

B Cell and Antibody Responses to Influenza A Virus in Human

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ABSTRACT

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Neutralising antibodies and antigen-specific B cells are important for protection against influenza A virus. However, the antigenic evolution of influenza A viruses has made a continuing challenge to the design of vaccine and the public health. The ability to generate cross-reactive response against influenza remains unclear in human. It is important to explore the antibody and B cell repertoire at single cell level.

The pandemic H1N1 and seasonal influenza vaccine induced robust antibody response in adults. However, pre- or co-vaccination with the seasonal vaccine led to a significantly reduced antibody response to pandemic H1N1 virus. Whether this interference has impact on subsequent infection rates remains undetermined. There observed substantial cross-reactive antibody response upon vaccination, as measured by HI, MN and B cell ELISpot assays. The antibody recognizing conserved proteins could be the main component of cross-reactivity against influenza A strains and subtypes. A significant expansion of influenza-specific MBC was observed after infection. Cross-reactive response was also noted in the MBC response. Importantly, a robust early-phase ASC response was detected in the peripheral blood upon influenza vaccination or infection. The size of ASC response significantly correlated with serum HI, MN and anti-HA IgG titre three weeks after

vaccination. The sequence analysis revealed that early-phase ASC accumulated high level of somatic mutations on Ig variable region and affinity maturation, as well as anti-influenza mAb, which suggested their origin from pre-existing MBC. Eight anti-influenza mAb were made from early-phase ASC, including one high-titre virus-neutralising HA1-specific, two other HA1-specific, one cross-reactive HA2-specific, and four cross-reactive NP-specific antibodies, indicating of the broad diversity of ASC repertoire.

In conclusion, this study demonstrated the properties of antibody and B cell responses to influenza A virus at serological, cellular and sequence level. The virus-neutralising and cross-reactive mAb derived from ASC could have therapeutic potential and their analysis might direct the vaccine design in the near future.

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For

Mum, Dad, Sister, and YuHsuan

ABBREVIATIONS

ADCC	Antibody Dependent Cell-mediated Cytotoxicity
APRIL	A Proliferation Inducing Ligand
ASC	Antibody Secreting plasma Cells
BAFF	B cell-Activating Factor of the TNF Family
BCMA	B Cell Maturation receptor
BFA	Brefeldin A
Blimp-1	B-Lymphocyte Induced Maturation Protein-1
BSA	Bovine Serum Albumin
CAIV	Cold Adapted Live Attenuated Vaccine
CDRs	Complementarity Determining Regions
CDR3	Complementarity Determining Region 3
CXCR	C-X-C chemokine Receptor
D	Diversity gene
DAB	3,3'-diaminobenzidine
DMEM	Dulbecco's Modified Eagle Medium
DNA	Deoxyribonucleic Acid
EBV	Epstein-Barr Virus
ELISA	Enzyme-Linked Immunosorbent Assay
ELISpot	Enzyme-Linked Immunosorbent spot
ER	Endocyttoplasmic Reticulum
FACS	Fluorescence Activated Cell Sorting
FBS	Fetal Bovine Serum
FCS	Foetal Calf Serum
FDA	U S Food and Drug Administration
FRs	Framework Regions
FSC	Forward Scatter
γ	Gamma

GBS	Guillian–Barré Syndrome
GMT	Geometric Mean Titre
H1N1 CA07	H1N1 A/California/07/09
H1N1 BR59	H1N1 A/Brisbane/59/07
H3N2 BR10	H3N2 A/Brisbane/10/07
H3N2 PR16	H3N2 A/Perth/16/09
H5N1 VN1194	H5N1 A/Vietnam/1194/04
H	Heavy chain
HA	Haemagglutinin
HAT	Human Airway Trypsin-like protease
HI	Haemagglutinin Inhibition
HIV	Human Immunodeficiency Virus
ICH	Immunocytochemistry
ICS	Intracellular Cytokine Staining
IFN	Interferon
Ig	Immunoglobulin
ISAAC	Immunospot Array Assay on a Chip
J	Joining gene
κ	Kappa chain
λ	Lambda chain
M1	Matrix 1
M2	Matrix 2
mAb	Monoclonal Antibodies
MADCAM1	Mucosa Vascular Addressin Cell Adhesion Molecule 1
MBC	Memory B Cells
MDCK	Madin-Darby Canine Kidney
MHC	Major Histocompatibility Complex
MN	Microneutralisation
NA	Neuraminidase

NK	Natural Killer
NLRs	NOD-Like Receptors
NP	Nucleoprotein
NS1	Non-Structural protein 1
NS2	Non-Structural protein 2
OAS	Original Antigenic Sin
OPD	σ -Phenylenediamine Dihydrochloride
PA	Polymerase Acidic protein
PAMPs	Pathogen-Associated Molecular Patterns
PB1	Polymerase Basic protein 1
PB2	Polymerase Basic protein 2
PBMC	Peripheral Blood Mononuclear Cells
PBS	Phosphate Buffered Saline
PCR	Polymerase Chain Reaction
PEI	Polyethylenimine
PMA	Phorbol 12-Myristate 13-Acetate
PRRs	Pattern Recognition Receptors
PWM	Pokeweed Mitogen
RBC	Red Blood Cells
RDE	Receptor Destroying Enzyme
RIG-1	Retinoic acid-Inducible Gene 1 proteins
RNA	Ribonucleic Acid
R/S ratio	the ratio of Replacement to Silent mutations
RT-PCR	Reverse Transcription Polymerase Chain Reaction
SAC	Staphylococcus Aureus Cowan Strain
SSC	Side Scatter
TCID	Tissue Culture Infectious Dose
Th1	CD4 T helper 1
TIV	Trivalent Inactivated Vaccine

TLRs	Toll-Like Receptors
TMPRSS2	Transmembrane Protease Serine S1 member 2
V	Variable gene
VCAM1	Vascular Cell Adhesion Molecule 1
vRNPs	viral Ribonucleoprotein complexes
WHO	World Health Organization

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CHAPTER 1

INTRODUCTION

1.1 B CELLS AND ANTIBODY RESPONSES IN ACUTE VIRAL INFECTION

Acute viral infections pose a major threat to human health. Smallpox caused an estimated 300-500 million of victims during the 20th century. Measles, another well-known acute virus in human, resulted in the death of hundreds of unvaccinated children. Moreover, the influenza A H1N1 pandemic in 2009 killed at least tens of thousands of infected people, from 2009 April to 2010 January.

The innate, humoral and cellular immunity are the major components of human immune system that work together to prevent human from harmful pathogens. In the view of humoral immunity, memory B and plasma cells determine the so-called antibody-mediated immunity with plasma cells providing continuous protection against pathogens through secretion of neutralising antibodies. Antibody-mediated immunity plays a crucial role in the protection against acute viral infection, such as the enteroviral infection. Most enteroviral infections cause mild illness in immunocompetent hosts, but are prone to becoming severe illness or fatal outcome in hosts with primary antibody deficiency (Fried *et al.*, 2009; Halliday *et al.*, 2003). Besides, it has been shown in breastfed infants that less enteroviral infections are significantly associated with a higher antibody level in maternal serum and breast milk (Sadeharju *et al.*, 2007). In addition, the eradication and control of several acute viral infection, e.g. smallpox, measles and yellow fever, by

vaccine-induced neutralising antibodies also showcases the importance of antibody-mediated immunity. (Amanna and Slifka, 2011; Edghill-Smite *et al.*, 2005; Law and Hangartner, 2008; Mason *et al.*, 1973; Samb *et al.*, 1995). However, the underlying mechanisms on how the antibodies elicited can confer protection remain to be elucidated.

