

Abstract

VON HOLLE, TARRA ALAINA. Influenza Receptor Binding Site Antibody Induction in Rhesus Macaques. (Under the direction of Scott Laster).

Despite annual updating of vaccine antigens, influenza is able to evade host immune responses and cause worldwide seasonal epidemics. The influenza virus demonstrates antigenic drift-gradual accumulation of mutations that allow the virus to escape immunological recognition. Current vaccination strategies do not focus immune responses on conserved regions, resulting in the need for annual vaccine updates. A "universal vaccine" strategy that can elicit broadly protective antibodies against influenza is needed. One such strategy focuses on eliciting antibodies against the surface glycoprotein hemagglutinin (HA), specifically against the HA head region. CH65, is a receptor binding site (RBS) HA head antibody found to mimic the contacts between HA and the sialic acid cell surface receptor for influenza. While CH65 was isolated from a vaccinated human, determining whether this class of antibodies can be elicited in model organisms is a crucial next step in vaccine development because it will establish a model system for testing future vaccine candidates. We studied rhesus macaques immunized with purified recombinant HA (H1 A/Solomon Islands/03/2006), using PBMCs for single cell sorting, and monoclonal antibody (mAb) isolation. We screened rhesus plasma and isolated mAbs using binding and blocking ELISA assays along with neutralization assays. Several isolated antibodies had characteristics similar to CH65 including neutralization and cross-blocking. Further studies are needed to evaluate the exact structural basis of CH65 blocking by these mAbs. The isolation of antibodies with CH65 blocking shows that rhesus macaques are a good model organism to use for influenza vaccine development.

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Influenza Receptor Binding Site Antibody Induction in Rhesus Macaques

by
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Dedication

This work is dedicated to my husband, Neil, who has been very supportive throughout this process; and to the rest of my family members who have always provided encouragement and support for all of my endeavors.

Biography

Tarra Alaina Von Holle (Buff) was born on September 27, 1989 and is the daughter of Todd Allen Buff and Teresa Carter Buff. She grew up in Maiden, North Carolina and attended Maiden High School. Tarra then attended North Carolina State University and graduated with a Bachelor of Science degree in Biological Sciences with minors in Genetics, and Microbiology. Upon graduation in 2011, she accepted a research technician position at Duke Human Vaccine Institute (DHVI) in Durham, North Carolina. Under the direction of Dr. Tony Moody she began working on studying the development of a vaccine for HIV. While performing this research she decided that she wanted to expand her knowledge in immunology as well as increase her research skills. In 2013, she was accepted into the Master of Science degree program in Comparative Biomedical Science at North Carolina State University where she is working to complete the requirements for her degree.

Acknowledgments

I would like to thank Dr. Tony Moody for allowing me to pursue this endeavor, and for the constant support and encouragement. I would also like to thank the members of my committee Drs. Scott Laster, Jonathan Fogle, and Sam Jones, for their support throughout my graduate experience. This work would not have been possible without the help of Kan Luo, Lawrence Armand, Rob Parks, Andrew Foulger, and Rachel Reed for providing technical assistance; and Thad Gurley for constant support and advice.

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List of Abbreviations

ADCC- Antibody Dependent Cellular Cytotoxicity

AF- Alexa Fluor

APC- allophycocyanin

bNAb- Broadly Neutralizing Antibody

BV- Brilliant Violet

cDNA- complementary DNA

CDR-H3- heavy-chain complementarity-determining region 3

dH₂O- distilled water

DMSO- Dimethyl Sulfoxide

DNA- deoxyribonucleic acid

ELISA- Enzyme Linked Immunosorbant Assay

FBS- Fetal Bovine Serum

FITC- fluorescein isothiocyanate

FL- Full length

HA - Hemagglutinin

HCL- hydrochloric acid

HEK- Human Embryonic Kidney

HIV- Human Immunodeficiency Virus

HRP- Horseradish Peroxidase

Ig- Immunoglobulin

IM- Intramuscular

LAIV- Live attenuated influenza vaccine

mAb- Monoclonal antibody

NP- Nucleoprotein (NP)

PBMCs-Peripheral Blood Mononuclear Cells

PBS- Phosphate-Buffered Saline

PCR- Polymerase Chain Reaction

PE- phycoerythrin

PerCP- peridinin-chlorophyll protein

RBS- Receptor binding site

RNA - Ribonucleic Acid

RT- reverse transcription

SIV- Simian Immunodeficiency Virus

TIV- Trivalent Inactivated Vaccine

TMB- Tetramethylbenzidine

TriH- Trimeric head

V2- variable loop 2

V_H- heavy chain V region

V_L- Light chain V region

WHO- World Health Organization

Chapter 1: Introduction

Seasonal influenza is responsible for an estimated 3 to 5 million cases of severe illness, and about 250,000 to 500,000 deaths worldwide per year (WHO). Influenza strains in the vaccine are chosen based on analysis of circulating strains in the tropics and southern hemisphere during the preceding year (WHO). Updated vaccine components are needed yearly due to the viral error prone replication mechanism in influenza viruses. Influenza has a segmented RNA genome encoding for its own RNA polymerase, which lacks an editing feature allowing errors in the new RNA genomes to occur (Air 2015). These errors, along with immunogenic pressure from infected humans lead to mutations in viral components causing antigenic drift and eventually viral escape over time.

Vaccines against influenza A and B viruses were first invented in the 1940s, and consisted of whole-virus inactivated vaccines grown in embryonated chicken eggs (Krammer 2015, Francis 1945, Salk 1945). Soon after these vaccines were discovered it became evident that influenza was caused by multiple strains, and antigenic drift compromised immune responses resulting in brief immunity to circulating strains, requiring reformulations of the vaccines every few years (Kilbourne 2011, Salk 1949, Allison 1977). The 1968 pandemic led to the development of the trivalent inactivated vaccines (TIVs), against influenza viruses and led to a reduction in illness in immunized individuals (Krammer 2015, Kilbourne 2011).

Current multivalent influenza vaccines contain either inactivated influenza antigens or live attenuated viruses (Soema 2015). Inactivated vaccines usually consist of two different types split viral constructs and subunit vaccines. Split viral construct vaccines are not adjuvanted and contain most or all of the viral proteins including hemagglutinin (HA),

neuraminidase (NA), matrix proteins, and some viral RNA. This type of vaccination can elicit immune responses against multiple viral components. In contrast, subunit vaccines only contain one or a few proteins, usually HA, and are generally combined with an adjuvant to enhance immune responses (Soema 2015). Alternatively, live attenuated vaccines (LAIV) are modified by passage in culture to adapt the virus to grow better in the cold and reduce pathogenicity of the virus at body temperatures. These vaccines are administered in the nasal passage, and mimic the natural route of infection allowing an increased localized mucosal immune response at the site of infection but without extension to the lower respiratory tract (Barria 2013). All of these vaccine types preferentially elicit immune responses to viral strains included in the vaccine.

Vaccine efficacy varies from season to season but recent studies have shown that vaccines can reduce the risk of flu illness by about 50-60% among the overall population when there is a good match between the circulating strains and those within the vaccine (CDC.gov). Mismatches between the vaccine strains and the circulating strains occur occasionally and are usually associated with lower vaccine efficacy (Krammer 2015, de Jong 2000). Vaccine effectiveness can vary based on many factors including but not limited to age, and health status. Recently, preliminary data on the effectiveness of the LAIV in children from 2 to 17 years old during the 2015-2016 season showed that the vaccine had a vaccine efficacy of 3%, indicating no benefit from vaccination, whereas inactivated vaccines had an estimated 63% vaccine efficacy (CDC.gov). This data shows the failure of the LAIV vaccine to protect against influenza infections, indicating that LAIV should not be used for the 2016-2017 influenza season (CDC.gov). In 2015, Helanterä et al. published infection

rates of 12% for vaccinated individuals, and 83% for unvaccinated individuals within an kidney transplant ward with an H1N1 outbreak, indicating that influenza vaccination can provide protection against infection for immunocompromised individuals, showing the need for vaccination in this population (Helanterä 2015). Despite annual vaccinations pandemic strains, such as the H1N1 strain that caused the 2009 pandemic, are still in existence. This viral strain was shown to have undergone antigenic shift, having originated in animals causing seasonal vaccines to be ineffective because of a lack of cross-protection (WHO). New vaccination strategies need to be developed to focus on creating a vaccine that has broad activity against multiple strains, and can withstand the yearly antigenic drift.

Vaccination strategies for universal coverage of influenza strains have focused on different influenza proteins including the M2 surface protein ion channel, and HA glycoprotein (Lee 2015, Schmidt 2015, Whittle 2011). Several strategies are focused on generating broadly neutralizing antibodies (bNAbs) against invariant epitopes, such as those present at the receptor binding site (RBS) on the surface of the HA "head", and at a conserved hydrophobic patch on the HA "stem" (Schmidt 2015, Knossow 2002, Ekiert 2009, Sui 2009, Corti 2011). It is hoped that these types of neutralizing antibodies will bind to a wide array of influenza subtypes and be able to prevent disease by blocking viral attachment to a cell, inhibiting the first step in viral infection, preventing conformational changes necessary for the virus life cycle, or by inhibiting the fusion function of HA (Air 2015).

The surface glycoprotein HA is trimeric and binds the viral receptor, sialic acid, promoting fusion that allows viral entry into the cells (Whittle 2011). HA is composed of a stalk that has relative sequence conservation between strains and subtypes and an

immunodominant globular head that can vary from strain to strain (DiLillo 2016). Antibodies can be generated against both of these regions; those against the stalk domain can often bind multiple strains and subtypes of HA, but those against the head region usually can react with only a single HA or highly similar HA strains (DiLillo 2016). However, tight packing of HA and neuraminidase on the surface of influenza virions reduces access of stalk-directed antibodies to those regions of HA on intact virions, resulting in poor neutralization and hemagglutination inhibition in standard assays. Recent work has shown that stalk-directed antibodies mediate protection by interacting with Fc receptors on effector cells (DiLillo 2016). Because of their ability to bind directly to HA on virions, HA head-directed antibodies are more easily detected in standard assays and can interfere with virion binding to target cells.

Isolating antibodies to either region capable of broad reactivity against influenza has been a focus of research in recent years, and has resulted in the identification of two bNAb epitopes. CR6261 is a stalk-directed mAb isolated using a phage displayed antibody library selected on recombinant H5 HA; this antibody neutralized various influenza subtypes (H1, H2, H5, H6, H8, and H9), and provided protection from lethal challenge with H1N1 and H5N1 in mice (Ekiert 2009). Vaccine strategies designed to elicit CR6261-like bNAbs are being tested in a phase 2 clinical trial (clinicaltrials.gov). In contrast, CH65 is an HA head bNAb, isolated from an individual who received the 2007 trivalent vaccine, using single plasma cell sorting (Moody 2011, Whittle 2011). CH65 has a 19-amino acid residue heavy-chain complementarity-determining region 3 (CDR-H3) and inserts into the receptor binding pocket, mimicking the interactions that are made by sialic acid (Whittle 2011). This mimicry

stems from key interactions from a critical dipeptide at the tip of the CDR-H3 (Schmidt 2015). The binding of CH65 will prevent influenza HA binding to target cells by blocking sialic acid, preventing cellular infection. A similar type of RBS mimicry has been observed for other viruses such as in Human Immunodeficiency Virus (HIV) such as with the VRC01 antibody. The VRC01 class of antibodies bind HIV envelope at the CD4 binding site and neutralizes ~ 90% of total HIV strains, and was demonstrated to give complete protection in several animal challenge studies (Lynch 2015). CH65 is the prototype of a class of antibodies against influenza that are analogous to VRC01 suggesting that strategies that can elicit CH65 like bNAbs are good targets for future vaccine design.

