Anti-Influenza Hyperimmune Immunoglobulin Enhances Fc-Functional Antibody Immunity During Human Influenza Infection

Hillary A. Vanderven,1,2 Kathleen Wragg,1 Fernanda Ana-Sosa-Batiz,1 Anne B. Kristensen,1 Sinthujan Jegaskanda,1 Adam K. Wheatley,1 Deborah Wentworth,1,3 Bruce D. Wines,1 P. Mark Hogarth,1,4 Steve Rockman,1,5 and Stephen J. Kent1,6; INSIGHT FLU005 Pilot Study Writing Group

1Department of Microbiology and Immunology, Peter Doherty Institute for Infection and Immunity, University of Melbourne, Victoria, Australia; 2Biomedicine, College of Public Health, Medical and Veterinary Sciences, James Cook University, Douglas, Queensland, Australia; 3Biostatistics Division, University of Minnesota, Minneapolis; and 4Burnet Institute, Melbourne, 5Seqirus Ltd, Parkville, 6Melbourne Sexual Health Centre and Department of Infectious Diseases, Alfred Health, Central Clinical School, Monash University, and Australian Research Council Centre of Excellence in Convergent Bio-Nano Science and Technology, University of Melbourne, Parkville, Victoria, Australia

**Background.** New treatments for severe influenza are needed. Passive transfer of influenza-specific hyperimmune pooled immunoglobulin (Flu-IVIG) boosts neutralizing antibody responses to past strains in influenza-infected subjects. The effect of Flu-IVIG on antibodies with Fc-mediated functions, which may target diverse influenza strains, is unclear.

**Methods.** We studied the capacity of Flu-IVIG, relative to standard IVIG, to bind to Fcy receptors and mediate antibody-dependent cellular cytotoxicity in vitro. The effect of Flu-IVIG infusion, compared to placebo infusion, was examined in serial plasma samples from 24 subjects with confirmed influenza infection in the INSIGHT FLU005 pilot study.

**Results.** Flu-IVIG contains higher concentrations of Fc-functional antibodies than IVIG against a diverse range of influenza hemagglutinins. Following infusion of Flu-IVIG into influenza-infected subjects, a transient increase in Fc-functional antibodies was present for 1–3 days against infecting and noninfecting strains of influenza.

**Conclusions.** Flu-IVIG contains antibodies with Fc-mediated functions against influenza virus, and passive transfer of Flu-IVIG increases anti-influenza Fc-functional antibodies in the plasma of influenza-infected subjects. Enhancement of Fc-functional antibodies to a diverse range of influenza strains suggests that Flu-IVIG infusion could prove useful in the context of novel influenza virus infections, when there may be minimal or no neutralizing antibodies in the Flu-IVIG preparation.

**Keywords.** influenza; immunoglobulin; ADCC; Fc receptor; passive transfer.

Alternative anti-influenza therapeutic strategies are urgently needed [1, 2]. One potential solution involves infusing pooled immunoglobulin from influenza-immune donors, termed influenza-specific hyperimmune immunoglobulin (Flu-IVIG) [3, 4]. There is some evidence from meta-analyses that passive transfer of antibody is a plausible therapy for severe influenza [5]. Recent placebo-controlled trials investigated convalescent plasma and hyperimmune IVIG during severe pandemic 2009 H1N1 (pH1N1) infection [3, 4]. Although reduced mortality and viral load was suggested, they lacked the design rigor to fully validate this approach.

Recently, a randomized, placebo-controlled INSIGHT FLU005 pilot study assessed Flu-IVIG for enhancing neutralizing antibodies in severe influenza infection [6]. Subjects infected with pH1N1 had greater hemagglutination inhibition (HAI) antibody titers against pH1N1 virus for approximately 3 days postinfusion with Flu-IVIG compared to the placebo controls [6]. While this indicates that Flu-IVIG could favorably modulate disease progression, a caveat concerns the utility of Flu-IVIG in future seasons. As Flu-IVIG is primarily manufactured in advance of future epidemics, there may be modest or no neutralizing activity against emerging strains.

HAI antibodies prevent viral attachment but usually target epitopes that accumulate point mutations and glycosylation, limiting specificity to small numbers of influenza strains [7–10]. Antibodies with Fc-mediated functions commonly target conserved epitopes and have the capacity to mediate complement-directed killing [11, 12], phagocytosis [13] and antibody-dependent cellular cytotoxicity (ADCC) toward influenza-infected cells [14–17]. Fc gamma receptor (FcγR) IIIa is expressed on the surface of innate immune cells such as natural killer (NK) cells, and binds the Fc portion of antigen-bound immunoglobulin G (IgG) on influenza-infected cells. Upon cross-linking of Fc-engaged FcγRIIIa molecules, NK cells release cytokines, perforin, and granzyme from intracellular stores. ADCC enhances clearance of influenza-infected cells in vitro and protects from lethal H1N1 challenge in...
mice [18–22]. Furthermore, cross-reactive, ADCC-mediating antibodies commonly exist in the absence of neutralizing antibodies [18, 19, 23].

