

Antibody-Dependent Cellular Cytotoxicity Is Associated with Control of Pandemic H1N1 Influenza Virus Infection of Macaques

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Emerging influenza viruses pose a serious risk to global human health. Recent studies in ferrets, macaques, and humans suggest that seasonal H1N1 (sH1N1) infection provides some cross-protection against 2009 pandemic influenza viruses (H1N1pdm), but the correlates of cross-protection are poorly understood. Here we show that seasonal infection of influenza-naïve Indian rhesus macaques (*Macaca mulatta*) with A/Kawasaki/173/2001 (sH1N1) virus induces antibodies capable of binding the hemagglutinin (HA) of both the homologous seasonal virus and the antigenically divergent A/California/04/2009 (H1N1pdm) strain in the absence of detectable H1N1pdm-specific neutralizing antibodies. These influenza virus-specific antibodies activated macaque NK cells to express both CD107a and gamma interferon (IFN- γ) in the presence of HA proteins from either sH1N1 or H1N1pdm viruses. Although influenza virus-specific antibody-dependent cellular cytotoxicity (ADCC)-mediated NK cell activation diminished in titer over time following sH1N1 infection, these cells expanded rapidly within 7 days following H1N1pdm exposure. Furthermore, we found that influenza virus-specific ADCC was present in bronchoalveolar lavage fluid and was able to activate lung NK cells. We concluded that infection with a seasonal influenza virus can induce antibodies that mediate ADCC capable of recognizing divergent influenza virus strains. Cross-reactive ADCC may provide a mechanism for reducing the severity of divergent influenza virus infections.

The seasonal influenza trivalent inactivated vaccine (TIV) is a crucial measure to reduce the incidence and severity of influenza virus infection (1–4). Vaccine-mediated protection against influenza disease is thought to depend on neutralizing hemagglutinin (HA)-specific antibodies, which mainly target the membrane-distal portion of the HA protein, preventing virions from binding to host cell sialic acids. However, current vaccines require long production schedules, hampering our ability to respond to emerging viruses. Furthermore, neutralizing antibodies (NAbs) induced by vaccination with seasonal influenza viruses provide little cross-protection from novel and/or divergent influenza virus strains (5–7). Novel approaches are therefore required to produce vaccines capable of inducing cross-protective immunity to a range of divergent influenza viruses.

Broadly neutralizing antibodies to influenza virus would be an ideal protective mechanism. Recent evidence suggests that at least some infected hosts are capable of producing such antibodies, which commonly target the membrane-proximal region of HA (stem) and either inhibit fusion of viral and endosomal membranes, inhibit viral egress, or inhibit HA maturation (8–12). At least some such antibodies are capable of neutralizing all 16 subtypes of influenza viruses *in vitro*, and they protect mice and ferrets against lethal challenges with H1N1 and H5N1 viruses, respectively (13, 14). However, broadly neutralizing antibodies are difficult to induce via vaccination, so other immune mechanisms may be necessary to provide broad vaccine-induced protection against influenza.

T cell responses can also aid in viral clearance and decrease the severity of influenza disease (15, 16). There is circumstantial evidence from human studies and animal models that T cells recognizing conserved epitopes can mediate heterotypic immunity against divergent influenza virus strains in the absence of cross-reactive NAbs (15–22), though this concept has so far been tested directly only in mice (21). Accordingly, a number of studies have

suggested that infection with seasonal influenza virus provides a level of protection against the swine-origin pandemic 2009 influenza virus (H1N1pdm) in the absence of NAbs, an effect which may be mediated by cross-reactive T cell responses (17, 23–28). However, the degree to which T cells can mediate effective, long-lived heterotypic immunity to influenza in humans remains unclear.

While most investigations of antibody-mediated immunity have focused on NAbs, nonneutralizing antibodies induced by prior influenza virus infection may also provide a level of protection against pandemic influenza virus. Recently, a study by Fang et al. showed that seasonal influenza virus infection provided some protection from pandemic influenza virus infection in mice and that this protection was mediated through a T cell-independent but B cell-dependent mechanism (29). Nonneutralizing effector functions such as phagocytosis (30, 31), complement activation (10), and antibody-dependent cellular cytotoxicity (ADCC) (32–34) may provide an alternative pathway of broad cross-protection. Nonneutralizing antibodies may recognize more conserved regions of influenza virus surface proteins, partially overcoming the NAb escape mediated by antigenic drift and antigenic shift (29).

NK cells are important mediators of innate immunity against influenza. NK cells infiltrate the lungs early during influenza virus infection and aid in direct viral clearance (35–37). NK cells can

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mediate effector functions via ADCC, by recognizing whole virus or viral antigens on the surfaces of infected cells. Once IgG1 antibodies are bound to these viral antigens, NK cells can in turn bind the antibodies via their CD16 receptor. This interaction induces signaling cascades leading to the release of perforin/granzymes as well as the secretion of antiviral mediators such as gamma interferon (IFN- γ) and tumor necrosis factor (TNF).

Influenza virus-specific ADCC studies have been limited over the past decade, most likely due to a lack of robust assays and reagents to characterize ADCC. We recently developed new assays to measure HIV-specific ADCC in humans by measuring ADCC-induced NK cell activation by flow cytometry, and we have applied these to influenza virus-specific ADCC (38, 39). We hypothesized that influenza virus-specific ADCC-mediating antibodies induced by prior influenza virus infection can bind to more conserved regions of influenza virus proteins, particularly HA and NA, and induce killing of influenza virus-infected cells through ADCC, thus providing cross-protection from a number of influenza virus strains. The induction of cross-reactive ADCC by prior influenza virus infection remains largely uncharacterized.

The generation of cross-protective influenza virus-specific ADCC responses is difficult to study in humans due to the ubiquitous and frequent exposure of people to multiple influenza viruses through vaccination and infection. Since the influenza virus antigen exposure history of human subjects is rarely known, it is difficult to determine whether specific prior infections provided protection from subsequent infections. The ferret influenza model has been important in the characterization of influenza virology and protection (18, 23), but ADCC is difficult to characterize in this model due to a lack of immunological reagents. Although murine models have been valuable for dissecting NAb and T cell immunity, the ability of influenza virus to induce ADCC in mice remains relatively undefined. The macaque model of influenza virus infection has recently gained popularity, as most human strains of influenza virus readily infect macaques, a large number of nonhuman primate reagents are being established, and macaques show similar pathology and immune responses to influenza virus to those seen in humans (17, 40–42). Therefore, the use of the macaque influenza model allowed us to dissect the induction and protective capacity of influenza virus-specific ADCC.

