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Cross-Reactive Influenza-Specific Antibody-Dependent Cellular Cytotoxicity Antibodies in the Absence of Neutralizing Antibodies

Sinthujan Jegaskanda,* Emma R. Job,* Marit Kramski,* Karen Laurie,[†] Gamze Isitman,* Robert de Rose,* Wendy R. Winnall,* Ivan Stratov,* Andrew G. Brooks,* Patrick C. Reading,*[†] and Stephen J. Kent*

A better understanding of immunity to influenza virus is needed to generate cross-protective vaccines. Engagement of Ab-dependent cellular cytotoxicity (ADCC) Abs by NK cells leads to killing of virus-infected cells and secretion of antiviral cytokines and chemokines. ADCC Abs may target more conserved influenza virus Ags compared with neutralizing Abs. There has been minimal interest in influenza-specific ADCC in recent decades. In this study, we developed novel assays to assess the specificity and function of influenza-specific ADCC Abs. We found that healthy influenza-seropositive young adults without detectable neutralizing Abs to the hemagglutinin of the 1968 H3N2 influenza strain (A/Aichi/2/1968) almost always had ADCC Abs that triggered NK cell activation and in vitro elimination of influenza-infected human blood and respiratory epithelial cells. Furthermore, we detected ADCC in the absence of neutralization to both the recent H1N1 pandemic strain (A/California/04/2009) as well as the avian H5N1 influenza hemagglutinin (A/Anhui/01/2005). We conclude that there is a remarkable degree of cross-reactivity of influenza-specific ADCC Abs in seropositive humans. Targeting cross-reactive influenza-specific ADCC epitopes by vaccination could lead to improved influenza vaccines. *The Journal of Immunology*, 2013, 190: 1837–1848.

Influenza pandemics cause worldwide alarm, morbidity, and mortality. Seasonal influenza virus infections cause >250 thousand deaths yearly, mostly in elderly and immune-compromised individuals (1). Although seasonally influenza virus infection can be prevented through vaccination, the effectiveness of this strategy is limited by the emergence of variant viruses resistant to Ab-mediated neutralization (2–4). Vaccination with trivalent influenza vaccines (comprising split inactivated preparations of appropriate H1N1, H3N2, and type B virus strains) primarily induces protective hemagglutinin (HA)-specific neutralizing Abs (5, 6). However, influenza surface HA proteins are highly susceptible to mutations, leading to loss of immune recognition overtime (antigenic drift), and therefore are required to be generated yearly (7, 8). Additionally, vaccination is thought to provide weak protection against emerging reassortant influenza viruses with novel

HA proteins (antigenic shift), which has historically led to significant worldwide influenza pandemics (9).

Influenza HA-specific Abs have been primarily characterized for their capacity to inhibit binding of the viral HA to cell surface sialic acid and/or to prevent viral fusion (10, 11). However, HA-specific binding Abs are likely to mediate other effector functions such as phagocytosis (12), complement activation (13, 14), and Ab-dependent cellular cytotoxicity (ADCC) (15–18). NK cells are an important component of the innate immune response mediating the clearance of virus-infected and transformed cells. The NK cell CD16 receptor binds the Fc portion of IgG1 Abs that in turn bind to Ags on virus-infected cells. This interaction induces both degranulation to release perforin/granzymes as well as secretion of antiviral cytokines such as IFN- γ and TNF- α . Nonneutralizing Abs have been shown to have an important role in several virus infection models (19–22).

There have been limited studies on influenza-specific ADCC Abs. In the late 1970s to early 1980s, Greenberg et al. and Hashimoto et al. (15, 16, 18) first demonstrated the presence of influenza-specific ADCC Abs using [⁵¹Cr] release assays. However, few studies followed in subsequent years, perhaps in part due to a lack of robust assays and reagents to characterize ADCC. There has been recent interest in the role of influenza-specific ADCC responses to the conserved M2 transmembrane viral protein expressed on the surface of virus-infected cells. Jegerlehner et al. (23) showed that partial protection was afforded by anti-M2 Abs in mice, which was mediated in part by ADCC. FcR knockout mice confirmed the importance of ADCC to M2 (24). There is a need to study influenza ADCC to conserved regions of other surface influenza proteins such as HA in human samples using improved assays.

To study influenza NK cell-mediated ADCC in more detail, we developed novel influenza ADCC assays to measure ADCC-inducing Abs in human and nonhuman primate samples. We

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Abbreviations used in this article: ADCC, Ab-dependent cellular cytotoxicity; ADCVE, Ab-dependent cell-associated viral elimination; HA, hemagglutinin; HI, hemagglutination inhibition; MN, microneutralization; MOI, multiplicity of infection; NP, nuclear protein; TCID₅₀, 50% tissue culture infectious dose.

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characterized ADCC responses to a 1968 H3N2 virus in adult plasma samples and found, in the absence of neutralizing Abs, ADCC-inducing Abs were both detectable and able to eliminate virus-infected PBMCs and respiratory epithelial cells in vitro. Furthermore, we observed ADCC responses to both pandemic H1N1 influenza virus and cross-reactive Abs to avian H5N1 proteins in the absence of neutralizing Abs, suggesting ADCC Abs to influenza can cross-react with multiple strains.

Materials and Methods

Ethics statement

The human studies were approved by the Alfred Hospital human research and ethics committee. All subjects provided written informed consent for blood samples.

Subjects

We recruited 10 healthy adults to donate blood samples. Seven of the subjects had been previously vaccinated with trivalent influenza vaccines one to nine times since 2003. The median age of the subjects was 30.5 y, range 22–52 y. We also obtained plasma from one influenza-seronegative infant, aged 5 y, and sera from seven influenza-seronegative pigtail macaques (*Macaca nemestrina*), aged 3–5 y. Studies on macaque samples were approved by the Commonwealth Scientific and Industrial Research Organization animal health ethics committee. Three of the macaques studied were subsequently infected with PR8 [A/Puerto Rico/8/1934 (H1N1)], followed by X-31 [A/Aichi/2/1968 (H3N2)] influenza viruses 4 wk apart (10^8 PFU via the respiratory tract). All three macaques became infected with both influenza viruses, as assessed by recovery of influenza RNA from respiratory tract samples and seroconversion, as previously described (25).

Influenza virus and virus proteins

The influenza viruses used in these studies were X-31, a reassortant virus expressing the HA and NA from A/Aichi/2/1968 (H3N2) with the internal genes from A/Puerto Rico/8/1934 (H1N1), and A/Auckland/01/2009 (A/California/07/2009-like 2009 pandemic H1N1). All infectious viruses were propagated in embryonated hen's eggs and stored at -80°C . Whole virus preparations had undetectable levels of endotoxin contamination (<0.06 EU/ml) as measured by *Limulus* amoebocyte lysate assay (Lonza, Walkersville, MD). Infectivity of viruses was quantified using the plaque assay on Madin-Darby canine kidney cells, as described previously (26). A sucrose gradient-purified, β -propiolactone-inactivated preparation of A/California/7/2009 virus (provided by CSL, Parkville, Australia) was used for hemagglutination inhibition (HI) assays. H3 (A/Aichi/2/1968), H1 (A/California/07/2009), and H5 (Anhui/01/2005) mammalian-expressed purified HA influenza virus proteins were purchased from Sinobiological (Shanghai, China).

HI assay

HI assays were performed on plasma samples, as previously described (27). Briefly, HI tests were performed by standard procedures in 96-well round-bottom microtest plates using 1% turkey erythrocytes. Plasma was titrated from a starting dilution of 1:10 to 1:1280 in PBS solution. Titers were expressed as the reciprocal of the highest dilution of plasma to completely inhibit 4 hemagglutinating units of virus.

Microneutralization assay

Viral titers were quantified by 50% tissue culture infectious dose (TCID₅₀) assay in 96-well flat-bottom plates, as described previously (28). Virus was detected by addition of 25 μl 1% turkey RBCs or by observation of cytopathic effects. Titers were calculated as described by Reed and Muench (29). The detection limit was 0.5 TCID₅₀ per 100 μl . For microneutralization (MN) assay, serum samples were assayed against egg-grown X-31 [A/Aichi/2/1968 (H3N2)] virus. Serum was inactivated at 56°C for 30 min, and then 2-fold dilutions of serum (from 1:10 to 1:1280) were mixed with 200 TCID₅₀ of each virus (1:1, \checkmark) and the samples were incubated at 35°C for 1 h. Serum/Virus mixes were added to washed Madin-Darby canine kidney–SIAT1 monolayers in 96-well flat-bottom plates and incubated at 35°C , 5% CO₂, for 1 h. Samples were replaced with serum-free medium containing 4 lg/ml trypsin, and plates were incubated for an additional 3 d. Virus was detected in the supernatant by the addition of 25 μl 1% turkey RBC. Wells containing fully hemagglutinated RBC were scored positive. Titres were expressed as the reciprocal of the highest dilution of

serum in which hemagglutination was prevented. Samples were analyzed in duplicate in both HI and MN assays. Duplicate titres differed by no more than 2-fold. Where there was a 2-fold discrepancy between titres, the lower value was used in analyses.

Anti-influenza virus protein IgG ELISA

Influenza-specific IgG in plasma were measured by direct ELISA. Wells of 96-well polyvinyl flat-bottom plates (Patttech) were coated overnight at 4°C with 100 μl /well of 200 ng/ml purified HA (H3; A/Aichi/2/1968 or H1; A/California/07/2009) or with PBS in coating buffer (pH 9.8; 2 mM Tris, 10 mM NaCl) as a background control. All subsequent steps were performed at room temperature. Wells were washed sequentially in PBST (PBS/0.1% Tween 20) and PBS and blocked with 5% casein in PBS for 2 h. After washing, 10-fold dilutions of plasma in 5% casein were added, incubated 4 h, and washed, and bound IgG was detected using HRP-conjugated rabbit anti-human IgG Ab (Sigma-Aldrich, St. Louis, MO). Color reactions were developed using 3,3'-5,5'-tetramethylbenzidine and stopped with 50 μl 1 M H₂SO₄. Absorbance was measured at 450 nm against a reference of 690 nm. The absorbance obtained for PBS background controls was subtracted from the corresponding HA absorbance values.