Given the broad cross-reactivity and protective effects of ADCC antibodies, it is conceivable that ADCC antibodies may increase the effectiveness of Flu-IVIG toward drifted seasonal strains of influenza. We characterized Flu-IVIG for Fc effector functions and studied the effect of Flu-IVIG on ADCC responses in 24 subjects with influenza infection.

**MATERIALS AND METHODS**

**Plasma Samples**

Plasma was obtained from the INSIGHT FLU005 pilot study (INSIGHT 005: Flu-IVIG Pilot, ClinicalTrials.gov identifier NCT02008578) [6]. Of 31 subjects enrolled, 24 had polymerase chain reaction (PCR)–confirmed influenza A or B and 4 serial (preinfusion and days 1, 3, and 7 postinfusion) plasma samples available. Most subjects also had a 1-hour time point (21/24) and a day 28 time point (21/24) available. Subjects randomly received Flu-IVIG (0.25 g/kg; n = 13) or saline placebo (n = 11).

**Intravenous Immunoglobulin**

The Flu-IVIG studied was manufactured by Emergent BioSolutions under contract to the National Institutes of Health. The lot of Flu-IVIG used in the INSIGHT FLU005 pilot study was prepared in 2013 and had reciprocal geometric mean HAI antibody titers of 1:640 against the A/California/7/2009(H1N1) pandemic influenza virus, 1:320 against the A/Victoria/361/2011(H3N2) influenza virus, and 1:160 against the B/Massachusetts/2/2012 influenza virus (see [6] for further details). We studied both the Flu-IVIG administered in the pilot study (prepared in 2013) and 3 additional, separately manufactured Flu-IVIG preparations (from 2014, 2015, and 2016, designated Flu-IVIG batches 1, 2, and 3 respectively) being used in an ongoing expanded clinical trial (INSIGHT FLU006, ClinicalTrials.gov identifier NCT02287467). Standard IVIGs manufactured in 2008 (prior to the 2009 pH1N1 pandemic), 2010, and 2016 were studied as comparators, with each containing pooled IgG from thousands of human immunodeficiency virus–negative donors. The IVIG prepared in 2016 is Intragam P (Seqirus Ltd) and we studied 3 additional batches of Intragam P (designated Intragam P batches 1–3). We also compared the Flu-IVIG from 2013, which was administered in the INSIGHT FLU005 pilot study, to a standard IVIG (Hizenta) also manufactured in 2013.

**Recombinant Proteins and Influenza Virus**

Recombinant influenza hemagglutinin (HA), nucleoprotein (NP), and simian immunodeficiency virus envelope proteins were purchased from Sinobiological. Neuraminidase (NA), and simian immunodeficiency virus–negative donors. The IVIG prepared in 2016 is Intragam P (Seqirus Ltd) and we studied 3 additional batches of Intragam P (designated Intragam P batches 1–3). We also compared the Flu-IVIG from 2013, which was administered in the INSIGHT FLU005 pilot study, to a standard IVIG (Hizenta) also manufactured in 2013.

Recombinant influenza hemagglutinin (HA), nucleoprotein (NP), and simian immunodeficiency virus envelope proteins were purchased from Sinobiological. Neuraminidase (NA) protein, purified from whole inactivated influenza virus as previously described [19, 24], was provided by Seqirus Ltd. A stabilized HA stem protein derived from A/California/07/2009(H1N1) was designed as previously described [25], expressed using transient transfection of mammalian Expi293 cells (ThermoFisher) and purified using Ni sepharose Excel (GE Healthcare). The A/California/07/2009 pandemic-like virus A/Auckland/01/2009 was used for infection of A549 cells.

**FcγRIIa and FcγRIIIa Dimeric Binding Enzyme-Linked Immunosorbent Assay**

To compare the capacity of IVIG preparations and patient plasma to cross-link FcγRs, a dimeric recombinant soluble FcγR (rsFcγR) binding enzyme-linked immunosorbent assay (ELISA) was employed, as described previously [26–28]. In the patient plasma samples, Intragam P 2016 was used as a positive control for FcγR cross-linking and to normalize between ELISA plates as previously described [27].