Recently, we showed that prior seasonal H1N1 (sH1N1) influenza virus infection partially protected macaques from H1N1pdm challenge (17). This protection was associated with cross-reactive T cells. Here we show that sH1N1 infection of the same macaques additionally induced cross-reactive ADCC-mediating antibodies against pandemic influenza virus in the absence of detectable neutralizing antibodies. Furthermore, these antibodies waned over time following sH1N1 clearance but were rapidly increased following H1N1pdm infection. H1N1pdm-specific antibodies induced robust activation of both blood and lung NK cells. Lastly, ADCC-mediating antibodies that recognize both seasonal and pandemic virus HA proteins can be found in the lung secretions of infected macaques. Together, our data provide evidence that cross-reactive, nonneutralizing antibodies could play a role in heterotypic immunity against influenza viruses.

MATERIALS AND METHODS

Macaques. Influenza-naïve Indian-origin rhesus macaques (*Macaca mulatta*) were used in this study, which was performed in accordance with the

guidelines of the United States National Research Council (43) and the Weatherall Report (44), under a protocol approved by the University of Wisconsin Graduate School Animal Care and Use Committee. Prior to any procedure, animals were anesthetized intramuscularly with ketamine or ketamine-medetomidine. Animals were inoculated with a total of 9×10^6 PFU of virus via the trachea, tonsils, and conjunctivae as described previously (17, 42, 45). Virus titers in nasal and tracheal secretions and bronchoalveolar lavage (BAL) fluid were determined using standard plaque assays. The present study used stored samples of EDTA-anticoagulated plasma, BAL fluid cells, and supernatant collected during these infections.

Influenza virus and virus proteins. Seasonal (A/Kawasaki/173/2001) and pandemic (A/California/04/2009) influenza virus stocks were expanded using Madin-Darby canine kidney (MDCK) cells as described previously (46, 47). Mammalian cell-expressed recombinant HA and neuraminidase (NA) proteins were purchased from Sinobiologicals (Shanghai, China).

HI assay. Hemagglutinin inhibition (HI) assays were performed using turkey red blood cells as previously described (48). Heat-inactivated receptor-destroying enzyme (RDE)-treated plasma was titrated from a starting dilution of either 1:8 or 1:10 to 1:1,280 in $1 \times$ phosphate-buffered saline (PBS). Titers were expressed as the reciprocals of the highest dilutions of plasma at which hemagglutination was prevented.

Microneutralization assay. Microneutralization assays were performed as described previously (48). Briefly, serum or plasma was heat inactivated at 56°C for 30 min prior to setting up the assay. Sixty microliters of Dulbecco's modified Eagle's medium (DMEM; HyClone, Logan, UT) was added to each well of a 96-well microtiter plate (Fisher Scientific, Pittsburgh, PA), and then an additional 48 μ l of DMEM was added to row A. Twelve microliters of heat-inactivated serum or plasma was then added to row A in quadruplicate, and 2-fold serial dilutions were performed by transferring 60 μ l from each row to the next. The final 60 μ l from the last row was discarded. The virus was diluted to 100 50% tissue culture infective doses (TCID₅₀) per 50 μ l in DMEM, and 60 μ l of diluted virus was added to each well containing serum or plasma dilutions, except for the mock infection wells. Sixty microliters of DMEM was added to all mock infection wells. Back titration of the test virus was performed by adding 438 μ l of dilute virus (100 TCID₅₀ per 50 μ l) to the first of a series of eight tubes. Next, 300 μ l of DMEM was added to each of the other seven tubes, and 138 μ l was transferred from the virus test dilution serially down the row of tubes. Sixty microliters of DMEM was added to each of the virus back-titration wells, and then 60 μ l of the respective back-titration mix was transferred to a microtiter plate. The virus-serum mixture was mixed and incubated at 37°C for 2 h. Confluent MDCK cells in a 96-well plate were washed twice with serum-free DMEM. After the 2-h incubation, 100 μ l from each virus-serum well was transferred to the corresponding well in the MDCK plate and incubated for an additional 2 h at 37°C. Following this incubation, the MDCK cells were washed with serum-free DMEM, and 200 μ l of complete DMEM (DMEM supplemented with 10% fetal bovine serum, L-glutamine, and penicillin-streptomycin [HyClone, Logan, UT]) containing tosylsulfonyl phenylalanyl chloromethyl ketone (TPCK)-trypsin at the appropriate concentration was added. The plate of MDCK cells was incubated for 3 days at 37°C and 5% CO₂ and then observed under an inverted microscope for viral cytopathic effect (CPE). The highest dilution of each serum completely protecting the cell sheet from CPE in at least 2 wells out of 4 was considered the viral antibody titer.

ADCC NK cell activation assay. We recently described novel influenza virus-specific ADCC assays in humans and adapted these to study macaque samples (57, 58). Wells of a 96-well enzyme-linked immunosorbent assay (ELISA) plate (Nunc, Rochester, NY) were coated overnight at 4°C with 600 ng/well of purified influenza virus protein in $1 \times$ PBS (HyClone, Logan, UT). Wells were washed repeatedly with $1 \times$ PBS and incubated with heat-inactivated EDTA-anticoagulated plasma (56°C for 60 min; diluted as indicated) for 2 h at 37°C. Plates were washed repeatedly

with $1 \times$ PBS, and then 10^6 freshly isolated naïve rhesus macaque peripheral blood mononuclear cells (PBMCs) were added to each well. PBMCs isolated from healthy naïve macaques by use of Ficoll-Paque (GE Healthcare, Madison, WI) were washed and resuspended in R10 medium (RPMI 1640 supplemented with 10% fetal calf serum, penicillin, streptomycin, and L-glutamine; HyClone, Logan, UT). In addition, anti-human CD107a–allophycocyanin (APC)–H7 (H4A3 clone; BD Bioscience, San Jose, CA), 5 μ g/ml brefeldin A (Sigma, St. Louis, MO), and 5 μ g/ml monensin (GolgiStop; BD Bioscience, San Jose, CA) were added to each well and incubated for 5 h at 37°C with 5% CO₂. Cells were then incubated with anti-CD3–Pacific Blue (SP34-2 clone; BD Bioscience, San Jose, CA), anti-CD14–phycoerythrin (PE)–Cy7 (M5E2 clone; BD Bioscience, San Jose, CA), and anti-NKG2A–APC (clone Z199; Beckman Coulter, Brea, CA) surface antibodies for 30 min at room temperature in the dark. Cells were then fixed with 1% paraformaldehyde (Sigma-Aldrich, St. Louis, MO) for 10 min and permeabilized using $1 \times$ FACS permeabilizing solution 2 (BD Bioscience, San Jose, CA) for 10 min. Finally, cells were incubated with anti-IFN- γ –AF700 (B27 clone; BD Bioscience) at room temperature for 1 h. Cells were fixed with 1% paraformaldehyde and acquired on an LSRII flow cytometer (BD Bioscience, San Jose, CA), with at least 200,000 lymphocyte events collected. Samples were analyzed using FlowJo, version 9.2 (TreeStar, Ashland, OR).

