MAJOR ARTICLE

Cross-Reactive Influenza-Specific Antibody-Dependent Cellular Cytotoxicity in Intravenous Immunoglobulin as a Potential Therapeutic Against Emerging Influenza Viruses

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Background. Intravenous immunoglobulin (IVIG) is a purified pool of human antibodies from thousands of donors that is used to prevent or treat primary immune deficiency, several infectious diseases, and autoimmune diseases. The antibodies that mediate antibody-dependent cellular cytotoxicity (ADCC) against heterologous influenza strains may be present in IVIG preparations.

Methods. We tested 8 IVIG preparations prior to the 2009 H1N1 swine-origin influenza pandemic and 10 IVIG preparations made after 2010 for their ability to mediate influenza-specific ADCC.

Results. ADCC mediating antibodies to A(H1N1)pdm09 hemagglutinin (HA) and neuraminidase (NA) were detected in IVIG preparations prior to the 2009-H1N1 pandemic. The HA-specific ADCC targeted both the HA1 and HA2 regions of A(H1N1)pdm09 HA and was capable of recognizing a broad range of HA proteins including those from recent avian influenza strains A(H5N1) and A(H7N9). The low but detectable ADCC recognition of A(H7N9) was likely due to rare individuals in the population contributing cross-reactive antibodies to IVIG.

Conclusions. IVIG preparations contain broadly cross-reactive ADCC mediating antibodies. IVIG may provide at least some level of protection for individuals at high risk of severe influenza disease, especially during influenza pandemics prior to the development of effective vaccines.

Keywords. ADCC; influenza; NK cells; swine-origin influenza virus; IVIG.

The emergence of a swine-origin H1N1 influenza (A(H1N1)pdm09) virus in 2009 resulted in 100,000 to 400,000 deaths worldwide [1–5]. In most cases the vaccine was available too late to reduce the peak of the pandemic [6–9]. With the threats of avian A(H7N9) and A(H5N1) pandemics looming, there is a need for new influenza therapies.

Antibodies with both neutralizing and nonneutralizing functions develop following natural influenza virus infection. Influenza hemagglutinin (HA)-specific antibodies neutralize by binding to epitopes surrounding the receptor-binding pocket and prevent sialic acid receptor binding, entry, or viral fusion. Nonneutralizing antibodies to influenza virus have the potential to mediate other effector functions including complement fixation [1, 2], phagocytosis [10, 11], and antibody-dependent cellular cytotoxicity (ADCC) [12–16]. ADCC mediating antibodies can bind to regions of influenza virus and mediate their activity through ligation of CD16 (FcγRIIIa) on effector cells such as natural killer (NK) cells [17, 18]. Recent mouse studies show ADCC function is critical for protection against lethal influenza virus challenge [19, 20].

Intravenous immunoglobulin (IVIG) is a pooled source of purified human immunoglobulin G (IgG) antibodies extracted from donor serum. IVIG is used

Received 8 March 2014; accepted 21 May 2014; electronically published 10 June 2014.

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The Journal of Infectious Diseases® 2014;210:1811–22

DOI: 10.1093/infdis/jiu334

to treat and prevent a range of infectious and autoimmune diseases [21–24]. Previous influenza virus infections of donors may result in IVIG preparations containing cross-reactive ADCC mediating antibodies. IVIG preparations, pooled from thousands of blood donors, provide an opportunity to study influenza antibodies at a population level. Recent studies suggest that IVIG preparations made prior to 2009 contain some level of A(H1N1)pdm09-specific neutralizing antibodies [25–27]. The administration of post-immune IVIG to patients suffering from severe A(H1N1)pdm09 influenza has therapeutic benefit if administered within 5 days of symptom onset [28]. However, the ability of human IVIG preparations to mediate cross-reactive ADCC against diverse influenza viruses is unknown. We hypothesized that cross-reactive ADCC mediating antibodies to A(H1N1)pdm09 virus are present in IVIG preparations made prior to the 2009 pandemic.

METHODS

IVIG Samples

IVIG preparations from 2004 to 2010 (Table 1, Sandoglobulin, CSL Behring, Australia) were prepared by cold ethanol fractionation process and are approximately 96% pure IgG. IVIG donors were human immunodeficiency virus (HIV)-antibody negative. The stock preparations are prepared to a concentration of 60–100 mg/mL. All IVIG samples were diluted to 10 mg/mL and heat-inactivated (56°C for 60 minutes).

Table 1. Intravenous Immunoglobulin (IVIG) Preparations and Hemagglutination inhibition (HI) Titer

	Sample No.	Manufactory Date	A/California/07/09 HI Titer[a]
Prior 2009	007	25 April 2004	32
	005	17 May 2004	32
	102	18 June 2004	16
	116	25 June 2004	16
	202	4 March 2005	16
	302	28 November 2005	128
	305	28 February 2008	128
	404	23 September 2008	256
Post 2009	1822	30 January 2010	256
	1823	03 February 2010	64
	1824	04 February 2010	256
	1825	15 February 2010	256
	1838	25 April 2010	256
	1841	19 May 2010	512
	1847	30 June 2010	512
	1848	07 July 2010	1024
	1860	06 September 2010	256
	1867	17 October 2010	256

[a] HI titer against A/California/07/09. The range of detection for HI was between 16 and 2048.

Additional Human Sera Samples

Sixty-two serum samples collected from healthy human subjects were kindly provided by the WHO Collaborating Centre on Influenza and the University of Melbourne. Samples were collected between 2012 and 2013 from individuals aged 20–89 (median 51, ages 21–30 n = 8, 31–40 n = 11, 41–50 n = 12, 51–60 n = 10, 61–70 n = 10, 71–80 n = 7, 81–90 n = 4). The Alfred Hospital and University of Melbourne ethics committees approved the use of human donor blood and plasma samples. Human influenza-seronegative (MBL) and seronegative pigtail macaque sera (naive sera) were used as additional negative controls as previously described [29].

Influenza Virus Antigens and HI Assay

Mammalian-cell expressed recombinant HA proteins were purchased from Sinobiologicals (Shanghai, China). Neuraminidase was purified by negative immunoaffinity based on a modified method as described [30]. HA2 was purified by solubilizing β propriolactone inactivated whole virus with buffered 4% Triton-X100 (2 hours, 37°C). Virus cores were removed using ultracentrifugation and residual detergent removed using Boibeads (Biorad). Neuaminidase protein was removed using a specific anti-N1 monoclonal antibody and affinity chromatography. Purified HA was reduced (10 mM DTT 30′ RT), and this reduced protein loaded on to a 10% bis Tris NuPAGE gel (Invitrogen Life Technology). Identified HA2 protein was excised from the gel and eluted by diffusion. Purity was confirmed by Western blotting and enzyme-linked immunosorbent assay (ELISA) using monoclonal antibodies reactive to HA2 (n = 3) and not reactive to H1, matrix, NP, and N1 antibodies. Hemagglutination inhibition (HI) assays were performed as previously described [31].