In the steady state, memory B cells (MBC) are thought to replenish antibody secreting plasma cells (ASC), which allows the maintenance of humoral immunity (Sallusto *et al.*, 2010). When the host is exposed to the same antigen, a secondary immune response is mediated by the MBC that would rapidly proliferate and differentiate into ASC (Sallusto *et al.*, 2010). It has been shown that the MBC response could be remarkably long-lived. For instance, after smallpox vaccination, the antigen-specific B cell memory could last for decades (Crotty *et al.*, 2003). In the future, for a variety of acute viruses, the relative contribution of the MBC response to protective immunity could be a prerequisite in developing a successful vaccine targeting at long-term humoral immunity.

Influenza virus is one of the major viruses causing acute infection in human and it occurs in distinct outbreaks of varying extent nearly every year. Based on the experiences from the sero-epidemiological survey and the convalescent plasma therapy, antibody-mediated immunity play a central role in the control of influenza infection (Hancock *et al.*, 2009; Hung *et al.*, 2011). The influenza vaccine is therefore produced annually to induce a potent antibody response in the population to match the circulating strain through the global effort. However, the evolving nature of influenza allows the escape of virus from pre-existing antibody immunity, thus reducing the effectiveness of

the yearly vaccination against influenza. A lot of questions about how the antigen-specific humoral immunity develops, maintains and confers protection upon vaccination or during the course of infection remain unanswered. Moreover, how the host's antibody-mediated immunity deals with considerable viral variations is poorly understood. Although serological tests are able to provide the fundamental information of elicited antibody response by vaccination or infection, it becomes increasingly important to explore the repertoire and function of antibody and B cell response at single cell level. The newly developed methods of making monoclonal antibodies (mAb) open the way to evaluate the B cell response at the molecular level. It is also intriguing to study the potential of mAb in preventing and treating viral infections, especially the probable pandemic virus, in the near future.

1.2 INFLUENZA A INFECTION

1.2.1 Virology

1.2.1.1 Viral structure

Influenza A virus belongs to the family Orthomyxoviridae which also includes other two genera of influenza virus, influenza B and C virus, among which only influenza A viruses are further classified into subtypes based on the two main surface proteins haemagglutinin and neuraminidase. Influenza B viruses are usually found only in humans, can cause morbidity and mortality among infected individuals, but generally produce less severe disease and epidemics than influenza A viruses. Influenza C viruses usually cause only mild upper respiratory tract illness in humans and do not cause epidemics or pandemics.

Influenza A virus is an enveloped virus with a genome constituted by negative sense, single-stranded, segmented RNA. The genome size is approximately 17 kb and there are eight segments which encoded for the 12 proteins: haemagglutinin (HA), neuraminidase (NA), matrix 1 (M1), matrix 2 (M2) nucleoprotein (NP), non-structural protein 1 (NS1), non-structural protein 2 (NS2), polymerase acidic protein (PA), polymerase basic protein 1 (PB1), PB1-F2, PB1-N40 and polymerase basic protein 2 (PB2) (Chen *et al.*, 2001; Cox *et al.*, 2004; Hay *et al.*, 1977; Lamb and Choppin, 1979; McGeoch *et al.*, 1976; Wise *et al.*, 2009) (figure 1.1).

The Influenza A viral particle is roughly spherical (80-120 nm diameter) or filamentous (~100 nm x 2 to 20 µm) (Choppin *et al.*, 1960; Kilbourne *et al.*, 1960; Rossman *et al.*, 2010). Its external layer consists of around 500 spike-like projections, most of which are the HA and NA proteins with a few trans-membrane M2 ion channels (figure 1.1). The HA and NA proteins are the major targets of neutralising antibody. As a result of immune pressure, the antigenic characteristics of HA and NA would have evolved and mutated over time. Moreover, like other RNA viruses, the lack of proofreading function by the viral RNA polymerase provides an intrinsic and strong source of mutations during replication. This accumulation of mutations in the HA, and to a lesser extent NA, is called antigenic drift, which provoke the constant evolution of influenza A viruses and this makes necessary the continuous update of vaccine strains. Depending on the antigenic differences, influenza A virus can be further subdivided into several subtypes; so far, sixteen subtypes of HA (H1-H16) and 9 subtypes of NA (N1-N9) have been identified.

Phylogenetically, HA and NA subtypes each could be classified into two

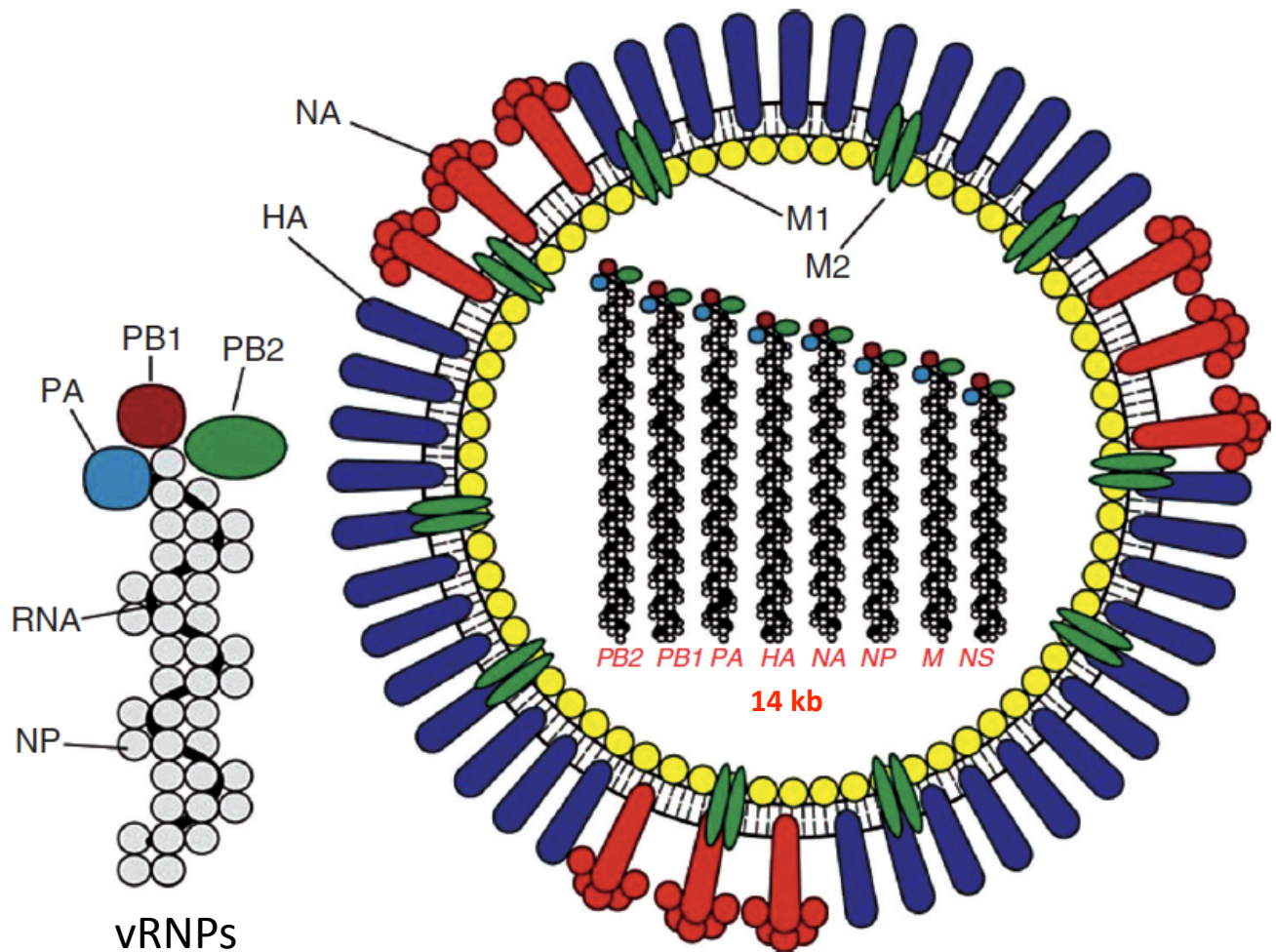


Figure 1.1. The structure of influenza A virus. HA, NA and M2 proteins are exposed on the outside of viral particles. The M1 proteins associate with the inner side of the envelope. The viral genome consists of eight negative-stranded RNA segments. The largest genomic segment is PB2 (2.3 kb), followed by PB1 (2.3 kb), PA (2.2 kb), HA (1.8 kb), NP (1.6 kb), NA (1.4 kb), M (1 kb), and NS (0.9 kb) gene, respectively. Each gene segment is packed as a helical ribonucleoprotein in complex with NP, PA, PB1 and PB2 proteins (vRNPs). [adapted from Cox *et al.*, 2004]

