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# Antibody-dependent NK cell degranulation as a marker for assessing antibody-dependent cytotoxicity against pandemic 2009 influenza A(H1N1) infection in human plasma and influenza-vaccinated transchromosomal bovine intravenous immunoglobulin therapy

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## ARTICLE INFO

### Keywords:

Influenza  
Antibody-dependent cellular cytotoxicity  
NK cell degranulation

## ABSTRACT

This study describes an antibody-dependent NK cell degranulation assay, as a biomarker to assess antibody-dependent cellular cytotoxicity (ADCC) response in influenza plasma and for antibody therapies against influenza infection. The concentration of neutralizing antibodies (NAbs) against the hemagglutinin receptor of influenza viruses is a current determinant in protection against infection, particularly following receipt of the seasonal influenza vaccine. However, this is a limited assessment of protection, because: (i) NAb titers that incur full protection vary; and (ii) NAb titers do not account for the entire breadth of antibody responses against viral infection. Previous reports have indicated that antibodies that prime ADCC play a vital role in controlling influenza infections, and thus should be quantified for assessing protection against influenza. This report demonstrates a non-radioactive assay that assesses NK cell activation as a marker of ADCC, in which NK cells interact with opsonized viral antigen expressed on the surface of infected Raji target cells resulting in effector cell degranulation (surrogate CD107a expression). A positive correlation was determined between HAI titers and sustained NK cell activation, although NK cell activation was seen in plasma samples with HAI titers below 40 and varied amongst samples with high HAI titers. Furthermore, sustained NK cell degranulation was determined for influenza-vaccinated transchromosomal bovine intravenous immunoglobulin, indicating the potential utility of this therapy for influenza treatment. We conclude that this assay is reproducible and relevant.

## 1. Introduction

Influenza is a significant cause of morbidity/mortality in human populations across the globe (Lavanchy, 1999). Influenza viruses are estimated to cause 3–5 million severe clinical infections yearly resulting in 250,000–500,000 fatalities (Stohr, 2002). Influenza A viruses, including H3N2 and H1N1 subtypes, are involved in seasonal epidemics with pandemic potential (Stohr, 2002). Immune-mediated protection against influenza viruses is thought to be largely dependent on neutralizing antibody (NAbs) specific against the membrane-distal portion of the viral envelope protein hemagglutinin (HA) preventing binding of virus to target cell sialic acids (Matlin et al., 1981).

Assessment of antibody (Ab) responses to specific influenza subtypes is an important diagnostic, epidemiological, and immunological tool (Cauchemez et al., 2012). Demonstration of serotype- and subserotype- (“subtype-”) specific Ab responses in individuals following

suspected infection or vaccination may be accomplished by a variety of methods. For influenza, the most widely used assay to assess antibody (IgG and otherwise) levels is the hemagglutination inhibition (HAI) assay, which provides a relative measure of the quantity of anti-HA Abs present in patient sera/plasma (Hobson et al., 1972; Katz et al., 2011). Additional assays that are employed include the neuraminidase inhibition (NI) and microneutralization (MN) assays that are used to detect neuraminidase-specific antibodies and the ability of plasma antibodies to neutralize infectivity of influenza virus respectively. The serological methods described above do not assess the entire breadth of the antibody mediated responses to influenza viruses. Additional serological and cellular effector functions include: (i) complement activation (Terajima et al., 2011); (ii) phagocytosis (El Bakkouri et al., 2011; Huber et al., 2001; Ana-Sosa-Batiz et al., 2016); and (iii) antibody-dependent cellular cytotoxicity (ADCC) (Greenberg et al., 1979; Jegaskanda et al., 2013a, 2016, 2014a,b, 2013b; Vanderven et al.,

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2016; Zhong et al., 2016; Co et al., 2014; Florek et al., 2014; Jegaskanda et al., 2013d; Srivastava et al., 2013; Jegaskanda et al., 2017, 2013c).

Anti-influenza NABs can directly or in combination with complement block or deactivate infectious viral particles prior to entry into host cells. However, if viral particles escape from NABs and establish infection inside target cells NABs may no longer be optimally effective, and immune effector cells are then required to recognize and eliminate virally infected cells. NK cells are an immune effector cell type that provides a first line of defense against infections and cell malignancies. NK cells can bind to cell surface antigen-Ab immune complexes via the Fc portion of the antibody. Fc $\gamma$ RIII is a receptor found on a number of different effector cells, importantly NK cells, and is involved in effector cell recognition of virally infected cells and induction of ADCC (Wren et al., 2013). CD107a (LAMP1) expression on NK cells has been used as a surrogate marker for the identification of natural killer cell activity (Alter et al., 2004).

ADCC against influenza has been demonstrated in a number of studies (Greenberg et al., 1979; Jegaskanda et al., 2013a, 2016, 2014a, b, 2013b; Vanderven et al., 2016; Zhong et al., 2016; Co et al., 2014; Florek et al., 2014; Jegaskanda et al., 2013d; Srivastava et al., 2013; Jegaskanda et al., 2017; Jegaskanda et al., 2013c; DiLillo et al., 2014; Cox et al., 2016). Assays based on radioactive chromium-labeling of target cells have been used to demonstrate the presence of antibodies capable of ADCC activity (Greenberg et al., 1979), and to demonstrate the presence of ADCC antibodies in clinical influenza serum samples (Co et al., 2014). Assays based on assessing CD107a expression of NK cells following incubation on serum-treated plates coated with influenza proteins have been conducted (Jegaskanda et al., 2013a, 2016, 2014a,b, 2013b; Vanderven et al., 2016; Zhong et al., 2016; Florek et al., 2014; Jegaskanda et al., 2013d; Jegaskanda et al., 2017, 2013c). These studies have largely used a flow-based ADCC NK cell activation assay using methods that are less physiologically relevant due to the removal of target cell and effector cell interaction. One influenza ADCC study utilized a flow-based method for assessing target cell lysis via membrane-labeling dyes following effector cell interaction (Srivastava et al., 2013). Influenza-infection of A549, an immortalized human epithelial cell line, has also been utilized for measuring ADCC Ab activity using CD107a expression of NK cells co-incubated with target cells (Jegaskanda et al., 2017, 2013c; DiLillo et al., 2014). Additionally, a bioassay to measure Fc $\gamma$ RIIIa activation by ADCC-Abs has also been conducted using influenza HA-transfected A549 cells following incubation with human plasma samples and Jurkat (effector) cells (Cox et al., 2016). Additional non-radioactive ADCC assays are becoming commercially available (Surowy et al., 2012).

ADCC-mediated clearance of virally infected cells and ADCC protection following vaccination or natural infection are potentially important immune mechanisms against influenza infection. Immunoglobulin-based therapeutic products such as human intravenous immunoglobulin (IVIG) should also be capable of stimulating an ADCC immune response to be maximally effective therapeutics. Previous studies have demonstrated the capacity of immunized bovine IgG to induce NK cell activation and mediate ADCC immunity to HIV infection (Kramski et al., 2012). Recently SAB Biotherapeutics has developed a transchromosomal (Tc) bovine system that replaces knocked out bovine immunoglobulin genes with a human artificial chromosome containing full germline sequences of human immunoglobulin (Kuroiwa et al., 2009; Matsushita et al., 2014, 2015; Sano et al., 2013). These Tc bovine are capable of producing full human antibodies, and have been used to produce polyclonal human IgG against hantavirus (Hooper et al., 2014), Middle East respiratory syndrome-related coronavirus (MERS-CoV) (Luke et al., 2016), and Ebola Zaire virus (Dye et al., 2016). Tc bovine hyperimmunized against pandemic strains of influenza also have the potential to produce a commercially viable IVIG product capable of neutralizing antibody and ADCC-antibody functions. As such, development of a standardized NK cell assay for detecting

ADCC activity against influenza viruses could aid in the assessment of: (i) population immunity; (ii) vaccine and therapeutic IVIG treatment efficacy; and (iii) possibly a clinical assay to indicate if an infected individual will or will not progress to severe illness.