In 2014, it was shown that HA stem and HA head antibodies provided protection via different mechanisms. HA stem antibodies were originally thought to provide protection by preventing fusion of the viral membranes, but more recently it was published that these antibodies are also important for binding HAs on the surface of infected cells initiating antibody dependent cellular cytotoxicity (ADCC) through binding the Fc receptors present on effector cells (Skehel 2000, El Bakkouri 2011, Jegerlehner 2004). DiLillo et al. published that the disruption of fusion by the anti-stalk antibodies may result in inefficient processing *in vivo*, mandating that need for FcγR involvement to provide effective protection *in vivo* (DiLillo 2014). Alternatively, HA head antibodies were not shown to interact with FcγR to initiate ADCC, although both HA antibodies have similar binding characteristics to the cell surface HA (DiLillo 2014). The lack of binding to FcγRs by HA antibodies was not simply due to steric hinderance or proximity, indicating changes in the Fc that affect its ability to engage FcγRs (DiLillo 2014). Further examination of antibodies against the HA head region

are needed to determine if all anti-head antibodies are able to neutralize influenza independently of Fc-FcγR interactions (DiLillo 2014).

Rhesus macaques have been useful models for studying a variety of viral infections including retroviruses, such as simian immunodeficiency virus (SIV) and HIV (Wiehe 2014). Rhesus macaques share ~ 93% genome homology with humans, and derive from a common ancestor that diverged from the evolutionary branch from which humans arose approximately 25 million years ago (Wiehe 2014, Gibbs 2007). Studies have shown that macaques are capable of producing antibodies with similar characteristics to those from humans. Kevin Wiehe et al. showed that variable loop 2 (V2) antibodies isolated from rhesus macaques immunized with the same regimen as in the human RV144 trial shared orthologous light chain genes involved in V2 K169 recognition (Wiehe 2014). Non-human primates (NHPs) have been used to study highly pathogenic influenza viral infections such as those caused by avian H5N1 and the 1918 pandemic viruses (Bouvier 2010). NHPs were shown to be susceptible to influenza infection, though this infection is less severe generally resulting in leukopenia, decrease in neutrophil population, and were shown to develop neutralizing antibodies 8-10 days post infection (Bouvier 2010). Given this information we wanted to determine if CH65-like antibodies could be elicited after vaccination with HA influenza in rhesus macaques, and to determine if these antibodies are generated through the same or different pathways. These animals were immunized intramuscularly with recombinant HA derived from H1 A/Solomon Islands/03/2006 along with an oil in water adjuvant, MF59. The purpose of this was to determine if rhesus macaques are a good immunological model for studying influenza, and whether they can be used in human vaccine development.

Chapter 2: Materials and Methods

Study animals and specimen collection. Six rhesus macaques were immunized with a intramuscular (IM) injection containing H1 A/Solomon Islands/03/2006 (Protein Sciences, Meriden, CT) in MF59 (Ott 1995) adjuvant at week 0, 8, 12, 16, and 20. Blood was drawn at the time of immunization and at weeks 2, 10, 14, 18, and 22.

Plasma and PBMCs Isolation. Plasma and peripheral blood mononuclear cells (PBMCs) were isolated from the blood. Briefly, the blood was centrifuged and plasma was removed and frozen; PBMCs were isolated using density-gradient centrifugation with FicollPaque (GE Healthcare, Waukesha, WI). After isolation, PBMCs were suspended in 10% dimethyl sulfoxide (DMSO) (Sigma, St. Louis., MI) in heat inactivated Fetal Bovine Serum (FBS) (Invitrogen, Carlsbad, CA) and frozen. Cells were stored in liquid nitrogen until use.

Flow Cytometry. Frozen PBMCs were thawed and stained with an antibody panel including the following antibodies: CD3 peridinin-chlorophyll protein (PerCP)-Cy5.5 (SP34-2), CD16 phycoerythrin (PE)-Cy7 (3G8), CD20 fluorescein isothiocyanate (FITC) (L27), IgM PE-Cy5 (G20-127) (all were from BD Biosciences, San Jose, CA), CD27 allophycocyanin (APC)-Cy7 (O323), CD14 brilliant violet (BV) 570 (M5E2) (both from Biolegend, San Diego, CA), IgD PE (polyclonal) (Southern Biotech., Birmingham, AL), CD8 PE-texas red (3B5) (from Invitrogen, Carlsbad, CA). The influenza proteins used to prepare antigen-specific B cell reagents were obtained from the laboratory of Stephen Harrison at Harvard and consisted of both full length (FL) and trimeric (TriH) HA constructs: H1 A/California/04/2009, H1

A/Solomon Islands/03/2006, H1 A/Massachusetts/01/1990. The influenza proteins from different viral strains were biotinylated and conjugated to Alexa Fluor (AF) 647, BV421, or AF488 fluochromes; the influenza reagents were included in the antibody panels to directly identify and isolate influenza specific cells. After staining with the antibody panel, cells were stained with Aqua Vital dye (Life Technologies, Grand Island, NY). Cells were index sorted on a BD FACSAria II (BD Biosciences, San Jose, CA) into 96 well PCR plates. Sorted cells were from the double positive or triple positive influenza specific live memory B cell population (Aqua Vital dye-, CD14 and CD16-, CD20 +, IgD-, influenza specific antigen pattern as described in figures below). Data was analyzed using FlowJo version 9.8.5 (Treestar, Ashland OR).

PCR and Sequencing. Single cells were sorted into 96 well PCR plates containing reverse transcription (RT) buffer (5 μ L of 5' first-strand cDNA buffer, 0.5 μ L of RNaseOUT (Invitrogen, Carlsbad, CA), 1.25 μ L of dithiothreitol, 0.0625 μ L Igepal CA-630 (Sigma, St. Louis, MO), 13.25 μ L of distilled H₂O (dH₂O; Invitrogen, Carlsbad, CA) (Moody 2015). The heavy and light genes were amplified using reverse transcription followed by nested PCR as described in (Liao 2009, Tiller 2008, Wardermann 2003, Wiehe 2014). PCR products of Ig V_H and V_L genes were purified by using a modified protocol as described (Liao 2011), that was optimized for amplifying rhesus antibodies (R.Z. unpublished data). Briefly, reverse transcription (RT) was performed using Superscript III reverse transcriptase (Invitrogen, Carlsbad, CA) using constant region primers for IgG, IgM, IgA, IgD, IgE, Ig κ , and Ig λ (Moody 2012, Wiehe 2014). Separate RT reactions were used to amplify cDNA using IgA1,

IgA2, IgD, IgG, IgM or Ig κ , and Ig λ with using either IgH, Ig κ , or Ig λ variable region primers using two rounds of PCR (Wiehe 2014). Sequence bases were determined using Phred (Ewing 1998, 1998), both forward and reverse strands were assembled based on an algorithm described in Kepler et al. 2010.

Clonal lineage Determination. Antibody sequences were grouped based on variable and joining region genes, and the third complementarity determining regions of the heavy (CDR H3) chains. To be classified as part of a clonal lineage antibodies had to have matching variable and joining region genes, as well as CDR-H3 loop lengths, and more than 70% homology in the CDR-H3 nucleotide sequences (Moody 2012). Clonal lineages were only identified if heavy and light chains satisfied all classifications criteria.

Small Scale Transfections and IgG purification. Antibody pairs from PCR, purification and sequencing were selected for small scale transfections as described (Liao 2009, 2011). Briefly, PCR products from gene isolation were used to construct linear transfection cassettes by overlapping PCR were transfected into a six well tissue culture plate (Becton Dickinson, Franklin Lakes, NJ) containing nearly confluent 293T human embryonic kidney cells (American Type Culture Collection, Manassas, VA). These expression cassettes were added to plated 293T cells (in 6 well culture plates), along with Buffer EC, enhancer, and Effectene (all from Qiagen, Hilden, Germany). Cells were then incubated for 72 - 96 hours at 37°C and 5% CO₂. Supernatants were gathered, centrifuged, and concentrated using 6mL concentrator tubes (Corning, Corning, NY). Concentrated supernatants were then purified using protein G

HP MultiTrap plates (GE Healthcare, Little Chalfont, United Kingdom) following the manufacturer's protocol.

Enzyme Linked Immunosorbant Assay (ELISA). Binding to HAs from various influenza strains (A/Wisconsin/67/05, A/Brisbane/59/07, A/Brisbane/10/07, A/Solomon Islands/03/06, A/Johannesburg/33/1994, A/Vietnam/1203/2004, A/California/04/09, A/Indonesia/05/2009 all HAs used in ELISA were from Protein Sciences, Meriden, CT) was assessed using an enzyme linked immunosorbant assay (ELISA) in a 384-well plate format. Plates were coated with the above listed HAs at a final concentration of 2 μ g/mL diluted in 0.1M NaHCO₃ (Sigma, St. Louis, MO) and incubated at 4°C overnight. After the overnight incubation the plates were washed using a 384-well plate washer (BioTek, Winooski, VT) with SuperWash (1X PBS-0.1% Tween20) and blocked for 1 hour using SuperBlock (40g Whey Protein, 150mL Goat Serum, 5 mL Tween20, 0.5g Sodium Azide, 40mL of 25X PBS, and bring up to 1L with DI water). After blocking the plates were washed and 10 μ L of diluted plasma (1:30 dilution in superblock), or 10 μ L of the transfection supernatants and positive (Ab2210), and negative (palivizumab) (MedImmune, LLC, Gaithersburg, MD) antibodies were directly added to the plates for 1 - 1.5 hours. Plates were washed and 10 μ L of horseradish peroxidase (HRP) conjugated secondary antibody (Jackson ImmunoResearch, West Grove, PA) diluted in Superblock (without sodium azide) at a 1:4000 dilution was added and incubated for 1 hour. The plates were washed and developed with 3,3',5,5'-Tetramethylbenzidine (TMB) substrate (KPL, Gaithersburg, MD), plates for 15 minutes. Development was stopped using 1% HCl

(Fisher Scientific, Pittsburgh, PA). Plates were read on a plate reader (Molecular Devices, Sunnyvale, CA) using 450nm wavelength.

ELISAs were used to determine IgG concentration of plasma and transfection supernatants. IgG concentration was determined using a similar protocol as stated above with some modifications. Goat anti-Rhesus (polyvalent IgG) antibody (Jackson ImmunoResearch, West Grove, PA) was diluted in 0.1M NaHCO₃ buffer at a 2 µg/mL final concentration to coat the 384 well ELISA plates. Plasma was diluted using 10 µL of a 1:5, 1:50, and 1:500 dilution, or 10 µL of diluted transfection supernatants. Positive and negative controls were 2F5, and palivizumab (MedImmune, LLC, Gaithersburg, MD) respectively. To determine IgG concentration within samples a standard curve of the 2F5 positive control was used.