groups. For HA, group 1 contains H1, H2, H5, H6, H8, H9, H11, H12, H13 and H16, and group 2 contains H3, H4, H7, H10, H14 and H15 (Air, 1981; Nobusawa *et al.*, 1991). For NA, group 1 contains N1, N4, N5 and N8, and group 2 contains N2, N3, N6, N7 and N9 (Russell *et al.*, 2006).

All subtypes of influenza A virus can be found in avian species, mainly waterfowl and shorebirds (Webster *et al.*, 1992). In contrast, so far, only H1N1, H2N2 and H3N2 subtype influenza A viruses have naturally circulated and caused pandemic in human population (table 1.1). Human infections with other subtypes, i.e., H5N1, H7N7, H9N2, and H7N3, have been reported but they are very rare (Fouchier *et al.*, 2004; Peiris *et al.*, 1999; Peiris *et al.*, 2007; Tweed *et al.*, 2004).

1.2.1.2 Life cycle

Influenza virus infects the human mainly via the respiratory tract. The airway columnar epithelial cells are the main targets of the viruses. The viral HA proteins play a vital role in conducting infection, as their attachment to cellular receptors could bring about the endocytosis of viral particles.

The head region of HA protein binds the sialic acid on carbohydrate side chains of glycoproteins and glycolipids in the cell membrane. This initiates the endocytosis of viral particle and the formation of endosomes (Gamblin and Skehel, 2010).

Once the virus enters the infected cells, the fusion of the viral envelope with the endosomal membrane takes place within 20 minutes after infection (Lakadamyali *et al.*, 2003; Sakai *et al.*, 2006). This process is necessary for

Table 1.1. Influenza pandemics since 1918* (Reid *et al.*, 2004; SOIA Novel *et al.*, 2009; Taubenberger *et al.*, 2005; Zimmer and Burke, 2009)

Year	Subtype	Estimate Mortality	Origin of gene							
			HA	NA	PA	PB1	PB2	NP	M	NS
1918	H1N1	50~100 million	avian	avian	avian	avian	avian	avian	avian	avian
1957	H2N2	1~4 million	avian	avian	human	avian	human	human	human	human
1968	H3N2	1 million	avian	human	human	avian	human	human	human	human
2009	H1N1	~18000	swine	swine	avian	human	avian	swine	swine	swine

* It is debatable whether 1977 "Russian Flu" was a pandemic or not, since it affected primarily only children and young adults under 23 years old. The origin of 1977 influenza virus could be a reemergence of 1950 H1N1 strain.

the virus to deliver its genome into the cytoplasm and to begin its replication cycle. The fusion activity is linked with the creation of low pH environment in the endosomal lumen, and is also mediated by the fusion peptide at the stem region of HA. The optimal pH value for fusion activity varies with different influenza strains, but a range between pH 5 to pH 6 is deemed suitable for most of influenza viruses. The acidification of the viral interior is facilitated by the M2 ion channel prior to fusion of the viral and endosomal membranes. Such drop in pH initiates the dissociation of the M1 and viral ribonucleoprotein complexes (vRNPs), which allows the release of viral genome into the cytoplasm (Martin and Helenius, 1991; Pielak and Chou, 2011).

vRNPs form the core of virus and are made up of the viral RNA genome, NP, PA, PB1 and PB2 proteins (figure 1.1). The nuclear localisation signals of NP regulate the nuclear transport of vRNPs (Ozawa *et al.*, 2007). In the nucleus of infected cells, the replication and transcription of viral RNA begins with the help of PA, PB1, and PB2 proteins (Engelhardt *et al.*, 2005; Li *et al.*, 2001). At the same time, NP and NS1 proteins would inhibit the further processing of cellular mRNA in the nucleus (Lu *et al.*, 1994; Momose *et al.*, 2001; Shimizu *et al.*, 1999). This facilitates the influenza virus utilizing the host cell's transcription machinery for its own benefits.

The envelope proteins (HA, NA, M2) are then translated in the cytoplasm and transported to the apical cellular membrane. Besides, the NP protein might interact with nuclear export components and help with the export of newly synthesized vRNPs from the nucleus (Nayak *et al.*, 2004; Portela and Digard, 2002). The M1 protein and vRNPs would be brought to the assembly site, where the vRNPs are packed into progeny virions with the interaction of M1

and other envelope proteins (Nayak *et al.*, 2004). While the assembled viral particles bud out from the host cell, the envelope NA protein, the receptor-destroying enzyme, helps removing sialic acids between HA and the host cell membrane. This process is important for the successful release of new viral particles and their further infectivity with neighbouring cells (Palese *et al.*, 1974).

In general, the respiratory viral load may reach the peak 2 to 3 days after infection and is accompanied by the onset of clinical illness. The viral shedding usually lasts 5 to 7 days and subsides with the resolution of clinical illness (Harper *et al.*, 2005; Leekha *et al.*, 2007). However, prolonged viral shedding could be observed in patients with a more severe illness, longer hospital stay or immunosuppression (Giannella *et al.*, 2011; To *et al.*, 2010).

1.2.1.3 Molecular characteristics of HA

It is important to understand the structural basis of the influenza HA protein because: Firstly, the structural conformations of HA are tightly linked to its function in the influenza virus life cycle; Secondly, the HA protein serves as two functional roles, receptor-binding and membrane fusion. The HA protein therefore becomes the target of both the neutralising antibody and the fusion inhibitor (Russell *et al.*, 2008).

During the process of viral replication, the HA0 polypeptide chain is translated within the endocytosolic reticulum (ER) of the host cell. In the ER, protein-folding catalysts, calnexin and calreticulin, would help the assembly and folding of newly synthesized HA0 single chain (Gething *et al.*, 1986; Hebert *et al.*, 1996). Three of the newly synthesized HA0 chains then interact to

generate a HA0 trimer in the ER (Copeland *et al.*, 1988), where HA0 trimer is subsequently transferred through the Golgi apparatus to the host cell surface. Prior to release from the host cell, each HA0 polypeptides is cleaved to form a disulphide-linked HA1/HA2 subunit (Wilson *et al.*, 1981). The cleavage of the HA0 is required for the initiation of membrane fusion and viral infectivity (Wiley and Skehel, 1987). Besides, the characteristic of cleavage site is related to the viral virulence. The x-ray crystal structure of HA reveals that the cleavage site generally forms a loop that extends from the stem region of HA0 (Chen *et al.*, 1998) (figure 1.2). For HA of most subtypes, the cleavage site between HA1 and HA2 contains single arginine residue which can only be cleaved extracellularly; nevertheless, for certain H5 and H7 subtypes, their cleavage sites have acquired polybasic amino acids that could be cleaved by ubiquitous endoproteases, which may explain the high virulence and infectivity of H5 and H7 (Steinhauer, 1999). Lately identified human airway trypsin-like protease (HAT) and transmembrane protease serine S1 member 2 (TMPRSS2) in human airways show proteolytic activity to HA, which provides further molecular evidence of influenza infectivity in the human airway (Böttcher-Friebertshäuser *et al.*, 2010).