To examine ADCC-Abs from a plasma panel collection, including Tc bovine IVIG, we utilized a high-throughput, non-radioactive, assay assessing influenza infection of Raji target cells and plasma Ab ability to activate NK effector cells as demonstrated by degranulation (CD107a surrogate expression). In this study we describe a reproducible and relevant method for assessing NK cell degranulation titer of plasma/sera samples. Using this assay we determined that Tc bovine IVIG had a high titer of NK cell degranulation antibodies, and thus represents a system that could be used as a countermeasure to rapidly produce fully human antibodies capable of eliciting ADCC responses against pandemic influenza.

## 2. Material and methods

### 2.1. Cell culture

Raji cells (American Type Culture Collection (ATCC), CCL-86; Manassas, VA) were grown in complete RPMI media (Gibco; Langley, OK) with 10% fetal bovine serum (FBS) (Quality Biological; Gaithersburg, MD) and 1% penicillin/streptomycin (P/S) (Corning; Corning, NY). Prior to infection with influenza, cells were washed with 1 x PBS, then resuspended in RPMI media with 1% Bovine Serum Albumin (BSA) (Fisher Scientific; Pittsburgh, PA), 1% P/S, and Tosyl phenylalanyl chloromethyl ketone (TPCK) (Accurate Chemical; Westbury, NY) at 2  $\mu$ g TPCK/mL media.

### 2.2. Effector cells

Blood was collected from healthy donors under a protocol approved by the Naval Medical Research Center Institutional Review Board in compliance with all applicable Federal regulations governing the protection of human subjects: HRPP# NMRC.2012.0008. Effector cells for assessing NK cell degranulation were the non-adherent fraction of peripheral blood mononuclear cells (PBMCs) from healthy donors. Donor PBMCs were screened for NK cell degranulation capacity in our lab as previously published (Sun et al., 2017). Utilizing data from that study, donor PBMCs with a degranulation capacity above 10% of background were utilized for all subsequent experiments described within this manuscript. The preparation of non-adherent PBMCs has been described previously (Sun et al., 2006). Briefly, cryopreserved whole PBMCs were thawed, washed and brought to 2  $\times$  10<sup>6</sup> cells/mL in culture medium, and were placed into a 6 well plate (Becton Dickinson MultiwellTM PrimariaTM 6 well; Becton Dickinson and Company, Franklin Lakes, NJ) at 3 mL/well. The plate was kept at 37 °C for 1–2 h to allow monocytes to adhere to the plate. After adherence, non-adherent cells were gently collected from the plate into a 15-mL or a 50-mL tube. The non-adherent cells were then washed once and were brought to 1  $\times$  10<sup>6</sup> cells/mL in culture medium. The cells were left in the 15-mL or 50-mL tube in a humidified 37 °C 5% CO<sub>2</sub> incubator overnight. Before adding the effectors to the target cells with plasma, they were washed once, counted and adjusted to 1  $\times$  10<sup>6</sup>/mL in culture medium.

### 2.3. Virus

H1N1 isolate A/California/07/2009 (H1N1pdm09) pandemic strain (Influenza Reagent Resource; Manassas, VA) virus was amplified in fertilized chicken eggs (Charles River Laboratories, Wilmington, MA). Eggs were incubated at 37 °C with humidity and 0% CO<sub>2</sub> for 48 h, before transfer to 4 °C for an additional 24 h. Allantoic fluid was then collected, spun down to remove debris, and titered with turkey red blood cells (Lampire Biological Laboratories; Pipersville, PA) to

determine hemagglutination units (HAU)/50  $\mu$ L – an HAU/50  $\mu$ L of 512 was determined.

#### 2.4. Infection

Infection rate of Raji cells was determined by incubating 100,000 cells/well on a 96 well cell culture-treated plate (Corning Costar; Corning, NY) with dilutions of virus representing HAU of 102, 34, 10, and 3 per well for 24 h in RPMI media with 1% BSA and TPCK. Supplementation with TPCK was based on previous usage for influenza A infectivity studies (Jegaskanda et al., 2017; Su et al., 2009). Cells were then washed two times with 1 x PBS and half were used for determination of cell surface expression of HA and half for intracellular expression of HA. For assessment of cell surface staining, 100  $\mu$ L of anti-HA monoclonal antibody (MAB8256, Millipore, Billerica, MA) was applied at a dilution of 1:200 for 30 min at room temperature. For assessment of intracellular expression, cells were fixed and permeabilized with BD Cytotfix/Cytoperm (BD Biosciences; San Jose, CA) for 20 min at room temperature and washed two times with PermWash prior to receiving antibody. All cells were then washed and stained with secondary FITC-conjugated goat  $\alpha$ -mouse antibody (BD Pharmingen; San Jose, CA) at a dilution of 1:500 for 30 min in the dark. Cells were washed then resuspended in 130  $\mu$ L of 1 x PBS and assessed by flow cytometry (Becton Dickinson FACS Canto II; San Jose, CA). Cells were assessed for mean fluorescent intensity (MFI) of HA expression of all cells.

#### 2.5. Plasma samples and HAI assay

Human plasma samples were obtained through the National Institute of Allergy and Infectious Diseases under a protocol approved by the Naval Medical Research Center Institutional Review Board in compliance with all applicable Federal regulations governing the protection of human subjects: HRPP# NMRC.2010.0012. These plasma samples were screened for levels of antibodies against the A/California/07/2009 (H1N1pdm09) HA receptor using the World Health Organization/Centers for Disease Control and Prevention HAI Assay previously described (Defang et al., 2012).

#### 2.6. The NK cell degranulation assay

NK cell degranulation was assessed by the expression of CD107a on effector cells using a modified method described previously (Sun et al., 2017; Chung et al., 2009). Briefly, on a 96 well U-bottom tissue culture-treated plate (Corning Costar), Raji cells were aliquoted at 100,000 cells/well and washed two times with PBS to remove residual FBS. Virus stock was diluted to a final concentration of 102 HAU per well with RPMI media with 1% BSA, P/S, and 2  $\mu$ g/mL TPCK, and aliquoted at 100  $\mu$ L/well. Plates were incubated 24 h at 37 °C with humidity and 5% CO<sub>2</sub>. Plates were then washed two times with PBS, and dilutions of 1:200, 1:1000, 1:5000, and 1:25000 of plasma (100  $\mu$ L/well) were applied and incubated on ice for 1.5 h. Plates were washed two times with PBS. Target cells treated with plasma were then divided into two parts, one for assessment of opsonization immediately after plasma treatment, and the other for determination of NK cell degranulation. For the opsonization plate, 100  $\mu$ L/well of PE-conjugated  $\alpha$ -human IgG (FC gamma-specific, 12-4998-82, EBioscience, San Diego, CA) diluted to 1:200 was added and the plate was incubated at room temperature for 30 min in the dark. Plates were washed and then antibody opsonization of infected cells was assessed via flow cytometry on the FACS Canto II (Becton Dickinson). For setting up the NK cell degranulation assay, effector cells were collected, counted, and reconstituted in complete RPMI media to a concentration of  $1 \times 10^6$  cells/mL. 100  $\mu$ L/well of effector cells were added to plasma-treated infected or uninfected target cells (Raji) at a ratio of 1:1 and incubated at 37 °C for 2 h. Media was removed and 20  $\mu$ L of antibody cocktail containing 1:20 of

$\alpha$ -CD56 (APC),  $\alpha$ -CD3 (PerCp),  $\alpha$ -CD16 (PE), and 1:10 of  $\alpha$ -CD107a (FITC) (BD Biosciences) were added to each well and incubated in the dark at room temperature for 30 min. Cells were then washed twice with PBS and resuspended in 130  $\mu$ L PBS. Cells were then assessed by flow cytometry. The CD3<sup>-</sup>CD56<sup>+</sup> NK cells were gated and the percentage of CD107a<sup>+</sup> cells within the NK cell population was analyzed. Effector cells not cultured with target cells were set as a negative control for background CD107a expression. Effector cells cultured with plasma-untreated target cells were set as baseline controls for Ab-independent NK cell activation.

