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## Research Paper

# Intravenous Immunoglobulin Protects Against Severe Pandemic Influenza Infection



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## ABSTRACT

Influenza is a highly contagious, acute, febrile respiratory infection that can have fatal consequences particularly in individuals with chronic illnesses. Sporadic reports suggest that intravenous immunoglobulin (IVIg) may be efficacious in the influenza setting. We investigated the potential of human IVIg to ameliorate influenza infection in ferrets exposed to either the pandemic H1N1/09 virus (pH1N1) or highly pathogenic avian influenza (H5N1). IVIg administered at the time of influenza virus exposure led to a significant reduction in lung viral load following pH1N1 challenge. In the lethal H5N1 model, the majority of animals given IVIg survived challenge in a dose dependent manner. Protection was also afforded by purified F(ab')<sub>2</sub> but not Fc fragments derived from IVIg, supporting a specific antibody-mediated mechanism of protection. We conclude that pre-pandemic IVIg can modulate serious influenza infection-associated mortality and morbidity. IVIg could be useful prophylactically in the event of a pandemic to protect vulnerable population groups and in the critical care setting as a first stage intervention.

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## 1. Introduction

Influenza is a highly contagious, acute, febrile respiratory infection that kills between 250,000 and 500,000 people worldwide annually (WHO, n.d.). These deaths occur primarily in individuals with known risk factors such as chronic respiratory and cardiovascular conditions. More recently, the H1N1 influenza pandemic of 2009 has also implicated morbid obesity as a risk factor for influenza-associated hospitalisation and death (Morgan et al., 2010), and like highly pathogenic avian influenza (HPAI), establishes deep lung infection in critical care patients (reviewed (Lapinsky, 2010)). Treatment options in

individuals with lower respiratory tract involvement or signs of systemic infection can be limited given the increase in isolation of anti-viral drug resistant strains (Pizzorno et al., 2011).

IVIg is prepared from the plasma collected from many thousands of donors, and is widely used for the treatment of inflammatory and autoimmune diseases (reviewed (Durandy et al., 2009)). IVIg and pathogen specific hyperimmune preparations are indicated to modify or prevent certain viral syndromes such as hepatitis A, hepatitis B, rabies, measles, cytomegalovirus and respiratory syncytial virus (Hemming, 2001).

For influenza, the limited published data support the use of convalescent sera to reduce mortality (Luke et al., 2010). This includes a recent meta-analysis of historical studies of patients during the 1918 pandemic (Luke et al., 2006), and sporadic reports of HPAI H5N1 infected individuals (Kong & Zhou, 2006; Zhou et al., 2007). A small study of severe pH1N1 virus pneumonia cases treated with convalescent plasma demonstrated a reduction in viral load, blunted serum cytokine response and lower mortality compared to age, sex and disease severity score matched controls (Hung et al., 2011). Data on the use of influenza hyperimmune serum in animal models (mice) is also limited (Luke et al., 2010), and these models are generally regarded as being unsuitable to evaluate the efficacy of modalities to treat serious influenza infection or to assess critical points of therapeutic intervention in humans.

**Abbreviations:** HI, haemagglutination inhibition; HPAI, highly pathogenic avian influenza; IVIg, intravenous immunoglobulin; NI, neuraminidase inhibition; WHO, World Health Organization; CSIRO, Commonwealth Scientific and Industrial Research Organization; ARRIVE, Animal Research: Reporting of In Vivo Experiments; CSL, Commonwealth Serum Laboratories; PBS, Phosphate Buffered Saline; PBMC, Peripheral Blood Mononuclear Cells; IFN, Interferon; IL, Interleukin; TNF, Tumor Necrosis Factor; RT-PCR, Reverse Transcriptase PCR.

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IVIg has been shown to contain antibodies that broadly cross-react with both Group 1 and 2 influenza A strains (Corti et al., 2010; Gioia et al., 2008; Garcia et al., 2009; Sui et al., 2011). This reactivity includes antibodies directed to the stem pocket of haemagglutinin (Sui et al., 2011). However, these cross-reactive antibodies to pandemic influenza strains in IVIg are present at low levels and whether they are able to modify influenza viruses not yet encountered by the population is unclear (Sui et al., 2011).

In this study we used a ferret infection model as previously reported (Rockman et al., 2012; Middleton et al., 2009) to investigate the disease modifying capacity of IVIg following challenge with two pandemic influenza viruses (pH1N1 and highly pathogenic H5N1). Due to their natural susceptibility to human influenza viruses, similar distribution of virus receptors and similar clinical signs, outbred ferrets are considered a relevant small animal model of human influenza infection (Smith & Sweet, 1988; van Riel et al., 2007). Herein we demonstrate that pre-pandemic IVIg prevents deep lung replication of influenza and protects against mortality and morbidity with pandemic viruses. Furthermore, we determined the mechanism of this protection is due to cross-reactive antibodies to influenza.

## 2. Methods

### 2.1. Ferrets

Healthy juvenile ferrets, <12 months old and weighing 800–1700 g. Ferrets were determined to be seronegative for the currently circulating seasonal strains (pH1N1, H3N2, B viruses) using standard hemagglutination inhibition (HI) tests prior to the commencement of the study. All experiments were performed under BSL3+ containment at the Australian Animal Health Laboratories (AAHL) with the approval of the CSIRO AAHL Animal Ethics Committee. Animal experimentation complied with ARRIVE guidelines and was conducted in accordance with Australian National Health and Medical Research Council guidelines on the care and use of animals for medical research which are aligned with UK and USA guidelines.

### 2.2. Viruses

The wildtype human influenza isolates of pH1N1 A/California/07/2009 and H5N1 A/Vietnam/1203/2004 were used as the challenge viruses as previously documented (Rockman et al., 2012).

### 2.3. IVIg Administration and Viral Challenge of Ferrets

IVIg was administered intravenously for the pH1N1 studies and by intraperitoneal injection for the H5N1 studies 2 h prior to viral challenge. In the pH1N1 studies, additional animals received IVIg intranasally either 2 h prior to challenge or 2 h after challenge. The dose of IVIg was 0.5 g/kg unless otherwise stated. Ferrets were challenged intranasally with  $10^6$  50% egg infectious doses ( $EID_{50}$ ) of virus in 0.5 mL under ketamine/medetomidine anesthesia (50:50, 0.1 mL/kg, reversed with atipemazole). The preparations of IVIg utilised were Intragram®P (6% w/v IgG), Privigen® (10% w/v IgG) and Hizentra® (20% w/v IgG). Homologous hyperimmune control serum for the pH1N1 studies was prepared in ferrets by priming with the CSL pH1N1 vaccine (60 µg HA/mL with 1 mg alum/mL) and boosting three weeks later by challenge with live virus ( $10^6$  TCID<sub>50</sub> H1N1 A/California/7/2009). Sera were harvested 14 days post challenge, pooled, and administered as per the treatment protocol.