**HA Stem IgG ELISA**

One hundred nanograms of HA stem protein in phosphate-buffered saline was coated on 96-well plates overnight, blocked with 5% bovine serum albumin for 2 hours at 37°C, then washed. Serial 2- to 4-fold dilutions of antibody were incubated for 2 hours at room temperature and washed. Rabbit antihuman IgG horseradish peroxidase (1:4000 dilution, Aligent) was incubated at room temperature for 1 hour then washed. 3,3,5,5-tetramethylbenzidine (TMB) was added, color developed, stopped with 1 M hydrochloric acid (HCl), and absorbance read at 450 nm.

**Antibody-Mediated NK Cell Activation**

As a measure of NK cell activation, CD107a externalization was quantified by flow cytometry in response to antibodies immobilized by plate-bound influenza antigen, as previously described [18]. To model NK cell activation in influenza infection, NK-92–FcγRIIa–green fluorescent protein (GFP) cell CD107a expression was measured when cultured with influenza virus–infected respiratory epithelial A549 cells, as previously described [29].

**ADCC of Influenza-Infected Cells**

A lactate dehydrogenase (LDH) assay was adapted to assess ADCC-mediated killing of influenza-infected cells [30, 31]. In brief, A549 cells were infected with pH1N1 virus (A/Auckland/01/2009, multiplicity of infection = 5) for 5 hours. A549 cells (20000) were then incubated 1:1 with NK-92–FcγRIIa–GFP cells and 2-fold dilutions of IVIG in triplicate for 4 hours. The Cytotox 96 kit (Promega) quantified supernatant LDH. Cytotoxicity was calculated: [(experimental – effector spontaneous – target spontaneous) / (maximum LDH – target spontaneous)]. Background killing was 2.3% without antibody.

**Statistical Analysis**

Analysis of covariance, with the baseline level as a covariate, was used to compare treatment differences at each time point. For titer data, analysis of covariance with the log-transformed
preinfusion titer as a covariate was used to compare treatment groups for log-transformed titer levels at each follow-up time point. Treatment differences for log-transformed titer levels were back-transformed to obtain geometric mean titers (GMTs). FcyRIIIa titers reported as <40 and >2560 were imputed as 20 and 5120, respectively. Statistical analyses were performed using SAS version 9.4 software.

RESULTS

**Flu-IVIG Contains More HA-Specific FcyR Cross-Linking Antibodies Than Commercially Available IVIGs**

Standard IVIG and Flu-IVIG preparations have been studied for treatment of severe influenza infections, but most analyses have been confined to neutralization assays [6, 32]. Antibodies with Fc-mediated effector functions commonly recognize a wide range of influenza strains and provide some protective immunity from influenza virus infection in vivo [21, 22, 33]. FcyRIIIa cross-linking antibodies in Flu-IVIG and 3 IVIG preparations from 2008, 2010, and 2016 were first tested against recombinant hemagglutinin (rHA) protein from the A/California/07/2009 (H1N1) pandemic swine influenza virus (pH1N1, Figure 1A). There were 5- to 9-fold higher concentrations of anti-pH1N1 HA FcyRIIIa dimer binding antibodies in Flu-IVIG compared to standard IVIG made in 2010 or 2016 and a 66-fold higher concentration than IVIG made in 2008, prior to the 2009 pandemic (Figure 1C).

Anti-influenza FcyRIIIa cross-linking antibodies mediating antibody-dependent phagocytosis (ADP) by alveolar macrophages has been highlighted as a possible mechanism of protection against heterologous influenza virus challenge in mice [33]. Flu-IVIG also demonstrated higher levels of FcyRIIIa cross-linking antibodies to the pH1N1 rHA compared to standard IVIGs (Figure 1B).