The HA1 subunit is a globular form protein outside the viral envelope and it contains the receptor-binding site and the major antigenic determinants. The receptor-binding site of HA1 is consisted of three conserved secondary structure, the 130-, 220- loops and the 190-alpha-helix at its edge, where the HA of virus would bind sialic acids of cell surface by hydrophobic interactions and by hydrogen bonds. Receptor-binding specificity and affinity are species-specific and tissue-linked (Rogers and Paulson, 1983; Shinya *et al.*, 2006;

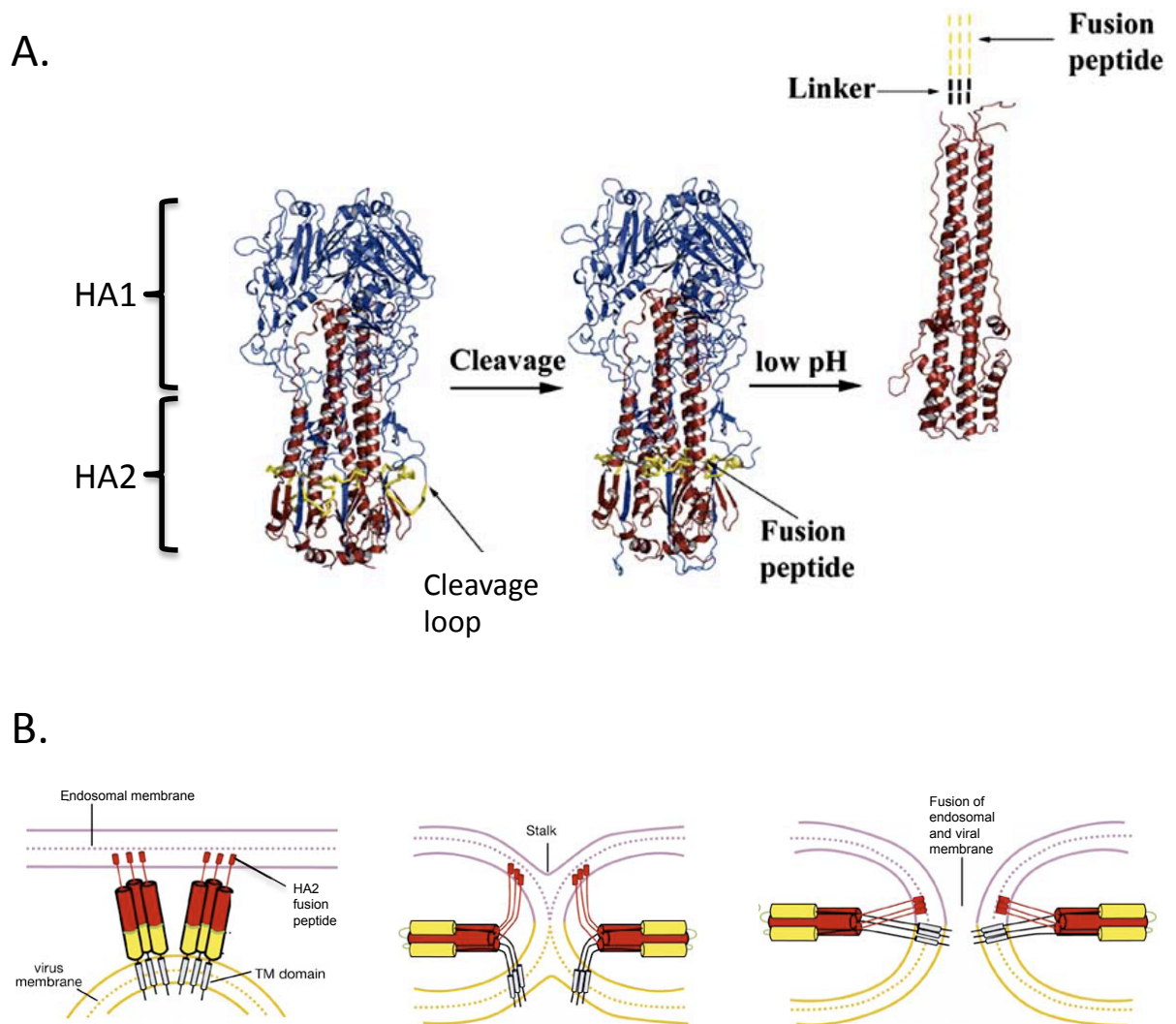


Figure 1.2. A. The structure of HA trimer. The HA1 subunit is depicted in blue, the HA2 subunit in red, and the fusion peptide in yellow. Before cleavage, the cleavage site forms a loop that extends from the stem region of HA0. The cleavage of HA0 liberates the fusion peptide of HA2, which reposition itself into the interior of HA0. At low pH, there is a conformational change that the fusion peptide becomes exposed and moves towards the endosomal membrane. B. The function of HA2 fusion peptide. The low-pH induced conformational change leads the fusion peptide to insert into the endosomal membrane. The pre-fusion stalk structure forms. Finally, the stalk melts and the membrane fusion completes. [adapted from Cross *et al.*, 2009]

Suzuki *et al.*, 2000). Avian influenza virus preferentially recognize sialic acids in α -2,3 linkage to galactose, which is predominantly displayed on avian intestinal tract. On the other hand, human influenza virus prefer to bind sialic acids in α -2,6 linkage to galactose, which is predominantly found on the human trachea and upper respiratory tract. Swine influenza virus could bind sialic acids in both linkage as both types of sialic acids could be found on the trachea of swine, which supported the theory that pigs might act as mixing vessels for both the avian and human influenza viruses (Ito *et al.*, 1998; Neumann *et al.*, 2009).

While the HA1 subunit is responsible for binding the host cell membrane, the HA2 subunit is responsible for the membrane fusion. A conserved and hydrophobic peptide, known as the fusion peptide, is a central motif in the membrane-fusion activity. In the uncleaved HA0, the fusion peptide is located at the bottom of cleavage loop. After cleavage, the fusion peptide would be precisely at the N-terminus of HA2, reposition itself to the interior of HA0 trimer and ready to conduct membrane-fusion activity (figure 1.2). Once the optimal low-pH environment is present, the fusion peptide would extrude from the interior position into the endosomal membrane, followed by the irreversible change of HA0 conformation and the formation of membrane fusion (Cross *et al.*, 2009; Maeda *et al.*, 1981; Skehel *et al.*, 1982) (figure 1.2).

1.2.2 Clinical manifestations

Influenza A virus is one of leading causes of infectious disease, mainly causing acute respiratory infection. In most infected individuals, acute respiratory illness with or without fever occurs; however, severe illness, i.e.

pneumonia, acute respiratory distress syndrome with high morbidity and mortality, could develop within elders, infants and people with chronic disorders (Harper *et al.*, 2005).

Upon acute influenza infection, bacterial super-infection is the well-known clinical complication and also one of the main causes of hospitalization and mortality. The most common infection-causing bacteria is *Streptococcus pneumoniae*, followed by *Staphylococcus aureus*, *Haemophilus influenzae*, and Group A Streptococcus (Harper *et al.*, 2005; Fiore *et al.*, 2010). The epidemiological evidence could trace back to 1918 flu, but the mechanism is poorly defined (Oxford, 2000). In mice model, it was suggested that influenza infection resulted in the removal of sialic acid or the alteration of receptor on respiratory epithelium, which allow the increased adherence of pneumococcus (McCullers and Rehg, 2002; Plotkowski *et al.*, 1986). The other study suggested that influenza infection causes the impaired NK cell response in lower respiratory tract, which might predispose the host to secondary bacterial infection (Small *et al.*, 2010).