ADCC NK Cell Activation Assays

We described ADCC assays that measure antibody-mediated NK cell activation [16]. Briefly 96-well ELISA plates (Nunc, Rochester, NY) were coated overnight at 4°C with purified influenza protein (400 ng/well) in phosphate-buffered saline (PBS). Wells were washed and incubated with heat-inactivated IVIG in fetal calf serum (FCS;starting concentration 10 mg/mL IgG) for 2 hours at 37°C. Plates were washed, and then 10^6 freshly isolated human peripheral blood mononuclear cells (PBMCs) from a healthy donor were added to each well. Brefeldin A (5 μg/mL, Sigma, St. Louis, MO) and Monensin (5 μg/mL, BD Bioscience, San Jose, CA) were added and incubated for 5 hours at 37°C. Cells were then labeled with anti-CD3-PerCP (clone SP34-2), anti-CD14-PE-Cy7 (clone M5E2) anti-CD56-APC (clone B159) and anti-CD107a-APC-H7 (clone H4A3, all from BD Bioscience) for 30 minutes. Cells were fixed, permeabilized, and incubated for 1 hour with anti-IFN-γ-AlexaFluor700 (clone B27). Cells fixed and acquired on an LSRII flow cytometer. Mammalian cell produced HIV-1_{AD8}

gp140 protein was included as a negative control antigen [29]. To reduce variability across assays, the same healthy blood donor was used as a source of fresh NK cells for all ADCC assays.

Antibody-Dependent Cellular Viral Elimination (ADCVE) Assay

We employed the recently described assay that measured antibody-mediated killing of influenza-infected respiratory epithelial cells [29]. Briefly, A549 cells were infected with A(H1N1)pdm09 for 1 hour at 37°C, 10%CO_2, washed 3 times, and incubated for a further 3 hours at 37°C, 5%CO_2. The cells were trypsinized, washed, and labeled with PE-conjugated anti-MHC I antibody (W6/32, eBioscience, San Diego, CA) for 20 minutes. Following washing, 6×10^5 infected A549 cells were combined with 2×10^5 freshly isolated human PBMCs with or without 10 μL of heat-inactivated plasma and incubated for 6 hours at 37°C. Cells were then stained with anti-CD3, anti-CD14 anti-CD56, and anti-CD107a as above, permeabilized, intracellularly stained with fluorescein isothiocyanate (FITC) conjugated anti-NP antibody (431 clone, Abam Cambridge, MA), fixed, and acquired by fluorescence activated cell sorting (FACS). Reductions in infected cells were calculated: (% of NP^+ cells or total number of NP^+ cells in the presence of plasma—% NP^+ cells or total number of NP^+ cells in the absence of plasma / % NP^+ cells or total number of NP^+ cells in the absence of plasma) ×100.

Statistical Analyses

Statistical analyses used Prism GraphPad Version 6 (GraphPad Software, San Diego, CA). All analyses were performed using nonparametric statistics due to sample sizes or skewed data. Analyses in Figures 4 and 5 were repeated using parametric statistics (paired *T*-tests or repeated measures 1-way ANOVAs followed by Tukey post hoc tests, data not shown) yielding the same results as nonparametric tests. Data in Figures 1*B*, 1*C*, 1*E*, 2, and 5*F* were analyzed using Mann–Whitney *U*-tests. Data in Figure 3*B* and 3*C* were analyzed by Kruskal–Wallis tests, followed by 3 Mann–Whitney *U*-tests and subsequent Bonferroni correction to the α-level, whereby differences of $P < .016$ are considered significantly different. Figure 5*C* and 5*D* were similarly analyzed but no adjustment was necessary. Data in Figure 4*A*–*E* were analyzed using Wilcoxon signed-rank tests. Data in Figure 4*F* and 4*G* were analyzed by Friedman tests followed by Dunn post hoc tests. Data in Figure 5*A* and 5*B* were analyzed by Friedman tests followed by Wilcoxon signed-rank tests and Bonferroni corrections leading to a new α-level of 0.016.

RESULTS

HA-specific ADCC Toward A(H1N1)pdm09 Virus is Present in IVIG Prior to 2009 Pandemic

To determine the level of preexisting ADCC-mediating antibodies toward A(H1N1)pdm09 virus, we evaluated a panel of 18 IVIG samples manufactured prior to and following the 2009 H1N1 pandemic (Table 1). There were modest titers of HI antibodies to A(H1N1)pdm09 virus in IVIGs manufactured prior to the 2009 H1N1 pandemic (mean HI titer of 70; range, 16–256), which increased significantly in IVIGs prepared following the 2009 H1N1 pandemic (mean HI titer of 364; range, 64–1024, $P < .001$).

To first assess A(H1N1)pdm09 HA-specific ADCC antibodies in IVIG prior to and following the 2009 H1N1 pandemic, we performed an antibody-mediated NK cell activation assay [29]. We measured NK cell activation (intracellular interferon γ [IFN-γ] and the surface degranulation marker CD107a expression) in the presence of HA proteins from the pandemic A(H1N1)pdm09 virus strain or seasonal H1N1 influenza vaccine strains (Figure 1*A*) comparing IVIG made from either prior to or following 2009. We found ADCC responses to all H1N1 strains within all IVIG preparations, with negligible background levels of NK cell activation against a control HIV protein (Figure 1*B* and 1*C*). There was no significant difference in ADCC-induced IFN-γ or CD107a expression by NK cells against all-seasonal H1N1 HA proteins (A/New Caledonia/20/99, A/Solomon Islands/3/06, A/Brisbane/59/07) when comparing IVIG manufactured prior to 2009 and post-2009 at a 10-mg/mL concentration ($P > .5$, Figure 1*B* and 1*C*). Upon endpoint titration, we observed similar titers of ADCC mediating antibodies to the 3 seasonal HA proteins tested on pre-2009 and post-2009 IVIG samples (Figure 1*D*). Further, there was only a trend toward increased titers of A(H1N1)pdm09 HA-specific antibodies in IVIG preparations made post-2009 (Figure 1*E*, $P = .136$).

Cross-reactive NA-specific ADCC Present in IVIG Prior to 2009

To further understand potential targets of ADCC antibodies towards influenza virus in IVIG, we measured NK cell activation in the presence of purified active NA protein from A(H1N1)pdm09 virus (A/California/04/2009) in the 18 IVIG preparations. Antibody-mediated NA-specific NK cell activation (both IFN-γ and CD107a expression) was detected in a subset of IVIG samples manufactured prior to 2009 (4–6/8) and in all IVIG samples made after 2009 (10/10 Figure 2*A* and 2*B*). The levels of NA-specific ADCC in IVIG was reduced in comparison to HA-specific ADCC, consistent with our previous observations on NA-specific ADCC following H1N1 influenza infection [32]. Endpoint titrations of the H1N1pdm NA-specific ADCC response found low (1:10 responses observed in 6 samples, Figure 2*A* and 2*B*) or undetectable (n = 2) ADCC titers in IVIG samples from prior to 2009 (median titer of <40) and a significant increase in titers in IVIG samples obtained following 2009 (median titer of 80, $P = .012$, Figure 2*C* and 2*D*). This suggests cross-reactive NA-specific ADCC mediating antibodies to the 2009 H1N1 virus were low prior to 2009 and were moderately boosted following the 2009-H1N1 pandemic. This slight rise in NA antibodies may be due to high level of NA expressed