ADCC NK cell activation assay

A schematic of the ADCC NK cell activation assay is shown in Fig. 1. Wells of a 96-well ELISA plate (Nunc, Rochester, NY) were coated overnight at 4°C with either whole virus (10^6 PFU/well) or purified influenza protein (200 ng/well) in $1\times$ PBS. Wells were washed five times with PBS and incubated with heat-inactivated plasma/sera (56°C for 60 min) (dilution as indicated) for 2 h at 37°C . There was no significant difference in Ab-mediated NK cell activation when either serum or plasma was used in the assay when tested across six separate donors (mean of 2.93% for serum, mean of 2.69% for plasma, $p = 0.38$). Plates were washed five times with PBS, and then 10^6 freshly isolated human PBMCs from a healthy donor were added to each well. PBMCs isolated from healthy human donors using Ficoll-Paque (GE Healthcare, Madison, WI) were washed and resuspended in RF10 media (RPMI 1640, supplemented with 10% FCS, penicillin, streptomycin, and *l*-glutamine; Life Technologies, Grand Island, NY), and 10^6 cells were added to washed ELISA wells. In addition, anti-human CD107a allophycocyanin-Cy7 Ab (H4B4 clone; BD Biosciences, San Jose, CA), 5 $\mu\text{g}/\text{ml}$ brefeldin A (Sigma-Aldrich), and 5 $\mu\text{g}/\text{ml}$ monensin (Golgi Stop; BD Biosciences) were added and incubated for 5 h at 37°C with 5% CO₂. Cells were then incubated with anti-human CD3 PerCP (clone SP34-4), anti-human CD14 PE-Cy7 (clone M5E2), and anti-human CD56 allophycocyanin (clone B159; all from BD Biosciences) for 30 min at room temperature in the dark. Cells were fixed with 1% formaldehyde (Sigma-Aldrich) for 10 min and permeabilized using $1\times$ FACS permeabilizing solution 2 for 10 min (BD Biosciences). Finally, cells were incubated at room temperature for 1 h with IFN- γ AF700 (clone B27; BD Biosciences). Cells were again fixed with 1% formaldehyde, and acquisition was performed on the LSRII flow cytometer (BD Biosciences) with up to 1×10^6 lymphocyte events collected. Samples were analyzed using FlowJo version 9.2 (Tree Star, Ashland, OR). To assess reproducibility of the assay, a single plasma sample was tested on five separate occasions with relatively low interassay variability, as follows: mean of $2.37 \pm 0.31\%$ HA-specific NK cell activation.

Ab-dependent cell-associated viral elimination assay using autologous PBMCs

A schematic for the PBMC Ab-dependent cell-associated viral elimination (ADCVE) assay is shown as Fig. 4A. Briefly, PBMCs were isolated, washed twice in RF10 media, washed once in PBS, and resuspended in PBS to remove FCS necessary to avoid any nonspecific inhibition of influenza infection (30). PBMCs were then infected with influenza virus at a multiplicity of infection (MOI) of 1 for 1 h at 37°C , 5% CO₂. Following incubation, PBMCs were washed four times with RF10 media. A total of 100 μl infected PBMCs (10^6 cells/well) was incubated in a 96-well U-bottom tissue culture plate with or without 100 μl heat-inactivated plasma for 6 h at 37°C , 5% CO₂. Following incubation, cells were incubated with Live/Dead Fixable Aqua Dead Cell Stain (Life Technologies) for 30 min at room temperature in the dark. Subsequently, cells were incubated with anti-human CD3 PerCP (clone SP34-2), anti-human CD14 PE-Cy7 (clone M5E2), and anti-human CD56 allophycocyanin (clone B159; all from BD Biosciences) for 30 min at room temperature in the dark. Cells were fixed in 1% formaldehyde for 10 min and permeabilized, as described above. Finally, cells were incubated at room temperature for 1 h with anti-influenza nuclear protein (NP) Ab (431; Abcam, Cambridge, MA). Cells

were again fixed with 1% formaldehyde (Sigma-Aldrich), acquired by flow cytometry, and analyzed, as above. Relative/Absolute reduction was calculated as follows: $(\% \text{ of NP}^+ \text{ cells or total number of NP}^+ \text{ cells in the presence of plasma} - \% \text{ NP}^+ \text{ cells or total number of NP}^+ \text{ cells in the absence of plasma}) / \% \text{ NP}^+ \text{ cells or total number of NP}^+ \text{ cells in the absence of plasma} \times 100$. NP staining was only detected upon permeabilization, and not surface staining, demonstrating minimal detection of influenza only adhering to the surface of infected cells.

ADCVE assay using a human respiratory epithelial cell line

A schematic of the following assay is shown as Fig. 5A. A >90% confluent T250 flask of A549 cells (American Type Culture Collection) was washed once with PBS and infected with influenza virus at a MOI of ~ 10 for 1 h at 37°C, 5% CO₂. Following incubation, the monolayer was washed three times with RF10 and incubated in RF10 for an additional 3 h at 37°C in 10% CO₂. Adherent cells were then treated with trypsin versene (Media Preparation Unit, University of Melbourne, Microbiology and Immunology) to generate single-cell suspensions and incubated for 8 min at 37°C in 5% CO₂. Infected A549 target cells were then washed and resuspended in a total volume of 200 μ l and incubated with 50 μ l anti-PE MHC class I Ab (clone W6/32; eBioscience, San Diego, CA) for 20 min at room temperature in the dark. Following washing, 600,000 infected A549 cells were combined with 200,000 freshly isolated human PBMCs in a 96-well U-bottom tissue culture plate with or without 10 μ l heat-inactivated plasma and incubated for 6 h at 37°C, 5% CO₂. Following incubation, cells were stained with anti-human CD3 PerCP (clone SP34-2), anti-human CD14 PE-Cy7 (clone M5E2), and anti-human CD56 allophycocyanin (clone B159; all from BD Biosciences) for 30 min at room temperature in the dark. Cells were fixed in 1% formaldehyde for 10 min and permeabilized, as described above. Finally, cells were incubated at room temperature for 1 h with anti-influenza NP Ab (431; Abcam). Cells were again fixed with 1% formaldehyde (Sigma-Aldrich), acquired by flow cytometry, and analyzed as above with the relative/absolute reduction calculated as follows: $(\% \text{ of NP}^+ \text{ cells or total number of NP}^+ \text{ cells in the presence of plasma} - \% \text{ NP}^+ \text{ cells or total number of NP}^+ \text{ cells in the absence of plasma}) / \% \text{ NP}^+ \text{ cells or total number of NP}^+ \text{ cells in the absence of plasma} \times 100$.

Statistical analyses

Statistical analyses were performed using SPSS version 18 software (IBM, Armonk, NY) and Prism GraphPad version 5 (GraphPad Software, San Diego, CA). Data in Figs. 2–7 were analyzed by Mann-Whitney *U* test using an α level of 0.05. Data in Fig. 4B were analyzed by two-way ANOVA, after log₁₀ transformation (Levene's test was insignificant, $p = 0.370$, after transformation), followed by Bonferroni multiple comparisons test using an α level of 0.05.

Results

Detection of NK cell-activating Abs toward influenza virus

Previous studies of influenza-specific ADCC Abs have primarily used cell line-based cytotoxicity assays or have been conducted in mouse models. To study influenza-specific ADCC Abs in humans and nonhuman primates, we modified our previously described HIV ADCC assays (31, 32) and measured intracellular IFN- γ expression and degranulation (CD107a expression) by CD3⁺CD56⁺ NK cells after incubation with immobilized influenza Ag/Ab complexes (ADCC NK cell activation assay; Fig. 1A). Human adult plasma Abs triggered clearly detectable IFN- γ ⁺/CD107a⁺ expression in NK cells incubated with X-31 influenza virus (which expresses H3N2 proteins from A/Aichi/2/1968 strain) compared with NK cells from the same donor in the absence of plasma (1.29 versus 0.09% of NK cells; Fig. 1B). Protein A-purified Ig from the same donor showed similar NK cell activation compared with the plasma sample with 1.32% of NK cells expressing both IFN- γ and CD107a (Fig. 1B). The protein G IgG-depleted flow through resulted in negligible NK cell activation (data not shown). Taken together these data emphasize the Ab-mediated mechanism of NK cell activation within this ADCC assay. To further address the specificity of ADCC for influenza, we assessed both HIV-1 gp140 envelope and influenza HA-specific ADCC in donors with or without HIV infection. Plasma from HIV-infected individuals mediated robust NK cell activation against immobilized gp140 Ag, whereas

HIV-uninfected individuals only exhibited background NK cell activation. Plasma from both HIV-infected and uninfected subjects resulted in NK cell activation to immobilized HA protein (Supplemental Fig. 1).

Influenza-exposed individuals possess nonneutralizing X-31 virus-binding Abs

The initial finding of NK cell-activating Abs to X-31 was interesting given that the plasma donor was 31 y of age and the H3 and N2 proteins from this strain circulated primarily in the late 1960s, before the 30-y-old subject was born. To determine whether the ADCC response to X-31 may represent a cross-reactive response to other influenza viruses, we recruited a cohort of 10 healthy adults aged 22–52 y. First, a sensitive protein ELISA was used to detect Abs that bound to purified X-31 HA. All subjects had absorbance readings >0.7 (mean absorbance 1.59), indicating high levels of X-31 HA-binding Abs (Fig. 2A). Because most human adults have Abs to one or more strains of influenza virus following natural infection, we used plasma from four pigtail influenza-naïve macaques (*M. nemestrina*) as negative controls. We recently showed that 3- to 5-y-old pigtail macaques are seronegative to influenza but seroconvert following experimental infection with influenza (25). As expected, plasma from naïve macaques did not contain Abs capable of binding the X-31 HA protein (median OD of 0.0885, significantly less than median OD 1.5745 of the human subjects, $p = 0.005$; Fig. 2A). Next, we tested adult human and naïve macaque plasma for neutralizing Abs by both hemagglutination inhibition (HI) and MN assay against X-31 virus. Neutralizing and HI titers toward X-31 were below detection in plasma from influenza-naïve macaque samples (HI ≤ 40 , 95% confidence interval 5%) and very low (9/10 adults ≤ 40 , 95% confidence interval 0.31–13.84%) in adult human plasma (Fig. 2B). Together, these data indicate the presence of influenza-binding Abs in the absence of neutralizing Abs.