Blocking ELISAs were used to determine the binding specificity of antibodies. The plates were coated overnight with A/Solomon Islands/03/2006. The washing, and blocking steps were the same as previously stated. The plasma was diluted at 1:30, or a starting concentration of 100µg/ml for monoclonal antibodies. The plasma and antibodies were diluted threefold over 11 wells. The diluted samples (10µL each) were added to the plates for 1 hour, including the positive and negative controls: CH65 and palivizumab (MedImmune, LLC, Gaithersburg, MD). The plates were washed and then 10µL/well of biotin conjugated CH65 was added to the plates and incubated for 1 hour. The plates were washed and 10 µL/well of Streptavidin (strep)-horseradish peroxidase (HRP) secondary diluted at 1:30000 (Thermo Scientific, Waltham, MA) was added to the plates and incubated for 1 hour. The

plates were washed and TMB substrate was added for 2 to 5 minutes until the optical density (OD) of the positive controls reached from 1 - 1.5. Development was stopped using 1% HCl and plates were read using 450nm.

Plasmids and Large Scale Transfections. Antibody DNA sequences were used in the construction and ordering of plasmids. Heavy and light chain sequences were synthesized and cloned into plasmids containing either human IgH, and IgK/L (Moody 2015), or rhesus IgG1/IgK/IgL constant regions (Zhang 2016). The large scale transfections use the ordered plasmids and 293T cells. The plasmids were combined with Expectifectamine (Life technologies, Carlsbad, CA) and added to the 5×10^6 - 7×10^6 293T cells to grow for 4 - 5 days in 37°C and 5% CO₂. After the 4 - 5 day incubation the cells were centrifuged and supernatants were concentrated using a Vivaflow 50 (Sartorius Stedium, US) concentrator and a Masterflex peristaltic pump and Easy Load II pump head (Cole Parmer, Vernon Hills, IL). Once the supernatants were concentrated they were purified using a protein A bead mixture, (Pierce, Fisher, Newark, DE) and purification columns (Biorad, Hercules, CA). Antibodies were concentrated using centrifugation in a Vivaspin Turbo 15 concentrator (Fisher, Waltham, MA) containing 1.5mL of Trizma HCl (Sigma, St. Louis, MO) performing a buffer exchange with 1X PBS (Gibco, Invitrogen, Carlsbad, CA) to reach a 1:400 dilution. The protein solution was filtered through a 0.22µm Millipore filter (Millipore, Billerica, MA) and centrifuged for 3000g at 4°C until all of the protein was filtered. Concentrations of the samples were then obtained by using a Nanodrop (Thermo Scientific, Waltham, MA). Proteins were stored at 4°C, or -80°C until use.

Neutralization Assays. Neutralization assays on plasma were performed as described in Moody et al. 2011. Neutralization assays on antibodies were performed using an adapted World Health Organization (WHO) ELISA based neutralization protocol. Briefly, antibodies were diluted two fold, and were incubated with dilutions of specific influenza viruses (A/Solomon Islands/03/2006, or A/Wisconsin/67/2005) at a 100 TCID₅₀/well in an ELISA plate for 1 hour at 37°C, Madin-Darby canine kidney (MDCK) cells were added to these wells at 1.5x10⁴ cells/well and incubated for 18 - 22 hours at 37°C. The plates and cells were washed and fixed (100mL 0.01 M PBS (pH7.2) (Invitrogen, Carlsbad, CA) using 400mL of acetone (Fisher scientific, Newark, DE). An anti-influenza A NP monoclonal antibody (United States Centers for Disease Control and Prevention, Atlanta, GA) was added at a 1:3000 concentration incubated at RT for 1 hour. The plate was washed and goat anti-mouse IgG HRP (Kirkegaard and Perry Laboratories Inc., Gaithersburg, MD) was diluted to 1:6000 and added and incubated for 1 hour at RT. The plates were washed and substrate (10mg *o*-phenylenediamine dihydrochloride (OPD) to each 20mL citrate buffer (Sigma, St. Louis, MO)) was added to each well. Plates were incubated for 5 - 10 minutes at RT and then stop solution (0.5N H₂SO₄) was added. The plates were read at 490nm.

Chapter 3: Results

Six rhesus macaques in this study received A/Solomon Islands/03/2006 in MF59 adjuvant at 5 different time points (Figure 1A). ELISA assays were used to determine specific binding of the plasma to various strains of influenza viruses. As shown in Figure 1B, plasma antibody bound multiple influenza A strains. The highest binding was shown against H1 influenza strains. These data demonstrate that immunizing rhesus macaques multiple times with an individual strain of influenza can generate antibodies that are capable of recognizing other influenza viruses. Plasma neutralization was the highest for the influenza strain the macaques were immunized with (A/Solomon Islands/03/2006), but the plasma showed significant neutralization against 8 other influenza strains compared to the pre-immunization control (Figure 1D). Figure 1C shows the CH65 blocking for these plasma samples. Multiple animals generated antibodies in their plasma that were capable of blocking CH65 binding, and these antibodies seemed to increase after multiple immunizations resulting in complete blocking by week 10. From these data two animals were selected that had high neutralization, binding, and blocking: 144-05 and 155-10 highlighted in blue and orange, respectively.

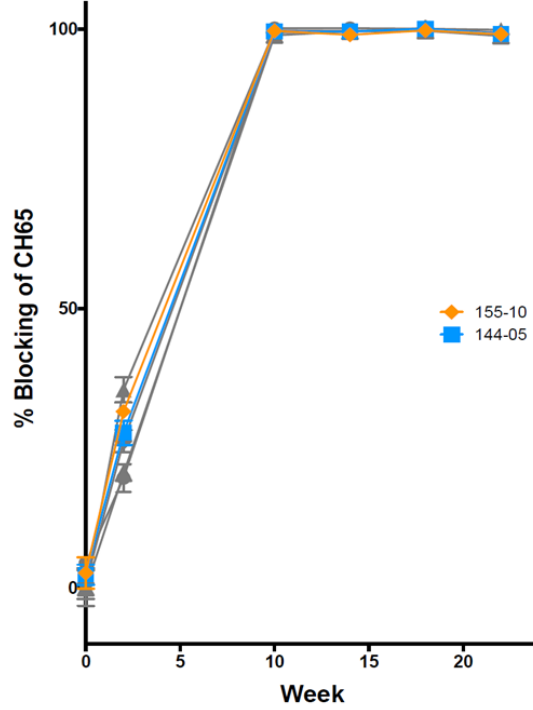
Figure 1: Influenza specific plasma screening. (A) Immunization sampling schedule for the 6 Rhesus Macaques; blood was processed for plasma and PBMC at the indicated time points. (B) ELISA binding of Post Bleed 3 (Week 14). Data is graphed using Log of Endpoint Titer, and the bars show the mean value of standard error of the mean (SEM). (C) Plasma CH65 blocking ELISA data for all time points. Positive and Negative control data not shown. Data is graphed using % blocking of CH65 calculated by the following equation: $100 - [(Sample\ OD / Average\ Total\ Binding) * 100]$. Average total binding calculated using negative control, palivizumab. (D) Plasma neutralization data Pre-immunized samples were only tested against the immunogenicity to the Solomon Islands viral strain, all other samples shown are from the post week 22 time point. Bars show mean and SEM. Colors indicate animals chosen for subsequent experiments.

A. Immunization Schedule in 9 Rhesus Macaques

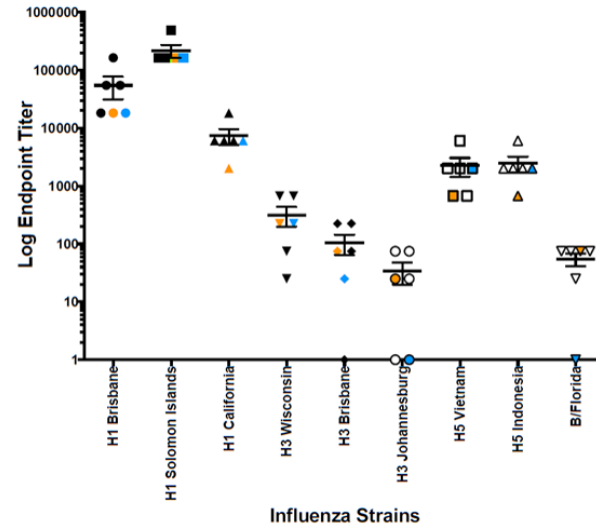
H1 A/Solomon Islands/03/2006 in MF59



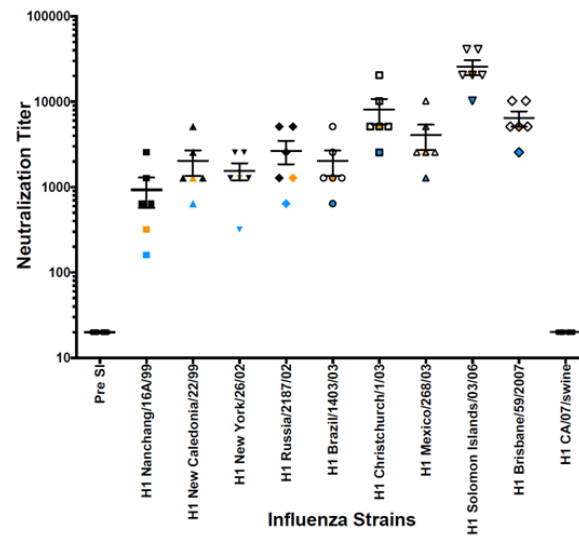
C.



B.