Neurological or gastrointestinal involvement was less seen in acute influenza infection. The gastrointestinal symptom could occur in avian H5N1 or swine influenza infection, but its cause remains undetermined.

1.2.3 Transmission

From human-to-human, influenza A virus is believed to transmit primarily through droplet and contact, although aerosol transmission is also a rarer possibility (Brankston *et al.*, 2007; Lowen *et al.*, 2006; Mubareka *et al.*, 2009). The transmission model is mainly provided in animal experiments. Generally,

influenza viruses infect human via the respiratory tract. In 1933, Wilson Smith intra-nasally challenged ferrets with human influenza viruses, demonstrating the causative origin of flu. Several human challenge studies also support the establishment of infection via intranasal inoculation of virus (Clements *et al.*, 1986; McMichael *et al.*, 1983; Treanor *et al.*, 1999; Treanor and Wright, 2003).

The human-to-human transmission is essential to bring about the outbreak of influenza in the community. On the other hand, animal-to-human transmission could be relevant to the emergence of adaptive influenza strains, to which most human have no pre-existing immunity.

Influenza A viruses could infect humans, birds, ducks, chickens, pigs, whales, horses, seals and other animals (Webster *et al.*, 1992). Interspecies transmission of influenza A virus, e.g., pig-to-human or bird-to-human transmission, is rarely reported. There is much debate about the transmission route, most possibly through contact spread

(<http://www.cdc.gov/flu/avian/gen-info/transmission.htm>). Although the animal-to-human transmission is less seen, it could lead to the spread of the reassorted influenza strain in human population. The exchange of genetic segments among influenza subtypes, i.e. genetic reassortment, a new and dangerous strain to which humans have no immunity may emerge. The reassortment could occur between human and bird influenza viruses in infected pigs, or between human and bird influenza viruses in infected humans. For instance, a reassorted influenza A virus consisting of a mix of swine, avian, and human viral gene segments emerges and causes a worldwide infection in 2009 (Zimmer and Burke, 2009) (table 1.1).

1.2.4 Epidemiology

The epidemiology of influenza infection usually presents in three forms. The endemic is mostly seen and usually results from the circulating seasonal strains which typically occurs between November and March in the northern hemisphere, and between April and September in the southern hemisphere (Viboud *et al.*, 1992). 5-15% of the world populations are affected with influenza illness in annual epidemics. The annual epidemics lead to the estimate of five million cases of severe illness and up to 500000 deaths around the world, according to the World Health Organization's (WHO) data (<http://www.who.int/mediacentre/factsheets/2003/fs211/en/>).

A pandemic may occur when a new influenza virus emerges against which the human population has no immunity. This new virus might begin with an epidemic in which the more cases and the quicker spread of infection are noted than an endemic, but an epidemic is generally restricted in a specific area or among a certain population group. However, a pandemic is a worldwide epidemic, which spreads quickly over a very wide geographical area and affects a huge number of people around the world.

Since 1918, there have been four known pandemics caused by influenza A viruses (table 1.1). 1918 pandemic leads to up to 500 million people infected, at least 20 million died and huge economic losses (Zimmer *et al.*, 2009). Not far ago, a novel swine-origin influenza A (H1N1) virus emerges early 2009, leads to a worldwide pandemic and results in over ten thousands death among population (SOIA Novel *et al.*, 2009).

1.2.5 Vaccination

At the present time, the primary strategy for reducing the effect of influenza is immunoprophylaxis with vaccine. Annually, WHO makes recommendations on the composition of the influenza vaccine in February for northern hemisphere and in September for southern hemisphere. There are more than 200 million vaccine doses manufactured each year based on the WHO recommendation. The major types of influenza vaccine licensed for human use are inactivated and live-attenuated vaccines. For decades, the annual seasonal influenza vaccine contains three circulating viral strains. For instance, the influenza vaccine used in the 2011-2012 northern hemisphere influenza season would contain an A/California/07/09 (H1N1)-like virus, an A/Perth/16/09 (H3N2)-like virus and a B/Brisbane/60/08-like virus.

1.2.5.1 Inactivated influenza vaccine

The first influenza pandemic was recorded in 1580, and the 1918 pandemic resulted in overwhelming morbidity and mortality around the world. However, not until 1933, the etiology of flu was not firstly identified. It was proved with modified Koch's postulates on ferret model at Rhodes Farm, Mill Hill (Smith *et al.*, 1933). Following the isolation of influenza virus and the development of viral culture techniques, several experimental influenza vaccines were produced late 1930s. Inactivated influenza vaccine has been available since the 1940s, and become the mainstay of seasonal influenza prevention, mainly due to its safety and stable effectiveness (Kilbourne *et al.*, 1974; Plotkin *et al.*, 2008).

1.2.5.1.1 Safety

The most common adverse effects of inactive influenza vaccines are local redness and sore arm of the injection site. These local reactions are usually mild and resolve within 2 days. The systemic reactions, such as fever, myalgia, arthralgia and headache, are rare.

With regard to Guillian–Barré Syndrome (GBS), a rare neurological disorder affecting the peripheral nerve system, has been associated with receipt of 1976 swine influenza vaccine. GBS is characterised by progressive ascending paralysis, paresthesia and dysesthesia and the underlined mechanism for association of GBS and inactivated influenza vaccine remains unclear. The current study reports that the incidence of GBS is approximately one case per million persons vaccinated (Juurlink *et al.*, 2006; Fiore *et al.*, 2010). In view of the substantially higher incidence of influenza-associated hospitalisation in high-risk groups (e.g., ~150 per 100,000 children under the age of 2), annual influenza vaccination is still recommended to those who are at high risk of severe influenza infections (Fiore *et al.*, 2010; Harper *et al.*, 2005).

1.2.5.1.2 Immunogenicity

Influenza vaccine primarily induces antibodies against influenza envelope proteins, HA and NA. Anti-HA antibodies, the main component of humoral immunity against influenza, could inhibit the infectivity of influenza virus and protect from illness (Skehel and Wiley, 2000). Anti-NA antibodies could not inhibit infection but may reduce the disease severity (Bright *et al.*, 2008).

Serum anti-HA antibody response is commonly measured by the haemagglutinin inhibition (HI) test and generally used to validate the immunogenicity of inactivated influenza vaccines. To meet the criteria for

influenza vaccine licensing in adults, one of the following three requirements must be achieved: (i) seroprotection, i.e., achievement of an HI titre of $\geq 1:40$ in $>70\%$ of subjects; (ii) seroconversion, i.e., a >4 -fold increase in the HI antibody titre, or reaching a titre of $>1:40$, in $>40\%$ of subjects; and (iii) a >2.5 -fold increase in geometric mean titres (GMT), according to the note of the European Union Committee for Human Medicinal Products and the European Centre for Disease Prevention and Control.

In general, the antibody response induced by inactivated influenza vaccine is strain-specific. This means that the vaccine strain needs constant update to provide the prompt protection, especially while novel or drifted strain emerges. It has been noted the antibody response to inactivated influenza is affected with age, with lower response among the elderly and the children. Currently, two doses of inactivated vaccine are recommended for children less than 9 years old, since some children in this age are relatively naïve to circulating strains of seasonal influenza virus. A higher dose vaccine (Fluzone high dose) is approved by the U S Food and Drug Administration (FDA) in 2009, which is an alternative inactivated vaccine for persons aged ≥ 65 years, and could induce a significant higher antibody response than standard vaccine in elders (Falsey *et al.*, 2009).

Inactivated vaccine could be poor immunogenic to naïve population.