#### 2.7. Statistical analysis

Statistics were performed using GraphPad Prism software (GraphPad; La Jolla, CA)

### 3. Results

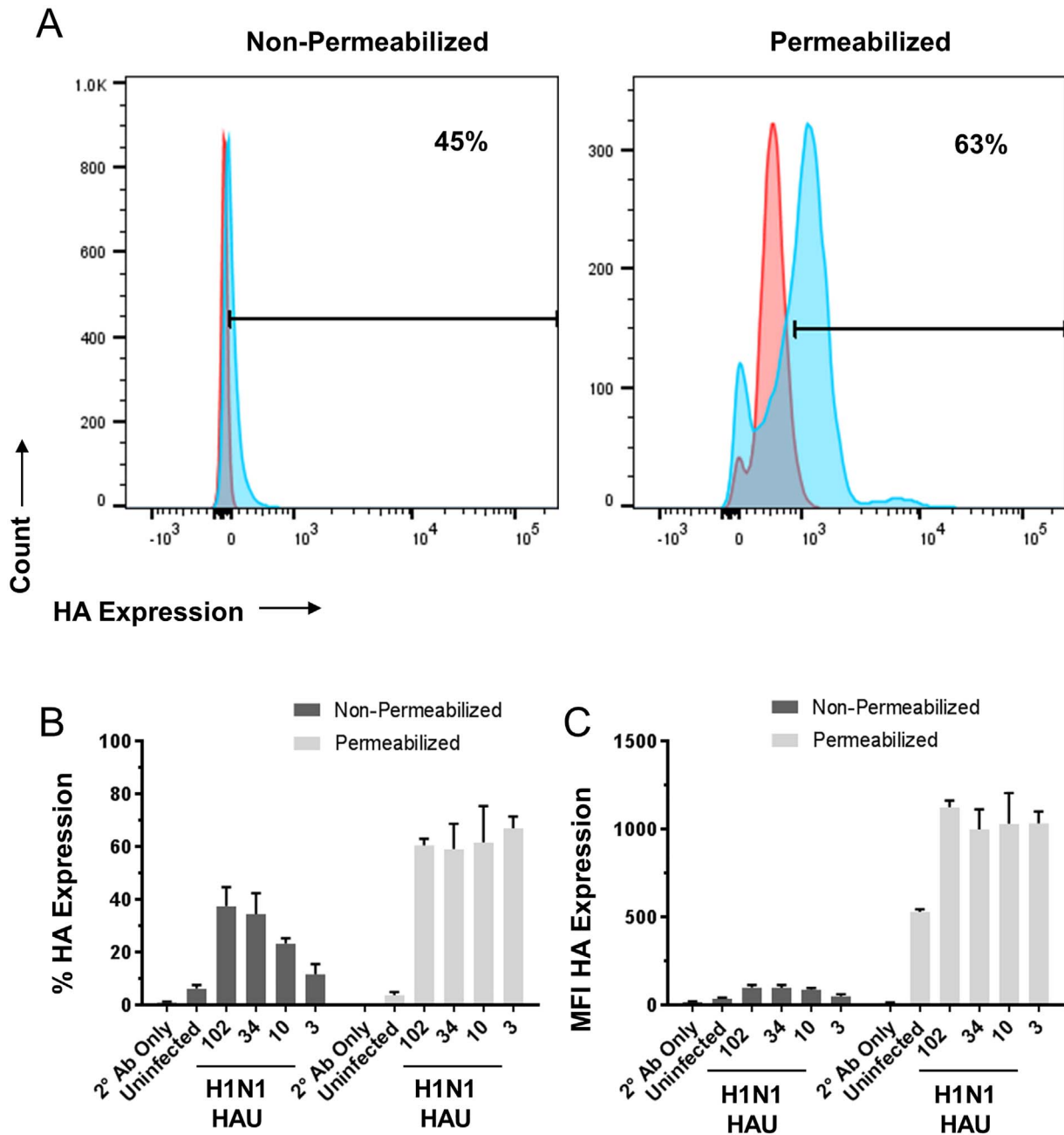
#### 3.1. H1N1 HA expression in influenza infected raji target cells

To determine if A/California/07/2009 (H1N1pdm09) could infect Raji target cells in our assay, we infected 100,000 Raji cells with dilutions of H1N1pdm09 stock virus representing HAU of 102, 34, 10, and 3, and incubated cells for 24 h. We assessed cells for viral antigen expression with an  $\alpha$ -HA MAb. Results indicated a rate of infection (Fig. 1) congruent with previous determinations (Srivastava et al., 2013). Cell populations were either stained right after incubation or fixed and permeabilized to compare overall HA expression vs. HA expression on cell surface (Fig. 1A). Both indicated HA expression at 24 h following virus incubation with cells; however, this tapered off in the non-permeabilized population with increasing dilution of virus (Fig. 1B). In contrast, the permeabilized population demonstrated a consistent rate of HA expression (~59–66%) in all dilutions. We believe that the tapering off of HA expression seen for the non-permeabilized cells is likely due to increased non-internalized virions present on the cell surface for the lower dilutions of virus compared to higher dilutions. This results in a higher cell surface staining for HA for lower dilutions of virus compared to higher dilutions without affecting overall intracellular levels of HA. In addition, the mean fluorescence intensity of HA expression was significantly higher in the permeabilized cells than the non-permeabilized cells at all dilutions of virus (Fig. 1C).

#### 3.2. Gating strategy for the NK cell degranulation assay and antigen opsonization and CD107a expression for viral and plasma dilution

We next elucidated opsonization of antigen expressed on the surface of infected target cells, and if this opsonization would activate NK effector cells. We assessed this across four dilutions of plasma and four dilutions of H1N1pdm09 virus infection. We selected two plasma samples that had high HAI titers against H1N1pdm09: Plasma1 (HAI 1280) and Plasma2 (HAI 640). The gating strategy for the assay is shown in Fig. 2A. NK cells (CD3<sup>-</sup>CD56<sup>+</sup>) could be distinguished from T (CD3<sup>+</sup>CD56<sup>-</sup>) and NKT (CD3<sup>+</sup>CD56<sup>+</sup>) cells. We co-stained NK cells with CD16 to further visualize the concomitant loss of CD16 during NK cell activation (determined by enhanced CD107a expression).

To assess opsonization, target cells were incubated with human plasma obtained from volunteer donors. Plasma1 demonstrated low opsonization as assessed by MFI of antigen (Ag)/Ab binding over all plasma and viral dilutions (Fig. 2B). In contrast, cells treated with Plasma2 demonstrated high opsonization (greater than 10000 MFI) remaining in the 1:1000 dilution of plasma and the 102 HAU dilution of virus (Fig. 2B). For Plasma2 opsonization did decrease at the 1:5000 dilution of plasma. We then assessed if opsonization would result in NK cell activation. We incubated effector cells with targets for 2 h at 37 °C, stained cells to select for the NK cell population, and determined NK cell activation by probing for expression of CD107a. Consistent with the



**Fig. 1.** Influenza infection of Raji cells and HA expression. Raji cells were infected with dilutions of H1N1pdm09, starting at 102 hemagglutination units (HAU) per 100,000 Raji cells, and infection rate was assessed for non-permeabilized (surface) and permeabilized cells (intracellular expression) by anti-hemagglutinin ( $\alpha$ -HA) monoclonal antibody. Controls include secondary (2°) antibody (Ab) only and uninfected cells. (A) Representative flow histograms showing expression of  $\alpha$ -HA for control uninfected cells (red) and cells infected with 102 HAU of influenza (blue). (B) Percentage of HA expressing cells and (C) mean fluorescence intensity (MFI) of HA expression for all cells for non-permeabilized cells and permeabilized cells. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