ELISA. Briefly, Immulon 2HB plates (Thermo Fisher Scientific, Waltham, MA) were coated with approximately 1 μ g of antigen in PBS at 4°C overnight. Antigen was removed, and plates were washed six times with PBS plus 0.05% Tween 20. Plates were then blocked with PBS plus 0.05% Tween 20 containing 1% bovine serum albumin (BSA). Plasma was diluted 1:100 in PBS, and 100 μ l was added to each well. Plates were incubated with diluted plasma at 37°C for 2 h and then washed six times with PBS plus 0.05% Tween 20. Mouse anti-human–horseradish peroxidase (BD Biosciences, San Jose, CA) was diluted 1:1,000 in PBS, and 100 μ l was added to each well and incubated at room temperature for 1 h. The plate was then washed six times with PBS plus 0.05% Tween 20, and SureBlue TMB Microwell peroxidase substrate (KPL, Gaithersburg, MD) was added and incubated at room temperature. When a blue color change was present in control wells, 100 μ l of 1 N HCl was added to each well to stop the reaction. Absorbance was measured at 450 nm.

Plaque reduction assay. The plaque reduction assay was performed as previously described (49). Protein G HP SpinTrap columns (GE Healthcare Life Sciences, Pittsburgh, PA) were used to isolate total IgG from plasmas obtained from rhesus monkeys challenged with seasonal H1N1 influenza virus according to the manufacturer's standard protocol, with slight modifications. Plasma was incubated with protein G Sepharose for 1 h with mild shaking, instead of the standard 5 min. Eluted IgG was buffer exchanged in PBS by using an Amicon Ultracel-30K centrifugal filter unit (Millipore, Billerica, MA) with a 30-kDa molecular mass cutoff in a swinging-bucket rotor. Protein concentrations were measured on a NanoDrop 2000 spectrophotometer at an absorbance of 280 nm. Stock A/California/04/2009 virus was diluted to 80 PFU/well and incubated with 3-fold serial dilutions of total IgG for 1 h at room temperature. Twelve-well plates seeded with MDCK cells were washed twice with PBS. Next, 300 μ l of antibody-virus mixture was absorbed onto MDCK cells for 45 min at 37°C. Virus-antibody mixtures were then aspirated off cells, which were washed once with PBS. One milliliter of agar overlay containing an appropriate antibody concentration and TPCK-trypsin was added to each well. Plates were incubated for 2 days at 37°C and then fixed with 10% formalin at room temperature for 1 h. Plaques were visualized and counted by eyesight.

Statistical analyses. Statistical analyses were performed using SPSS, version 18, software (IBM, Armonk, NY) and Graphpad Prism, version 4 (GraphPad Software Inc., San Diego, CA). Data in Fig. 3 were statistically analyzed by the Wilcoxon signed-rank test, with an alpha level of 0.05. Data in Fig. 4A and B and 5A and B were analyzed using Mann-Whitney U tests.

TABLE 1 Summary of H1N1pdm-specific macaque HI titers, neutralization titers, and ELISA binding prior to H1N1 challenge

Animal group and no.	Prechallenge HI titer ^a	Prechallenge neutralization titer ^b	Plaque reduction neutralization titer ^{c,d}	ELISA OD ^e
Animals primed with sH1N1 infection				
rh2306	<10	<10	>150	1.316
r02108	<10	<10	>150	1.405
r01072	<10	<10	>150	0.472
r02027	<10	<10	>150	0.537
r03079	<10	<10	>150	0.473
Naïve animals				
r02002	<10	<10	ND	0.145
r03087	<10	<10	ND	0.149
r03089	<10	<10	ND	0.153
r03137	<10	<10	ND	0.152
r04052	<10	<10	>150	0.254
r04077	<10	<10	>150	0.172
r05092	<10	<10	ND	0.144

^a Serum HI titer against pandemic A/California/04/2009 virus on day of H1N1pdm challenge.

^b Serum microneutralization titer on day of H1N1pdm challenge.

^c Concentration of IgG (μ g/ml) required to inhibit 50% of A/California/04/2009 plaques. ND, not determined.

^d As a positive control, 5.5 μ g/ml was required to achieve 50% inhibition for animal rh2306 at 21 days post-H1N1pdm challenge.

^e Sample optical density (OD) at 450 nm against purified HA protein from A/California/06/2009 at day 7 post-sH1N1 infection.

RESULTS

Detection of binding but nonneutralizing cross-reactive influenza virus antibodies. We recently showed that prior infection of macaques with a seasonal H1N1 influenza virus strain resulted in partial protection from challenge with a swine-origin pandemic H1N1 influenza virus (17). During this study, five macaques were “primed” by intranasal infection with seasonal influenza virus A/Kawasaki/173/2001 (sH1N1) and subsequently infected with the pandemic virus A/California/04/2009 (H1N1pdm). The HA amino acid sequences of these two viruses are less than 80% identical. Virus titers following the H1N1pdm challenge were compared to those of seven influenza-naïve macaques infected with the same stock of H1N1pdm virus. The “primed” animals cleared H1N1pdm infection significantly faster than the naïve animals. This clearance was associated with a rapid expansion of cross-reactive T cell responses, while antibodies capable of neutralizing H1N1pdm were not detectable (by HI or microneutralization assay) until after all animals had cleared infection (Table 1). To further exclude the presence of stalk-reactive neutralizing antibodies to H1N1pdm HA prior to the H1N1pdm challenge, we also performed a plaque reduction neutralization assay (49). No plaque-reducing neutralizing antibodies were present prior to challenge (Table 1).