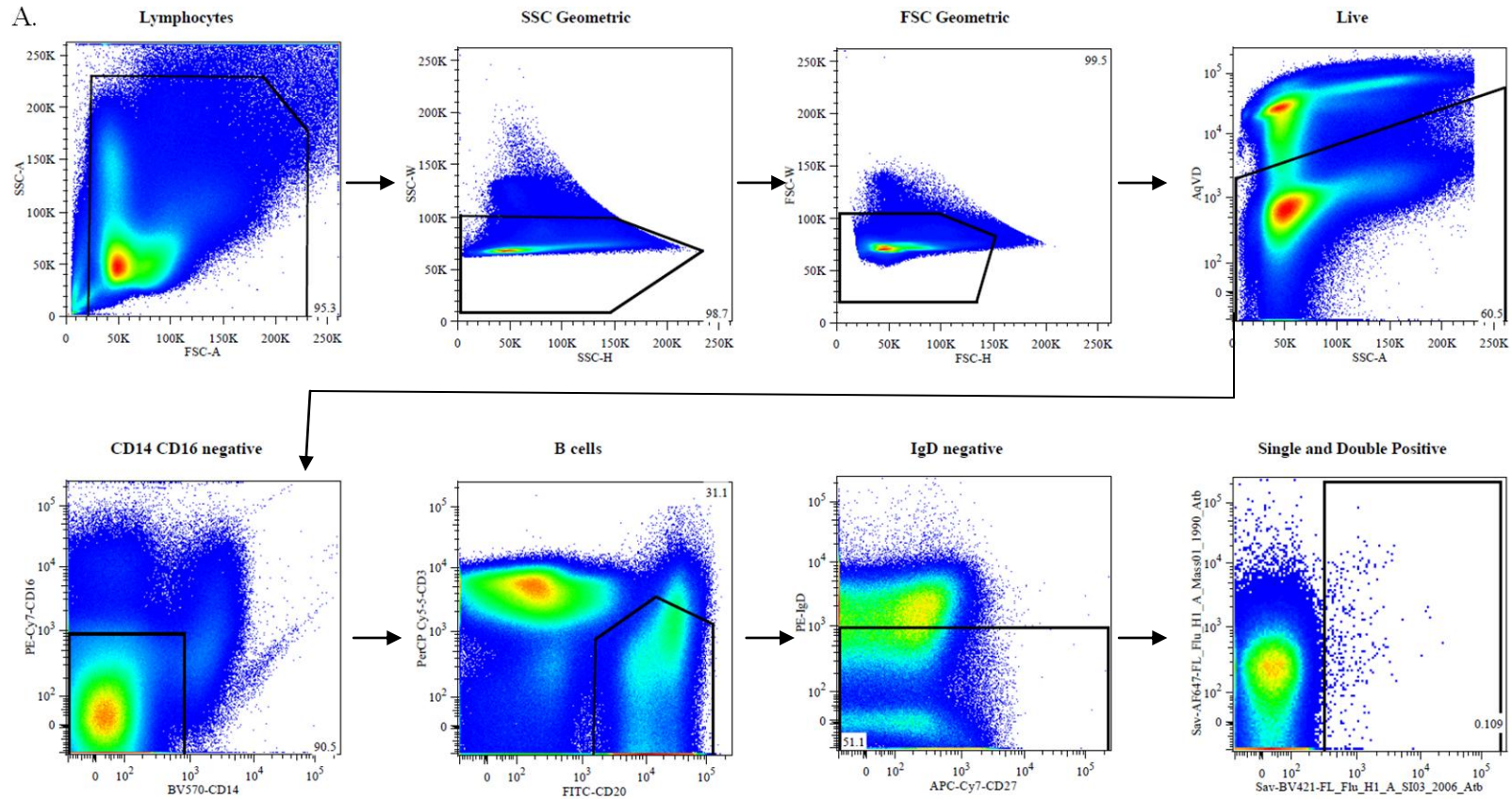


D.



Broadly neutralizing RBS antibodies isolated from a vaccinated human patient were reported in 2011 (Moody 2011, Whittle 2011). We used a similar strategy to determine if rhesus macaques immunized repeatedly with a single strain of influenza could generate similar broadly neutralizing antibodies. PBMCs from animal 155-10 at the week 22 time point were stained with an antibody panel including B and T cell markers along with various influenza specific reagents in order to identify and isolate influenza specific cells. Figure 2A shows the gating strategy used in this experiment. PBMCs from 155-10 were sorted using a single and double positive gate, specifically gating for cells specific for both A/Solomon Islands/03/2006 and A/Massachusetts/01/1990 (Figure 2A). Cells of interest were live, IgD negative B cells. Cells were sorted into 96 well plates containing RNA stabilization buffer and stored at - 80°C until use. Plates were then thawed and RT PCR was performed using a modified protocol reported by Liao et. al. in 2009 to use primers specific for rhesus macaques (Wiehe 2014). Figure 2B shows the numbers of: cells sorted, functional antibodies, and clonal lineages. From clonal lineage analysis we determined that there were 5 clonal lineages isolated from 155-10 (Figure 2B). These lineages from 155-10 had 2-7 consistent with the number of sorted cells from these experiments.

Figure 2: Flow cytometric gating analysis for 155-10. (A) Flow cytometry data for 155-10 shows the gating strategy used for sorting. Cells from the following populations: Lymphs, Singlets, Live, CD14 CD16 negative, B cells, IgD negative all, and the single and double positive A/Solomon Islands/03/2006 population. (B) Shows the conjugates used, number of sorted events, functional antibodies isolated and clonal lineage information.

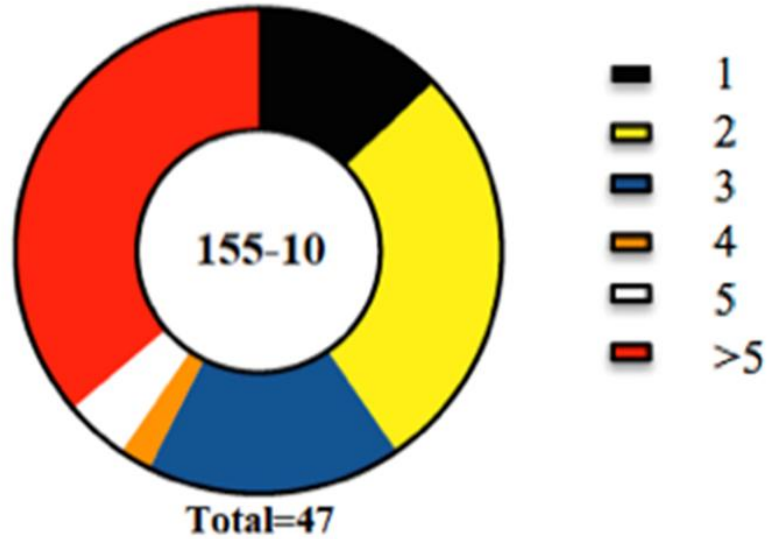


B.

MID	Conjugates used	Timepoint (Weeks)	# Sorted Events	# Functional Antibodies	# Clonal Lineages	# Members in Clonal Lineages
155-10	FL_Flu_H1_A_Mass01_1990 AF647 FL_Flu_H1_A_SI03_2006 BV421	22	232	62 (27%)	5	2 to 7

The binding of these antibodies was then assessed by ELISA plates coated with various influenza A strains including: A/Wisconsin/67/05, A/Brisbane/59/07, A/Brisbane/10/07, A/Solomon Islands/03/06, A/Johannesburg/33/1994, A/Vietnam/1203/2004, A/California/04/09, A/Indonesia/05/2009. Antibody supernatants generated from small scale transfections were used in these binding assays. Figure 3 shows the binding of these antibodies varied. Antibodies from 155-10 have binding to multiple strains of influenza ranging from 1 to more than 5 strains. To determine the specificity of the influenza strain binding, viruses were separated based on subtype (H1, H3, and H5) (Figure 3B). Influenza binding seemed to be highest for H1 only strains, but binding was also shown to H1+H5 strains, and for all strains present (Figure 3B). Showing that some of the antibodies isolated had high cross strain binding.

A. Counts of Reactive Strains



B. Counts of Subtype Reactivity

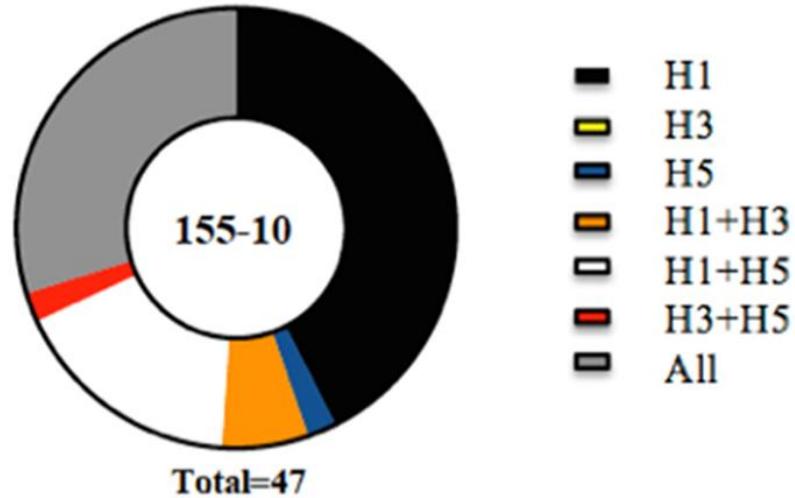


Figure 3: Influenza binding characteristics for antibodies isolated from 155-10.

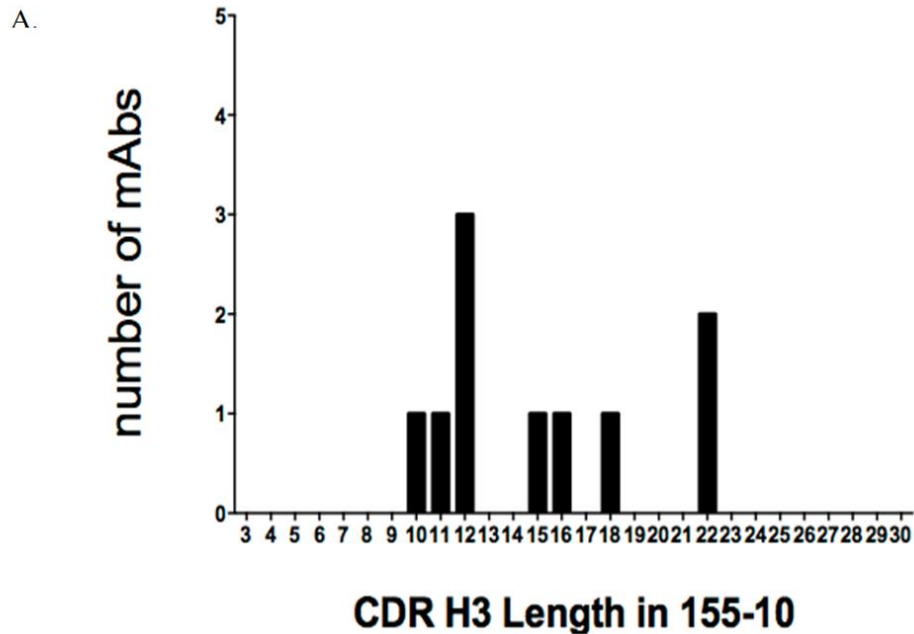
Antibody supernatants were screened for binding by ELISA against multiple influenza antigens from H1, H3, and H5 strains. (A) The number of antigens bound by antibodies are indicated based on color. (B) HA strains were separated based on subtype and subtype specific binding patterns are indicated by color.

The characteristics of binding antibodies are shown in Table I and Figures 4 and 5. Gene usage, CDR3 sequence length, and original antibody isotypes are shown in Table I. We then used these data to identify antibodies that had similar characteristics to CH65, as defined in Schmidt et al. 2015. These characteristics were a CDR-H3 of 19 ± 4 , central critical dipeptide with a hydrophobic amino acid next to an acidic amino acid (X(D/E)), and a cluster of aromatic residues between the central X(D/E) motif and the C terminus of the CDR-H3 (Schmidt 2015). The presence of these characteristics were shown to be important for the insertion of the CDR-H3 loop into the receptor binding pocket on the surface of HA, without these characteristics proper insertion and binding would not have been possible (Schmidt 2015). When examining sequences from antibodies with positive binding, we found 10 sequences from 155-10 similar to CH65 (Table I, Figure 4, 5). The CDR-H3 lengths ranged from 10 to 22, with the majority of antibodies having a CDR-H3 length above 15 amino acids long (Figure 4A). The JH6 gene in humans contains a trinucleotide repeated motif that encodes for a tyrosine repeat (TAC or TAT) that resulted in the observed cluster of aromatic residues found in the CH65 lineage, shown to be important for proper CDR-H3 loop formation (Schmidt 2015). This repeat is also found in 2 of the rhesus macaque antibodies isolated, Ab910798, and Ab910810 (Table I, Figure 5). Most of the isolated rhesus antibodies did not use the JH6 gene, instead they were from the JH4 gene family (Table I, Figure 5). Along with the presence of the JH6 gene usage, several of these antibodies also had dipeptides that might account for binding, though these dipeptides were not centrally located as in Schmidt et al. (2015) (Figure 4B). The observed dipeptides also varied from those seen in Schmidt et. al, in that the hydrophobic residue V(D/E) observed in

humans was replaced by the smaller glycine G(D/E) in our mAbs isolated from rhesus macaques (Schmidt 2015) (Figure 4B). Although, glycine is not a hydrophobic amino acid, its lack of a side chain would not be expected to interfere sterically with insertion of the CDR-H3 loop in the sialic acid binding pocket. Our binding data suggest that these amino acids could have the same functions as the motif of human RBS antibodies.