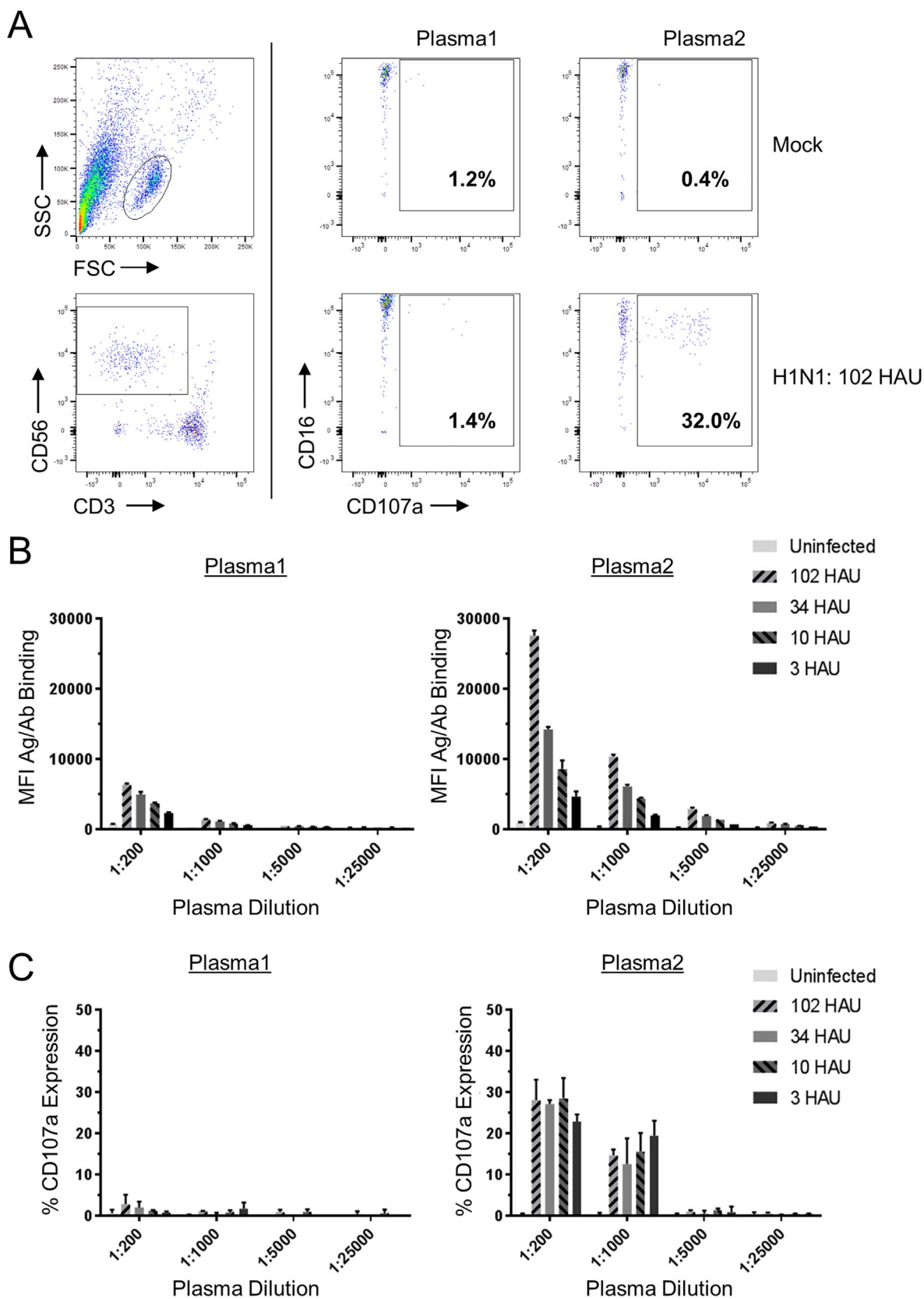
opsonization data, there was significant CD107a expression in cells incubated with Plasma2. In contrast, for cells incubated with Plasma1 expression did not rise above baseline levels (Fig. 2C). Taken together, these data suggest that NK cells are activated by interacting with opsonized antigens expressed on the surface of infected cells.

### 3.3. Reproducibility of human plasma Ag/Ab opsonization and NK cell degranulation titer determination

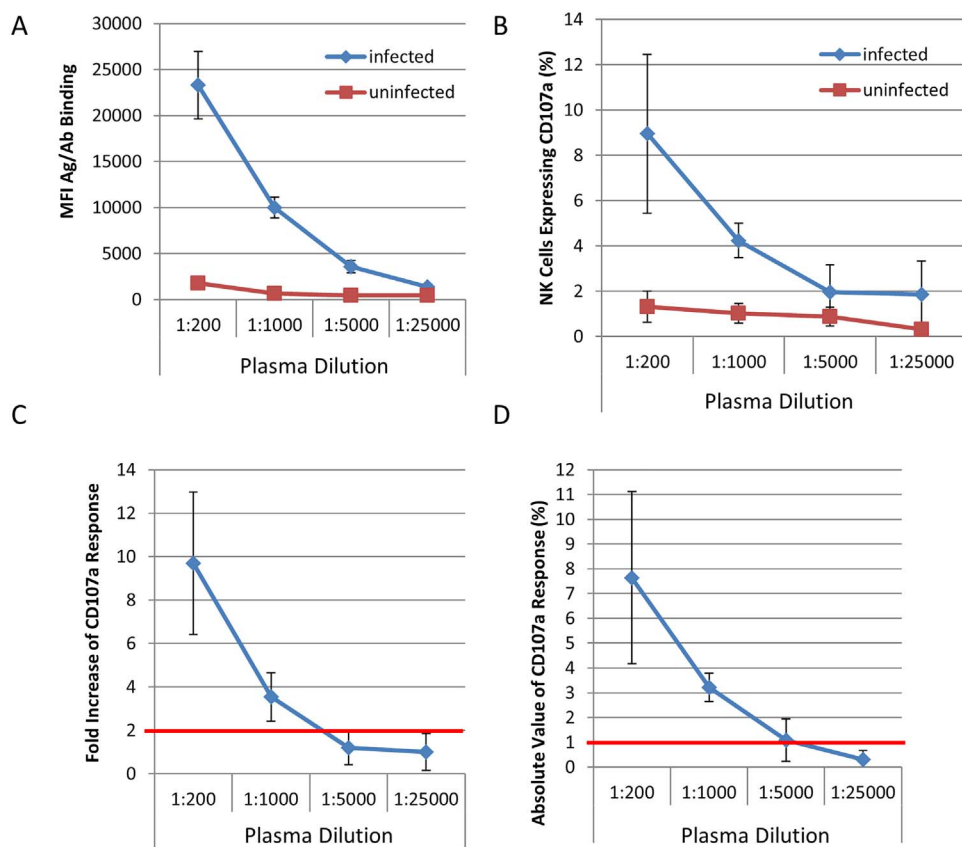
For the remaining experiments we standardized our infection of target cells at 102 HAU of H1N1pdm09 per 100,000 cells. We selected one plasma sample (HAI 640) and tested both opsonization (Fig. 3A) and NK cell degranulation (Fig. 3B) reproducibility over three

experiments for infected and uninfected target cells. The titer of Abs capable of stimulating NK cell activation was assessed by determining the fold increase of the NK cell response (Fig. 3C) and the absolute value of the NK cell response (Fig. 3D). Fold increase of NK cell response was determined by the fold increase of the CD107a NK cell response for infected cells compared to uninfected cells at each dilution. The absolute value of the CD107a NK cell response was determined as the percentage of NK cells expressing CD107a for infected cells minus the percentage for uninfected cells. The highest dilution of plasma that had both a two-fold increase of CD107a expression and an absolute value of CD107a over one was determined as the titer of NK cell degranulation.





**Fig. 2.** Gating strategy and opsonization/NK cell response (CD107a assay) for influenza infected Raji cells. (A) Gating strategy for CD107a expression by NK effector cells. Effector cells were gated by side scatter (SSC) and forward scatter (FSC), and then the CD3<sup>-</sup>CD56<sup>+</sup> NK cells were gated and the percentage of CD107a<sup>+</sup> cells within the NK cell population was analyzed. Mock infected and H1N1 infected cells are shown. Concomitant loss of CD16 during NK cell activation is also demonstrated. Hemagglutination units = HAU. (B) Two samples of human plasma (Plasma1 and Plasma2) known to have high titers of neutralizing antibodies against H1N1 were assessed for opsonized antigen, by mean fluorescent intensity (MFI) of antigen/antibody (Ag/Ab) binding, on the surface of Raji cells over a dilution of both H1N1pdm09 virus and incubated plasma. (C) NK cell degranulation activity of the same two human plasma samples indicating NK cell effector activity against Plasma2 but not Plasma1.