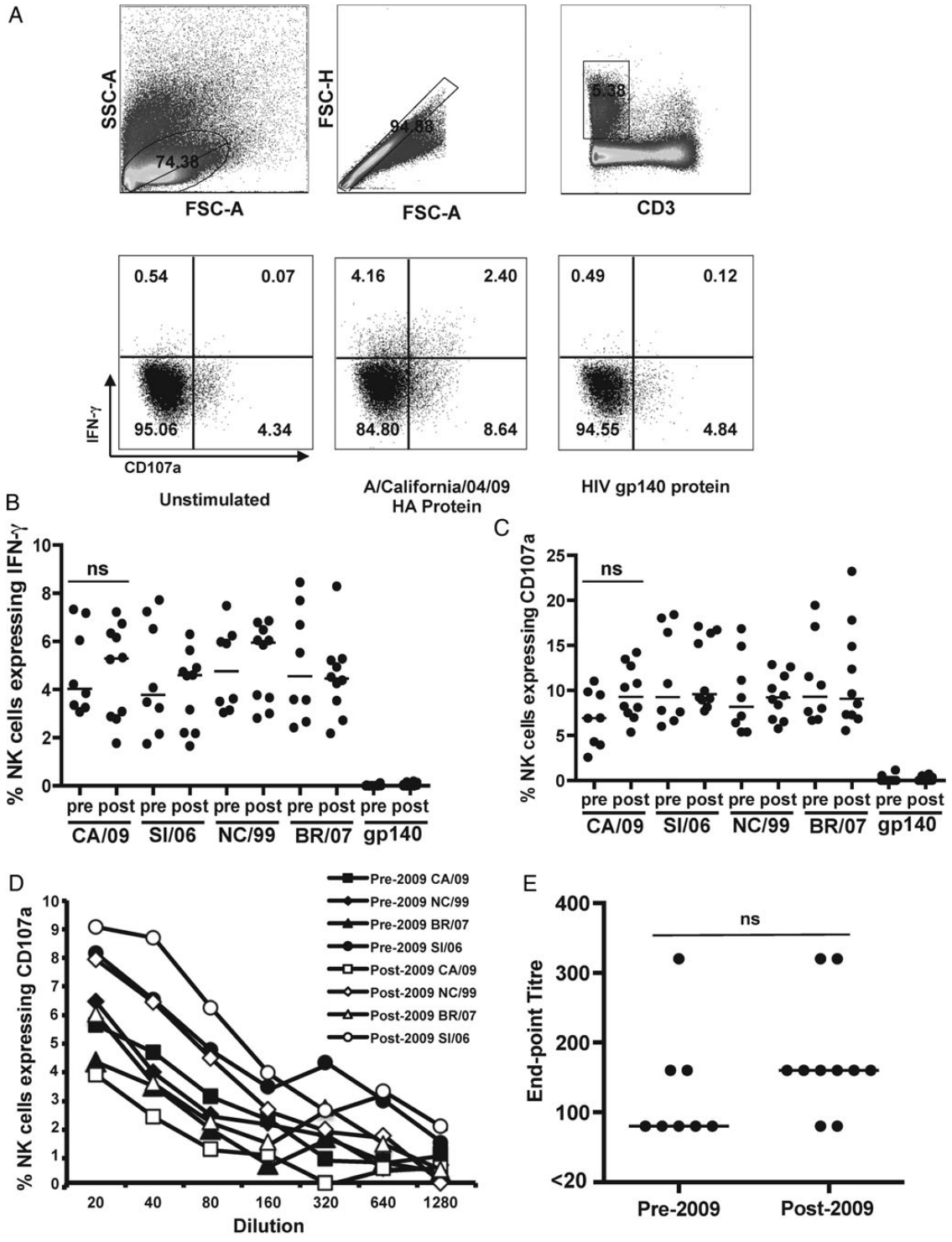

Figure 1. ADCC mediated NK cell activation to HA protein using manufactured IVIG. *A*, Gating strategy of NK cell activation assay, surface CD107a and intracellular expression of IFN-γ was determined in $CD3^{-}CD56^{+}$ lymphocytes. NK cell expression of IFN-γ (*B*) and CD107a (*C*) in response to A/California/04/2009 (CA/09) HA protein or seasonal H1N1 HA proteins from A/Solomon Islands/3/2006 (SI/06), A/New Caledonia/20/1999 (NC/99), A/Brisbane/59/2007 (BR/07) and HIV gp140 (AD8) comparing IVIG preparations made pre-2009 (n = 8) and post-2009 (n = 10; 10 mg/mL). ADCC antibody endpoint titers to A/California/04/2009 (CA/09) HA protein or seasonal HA proteins (*D*) were determined by measured NK cells expressing CD107a at 2-fold dilutions of IVIG preparations made pre-2009 and post-2009 (1:20-1:1280), a threshold of 2 times background (with IVIG but without HA protein) was used to determine endpoint. Endpoint titers against A/California/04/2009 using IVIG preparations made pre-2009 (n = 8) and post-2009 (n = 10, *E*). All samples were corrected for background based on their response to well containing IVIG but with no plate-bound antigen. Lines represent the median, and pre-2009 vs post-2009 IVIG samples were compared using individual Mann–Whitney *U*-tests. Abbreviations: ADCC, antibody-dependent cellular cytotoxicity; HA, hemagglutinin; IVIG, intravenous immunoglobulin; NK, natural killer.