Compared to one dose of $15\mu\text{g}$ HA standard seasonal vaccine, two $30\mu\text{g}$ doses of H5N1 vaccine are unable to induce acceptable antibody response in adults (Bresson *et al.*, 2006). A booster dose with either adjuvant or high dose HA is necessary to overcome this obstacle (Bresson *et al.*, 2006; Lin *et al.*, 2006; Treanor *et al.*, 2006).

The use of adjuvant could improve the immunogenic performance of influenza vaccines (Dormitzer *et al.*, 2011). The adjuvant-enhanced immune response is possibly related to the enhancement of antigen presentation by recruiting inflammatory cells and activation their innate immune signals. For instance, the MF59 adjuvant significantly enhances the immunogenicity of influenza vaccines in the elderly, who typically respond poorly to traditional influenza vaccines, owing to age-related impairment of their immune systems (immunosenescence) (Goodwin *et al.*, 2006). Recently, MF59 has been shown to be safe in a seasonal influenza vaccine in infants and children and increased vaccine efficacy from 43 to 89% (Vesikari *et al.*, 2009a; Vesikari *et al.*, 2009b). The adjuvanted H5N1 vaccine could not only increase the antibody level and amplify the antibody repertoire against HA and NA, but also induce the higher and broader influenza-specific CD4 T cell response (Galli *et al.*, 2009b; Khurana *et al.*, 2010).

1.2.5.1.3 Efficacy and effectiveness

In general, the seasonal inactivated vaccine could achieve satisfactory efficacy rate in human (Bridges *et al.*, 2000; Fiore *et al.*, 2010). In a randomized, double-blinded and placebo-controlled study, the estimated efficacy of inactivated influenza vaccine against nasal challenge of wild-type virus was about 70% (Treanor *et al.*, 1999). Recently, several studies demonstrate that the inactivated influenza vaccine could effectively prevent infection by 70~80% (Castilla *et al.*, 2011; Song *et al.*, 2011).

The efficacy or effectiveness of the inactivated influenza vaccine is mainly affected by age, immune status of the vaccinee and the antigenic match

between vaccine strains and circulating strains (Bridges *et al.*, 2000; Fiore *et al.*, 2010; Govaert *et al.*, 1994; Jefferson *et al.*, 2008; Jefferson *et al.*, 2010; Rivetti *et al.*, 2006). It has been reported that unmatched vaccine has poor efficacy against influenza illness (Bridges *et al.*, 2000). The efficacy has also been reported lower in elders, though the randomized vaccine trial for elders is few. A randomized, double-blinded and placebo-controlled study shows that the efficacy against influenza illness is less than 60 % in the elders aged over 65 (Govaert *et al.*, 1994). In children, the influenza vaccine is efficacious to those older than two years old, but there is not enough evidence for those less than two years old (Jefferson *et al.*, 2008).

Theoretically, the most persuasive evidence of vaccine efficacy should be gathered using randomized and placebo-controlled trial combining with the outcome measurement of laboratory-confirmed influenza illness. However, it has to be pointed out that, for those recommended to receive vaccine annually, such vaccine trials are usually unable to conduct ethically. The important vaccine effectiveness or efficacy data mainly come from observational studies that assess outcomes of laboratory-confirmed influenza illness.

1.2.5.2 Live attenuated influenza vaccine

Initially, the influenza vaccine studies are merely conducted on animals. Wilson Smith and Thomas Francis demonstrated that animals (ferret and mice) immunized with live influenza virus could generate neutralising antibodies and gain protection against severe illness of flu. Very soon, two

human vaccine trials were conducted in USA, showing protective potential of live influenza vaccines (Francis and Magill, 1937; Stokes *et al.*, 1937).

At the early stage of influenza vaccine development, the live vaccine is the favor due to its relatively small dosage, being painless, and mucosal immunogenicity (Kilbourne *et al.*, 1974). However, the selection of attenuated live virus is tedious and unpredictable (Tyrrell and Beare, 1969). The attenuated influenza virus is acquired by serial passage (Burnet and Bull, 1943), which would usually reduce the human pathogenicity and infectivity, but enhanced virulence of passaged virus has also been described (Beare *et al.*, 1968). There lacks the reliable laboratory marker to monitor the antigenic and virulent changes of passaged virus, but only depending on volunteer trials.

The hybrid (recombination) technique uses an avirulent influenza virus, A/PR/8/34 (H0N1), and a virulent influenza virus harboring the surface antigens of the virulent strain, sufficiently produces the safe and attenuated vaccine strain. This technique provides the quickest known method of attenuation for influenza vaccine strain (Kilbourne, 1969, Beare and Hall, 1971; McCahon and Schild, 1972). Nasal inoculation of live attenuated influenza vaccine confers a potent protection (Beare *et al.*, 1968). The first vaccine trials using live-attenuated recombinant virus in adults demonstrated the satisfactory serum and nasal antibody response (Minor *et al.*, 1975).

The development of temperature-sensitive, cold-adapted viral strain also promotes the generation of stable and safe live attenuated influenza vaccine (Maassab, 1967; Maassab, 1969; Maassab *et al.*, 1982). This live vaccine

virus grow well at 25°C and poorly at 37°C, which allows its efficient replication in the nasal cavity and also prevents its growth in the lower airway of vaccinee, successfully making the live influenza vaccine both immunogenic and safe. In 2003, a cold-adapted, live-attenuated influenza vaccine was therefore firstly licensed by FDA (Fiore *et al.*, 2009; Plotkin *et al.*, 2008).

In brief, so far, trivalent inactivated (TIV) and cold-adapted, live-attenuated vaccines (CAIV) are two major types of commercial influenza vaccines. They have different routes of administration and different age groups they are approved for. A precise correlation between protective immunity and serum HI antibody for the CAIV remains to be determined (Beyer *et al.*, 2002; Clover *et al.*, 1991). The CAIV could elicit significant nasal IgA antibody and cytotoxic T cell response, which may provide protection (Belshe *et al.*, 2000; Beyer *et al.*, 2002). Generally, they have no significant overall difference in immunogenicity or efficacy (Beyer *et al.*, 2002; Dormitzer *et al.*, 2011). However, CAIV is reported to be more efficacious in children or in those having prior flu exposure (Eick *et al.*, 2009; Rhorer *et al.*, 2009).

1.3 IMMUNE RESPONSES TO INFLUENZA A VIRUS

Although annual vaccination greatly reduces the burden of influenza disease and related death, the newly emerging strains that are not covered by annual vaccination component continues to threaten the public health. After the influenza vaccine was introduced in 1950s, pandemics of worldwide scale still occurred in 1957, 1968 and 2009, besides, epidemics also occurred unexpectedly. For example, in 2007-2008 flu season, a newly drifted A/Brisbane/10/07-like virus caused the epidemic and the influenza vaccine

that year was less than 50% effective (CDC, 2008). Early 2009, a triple-reassortant H1N1 influenza A virus of swine origin brought about a pandemic and the previous seasonal vaccination confers very limited protection (CDC, 2009a; CDC, 2009b).

In the protective immunity against influenza, neutralising antibodies against envelope antigens, induced by vaccination or natural infection, play a critical role and also serve as a protective parameter in vaccination. However, in the absence of neutralising antibody when new viral strains emerge or diminished antibody level, innate immunity and T cell mediated immunity are critical in mitigating virus shedding and disease severity.