### 2.4. Monitoring

Animals were visually examined twice daily following challenge and general clinical observations recorded on a detailed clinical signs sheet. Behavioural observations were rated using a five-level activity score, as previously described (Rockman et al., 2012). Animals were weighed

and rectal temperatures taken while under sedation at the time of challenge, and at days 3, 5, 7, and 14 post-challenge. Ferrets were euthanised 14 days after challenge or upon reaching a predetermined humane end point defined as either a 10% body weight loss or exhibition of signs consistent with involvement of other organ systems (e.g. tremour or abdominal guarding). Detailed activity assessments were performed in the first cohort of animals studied in each set of experiments but not in subsequent cohorts of animals studied for each experimental study.

### 2.5. Sampling

Blood samples were collected prior to viral challenge/administration of IVIg, and where appropriate at 14 days post challenge to confirm exposure to the challenge virus. Blood was collected from anaesthetised animals via the jugular or axillary veins. Nasal washes were taken into 1 mL of PBS on days 3, 5, and 7 post-challenge for virus isolation.

### 2.6. Immunological and Virological Evaluation

Serum samples were heat inactivated at 56 °C for 1 h prior to testing. The samples were assessed by HI using chicken red blood cells, and virus neutralization (VN) by standard methods (Rockman et al., 2012). Determination of IVIg reactivity to the HA cleavage site was based on the method of Sui et al. (2011) except that in-house characterised cleavage site monoclonal antibodies were utilised (Sui et al., 2011). Detection of IVIg reaction to recombinant H5 protein, split virion H5N1 material and purified N1 protein was performed by a capture enzyme linked immune assay. Microtitre plates (Nunc, New York, USA) were coated with target antigen diluted in 0.05 M carbonate buffer, and incubated at room temperature (RT) overnight. Plates were blocked for non-specific binding using 1% (w/v) sodium casein solution in calcium- and magnesium-free PBS (PBS-). Serial two fold dilutions of IVIg were added to the plate, in duplicate, and incubated for 1 h at RT. Plates were washed with PBS-containing 0.05% Tween-20 (v/v) (Sigma, California, USA) and incubated with HRP-conjugated mouse anti-human antibody (molecular Probes) for 1 h at RT, washed, and then incubated with peroxidase substrate (KPL, Maryland, USA) as recommended. Substrate development was ceased by addition of 0.55 M H<sub>2</sub>SO<sub>4</sub>. The optical density (OD) was measured using a plate reader (Tecan, Mannedorf, Switzerland) with filters suitable to detect 450 nm. For inhibition of NA activity, samples were standardised by protein concentration before serial two fold dilutions in assay buffer (32.5 mM MES, pH 6.5; 4 mM CaCl<sub>2</sub>, 0.1% NP-40 and 0.3 mg/mL BSA) and activity determined by fluorometric assay with 2'-(4-methylumbelliferyl)-e-D-N-acetylneuraminic acid (Potier et al., 1979; Wetherall et al., 2003) using a TECAN infinite M200 fluorometer, with Magellan 7.0 software. A negative control of buffer only showed a lack of NA activity inhibition (similar to Fc antibody fragment alone) and previous work showed anti-NA monoclonal antibodies and drug inhibitors of NA act as positive controls (data not shown and (Potier et al., 1979; Wetherall et al., 2003)). The neuraminidase inhibition (NI) titre was defined as the reciprocal dilution of IVIg at which NA activity was inhibited by 50%.

### 2.7. Generation of IVIg Fragments

Three preparations of IVIg were utilised in these experiments Intragram®P (CSL Behring Australia), Privigen® (CSL Behring, Bern) and Hizentra® (CSL Behring, Bern). Hizentra® (20% w/v IgG) was used as the starting material for the generation of F(ab')<sub>2</sub> and Fc fragments from IVIg. Enzymatic digestion of IVIg was performed using immobilised pepsin and papain (Pierce, Rockford, USA) according to the manufacturer's instructions. Chromatography media were obtained from GE Healthcare Bio-Sciences AB (Uppsala, Sweden).

To prepare the F(ab')<sub>2</sub> fragments, IVIg in 0.1 M citrate pH 3.5 was digested with 80 mg/mL of settled immobilised pepsin gel for 2 h at 37 °C. The F(ab')<sub>2</sub> fragments were purified by size exclusion

chromatography (SEC) on a Superdex 200 column equilibrated with Dulbecco's phosphate buffered saline. Fractions containing high purity F(ab')<sub>2</sub> were pooled and concentrated to 77 mg/mL using centrifugal filter devices.

To prepare the Fc fragments, IVIg in 20 mM sodium phosphate, 10 mM EDTA, 20 mM cysteine pH 6.5 was digested with 40 mg/mL of immobilised papain gel for 20 h at 37 °C. The Fc fragments were then purified on a Protein A column and eluted with 50 mM acetic acid, 10 mM NaCl pH 3.0. Undigested antibody was separated from the Fc fragments using SEC on a Superdex 200 column equilibrated with Dulbecco's phosphate buffered saline. Fractions containing high purity Fc were pooled and concentrated to 84 mg/mL using centrifugal filter devices. The purity of IVIg-derived F(ab')<sub>2</sub> and Fc fragments was assessed by SDS-PAGE analysis and HPLC- SEC. Endotoxin levels were measured using the Kinetic-QCL commercial assay (Lonza, Basel, Switzerland) and found to be <0.009 EU/mg.