The low neutralizing titres of human plasma against X-31 indicate that recent infection with this (or an antigenically closely related) virus was unlikely. Thus, the detection of ADCC to X-31 using the NK cell activation assay in the initial subject tested shown in Fig. 1 was surprising. To further investigate, we tested plasma from all 10 cohort members and the naïve macaque controls in the NK cell activation assay, measuring the levels of both intracellular cytokine (IFN- γ) expression and the degranulation marker CD107a in response to immobilized X-31 whole virus (Fig. 2C, 2D). Remarkably, plasma from all 10 adult humans elicited robust NK cell IFN- γ (median of 2.37%) and CD107a expression (median of 4.20%) responses from NK cells in the presence of X-31 virus, which were significantly higher than those of influenza-naïve macaques (IFN- γ : macaque median 0.0345%, $p = 0.005$; CD107a: macaque median 0.05%, $p = 0.005$; Fig. 2C).

Because prior influenza infection is virtually ubiquitous in adults and we found influenza-specific ADCC was very common in adult humans, it becomes problematic to define an appropriate ADCC-negative control. As the total IgG content of human and macaque sera is similar [ranging from 5.3 to 16.3 mg/ml in adults and 9.7 to 17.3 mg/ml in macaques (33, 34)], we first tested three pigtail macaque sera known to be influenza seronegative as a negative control. We found that ADCC-mediated NK cell activation was very low in the macaque sera samples (Supplemental Fig. 2A). Additionally, to confirm that macaque plasma can mediate Ab-induced NK cell activation, we tested plasma from three macaques prior to (Pre-Flu) and following sequential infection (Post-Flu) with PR8 and X-31 viruses (Supplemental Fig. 2A). All animals seroconverted to PR8 virus and had HI titres of >40 toward PR8 virus (Supplemental Fig. 2B). As expected, we detected robust Ab-dependent NK cell IFN- γ expression only after influenza in-

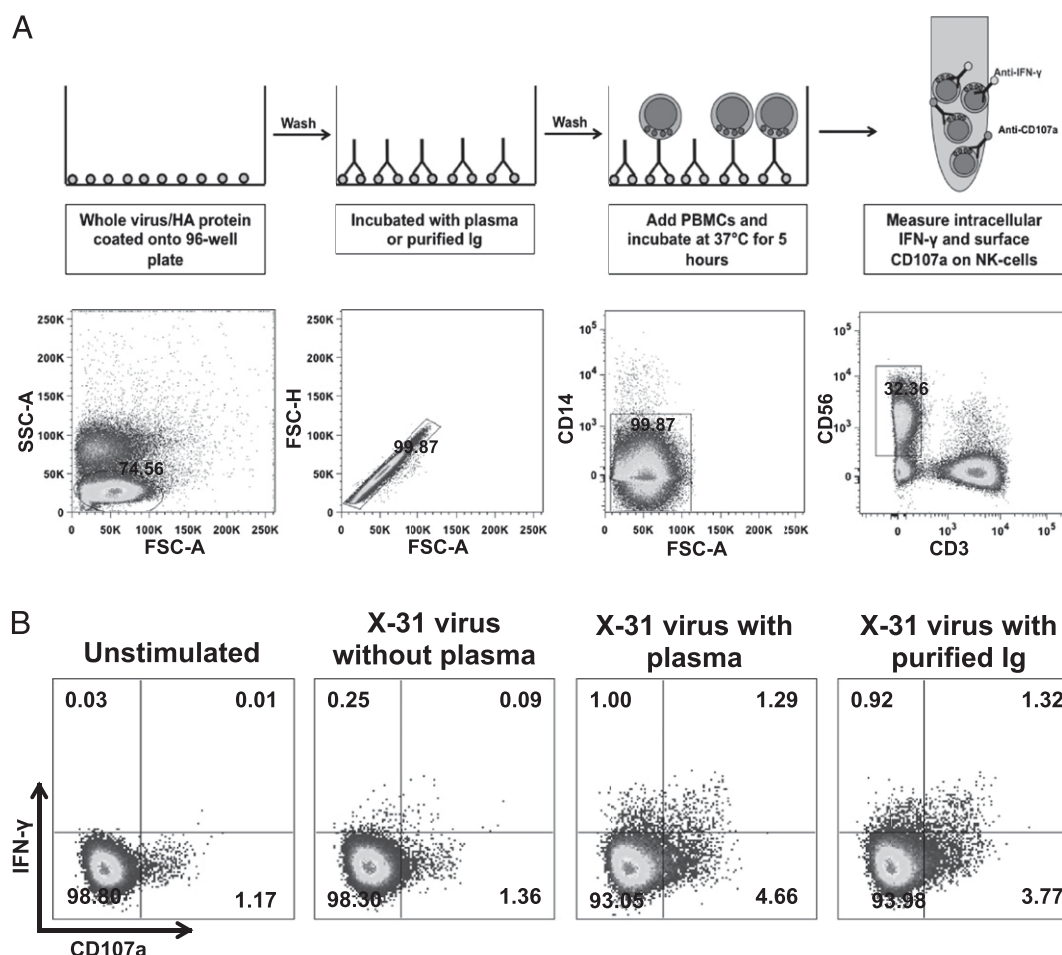


FIGURE 1. ADCC NK cell activation assay using plate-bound Ag–Ab complexes. **(A)** Assay setup and gating strategy for flow cytometry analysis. Influenza virus (10^6 PFU/well) or viral protein (200 ng/well) was coated onto a 96-well plate, washed, and incubated with either plasma or protein A-purified Ig. Healthy donor PBMCs were added and incubated for 5 h in the presence of CD107a, monensin, and brefeldin A. Cells were stained for cell surface expression of CD14, CD3, and CD56 and intracellularly stained for IFN- γ prior to analysis by flow cytometry. Lymphocytes by size/granularity (FSC-A versus SSC-A), ensuring they were single cells (FSC-A versus FSC-H), excluding monocytes (CD14 $^{-}$) and using the markers CD3 $^{-}$ CD56 $^{+}$ cells. **(B)** Influenza virus-mediated NK cell activation in the presence of human Ig. PBMC were incubated with X-31 virus in the presence or absence of heat-inactivated plasma or with purified Ig from an influenza-exposed adult. The proportion of CD3 $^{-}$ CD56 $^{+}$ expressing CD107a and/or IFN- γ is shown.

fection (mean of 1.9% Post-Flu), but not prior to infection (mean of 0.17% Pre-Flu; Supplemental Fig. 1A).

Characterization of NK cell-activating Abs toward influenza X-31 HA proteins in influenza-exposed individuals

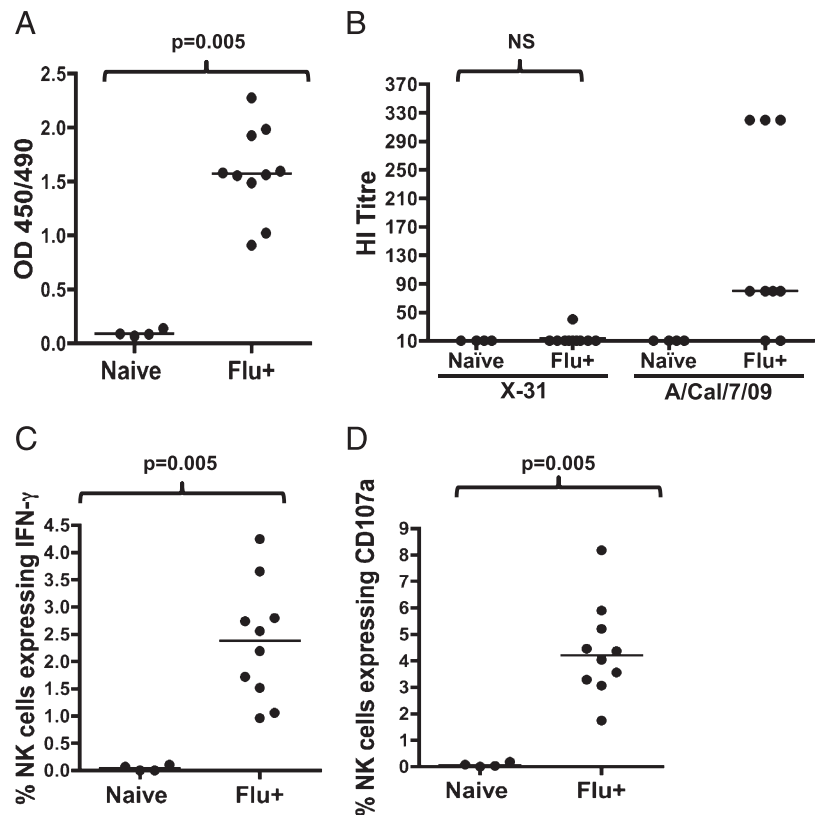
HA is the predominant glycoprotein expressed on the surface of influenza virions. Therefore, we hypothesized that responses to H3 HA protein of X-31 were likely to constitute a significant proportion of the overall NK cell response to X-31 virus. To directly assess H3 HA-specific activating Abs, we measured NK cell activation in response to plate-bound purified X-31 HA H3 protein. We observed substantial NK cell IFN- γ /CD107a expression to X-31 HA in the presence of human plasma (0.77% versus 0.00%, in the absence of plasma; Fig. 3A). X-31 HA protein induced NK cell activation (both IFN- γ and CD107a expression) in all 10 of our adult human subjects examined (IFN- γ median 1.8285%, CD107a median 4.70%), which was significantly higher than the responses induced by plasma from influenza-naïve macaques (IFN- γ : macaque median 0.0585%, $p = 0.005$; CD107a: macaque median 0.375%, $p = 0.005$; Fig. 3B).