1.3.1 Innate immunity

Innate immune system is the first line of defense against influenza A virus, despite its lack of specificity and memory. Natural killer (NK) cells, alveolar macrophage and dendritic cells play the major role in the control of viral replication and regulation of adaptive immune responses following the influenza A infection (McGill *et al.*, 2009). These innate immune cells attack influenza virus mainly through the potent cytotoxic activities and robust production of inflammatory cytokines. It has been shown NK cells recognize virus-infected cells through the natural cytotoxicity receptors NKp46 and NKp44 and help to clear infected cells (Mandelboim *et al.*, 2001; McGill *et al.*, 2009). The alveolar macrophages and dendritic cells are recruited in the airways upon influenza infection. Alveolar macrophages help to clear apoptotic host cells and limit viral spread and dendritic cells, with the primary function of antigen presentation, play an important role in connecting the

innate and adaptive immune response against virus (Hashimoto *et al.*, 2007). The antiviral response are mainly initiated upon the interaction between the pathogen-associated molecular patterns (PAMPs), such as viral nucleic acids, and pattern recognition receptors (PRRs), including Toll-like receptors (TLRs), retinoic acid-inducible gene 1 proteins (RIG-1), and the NOD-like receptors (NLRs) (Ehrhardt *et al.*, 2010; Wolff and Ludwig, 2009). The mechanisms of viral recognition leading to cytokine production are largely unclear. TLRs have been explored as key transducers of type I interferon (IFN) during viral infections. The expression of IFN- α and IFN- β could induce the transcription of several antiviral acting genes, many of which protect uninfected cells upon the infection (Ehrhardt *et al.*, 2010). However, the excessive innate immune response could result in enhanced morbidity and mortality. Dysregulated cytokine response observed in patients with high pathogenic H5N1 infection is suggested to mediate the lung pathology following infection, although the underlined mechanism remains unclear (de Jong *et al.*, 2006; Peiris *et al.*, 2004; Peiris *et al.*, 2009).

1.3.2 Adaptive immunity

In contrast to the constitutive and non-specific response conferred by innate immunity, the adaptive immunity involves a regulated response between antigen-presenting cells and T and B lymphocytes, and the generation of antigen-specific immunological memory. T and B cells are the major effector cells of adaptive immunity, which is composed of cellular immunity and humoral immunity, respectively. T and B cells mature in thymus or bone marrow, where through the somatic rearrangement of the array of gene segments, a broadly diverse repertoire of receptor capable of recognizing

potentially various antigens is generated. The mature T and B cells interact with antigens in the secondary lymphoid organs and develop into antigen-specific memory cells. Upon encountering subsequent stimulation of the same antigen, the memory cells are quickly activated and provide a robust protective response (Bonilla and Oettgen, 2010; Sallusto *et al.*, 2010).

1.3.2.1 T cell response

Mature T cells are activated through the recognition of processed peptides bound to the major histocompatibility complex (MHC) molecules by T cell receptors. For instance, CD4 T cells respond to 12-18 mers peptides complexed with MHC-class II molecules, while CD8 T cells to 9- or 10-mer peptides complexed with MHC-class I (Stern *et al.*, 1994; Townsend *et al.*, 1985). Importantly, it has been observed influenza-specific CD8 T cells recognize relevant peptide antigen bound to MHC-class I through heterodimeric T cell receptor, which in turn triggers the specific lysis of virus-infected cells and the secretion of cytokines (Bastin *et al.*, 1987; Townsend *et al.*, 1985).

Both CD4 and CD8 T cells mediate the control of influenza infection (Woodland, 2003). In the first few days of infection, CD8 T cell can confer partial protection against influenza virus by promoting viral clearance and reducing disease severity in the absence of antibody immunity (McMichael *et al.*, 1983). Upon infection, CD4 T cells promote the generation of virus-specific CD8 T cells. Besides, CD4 T helper 1 (Th1) cells are thought to provide protective immunity by modulating anti-viral cytokine response. Importantly, pre-existing CD4 T cells could be cytotoxic and associated with lower virus

shedding and less severe illness in human challenged with influenza virus (personal communication with Cecilia Chui).

T cell response might contribute to the cross-protection against influenza viruses. A study using archival records before and during the 1957 H2N2 pandemic, demonstrated that adults with previous flu history had a significantly lower incidence of H2N2 infection than children. Since H2N2 influenza virus was an emerging new subtype (during the pandemic which a shift from subtype H1N1 to H2N2 occurred), the immunity induced by earlier strains was less possibly to be H2N2-specific in those adults. Moreover, no difference of pre-existing antibody level was found among adult and children population. Other protective factors, such as T cell immunity established by previously accumulated infections are thought to play a role in the cross-reactive immunity (Epstein *et al.*, 2006).

1.3.2.2. Humoral response

The development of antigen-specific B cell takes place in secondary lymphoid organs where mature B cells are stimulated by antigen presenting cells bearing viral antigens. Primed B cells interact with follicular helper T cells and then develop into ASC and MBC (Crotty and Ahmed, 2004; Lambert *et al.*, 2005; LeBien and Tedder, 2008; McHeyzer-Williams and McHeyzer-Williams, 2005; Pelletier and McHeyzer-Williams, 2009) (figure 1.3). ASC are the primary source of serum antibody and could generate up to 20,000 molecules of immunoglobulin (Ig) per second (Amanua and Slifka, 2011; Helmreich *et al.*, 1961; Helmreich *et al.*, 1962). MBC circulate between peripheral blood and secondary lymphoid tissues, and could rapidly differentiate into ASC when the

