Anti-influenza hyperimmune immunoglobulin enhances Fc-functional antibody immunity during human influenza infection

Running title: Flu-IVIG enhances FcγR-mediated immunity in influenza infection

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Summary: Anti-influenza hyperimmune immunoglobulin (Flu-IVIG) generally contained higher concentrations of influenza-specific Fc-functional antibodies than standard intravenous

immunoglobulins (IVIGs) against an array of influenza virus strains and subtypes. Passive infusion of Flu-IVIG into influenza-infected patients transiently boosted Fc-functional antibodies early after infusion.

Abstract

Background: New treatments for severe influenza are needed. Passive transfer of influenza-specific hyperimmune pooled immunoglobulin (Flu-IVIG) boosts neutralising 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 Fcγ 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 non-infecting 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 neutralising antibodies in the Flu-IVIG preparation.

Keywords: Influenza, immunoglobulin, ADCC, Fc receptor, passive transfer.

Footnote page:

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Introduction

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 G (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 rigour to fully validate this approach.

Recently, a randomised, placebo-controlled INSIGHT FLU005 pilot study assessed Flu-IVIG for enhancing neutralising antibodies in severe influenza infection [6]. Subjects infected with pH1N1 had greater hemagglutination inhibition (HAI) antibody titres against pH1N1 virus for ~3 days post-infusion with Flu-IVIG compared to the placebo controls [6]. Whilst this indicates that Flu-IVIG could

favourably 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 neutralising 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 IIIa (FcγRIIIa) is expressed on the surface of innate immune cells such as NK cells, and binds the Fc portion of antigen-bound IgG on influenza-infected cells. Upon cross-linking of Fc-engaged FcγRIIIa molecules, NK cells release of 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 neutralising 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 towards drifted seasonal strains of influenza. We characterised 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 #NCT02008578) [6]. Of 31 subjects enrolled, 24 had PCR-confirmed influenza A or B

and four serial (pre-infusion, day 1, 3 and 7 post-infusion) plasma samples available. Most subjects also had a 1 hour time point (21/24) and a day 28 time point (21/24) available. Subjects were randomly received Flu-IVIG (0.25g/kg; n=13) or saline placebo (n=11).

Intravenous immunoglobulin

The Flu-IVIG studied was manufactured by Emergent BioSolutions (Winnipeg, Canada) 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 titres 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 three 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 #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 HIV-negative donors. The IVIG prepared in 2016 is Intragam P (Seqirus) and we studied three 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 (Hizentra) also manufactured in 2013.

Recombinant proteins and influenza virus

Recombinant influenza hemagglutinin (HA), nucleoprotein (NP) and SIV envelope proteins were purchased from Sinobiological (Shanghai, China). Neuraminidase (NA) protein, purified from whole inactivated influenza virus as previously described [19, 24], was provided by Seqirus Ltd. A stabilised

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 ELISA

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

HA stem IgG ELISA

100ng of HA stem protein in PBS was coated on 96-well plates overnight, blocked with 5% BSA for 2h at 37°C then washed. Serial 2-4 fold dilutions of antibody were incubated for 2h at room temperature and washed. Rabbit anti-human IgG HRP (1:4000 dilution, Aligent) was incubated at room temperature for 1h then washed. TMB was added, colour developed, stopped with 1M HCl and absorbance read at 450nm.

Antibody-mediated NK cell activation

As a measure of NK cell activation, CD107a externalisation was quantified by flow cytometry in response to antibodies immobilised by plate bound influenza antigen, as previously described [18]. To model NK cell activation in influenza infection, NK-92-FcγRIIIa-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]. Briefly, A549 cells were infected with pH1N1virus (A/Auckland/01/2009, MOI=5) for 5 hours. A549 cells (20,000) were then incubated 1:1 with NK-92-FcγRIIIa-GFP cells and 2-fold dilutions of IVIG in triplicate for 4 hours. The Cytotox 96 kit (Promega, Madison, WI) 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 titre data, analysis of covariance with the log-transformed pre-infusion titre as a covariate was used to compare treatment groups for log-transformed titre levels at each follow-up time point. Treatment differences for log-transformed titre levels were back transformed to obtain geometric mean titres (GMTs). FcγRIIIa titres reported as <40 and >2560 were imputed as 20 and 5120 respectively. Statistical analyses were performed using SAS, version 9.4.