Plasma from influenza-naïve macaques served as a useful negative control; however, it was important to confirm that plasma from influenza-naïve humans did not induce ADCC NK cell ac-

tivation. However, given the ubiquity of influenza infections in humans, such samples are rare in adults. We obtained a small amount of serum from a 5-y-old child who was seronegative to influenza by ELISA and who had no detectable HI Abs to recently circulating strains of type A or type B influenza viruses (data not shown). This sample of human influenza-seronegative serum did not induce significant activation of NK cells (0.01% of NK cells expressing IFN- γ and CD107a; Supplemental Fig. 3). Additionally, commercially available influenza-negative serum (MBL, Woburn, MA) at a 1:1 dilution was found to result in <0.2% of NK cells expressing CD107a in the presence of X-31 HA protein, whereas using influenza-exposed plasma at the same dilution, 3.79% of NK cells expressed CD107a.

The proportion of activated NK cells measured by the NK cell activation assay at a single Ab dilution may not necessarily reflect the serum titer of ADCC Abs. We therefore titrated the plasma against X-31 HA protein to determine ADCC titers and compared these with HI titers and MN titres obtained using X-31 virus (Table I). Responses were considered positive when the magnitude of NK cell activation was above the background levels (i.e., NK cells without Ag but with plasma) defined by CD107a expression. Most individuals had both neutralizing and HI titers of <40, whereas ADCC Ab titers required for NK cell activation were >40 in 8

FIGURE 2. Influenza-specific ADCC in the absence of HI-neutralizing Abs in influenza-exposed individuals. Anti-influenza Abs in plasma from 10 influenza-exposed (Flu⁺) adults and 4 influenza (naive) pigtail macaques were detected by **(A)** protein-ELISA using purified X-31 (A/Aichi/2/1968) HA protein (H3) and plasma (1:100), as described in *Materials and Methods*. **(B)** Serum HI titers against X-31 virus (A/Aichi/2/1968) and Cal/09 virus (A/California/07/09). **(C)** IFN- γ and **(D)** CD107a expression by CD3⁺CD56⁺ NK cells following incubation with 10×7 PFU X-31 virus in the presence of influenza-exposed (Flu⁺) or naive plasma. Values for protein ELISA and NK cell activation assay were all background subtracted. Influenza-exposed and naive samples for all assays were compared using a Mann-Whitney *U* test where $p < 0.05$ was considered significant. Lines on the graphs denote median values.



of 10 of our human cohort plasma samples (Table I). As expected, plasma samples from influenza-naive macaques all maintained ADCC titers of <10 .

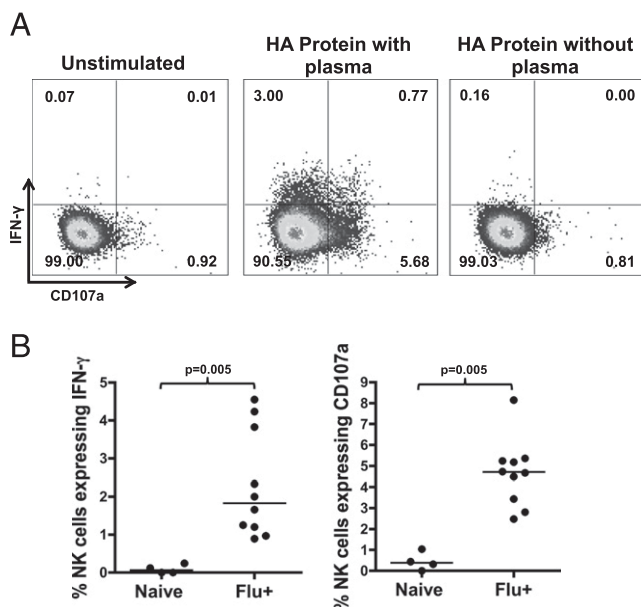


FIGURE 3. Influenza-specific ADCC toward H3 HA protein. **(A)** NK cell activation to X-31 (A/Aichi/2/1968) HA protein (H3) in the presence or absence of plasma. Representative dot plots of IFN- γ and CD107a expression by CD3⁺CD56⁺ NK cells are shown. **(B)** ADCC plate-bound NK cell activation assay was used for IFN- γ (left) and CD107a (right) expression by CD3⁺CD56⁺ NK cells following incubation with plasma from 10 influenza-exposed (Flu⁺) adults or 4 influenza-naive pigtail macaques. Values shown were background subtracted. Naive samples and Flu⁺ samples were compared using a Mann-Whitney *U* test where $p < 0.05$ was considered significant. Lines on the graphs denote median values.

Measuring the reduction of virus-infected autologous PBMCs in the presence of ADCC influenza-specific Abs

The ability of Abs to activate NK cells is an important measure of their function, but does not directly measure their capacity of NK cells to clear virus-infected cells. To further evaluate the functional capacity of X-31 influenza-specific ADCC Abs, we developed a novel assay to measure clearance of virus-infected cells in autologous human blood samples. Target cells were generated by incubating PBMCs with influenza virus for 1 h, washing to remove free virus, and then incubating cells in the presence or absence of plasma containing ADCC Abs. Leukocyte populations in PBMC were identified by FACS and the percentage of virus-infected cells quantified by intracellular staining for influenza NP. Concomitantly, NK cell degranulation was assessed by detection of cell surface expression of CD107a (Fig. 4A). This ADCVE assay measures the proportion of NP-expressing infected T cells in the presence of autologous plasma minus the proportion in the absence of plasma. The presence and activity of ADCC Abs in plasma samples from different individuals can then be compared. Because cells are only allowed to incubate for 7 h in the absence of trypsin, the assay measures clearance of infected cells rather than restriction of a spreading infection. Furthermore, because the assay system only uses autologous cells within the same PBMC donor, background killing of virus-infected cells by direct NK cell killing is reduced.

The efficiency of influenza infection of PBMCs is comparatively low; a maximum infection of 21% of T cells, 49.4% of CD14⁺ monocytes, and 28.7% of CD19⁺ B cells was observed. Incubation of influenza-infected PBMC in the presence of plasma or purified Ig resulted in substantially decreased numbers of influenza NP-expressing (infected) T and B cells, but no significant reduction in infected monocytes or NK cells ($F_{(3,48)} = 26.7$, $p < 0.001$; Fig. 4B). The lack of significant reduction in monocytes may be due to adherence of infected monocytes, monocyte cell death, or intrinsic

Table I. Serum-neutralizing and ADCC Ab titers to X-31 (H3N2) virus

Sample	HI Titer ^a	Neutralizing Titer ^b	ADCC-Ab Titer ^c
Flu ⁺ 1	<10	ND	80
Flu ⁺ 2	10	<40	80
Flu ⁺ 3	<10	<40	20
Flu ⁺ 4	40	80	80
Flu ⁺ 5	<10	<40	>160
Flu ⁺ 6	10	<40	>160
Flu ⁺ 7	<10	<40	40
Flu ⁺ 8	<10	<40	40
Flu ⁺ 9	<10	<40	>160
Flu ⁺ 10	<10	<40	2
Flu naive 1	<10	<40	<10
Flu naive 2	<10	<40	<10
Flu naive 3	<10	<40	<10
Flu naive 4	<10	<40	<10

^aHI titer against X-31 virus. The range of detection for HI was between 10 and 1280.

^bMicroneutralization titer against X-31 virus.

^cADCC NK cell activation assay against X-31 (H3N2) virus was performed using donor PBMCs and influenza-exposed (Flu⁺) human and influenza (Flu) naive pigtail macaque plasma. NK cell activation was indicated as the CD107a expression by NK cells. Samples were found to be positive when NK cell activation was higher than the mean of background signal (with plasma but without Ag) + 2 SDs (human and macaque samples were analyzed separately).

resistance of monocytes to ADCC-mediated killing. Reduction of NP-expressing cells was associated with CD107a expression by NK cells (Fig. 4C), but not by CD107a expression from T cells in the same samples (data not shown). CD107a expression is an indirect marker of cytotoxicity and correlates with a reduction in granzyme B expression on NK and T cells (35–37). This ADCVE assay allows us to measure the functional capability of ADCC Abs and NK cells to reduce virus-infected T cells in vitro.

The development of this ADCVE assay allowed us to assess the ability of human plasma from our cohort of humans with low or undetectable levels of neutralizing Abs to mediate elimination of X-31-infected cells in vitro. Significant reduction in both the frequency and absolute number of X-31-infected T cells was observed in the presence of the adult human plasma samples (median relative reduction of 35.7% and median absolute reduction of 38.5%), which was significantly higher than the reduction in the presence of influenza-naive macaque plasma (median relative reduction of 1.7% and median absolute reduction of 0%, $p < 0.05$; Fig. 4D). Furthermore, the reduction was concomitant with significantly higher levels of CD107a expression on NK cells in the presence of adult human plasma (median 46.3%) compared with the influenza-naive macaque samples (median for macaques: 21.8%, $p = 0.005$; Fig. 4D). We also tested sera from an influenza-naive human child described above and observed no significant reduction in the relative and absolute number of virus-infected cells or CD107a expression on NK cells was found (3.4% relative reduction of infected cells with 0% CD107a expression on NK cells). Thus, adult plasma contain X-31 HA-binding Abs that can mediate specific activation of NK cells and clear virus-infected cells in vitro, in the absence of detectable neutralizing Abs.

Measuring the reduction of virus-infected respiratory epithelial cells in the presence of ADCC Abs

The above PBMC model for assessing the ability of ADCC Abs to clear virus-infected cells has the advantage of being an autologous system. However, blood leukocytes are generally not targets of influenza virus infection. To assess the ability of cross-reactive ADCC Abs to clear virus-infected respiratory cells, we modified our ADCVE assay to use a respiratory cell line (A549) infected with influenza virus as target cells. The respiratory epithelial cells were incubated with influenza virus at a MOI of ~10 for 4 h, and

then washed to remove any free virus. Infected respiratory epithelial cells were labeled and incubated with PBMCs at an E:T ratio of 1:3, in either the presence or absence of plasma containing ADCC Abs. The percentage of virus-infected cells was quantified by intracellular staining for influenza NP, as described previously. Fig. 5A shows the setup of this assay.