**Figure 1.** Greater Fc gamma receptor (FcγR) cross-linking by anti-hemagglutinin (HA) antibodies in influenza-specific hyperimmune immunoglobulin (Flu-IVIG). Recombinant soluble (rs) FcγR dimer ELISAs were used to compare Flu-IVIG made during 2013 to standard IVIGs made in 2008 (prior to pandemic H1N1 [pH1N1]), 2010, and 2016. Dimeric rsFcγRIIIa (A) and dimeric rsFcγRIIIa (B) binding antibodies (optical density [OD]) against pH1N1 recombinant hemagglutinin (rHA) protein (A/California/07/2009 strain) are shown in 4 different IVIG preparations. The (1 / effective concentration 50 [EC50]) × 10^3 values for dimeric rsFcγRIIIa (C) and dimeric rsFcγRIIIa (D) binding antibodies against diverse rHA proteins were calculated for Flu-IVIG and 3 standard IVIG preparations. Fold increase in the concentration of FcyR cross-linking antibodies in Flu-IVIG relative to standard IVIGs was calculated by dividing the (1 / EC50) × 10^3 value for Flu-IVIG by the (1 / EC50) × 10^3 value for standard IVIG (μg/mL)^{-3} and IVIG 2008 (1 / EC50) × 10^3 = 4.56 (μg/mL)^{-3}, so 23.55 / 4.56 = 5.2-fold increase. Fold increases in the concentration of FcyR cross-linking antibodies in Flu-IVIG relative to standard IVIGs prepared between 2008 and 2016 are shown. All IVIG preparations were also tested for binding against an irrelevant simian immunodeficiency virus glycoprotein 120, the mean background was calculated and multiplied by 3 to give a threshold of detection represented by the dotted line. Error bars represent standard deviation of the mean of duplicate wells in the FcγR dimer ELISA.
To assess breadth of responses, we tested FcγRIIIa dimer binding antibodies in Flu-IVIG against rHA proteins from 13 different influenza viruses (Figure 1C). Flu-IVIG contained higher concentrations of FcγRIIIa cross-linking antibodies against a variety of different rHA proteins by both effective concentration 50 (EC_{50}) (Figure 1C) and endpoint titer (Supplementary Figure 1A) than IVIGs prepared in 2008, 2010, and 2016. Similarly, Flu-IVIG contained more FcγRIIIa cross-linking antibodies against rHAs from 3 seasonal influenza virus strains circulating in 2013–2014 (Figure 1D and Supplementary Figure 1B).

Batch-to-batch variation of FcγRIIIa cross-linking antibodies against 3 influenza rHA proteins was low across 4 Flu-IVIGs made from 2013 to 2016 (Supplementary Figure 2A). Similarly, 4 batches of the standard IVIG Intragram P (IVIG 2016 [which is Intragram P] and 3 additional Intragram P batches 1–3) also had relatively uniform FcγRIIIa dimer binding antibodies against 3 influenza rHA proteins (Supplementary Figure 2B). As Flu-IVIG was prepared in 2013, we compared Flu-IVIG to IVIG prepared during 2013 (Hizentra) for FcγRIIIa cross-linking antibodies to 3 influenza strains circulating in 2013–2014 (Supplementary Figure 2C). The 2013 Flu-IVIG contained greater quantities of FcγRIIIa cross-linking antibodies than the 2013 IVIG.

**Flu-IVIG Mediates ADCC**

Engaging FcyRs on NK cells can result in effector functions, including expression of the degranulation marker CD107a and ADCC of influenza-infected cells. We performed NK cell activation and ADCC assays against rHA proteins or influenza-infected cells. Flu-IVIG demonstrated higher concentrations of NK cell activating antibodies against rHAs from three 2013–2014 influenza strains than IVIG preparations from 2008, 2010, and 2016 (Figure 2A and 2D), corroborating the FcγR binding data above.

Influenza-infected cells express a range of influenza proteins other than HA. NK cell activation assays were therefore performed using a respiratory epithelial cell line infected with pH1N1. Flu-IVIG contained more influenza-specific NK cell activating antibodies than IVIG preparations from 2008 and 2016 by EC_{50} but nearly identical EC_{50}s were calculated for Flu-IVIG and an IVIG prepared in 2010 (Figure 2B and 2D). This suggests that influenza proteins besides HA may be involved in antibody-dependent NK cell activation and ADCC. We therefore tested the 4 IVIG preparations against 2 other known protein targets of influenza-specific ADCC (NA and NP), using the dimeric FcγRIIIa binding ELISA and the NK cell activation assay (Supplementary Figure 3). Flu-IVIG had the highest concentration of FcγRIIIa cross-linking and NK cell activating antibodies against the pH1N1 NA protein. Although lower than Flu-IVIG, standard IVIG preparations from 2010 and 2016 contained readily detectable NA-specific FcγRIIIa dimer binding and NK cell activating antibodies. IVIG prepared prior to the 2009 pandemic in 2008 only demonstrated modest levels of pH1N1 NA-specific FcγRIIIa cross-linking and NK cell activating antibodies (Supplementary Figure 3A and 3C). Likewise, NP-specific FcγRIIIa binding and NK cell activation were slightly higher in Flu-IVIG than standard IVIGs from 2008 and 2016, but almost identical to the 2010 preparation (Supplementary Figure 3B and 3D).