### 2.8. Cytokine mRNA Analysis

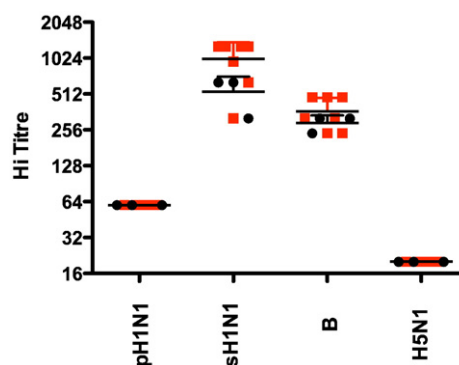
Ferret RNA was purified from PBMCs (buffy coat) using a RNeasy mini kit (Qiagen part no 74106) and eluted in 20 µL RNAase-free water. Probe and primers specific for ferret IL-10 have previously been described (Nakata et al., 2009). Primers specific for ferret IFN-γ and TNF-α were designed based on published sequences (Svitek & von Messling, 2007). To design primers specific for ferret perforin, we used primers specific for the canine perforin gene (Neta et al., 2010) and amplified the ferret perforin gene fragment from RNA isolated from ferret PMBCs. The nucleotide sequence of this fragment was then determined and used to design primers specific for ferret perforin. Real-time PCR was performed using an Applied Biosystems 7500 Real Time PCR System. For detection of ferret IL-10, the specific probe and primers were used with a Taqman One-step RT-PCR Master mix reagents kit (ABI, part no. 4309169). For detection of ferret IFN-γ, TNF-α and perforin the specific primers were used with a Quantitect SYBR Green RT-PCR kit (Qiagen, part no. 204243).

## 3. Results

### 3.1. IVIg Produced From Plasma Harvested Prior to the 2009 pH1N1 Influenza Pandemic Prevents Virus Replication in the Lungs of Ferrets Challenged With pH1N1 Virus

Although the H1N1/09 virus was a novel virus it shares some antigenicity with prior influenza strains. Indeed, the haemagglutination inhibition (HI) titre was 64 (equating to a level 94 µg/mL) for both IVIg (Intragram®P) prepared from plasma collected in 2004 and 2009 just prior to the identification of the pandemic H1N1/09 virus. As expected both the 2004 and 2009 IVIg preparations displayed considerable reactivity against the more recent seasonal H1N1 (A/Brisbane/59/2007) and B (B/Brisbane/60/2008) strains with mean HI titres of 512 (750 µg/mL) and 256 (375 µg/mL) respectively. In contrast, neither the 2004 or 2009 IVIg preparations demonstrated reactivity against the H5N1 (A/Indonesia/1/05) strain (Fig. 1).

To investigate the ability of IVIg harvested prior to a pandemic outbreak to protect against a pandemic viral challenge, ferrets (*n* = 3 per group) were given pre-pandemic IVIg (Intragram® P 2004) intravenously (jugular vein) 2 h prior to challenge, or IVIg intranasally either 2 h prior to or 2 h post challenge. The intranasal route was examined because of a prior report demonstrating that IVIg is protective when delivered intranasally to mice prior to influenza challenge, albeit at a dose of IVIg 80–400 times the human dose/kg equivalent (Ramisse et al., 1998). A negative control group of animals was given diluent only intravenously 2 prior to challenge. A positive control group was given homologous (pH1N1) hyperimmune serum (5 mL by intraperitoneal route; HI titre 1:640).

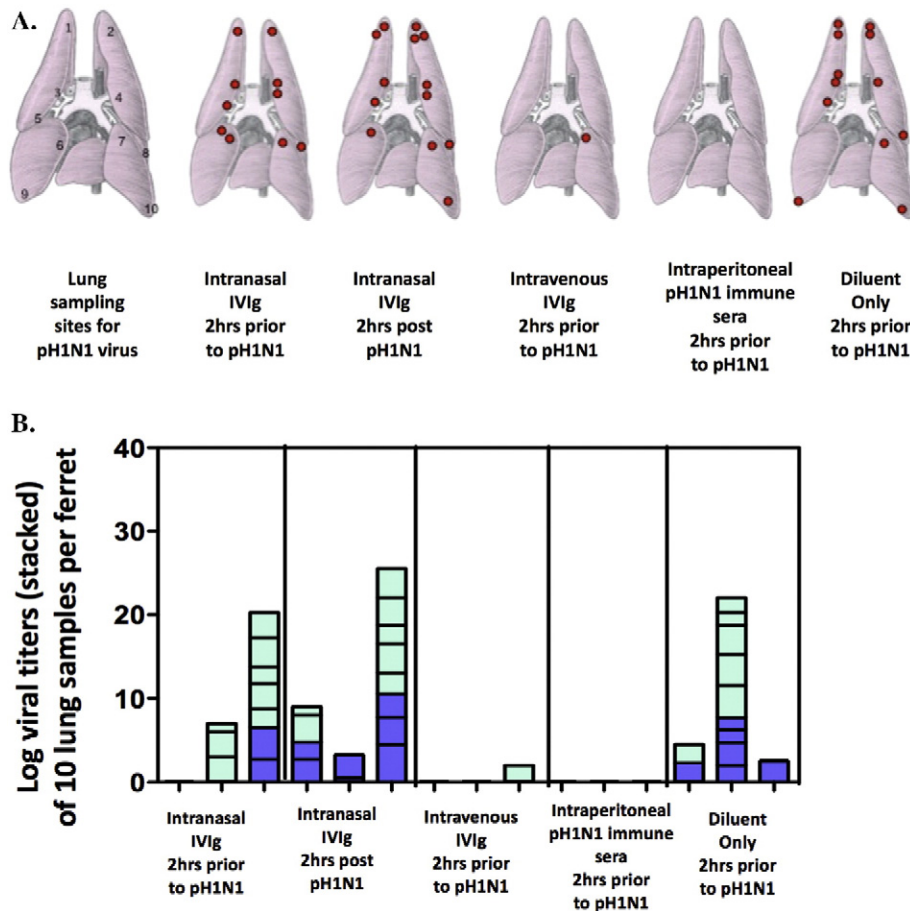


**Fig. 1.** Reactivity of IVIg against seasonal and pandemic strains. IVIg prepared in 2004 (black dots) or 2009 prior to the appearance of the swine origin H1N1 pandemic (red squares) was titred using 2 fold dilutions and tested in a standard HI assay against the 2009 swine origin pandemic strain (pH1N1), seasonal H1N1 (sH1N1:A/Brisbane/59/2007), B (B/Brisbane/60/2008), and pandemic H5N1 (A/Indonesia/5/2005) strains. Assays were performed in triplicate.