Incubation of infected respiratory epithelial cells with human adult plasma resulted in a reduction in both the frequency and total number of virus-infected respiratory epithelial cells. An associated increase in NK cell CD107a expression was also observed (Fig. 5B). No reduction in influenza-infected respiratory epithelial cells was observed in the presence of influenza-seronegative macaque plasma. Measurement of virus reduction across the 10 subjects showed a significant reduction in both the relative frequency and absolute numbers of infected respiratory epithelial cells in the presence of healthy adult plasma (Fig. 5C; median relative viral reduction of 18.2% and median absolute reduction of 61.0%, $p < 0.05$, respectively). The reduction in the number of virus-infected respiratory epithelial cells was associated with a significant increase in CD107a expression on NK cells (Fig. 5C; median CD107a expression of 24.9%, $p < 0.005$). This suggests that cross-reactive ADCC Abs can mediate in vitro elimination of influenza-infected respiratory cells in the absence of neutralizing Abs.

ADCC responses to pandemic H1N1 virus and proteins

The 2009 H1N1 influenza pandemic virus led to significant global infection, yet disease was mostly mild (38). Cross-reactive immune responses may have reduced the capacity of this novel influenza virus to cause disease. Therefore, we evaluated whether healthy human adults have NK cell-activating ADCC Abs toward the 2009 pandemic H1N1 virus. We used the ADCC NK cell activation assay to measure NK cell activation toward pandemic H1N1 virus strain Auck/09 or purified HA protein from the closely related Cal/09 strain. The addition of human plasma to PBMCs resulted in significant activation of NK cells in the presence of Auck/09 virus or purified Cal/09 HA protein with 0.61 and 0.83% of NK cells expressing both IFN- γ^+ /CD107a⁺, respectively. All 10 human plasma samples tested showed substantial NK cell activation when exposed to either Auck/09 virus or Cal/09 HA (Auck/09 virus: IFN- γ median 1.09%, CD107a median 3.09%; Cal/09 HA protein: IFN- γ median 1.89%, CD107a median 3.11%). As expected, significantly less NK cell activation was induced in the influenza-naive macaque plasma samples compared with the human plasma samples (all $p = 0.005$; Fig. 6B, 6C).

Although influenza-specific ADCC Abs toward pandemic H1N1 virus and proteins were detected in our healthy adult cohort, it is likely that many were recently exposed to this virus. We therefore investigated whether some individuals might have high levels of pandemic influenza H1N1 ADCC Abs in the absence of neutralizing Abs. Of interest, 7 of 10 of the healthy adults had significant levels of ADCC Abs (with ADCC titers >40); however, we could not detect HI titres against Cal/09 in 2 of 7 of the ADCC Ab-positive samples (Table II). This suggests that pandemic H1N1-specific ADCC Abs can be present in the absence of neutralizing Abs in at least a subset of healthy adults.

Cross-reactive ADCC Abs to avian H5 protein

The emergence and spread of novel influenza viruses in the human population are a major threat to global health. Our previous results suggest that ADCC Abs to X-31 and to pandemic H1N1 strains can be detected in the absence of neutralizing Abs. However, in the above studies, it is difficult to completely exclude the possibility that previous infection or vaccination with those strains resulted in the prolonged persistence of ADCC Abs, whereas neutralizing Abs

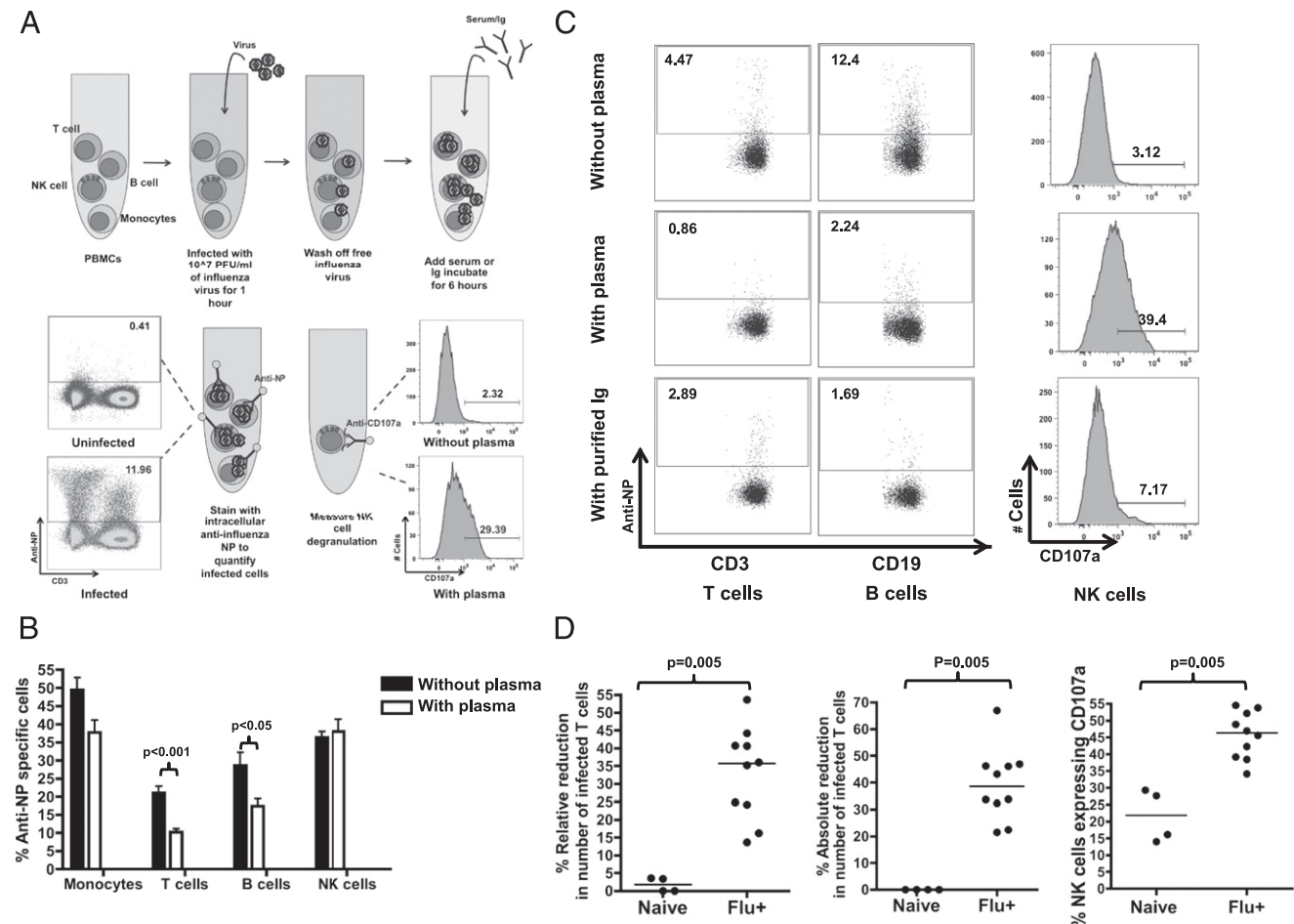


FIGURE 4. ADCVE assay. **(A)** Assay setup and analysis. Donor PBMCs were incubated with a final concentration of 10^7 PFU/ml X-31 (A/Aichi/2/1968) influenza virus for 1 h at 37°C . PBMCs were then washed four times to remove unbound virus and incubated with plasma or purified Ig for 6 h at 37°C . After incubation, the percentage of virus-infected cells was determined based on intracellular expression of viral NP and activation of NK cells by surface expression of CD107a for each given sample. **(B)** X-31 infection of CD14⁺CD3⁺ monocytes, CD19⁺ B cells, CD3⁺ T cells, or CD56⁺CD3⁺ NK cells following incubation with plasma (from 10 influenza-seropositive donors) or without plasma ($n = 4$). Data show the mean \pm SEM percent infection and were analyzed by two-way ANOVA after \log_{10} transformation. **(C)** Concomitant reduction of virus-infected cells and NK cell activation in the presence of Ig at 6 h postinfection with X-31, intracellular expression of viral NP in T cells, and B cells that had been incubated in media alone or in media supplemented with plasma or purified Ig from the same donor. NK cell activation was assessed by the cell surface expression of CD107a expression by CD3⁺CD56⁺ NK cells. **(D)** Relative and absolute reduction of virus-infected T cells was measured in the presence of plasma from influenza-exposed adult plasma ($n = 10$) or plasma from influenza-naïve macaques ($n = 4$). Data from influenza-exposed and naïve samples were compared using a Mann-Whitney U test where $p < 0.05$ was considered significant. Lines on the graphs denote median values.

waned more rapidly. Exposure to avian H5N1 virus is likely to be exceedingly rare, with limited human cases described worldwide. To further investigate the concept of cross-reactive influenza-specific ADCC immunity, we tested ADCC responses toward H5 HA protein (Anhui/1/2005) in our cohort of healthy human adults. We observed robust Ab-mediated activation of NK cells when exposed to H5 protein in the presence of plasma (2.06% in the presence of plasma compared with 0.03% IFN- γ ⁺/CD107a⁺ NK cells in the absence of plasma; Fig. 7A). ADCC-mediated NK cell activation was detected in all 10 human sera (IFN- γ median 3.11%; CD107a median 5.99%), but not using plasma from four influenza-naïve macaques (macaque IFN- γ median 0%, $p = 0.004$; macaque CD107a median 0%, $p = 0.004$). These results suggest that previous exposure to one or more subtypes of influenza can elicit ADCC Abs that can cross-react with a range of distinct influenza subtypes.