Results

Flu-IVIG contains more HA-specific FcγR 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 neutralisation assays [6, 32]. Antibodies with Fc-mediated effector functions commonly recognise a wide range of influenza strains and provide some protective immunity from influenza virus infection *in vivo* [21, 22, 33]. FcγRIIIa cross-linking antibodies in Flu-IVIG and three IVIG preparations from 2008, 2010 and 2016 were first tested against recombinant HA (rHA) protein from the A/California/07/2009(H1N1) pandemic swine influenza virus (pH1N1, Figure 1A). There were 5-9 fold higher concentrations of anti-pH1N1 HA FcγRIIIa 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 FcγRIIa 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 FcγRIIa cross-linking antibodies to the pH1N1 rHA compared to standard IVIGs (Figure 1B).

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 EC50 (Figure 1C) and endpoint titre (Figure S1A) than IVIGs prepared in 2008, 2010 and 2016. Similarly, Flu-IVIG contained FcγRIIa cross-linking antibodies against rHAs from three seasonal influenza virus strains circulating in 2013-2014 (Figure 1D and Figure S1B).

Batch-to-batch variation of FcγRIIIa cross-linking antibodies against three influenza rHA proteins was low across four Flu-IVIGs made from 2013-2016 (Figure S2A). Similarly, four batches of the standard IVIG Intragam P [IVIG 2016 (which is Intragam P) and three additional Intragam P batches 1-3] also had relatively uniform FcγRIIIa dimer binding antibodies against three influenza rHA proteins (Figure S2B). Since Flu-IVIG was prepared in 2013, we compared Flu-IVIG to IVIG prepared during 2013 (Hizentra) for FcγRIIIa cross-linking antibodies to three influenza strains circulating in 2013-2014 (Figure S2C). The 2013 Flu-IVIG contained greater quantities of FcγRIIIa cross-linking antibodies than the 2013 IVIG.

Flu-IVIG mediates antibody-dependent cellular cytotoxicity (ADCC)

Engaging FcγRs 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, D), 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 EC50, but nearly identical EC50s were calculated for Flu-IVIG and an IVIG prepared in 2010 (Figure 2B, D). This suggests influenza proteins besides HA may be involved in antibody-dependent NK cell activation and ADCC. We therefore tested the four IVIG preparations against two other known protein targets of influenza-specific ADCC, NA and NP, using the dimeric FcγRIIIa

binding ELISA and the NK cell activation assay (Figure S3). 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 (Figure S3A and S3C). 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 (Figure S3B and S3D).

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, D). 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 analysing 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 (1h) and 1 day (1d) post-infusion than placebo recipients, but not at 3d, 7d and 28d post-infusion (Figure 4A, B).

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 titres of HA-specific FcγRIIIa cross-linking antibodies at 1h and 1d post-infusion (Figure 4C), with Flu-IVIG/placebo ratios of 5.74 (95%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) at 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 1h and 1d post-infusion than placebo subjects (Figure S4).

Flu-IVIG increases antibody-dependent NK cell activation early post-infusion

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 1h and 1d post-infusion than placebo recipients (Figure 5A). The Flu-IVIG group also trended towards a higher GMT of NK cell activating antibodies against pH1N1-infected cells relative to the control group (p=0.06; Figure 5B), with a Flu-IVIG/placebo ratio of 2.48 (95%CI: 0.96-6.43). There was no significant difference between Flu-IVIG and placebo infused patients beyond 1d post-infusion (Figure 5A, B).

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, D). FcγRIIa dimer ELISAs were therefore performed on plasma samples from the 15 pH1N1-infected subjects. At 1h and 1d post-infusion 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 FcγRIIIa cross-linking antibodies against diverse influenza virus HAs

We showed above (Figures 1C, 1D, 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 FcγRIIIa cross-linking antibodies against heterologous group 1 rHAs (H2 and H5 subtypes) at 1h and 1d post-infusion (Figure 7A), although the responses were modest to H5. Humoral responses to group 1 rHAs started to emerge by day 3,

suggesting rapid activation of cross-reactive memory B-cells by infection. FcγRIIIa cross-linking antibodies against antigenically divergent group 2 HAs (H3 and H4 subtypes) were also higher in the Flu-IVIG group than the control group at 1h, 1d and 3d post-infusion (Figure 7B). Modest antibody responses arose relatively late (between day 3 and day 7 post-infusion) in pH1N1-infected placebo recipients, consistent with a weakly cross-reactive humoral response to group 2 rHAs. Finally, FcγRIIIa cross-linking antibodies against the rHA of an influenza B virus (B/Phuket/3073/2013) were significantly increased in the Flu-IVIG group compared to the control group at 1h, 1d, 3d and 7d post-infusion (Figure 7C) with no response detected in the pH1N1-infected placebo recipients. By 28d post-infusion the Flu-IVIG group no longer demonstrated higher levels of FcγRIIIa cross-linking antibody against the rHA of B/Phuket/3073/2013 influenza virus (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 randomised 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 titres in treated patients compared to 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]. 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 2008-2016. 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 like 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 0.9-3.1 fold different than Flu-IVIG by EC50). 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 *neutralising* 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 influenza virus strains. In mouse models,

