al. (2015) High-affinity H7 head and stalk domain-specific antibody responses to an inactivated influenza H7N7 vaccine after priming with live attenuated influenza vaccine. *J Infect Dis* 212(8):1270–1278.
8. Pica N, et al. (2012) Hemagglutinin stalk antibodies elicited by the 2009 pandemic influenza virus as a mechanism for the extinction of seasonal H1N1 viruses. *Proc Natl Acad Sci USA* 109(7):2573–2578.
9. Kramer F, et al. (2014) H3 stalk-based chimeric hemagglutinin influenza virus constructs protect mice from H7N9 challenge. *J Virol* 88(4):2340–2343.
10. Kramer F, et al. (2014) Assessment of influenza virus hemagglutinin stalk-based immunity in ferrets. *J Virol* 88(8):3432–3442.
11. Steel J, et al. (2010) Influenza antibodies in the absence of neutralizing antibodies. *J Virol* 84(1):10008–1010.
12. Wohlbidl TJ, et al. (2015) Vaccination with soluble headless hemagglutinin protects mice from challenge with divergent influenza viruses. *Vaccine* 33(29):3314–3321.
13. Yassine HM, et al. (2015) Hemagglutinin-stem nanoparticles generate heterosubtypic influenza protection. *Nat Med* 21(8):1065–1070.
14. Impagliazzo A, et al. (2015) A stable trimeric influenza hemagglutinin stem as a broadly protective immunogen. *Science* 349(6254):1301–1306.
15. Kramer F, Palese P (2014) Universal influenza virus vaccines: Need for clinical trials. *Nat Immunol* 15(1):1–3.
16. Brandenburg B, et al. (2013) Mechanisms of hemagglutinin targeted influenza virus neutralization. *PLoS One* 8(12):e80034.
17. He W, et al. (2015) Broadly neutralizing anti-influenza virus antibodies: Enhancement of neutralizing potency in polyclonal mixtures and IgA backbones. *J Virol* 89(7):3610–3618.
18. He W, Mullarkey CE, Miller MS (2015) Measuring the neutralization potency of influenza A virus hemagglutinin stalk/stem-binding antibodies in polyclonal prepara-
19. Di Lillo DI, Tan GS, Brower, et al. (2014) Broadly neutralizing hemagglutinin stalk-specific antibodies require FcγR interactions for protection against influenza virus in vivo. *Nat Med* 20(2):143–151.
20. Kramski M, Stratov I, Kent SJ (2015) The role of HIV-specific antibody-dependent cellular cytotoxicity in HIV prevention and the influence of the HIV-1 Vpu protein. *AIDS* 29(12):137–144.
21. Jegaskanda S, Reading PC, Kent SJ (2014) Influenza-specific antibody-dependent cellular cytotoxicity: Toward a universal influenza vaccine. *J Immunol* 193(2):469–475.
22. Di Lillo DI, Ravetch JV (2014) Differential Fc receptor engagement drives an anti-
23. Tsai TL, et al. (2014) Characterization of variable-region genes and shared cross-reactive idiotypes of antibodies specific for antigens of various influenza viruses. *Viral Immunol* 1(1):1–12.
24. Kiessling K, et al. (2012) Cross-neutralization of influenza A viruses mediated by a single antibody loop. *Nature* 497(7447):526–532.
25. Kiessling K, et al. (2015) Antibodies to influenza virus neuraminidase: An independent correlate of protection. *J Infect Dis* 212(8):1191–1199.
26. Jegaskanda S, et al. (2014) Cross-reactive influenza-specific antibody-dependent cellular cytotoxicity in intravenous immunoglobulin as a potential therapeutic against emerging influenza viruses. *J Infect Dis* 210(1):1811–1822.
27. Jegaskanda S, et al. (2013) Age-associated cross-reactive antibody-dependent cellular cytotoxicity toward 2009 pandemic influenza A virus subtype H1N1. *J Infect Dis* 207(10):1051–1061.
28. Jegaskanda S, Weinifurtner JT, Friedrich TC, Kent SJ (2013) Antibody-dependent cellular cytotoxicity is associated with control of pandemic H1N1 influenza virus infection of macaques. *J Virol* 87(10):5512–5522.
29. Jegaskanda S, et al. (2013) Cross-reactive influenza-specific antibody-dependent cellular cytotoxicity antibodies dominate the human B cell response against 2009 pandemic H1N1 influenza virus infection. *J Virol* 87(10):5512–5522.
30. Henry Dunand CJ, et al. (2016) Both neutralizing and non-neutralizing human H7N9 antibodies protect against lethal H10N8 influenza virus infection in mice. *J Virol* 90(2):851–861.
31. Jegaskanda S, et al. (2014) Broadly cross-reactive antibodies dominate the human B cell response against 2009 pandemic H1N1 influenza virus infection. *J Exp Med* 208(1):181–193.
32. Jegaskanda S, et al. (2013) Age-associated cross-reactive antibody-dependent cellular cytotoxicity toward 2009 pandemic influenza A virus subtype H1N1. *J Infect Dis* 207(10):1051–1061.
33. Jegaskanda S, Weinifurtner JT, Friedrich TC, Kent SJ (2013) Antibody-dependent cellular cytotoxicity in intravenous immunoglobulin as a potential therapeutic against emerging influenza viruses. *J Infect Dis* 210(1):1811–1822.
34. Jegaskanda S, Weinifurtner JT, Friedrich TC, Kent SJ (2013) Antibody-dependent cellular cytotoxicity in intravenous immunoglobulin as a potential therapeutic against emerging influenza viruses. *J Infect Dis* 210(1):1811–1822.
35. Henningt RJ, et al. (2012) Membrane-bound IL-21 promotes sustained ex vivo proliferation of human natural killer cells. *PLOS One* 7(1):e30264.