Discussion

Neutralizing Abs are a major correlate of influenza virus-protective immunity. However, more recent studies have implicated non-neutralizing Abs in providing a broader level of protection (39, 40).

Characterizing the functional capacities of nonneutralizing influenza-specific Abs has been difficult due to the lack of robust assays. We developed two novel flow cytometry-based ADCC assays and showed that influenza-specific ADCC Abs toward X-31 virus (A/Aichi/2/1968) exist in the absence of detectable neutralizing Abs. We also detected ADCC Abs that reacted against pandemic H1N1 and avian H5N1 HA proteins in our adult cohort. These ADCC Abs have the ability to induce NK cell activation, leading to the release of the antiviral cytokine IFN- γ and the expression of CD107a, an indirect marker of killing. Additionally, the X-31-specific ADCC Abs were shown to be able to clear influenza-infected PBMCs and respiratory epithelial in vitro using our novel ADCVE assay.

Little is known about the function of cross-reactive non-neutralizing Abs in influenza infection. Although some previous studies suggest nonneutralizing Abs are involved in immunopathology, others suggest they are important in protection (13, 41). Several recent studies have highlighted a potential role for non-neutralizing Abs induced by prior seasonal influenza infection in providing a level of protection from pandemic influenza exposure

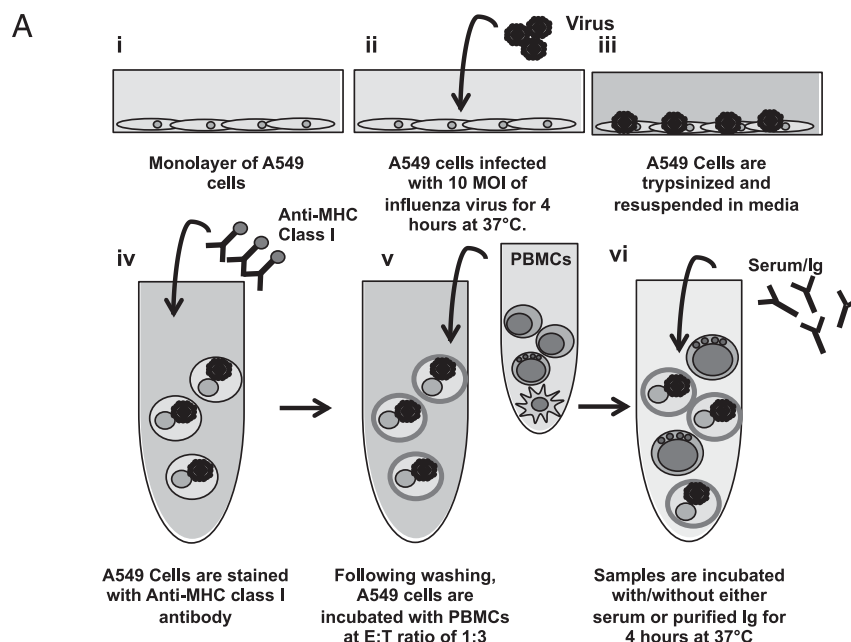
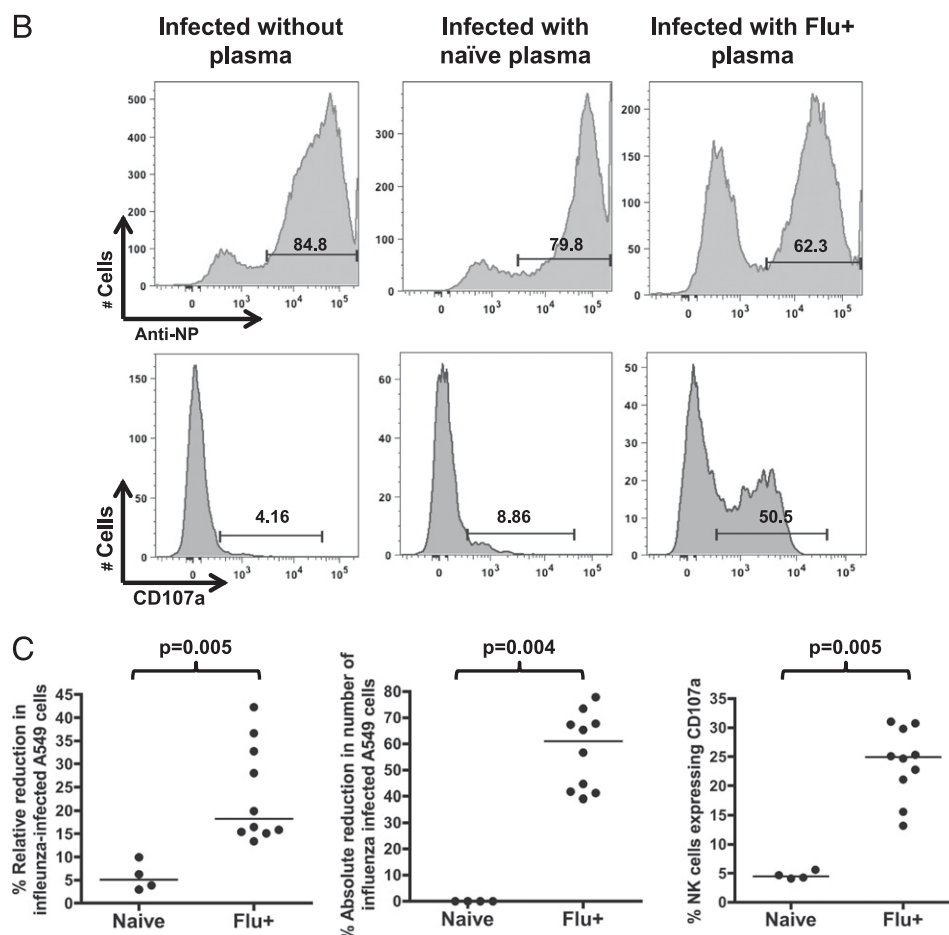


FIGURE 5. Elimination of virus-infected respiratory epithelial cells by NK cells in the presence of plasma from influenza-exposed adults. **(A)** Assay setup and analysis. Confluent monolayer of A549 cells was infected for 1 h with X-31 influenza, washed, and incubated for 3 h at 37°C (i, ii). Infected A549 cells were then trypsinized and resuspended in media (iii). A549 cells were then labeled with PE-conjugated anti-MHC class I Abs (iv) and incubated with purified PBMCs at E:T ratio of 1:3 (v), with or without serum for 4 h at 37°C (vi). The numbers of infected A549 cells were measured by gating on PE-fluorescing cells, and infection was measured by anti-NP staining. The relative frequency reduction and absolute number reduction of infected A549 cells were then calculated. **(B)** Reduction in the number and frequency of virus-infected A549 cells in the presence of naive and healthy adult plasma (Flu⁺ plasma). The CD107a expression was assessed on CD3⁺CD56⁺ NK cells. **(C)** Relative and absolute reduction of virus-infected A549 cells in the presence of plasma from influenza-exposed adult plasma ($n = 10$) or plasma from influenza-naïve macaques ($n = 4$). Data from influenza-exposed and naïve samples were compared using a Mann-Whitney U test where $p < 0.05$ was considered significant. Lines on the graphs denote median values.



(39, 42–44). A recent study by Fang et al. (39) showed that seasonal infection with seasonal A/Brisbane/59/2007 H1N1 virus in mice provided protection from subsequent infection with pandemic A/Mexico/4108/2009 H1N1 virus via cross-reactive non-neutralizing Abs. This protection did not require CD8 T cells, but rather was B cell dependent. We speculate that ADCC Abs against pandemic influenza H1N1 virus in our adult cohort may provide a

level of cross-protection. Weinfurter et al. (42) showed that protection of macaques from pandemic H1N1 can be mediated by seasonal H1N1 infection. Although CTL responses most likely played a role in the cross-protection observed in this macaque model, the authors also showed the presence of cross-reactive non-neutralizing Abs to pandemic H1N1 arises very early during infection, but only in macaques with prior seasonal infection. Further