Table I: Isolated influenza specific sequence information for 155-10. Antibody sequences were analyzed using Clonalyst to determine V, D, and J chain usage; along with the above listed characteristics. Sequences are sorted based on CDR3 chain length and the J chain usage.

Donor	Week	Ab ID	ID	VH _{or} L		J	CDR3 length	CDR3 H3 Sequence Translation	Isotype	Mutated
				V	D					
155-10	22	Ab910798	H910798	IGHV3-A*01	IGHD6-15*01	IGHJ6-1*01	22	CSKDGGEDTVATVGGGYYYGLDS	G2	0.11
155-10	22	Ab910798	L905983	IGLV3-J*01		IGLJ2-2*01	11	CQVWDFSSDHRV		0.07
155-10	22	Ab910810	H910810	IGHV3-A*01	IGHD6-15*01	IGHJ6-1*01	22	CSKDGGEDTVATVGGGYYYGLDS	G1	0.10
155-10	22	Ab910810	L905991	IGLV3-J*01		IGLJ2-2*01	11	CQVWDFSSDHRVF		0.06
155-10	22	Ab910704	H910704	IGHV3-Y*01	IGHD3-5*01	IGHJ5-1*01	18	CVRDGPYYGDVYEQWFGV	G2	0.06
155-10	22	Ab910704	K905357	IGKV1-22*01		IGKJ2-4*1	9	CLQYSKSPYS		0.08
155-10	22	Ab910806.2	H910806.2	IGHV4-L*03	IGHD4-22*01	IGHJ4-1*01	16	CARIRGDEFHNYGDFDS	G2	0.08
155-10	22	Ab910806.2	L905990	IGLV2-A*01		IGLJ2-2*01	10	CSSYTTSGTWV		0.06
155-10	22	Ab910710	H910710	IGHV4-F*02	IGHD6-15*01	IGHJ4-1*01	15	CARDTCTVGRDCNFDC	G2	0.02
155-10	22	Ab910710	K905360	IGKV1-S6*01		IGKJ1-5*1	9	CQQGYNTPWT		0.02
155-10	22	Ab910801	H910801	IGHV4-B*01	IGHD6-19*01	IGHJ4-1*01	10	CATIVVAGFDS	G2	0.041509
155-10	22	Ab910801	L905985	IGLV3-D*01		IGLJ1-LC1*01	13	CSTSFSGLSWQYI		0.042017
155-10	22	Ab910803	H910803	IGHV4-D*01	IGHD2-11*01	IGHJ4-1*01	12	CARQKMGWNRVDF	G4	0.08
155-10	22	Ab910803	L905987	IGLV3-B*01		IGLJ2-2*01	10	CFLYMGXXXXL		0.07
155-10	22	Ab910803_L2Rh (Ab910806)	H910803	IGHV4-D*01	IGHD2-11*01	IGHJ4-1*01	12	CARQKMGWNRVDF	G4	0.08
155-10	22	Ab910803_L2Rh (Ab910806)	L905990	IGLV2-A*01		IGLJ2-2*01	10	CSSYTTSGTWV		0.06
155-10	22	Ab910688	H910688	IGHV4-F*01	IGHD2C-16*01	IGHJ4-1*01	12	CAKEEGFYKYLDI	G2	0.08
155-10	22	Ab910688	L905913	IGLV1-B*01		IGLJ1-LC1*01	11	CGAWDSSLRAYI		0.09
155-10	22	Ab910727	H910727	IGHV3-C*01	IGHD5-6*01	IGHJ4-1*01	11	CARFGNSNHFDY	G2	0.06
155-10	22	Ab910727	L905939	IGLV6-A*02		IGLJ2-2*01	10	CQSADGSHNRV		0.02



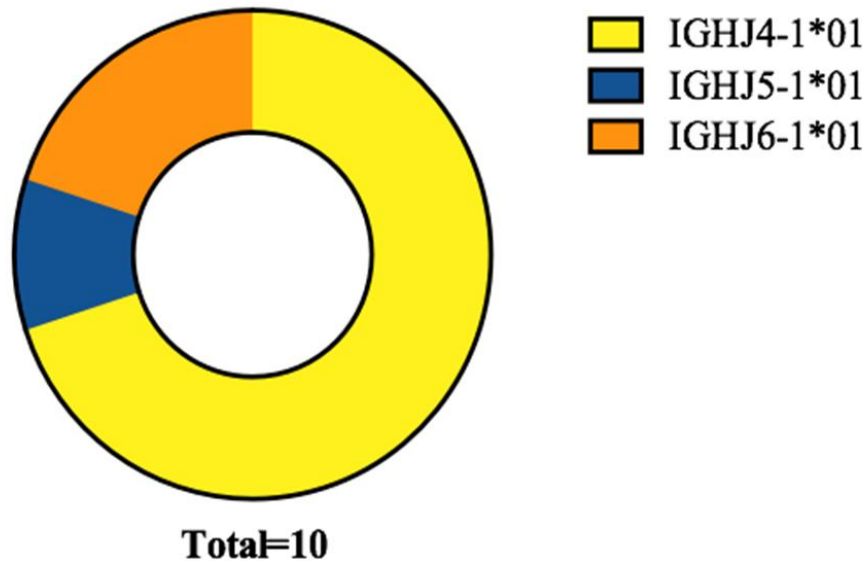
B.

>H910688	CAKEEGFYKY	LT
>H910704	CVRDGPYYGD	VYEQGWFGV
>H910710	CARDTCTVGR	DCNFDC
>H910727	CARFGNSNHF	DY
>H910798	CSKDG GE DTV	ATVGGGYYYG LDS
>H910801	CATIVVAGFD	S
>H910803	CARQKMGWNR	VDF
>H910806.2	CARIR G DEFH	NYGDFDS
>H910810	CSKDG GE DTV	ATVGGGYYYG LDS
>CH65 heavy	CARGGLEPRS	VD YYYYGMDV
>CH66 heavy	CARGGLEPRS	VD YYYYGMDV
>CH67 heavy	CARAGLEPRS	VD YYFYGLDV

Figure 4. CDR-H3 length and sequence characterization for antibodies from 155-10.

(A) Antibodies graphed based on CDR H3 length. (B) Antibody CDR H3 sequences with putative dipeptides highlighted in red. CH65, CH66, and CH67 included in data to highlight the canonical dipeptide sequences.

A. **JH Gene Usage**



B

Heavy Chain	JH Gene	Sequence
>H910688	J _H 4	AGGAGGGCTT CTACAAGTA
>H910704	J _H 5	CGGGGATGTC TACGAACAGG GGTG
>H910710	J _H 4	GGGTAGAGAC TGTAAC TTTG
>H910727	J _H 4	TGGAAACAGC AACCACTTTG ACT
>H910798	J _H 6	TACAGTGGCT ACAGTTGGGG GGGGG TATTA CTACGGTT
>H910801	J _H 4	GTGGTGGCTG GCTTTG
>H910803	J _H 4	AGATGGGCTG GAACAGAG
>H910806.2	J _H 4	AGATGAATTC CATAACTACG GGGA
>H910810	J _H 6	TACAGTGGCT ACAGTTGGGG GGGGG TATTA CTACGGTT
CH65 heavy	J _H 6	CCGATCTGTA GACT TACTACT ATTATGGTA
CH66 heavy	J _H 6	TCGATCTGTA GACT TACTACT ATTATGGTA
CH67 heavy	J _H 6	ACGATCCGTA GACT TACTACT TCTACGGTT

Figure 5: JH gene usage for influenza specific antibodies isolated from 155-10.

(A) Antibody JH gene usage are differentiated based on colors. (B) JH antibody sequences shown, with sequences encoding the tyrosine repeat present in JH6 antibodies highlighted in red. CH65, CH66, and CH67 antibodies are included to show the human tyrosine repeat motif.

Figure 6A shows the blocking data for these antibodies. Three of the screened antibodies had blocking comparable to our CH65 control (Figure 6A). Two of the three CH65 blocking antibodies were from the same clonal lineage, Ab910798, and Ab910810 (Table I). The third antibody had different VH and JH gene usage, IGHV4-L*03 and IGHJ4-1*01, instead of IGHV3-A*01 and IGHJ6-1*01 (Table I). Surprisingly, Ab910806.2 showed high blocking for CH65 and was not a JH6 antibody, instead it was from the JH4 family. This data suggests that this antibody may have a different mode of binding leading to this antibody having the ability to block CH65. Data from Ab910806.2 and Ab910803_L2RH (Ab910806) indicates that the light chain used does not appear to be restricted to have CH65 blocking ability (Table I). These antibodies share a light chain but have variable heavy chains that lead to CH65 blocking in one antibody and not the other. Schmidt et al. showed that contacts are made between CH65 and HA through the heavy chain specifically the CDR-H3 region, interactions are also made with the CDR-L1 and -L3 of the light chain. (Whittle 2011). These data show that the main contacts with the RBS and HA are made through the interaction with the CDR-H3 projecting into the RBS, though contacts with the light chain are also important for proper binding. These results suggest that CH65-like antibodies can be isolated from rhesus macaques after repeated immunization with a single vaccine strain.

Interestingly, despite the ability of these antibodies to bind multiple HA strains, we observed that in flow cytometry the cells from which these antibodies were isolated did not bind to both HAs used in sorting (Figure 6). This indicates that more generous gating strategies may be needed for future studies.