broadly neutralising 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 1h to 1d post-infusion suggesting Flu-IVIG treatment boosts ADCC and ADP activity for 1-2 days before the natural humoral response to infection catches up (at 3d post-infusion). Analysis of FcγR cross-linking antibodies against influenza B in subjects infected with pH1N1 influenza A virus showed detectable responses for at least one week after Flu-IVIG infusion.

A small human influenza challenge study showed that high titres 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 underway (FLU006, clinicaltrials.gov #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 neutralising antibodies.

Fc-functional antibodies develop rapidly to influenza infection and by 3d post-infusion there was no significant difference between the Flu-IVIG and placebo groups. The rapid induction of HA-specific Fc-functional antibodies suggests recall of pre-existing 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-naïve patients, such as unvaccinated children, during influenza infection. Broadly binding Fc-functional antibodies capable of recognizing

group 2 influenza A virus HAs (H3 and H4 subtypes) were also generated during the humoral immune response to pH1N1 virus infection in control subjects, however this occurred at least two days later (after 3d post-infusion) 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 naïve 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 (1h and 1d post-infusion) and increased in placebo infused subjects by 3d.

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 titres from 1h to 3d post-infusion, at least three 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 1h to 1d post-infusion; 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 neutralising antibodies are produced [40].

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Potential conflicts of interest

Steven Rockman is an employee of Seqirus Ltd, a CSL company that manufactures and markets intravenous immunoglobulin (IVIG). The other authors have no conflicts of interest to declare. 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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Figure Legends

Figure 1.

Greater FcγR cross-linking by anti-HA antibodies in Flu-IVIG. RsFcγR dimer ELISAs were used to compare Flu-IVIG made during 2013 to standard IVIGs made in 2008 (prior to pH1N1), 2010 and 2016. Dimeric rsFcγRIIIa **(A)** and dimeric rsFcγRIIa **(B)** binding antibodies against pH1N1 rHA (A/California/07/2009 strain) are shown in four different IVIG preparations. The $(1/EC50)\times10^3$ values for dimeric rsFcγRIIIa **(C)** and dimeric rsFcγRIIa **(D)** binding antibodies against diverse rHA proteins were calculated for Flu-IVIG and three standard IVIG preparations. Fold increase in the concentration of FcγR cross-linking antibodies in Flu-IVIG relative to standard IVIGs was calculated by dividing the $(1/EC50)\times10^3$ value for Flu-IVIG by the $(1/EC50)\times10^3$ value for standard IVIG [eg. Flu-IVIG $(1/EC50)\times10^3$= 23.55 $(\mu g/ml)^{-3}$ and IVIG 2008 $(1/EC50)\times10^3$= 4.56 $(\mu g/ml)^{-3}$ so 23.55/4.56 = 5.2 fold increase]. Fold increases in the concentration of FcγR cross-linking antibodies in Flu-IVIG relative to standard IVIGs prepared between 2008-2016 are shown. All IVIG preparations were also tested for binding against an irrelevant SIV gp120 protein, the mean background was calculated and multiplied by three to give a threshold of detection represented by the dotted line. Error bars represent standard deviation (SD) of the mean of duplicate wells in the FcγR dimer ELISA.

Figure 2.

Enhanced Fc-functional anti-influenza antibodies in Flu-IVIG. NK cell activation and ADCC assays were used to study Flu-IVIG compared to standard IVIGs. Antibody-dependent NK cell activation, as measured by the proportion of an NK-like cell line expressing the degranulation marker CD107a by flow cytometry, was assessed to **(A)** pH1N1 rHA protein (A/California/07/2009 strain) and **(B)** pH1N1-infected A549 cells (A/Auckland/01/2009 strain). **(C)** ADCC was measured by LDH release

from pH1N1-infected A549 cells (A/Auckland/01/2009 strain). NK cell activation **(A and B)** or target cell killing **(C)** in the absence of IVIG was also tested in each assay and multiplied by three to give a threshold of detection represented by the dotted line. **(D)** The $(1/EC50)\times10^3$ values for ADCC antibodies against diverse rHA proteins and A/Auckland/01/2009(H1N1)-infected target cells were calculated for Flu-IVIG and three standard IVIG preparations as described in Figure 1. Fold increases in the concentration of ADCC antibodies in Flu-IVIG relative to standard IVIGs prepared between 2008-2016 are shown.