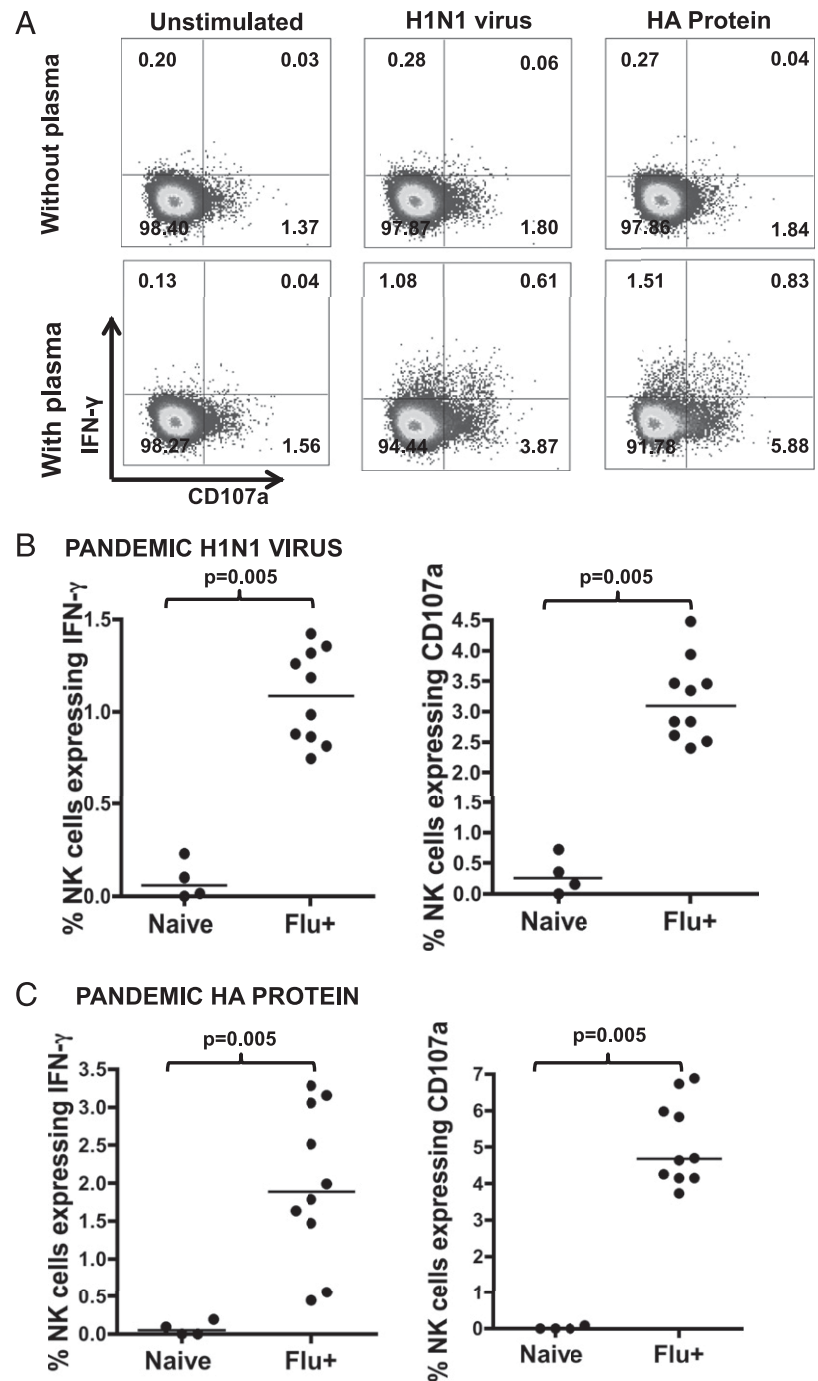


FIGURE 6. Influenza ADCC toward pandemic H1N1 virus and proteins. **(A)** Activation of CD3⁺CD56⁺ NK cells by pandemic H1N1 (Auck/09) virus and H1 (Cal/09) protein in the presence of influenza-seropositive plasma. **(B and C)** Plasma from 10 influenza-exposed (Flu⁺) adults and 4 influenza (naive) pigtail macaques were assessed for reactivity to pandemic H1N1 virus (B) and pandemic H1 HA protein (C) using the NK cell activation assay. The IFN- γ (left) and CD107a (right) expression by CD3⁺CD56⁺ NK cells was determined. Values of NK cell activation were all background subtracted, and influenza-exposed and naive samples for all assays were compared using a Mann–Whitney *U* test where $p < 0.05$ was considered significant. Lines on the graphs denote median values.

studies of ADCC Abs in both animal models and expanded studies in humans with characterized cross-protective immunity to influenza are now warranted.

The isolation of influenza-specific Abs able to neutralize all 16 known subtypes of influenza A viruses has been a major challenge. The conservation of neutralizing epitopes is mostly restricted to the membrane-distal subdomain of the influenza HA protein, the HA1 region (45–47). Effective nonneutralizing Abs may not be limited by the same epitope constraints placed on neutralizing Abs because they do not need to stop virus binding or entry. In preliminary studies, we found that ADCC responses to the HA1 region of the influenza protein induce a lower level of NK cell activation than the full-length HA protein (data not shown). This implies that ADCC Abs can also target non-HA1 regions within HA and such responses could be more cross-protective (48, 49). Indeed, other

surface influenza proteins, including the NA and M2 protein, are also likely to be targets for ADCC (49). Vaccination studies performed using influenza M2 protein suggest a protective role mediated by nonneutralizing Abs (50–52). The M2-specific Abs were shown to protect against both homologous and heterologous influenza virus challenge in the mouse model. A study by Jegerlehner et al. (23) described the protective role of M2 ADCC-mediating Abs, showing that they exhibited poor protection and a lower degree of in vivo clearance. The protection mediated by M2 Abs has been shown to be dependent on FcRs, implying an ADCC mechanism (24).

The importance of ADCC has become more prominent in a number of fields, including cancer therapy and chronic viral infections. Tumor-specific mAbs are more effective when designed to elicit ADCC functions (53). In addition, HIV- and SIV-specific

Table II. Serum HI titer and ADCC Abs to pandemic (H1N1) virus

Sample	HI Titer ^a	ADCC–Ab Titer ^b
Flu ⁺ 1	<10	80
Flu ⁺ 2	320	80
Flu ⁺ 3	<10	<10
Flu ⁺ 4	80	80
Flu ⁺ 5	320	>160
Flu ⁺ 6	80	80
Flu ⁺ 7	320	20
Flu ⁺ 8	80	10
Flu ⁺ 9	20	80
Flu ⁺ 10	<10	>160
Flu naive 1	<10	<10
Flu naive 2	<10	<10
Flu naive 3	<10	<10
Flu naive 4	<10	<10

^aHI titer against A/California/07/2009 IZP. The range of detection for HI was between 10 and 1280.

^bADCC NK cell activation assay against A/California/04/2009 HA protein was performed using donor PBMCs and influenza-exposed (Flu⁺) human and influenza (Flu) naive pigtail macaque plasma (undiluted). NK cell activation was indicated as the CD107a expression by NK cells. Samples were found to be positive when NK cell activation was higher than the mean of background signal (with plasma but without Ag) + 2 SDs (human and macaque samples were analyzed separately).

ADCC Abs have been implicated in providing protection in macaque and human HIV/SIV vaccine studies (54–56). A major constraint in measuring NK cell-mediated ADCC are the high background levels of NK cell activation due to the presentation of epitopes on foreign target cell lines and the limited presentation of some Ags on the surface of target cells. Our NK cell activation ADCC assay uses autologous PBMCs in which background levels of NK cell activation are low and specific conformational Ags can be used to assess ADCC activity. This should aid in the assessment of ADCC Abs during influenza vaccine studies, but could also assist in characterization of ADCC Abs in other viral diseases such as HIV, hepatitis C, or dengue virus.

The near ubiquitous cross-reactive influenza-specific ADCC responses in healthy adults presumably reflect the cumulative effect of multiple prior influenza infections. Our study of only 10 healthy subjects identified high levels of broadly cross-reactive influenza-specific ADCC Abs. Larger cohort studies should provide additional insights. Defining influenza-negative controls is difficult due to the pervasive nature of influenza infection. We were able to study a small amount of sera (40 μ l) from only one influenza-naive infant, limiting our experiments with this sample to be also performed at a lower (1:10) dilution. We therefore included influenza-seronegative macaque sera as the controls for our assays. Macaque plasma contains a similar Ig composition to human sera, and sera from influenza-infected macaques produce comparable NK cell activation to human sera.

The comparative roles of NK cells versus other Fc-bearing cells in mediating influenza-specific ADCC in vitro and in vivo remain to be clarified. We detected influenza ADCC Abs in humans by using NK cell activation; however, this is not the only CD16 receptor-bearing cell type. Other cell types such as monocytes also may mediate influenza-specific ADCC in vivo and in vitro (57). Furthermore, particular subsets of KIR-expressing NK cells may most effectively mediate influenza-specific ADCC (58). Future studies should define the relative contributions of NK cell subsets and other Fc-bearing cell types in the mediation of influenza-specific ADCC.

In summary, important assays to measure influenza-specific ADCC Abs in humans were developed and used to show that influenza-specific ADCC Abs to influenza exist in the absence of detectable neutralizing Abs. Influenza-specific ADCC Abs were

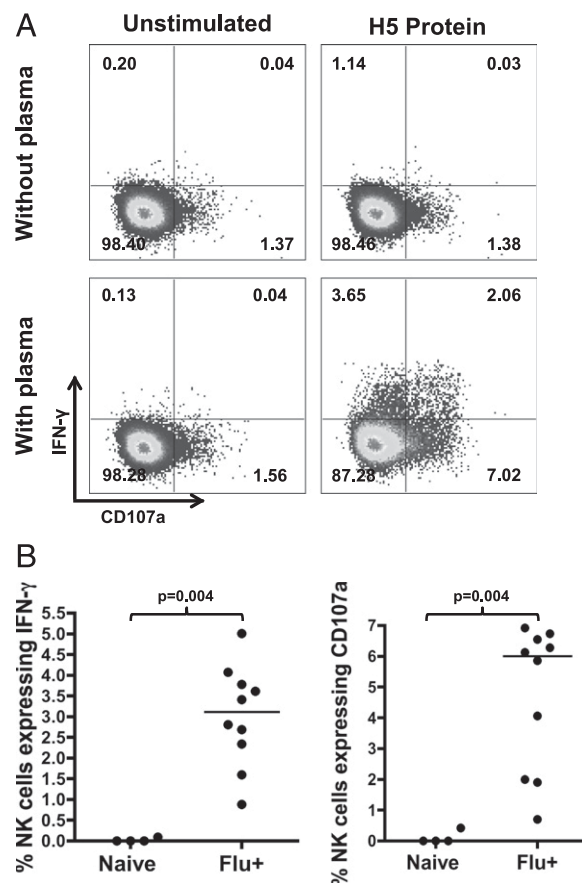


FIGURE 7. Influenza ADCC toward HA of avian H5N1 virus. **(A)** Activation of CD3⁺CD56⁺ NK cells by H5 (Anhui/01/2005) protein in the presence of influenza-exposed plasma. **(B)** Plasma from 10 influenza-exposed (Flu⁺) adults and 4 influenza (naive) pigtail macaques were assessed for reactivity to avian H5 protein using NK cell activation assay. IFN- γ (left) and CD107a (right) expression by CD3⁺CD56⁺ NK cells was measured for both groups. Values of NK cell activation were all background subtracted, and influenza-exposed and naive samples for all assays were compared using a Mann–Whitney *U* test where *p* < 0.05 was considered significant. Lines on the graphs denote median values.

shown to not only induce robust activation of NK cells, but also mediate the clearance of influenza-infected blood and respiratory epithelial cells in vitro. Influenza-specific ADCC Abs that recognize pandemic H1N1 and avian H5N1 are also commonly present in healthy adults, suggesting a possible mechanism of cross-protective immunity. Further studies of influenza infection and vaccination in humans and nonhuman primate models will provide insights into the protective role of influenza ADCC Abs against a broad range of influenza virus infections.