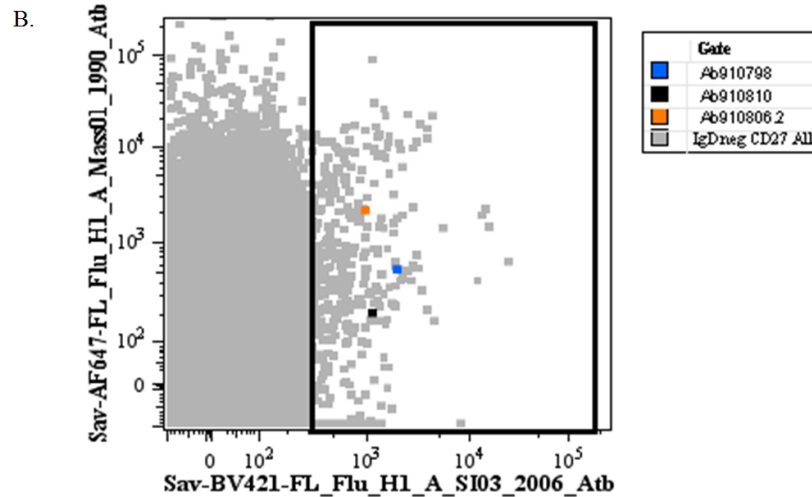
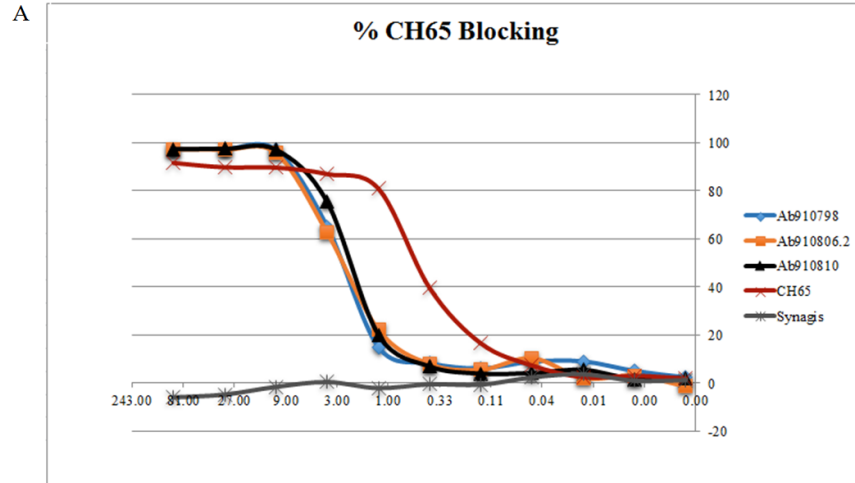


Figure 6: CH65 blocking antibodies isolated from 155-10. Purified antibodies were screened using the CH65 blocking ELISA assay. (A) Shows the data of 3 antibodies compared to the positive control, CH65, and negative control, palivizumab. (B) Shows the positions of the 3 blocking antibodies from the index sorted flow cytometry data.

We selected 4 mAbs from 155-10 to determine if they were capable of neutralizing influenza. Plasmids for these antibodies were transfected into 293i cells. One of the plasmids, Ab910810, did not express when transfected into 293i cells, which was unfortunate because it had high CH65 blocking activity when tested from the small scale transfection product. This antibody is being studied further to determine if other methods of gene isolation and expression will result in a higher antibody yield. Of the 4 antibodies tested, only 2 showed neutralization against the H1 strain, likely because the animals were immunized with (A/Solomon Islands/03/2006), and had not been exposed to the H3 A/Wisconsin/67/2005 strain (Table II). Similar neutralization activity was shown for both rhesus antibodies and humanized version of the same antibodies. The neutralizing antibodies both had long CDR-H3 regions, and the putative dipeptide but only one of the antibodies was from the JH6 lineage. These data suggest that the dipeptide motif is more important for CH65 blocking and strain neutralization than is the JH6 gene usage, although other mechanisms of CH65 blocking such as steric hindrance cannot be excluded. Other strains of influenza are currently being used to test the neutralization capacity of these 2 neutralizing antibodies.

Table II: Influenza specific neutralizing antibodies from 155-10. Antibodies were screened for neutralization against two strains, A/Solomon Islands/03/2006, and A/Wisconsin/67/2005. CH65, and Ab2210 were positive controls; and AB900294RH_AAA was the negative control. Endpoint titers reported as the highest dilution to achieve $\geq 50\%$ neutralization. Information regarding antibody JH, CDR-H3 are listed as indicated for each antibody.

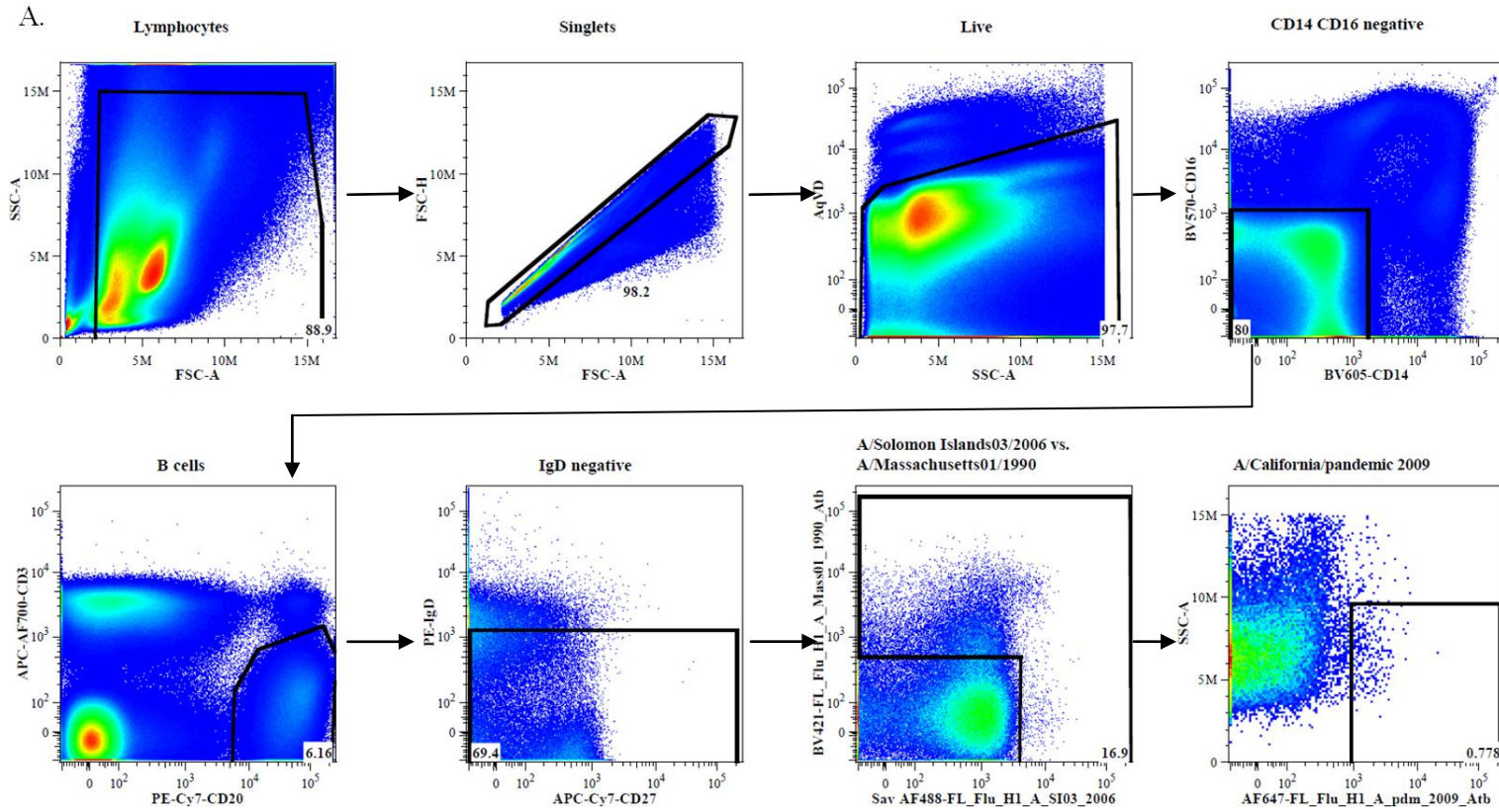
Antibody	J _H Chain Usage	CDR H3 Length	Critical Dipeptide Present (Y/N)	Treatment (Test Virus of Interest)			
				A/Solomon Islands/03/2006		A/Wisconsin/67/2005	
				Rhesus Antibodies	Human Antibodies	Rhesus Antibodies	Human Antibodies
Ab910798Rh	IGHJ6-1*01	22	Y	$\leq 0.05 \mu\text{g/mL}$	0.024 $\mu\text{g/mL}$	> 50 $\mu\text{g/mL}$	>50 $\mu\text{g/mL}$
Ab910803Rh	IGHJ4-1*01	12	N	> 50 $\mu\text{g/mL}$	>50 $\mu\text{g/mL}$	> 50 $\mu\text{g/mL}$	> 50 $\mu\text{g/mL}$
Ab910803_L2Rh	IGHJ4-1*01	12	N	> 50 $\mu\text{g/mL}$	> 50 $\mu\text{g/mL}$	> 50 $\mu\text{g/mL}$	>50 $\mu\text{g/mL}$
Ab910806.2Rh	IGHJ4-1*01	16	Y	0.10 $\mu\text{g/mL}$	0.098 $\mu\text{g/mL}$	> 50 $\mu\text{g/mL}$	> 50 $\mu\text{g/mL}$
CH65	IGHJ6-1*01	19	Y	0.01 $\mu\text{g/mL}$	0.016 $\mu\text{g/mL}$	N/A	N/A
DH253 Ab2210	IGHJ3-1*01	10	N	N/A	N/A	3.13 $\mu\text{g/mL}$	3.125 $\mu\text{g/mL}$
AB900294RH_AAA (Negative Ctrl)	IGHJ1-1*01	7	N	> 50 $\mu\text{g/mL}$	> 50 $\mu\text{g/mL}$	> 50 $\mu\text{g/mL}$	> 50 $\mu\text{g/mL}$

Another question for vaccine development is how quickly can we elicit broadly reactive antibodies that might have cross-strain reactivity against influenza. For this reason, we stained PBMCs from animal 144-05 from an earlier time point at week 10, with 3 different H1 influenza specific reagents. For this experiment cells were sorted based on positivity to A/Solomon Islands/03/2006, A/Massachusetts/03/1990, and A/California/07/2009 strains (Figure 7A). This set of HA proteins were chosen based on the recent observation that influenza antibodies against the RBS show a strong degree of imprinting by earlier strains (Schmidt 2015). From this experiment, we isolated 10 clonal lineages with 2 - 4 members each (Figure 7B). Antibodies were screened in ELISA assays for cross influenza strain reactivity (Figure 8A-B). These antibodies from 144-05 at the week 10 time point were evenly split between having high binding to a single strain and multiple subtypes of influenza (Figure 8B), with a greater degree of cross strain binding between influenza A H1 and H5 strains compared to H3 strains (Figure 8B). At this time point, most antibodies showed binding to multiple HA strains (Figure 8A). From the binding and CDR-H3 characteristics used to select antibodies from 155-10, 12 antibodies were selected for further screening. Antibody sequences were analyzed for CDR-H3 length, for the putative dipeptide sequence and for JH gene usage (Figure 9, 10). Even though some CH65 like characteristics were present, none of the antibodies tested blocked CH65 binding to HA (Figure 6). This lack of blocking could be from the difference in the number of immunizations received, 144-05 only received 2 at the week 10 time point compared to 5 at

the week 22 time point in 155-10, thus these antibodies may not yet have had sufficient selection to induce higher affinity cross-blocking activity.