ADCC assays were performed to assess killing of pH1N1-infected respiratory epithelial cells in the presence of Flu-IVIG or IVIG. Flu-IVIG demonstrated higher concentrations of ADCC-mediating antibodies compared to IVIG (Figure 2C and 2D). Overall, Flu-IVIG contained higher levels of influenza-specific FcγR cross-linking, NK cell activating, and ADCC antibodies compared to standard IVIG in vitro, suggesting that this preparation will be useful for analyzing enhanced Fc-functional antibodies in subjects with influenza infections in vivo.

**Flu-IVIG Contains HA Stem-Specific Antibodies**

Monoclonal antibodies targeting the HA stem mediate potent ADCC in vitro [34], and we therefore assessed HA stem antibodies. Flu-IVIG and the standard IVIG from 2010 demonstrated higher concentrations of pH1N1 HA stem-specific antibodies than IVIGs prepared in 2008 and 2016 by IgG ELISA, FcγRIIIa dimer ELISA, and NK cell activation assay (Figure 3A–C). The higher levels of HA stem antibodies in the 2010 IVIG presumably reflect recent infections with pH1N1 in the human population [35].

**Flu-IVIG Enhances FcγRIIIa Cross-Linking Antibodies Early After Infusion**

Fc-functional antibodies are readily detectable in concentrated IVIG preparations [19], but to provide clinical benefits these antibodies must be present after in vivo administration. We studied samples from 24 subjects from the INSIGHT FLU005 pilot study, first examining the 15 subjects enrolled that were naturally infected with pH1N1-like viruses. FcγRIIIa dimer ELISAs were performed with plasma from patients infused with either Flu-IVIG (8 patients) or placebo (7 patients). Plasma from recipients of Flu-IVIG demonstrated significantly more FcγRIIIa cross-linking antibodies against the pH1N1 rHA at 1 hour and 1 day postinfusion than placebo recipients, but not at 3 days, 7 days, and 28 days postinfusion (Figure 4A and 4B). The rsFcγRIIIa dimer ELISA was also performed using plasma samples from all 24 patients with PCR-confirmed influenza infections: 15 patients infected with pH1N1, 3 patients infected with H3N2, and 6 patients infected with influenza B. As with the pH1N1-infected subjects alone, plasma samples from the Flu-IVIG group contained significantly higher titers of HA-specific FcγRIIIa cross-linking antibodies at 1 hour and 1 day postinfusion (Figure 4C), with Flu-IVIG/placebo ratios of 5.74 (95% confidence interval [CI], 3.52–9.35) and 3.77 (95% CI, 2.34–6.07), respectively. Collectively, these data suggest that
Flu-IVIG infusion elevated HA-specific FcγRIIIa cross-linking antibody levels relative to the natural humoral immune response (placebo) early after infusion. We also assessed HA stem antibodies in the 15 patients with pH1N1-like infections. Flu-IVIG recipients had significantly higher HA stem-specific IgG at 1 hour and 1 day postinfusion than placebo subjects (Supplementary Figure 4).

Flu-IVIG Increases Antibody-Dependent NK Cell Activation Early Postinfusion

ADCC-mediating antibodies are linked to less severe disease [29]. We determined whether plasma from Flu-IVIG–infused patients contains antibodies capable of stimulating NK cell degranulation. Flu-IVIG infused subjects with pH1N1 infections demonstrated greater HA-specific NK cell activating antibodies at 1 hour and 1 day postinfusion than placebo recipients (Figure 5A). The Flu-IVIG group also trended toward a higher GMT of NK cell activating antibodies against pH1N1-infected cells relative to the control group (P = .06; Figure 5B), with a Flu-IVIG/placebo ratio of 2.48 (95% CI, 96–6.43). There was no significant difference between Flu-IVIG and placebo-infused patients beyond 1 day postinfusion (Figure 5A and 5B).

Flu-IVIG Infusion Increases HA-Specific FcγRIIa Cross-Linking Antibodies

Above we showed that Flu-IVIG also contains FcγRIIa cross-linking antibodies, which may mediate ADP of influenza virions or infected cells (Figure 1B and 1D). FcγRIIa dimer ELISAs were therefore performed on plasma samples from the 15 pH1N1-infected subjects. At 1 hour and 1 day postinfusion, plasma samples from the Flu-IVIG infused patients demonstrated greater HA-specific FcγRIIa cross-linking than plasma samples from the control group (Figure 6).