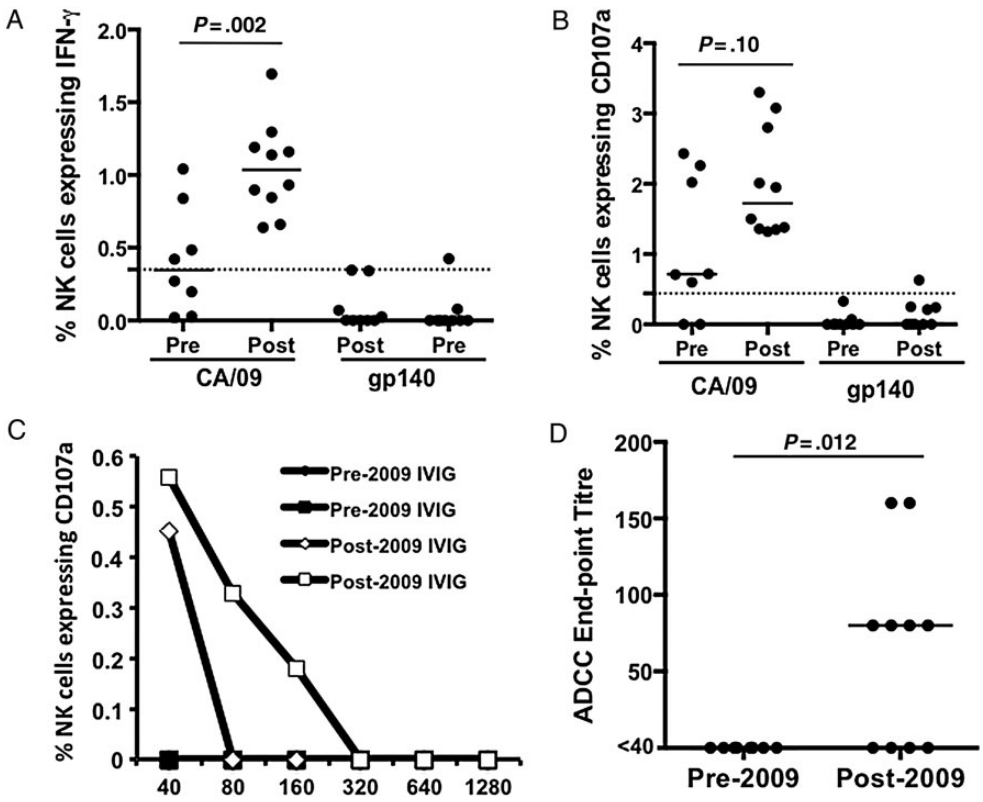

Figure 2. ADCC mediated NK cell activation to NA protein using manufactured IVIG. The NK cell expression of IFN-γ (*A*) and CD107a (*B*) in response to plate-bound NA from A/California/04/2009 (CA/09) and HIV gp140 (AD8) proteins in the presence of IVIG preparations pre-2009 (n = 8) or post-2009 (n = 10; 10 mg/mL). Dotted line represents the threshold for positive responses (mean + 2 SD of IVIG with gp140) and is deemed the threshold for positive responses. ADCC antibody endpoint titers to A(H1N1)pdm09 (A/California/04/2009) NA were determined by measured NK cells expressing CD107a at 2-fold dilutions of IVIG preparations made pre-2009 and post-2009 (1:40-1:1280), a threshold of 2 times background (with IVIG but without NA protein) was used to determine endpoint. Representative endpoint titrations of NA specific ADCC responses in 2 pre-2009 IVIG samples and 2 post-2009 IVIG samples is shown (*C*). NA-specific ADCC titers in pre-2009 (n = 8) and post-2009 (n = 10) IVIG preparations (*D*). Lines represent the median, and pre-2009 vs post-2009 IVIG samples were compared using Mann–Whitney *U*-tests. All samples were corrected for background based on their response to well containing IVIG but with no plate-bound antigen. Abbreviations: ADCC, antibody-dependent cellular cytotoxicity; HA, hemagglutinin; HIV, human immunodeficiency virus; IFN, interferon; IVIG, intravenous immunoglobulin; NA, neuraminidase; NK, natural killer; SD, standard deviation.

by the A(H1N1)pdm09 virus (A/California/07/2009) compared to previous H1N1 seasonal strains (Rockman unpublished observation).

Elimination of A(H1N1)pdm09-infected Respiratory Cells by Pre-2009 IVIG

We next evaluated the ability of ADCC mediating antibodies in IVIG to eliminate A(H1N1)pdm09-infected airway epithelial cells. IVIG preparations from both prior to and after 2009 had substantial capacity to eliminate A(H1N1)pdm09-infected airway epithelial cells. Figure 3*A* shows representative FACS plots of the frequency of influenza nucleoprotein containing respiratory cells in the presence of healthy donor NK cells and either naive human sera (61%), naive macaque sera (62.1%), IVIG generated prior to the 2009 (51.5%) or IVIG generated post the 2009 (49.9%, upper panel). The 8 IVIG samples prepared prior to 2009 were all capable of eliminating A(H1N1) pdm09-infected respiratory cells compared to influenza-naive sera ($P = .004$, Figure 3*B*). The 10 IVIG samples prepared following the 2009 H1N1 pandemic had a further enhanced capacity to clear A(H1N1)pdm09-infected respiratory cells ($P < .001$, Figure 3*B*). Incubation of all 18 IVIG samples with the A (H1N1)pdm09-infected respiratory cells and PBMCs resulted in a marked increase in expression of the degranulation marker CD107a on NK cells (Figure 3*A* lower panel and 3*C*). Thus, ADCC mediating antibodies found in IVIG both prior to and following the 2009 H1N1 pandemic can both mediate NK cell activation and, importantly, eliminate A(H1N1)pdm09-infected respiratory cells.

IVIG Recognition of HA1 and Conserved Regions of HA2 Stem by ADCC

The broad cross-reactivity of HA-specific ADCC in IVIG suggests the recognition of conserved HA sequences. The HA0