All 15 animals were challenged intranasally with 10<sup>6.2</sup> EID<sub>50</sub> wildtype A/California/07/2009 (pH1N1) and demonstrated little disruption to activity, slight weight decrease, and only a transient rise in temperature over the 5 day observation period, which is consistent with our previous observations (Rockman et al., 2012). In this experiment the animals were euthanised at Day 5, the lungs excised and lung tissue blocks taken from 10 different sites for each animal, which were then homogenised and titrated individually for virus content as previously described (Rockman et al., 2012). Sample sites included all lung lobes and both proximal lung tissue adjacent to the hilus of the lung (six sites) and more distal tissue at the periphery of the lungs (4 sites). There was a marked reduction in the number of lung sites sampled that were found to contain virus in ferrets that were administered either IVIg intravenously or pH1N1 hyperimmune serum at the time of challenge with pH1N1/09, compared to diluent treated controls (Fig. 2A). In contrast the number of virus positive lung samples in ferrets administered IVIg via the IN route, either 2 h prior to or 2 h post challenge was similar to the control diluent-treated animals. Consistent with this observation, there was a significant difference between the level of virus isolated from the lungs of ferrets administered IVIg or pH1N1 hyperimmune serum intravenously versus animals given IVIg intranasally or diluent as a control (*P* < 0.001 and *P* < 0.05 respectively Kruskal-Wallis test, Fig. 2B). Although there was a significant effect of IVIg and immune sera on lung pH1N1 replication, there was no effect of either IVIg or immune sera on upper respiratory levels of pH1N1 replication in this model (Supplementary Table 1).

### 3.2. IVIg Prevents Significant Morbidity and Mortality in Ferrets Following Challenge with Highly Pathogenic Avian H5N1 Influenza

The ability of IVIg to protect ferrets against challenge with pH1N1 virus may be due to the presence of cross-reactive antibodies in some of the plasma donors, and hence IVIg, generated as a consequence of previous exposure either via infection or with vaccination against seasonal influenza strains (Rockman et al., 2013). To further investigate the ability of IVIg to protect against a potential pandemic influenza virus, IVIg was administered to ferrets at the time of challenge with the lethal avian H5N1 influenza strain. Prior to administration the IVIg (Privagen®) was tested in a standard haemagglutination inhibition (HI) assay for reactivity against the A/Vietnam/1194/2004 H5N1 strain and found to be below the detection limits of this assay (data not shown). Four animals were administered 0.5 g/kg of IVIg by intraperitoneal injection or a similar volume of diluent as a control at the time of challenge with 10<sup>6</sup> EID<sub>50</sub> of the A/Vietnam/1194/2004 H5N1 strain. All challenged animals demonstrated a rise in temperature post challenge



**Fig. 2.** Reduced pH1N1 lung viral loads in ferrets administered intravenous IVIg. Pulmonary viral loads in the lungs of 15 swine origin 2009 H1N1 (pH1N1) challenged ferrets. Ten sites within the lungs of each ferret (5 groups,  $n = 3$  per group) were sampled as shown in the upper left panel of (A): 1, left apical distal; 2, left apical proximal; 3, left apical caudal hilar; 4, left diaphragmatic distal; 5, left diaphragmatic proximal; 6, right apical distal; 7, right apical proximal; 8, right intermediate; 9, right diaphragmatic distal; 10, right diaphragmatic proximal. The 5 groups were intranasal IVIg administered 2 h prior to challenge, intranasally IVIg administered 2 h after challenge, intravenous IVIg administered 2 h prior to challenge, pH1N1 immune sera administered intraperitoneally 2 h prior to challenge and diluent only administered 2 h prior to challenge. The location of samples that contained virus for each experimental group are shown as a filled circle in (A). The log viral titres (expressed as  $\log_{10}$  TCID<sub>50</sub>/0.1 mL) for each positive sample are plotted for each ferret as a stacking graph (B). Proximal sites are in light green, distal sites in dark blue.

by Day 3 (average 40.43 °C; range 39.7–41 °C), which in the IVIg group returned to normal within 48 h (Fig. 3A). Slight weight loss was observed in the IVIg-treated animals on day 3 post challenge (average 3.1%; range 1.8–6.3%), with most animals returning to pre-challenge levels by the end of the study (Fig. 3B), and remaining active throughout the study period (Fig. 3D). In contrast three of the control animals were euthanised on day 4 (the first black symbol in Fig. 3D indicates the day of euthanasia) and 1 on day 5 post-challenge as a consequence of 10% or greater loss in body weight (Fig. 3C,D). The survival in the IVIg group compared to buffer controls was significant ( $p = 0.0058$ , Log-Rank (Mantel-Cox) Test,  $p = 0.0097$ , Gehan-Breslow-Wilcoxon Test, Fig. 3C). There was also a striking contrast in the activity of IVIg-treated and control ferrets post challenge. Whereas animals that were given IVIg remained active throughout the experimental period, with only a transient modest reduction in activity, the activity of control animals rapidly declined by day 3 post-challenge, which paralleled their weight loss (Fig. 3D).

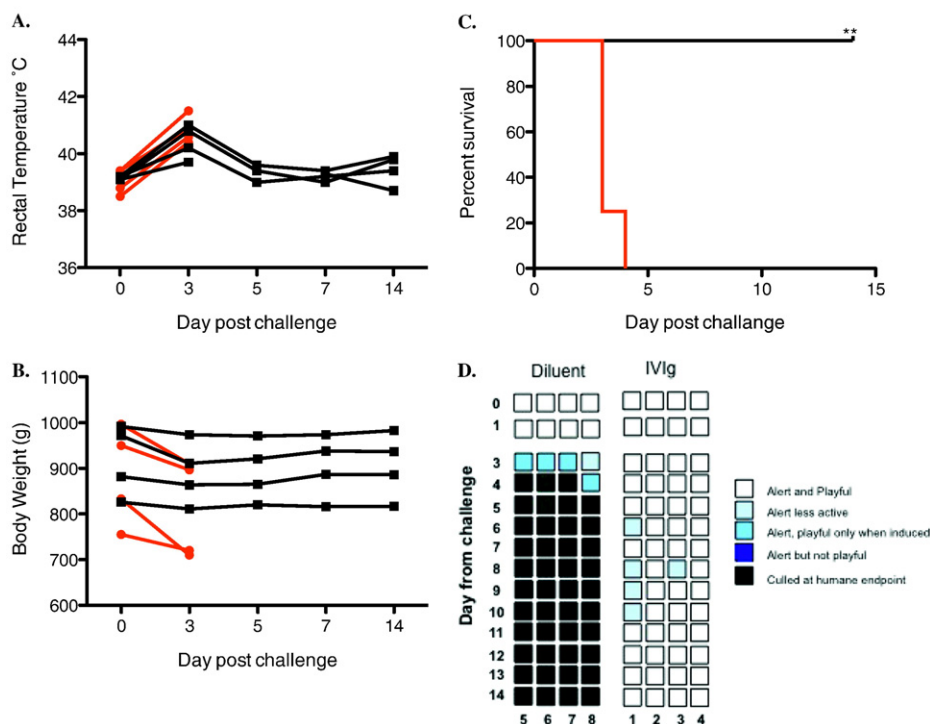
### 3.3. Effect of Dose of IVIg on Protection of Ferrets From H5N1 Influenza