Flu-IVIG Infusion Enhances FcγRIIa Cross-Linking Antibodies Against Diverse Influenza Virus HAs

We showed above (Figures 1C, 1D, and 2D) that Flu-IVIG contains ADCC-mediating antibodies capable of binding to different strains and subtypes of influenza virus. The breadth
of FcγRIIIa cross-linking antibodies was assessed in plasma samples from patients infected with pH1N1 viruses. As with the homologous H1 subtype, Flu-IVIG infusion increased **Figure 3.** Influenza-specific hyperimmune immunoglobulin (Flu-IVIG) contains hemagglutinin (HA) stem-specific antibodies. Immunoglobulin G (IgG) enzyme-linked immunosorbent assays (ELISAs), recombinant soluble Fc gamma receptor (rsFcγR) dimer ELISAs, and natural killer (NK) cell activation assays were used to compare Flu-IVIG made during 2013 to standard IVIGs made in 2008 (prior to pandemic H1N1 [pH1N1]), 2010, and 2016. IgG antibodies (optical density [OD]) (A), dimeric rsFcγRIIIa binding antibodies (OD) (B), and NK cell activating antibodies (C) against the pH1N1 HA stem are shown in the 4 different IVIG preparations. All IVIG preparations were also tested for dimeric rsFcγRIIIa binding antibodies against an irrelevant simian immunodeficiency virus glycoprotein 120; the mean background was calculated and multiplied by 3 to give a threshold of detection represented by the dotted line. Error bars represent standard deviation of the mean of duplicate wells in the FcγR dimer ELISA.

**Figure 4.** Increased Fc gamma receptor (FcγR) IIIa cross-linking antibodies against hemagglutinin (HA) following infusion of influenza-specific hyperimmune immunoglobulin (Flu-IVIG) into subjects with influenza infection. The recombinant soluble (rs) FcγRIIIa dimer enzyme-linked immunosorbent assays were used to examine serial plasma samples from 24 influenza-infected subjects (15 of whom had pandemic H1N1 [pH1N1] infections) randomized to receive Flu-IVIG or placebo. (A) Means of dimeric rsFcγRIIIa binding antibodies against pH1N1 recombinant HA (rHA) in 24 influenza-infected subjects. GMTs of dimeric rsFcγRIIIa binding antibodies against pH1N1 recombinant HA (rHA) protein (at a 1:40 dilution of plasma) in the 15 pH1N1-infected subjects are shown. Preinfusion antibody levels for each individual patient were subtracted from that patient's follow-up samples to give change from baseline at every time point, and the adjusted mean changes from baseline (preinfusion) are graphed. Mean optical density (OD) of preinfusion samples was 0.22 for controls and 0.22 for the Flu-IVIG group. (B) Geometric mean titers (GMTs) of dimeric rsFcγRIIIa binding antibodies against pH1N1 rHA in the 15 pH1N1-infected subjects are graphed. GMTs for postinfusion time points are shown adjusted for baseline or preinfusion titer; the mean GMTs for preinfusion samples were 59 for the control group and 62 for the Flu-IVIG group. (C) GMTs of dimeric rsFcγRIIIa binding antibodies against pH1N1 rHA in 24 influenza-infected subjects (15 had pH1N1, 3 had H3N2, and 6 had B infection) are shown. As before, postinfusion GMTs were adjusted for preinfusion titers, which were 80 for the control group and 65 for the Flu-IVIG group. All plasma samples were also tested for dimeric rsFcγRIIIa binding antibodies against an irrelevant simian immunodeficiency virus type 1 glycoprotein 120, and background was subtracted for each plasma sample. Error bars represent standard error of the mean. ***P < .001.
Finally, FcγRIIIa cross-linking antibodies against the rHA of B/Phuket/3073/2013 influenza virus demonstrated higher levels of FcγRIIIA cross-linking antibodies against group 2 rHAs (H3 and H4 subtypes) at 1 hour, 1 day, 3 days, and 7 days postinfusion (Figure 7C), likely reflecting the eventual decay of the infused Flu-IVIG.

DISCUSSION

There has long been interest in passively transferred antibodies to treat influenza, but in-depth laboratory analyses of randomized controlled trials are few. Treatment of severe human influenza with convalescent blood products significantly reduced mortality during the 1918 and 2009 pandemics [3–5]. Commercially available IVIG preparations have also been studied for treatment of influenza [4, 36] but, unlike convalescent plasma, they are not enriched in influenza-specific antibodies.

Flu-IVIG infusion increases serum HAI antibody titers in treated patients compared with placebo-infused controls [6]. However, because therapeutic Flu-IVIG is administered after influenza infection is established, Fc-mediated antibody functions may also be important for clearance of influenza-infected cells. IVIG preparations have been shown to contain broadly reactive antibodies with ADCC and ADP activity [19, 37].