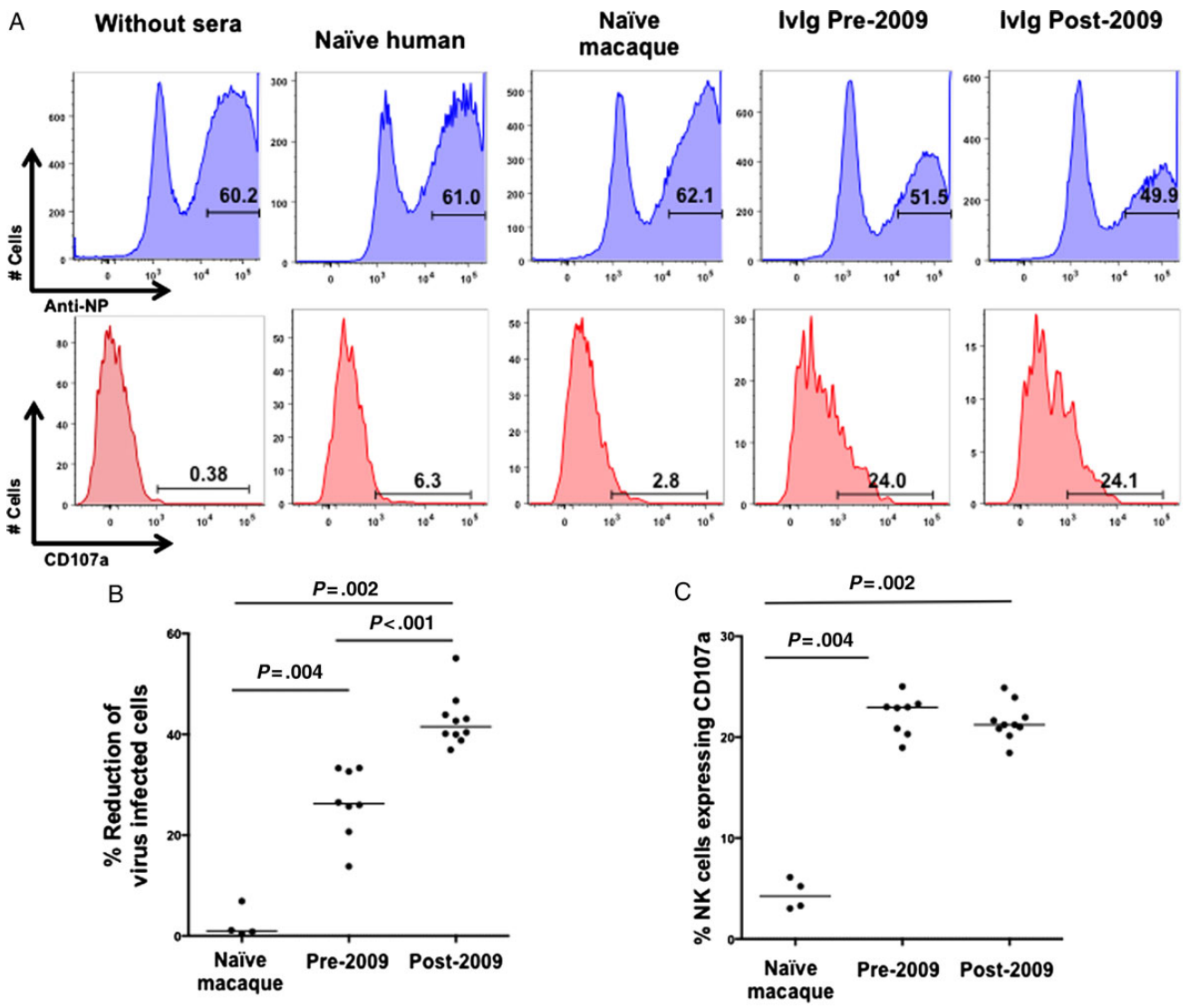

Figure 3. ADCC-mediated elimination of influenza infected respiratory cells. Human airway epithelial cell line (A549) was infected with influenza virus (A/Auckland/01/2009-PR8) at a MOI of 10. After 4 hours incubation, infected or uninfected A549 cells were combined with healthy donor PBMCs at 1:3 (E:T ratio) in the presence or absence of naïve human sera, naïve macaque sera, pre-2009 IVIG (1 mg/mL) or post-2009 IVIG (1 mg/mL). Representative plots of A549 cells expressing influenza virus NP (*top*) and $CD56^+ CD3^-$ NK cells expressing CD107a, with/without naive human sera, naive macaque sera, pre-2009 IVIG or post-2009 IVIG (*A*). The reduction in A549 cells (*B*, naive macaque sera n = 4, n = 8 for pre-2009 IVIG and n = 10 for post-2009 IVIG) and NK cell CD107a expression (*C*, naive macaque sera n = 4, n = 8 for pre-2009 IVIG and n = 10 for post-2009 IVIG) was measured in the presence of naive macaque sera or IVIG preparations manufactured pre-2009 or post-2009. Lines represent the median, and groups were compared by Kruskal–Wallis tests ($P < .001$ for both *B* and *C*), followed by Mann–Whitney *U*-tests. A Bonferroni correction was applied to the post hoc tests, making *P* values < .016 considered significantly different. Abbreviations: ADCC, antibody-dependent cellular cytotoxicity; IVIG, intravenous immunoglobulin; MOI, multiplicity of infection; NK, natural killer; PBMC, peripheral blood mononuclear cell.

protein can be divided into 2 regions, HA1 and HA2. The HA2 region is more conserved and a target for broadly cross-reactive neutralizing antibodies [33–35]. To determine the regions targeted by HA-specific ADCC we measured NK cell activation to whole HA compared to the HA1 protein using IVIG preparation made prior to 2009 and post-2009 at a 10 mg/mL concentration of IVIG. At 10 mg/mL IVIG, we observed no difference in NK cell activation toward whole HA0 protein or HA1 protein (either IFN-γ or CD107a expression) in the presence of IVIG preparations ($P > .05$, Figure 4*A* and 4*B*–IVIG samples pre- and post 2009 are shown as squares and circles, respectively, in the figure). In endpoint titration experiments, however, we observed that IVIG samples had significantly lower ADCC titers to HA1 protein (A/California/04/2009)

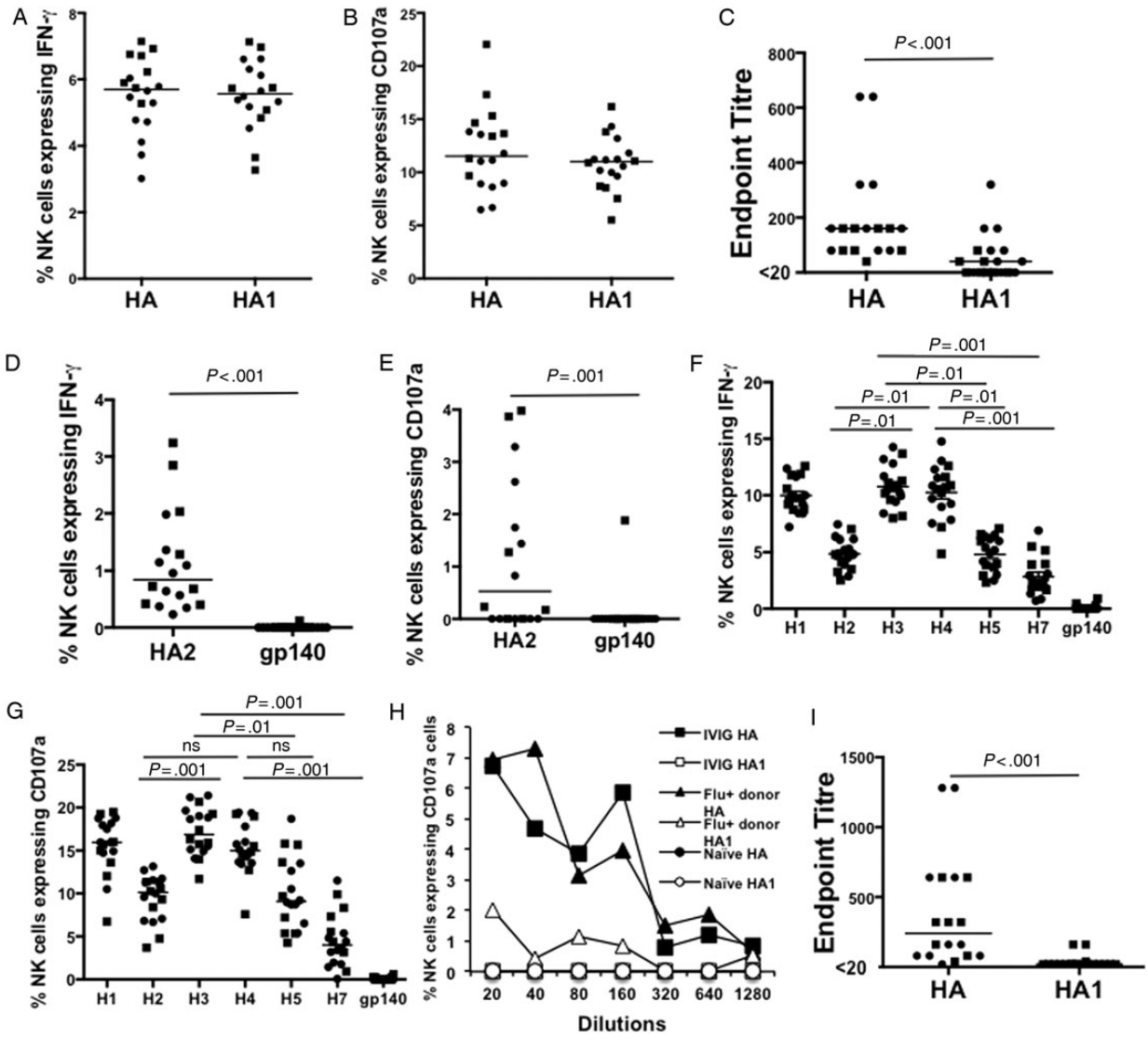

Figure 4. Cross-reactivity of ADCC mediating antibodies in commercial IVIG. NK cell expression of IFN-γ (*A*) and CD107a (*B*) in response to whole HA (A/California/04/2009) vs HA1 (A/California/04/2009) using IVIG preparations made pre-2009 (*square*) and post-2009 (*circle*; n = 18). Comparison of the ADCC antibody endpoint titers to whole HA (A/California/04/2009) vs ADCC antibody endpoint titers to HA1 (A/California/04/2009) were determined by measured NK cells expressing CD107a at 2-fold dilutions of IVIG preparations made pre-2009 (*square*) and post-2009 (*circle*; n = 18, starting concentration 10 mg/mL, 1:20-1:1280) a threshold of 2 times background (with IVIG but without HA/HA1 protein) was used to determine endpoint (*C*). NK cell expression of IFN-γ (*D*) and CD107a (*E*) in response to plate-bound HA2 from A/California/04/2009 and HIV gp140 (AD8) proteins in the presence of IVIG preparations pre-2009 (*square*) and post-2009 (*circle*; n = 18; 10 mg/mL). NK cell expression of IFN-γ (*F*) and CD107a (*G*) in response to H1 A/California/04/2009, H2 (A/Japan/305/1957), H3 (A/Brisbane/10/2007), H4 (A/Swine/Ontario/01911–1/1999), H5 (A/Vietnam/1203/2004), or H7 (A/Anhui/01/2013) HA proteins using IVIG preparations made pre-2009 (*square*) and post-2009 (*circle*). Endpoint titration of pre-2009 IVIG, post-2009 IVIG, healthy donor (flu+ donor) sera or naive macaque sera against A/Swine/Ortario/01911-1/1999 whole HA or HA1 proteins (*H*). ADCC antibody endpoint titers to whole HA (A/Swine/Ortario/01911-1/1999) vs endpoint titers to HA1 (A/Swine/Ortario/01911-1/1999) using IVIG preparations made pre-2009 (*square*) and post-2009 (*circle*; n = 18, starting concentration 10 mg/mL, 1:20-1:1280) a threshold of 2 times background (with IVIG but without HA/HA1 protein) was used to determine endpoint (*I*). All samples were corrected for background based on their response to a well containing IVIG but with no plate-bound antigen. Lines represent the median. Groups in *A* to *E* were compared using Wilcoxon signed-rank tests. Groups in *F* and *G* were compared using Friedman tests ($P < .001$ for both *F* and *G*) followed by Dunn post hoc tests. Abbreviations: ADCC, antibody-dependent cellular cytotoxicity; HA, hemagglutinin; HIV, human immunodeficiency virus; IFN, interferon; IVIG, intravenous immunoglobulin; NK, natural killer.