**Fig. 3.** Reproducibility of binding of patient plasma to influenza infected cells and NK cell response. Plasma2 sample was used in three separate assays to assess reproducibility of opsonization and the CD107a NK cell degranulation assay. (A) Results for mean fluorescent intensity (MFI) of Ag/Ab opsonization over plasma dilution for uninfected and infected cells. (B) Results for the percentage of NK cells expressing CD107a over plasma dilution for uninfected and infected cells. (C) The fold increase of the CD107a NK cell response for infected cells compared to uninfected cells at each dilution. Red line indicates cut-off of two-fold increase. (D) The absolute value of the CD107a NK cell response expressed as the percentage of NK cells expressing CD107a for infected cells minus the percentage for uninfected cells. Red line indicates cut-off of one. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

**3.4. Assessment of opsonization and NK cell degranulation titer for a human plasma sample set**

We selected 26 samples of human plasma. HAI titers against A/California/07/2009 ranged from 5 to 1280, organized in low (HAI 5-50), middle (HAI 160-202), and high (HAI 320-1280) groups (Table 1). Opsonization (presented as binding intensity) to uninfected cells was consistently low. For infected cells, we observed a MFI of 5000 or higher for opsonization of antigen in the 1:200 dilutions in all plasma except for Low10, High1, and High8 (Fig. 4A). In 1:1000 or higher dilutions, opsonization tapered off dramatically in cells treated with the low titer and middle titer plasma. This phenomenon occurred to a lesser extent with the middle titer group. We observed higher opsonization in the 1:200 dilutions for most of the samples in the high titer group, and continued to see significant opsonization in the higher dilutions with the exception of High1 and High8 plasmas, which consistently showed diminished opsonization akin to the samples with low titer (Fig. 4A).

In order to assess NK cell degranulation we incubated target cells in the same conditions as above, followed by incubation with effector cells for two hours. NK cell activity was determined by the percentage of NK cells expressing CD107a (Fig. 4B). Two of the ten samples from the low titer group (Low6 and Low8) had up to 10% of NK cells expressing CD107a in the 1:200 dilutions. This was reduced to around baseline levels in all subsequent dilutions. Five of the eight middle titer plasma samples (Mid1, Mid4, Mid5, Mid6, Mid8) had at least 10% of NK cells expressing CD107a in the 1:200 dilutions. NK cell degranulation varied particularly widely among the high titer samples. Three of the eight high titer human plasma samples (High4, High5, High6) had at least 10% of NK cells expressing CD107a in the 1:200 dilutions, and we continued to observe high CD107a expression in the higher dilutions of these samples. High8 (HAI 1280) demonstrated both low opsonization and low NK cell degranulation.

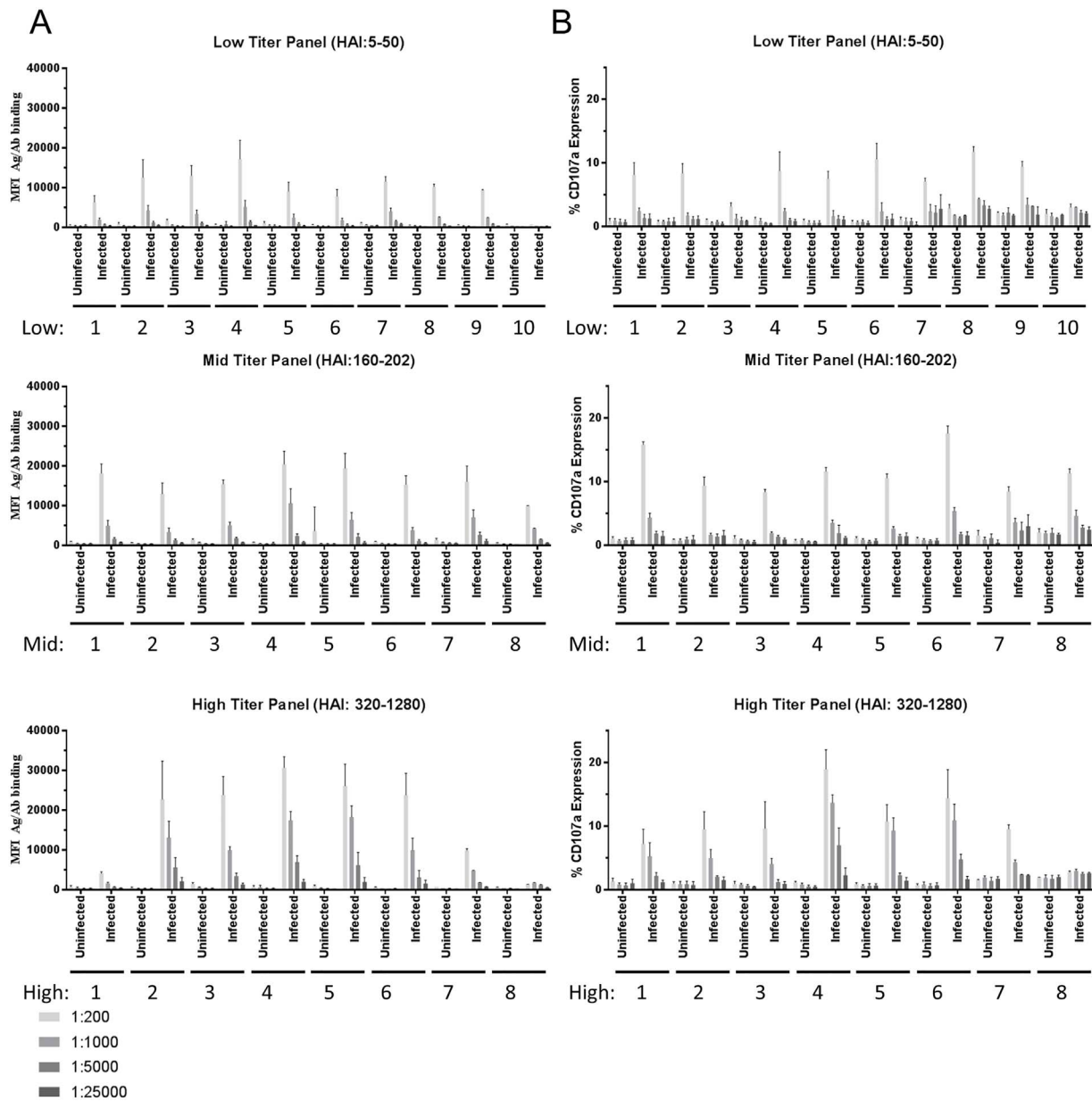
For normalizing the data, NK cell activity was also presented as fold increase and absolute value for infected cells compared to uninfected

**Table 1**

Human plasma sample set and transchromosomic (Tc) bovine IVIG. NK cell titer is assessed as the highest plasma dilution with an absolute value for CD107a expression by NK cell greater than one and a fold-change of CD107a expression by NK cells greater than two compared to uninfected cells. Hemagglutination inhibition = HAI.

Plasma ID	HAI Titer	NK Cell Titer
Low1	20	1:200
Low2	40	1:200
Low3	40	1:200
Low4	40	1:200
Low5	40	1:200
Low6	40	1:1000
Low7	50	1:1000
Low8	40	1:200
Low9	50	1:200
Low10	5	< 1:200
Mid1	160	1:1000
Mid2	160	1:200
Mid3	160	1:200
Mid4	160	1:5000
Mid5	160	1:1000
Mid6	160	1:1000
Mid7	202	1:1000
Mid8	202	1:200
High1	1280	1:5000
High2	1280	1:5000
High3	640	1:1000
High4	1280	1:25,000
High5	1280	1:5000
High6	1280	1:5000
High7	640	1:200
High8	1280	< 1:200
Tc Bovine IVIG	1280	1:5000

cells. Heat map analysis (Fig. 5A) of the CD107a expression fold increase for infected cells compared to uninfected cells demonstrated a trend towards more sustained (greater than two-fold) fold increase at



**Fig. 4.** Overall plasma panel results for opsonization and NK cell degranulation. Four data points (two bio-replicates done in duplicate) for each sample at each dilution were averaged together. (A) Results for mean fluorescent intensity (MFI) of Ag/Ab opsonization for the low, middle, and high titer groups. Hemagglutination inhibition = HAI. (B) Results for the percentage of NK cells expressing CD107a for the low, middle, and high titer groups. Plasma dilutions; light grey bars = 1:200; second lightest grey bar = 1:1000, second darkest grey bar = 1:5000, and darkest grey bar = 1:25,000).

higher dilutions for high HAI titer plasmas compared to low or middle titer samples. At a 1:200 dilution of plasma the NK cell degranulation assay demonstrated a positive correlation ( $r = 0.8526$ ) between the absolute value of CD107a response and the fold increase of CD107a response (Fig. 5B). At serum dilutions of 1:1000 and 1:5000, the correlation between the absolute value and the fold increase were both  $r = 0.97$ . This indicates a relationship between the two values and the utility of both for determination of the NK cell titer. MFI of Ag/Ab binding/opsonization on target cells also was positively correlated with fold increase of CD107a response at 1:200 dilution of plasma (Fig. 5C). This indicates the importance of Ag/Ab binding for elicitation of NK cell degranulation.