In order to investigate the dose of IVIg that is required to confer protection from a lethal H5N1 infection and to determine the appropriate IVIg dose for mechanistic studies, 4 different doses of IVIg (0.5, 0.25, 0.125 g/kg or no IVIg) were administered to a total of 23 animals in

this model. All 8 diluent control animals not receiving IVIg succumbed to H5N1 infection whereas only 1 of 7 animals given the 0.5 g/kg IVIg dose succumbed to infection (Fig. 4A). The 0.25 and 0.125 g/kg groups had an intermediate outcome, with 1 of 4 and 2 of 4 succumbing to H5N1 infection.

To corroborate the survival data above, we took detailed activity assessments in a subset of 15 of the 23 animals studied. Consistent with the survival data, 2 of the 3 animals studied in the 0.5 g/kg IVIg group remained disease free throughout the 15 day study period, and the third animal (ferret #31) demonstrated neurological symptoms on day 10 post H5N1 challenge and was euthanised (Fig. 4B). Similarly 3 of the 4 ferrets that received 0.25 g IVIg/kg survived lethal H5N1 challenge with little or no impact on activity. The fourth animal in this group was euthanised on Day 6 post challenge due to a >10% loss in body weight. In the 0.125 g IVIg/kg treated group, 2 of the 4 animals survived, with little or no impact on activity and the other two animals were euthanised on Day 5 and 7 post challenge. Consistent with the previous study, all of the ferrets in the control diluent-treated group were euthanised after reaching humane endpoints (4 on day 3 and 1 on days 4, 5, 6 and 14 post challenge, Fig. 4A).

To examine whether the IVIg also limits viral levels in the upper respiratory tract in addition to preventing disease outcomes, we also studied nasal wash viral titres. The viral titres in nasal washes at Day 3 post H5N1 challenge were found to broadly correlate negatively with the



**Fig. 3.** IVIg protects ferrets against H5N1 challenge. Ferrets ( $n = 4/\text{group}$ ) were administered IVIg (black lines) or diluent control (red lines) intraperitoneally and challenged with  $10^6$  EID<sub>50</sub> of A/Vietnam/1194/2004 on Day 0 and observed for 14 days unless they reached a humane end point and were euthanised. (A) Rectal Temperature, (B) Weight, (C) Percentage survival, (D) Activity scores. Activity scores are depicted for each ferret by a strip of colored squares corresponding to the daily activity of the animal as indicated in the legend. Squares that are black indicate that the animal was euthanised as a consequence of reaching a pre-determined humane end point as defined in the methods. Data presented is for individual ferrets.

dose of IVIg administered (Fig. 4C). However, the only statistically significant difference was between the 0.5 g IVIg/kg and diluent control groups (One way ANOVA with Tukey's multiple comparison test  $p < 0.05$ , Fig. 4C). Viral titrations were not performed at latter time points (Day 5, 7) because the number of animals remaining in some of the groups was too small to allow a meaningful comparison.

#### 3.4. F(ab)<sub>2</sub> but not Fc Fragments of IVIg Protect Ferrets Against Lethal H5N1 Challenge

To investigate the mechanism by which IVIg conferred protection against lethal H5N1 challenge purified F(ab)<sub>2</sub> and Fc fragments derived from IVIg, at equimolar levels to the intact IVIg used in the preceding experiments (0.5 g/kg) were administered to ferrets at the time of challenge with H5N1 HPAI. We studied a total of 38 animals as shown in Fig. 5. Overall 8/10 (80%) of the F(ab)<sub>2</sub>-treated animals survived lethal HPAI challenge, which was significantly greater ( $p = 0.0015$ , Log-ranked Mantel-Cox) than the Fc-treated and diluent control groups with only 3/18 (17%) and 1/10 (10%) survivors respectively. Detailed activity assessments in a subset of 12 animals studied supported the conclusions of the mortality outcome (data not shown).

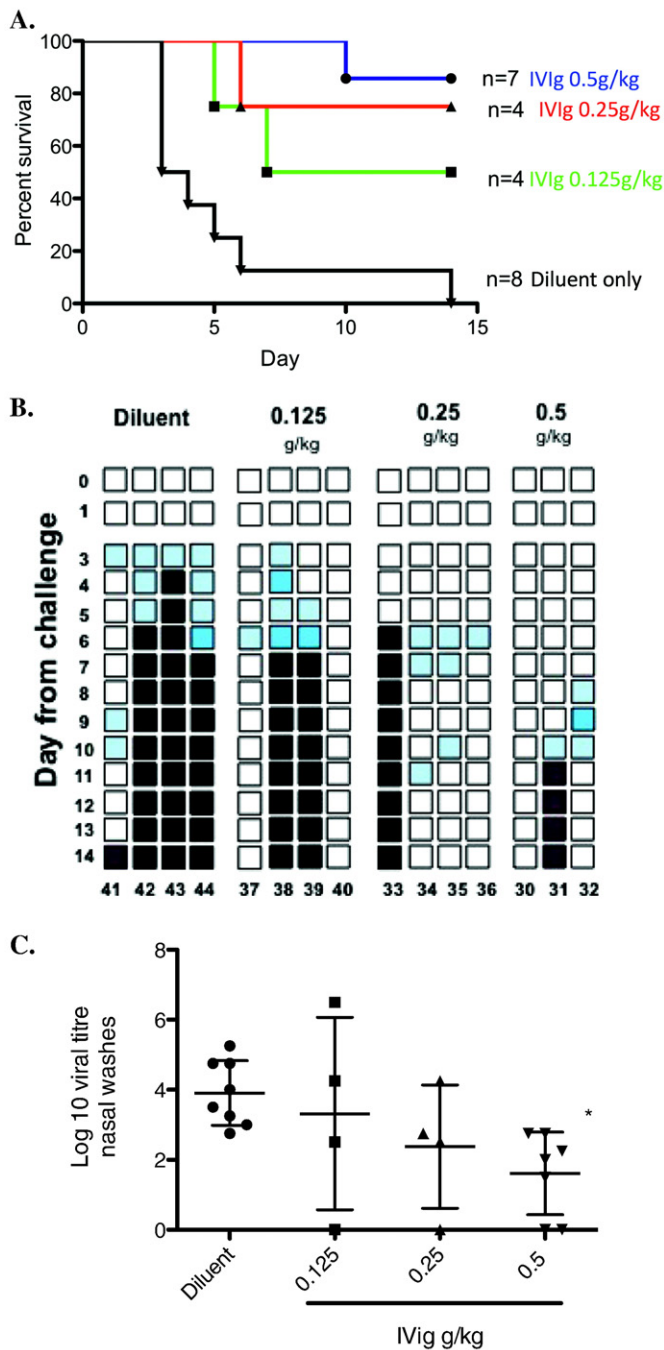
#### 3.5. IVIg Displays Functional Anti-neuraminidase Inhibition to H5N1