compared to ADCC titers to HA0 protein ($P < .001$, Figure 4*C*). This suggests that a proportion of the ADCC response is directed toward the HA1 proportion.

The measurement of influenza-specific antibodies toward HA2 is made difficult by the availability of HA2 protein in native conformation. The only available HA2 protein from A/California/04/

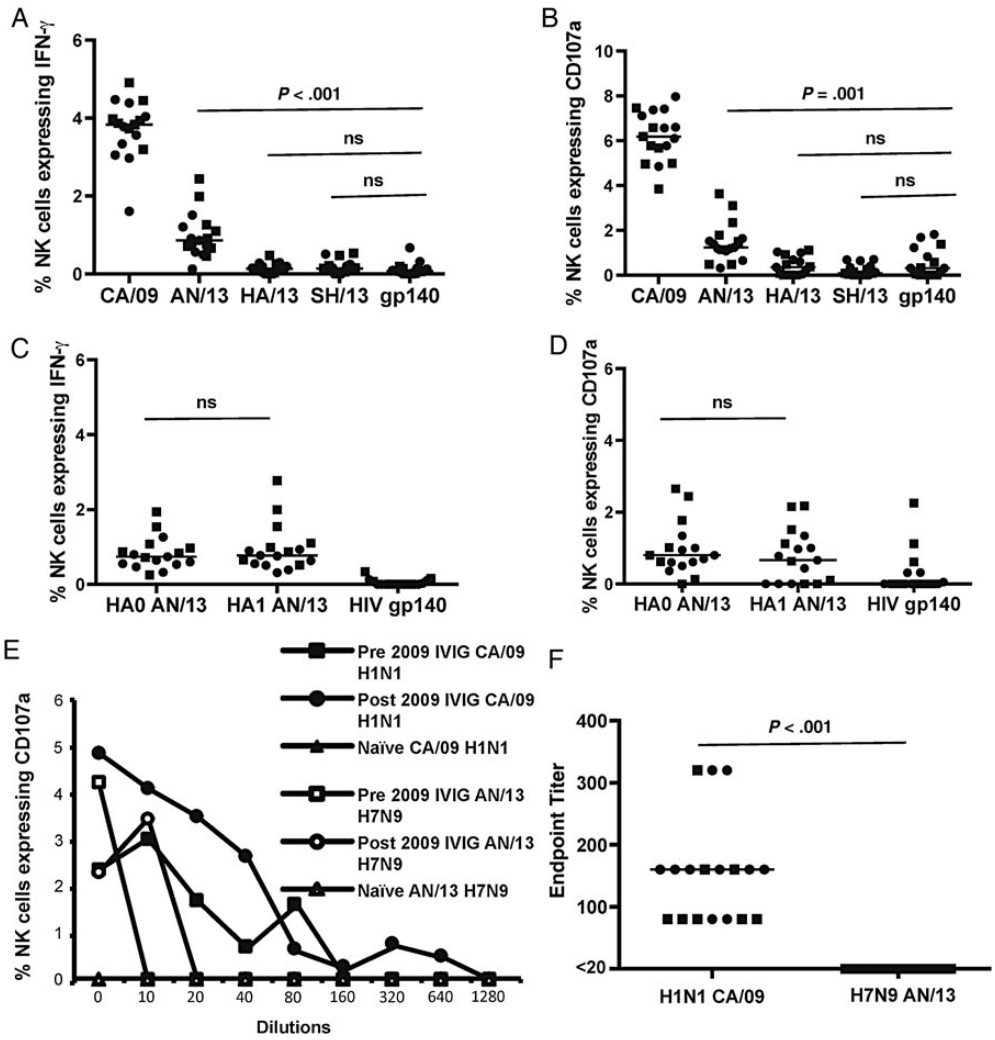

Figure 5. H7N9-specific ADCC mediating antibodies. NK cell expression of IFN-γ (*A*) and CD107a (*B*) in response to H1 A/California/04/2009 (CA/09), H7 Anhui/01/2013 (AN13), A/Hangzou/01/13 (HA/13), A/Shanghai/01/2013, and HIV gp140 (AD8) using IVIG preparations made pre-2009 (*square*) and post-2009 (*circle*). NK cell expression of IFN-γ (*C*) and CD107a (*D*) in response to HA0 and HA1 proteins from H7N9 A/Anhui/01/2013 and HIV gp140 (AD8) using IVIG preparations made pre-2009 (*square*) and post-2009 (*circle*). ADCC antibody endpoint titers to A(H1N1)pdm09 (A/California/04/2009) and H7N9 (A/Anhui/01/2013) HA were determined by measured NK cells expressing CD107a at 2-fold dilutions of either pre-2009 IVIG, post-2009 IVIG, and naive macaque sera (starting concentration 10 mg/mL, Neat-1:1280; *E*). Comparison of the ADCC antibody endpoint titers to A(H1N1)pdm09 (A/California/04/2009) HA vs ADCC antibody endpoint titers to H7N9 (A/Anhui/01/2013) HA using IVIG preparations made pre-2009 (*square*) and post-2009 (*circle*; n = 18, starting concentration 10 mg/mL, 1:20-1:1280) a threshold of 2 times background (with IVIG but without HA/HA1 protein) was used to determine endpoint (*F*). Lines represent the median. Groups in *A* to *B* were compared using Wilcoxon signed-rank tests. Groups were compared using Friedman tests ($P < .001$ for both *A* and *B*) followed by 3 Wilcoxon signed-rank tests, or Kruskal–Wallis tests ($P < .001$ for *C* and $P = .0254$ for *D*) followed by 3 Mann–Whitney *U*-tests. For *A* to *C*, a Bonferroni correction was applied to the post hoc tests, making *P* values < .016 considered significantly different. In *F*, groups were compared using a Mann–Whitney *U*-Test. Abbreviations: ADCC, antibody-dependent cellular cytotoxicity; HA, hemagglutinin; IVIG, intravenous immunoglobulin; NK, natural killer.

2009 was purified from split whole virion preparations following detergent solubilization, negative immune affinity chromatography, and gel electrophoresis. The stability of HA2 protein was confirmed by enzyme immunoassay (EIA) using IVIG preparations and anti-HA2 monoclonal antibodies (n = 3, data not shown). Using this purified HA2 protein, we detected moderate antibody-mediated NK cell activation (both IFN-γ and CD107a expression) toward HA2 in IVIG preparations compared to HIV gp140 protein controls ($P < .005$, Figure 4*D* and 4*E*).