Interestingly, we also noted that the sH1N1 influenza virus infection induced antibodies that bound H1N1pdm HA by ELISA. These binding but nonneutralizing antibodies were detectable in “primed” animals at the time of H1N1pdm challenge and increased in titer within the first 7 days of infection. This suggested that nonneutralizing antibodies could also play a role in the resolution of the H1N1pdm infection, potentially by mediating an effector function such as ADCC through NK cells (Table 1) (17). We therefore modified our previously described assays for detection of HIV-specific ADCC in humans to measure influenza virus-

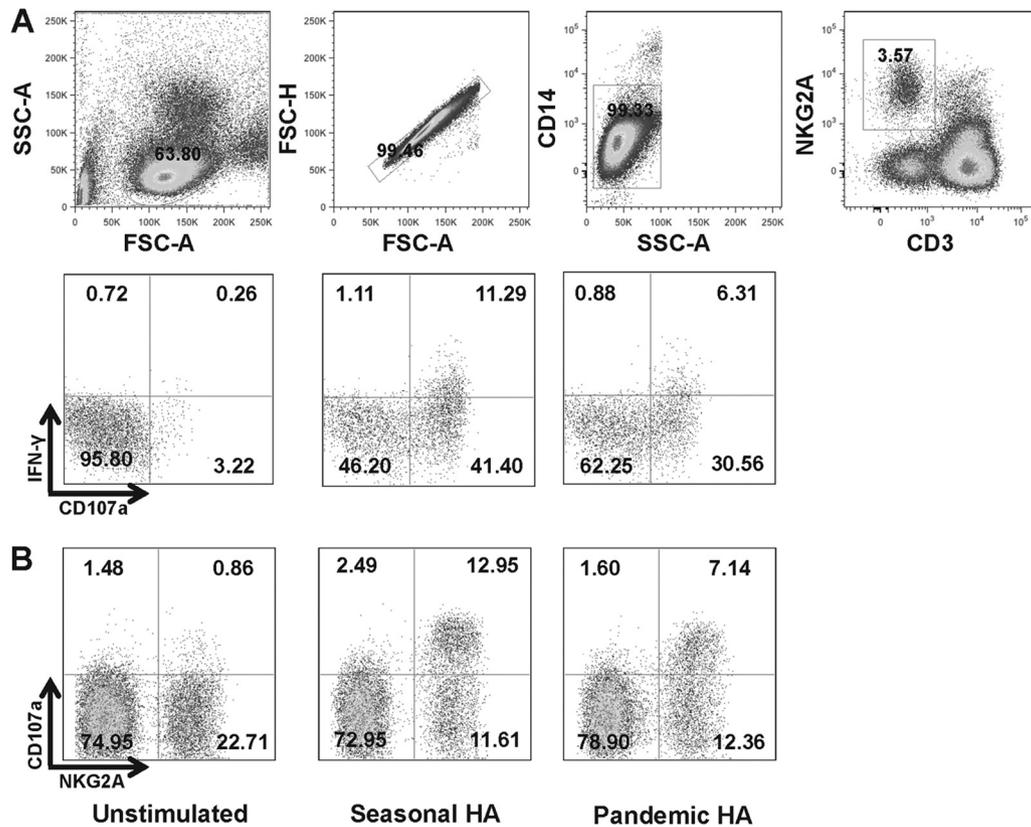


FIG 1 Activation of macaque NK cells by use of immobilized HA protein and plasmas from influenza virus-infected macaques. We modified our previously described assay for detecting ADCC-mediating antibodies for use with influenza virus-infected macaques. (A) Gating strategy for measuring NK cell IFN- γ and CD107a expression. Macaque PBMCs were stimulated with either seasonal or pandemic virus HA protein in the presence of plasma taken from a “primed” animal on day 7 after infection with H1N1pdm. (B) CD107a expression is mostly restricted to CD3⁻ NKG2A⁺ NK cells.

specific ADCC in macaques (38, 39, 50). We assessed rhesus macaque CD3⁻ CD14⁻ NKG2A⁺ NK cells in peripheral blood for expression of intracellular IFN- γ and surface CD107a (a degranulation marker) in the presence of immobilized influenza virus HA protein from the sH1N1 virus New Caledonia/20/1999 or the H1N1pdm virus A/California/04/2009. The HA of A/New Caledonia/20/1999 is 98% identical at the amino acid level to that of the sH1N1 virus used to infect the 5 “primed” macaques. Figure 1A shows the assay setup and gating strategy. NK cell activation in macaques was predominantly restricted to CD3⁻ CD14⁻ NKG2A⁺ NK cells, confirming that these markers sufficiently encompassed most of the ADCC-responding NK cells (Fig. 1B).

sH1N1 influenza virus infection induces HA-specific ADCC-mediating antibodies that cross-react with H1N1pdm virus. We hypothesized that some of the binding but nonneutralizing antibodies to H1N1pdm induced by sH1N1 infection could mediate ADCC. We therefore characterized the NK cell-activating activities of antibodies against both sH1N1 and H1N1pdm HA proteins prior to and following sH1N1 infection. We measured the levels of antibody-mediated NK cell activation (both intracellular IFN- γ and the cell surface degranulation marker CD107a) in response to the immobilized sH1N1 or H1N1pdm HA protein. Macaque plasma samples collected prior to sH1N1 infection induced negligible NK cell activation in response to either seasonal or pandemic influenza virus HA protein (median IFN- γ and CD107a expression by 0% of NK cells in the presence of either HA) (Fig. 2A

and C). However, plasmas from all “primed” animals taken 2 weeks after sH1N1 infection induced expression of both IFN- γ and CD107a on NK cells in the presence of both seasonal (median IFN- γ and CD107a expression by 4.1% and 33.8% of NK cells, respectively) and pandemic (median IFN- γ and CD107a expression by 4.3% and 40.5% of NK cells, respectively) influenza virus HA proteins (Fig. 2B and D). Remarkably, for each individual animal, levels of IFN- γ and CD107a expression by NK cells were almost equivalent in the presence of either the seasonal or pandemic virus HA protein. These results indicate that sH1N1 influenza virus infection can elicit antibodies capable of binding HA proteins from divergent viruses.

We hypothesized that the titers of these antibodies would wane with time and therefore tested plasma samples taken at least 4 months after sH1N1 infection. Antibodies in these samples stimulated significantly less IFN- γ expression by NK cells in the presence of HA protein from either sH1N1 or H1N1pdm ($P = 0.043$ by the Wilcoxon signed-rank test) (Fig. 3). Antibody-induced NK cell CD107a expression also showed a similar trend of waning over time, but the difference did not reach statistical significance ($P = 0.08$ by the Wilcoxon signed-rank test). This suggests that the cross-reactive antibodies stimulated by sH1N1 infection decline over time following viral clearance.

sH1N1 and H1N1pdm HA-specific ADCC-mediating antibodies expand rapidly following H1N1pdm infection. If pre-existing cross-reactive ADCC-mediating antibodies were stimu-