### 3.5. Demonstration of the NK cell degranulation assay for Tc bovine IVIG therapeutic

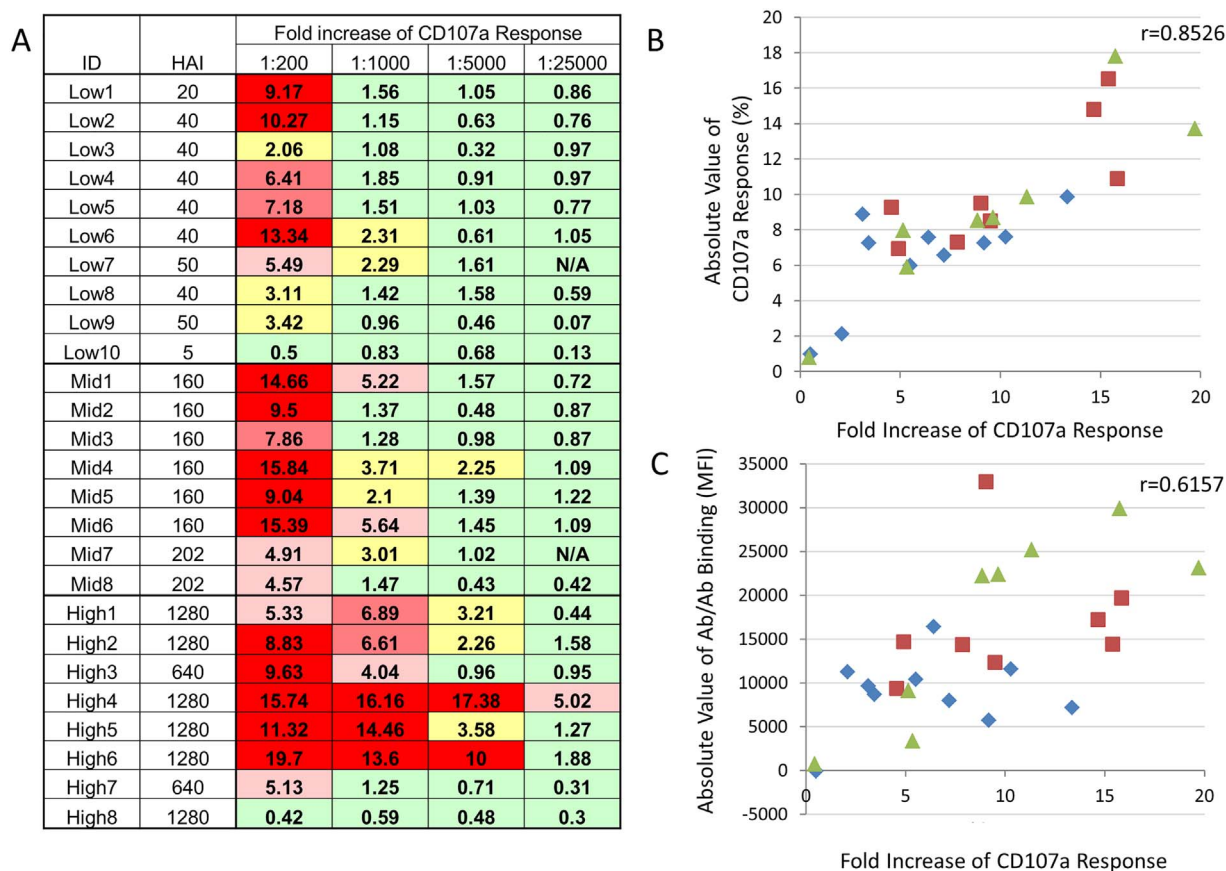
Using a Tc bovine IVIG sample (HAI 1280) we determined

opsonization of infected and uninfected Raji cells in our assay (Fig. 6A). NK cell CD107a expression for infected and uninfected target cells was determined over dilution of IVIG (Fig. 6B). At 1:200 dilution Tc bovine IVIG demonstrated just under 10% NK cell degranulation when cultured with infected target cells. Using this information, the NK cell degranulation titer for Tc bovine IVIG was determined to be 1:5000 (Table 1). The titer of Abs capable of stimulating NK cell activation was assessed by determining the fold increase of the NK cell response (Fig. 6C) and the absolute value of the NK cell response (Fig. 6D).

### 3.6. Correlation between HAI titer and NK cell degranulation titer

Using titer information for the human plasma sample set and the Tc bovine IVIG we conducted a Spearman correlation analysis and determined that a positive relationship exists between HAI titer and NK cell degranulation titer (Fig. 7). Taken together, this data suggests that a positive correlation exists between HAI titers and sustained NK cell





**Fig. 5.** NK cell degranulation assay using human plasma analysis. (A) Heat map analysis of the average fold increase of the CD107a NK cell response for infected cells compared to matched uninfected controls at each dilution of plasma. Green is for values  $< 2$ ; yellow is for values  $\geq 2$ ; pink is for values  $\geq 4$ ; light red is for values  $\geq 6$ ; and dark red is for values  $\geq 8$ . N/A = not available as the value was zero or negative. (B–C) Using 1:200 dilution of plasma an analysis of the NK cell degranulation assay was performed for all human plasma samples. Green triangles, high HAI titer; red squares, middle HAI titer; and blue diamonds, low HAI titer. (B) The absolute value of the CD107a NK cell response, expressed as the percentage of NK cells expressing CD107a for infected cells minus the percentage for uninfected cells, plotted on the y-axis and the fold increase of the CD107a NK cell response for infected cells compared to uninfected cells plotted on the x-axis. Statistical results from a non-parametric Pearson correlation analysis (two-tailed) demonstrated a positive correlation. (C) The absolute value of mean fluorescent intensity (MFI) of Ag/Ab binding, expressed as the MFI for infected cells minus the MFI for uninfected cells, plotted on the y-axis and the fold increase of the CD107a NK cell response for infected cells compared to uninfected cells plotted on the x-axis. Statistical results from a non-parametric Pearson correlation analysis (two-tailed) demonstrated a positive correlation. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

activation, although NK cell activation can be detected in plasma samples with HAI titers below 40 (particularly at low plasma dilutions) and varied amongst samples with high HAI titers.