Figure 3.

Flu-IVIG contains HA stem-specific antibodies. IgG ELISAs, rsFcγR dimer ELISAs and NK cell activation assays were used to compare Flu-IVIG made during 2013 to standard IVIGs made in 2008 (prior to pH1N1), 2010 and 2016. IgG antibodies **(A)**, dimeric rsFcγRIIIa binding antibodies **(B)** and NK cell activating antibodies **(C)** against the pH1N1 HA stem are shown in the four different IVIG preparations. All IVIG preparations were also tested for binding against an irrelevant SIV gp120 protein, the mean background was calculated and multiplied by three to give a threshold of detection represented by the dotted line. Error bars represent standard deviation (SD) of the mean of duplicate wells in the FcγR dimer ELISA.

Figure 4.

Increased FcγRIIIa cross-linking antibodies against HA following infusion of Flu-IVIG into subjects with influenza infection. RsFcγRIIIa dimer ELISAs were used to examine serial plasma samples from 24 influenza-infected subjects (15 of whom had pH1N1 infections) randomised to receive Flu-IVIG or placebo. **(A)** Means of dimeric rsFcγRIIIa binding antibodies against pH1N1 rHA (at a 1:40 dilution of plasma) in the 15 pH1N1-

infected subjects are shown. Pre-infusion 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 (pre-infusion) are graphed. Mean O.D. of pre-infusion samples was 0.22 for controls and 0.22 for the Flu-IVIG group. **(B)** Geometric mean titres (GMTs) of dimeric rsFcγRIIIa binding antibodies against pH1N1 rHA in the 15 pH1N1-infected subjects are graphed. GMTs for post-infusion time points are shown adjusted for baseline or pre-infusion titre, the mean GMTs for pre-infusion 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, post-infusion GMTs were adjusted for pre-infusion titres, 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 SIV-1 protein gp120 and background was subtracted for each plasma sample. Error bars represent standard error (SE) of the mean. *** = $p < 0.001$

Figure 5.

Enhanced influenza-specific antibody-mediated NK cell activation following Flu-IVIG infusion. NK cell activation assays measuring CD107a expression were used to study serial plasma samples from 15 subjects with pH1N1 infections randomized to receive either Flu-IVIG or placebo. **(A)** Mean changes from pre-infusion or baseline for NK cell activating antibodies to pH1N1 rHA and **(B)** GMTs of NK cell activating antibodies to pH1N1-infected A549 cells are shown. Data in A and B are adjusted for pre-infusion antibody levels. All plasma samples were also tested for antibody-mediated NK cell activation against an irrelevant SIV-1 protein gp120 and background was subtracted for each

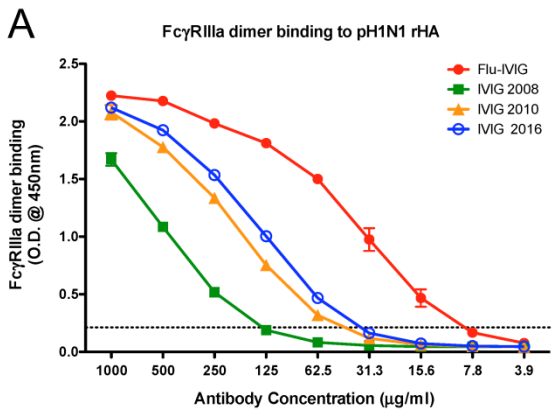
individual sample. Error bars represent standard error (SE) of the mean. *** = p < 0.001, ** = p < 0.01

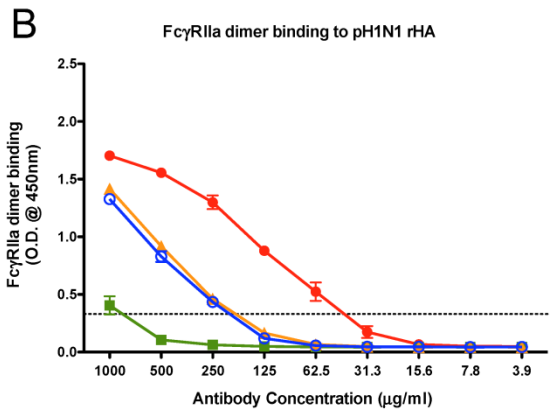
Figure 6.