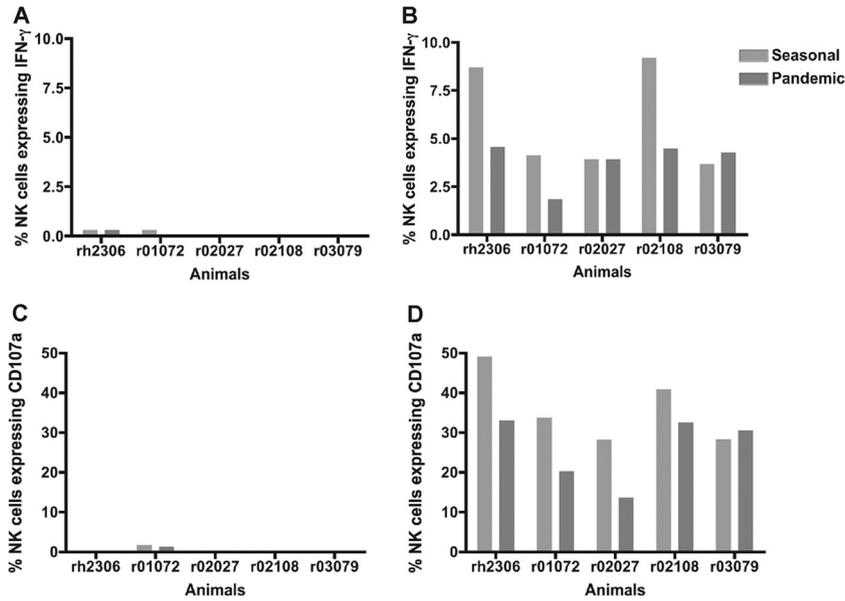


FIG 2 Infection with sH1N1 induces cross-reactive ADCC-mediated antibodies against H1N1pdm. Using the NK cell activation assay, we compared the abilities of plasma samples prior to (A and B) and 2 weeks after (C and D) sH1N1 infection to bind HA proteins from seasonal (light gray bars) and pandemic (dark gray bars) influenza viruses and to induce expression of IFN-γ or CD107a by NK cells.

lated by sH1N1 infection, one might expect H1N1pdm challenge to result in a rapid increase in anamnestic ADCC responses. We first tested the ability of macaque plasma obtained 28 days after H1N1pdm infection to stimulate NK cells in the presence of either the sH1N1 or H1N1pdm HA protein. We found robust NK cell activation in both “primed” and “naïve” animals in the presence

of HA proteins from both viruses. There was a significantly greater NK cell expression of both IFN-γ and CD107a in response to the sH1N1 HA protein in “primed” animals than in “naïve” animals ($P = 0.003$ by the Mann-Whitney test) (Fig. 4A and B). However, there was no significant difference in NK cell activation between “primed” and “naïve” animals in the presence of the H1N1pdm HA protein. This suggests that the H1N1pdm infection boosted preexisting antibody responses in “primed” animals, although by day 28, levels of H1N1pdm-specific ADCC were similar in both groups of infected animals.

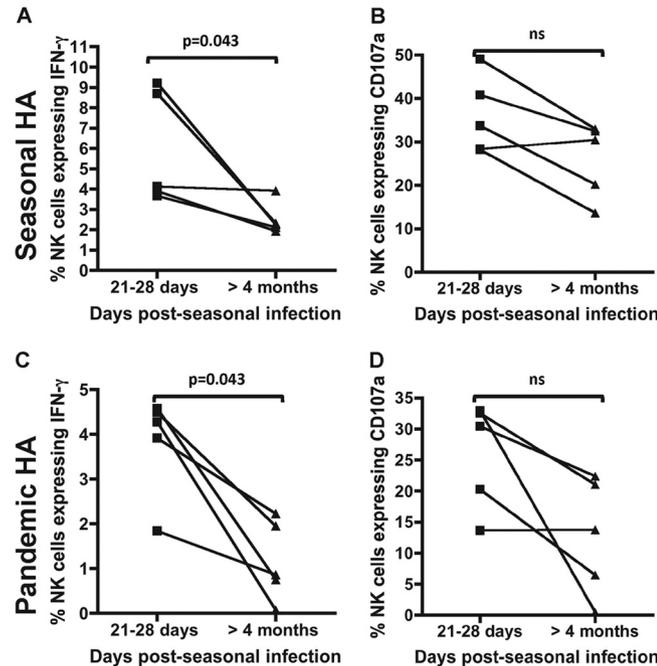


FIG 3 Cross-reactive ADCC-mediated antibodies wane over time. We obtained plasmas from sH1N1-infected macaques either 3 to 4 weeks or at least 4 months after sH1N1 infection and measured the ability of antibodies in these plasmas to stimulate IFN-γ and CD107a expression by NK cells in the presence of either sH1N1 HA (A and B) or H1N1pdm HA (C and D).

To further evaluate the kinetics of ADCC responses at much earlier time points throughout pandemic influenza virus infection, we measured NK cell activation in the presence of sH1N1 or H1N1pdm HA by using plasmas sampled through the first 7 days after H1N1pdm challenge. We tested plasma obtained at day 0 (just prior to H1N1pdm infection) and serial samples obtained 2, 3, 4, 5, and 7 days after H1N1pdm infection. For most “primed” animals, we observed an increase in the ability of H1N1pdm HA-specific antibodies in plasma to activate NK cells (both IFN-γ and CD107a expression) around 4 to 5 days after H1N1pdm infection (Fig. 4C and D, gray traces). In contrast, there was no noticeable increase in ADCC activity through the first 7 days of H1N1pdm infection in the two naïve animals tested (Fig. 4C and D, black traces).

The proportion of NK cells activated by antibodies in undiluted plasma is one measure of ADCC activity, but endpoint titrations provide additional measures of the strength of ADCC responses and allow comparisons with NAb titers. We therefore tested the ability of serial dilutions of plasma samples to stimulate NK cells in the presence of immobilized HA protein from H1N1pdm. On the day of H1N1pdm challenge, approximately 4 months after sH1N1 infection in “primed” animals, endpoint titers of detectable NK cell IFN-γ expression were no greater than 1:80 (Fig. 4E). Interestingly, however, within 1 week after challenge with H1N1pdm, plasmas from the “primed” animals con-