To further investigate the mechanism of protection, IVIg and F(ab)<sub>2</sub> fragments of IVIg were tested for reactivity to H5N1 influenza by HI and virus microneutralisation (MN) assay. No reactivity could be detected in a haemagglutination inhibition assay to purified H5N1 virus with either IVIg or the F(ab)<sub>2</sub> fragments of IVIg. Similarly, no H5N1 reactivity was detected with the F(ab)<sub>2</sub> or Fc preparations using a Vero cell-based microneutralisation (MN) assay. In contrast IVIg demonstrated a low level of reactivity (titre 8 or 200 µg/mL) against H5N1 in the MN assay.

Antibodies to the HA stalk can cross react and neutralise a range of influenza viruses (Wang et al., 2016; DiLillo et al., 2014; Krammer et al., 2012). They are present at much lower levels than HA head antibodies but anti-stalk mAbs can be cloned from human subjects (Wang et al., 2016). To determine reactivity to the stalk region of HA, IVIg and fragments of IVIg were reacted with a recombinant H5 protein (A/Vietnam/1203/04) in a solid phase enzyme linked assay. No reactivity was observed with either whole or fragmented IVIg in this assay, whereas monoclonal antibodies raised to and specific for the stalk region peptides were positive (data not shown). This analysis indicated that the level of anti-haemagglutinin in IVIg, including stalk region antibodies, that can cross react with H5 is low, consistent with previous findings (Sui et al., 2011; Rockman et al., 2013).

We and others have recently published the results of studies demonstrating the ability of anti-neuraminidase antibodies generated in response to a seasonal H1N1 strain to protect ferrets against challenge with avian H5N1 (Rockman et al., 2013; Easterbrook et al., 2012; Sandbulte et al., 2007). To analyse the level of neuraminidase antibody reactivity in IVIg, neuraminidase (N1) was immunoaffinity purified from A/California/07/2009 (H1N1). This purified N1 and detergent split A/Vietnam/1194/2004 (H5N1) were then tested in a fluorescence-based sialic acid substrate assay (MUNANA), in the presence of two fold dilutions of IVIg or fragments thereof to determine neuraminidase inhibition activity. IVIg and the F(ab)<sub>2</sub> fragment of IVIg had similar levels of neuraminidase inhibitory activity against purified N1 (Fig. 6A). The concentration of IVIg and the F(ab)<sub>2</sub> fragments at which neuraminidase activity was inhibited by 50% was 1.8 mg/mL and 1.2 mg/mL respectively, whereas the Fc fragments of IVIg did not demonstrate any NA inhibitory activity at any concentration tested (up to 10 mg/mL).

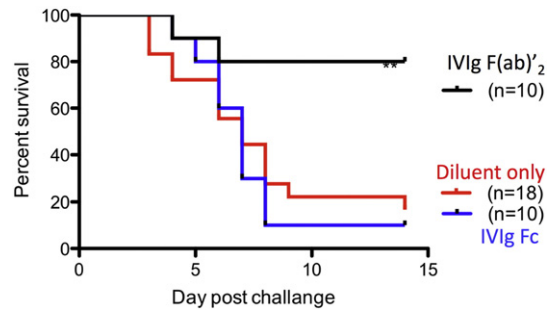
IVIg and F(ab)<sub>2</sub> fragments of IVIg also inhibited the NA activity of the split H5N1 A/Vietnam/1194/2004 virus preparation, the NA of which is identical to the challenge strain (A/Vietnam/1203/2004). In contrast, the Fc fragments of IVIg did not demonstrate any neuraminidase inhibition activity.



**Fig. 4.** Effect of IVIg dose on protection of ferrets against H5N1 challenge. Ferrets were challenged with  $10^6$  EID<sub>50</sub> of A/Vietnam/1194/2004 on Day 0 and were observed for 14 days unless they reached a humane end point and were euthanised. (A) Percentage survival across 23 ferrets. 0.125 g/Kg IVIg (square, in green), 0.25 g/kg IVIg (triangle, in red), 0.5 g/kg IVIg (circle, in blue) and diluent (upside down triangle, black). The number of animals in each group is indicated. A one way ANOVA with Tukey's multiple comparison test showed significant protection in the 0.5 g/kg IVIg group compared to diluent controls ( $p < 0.05$ ). (B) Activity scores (as described Fig. 2) in a subset of 15 animals. Data is for individual ferrets. (C) Log Viral titres from nasal washes from all 23 ferrets taken at Day 3.