Figure 5. Enhanced influenza-specific antibody-mediated natural killer (NK) cell activation following influenza-specific hyperimmune immunoglobulin (Flu-IVIG) infusion. NK cell activation assays measuring CD107a expression were used to study serial plasma samples from 15 subjects with pandemic H1N1 (pH1N1) infections randomized to receive either Flu-IVIG or placebo. Mean changes from preinfusion or baseline for NK cell activating antibodies to pH1N1 recombinant hemagglutinin (rHA) protein (A) and geometric mean titers (GMTs) of NK cell activating antibodies to pH1N1-infected A549 cells (B) are shown. Data in A and B are adjusted for preinfusion antibody levels. All plasma samples were also tested for antibody-mediated NK cell activation against an irrelevant simian immunodeficiency virus type 1 glycoprotein 120, and background was subtracted for each individual sample. Error bars represent standard error of the mean. **P < .01, ***P < .001.

Figure 6. Greater Fc gamma receptor (FcγR) IIa cross-linking antibodies against hemagglutinin (HA) following infusion of influenza-specific hyperimmune immunoglobulin (Flu-IVIG) into subjects with influenza infection. A recombinant soluble (rs) FcγRlla dimer enzyme-linked immunosorbent assay was used to examine serial plasma samples from 15 subjects with pandemic H1N1 (pH1N1) infections randomized to receive Flu-IVIG or placebo. As in Figure 4A, preinfusion antibody levels for each individual patient were subtracted from that patient’s follow-up samples to give change from baseline at every time point, and the adjusted mean changes from baseline are shown for dimeric rsFcγRIIa binding antibodies (optical density [OD]) against pH1N1 recombinant HA (at a 1:40 plasma dilution). All plasma samples were also tested for dimeric rsFcγRIIa binding antibodies against an irrelevant simian immunodeficiency virus type 1 glycoprotein 120, and background was subtracted for each plasma sample. Error bars represent standard error of the mean. ***P < .001.

Flu-IVIG Enhances FcyR-Mediated Immunity in Influenza Infection • JID 2018:XX (XXXXX) • 7
Similarly, we found that Flu-IVIG contained Fc-functional antibodies capable of binding to many different strains and subtypes of influenza virus including H1, H2, H3, H4, H5, H7, B/Yamagata, and B/Victoria. In general, Flu-IVIG preparations contained higher concentrations of HA-specific FcγR cross-linking and ADCC-mediating antibodies than IVIG preparations from 2008 to 2016. Flu-IVIG also demonstrated pH1N1 HA stem-specific antibody levels comparable to those measured in a 2010 IVIG manufactured shortly after the 2009 pandemic. Functional ADCC assays performed with influenza-infected cells demonstrated a more modest enhancement of Flu-IVIG over standard IVIG preparations, suggesting involvement of antibodies against other influenza proteins such as NP and NA [21, 28, 38, 39]. FcγRIIIa cross-linking by NP antibodies was high and relatively consistent between the Flu-IVIG and IVIG preparations, whereas Flu-IVIG contained more...
NA-specific FcγRIIIa cross-linking antibodies than the other IVIG preparations. These results suggest that antibodies against NP and possibly other influenza antigens may be responsible for increasing the influenza-specific ADCC activity of standard IVIGs to a similar level as Flu-IVIG (between 0.9- and 3.1-fold different than Flu-IVIG by EC$_{50}$). Overall, Flu-IVIG appears to contain equal or greater concentrations of Fc-functional antibodies when directly compared to commercially available IVIG preparations across time. However, future experiments in animal models and human efficacy studies directly comparing Flu-IVIG to standard IVIG are required to determine if Flu-IVIG is a more effective treatment for influenza than standard IVIGs.

Like current vaccines and standard IVIG, Flu-IVIG will typically be manufactured months ahead of clinical use. Flu-IVIG may therefore contain suboptimal levels of strain-specific neutralizing antibodies against antigenically drifted, pandemic, or emerging zoonotic strains of influenza. We show here, however, that Flu-IVIG preparations contain antibodies with Fc-mediated effector functions that are capable of binding to a diverse array of influenza virus strains. In mouse models, broadly neutralizing HA antibodies require Fc-mediated functions to provide protection from heterologous influenza virus infection in vivo [21, 22]. Furthermore, IVIG treatment reduced morbidity and mortality in ferrets following lethal influenza challenge with an H5N1 virus despite the absence of detectable HAI antibodies in the IVIG [36]. Flu-IVIG infusion resulted in increased Fc-functional antibodies from 1 hour to 1 day postinfusion, suggesting that Flu-IVIG treatment boosts ADCC and ADP activity for 1–2 days before the natural humoral response to infection catches up (at 3 days postinfusion). Analysis of FcγR cross-linking antibodies against influenza B in subjects infected with pH1N1 influenza A virus showed detectable responses for at least 1 week after Flu-IVIG infusion.