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Disclosures

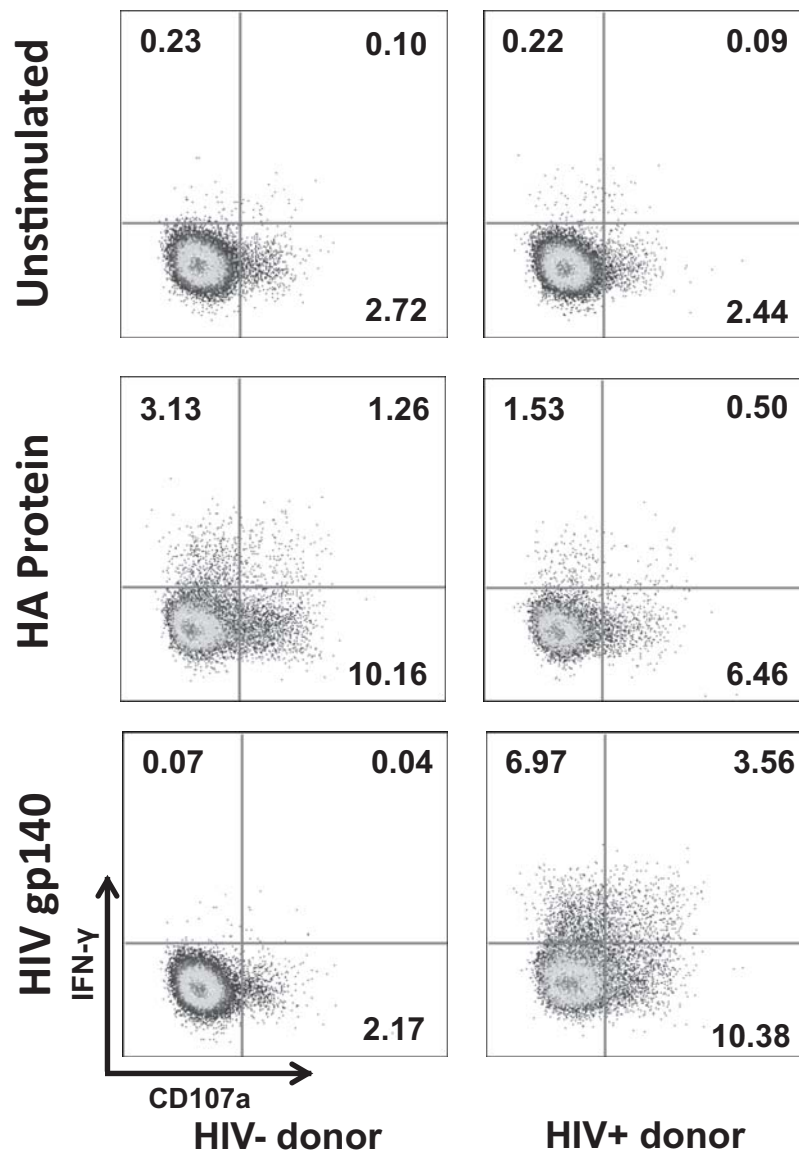
The authors have no financial conflicts of interest.

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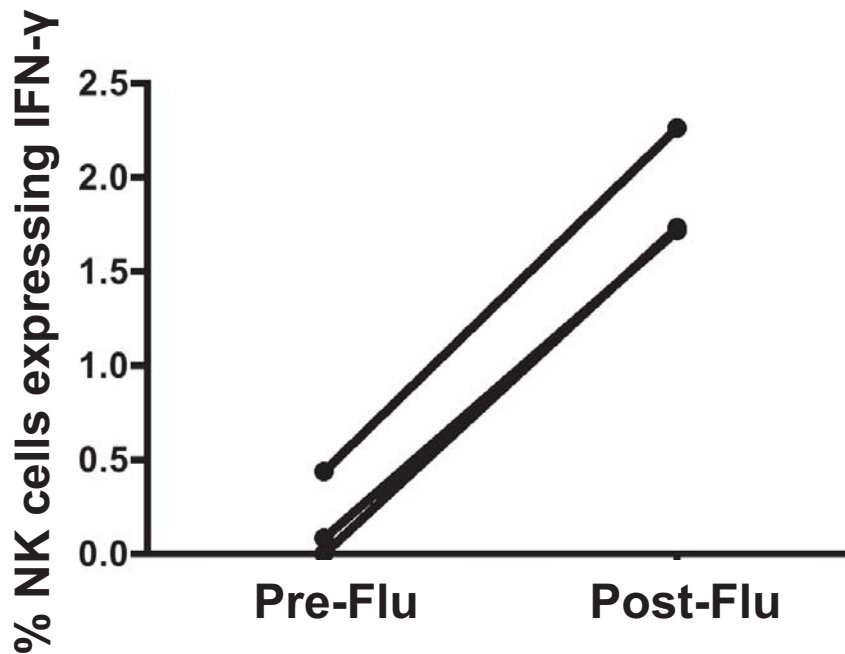
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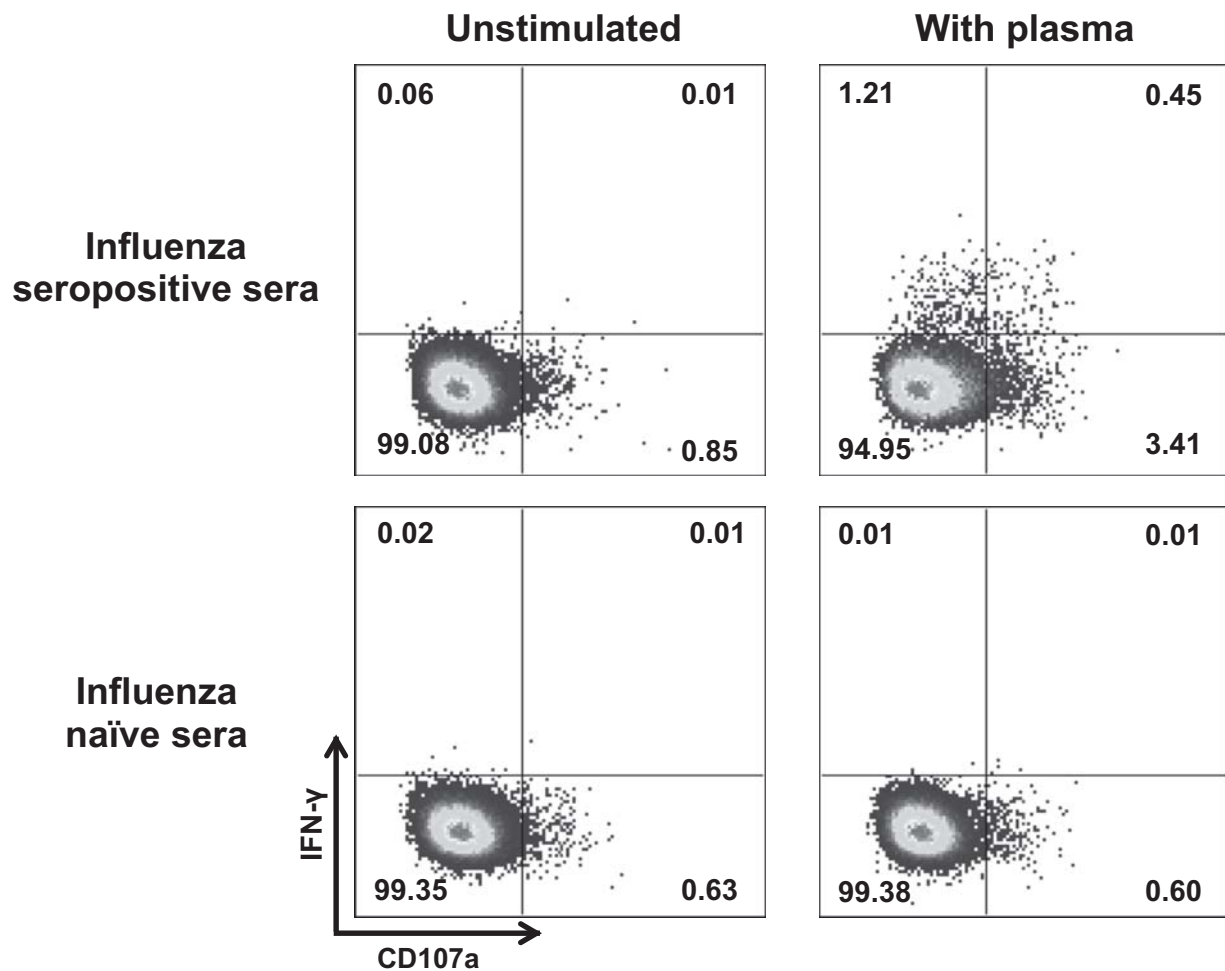


Supplementary Fig 1. ADCC towards HIV and influenza proteins. Activation of CD3-CD56⁺ NK cells measured against either HA protein (PR8) or HIV gp140 (AD8), in the presence of either HIV negative or HIV positive donor plasma.

A**B**

	PR8 HI Titre	
	Pre	Post
19351	<10	1280
19350	<10	160
C0933	<10	80

Supplementary Fig 2. Influenza-ADCC towards PR8 virus in vaccinated macaques. Activation of CD3-CD56⁺ NK cells against PR8 influenza virus in the presence of purified Ig from pigtail macaques (n=3) before (pre-Flu) and after infection (post-Flu) with PR8 virus **(A)**. Animals were administered 2 doses of 10^8 pfu of both PR8 influenza virus and X-31 influenza virus via the respiratory tract at ~4 weekly intervals and sera studied 4 weeks after the last infection **(B)** HI Titre against PR8 influenza virus for animals tested either at day 0 (Pre) or day 133 (post) post influenza infection.



Supplementary Fig 3. Lack of influenza-ADCC antibodies in naïve human plasma sample. Activation of CD3-CD56⁺ NK cells against influenza H3 protein using plasma (1:10 dilution) from influenza naïve human or influenza exposed human.