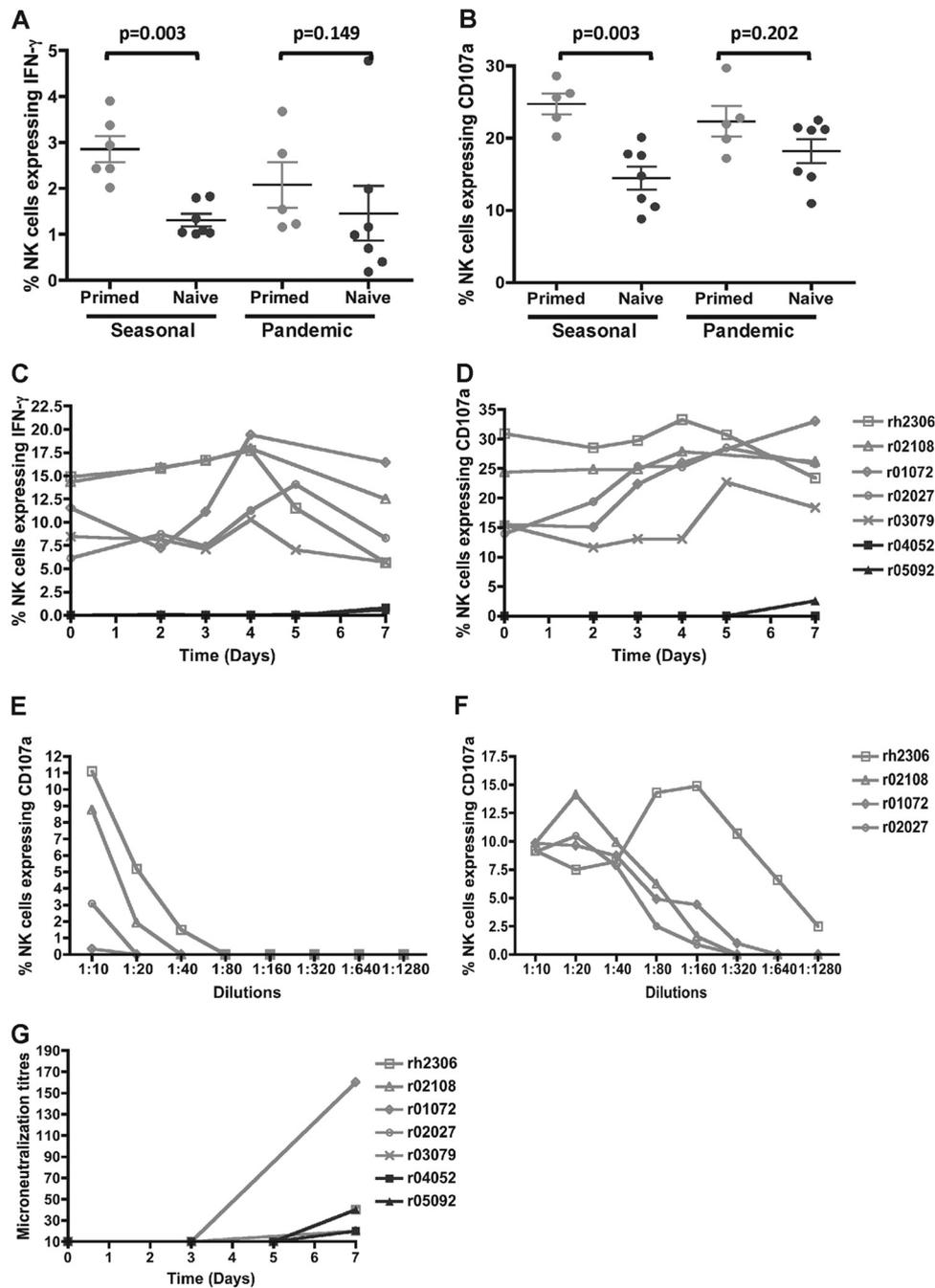


FIG 4 Cross-reactive ADCC-mediating antibodies are detectable within days of H1N1pdm challenge in “primed” but not naïve macaques. We compared the abilities of plasmas sampled from sH1N1-primed and naïve animals after H1N1pdm challenge to stimulate NK cells in the presence of the H1N1pdm HA protein. By 28 days after H1N1pdm challenge, animals in both the “primed” and naïve groups had antibodies capable of stimulating NK cells to produce IFN- γ (A) and CD107a (B) in the presence of H1N1pdm HA, but within the first 7 days of infection, only plasmas from “primed” animals contained detectable ADCC-mediating antibodies (C and D). To examine the concentrations of ADCC-mediating antibodies early after infection in primed animals, we tested the ability of serial dilutions of plasma samples taken on day 0 (E) or day 7 (F) after challenge to stimulate NK cell production of CD107a in the presence of H1N1pdm HA. (G) Titers of NAbs against A/California/04/2009 virus (H1N1pdm) at days 0, 3, 5, and 7 postchallenge, detected by microneutralization assay.

tained much higher titers ($\geq 1:320$) of antibodies capable of stimulating NK cell expression of IFN- γ in the presence of H1N1pdm HA (Fig. 4F). Notably, this rise in H1N1pdm HA-specific ADCC occurred during the period in which H1N1pdm virus titers declined in infected “primed” animals. These observations are therefore consistent with a role for ADCC in assisting in the control of H1N1pdm challenge.

NAbs are regarded as an important measure of protective immunity toward influenza virus, but the kinetics of induction of NAbs in comparison to nonneutralizing antibodies are not well characterized. To compare the relative titers of NAbs and ADCC-mediating antibodies in early H1N1pdm infection, we measured NAbs in macaque sera at days 0, 3, 5, and 7 postinfection and compared their titers to those of ADCC-mediating antibodies. By

TABLE 2 Summary of antibodies 7 days after H1N1pdm challenge

Animal group and no.	HI titer ^a	Microneutralization titer ^b	ADCC antibody titer ^c
Primed animals			
rh2306	<10	40	>1,280
r02108	<10	20	320
r01072	<10	160	640
r02027	<10	20	320
r03079	<10	40	ND
Naïve animals			
r02002	<10	10	<10
r03087	<10	80	<10
r03089	<10	40	ND
r03137	<10	20	ND
r04052	<10	20	ND
r04077	<10	20	ND
r05092	<10	40	ND

^a Serum antibody titer against pandemic A/California/04/2009 (H1N1pdm) virus, detected using an HI assay.

^b Serum antibody titer against H1N1pdm, detected using a microneutralization assay.

^c Serum ADCC antibody titer against the H1N1pdm HA protein. The endpoint titer was deemed positive only when NK cell activation was 3-fold higher than the background level. ND, not determined.

day 7 of H1N1pdm infection, H1N1pdm-specific NAb were detectable only by using the sensitive microneutralization assay and were undetectable using the HI assay (Table 2). In contrast, H1N1pdm-specific NAb were not detectable by microneutralization at day 5 post-H1N1pdm infection (Table 2 and Fig. 4G). Cross-reactive ADCC-mediating antibody titers increased following day 4 to 5 postchallenge. Additionally, at day 7 postchallenge, animals had higher levels of ADCC-mediating antibodies than NAb. In “primed” animals on day 7, NAb had a maximum titer of 1:160 (median titer, 1:40), whereas the ADCC-mediating antibody titers for all animals tested were mostly above 1:320 (Fig. 4G and Table 2). Both NAb and ADCC responses were detectable at day 7 post-H1N1pdm infection for “naïve” animals. Together, these data suggest that priming by prior influenza virus infection aids in the induction of cross-reactive ADCC-mediating antibodies but not cross-reactive NAb. The induction and expansion of cross-reactive ADCC-mediating antibodies to pandemic influenza virus may contribute to protection from influenza virus infection.