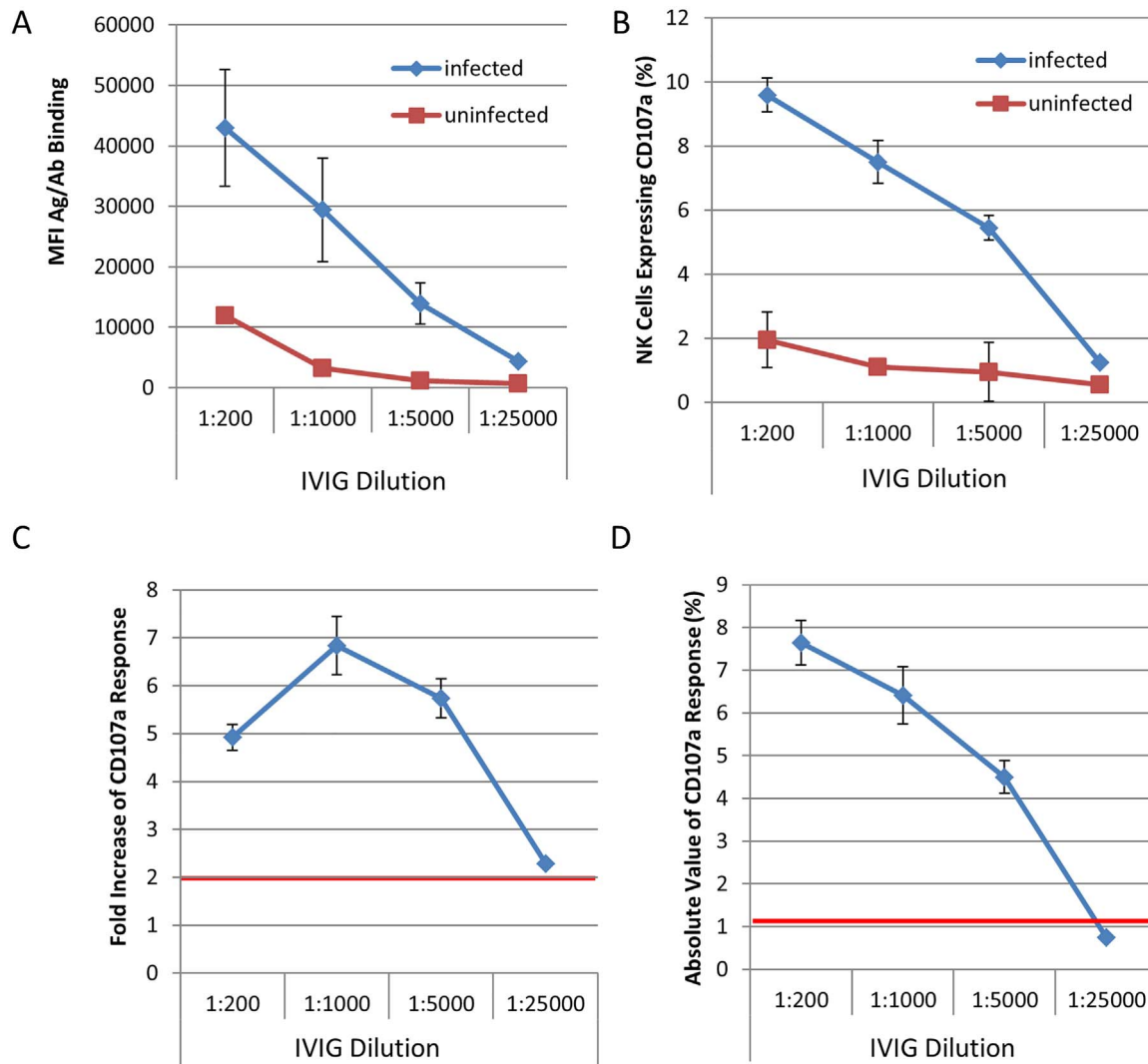
#### 4. Discussion

In this report, we demonstrate a non-radioactive assay for determining concentrations of ADCC NK cell activating antibodies in human serum or plasma by their ability to activate NK cells as demonstrated by degranulation (CD107a expression). We further support that the titer of HA hemagglutination antibodies positively correlates with NK cell activation in our sample set, although NK activation varied widely amongst low, middle, and high HAI titer plasma samples. Furthermore, we report that influenza-vaccinated Tc bovine IVIG was determined to have sustained antibody titers capable of eliciting NK cell degranulation.

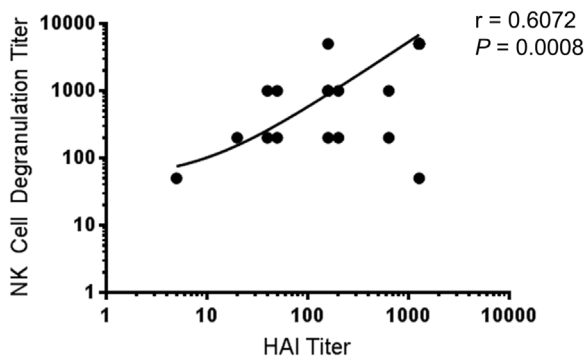
Titers of hemagglutination antibodies against the globular head of the HA receptor of influenza viruses is a current correlate of protection from influenza virus infection. Generally, a titer of 1:40 is considered protective (Co et al., 2014; Baz et al., 2013). However, this varies widely amongst individuals. Children, in particular, usually have experienced less exposure, and tend to require higher titers of around 1:110 to incur a 50% protective rate (Black et al., 2011). Protective titers can vary even among individuals in similar age ranges (Co et al., 2014). The HAI assay, which screens for hemagglutination inhibition, is

the current means to predict protection. However, this metric has its limitations. In addition to the aforementioned unpredictable and varying range of titers required to incur immunity among individuals, HAI assays determine levels of hemagglutination against the HA globular head alone. The activities of neutralizing and non-neutralizing antibodies against the HA stalk, or other viral proteins such as the ion channel matrix protein, nucleoprotein or neuraminidase are not detected by this assay. Routine evaluation of vaccine-induced antibody responses and determination of protective titers will potentially benefit from utilizing additional influenza assays such as NI, MN, and ADCC assays. The MN assay, for instance, has demonstrated utility for evaluating influenza serostatus of individuals and evaluating protective titers against seasonal influenza (Verschoor et al., 2015). Importantly, other immune responses, such as ADCC, have been established as vital in providing protection against influenza viruses (Greenberg et al., 1979; Jegaskanda et al., 2013a, 2016, 2014a,b, 2013b; Co et al., 2014; Jegaskanda et al., 2013d; Srivastava et al., 2013; DiLillo et al., 2014; Simhadri et al., 2015).

We have demonstrated an NK cell degranulation assay, as a metric for ADCC activity, utilizing influenza-infected Raji cells as targets. Consistent with previous reports utilizing Raji cells as targets for ADCC activity, we have found Raji cells to have high percentage of infection by influenza (Srivastava et al., 2013). We have shown that Raji cells are infected by H1N1pdm09 and express HA antigens at a high rate ( $\sim 62.3\%$ ). Opsonization results comparing infected and uninfected



**Fig. 6.** Influenza-vaccinated transchromosomal bovine IVIG therapy for influenza demonstrates opsonization and NK cell degranulation using this assay. (A) Results for mean fluorescent intensity (MFI) of Ag/Ab opsonization over IVIG dilution for uninfected and infected cells. (B) Results for the percentage of NK cells expressing CD107a over IVIG dilution for uninfected and infected cells. (C) The fold increase of the CD107a NK cell response for infected cells compared to uninfected cells. Red line indicates cut-off of two-fold increase. (D) The absolute value of the CD107a NK cell response, expressed as the percentage of NK cells expressing CD107a for infected cells minus the percentage for uninfected cells. Red line indicates cut-off of one. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)



**Fig. 7.** Correlation of HAI titer and NK cell degranulation titer for human plasma set and the transchromosomal bovine IVIG therapeutic. NK cell titer is assessed as the highest plasma dilution with an absolute value for CD107a expression by NK cell greater than one and a fold-change of CD107a expression by NK cells compared to uninfected cells greater than two. NK cell titer is compared to hemagglutination inhibition (HAI) titer. Correlation coefficient,  $r$ , equals 0.6072 and  $p$  value equals 0.0008 indicating a positive correlation. All 27 samples are plotted (several samples are overlapping). For Low10 and High8 NK cell degranulation titer was set at 50. Statistical results are from a non-parametric Spearman correlation analysis (two-tailed).

cells demonstrate expression of viral antigens on the cell surface of infected cells. It should be noted that Raji cells are an Epstein-Barr virus-transformed human cell line of hematopoietic origin and are not important natural targets of influenza virus. We utilized Raji cells, instead of A549 cells, which have previously been used for influenza ADCC assays, as they are non-adherent and are convenient for high-throughput flow-based assays. A commercially available non-radioactive ADCC reporter assay has been recently developed by Promega (ADCC Reporter Bioassay Core Kit) (Surowy et al., 2012), which has utilized Jurkat effector cells and Raji target cells, further demonstrating the utility of Raji cells for ADCC assays.