### 3.6. Cross Reactive Antibodies to H5N1 Responsible for Protection

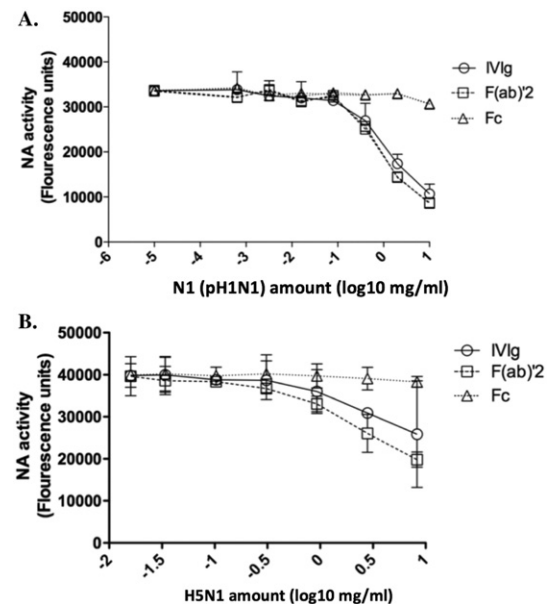
The lack of HI reactivity to H5N1 and the high level of cross reactive NA antibodies suggested that anti-NA antibodies may be responsible for the observed protection with IVIg in the H5N1 model. In order to further investigate this IVIg (Hizentra®) was adsorbed 1:1 ( $n = 3$  w:v) with inactivated H1N1 (A/California/7/2009) or H3N2 (A/Victoria/210/



**Fig. 5.** F(ab)<sub>2</sub> but not Fc fragments of IVIg protect ferrets against lethal H5N1 challenge. Ferrets were challenged with  $10^6$  EID<sub>50</sub> of A/Vietnam/1194/2004 on Day 0 and were observed for 14 days unless they reached a humane end point and were euthanised. Cumulative survival across of a total of 38 animals, Fab<sub>2</sub> (black line), Fc (blue line) and diluent (red line). Survival in the F(ab)<sub>2</sub> group was significantly greater ( $p = 0.0015$ , Log-ranked Mantel-Cox) than the Fc-treated and diluent control groups.

2009) whole virus. The H1N1 adsorbed IVIg displayed no in vitro inhibition of NA post adsorption, whereas the HI activity to H1 and H3 was reduced from a titre 80 to 16 and >64 to <1 respectively. The majority of ferrets administered adsorbed IVIg (H1N1 and H3N2) survived challenge with highly pathogenic H5N1 (2/3 and 4/6 survivors respectively) whereas the majority of animals given diluent (5/6) succumbed to the challenge.

To determine the level of remaining cross reactive antibodies in adsorbed IVIg compared to unadsorbed IVIg, an enzyme linked immunoassay was performed on split H5N1 (A/Vietnam/1194/2003) material and immunoaffinity purified N1 protein from A/California/07/2009. The 50% titre of the H1N1 adsorbed IVIg was >1/20,000 to H5N1 and >1/1000 to purified N1 (H3N2 > 1/18,500 and >1/1300 respectively) (data not shown). This indicated a 6.2 fold and 4.6 fold decrease in reactivity to H5N1 or the purified N1 protein respectively compared to unadsorbed IVIg. The H3N2 adsorbed IVIg displayed a similarly reduced reactivity (8.6 and 4.1 fold respectively). This data suggests that post absorption IVIg still contains a significant level of non-neutralising cross reactive antibodies which are sufficient to afford protection in this



**Fig. 6.** IVIg contains antibodies that inhibit the neuraminidase activity of purified N1 and H5N1 virus. IVIg and fragments of IVIg were titrated for NA inhibition activity against purified N1 (A/California/07/2009) (A) and split virion H5N1 (A/Vietnam/1194/2003) (B). The mean and standard deviation of triplicate dilutions is indicated.

model, which is consistent with our previous finding that ferrets vaccinated with seasonal H1N1 strains are protected against lethal H5N1 challenge (Rockman et al., 2013).

### 3.7. Induction of IL-10 Following IVIg Does Not Correlate With Protection in the H5N1 Model

It is well established that IVIg possesses anti-inflammatory and immunomodulatory capabilities (reviewed (Aschermann et al., 2010)), and IL-10 induction has been recently shown to be the key mechanism by which IVIg protects against fatal HSV encephalitis in mice (Ramakrishna et al., 2011). IL-10 has also been proposed to control lung inflammation during acute influenza infection by an effector T cell mechanism (Sun et al., 2009). To investigate if cytokine induction in response to IVIg plays a role in protection against challenge with HPAI, mRNA was isolated from peripheral blood leukocytes on the day immediately prior to challenge and days 3 and 5 post challenge, and the level of mRNA for cytokines (IL-10, IFN $\gamma$ , TNF $\alpha$ ) and perforin determined by RT-PCR. The level of IL-10 mRNA was 50, 86 and 1027 fold higher (average of the Day 3 and Day 5 fold increase) when compared to the diluent control group, in the Fc fragment, F(ab')<sub>2</sub> fragment and IVIg treatment groups respectively (Supplementary Fig. 1). Although there was a slightly higher increase in IL-10 mRNA in the Fc fragment group than the F(ab')<sub>2</sub> fragment group, the increase in IL-10 mRNA level was only significant ( $p < 0.05$ ) at Day 5 for IVIg compared to diluent (Bonferroni post test analysis, 2 way ANOVA). No increase over baseline was observed for IFN $\gamma$ , TNF $\alpha$  or perforin mRNA transcripts (data not shown). The finding that IL-10 expression was increased in response to treatment with both the Fc and F(ab')<sub>2</sub> fragments of IVIg (albeit with a marginally higher increase in the F(ab')<sub>2</sub> fragment group), with only the latter providing any protection against lethal HPAI challenge suggests that IL-10 is not the primary mechanism for protection in this model. Taken together with the observation that protection is only observed with IVIg and F(ab')<sub>2</sub> fragments of IVIg, this strongly implicates influenza-specific antibody reactivity as being critical for protection.

## 4. Discussion

This study in the ferret model demonstrates the efficacy of administration of a single dose of IVIg in preventing significant viral replication in the lung after challenge with the 2009 H1N1 pandemic strain, and significant reduction in morbidity and mortality following exposure to an otherwise lethal challenge with H5N1. We have previously proposed that there may be a benefit of administration of hyperimmune serum during the earliest febrile stage of disease prior to the onset of other constitutional symptoms (Rockman et al., 2012). These studies suggest the possibility that a single dose of IVIg may also provide a beneficial effect in delaying the systemic consequences of HPAI influenza infection.

The serological analysis of IVIg harvested prior to the 2009 pandemic indicated that there was low level cross reactivity with the 2009 H1N1 pandemic strain. The finding that this IVIg could limit lung viral replication when the animal was challenged with this strain is consistent with reports in man and animal models, that prior seasonal influenza exposure (vaccine or infection) was associated with some protection against a pandemic strain (Rockman et al., 2012; Steel et al., 2010; Marcelin et al., 2011; Garcia-Garcia et al., 2009; Johns et al., 2010).