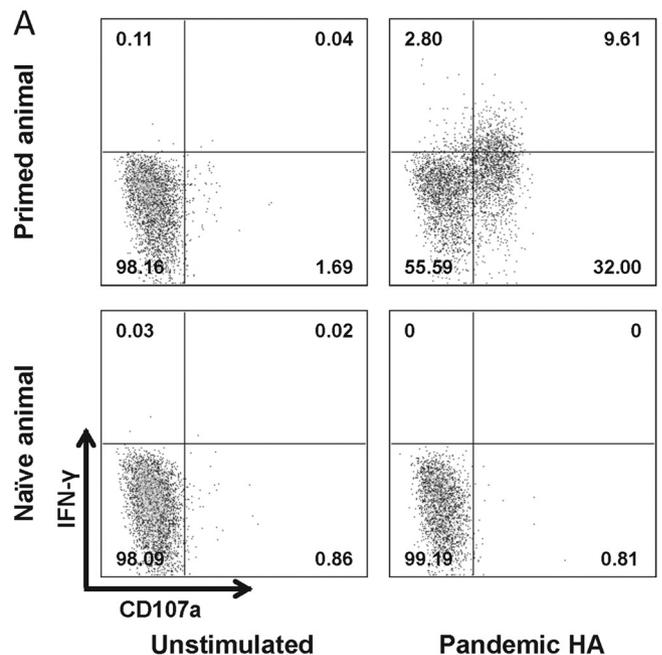
H1N1pdm HA-specific ADCC-mediating antibodies are present in the lungs of “primed” animals within 7 days of H1N1pdm challenge. The ADCC assays described above focused on antibodies present in plasma. While such samples provide a readily accessible measure of virus-specific immunity, it is important that in order to be effective at controlling virus replication, influenza virus-specific ADCC must be present in the respiratory tract. We previously showed that H1N1pdm replicates to high titers in the lower respiratory tract of macaques. Since influenza virus replication at this anatomical site is associated with severe pathogenic sequelae, we reasoned that the presence of ADCC-mediating antibodies in the lower respiratory tract might provide a particularly effective measure of their potential role in cross-protection against severe disease caused by divergent pandemic influenza viruses.

We therefore asked whether BAL fluid samples obtained after H1N1pdm infection of both primed and naïve animals contained

antibodies capable of stimulating NK cells in the presence of H1N1pdm HA. Strikingly, 7 days after H1N1pdm challenge, antibodies in BAL fluid from “primed” animals elicited production of both IFN- γ and CD107a by NK cells in the presence of immobilized H1N1pdm HA protein. In contrast, we detected no NK cell activation in the same assay when BAL fluids from naïve animals were used (Fig. 5A).

Elimination of virus-infected cells *in vivo* via ADCC would also require that NK cells in the lungs be sensitive to stimulation with ADCC-mediating antibodies. We therefore measured IFN- γ and CD107a expression by NK cells isolated by BAL from healthy macaques when the cells were stimulated with H1N1pdm HA protein in the presence of plasma taken 7 days after H1N1pdm infection. Indeed, lung NK cells were capable of activation in the presence of H1N1pdm-specific ADCC-mediating antibodies (Fig. 5B).

BAL Fluid



BAL NK cells

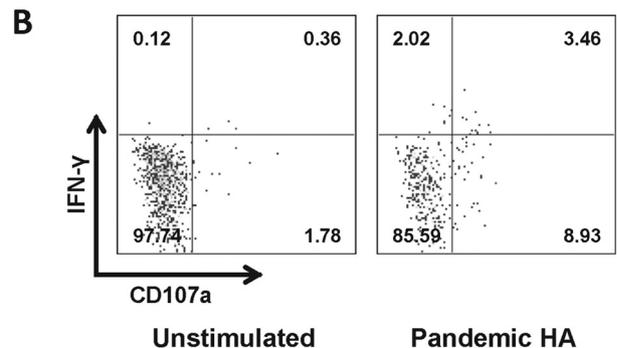


FIG 5 Macaque lung antibodies and NK cells are capable of ADCC-associated effector functions. (A) BAL fluid from “primed” or naïve animals taken 7 days after pandemic virus infection was used to stimulate peripheral NK cells to produce IFN- γ and CD107a in the presence of the H1N1pdm HA protein. (B) BAL fluid NK cells from an influenza-naïve macaque were stimulated using plasma from “primed” animals 7 days after pandemic virus infection, in the presence of the H1N1pdm HA protein.

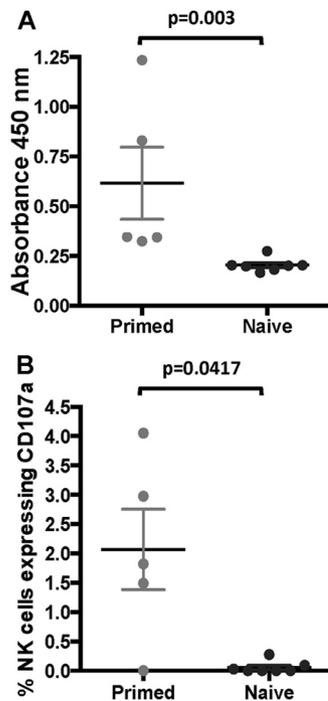


FIG 6 Cross-reactive NA-specific ADCC-mediated antibodies are detectable in sH1N1-primed but not naïve macaques. (A) We assessed the binding of antibodies to recombinant NA (A/California/04/09) via ELISA, using plasmas from sH1N1-primed and naïve animals prior to H1N1pdm challenge. (B) We compared the abilities of plasmas sampled from sH1N1-primed and naïve animals prior to H1N1pdm challenge to activate NK cells to express CD107a in the presence of the H1N1pdm NA protein.