Greater FcγIIa cross-linking antibodies against HA following infusion of Flu-IVIG into subjects with influenza infection. A rsFcγRIIa dimer ELISA was used to examine serial plasma samples from 15 subjects with pH1N1 infections randomized to receive Flu-IVIG or placebo. As in Figure 4A, pre-infusion 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 against pH1N1 rHA (at a 1:40 plasma dilution). All plasma samples were also tested for dimeric rsFcγRIIa binding antibodies against an irrelevant SIV-1 protein gp120 and background was subtracted for each plasma sample. Error bars represent standard error (SE) of the mean. *** = p < 0.001

Figure 7. Increased FcγIIIa cross-linking antibodies against HAs from diverse strains of influenza virus following Flu-IVIG infusion. RsFcγRIIIa dimer ELISAs were used to study serial plasma samples from 15 subjects with pH1N1 infections randomized to receive either Flu-IVIG or placebo. Mean changes from pre-infusion or baseline for dimeric rsFcγRIIIa binding antibodies to rHAs are shown. The rHAs represent the following strains: **(A)** H2N2 A/Japan/305/1957 and H5N1 A/Vietnam/1194/2004, **(B)** H3N2 A/Texas/50/2012 and H4N6 A/Swine/Ontario/01911-/1999, **(C)** B/Phuket/3073/2013. Error bars represent standard error (SE) of the mean. *** = p < 0.001, ** = p < 0.01, * = p < 0.05

Figure 1 :





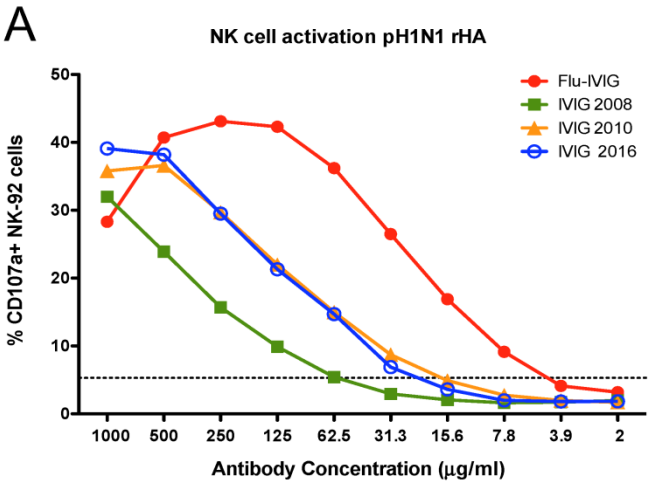
C

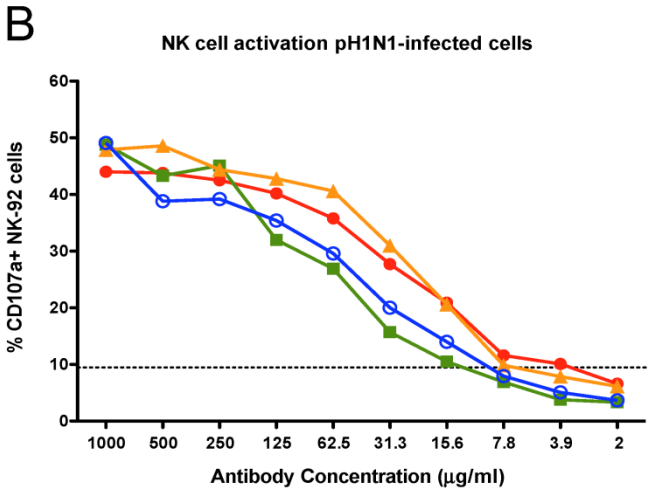
HA protein	(1/EC50)x10^3 (µg/ml)$^{-3}$	Fold increase in the concentration of FcγRIIIa cross-linking antibodies in Flu-IVIG relative to standard IVIGs		
	Flu-IVIG	IVIG 2008	IVIG 2010	IVIG 2016
H1 A/New Caledonia/20/1999	23.5	5.2	2.3	4.3
H1 A/Solomon Islands/03/2006	36.2	5.2	2.6	4.1
H1 A/Brisbane/59/2007	23.0	7.3	2.6	4.6
H1 California/07/2009	29.6	66.3	8.7	5.7
H3 A/Brisbane/10/2007	12.5	12.0	3.3	2.9
H3 A/Perth/16/2009	31.2	22.1	5.2	3.0
H3 A/Switzerland/9715293/2013	6.8	12.5	4.4	2.8
H3 A/Texas/50/2012	23.5	15.3	4.3	2.2
HA B/Florida/04/2006	8.7	6.8	2.5	1.9
HA B/Massachusetts/03/2010	13.1	2.9	1.5	1.6
HA B/Phuket/3073/2013	16.4	6.5	3.4	2.2
H2 A/Japan/305/1957	3.5	3.4	1.0	1.3
H4 A/Swine/Ontario/01911-1/1999	2.0	5.5	2.7	2.9