ADCC Antibodies in IVIG Cross-react to Divergent Influenza Strains

Influenza virus HA glycoproteins are classified phylogenetically into group 1 (H1, H2, H5) or group 2 (H3, H4, H7) [36]. The

HA proteins from A(H7N9) and A(H5N1) are of interest given their pandemic potential [37–39]. IVIG is known to contain low-level broadly cross-reactive antibodies to both group 1 and 2 influenza A strains [35, 40–42]. This may include antibodies to the HA-stem region, directed primarily to the alpha-helical region between HA1-HA2 [20]. We detected IVIG-mediated NK cell activation toward all HA proteins tested including H7 and H5, without observable difference in ADCC activity-comparing IVIG samples from before or after 2009 (Figure 4*F* and 4*G*). The median NK cell activation (% NK cells expressing IFN-γ) induced by the 18 IVIGs to H3 and H4 was significantly higher than that induced by H2, H5, and H7 proteins (Figure 4*F*). Similarly, NK cell expression of CD107a induced by the IVIGs to H3 was significantly higher than that induced by H2, H5, and H7 (Figure 4*G*). Endpoint titration of IVIG against a H4 whole HA protein and HA1 protein suggested there was a significant difference between ADCC responses measured against whole HA protein and HA1 protein using IVIG manufactured prior to 2009 and post-2009 (Figure 4*H* and 4*I*, $P < .001$).

ADCC Recognition of H7N9 HA by IVIG

The recent outbreak of H7N9 influenza in humans highlights the need for new influenza therapeutics [38, 43]. Although ADCC recognition of H7 protein in IVIG was low, even low-level cross-reactive ADCC may be useful in treating severe influenza virus infection. To determine the range of cross-reactivity we measured ADCC-mediated NK cell activation towards A(H7N9) HA proteins from A/Anhui/1/2013, A/Shanghai/01/2013 and A/Hangzhou/01/2013. There was low-level ADCC response towards A/Anhui/01/2013 HA protein but no ADCC responses against A/Shanghai/01/2013 and A/Hangzhou/01/2013 (Figure 5*A* and 5*B*). We detected similar ADCC responses towards HA1 and HA0 proteins from A/Anhui/01/2013, suggesting these antibodies are at least partially directed towards the HA1 portion of H7N9 HA (Figure 5*C* and 5*D*). To quantitate H7-specific ADCC in IVIG further we determined end-point titers toward A/Anhui/01/2013 in comparison to A(H1N1)pdm09. We found very low titers (<20) of ADCC mediating antibodies toward H7N9 HA protein in contrast to A(H1N1)pdm09 HA (median, 155; range, 80–320; Figure 5*E* and 5*F*).

ADCC Recognition of H7N9 HA by Healthy Donors

The low level of ADCC mediating antibodies toward H7N9 HA protein suggests that either cross-reactive ADCC antibodies are found at low concentration in multiple individuals or rare donors in the population contribute significantly titers of H7N9 HA-specific ADCC. We therefore evaluated serum obtained in 2012–2013 from 62 healthy plasma donors aged 20–89 for NK cell activation against H7 proteins (A/Anhui/01/2013 and A/Shanghai/01/2013). High antibody-mediated NK cell activation was observed against A(H1N1)pdm09 HA protein in most serum samples (mean % NK cells expressing IFN-γ or CD107a of 3.83, 5.26, respectively); however, only 1 of the 62 individuals tested had high NK cell activation toward H7N9 HA protein (% NK cells expressing IFN-γ or CD107a of 6.22, 8.85 respectively, open circle in Figure 6*A* and 6*B*).

To further investigate the induction of this ADCC response to H7N9 in this donor, we obtained stored plasma samples from 4 separate time-points (2009, 2010, 2011, 2013). Lower ADCC responses to the H7N9 A/Anhui/01/2013 strain were observed in 2009 but increased during 2010 (0.81%–10.80% for IFN-γ and 1.39%–16.41% for CD107a expression, respectively; Figure 6*C* and 6*D*). Endpoint titrations showed a marked increase in the titer of H7N9-specific ADCC antibodies from 1:20 to >1280 from the 2009 to 2010 time-points (Figure 6*E*).

The marked increase in H7N9-specific ADCC in 2010 suggests either an infection with H7N9 had occurred or that an infection/vaccination with another influenza virus in 2009–2010 led to cross-reactive ADCC antibodies. To evaluate this, we assessed anti-influenza neutralizing antibody responses because such responses are typically more specific for individual infections. We found increase in HI titers to A/California/04/2009 (<10 in 2009 to 40 in 2010) without changes to HI levels for 3 H1N1 (A/Brisbane/59/07, A/Fukashima/141/2006, A/California/04/2009) and 3 H3N2 (A/Aichi/02/1968, A/Perth/16/2009, A/Brisbane/10/2007) circulating strains tested (not shown). Further, we confirmed the lack of H7N9 neutralizing antibodies using a sensitive microneutralization assay (not shown). These results suggest an influenza virus infection between 2009 and 2010, probably A(H1N1)pdm09, resulted in ADCC antibodies capable of recognizing H7N9 HA.

DISCUSSION

Improved antiviral therapies that provide broad protection from diverse influenza virus infections are needed [3–9]. We found IVIG preparations made prior to 2009 had substantial ADCC activity to HA and NA proteins of the A(H1N1)pdm09 virus. These ADCC mediating antibodies within IVIG activated NK cells were present at moderate titers and could eliminate A(H1N1)pdm09-infected respiratory cells in vitro. Further evaluation of ADCC mediating antibodies in IVIG as an immunoprophylaxis or immunotherapy against influenza virus infection is warranted, particularly where effective vaccines or other therapies are not available.

Antibodies capable of nonneutralizing function may have a broader degree of cross-reactivity than their neutralizing counterparts [29]. We found IVIG preparations contained ADCC mediating antibodies reactive against group 1 and group 2 influenza virus HA proteins including avian H5N1 and H7N9. This is consistent with our studies showing cross-reactive ADCC antibodies in healthy subjects [20, 29]. Several broadly neutralizing