H1N1pdm NA-specific ADCC-mediated antibodies are found in “primed” animals prior to H1N1pdm challenge. The presence of cross-reactive HA-specific ADCC-mediated antibodies in “primed” animals does not exclude a role for ADCC mediated by antibodies directed against other influenza virus antigens. As an envelope protein, NA in particular may also be a target of cross-reactive ADCC-mediated antibodies. We therefore used ELISAs to compare the levels of antibodies capable of binding the H1N1pdm NA protein in plasmas from primed and naïve animals prior to H1N1pdm challenge. We found that all 5 of the sH1N1 “primed” animals had detectable NA-binding antibodies (Fig. 6A). Indeed, for 4 of 5 “primed” animals, plasma samples taken after sH1N1 infection but before H1N1pdm challenge harbored antibodies capable of mediating ADCC in the presence of plate-bound NA protein (Fig. 6B). This result suggests that cross-reactive NA-specific antibodies may also contribute to protective immunity against divergent influenza virus strains.

DISCUSSION

A growing body of evidence suggests that prior infection with seasonal influenza virus strains leads to partial protection from divergent strains, although the mechanism(s) remains unclear. Cross-reactive T cell responses are likely to play an important role, although contributions from other immune responses may also be important, particularly in the very early control of infection. The NAb stimulated by infection with recent seasonal influenza viruses provide little or no neutralizing activity against antigenically distant influenza viruses; this was true for the swine-origin

H1N1pdm pandemic. Indeed, in our macaque study, infection with A/Kawasaki/173/2001 sH1N1 induced no detectable NABs against the H1N1pdm influenza virus, despite inducing partially protective immunity. However, we did detect binding antibodies induced by the sH1N1 infection that were capable of binding HA and NA from the H1N1pdm virus, suggesting a possible role for these nonneutralizing antibodies in protection. We found that HA-specific antibodies capable of activating NK cells induced by sH1N1 infection could also activate NK cells in the presence of H1N1pdm HA and NA. Moreover, these HA-specific ADCC-mediated antibodies rapidly expanded in sH1N1-primed animals within 5 days of H1N1pdm exposure, a time point at which H1N1pdm replication was being brought under control in “primed” animals and at which H1N1pdm-specific NABs remained undetectable by sensitive microneutralization assays. Importantly, ADCC was detectable in BAL fluid and was capable of activating lung NK cells. Our results suggest that cross-reactive ADCC induced by prior seasonal influenza virus infection could contribute to the partial protection from divergent influenza virus infections.

Previous exposure to seasonal influenza viruses, even decades in the past, may provide partial immunity to diverse and emerging influenza virus strains in humans (23, 51). This concept is supported by a recent study by O’Donnell et al., who measured protection provided by antigenically distant seasonal H1N1 influenza virus strains against pandemic influenza virus challenge in ferrets (23). Although “priming” with viruses from 1930 to 1976 protected ferrets against H1N1pdm challenge, there was very little cross-reactive NAb activity measured by HI or microneutralization assays. However, ELISAs showed that sH1N1 viruses that protected ferrets against H1N1pdm challenge induced higher levels of IgG antibodies capable of binding H1N1pdm HA than did sH1N1 viruses that did not induce cross-protective immunity. Similarly, in our studies, we saw an increase in cross-reactive binding antibodies following sH1N1 infection. Additionally, we showed that some of the binding but nonneutralizing antibodies induced by sH1N1 infection have ADCC-associated activity. These ADCC-mediated antibodies robustly activate macaque NK cells, causing them to produce the antiviral cytokine IFN- γ and to degranulate, as indicated by CD107a expression.

A critical issue in protection from influenza is the swiftness of the immune response, since influenza disease progresses rapidly, within days of infection. Memory T cell responses to influenza viruses can require several days to expand and contribute to virus control. ADCC-mediated antibodies are potentially attractive, since the innate effector cells (NK cells, monocytes, and neutrophils) that mediate ADCC do not require anamnestic expansion. In the setting of HIV infection, we have shown more rapid *in vitro* expression of effector molecules by ADCC-activated NK cells than activation of CD8⁺ T cells (52). The rapid infiltration of NK cells into lungs observed after influenza virus infection is consistent with a role for ADCC and/or other innate immune responses in the early control of virus replication.

Although this is the first description of influenza virus-specific ADCC in nonhuman primates, we recognize that there are several limitations to our study. First, we recognize that it is difficult to separate the roles of ADCC and T cells in controlling H1N1pdm replication, although recent experiments with humans suggest a limited role for T cell immunity (53). Future experiments using passive antibody transfer, adoptive T cell transfer, and antibody-

mediated depletion of lymphocyte subsets will help to delineate the distinct roles of these cells in mediating cross-protection. Major histocompatibility complex (MHC)-matched primate models have recently been developed that should assist in this work (54, 55). Second, we studied a limited number of animals (12 in total), making it difficult to correlate specific immune responses with heightened protection. Expanded studies will be required to better define the correlates of protection. Third, although we observed marked antibody-mediated NK cell activation in response to purified influenza virus antigens, further development of novel assays will be required to illustrate ADCC-mediated clearance of influenza virus-infected cells. The requirement to identify appropriate influenza virus-infected target cells that are not highly susceptible to direct NK cell killing makes this a challenging task. Fourth, our work does not address a role for vaccine-induced ADCC in controlling influenza virus infections. Vaccine regimens that induce ADCC responses will be of great interest. Fifth, we studied primarily HA-specific ADCC antibodies, although we also found that cross-reactive H1N1pdm NA-specific ADCC-mediating antibodies are induced following sH1N1 infection (Fig. 6). We cannot exclude the possibility that M2-specific ADCC-mediating antibodies may also assist in controlling influenza virus replication. M2-specific ADCC responses have been shown to mediate partial protective immunity to influenza viruses in murine models (32, 56). Sixth, we have not defined the regions or epitopes within HA or other proteins that are targeted by the antibodies described here. Recent work suggests that antibodies targeting the stem of HA can be broadly cross neutralizing (8, 14). Indeed, NABs such as these may also have an ADCC function. NABs in combination with ADCC-mediating antibodies are likely to provide the broadest protection against influenza virus infection. Further studies and characterization of influenza virus-specific ADCC responses in human and animal models will provide insights for future influenza vaccine initiatives.

In summary, we have investigated the correlates of cross-protective immunity against pandemic influenza viruses in a translational nonhuman primate model. Infection with sH1N1 generated antibodies capable of binding but not neutralizing H1N1pdm. These binding but nonneutralizing antibodies had ADCC activity that activated NK cells to produce both IFN- γ and CD107a in the presence of the H1N1pdm HA protein. Although ADCC activity waned with time after sH1N1 infection, it rapidly expanded within days of infection with H1N1pdm. The presence in the respiratory tract of both ADCC-mediating antibodies and NK cells suggests that ADCC may be a potential mechanism of cross-protection against divergent influenza viruses. Further studies in understanding the role of ADCC in protective immunity against influenza virus infection may provide valuable insights into future vaccine design.

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