In this study, differences found between the cells isolated from two different animals at varying time points show that the affinity maturation process is essential in the development of effective antibody responses (Gitlin 2015). Affinity maturation takes place in the germinal centers and is the process where antibodies gain increased affinity, avidity, and anti-pathogen activity as a result of somatic hypermutation (SHM) (Doria-Rose 2015). In this study we specifically selected on memory B cells from the IgD negative population (Figure 7A). Due to live cell sorting, some cells from the IgD positive population, naive cells, were included in the sorted cell population. Cells within the memory population showed specificity to the viral strain the animals were immunized with (A/Solomon Islands/03/2006), along with binding to the two other strains (A/Massachusetts/01/1990, and A/California/pandemic/2009) (Figure 7A). Even though these cells had binding they did not have the proper mutations including the long CDR-H3, and dipeptide necessary for proper binding of HA. One antibody Ab910950 had the presence of a dipeptide, and a JH6 gene segment, though these characteristics differed from other CH65 blocking antibodies isolated from 155-10 (Table III, Figure 9, Figure 10). The CDR-H3 region consisted of only 13 amino acids, and had a dipeptide closer to the N terminus of the CDR-H3 region showing that a longer CDR-H3 length and proper dipeptide positioning are critical for CH65 blocking (Figure 9, Figure 10). In order to determine if these types of antibodies can be isolated from animal 144-05 further studies will be needed on samples from later time points.

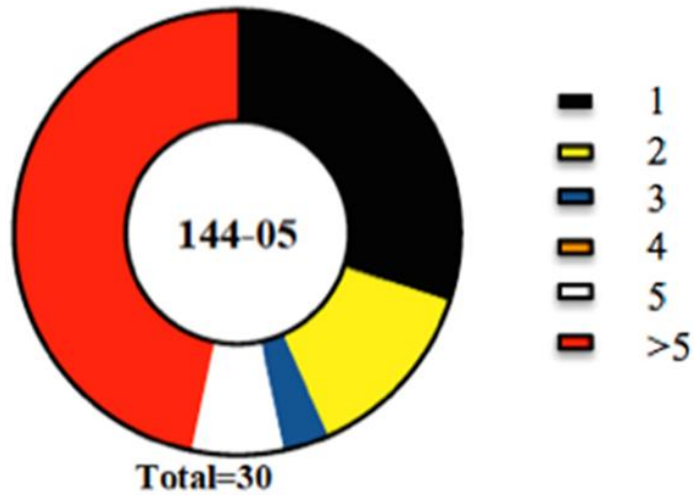
Figure 7: Flow cytometric gating analysis for 144-05. (A) Flow cytometry data for 144-05 shows the gating strategy used for sorting. Cells from the following populations: Lymphs, Singlets, Live, CD14 CD16 negative, B cells, IgD negative all, the single and double positive A/Solomon Islands/03/2006 and A/Massachusetts/01/1990, and then positive A/California/pandemic 2009 population. (B) Shows the conjugates used, number of sorted events, functional antibodies isolated and clonal lineage information.



B.

MID	Conjugates used	Timepoint (Weeks)	# Sorted Events	# Functional Antibodies	# Clonal Lineages	# Members in Clonal Lineages
144-05	FL_Flu_H1_A_SI03_2006 AF488	10	80	30 (38%)	10	2 to 4
	FL_Flu_H1_A_Mass01_1990 BV421					
	FL_Flu_H1_A_SI03_2006 AF488	10	316	125 (40%)		
	FL_Flu_H1_A_Mass01_1990 BV421					
FL_Flu_H1_A_pdm_2009 AF647						

A. **Counts of Reactive Strains**



B. **Counts of Subtype Reactivity**

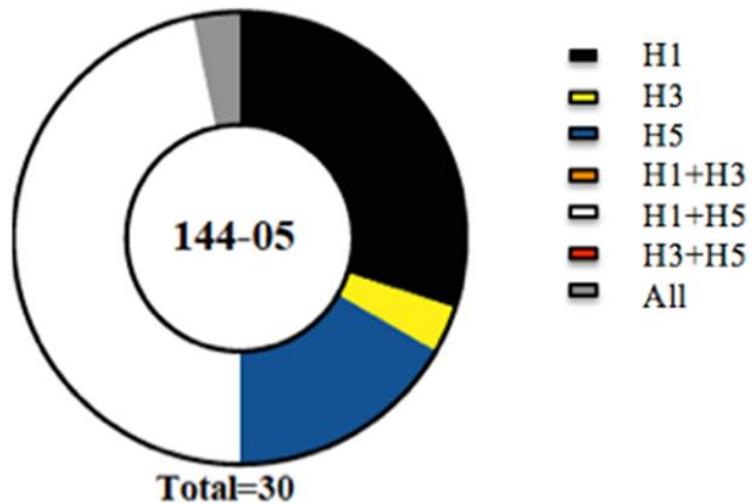
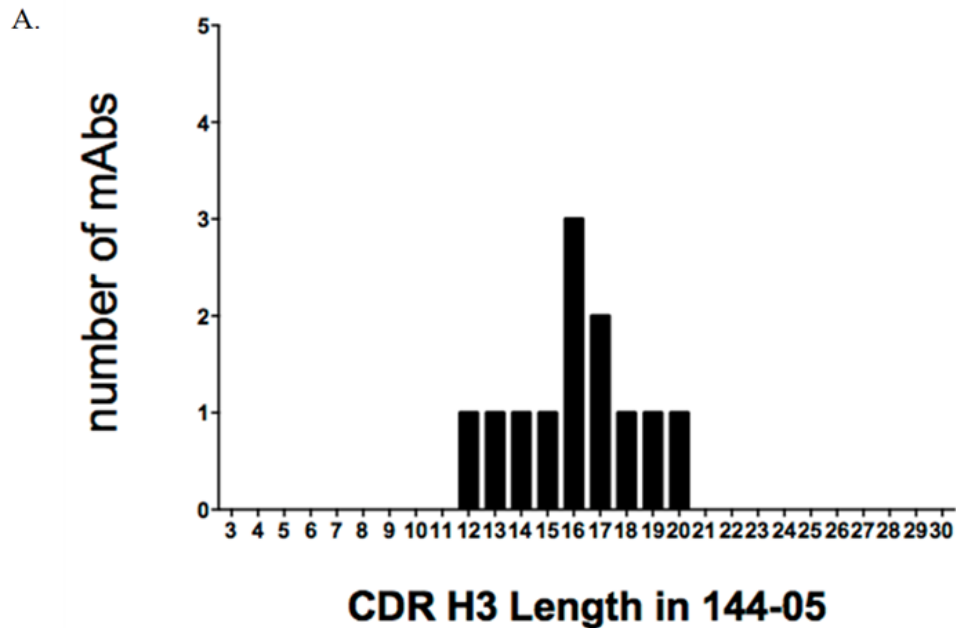


Figure 8: Influenza binding characteristics for antibodies isolated from 144-05.

Antibody supernatants were screened for binding by ELISA against multiple influenza antigens from H1, H3, and H5 strains. (A) The number of antigens bound by antibodies are indicated based on color. (B) HA strains were separated based on subtype and subtype specific binding patterns are indicated by color.

Table III: Isolated influenza specific sequence information. Antibody sequences were analyzed using Clonality to determine V, D, and J chain usage; along with the above listed characteristics. Sequences are sorted based on CDR3-H3 chain length and the J chain usage.

VH _{orL}										
Donor	Week	Ab ID	ID	V	D	J	CDR3 length	CDR3 H3 Sequence Translation	Isotype	Mutated
144-05	10	Ab910966	H910966	IGHV3-H*01	IGHD2-11*01	IGHJ1-1*01	20	CARAGHCSSTYCSEQSDAFDF	G1	0.09
144-05	10	Ab910966	L906061	IGLV1-D*01		IGLJ2-2*01	10	CCSYAGKYTYI		0.06
144-05	10	Ab911109	H911109	IGHV4-L*03	IGHD2-9*01	IGHJ5-1*01	19	CARLFCSSSTICYASYNRFDV	G2	0.05
144-05	10	Ab911109	K905561	IGKV1-S9*01		IGKJ2-4*1	9	CQQSYGTPYS		0.07
144-05	10	Ab910974	H910974	IGHV4-F*01	IGHD2-16*01	IGHJ4-1*01	18	SARQYCSGAVCSEHNRFDV	G2	0.09
144-05	10	Ab910974	K905495	IGKV1-20*01		IGKJ3-2*1	9	CQQYYSFPYS		0.06
144-05	10	Ab911093	H911093	IGHV4-L*03	IGHD2-4*01	IGHJ5-1*01	17	CTRPSVFLVIRYNRFDV	G1	0.05
144-05	10	Ab911093	K905553	IGKV1-S22*01		IGKJ4-LC1*20	9	CQQDYSYPIT		0.05
144-05	10	Ab911017	H911017	IGHV4-K*01	IGHD3-12*01	IGHJ4-1*01	17	CARERGCSTYCFYFFDY	G1	0.06
144-05	10	Ab911017	L906084	IGLV1-D*01		IGLJ1-LC1*01	11	CLSVDSSLSAYI		0.09
144-05	10	Ab911115	H911115	IGHV4-L*03	IGHD2C-16*01	IGHJ5-1*01	16	CARQTRKTVGTTGRFDA	G2	0.08
144-05	10	Ab911115	K905565	IGKV1-13*01		IGKJ4-LC1*20	9	CLQGNTKPLT		0.05
144-05	10	Ab910970	H910970	IGHV3-W*02	IGHD4C-22*01	IGHJ4-1*01	16	CARPSRRTVGTVNFDDY	G1	0.06
144-05	10	Ab910970	K905494	IGKV1-S1*01		IGKJ2-4*1	9	CQQHNSYPFT		0.05
144-05	10	Ab911106	H911106	IGHV4-L*03	IGHD3-12*01	IGHJ4-1*01	16	CARDYCSGIYCYVGFDC	G2	0.06
144-05	10	Ab911106	L906121	IGLV2-D*02		IGLJ2-2*01	10	CGSYAGSNTFL		0.06
144-05	10	Ab911071	H911071	IGHV3-O*01	IGHD3-5*01	IGHJ4-1*01	15	CSREGGRLRG*LRIS	G2	0.16
144-05	10	Ab911071	K905533	IGKV1-11*01		IGKJ3-2*1	9	CQQHNSYPFT		0.05
144-05	10	Ab910989	H910989	IGHV3-D*01	IGHD2C-16*01	IGHJ4-1*01	14	CARGPVLPGSGGFDV	G2	0.08
144-05	10	Ab910989	K905538	IGKV1-20*01		IGKJ1-5*1	9	CQQYHAYPPT		0.14
144-05	10	Ab910950	H910950	IGHV3-D*01	IGHD4C-22*01	IGHJ6-1*01	13	CARAGDGGSYYYGLDS	A1	0.09
144-05	10	Ab910950	K905474	IGKV3-S3*01		IGKJ1-5*1	9	CAGDGGSYYYGLDS		0.10
144-05	10	Ab910987	H910987	IGHV3-B*01	IGHD3-21*01	IGHJ4-1*01	12	CARERGSYGRDY	G2	0.08
144-05	10	Ab910987	L906071	IGLV2-E*02		IGLJ1-LC1*01	6	VCSTCAPA		0.15

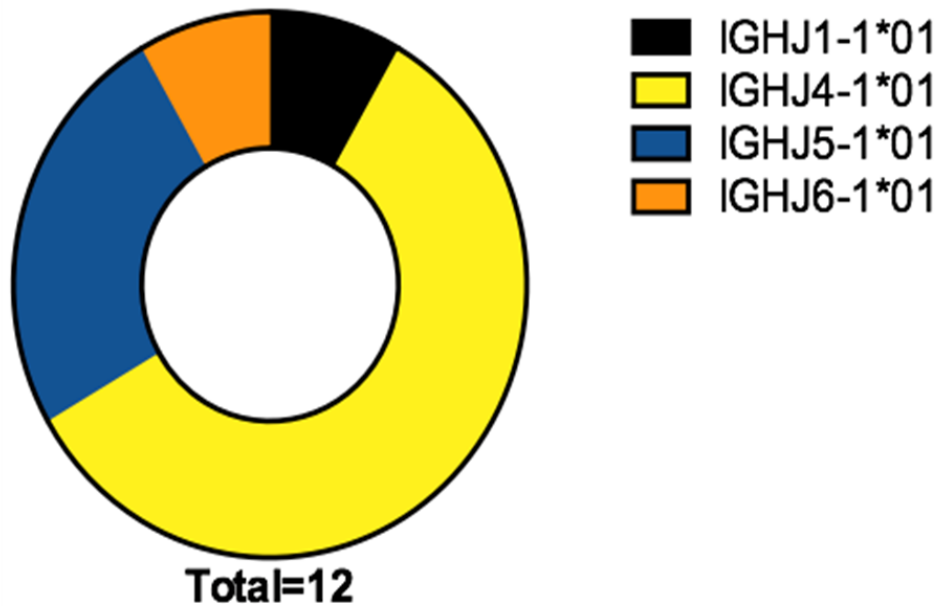


B.