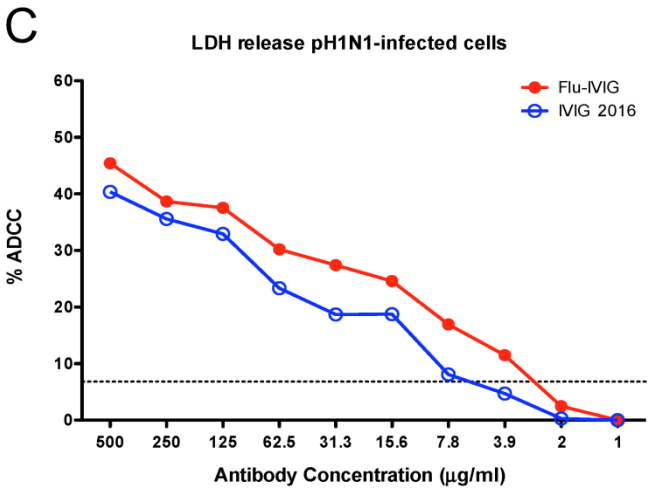
D

HA protein	(1/EC50)x10^3 (µg/ml)$^{-3}$	Fold increase in the concentration of FcγRIIa cross-linking antibodies in Flu-IVIG relative to standard IVIGs		
	Flu-IVIG	IVIG 2008	IVIG 2010	IVIG 2016
H1 California/07/2009	6.5	Not calculable	13.9	17.4
H3 A/Texas/50/2012	15.8	194.1	3.6	3.8
HA B/Phuket/3073/2013	7.7	9.6	2.9	2.9

Figure 2 :