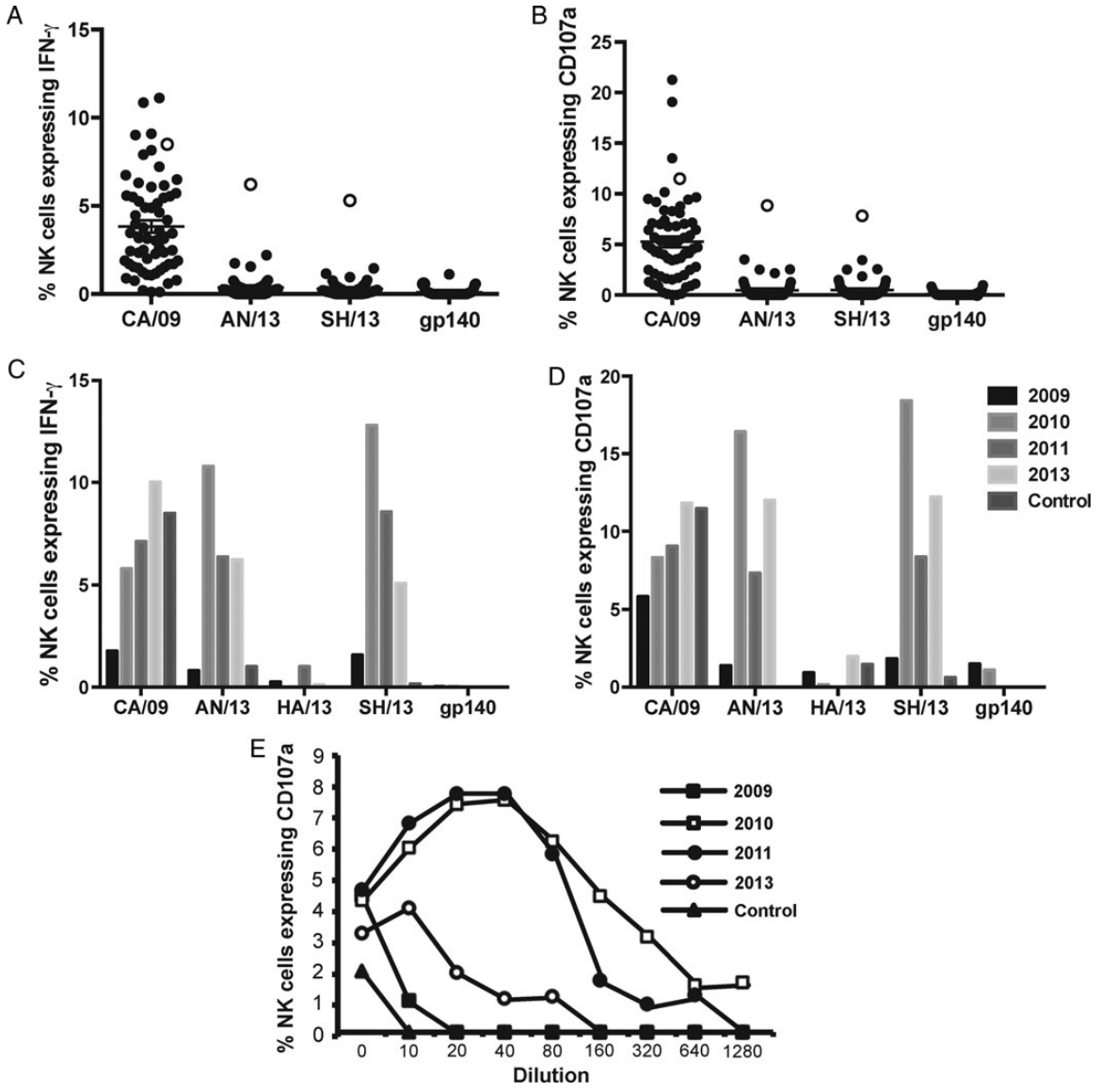

Figure 6. Detecting H7N9 cross-reactive ADCC in healthy donor serum. NK cell expression of IFN-γ (*A*) and CD107a (*B*) in response to H1 A/California/04/2009 (CA/09), H7 Anhui/01/2013 (AN13), A/Shanghai/01/2013, and HIV gp140 (AD8) using serum samples from 62 healthy donors. NK cell expression of IFN-γ (*C*) and CD107a (*D*) in response to H1 A/California/04/2009 (CA/09), H7 Anhui/01/2013 (AN13), A/Hangzou/01/13 (HA/13), A/Shanghai/01/2013 and HIV gp140 (AD8) using healthy donor sera from 4-separate time-points (2009, 2010, 2011, 2013) and healthy control sera. ADCC antibody endpoint titers to H7N9 (A/Anhui/01/2013) HA were determined by measured NK cells expressing CD107a at 2-fold dilutions of donor sera from 4 separate time-points (2009, *closed squares*; 2010, *open squares*; 2011, *closed circles*; 2013, *open circles*) and healthy control sera (*triangles*, concentrations tested Neat-1:1280). Abbreviations: ADCC, antibody-dependent cellular cytotoxicity; HA, hemagglutinin; HIV, human immunodeficiency virus; IFN, interferon; IVIG, intravenous immunoglobulin; NK, natural killer.

antibodies against stem-regions of HA have robust ADCC function in mice [19, 36].

Further experiments on the efficacy of ADCC mediating antibodies within IVIG in animal models of influenza are warranted. Indeed, we recently found that a human dose equivalent of IVIG (produced from plasma collected in 2004) administered at the time of influenza virus exposure demonstrated a significant reduction in lung viral load in mice (data not shown). Furthermore, in a lethal H5N1 influenza ferret model, a majority of IVIG-treated animals survived challenge (Rockman et al, in preparation). Dissecting immune correlates of IVIG-induced protection from influenza may aid designing improved influenza vaccines.

Our studies indicate cross-reactive ADCC antibodies toward H5N1 and H7N9 are present within IVIG at low concentrations. This is likely due to only a small number of individual IVIG donors contributing H7N9 cross-reactive ADCC antibodies. The generation of rare subsets of cross-reactive influenza-

specific antibodies has been previously discussed in other studies [44]. The isolation of antibodies from such donors could lead to the generation of more potent antibody preparations [28].

We recognize that there are limitations to our study that warrant future investigations. (1) Our data do not show that influenza ADCC antibodies in IVIG are protective in vivo. Recent studies with broadly neutralizing stem-antibodies suggest that ADCC activity is essential for in vivo potency in mice [19]. Studies on the direct role of cross-reactive ADCC mediating antibodies in IVIG for in vivo protection against emerging influenza strains are necessary. (2) The poorer conformational stability of HA1 and HA2 proteins compared to HA0 proteins used in this study may underestimate endpoint ADCC titers to these proteins. While we show as a proof-of-principle that HA2 region can be a target of ADCC mediating antibodies, further measures of the relative contribution of ADCC antibodies to each region and the mapping of specific ADCC epitopes is warranted. (3) We show IVIG contains ADCC antibodies that can kill A (H1N1)pdm09-infected cells. However, further investigation of the ability of ADCC antibodies in IVIG to kill cells infected with a wider range of IAVs is needed. (4) Although we identified 1 individual subject out of 62 screened that had H7-specific ADCC, this does not definitively show that these rare individuals are contributing the H7-specific ADCC within IVIG. Analyses of sera from larger numbers of subjects are warranted.

In summary, IVIG preparations made prior to the 2009-H1N1 pandemic contain cross-reactive ADCC mediating antibodies towards A(H1N1)pdm09 HA and NA proteins. Further, there is cross-reactive ADCC mediating antibodies against avian origin influenza strains H5N1 and H7N9 within IVIG. These studies suggest the possible utility of IVIG as a therapy during influenza pandemics.

Notes

Acknowledgments. The authors are grateful to Sarina Camuglia at CSL for assistance with multiple reagents. They thank Dr Patrick C. Reading at WHO Collaborating Centre for Reference and Research on Influenza (VIDRL) and Dr Rob Center at University of Melbourne Microbiology and Immunology for assistance with reagents.

Financial support. Australian National Health and Medical Research Council (NHMRC) awards 628331, 1023294, and 510488. The Melbourne WHO Collaborating Centre for Reference and Research on Influenza is supported by the Australian Government Department of Health.

Potential conflict of interest. S. R. and K. V. are employees of bioCSL Ltd, a company that manufactures IVIG preparations. K. V. and S. R. are employees of bioCSL Ltd, a subsidiary of CSL Ltd, that manufactures intravenous immunoglobulin. All other authors report no potential conflicts.

All authors have submitted the ICMJE Form for Disclosure of Potential Conflicts of Interest. Conflicts that the editors consider relevant to the content of the manuscript have been disclosed.

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