A small human influenza challenge study showed that high titers of ADCC-mediating antibodies were associated with reduced influenza virus shedding and less severe disease [29]. This INSIGHT FLU005 Flu-IVIG pilot study was not powered to assess whether the transient rise in Fc-functional antibodies modulated influenza disease severity or mortality and a larger study is currently under way (FLU006, ClinicalTrials.gov identifier NCT02287467). We speculate that the greatest benefit of Fc-functional antibodies in Flu-IVIG may be in subjects infected with antigenically drifted or novel influenza viruses to which Flu-IVIG does not contain neutralizing antibodies.

Fc-functional antibodies develop rapidly to influenza infection and by 3 days postinfusion, there was no significant difference between the Flu-IVIG and placebo groups. The rapid induction of HA-specific Fc-functional antibodies suggests recall of preexisting cross-reactive memory B-cells. Antigen recognition by these memory B cells stimulates differentiation into antibody-secreting plasma cells, which produce cross-reactive Fc-functional antibodies. Based on these results, it could be speculated that Flu-IVIG treatment may be more beneficial to influenza-naive patients, such as unvaccinated children, during influenza infection. Broadly binding Fc-functional antibodies capable of recognizing group 2 influenza A virus HA(H3 and H4 subtypes) were also generated during the humoral immune response to pH1N1 virus infection in control subjects; however, this occurred at least 2 days later (after 3 days postinfusion) than production of Fc-functional antibodies against group 1 influenza virus HAs. The later induction of group 2 reactive Fc-functional antibodies could suggest activation of naive B cells that recognize HA epitopes conserved across group 1 and 2 influenza A viruses, such as the HA stem. Indeed, HA stem-specific antibodies were boosted early after Flu-IVIG infusion (1 hour and 1 day postinfusion) and increased in placebo-infused subjects by 3 days.

Previous work performed with plasma samples from the INSIGHT FLU005 pilot study showed that Flu-IVIG administration resulted in a spike in strain-specific HAI antibody titers from 1 hour to 3 days postinfusion, at least 3 days earlier than the HAI antibody response to natural infection [6]. In this study, we found that Flu-IVIG infusion caused an increase in HA-specific ADCC-mediating antibodies from 1 hour to 1 day postinfusion; this is 1–2 days earlier than the ADCC response to natural infection and approximately 2–4 days earlier than strain-specific HAI antibodies [6]. This is broadly consistent with our previous data on patients hospitalized with severe influenza due to either seasonal or avian H7N9 strains [31]. We speculate that the early presence of cross-reactive ADCC antibodies may be important for partial control of virus replication and spread until higher concentrations of strain-specific neutralizing antibodies are produced [40].

**Supplementary Data**

Supplementary materials are available at *The Journal of Infectious Diseases* online. Consisting of data provided by the authors to benefit the reader, the posted materials are not copyedited and are the sole responsibility of the authors, so questions or comments should be addressed to the corresponding author.

**Notes**

**Acknowledgments.** We thank all of the patients who participated in this study.

**International Network for Strategic Initiatives in Global HIV Trials (INSIGHT) FLU005 Pilot Study Writing Group.**

Sean Emery (Faculty of Medicine, University of Queensland, Brisbane, and Kirby Institute, University of New South Wales, Sydney, Australia); H. Clifford Lane (National Institute of Allergy and Infectious Diseases [NIAID], Bethesda, Maryland); Janaki Amin (Macquarie University, Sydney, New South Wales, Australia); Richard T. Davey Jr (NIAID); Eduardo Fernandez-Cruz (Inmunología Clinica, Hospital General Universitario Gregorio Marañón, Madrid, Spain); Norman P. Markowitz.
Financial support. This work was supported by the Australian National Health and Medical Research Council (award number 1052979 to S. J. K.) and the NIAID Intramural Research Program and the NIAID Division of Clinical Research, through a contract with the University of Minnesota (Leidos prime contract HHSN261200800001E).

Potential conflicts of interest. S. R. is an employee of Seqirus Ltd, a CSL Ltd company that manufactures and markets 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.

References


40. Jegaskanda S, Vanderven HA, Wheatley AK, Kent SJ. Fc or not Fc; that is the question: antibody Fc-receptor interactions are key to universal influenza vaccine design. Hum Vaccin Immunother 2017; 13:1–9.