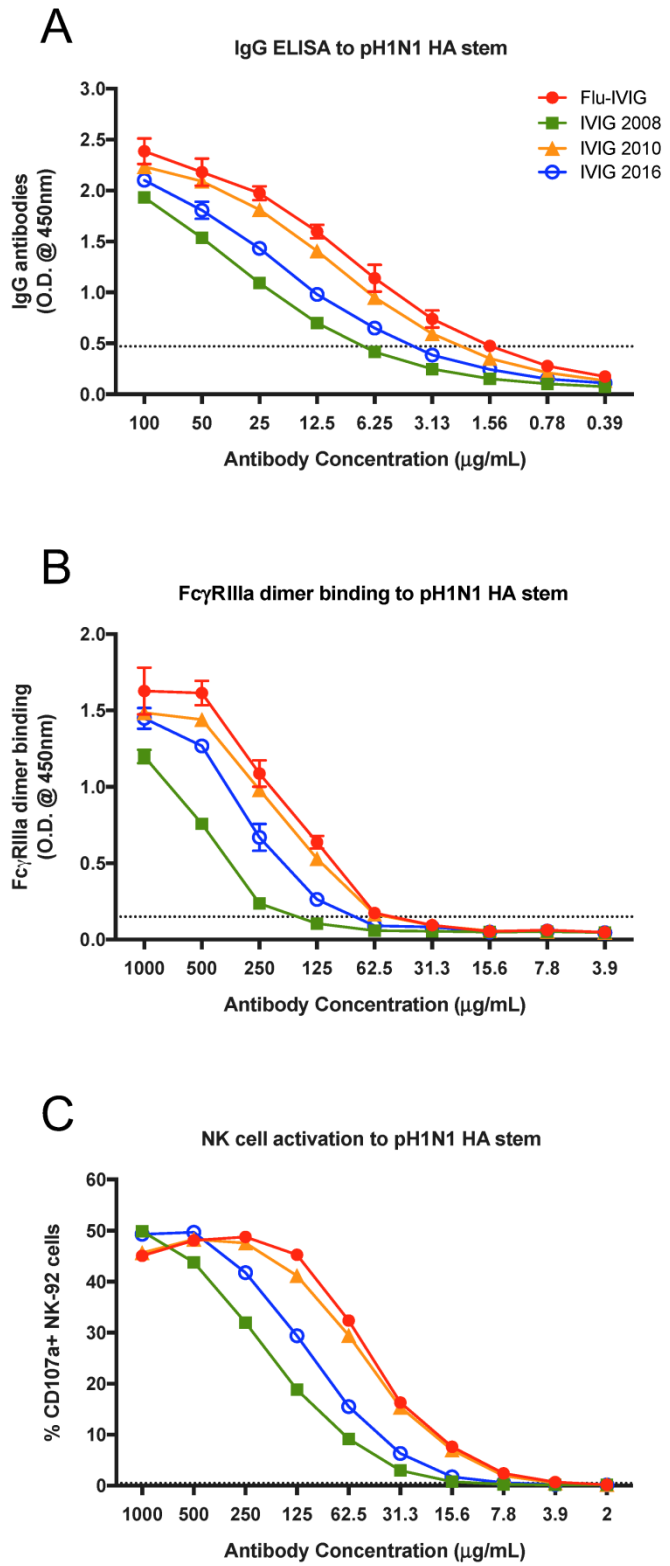




D

HA protein or virus	(1/EC50)x10^3 (µg/ml)$^{-3}$	Fold increase in the concentration of ADCC antibodies in Flu-IVIG relative to standard IVIGs		
	Flu-IVIG	IVIG 2008	IVIG 2010	IVIG 2016
NKCAA H1 California/07/2009 protein	74.0	44.5	8.7	10.8
NKCAA H3 A/Texas/50/2012 protein	21.1	13.2	2.1	3.0
NKCAA HA B/Phuket/3073/2013 protein	18.7	7.7	1.3	1.5
NKCAA A/Auckland/01/2009-infected A549 cells	45.8	3.1	0.9	2.0
LDH release from A/Auckland/01/2009-infected A549 cells	91.7			2.5

Figure 3 :



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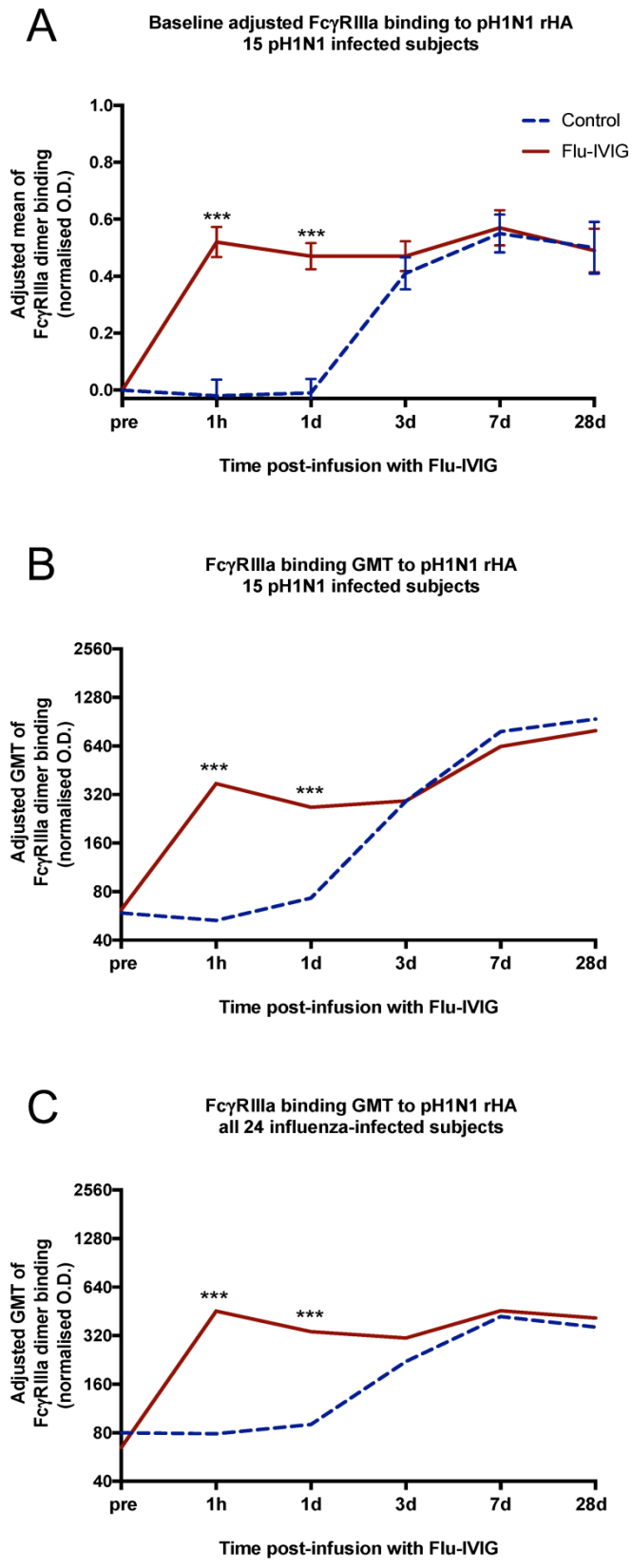