The assay described in this report is relevant as we explore NK cell degranulation (CD107a expression) as the result of binding with opsonized antigen on the surface of infected target cells. Previously, NK cell activity/ADCC activity has been detected using chromium-51 ( $^{51}\text{Cr}$ ) labeling (Greenberg et al., 1979; Co et al., 2014) or by sera/plasma-treating ELISA plates coated with purified viral proteins (Jegaskanda et al., 2013a, 2016, 2014a,b, 2013b; Vanderven et al., 2016; Zhong et al., 2016; Florek et al., 2014; Jegaskanda et al., 2013d), including the use of Fc $\gamma$ R dimer-binding assays (Kristensen et al., 2016; Wines et al., 2016). Major advantages the method described herein has over  $^{51}\text{Cr}$  labeling includes the removal of radioactive material and increased

sensitivity as  $^{51}\text{Cr}$  assays often demonstrate low sensitivity and spontaneous release of the isotope from non-lysed target cells (Zaritskaya et al., 2010). ELISA-based approaches lend themselves well to high-throughput assays. While the method of coating ELISA plates with purified antigen also removes the radioactivity hazard, it also has limitations. The plate-coating method is an excellent means to select for specific antibodies against a given antigen in serum or plasma; however, it removes effector cell interaction with opsonized antigen on an infected cell. Our method returns this cell to cell interaction using Raji cells as the target. In addition, other components in plasma, as well as on the cell surface, may affect binding affinity between the Fc region and the receptors on effector cells – variables that are removed altogether when antibodies are purified and/or presented with purified antigen alone (Vandervan et al., 2016; Srivastava et al., 2013; Wren et al., 2013). NK cell activation is controlled by a balance of inhibitory and activating signals (Moretta et al., 2001), and a further advantage of the method described herein is that it could allow for future studies that more deeply assess this NK cell activation/inhibition balance in the setting of influenza-infected target cells and ADCC. Our use of a target cell provides a sound model for assessing the process of ADCC priming in the context of infection with influenza. Furthermore, this method is consistent with reproducibility demonstrated for both opsonization and CD107a degranulation of effector cells (Fig. 3). Future studies will be needed to determine how well the Raji target cell assay described herein correlates with ELISA-based, including Fc $\gamma$ R dimer-binding, assays.

Using our plasma set we demonstrated a positive correlation between NK cell degranulation and HAI titer. However, it should be noted that this correlation was conducted with a relatively small set of samples, and that the relationship between HAI titer and NK cell degranulation titer is more complicated than the demonstrated correlation herein presented. In our set, plasma with higher HAI titer tended to demonstrate higher ability to sustain NK cell activity/NK degranulation titer. However, NK cell activation was observed in plasma with HAI titers lower than the suggested protective titer of HAI 40. In addition, NK cell activation varied in persons with high HAI titers with endpoint titers ranging from < 1:200 to 1:25,000. This is consistent with what has been previously reported in regards to a weak correlation between HAI and ADCC antibody titers (Zhong et al., 2016; Co et al., 2014), in addition to what we have observed over multiple experiments. Of particular note, Plasma1 (HAI 1280) and Plasma2 (HAI 640) had vastly different abilities to stimulate NK cell degranulation. Despite having a higher HAI titer than Plasma2, Plasma1 was a poorer inducer of ADCC. This suggests that HAI titers do not strongly correlate with ADCC titers. Recent evidence suggests that HAI-inducing antibodies do not optimally induce ADCC activity (He et al., 2016; Leon et al., 2016). There have also been reports indicating the presence of cross-reactive ADCC-mediating Ab titers in influenza-seropositive individuals without detectable NAb titers (Jegaskanda et al., 2013a; Jegaskanda et al., 2013c; Terajima et al., 2015). In aggregate these reports suggests that HAI, a surrogate for NAb titer, does not in all cases correlate with ADCC-mediating Ab titer, and HAI-inducing antibodies may in fact block the induction of ADCC by other antibodies (He et al., 2016). The correlation we determined between HAI titer and NK cell degranulation titer may be attributed to the overall higher titers of influenza-specific antibodies present in the high HAI samples compared to the low HAI samples. Finally, we have observed a mild correlation between Ag/Ab binding intensity to the surface of infected cells and the NK cell degranulation activity. This suggests that for serum/plasma with or without a protective HAI titer, the opsonization to infected cells is essential for the sample's ADCC activity.

Pandemic 2009 influenza A(H1N1) has demonstrated mortality for critically ill patients of up to 15% (Webb et al., 2009). As such development of alternative therapies, in addition to vaccination, is warranted for preparation for future pandemic strains of influenza. Influenza passive immunotherapy treatments include use of convalescent plasma,

hyperimmune immunoglobulins, or monoclonal antibody therapies. Convalescent patient blood products have demonstrated utility in reducing mortality associated with the 1918 Spanish influenza pandemic (Luke et al., 2006) and for treatment of H5N1 influenza (Zhou et al., 2007). In response to the rapid need to generate human antibodies for passive immunotherapy against pandemic strains of influenza monoclonal antibodies, convalescent plasma, and hyperimmune human-derived immunoglobulins are not ideal due to economic, logistic, and time factors. One potential source for rapidly producing fully human polyclonal antibodies for passive immunotherapy is the Tc bovine system. Vaccinated Tc bovines can produce convalescent plasma pools from which human IgG can be purified (150–600 g of Tc human IgG per animal per month). Using our assay and assessing an influenza-vaccinated Tc bovine IVIG therapeutic, NK cell degranulation was specific for virally-infected cells and demonstrated a high titer of NK degranulation antibodies (1:5000). Any additional benefits of influenza-vaccinated Tc bovine IVIG therapy compared to human IVIG therapeutics and bovine colostrum IgG therapeutics will need to be further assessed. However, influenza-vaccinated Tc bovine IVIG therapy deserves additional evaluation as a potential therapeutic for critically ill H1N1-infected patients. This therapy has the ability to initiate ADCC antibody responses, which is an important mechanism of protection in already infected patients.

The NK cell degranulation assay described here has relevance to global surveillance of influenza. ADCC-mediated responses to influenza can recognize a broader range of influenza strains than neutralizing antibodies, that generally target a more highly variable region of influenza proteins (Jegaskanda et al., 2014a). ADCC-mediating antibodies have been demonstrated to the 2009 novel swine-origin H1N1 influenza virus (A(H1N1)pdm09) prior to the 2009 pandemic in individuals greater than 45 years of age who did not have detectable HAI titers (Jegaskanda et al., 2013a). Furthermore, in studies where NAbs were not detected to A(H1N1)pdm09, non-NAbs titers correlated with protection from severe infection (O'Donnell et al., 2012; Weinfurter et al., 2011). This demonstrates the utility of assays for assessing ADCC for global influenza surveillance and population immunity. Use of ADCC assays may also drive a shift away from solely assessing the role of neutralizing or HAI-mediating Abs for vaccine efficacy and antibody passive immunotherapeutic assessment. ADCC-mediating Abs can target internal proteins of influenza virus (Vandervan et al., 2016). Targets of ADCC include: (i) HA (Zhong et al., 2016); (ii) the intracellular nucleoprotein antigen (Jegaskanda et al., 2017), that has been shown to be transiently expressed on virally infected cell surface (Yewdell et al., 1981); and (iii) neurominidase and matrix-2 proteins (Terajima et al., 2015). Vaccine induction or therapeutic production of antibodies that target influenza internal proteins via ADCC response may prove to be a new development for influenza vaccine/treatment design.

## 5. Conclusions

In conclusion, ADCC is a vital immune response in controlling influenza infections, and is a metric that should be assessed for broad spectrum influenza vaccines or influenza passive immunotherapy approaches. ADCC assessment is also a vital tool for conducting serosurveillance of influenza investigations in order to better assess the breadth of antibody responses to epidemic/pandemic and seasonal influenza. This could be especially relevant in assessing influenza exposure or protective immune response in non/low-neutralizing patient plasma. The NK cell activation assay demonstrated in this report could be of use: (i) for future research investigations into the role of ADCC against influenza, possibly predicting patient clinical response; (ii) as a metric of vaccine immune response; (iii) as a tool to assess ADCC activity in patient plasma globally; and (iv) as a tool to assess immunoglobulin products such as Tc bovine IVIG.



## Disclaimers

The views expressed in this article are those of the author and do not necessarily reflect the official policy or position of the Department of the Navy, Department of Defense, nor the US Government.

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## Funding

This work was supported by work unit number A1404, Global Emerging Infections Surveillance and Response System.

## Conflicts of interest

Authors declare no conflicts of interest.

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