>H910950	CARA GD GGSY YYGLDS
>H910966	CARAGHCSST YCSEQSDAFD F
>H910970	CARPSRRTVG TVNFFDY
>H910974	SARQYCSGAV CSEHNRFDV
>H910987	CARERGYSGY RDY
>H910989	CARGPVLPGS GGFDV
>H911017	CARERGCSTY YCFYYFDY
>H911071	CSREGGRLRG LRIKS
>H911093	CTRPSVFGLV IRYNRFDV
>H911106	CARDYCSGIY CYVGFDC
>H911109	CARLFCSSTY CYASYNRFDV
>H911115	CARQTRKTVG TTGRFDA
>CH65 heavy	CARGGLEPRS VD Y YYYGMDV
>CH66 heavy	CARGGLEPRS VD Y YYYGMDV
>CH67 heavy	CARAGLEPRS VD Y YFYGLDV

Figure 9. CDR-H3 length and sequence characterization for antibodies isolated from 144-05. (A) Antibodies graphed based on CDR H3 length. B: Antibody CDR-H3 sequences with putative dipeptides highlighted in red. CH65, CH66, and CH67 included in data to highlight the canonical dipeptide sequences.

A. **JH Gene Usage**



B.

Heavy Chain	JH Gene	Sequence
>H910950	J _H 6	TGGAGGTAGT TATTACTACG GTT
>H910966	J _H 1	AGTAGTACTT ACTGCTCCGA ACAATCTGAT GC
>H910970	J _H 4	AGGACAG-TG GGTACAGTTA ATTT
>H910974	J _H 4	AGTGGTGCTG TCTGCTCAGA ACATAACCG
>H910987	J _H 4	GAGGGGATAC AGTGGGTACA GGG
>H910989	J _H 4	TTCCGGGGTC GGGGGG
>H911017	J _H 4	TGTAGTGGTA TTTACTGCTT CTA
>H911071	J _H 4	GATTACGAGG ATGATTACGG AT
>H911093	J _H 5	CTTTGGACTG GTTATTCGGT ACAACCG
>H911106	J _H 4	TGTAGTGGTA TTTACTGCTA TGTCGG
>H911109	J _H 5	AGTAGTACTT ACTGCTACGC CTCGTACAAC CG
>H911115	J _H 5	AAAACCGTGG GAACTACAGG GAG
CH65 heavy	J _H 6	CCGATCTGTA GACT TACTACT ATTAT GGTA
CH66 heavy	J _H 6	TCGATCTGTA GACT TACTACT ATTAT GGTA
CH67 heavy	J _H 6	ACGATCCGTA GACT TACTACT TCT TAC GGTT

Figure 10: JH gene usage for influenza specific antibodies isolated from 144-05.

(A) Antibody JH gene usage differentiated based on colors. (B) JH antibody sequences shown, with sequences encoding the tyrosine repeat present in JH6 antibodies highlighted in red. CH65, CH66, and CH67 antibodies are included to show the human tyrosine repeat motif.

Chapter 4: Discussion

Current influenza vaccination strategies result in limited protection against viral strains circulating within human populations and need yearly re-administration and updating. No cross-protection is seen between influenza strains in both natural influenza infection and vaccination (Nabel 2010). One explanation for this is that the predominant components of the virus that the immune system sees upon infection are those that change with each emerging strain which reduces the likelihood of protection induced by prior infections (Nabel 2010). Indicating a need for the development of novel vaccine strategies that will prevent infection against multiple subtypes and clades of influenza viruses. In order to achieve broad protection against influenza viruses, vaccines may need to focus on eliciting antibodies to conserved portions of the influenza virion, such as the RBS, which is necessary for virus attachment and infection. Understanding the development of antibodies like CH65 may permit better vaccine designs that can more quickly and reliably elicit those antibodies (Schmidt 2015). Previous work in humans has shown that influenza infection or exposure occurs very early in life, with nearly all people showing evidence of influenza exposure by 6 years of age (Bodewes 2011). Despite yearly vaccinations and other exposures individuals often produce antibodies against epitopes expressed on the first influenza virus they were exposed to, and it has been shown that existing antibodies are capable of suppressing naïve B cell responses for novel antigens (Schmidt 2015, Janeway 2012). This phenomenon is called original antigenic sin (OAS) and is generally thought of having negative implications because though it is a memory response it is not one that is desired. Vaccination also initiates primary responses along with other recall or memory responses. Affinity maturation

results from vaccination causing memory cells to re-enter the germinal centers and undergo new rounds of somatic hypermutation and selection increasing antibody affinity and the generation of bNAbs (Janeway 2012). This process indicates that the immune response to vaccination is a dynamic process. It is impractical to test novel vaccine strategies in humans, and for that reason effective animal models need to be used. Mouse models are attractive given cost considerations, but they may not be as effective as other animal models that share closer genome homology to humans. Currently, rhesus macaques have been used as an animal model for studying SIV, and HIV vaccine strategies. Rhesus macaques have ~93% genome similarity with humans and prior work has shown that they can respond to some epitopes in the same manner as humans (Gibbs 2007, Wiehe 2014). Rhesus macaques have also been shown to develop similar pathology and immune response to influenza as seen in humans (Weinfurter 2011, Shinya 2012, Giles 2012, Jegaskanda 2012, Sinthujan 2013). Our study has extended that work, showing that rhesus macaques have promise for influenza vaccine design.

After serial immunizations with a single influenza strain (A/Solomon Islands/03/2006) we isolated antibodies from rhesus macaques some of which were similar to CH65. Two of the three isolated CH65 blocking antibodies were clonally related and used the JH6 gene segment that had been shown to be important for CDR-H3 loop interactions with the HA RBS (Schmidt 2015, Table I). The other antibody, Ab910806.2, had a different VH (IGHV4-L*03) and JH (IGHJ4-1*01) chains (Table I). The IGHJ4-1*01 gene segment, does not have the tyrosine repeat as seen in IGHJ6-1*01, showing that blocking is not completely dependent upon this tyrosine repeat (Figure 5). The differing VH gene usage is

consistent with the findings of Schmidt et. al. in 2015 showing that CH65 like antibodies can arise from various germlines and develop independently (2015). Even though the RBS antibodies developed from diverse germlines the one common feature they shared was the JH6 gene segment, showing that this segment was universally found in all of their antibodies (Schmidt 2015). Our findings on the other hand show that these antibodies were isolated from diverse germlines including differing JH gene segments. Indicating that the rhesus isolated antibodies did not depend on this JH6 gene these findings demonstrate that antibodies that can block known RBS antibodies can arise in rhesus macaques from multiple different gene families. Structural studies of these rhesus antibodies will be needed to determine if the rhesus antibodies have the same mode of binding to HA as the human CH65-like antibodies.

Isolated antibodies were capable of binding various influenza strains from different subtypes (Figure 3A, 3B). All of the CH65 blocking antibodies in our study had CDR-H3 loops ranging from 16-22 amino acids long, and a non-central dipeptide (Table I, Figure 5). The presence of both of these characteristics seemed to be necessary for H1 neutralization activity as shown in Table II. Since these antibodies did not show neutralization activity for the H3 strain A/Wisconsin/67/2005, additional strains are being tested to determine if these antibodies are able to neutralize other H1 strains as well as those from other subtypes.

In HIV, bNAbs can be isolated during chronic infection, but are not normally present during the acute phase of infection and have not been reliably elicited by vaccination (Haynes 2013, Haynes 2014) . Broadly neutralizing antibodies against the HIV envelope that are capable of neutralizing a broad range of HIV-1 isolates have not successfully been

induced in high titers by vaccination, and are only present in 20% of individuals only after 4 or more years of infection (Haynes 2013). In contrast, our study showed that influenza antibodies similar to CH65 are acquired after only 22 weeks of repeated immunizations. All three of the CH65 blocking antibodies were isolated from animal 155-10 at the week 22 time point, and not from animal 144-05 at week 10. At week 22, the animals had received 5 immunizations with A/Solomon Islands/03/2006 with the last immunization occurring on week 20. This repeated stimulation can cause memory B cells to re-enter the germinal centers to undergo new rounds of somatic hypermutation and selection, leading to changes in antibody structure and formation of long CDR-H3 regions (Victoria 2012, De Silva 2015, Haynes 2012). The 5 immunizations compared to the 2 that animal 144-05 had received by the week 10 time point may have been the reason that blocking antibodies were not isolated from this animal. The 2 immunizations received by 144-05 may not have been enough for the antibodies to undergo further rounds of affinity maturation to generate the changes needed for to have CH65 blocking ability. These questions will not be answered until samples from later time points are screened for animal 144-05.

Together these data show that antibodies similar to CH65 can be isolated from rhesus macaques with immunization of a single influenza strain and no prior influenza exposure. Generating these antibodies without prior exposure is an important discovery that demonstrates that the generation of these antibodies is not dependent on prior exposure to other influenza viruses, and that these antibodies can develop with repeated immunizations. In this study the animals were only ever exposed to one strain of influenza (A/Solomon Islands/03/2006), but were still capable of binding antigens present in other viral strains. This

raises the hypothesis that repetitive exposure may allow the generation of cross strain specific and neutralizing antibodies through antibody affinity maturation. It is not known whether a carefully designed prime-boost strategy with different HA strains could elicit antibodies with even greater breadth, and work to investigate this possibility is ongoing.

In conclusion, this study shows that rhesus macaques are a good immunological model for influenza vaccination. Antibodies isolated during this study are similar to CH65, and need to undergo further studies including screening against a broader range of influenza viruses to determine neutralization capabilities, and to confirm the structural basis for blocking of RBS-directed mAb binding. ADCC studies will also be beneficial to determine if any of these antibodies are capable of binding the Fc γ R of effector cells. Other antibodies isolated in this study that were not CH65-like but had cross-strain reactivity will also undergo further screening to determine if these antibodies are more similar to HA stem antibodies, or if they are specific for some other epitope. Further characterization of these antibodies will provide beneficial results for future vaccination studies.

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