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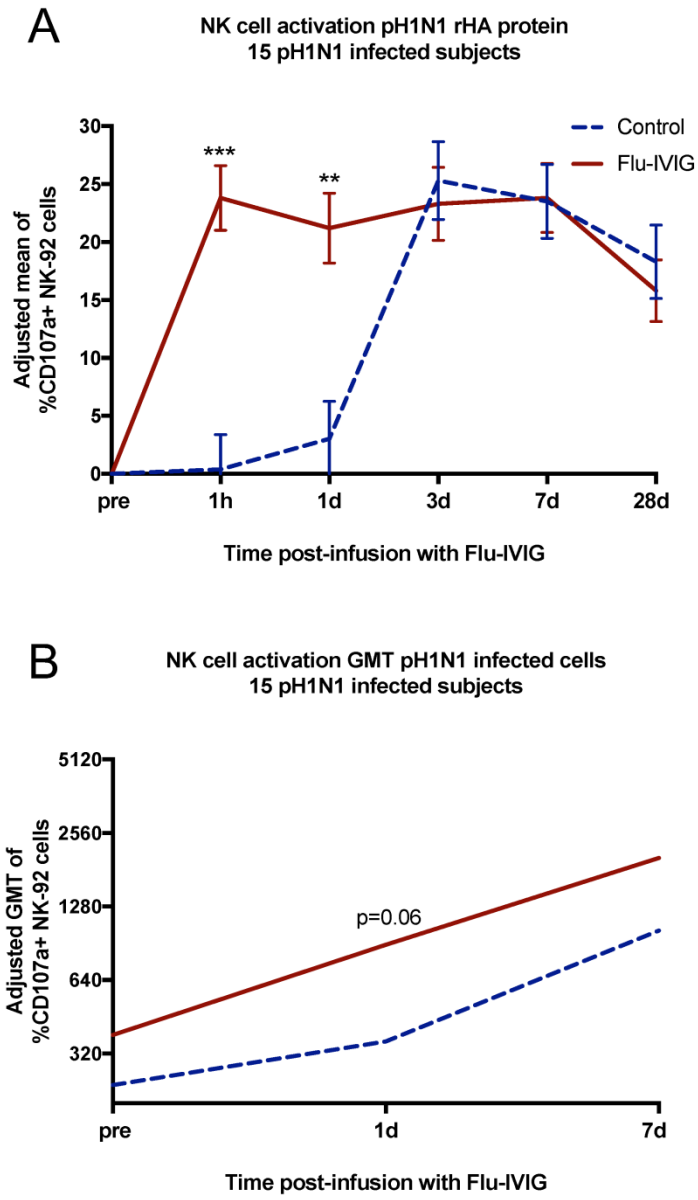

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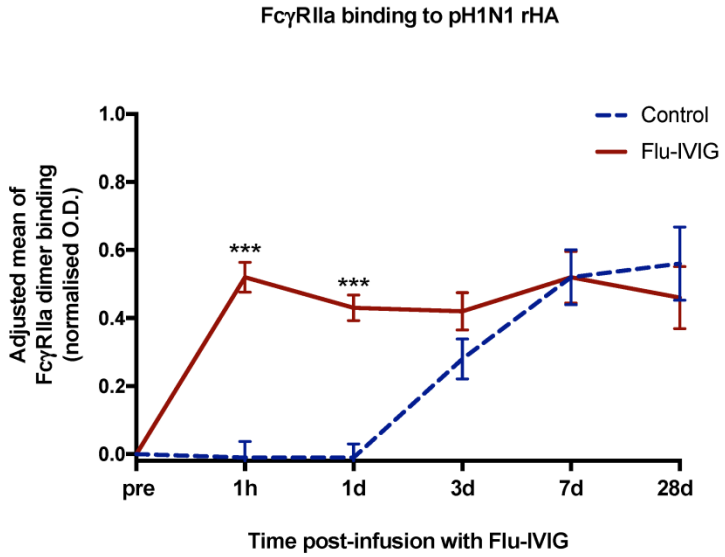

Figure 7 :