To further understand the mechanism of IVIg protection and the potential of IVIg application in a pandemic, we investigated a second pandemic model, avian influenza H5N1 (HPAI), where there was no prior exposure of the plasma donors from which the IVIg was derived and therefore limited cross-reactivity to HA as determined by classical serological methods. The finding that the F(ab')<sub>2</sub> but not the Fc fragments of IVIg also protected in these challenge models indicated that complement or Fc gamma receptor bearing cells were not required for protection in this model, and therefore that a specific cross-reactive antibody

was most probably responsible. There is recent evidence that anti-influenza antibodies with Fc-mediated functions, such as antibody-dependent cellular cytotoxicity or antibody-dependent phagocytosis are present in IVIg and that these antibodies can assist in protective immunity from influenza (DiLillo et al., 2016; Jegaskanda et al., 2013a; Jegaskanda et al., 2013b; Jegaskanda et al., 2013c). However, there may be limited ability of human antibodies to bind Fc receptors of ferrets and human or non-human primate models with more conserved Fc receptors may ultimately be required to assess whether the Fc component of human IVIg can assist in protective immunity. We are currently studying the capacity of ferret Fc receptors to bind human antibodies to more fully understand the role of IVIg in protection of ferrets from influenza.

We found that the IVIg we studied (made prior to the 2009 pH1N1 pandemic) demonstrated little cross reactivity to H5 in a HI test, but a significant level of inhibition against purified NA. However, when IVIg was depleted of the NA antibody reactivity, it still retained the capacity to protect ferrets against H5N1 challenge. We speculate that the protection we observed is a consequence of the demonstrated high level of cross reactive non-neutralising antibodies. This is consistent with our own and other recent vaccine studies (Rockman et al., 2012; Rockman et al., 2013; Easterbrook et al., 2012; Sandbulte et al., 2007).

Low levels of anti-stalk monoclonal antibodies may also have contributed to the protection of ferrets from pandemic influenza strains we observed. Monoclonal anti-stalk antibodies have protected ferrets from a number of influenza strains (Kanekiyo et al., 2013; Nachbagauer et al., 2015). It will be of interest to directly compare such monoclonal antibodies with IVIg in future studies. Sialylation of antibodies has recently been identified as an important factor in the immunomodulatory activity of IVIg and may play a role in neutralization of influenza and act as a decoy receptor for influenza (Huang et al., 2016; Schwab & Nimmerjahn, 2014). Future studies examining the protective efficacy of de-sialylated IVIg against pandemic influenza are warranted.

Although we cannot rule out the possibility that the induction of immunomodulatory cytokines such as IL-10 in response to IVIg may contribute to the observed protection, in the models of pandemic influenza presented herein, the data suggest that the mechanism of protection appears to be due to cross reactive influenza antibody rather than through an immunomodulatory mechanism. We speculate that IVIg preparations manufactured after the pH1N1 pandemic of 2009 may have even greater protective efficacy against H5N1 infections given the partial cross-reactivity of immune responses to pH1N1 and H5N1 (Rockman et al., 2013; Jegaskanda et al., 2014).

At this time, specific reports of the use of IVIg in human cases of influenza, while promising, are limited (Luke et al., 2010; Chong et al., 2011; Zhang et al., 2010; Dubnov-Raz et al., 2011). Studies relating to the treatment or prevention of influenza have reported reduced mortality in patients transfused with convalescent human blood products derived from the plasma of individuals who have been infected with the same strain and subsequently recovered (reviewed (Luke et al., 2010; Luke et al., 2006)). While these reports are promising, the utility of using post convalescent sera is limited due to the significant time delay that would be encountered whilst suitable donors, in sufficient numbers are identified and sera is collected and processed. Overall this would take many months and severely limit the usefulness of this approach, particularly early in a pandemic, when the impact of such a treatment modality would be maximal. Importantly, in the studies presented in regard to both models of pandemic influenza, the IVIg used was not selected for challenge strain-specific activity. The IVIg used in our ferret studies was harvested from the general population that had no prior exposure to either pandemic virus. The advantage of IVIg in a pandemic setting is that the product is already registered and readily available.

Clearly, most benefit would be derived from passive treatment with IVIg if it were provided at the earliest time point from contact with an

infectious dose of virus as possible. Once HPAI infection is well established, including replication in peripheral organs such as brain and pancreas, it is likely that comparatively larger amounts of IVIg would be required as a consequence of the exponentially increasing viral load. Due to the low and sporadic incidence of HPAI H5N1, it is difficult to perform randomised controlled trials in humans. A randomised trial in the setting of seasonal influenza of a hyperimmune influenza IVIg preparation is ongoing, with early evidence from a pilot trial that it this IVIg is safe and can boost HI antibody levels in infected subjects (Group IFIPS, 2016).

The threat of HPAI H5N1 or another severe pandemic influenza virus arises from the continual circulation and reassortment of influenza in avian, pig and human populations. The impact of a pandemic event is determined by the level of population immunity to the pandemic strain. Our data suggests that following exposure through either vaccination or infection, a level of endogenous antibody cross reactivity to highly pathogenic influenza strains occurs in the community, and that this is reflected in the donor-derived plasma pools that are used to prepare IVIg. The data presented also suggests that the cross reactive antibodies in IVIg may provide a level of protection against certain pandemic strains including the persistent circulation of HPAI H5N1.

In summary, these studies in the ferret model suggest that human IVIg may be effective in preventing serious influenza infection and provides a possible alternative treatment option requiring confirmation in human clinical trials.

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#### Author Contribution

SR: study design, data collection, data analysis, data interpretation, writing. SL: data collection, data analysis, data interpretation. SC: data collection, data analysis, data interpretation. KV: data collection, data analysis, data interpretation. ST: study design, data analysis, data interpretation. LF: study design, data analysis, data interpretation. SM: study design, data analysis, data interpretation. MP: study design, data interpretation, writing. DM: study design, data analysis, data interpretation, interpretation. SJK: data interpretation, writing. DM: study design, data interpretation, writing.

#### Conflict of Interest

Shirley Taylor, Lou Fabri and Martin Pearse are employees of CSL Ltd.; Sylvia Meischer, Darryl Maher are employees of CSL Behring and Sarina Camuglia, Kirsten Vandenberg and Steven Rockman are employees of Seqirus, a CSL company. CSL Behring manufactures IVIG.

#### Appendix A. Supplementary data

Supplementary data to this article can be found online at <http://dx.doi.org/10.1016/j.ebiom.2017.04.010>.

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