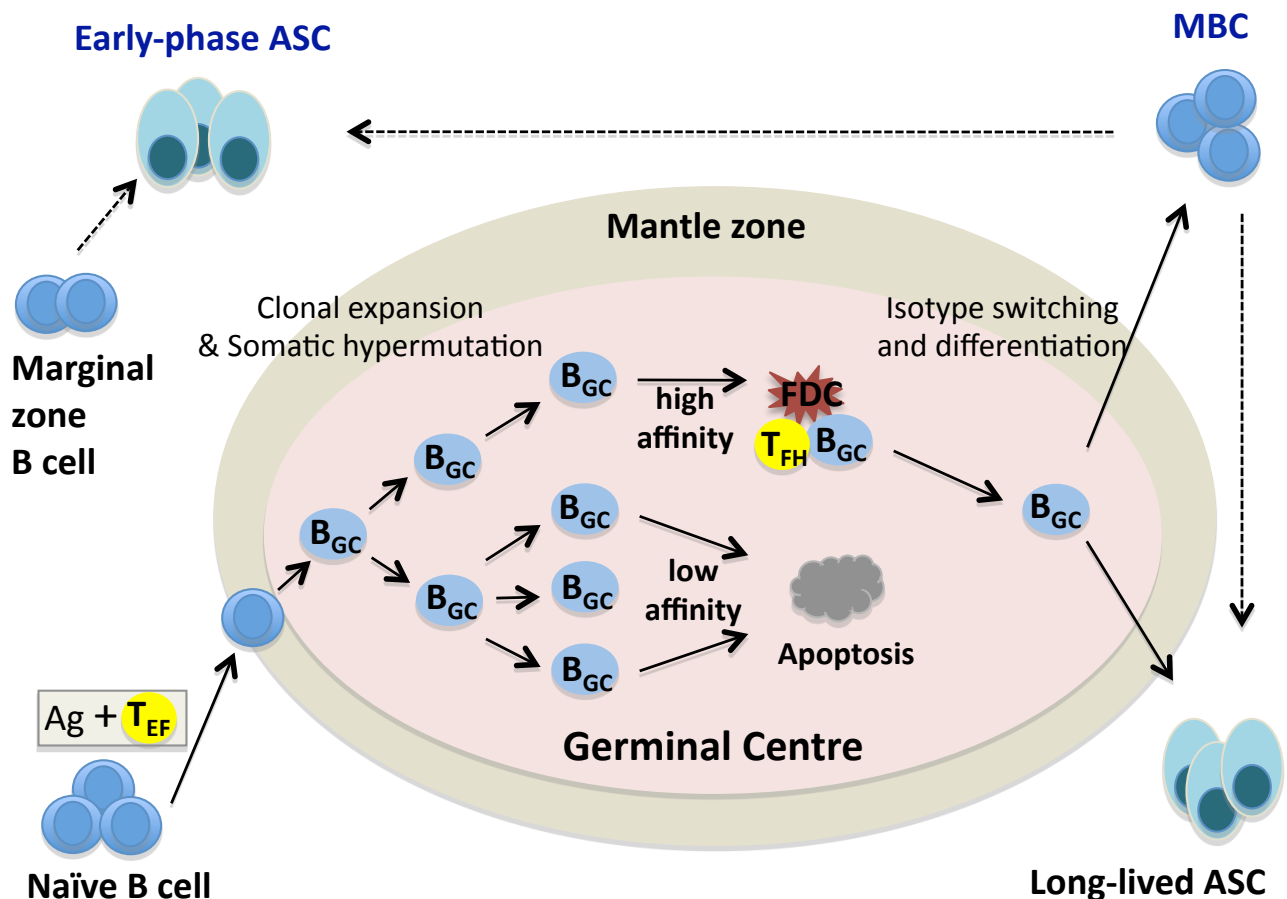


Figure 1.3. Development of antigen-specific MBC and ASC in the T-cell dependent humoral response. In the secondary lymphoid organs (spleens, mucosal-associated lymphoid tissues and lymph nodes, etc.) , mature naïve B cells are activated initially by antigens (Ag) and extrafollicular T cells (T_{EF}), which leads to the germinal centre development. During the germinal centre reaction, antigen-primed B cells (B_{GC}) undergo clonal expansion and somatic hypermutation followed by affinity maturation with the help of follicular dendritic cells (FDC). Follicular helper T cells (T_{FH}) promote the formation of germinal centre, the isotype switching of germinal centre B cells and the differentiation of high-affinity B cells into MBC and ASC. MBC circulate between peripheral blood and secondary lymphoid tissues, might replenish long-lived ASC and might rapidly differentiate into early-phase ASC when the host encounters the same antigen in the future (Crotty and Ahmed, 2004; Lambert *et al.*, 2005; LeBien and Tedder, 2008; McHeyzer-Williams and McHeyzer-William, 2005; Pelletier and McHeyzer-William, 2009). In mice, marginal zone B cells play a role in the induction of early-phase ASC to influenza virus. However, the importance of marginal zone B cells in human viral infection remains undetermined (Weill *et al.*, 2009).

host encounters the same viral pathogen in the future (LeBien and Tedder, 2008).

1.3.2.2.1 Antibody mediated immunity

In 2009, swine-origin H1N1 pandemic causes disproportionately higher mortality and hospitalization rates among young people, compared with the elders aged over 65 (CDC, 2010). This phenomenon highlights the importance of pre-existing antibody immunity in elders born before 1950 (Hancock *et al.*, 2009; Xu *et al.*, 2010). The role of antibody immunity is further emphasized by the experiences from successful passive immunotherapy in the treatment of severe influenza illness. Previously, there had been the meta-analysis report or case report suggesting that the convalescent plasma therapy was associated with reduced morbidity and mortality among severe influenza patients (Luke *et al.*, 2006; Zhou *et al.*, 2007). Lately, a multicentre and case-control study demonstrates that the convalescent plasma containing high-titre neutralising antibodies could effectively reduce both the respiratory viral load and the mortality of severe patients (Hung *et al.*, 2011). These results not only support a viable option for the treatment of influenza A infection, but also indicate a future need of antibody-based therapy.

It has been mentioned previously, that the envelope HA protein is the target of anti-influenza neutralising antibodies (figure 1.4). The molecular interaction between the monoclonal neutralising antibody (Fab fragments) and the HA protein have been previously described (Bizebard *et al.*, 1995; Fleury *et al.*, 1999; Karlsson Hedestam *et al.*, 2008; Knossow *et al.*, 2002; Xu *et al.*, 2010).

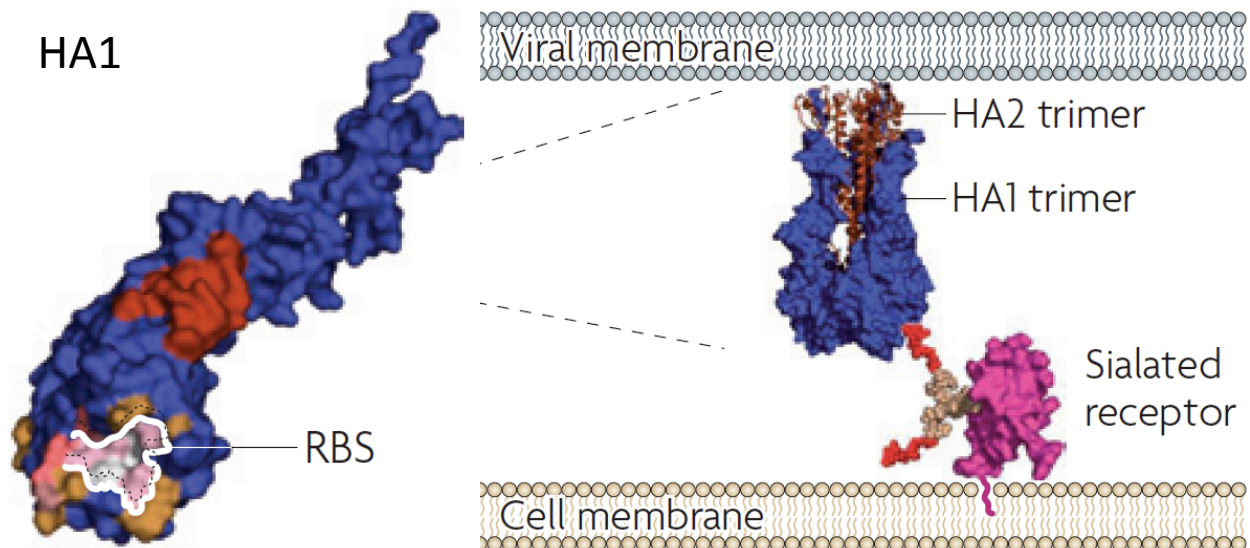


Figure 1.4. The receptor-binding site (RBS) and the antibody-binding sites (or the antigenic determinants). The influenza HA1 trimer binds sialic acids on host cell membrane and initiates the viral infection. Following the endocytosis of influenza virus, the HA2 trimer mediates the fusion of endosomal and viral membrane, which allows the viral entry. In the humoral immunity against influenza virus, the HA1 protein is the main target of anti-influenza antibodies. Several antibody-binding sites have been reported and are depicted in pink, brown, white, or red color in the left figure. The receptor-binding site is highlighted as well. The anti-influenza antibody binds over the receptor-binding site, could interfere the virus-cell attachment and therefore inhibits the viral infectivity. [adapted from Karlsson Hedestam *et al.*, 2008]

The crystallographic study shows that the antibody binds over the receptor-binding site, and therefore blocks the virus-cell attachment. The inhibition of virus-cell attachment could lead to the loss of viral infectivity *in vitro* (Knossow *et al.*, 2002). The monoclonal neutralising antibody, which binds the distant receptor-binding site, could neutralise viral infectivity as well, while the size of antibody is large enough to interfere with the host cell receptor.

Despite the presence of neutralising antibodies against receptor-binding HA subunit, the influenza virus could have continuously evolved through antigenic drift and escaped from the existing antibody immunity. The majority of escape mutants contain single amino acid substitutions at the HA1 subunit. The mutation could have increased the binding avidity between the mutant virus and host cell surface, and allowed the escape of the mutant virus from polyclonal neutralizing antibodies simultaneously (Hensley *et al.*, 2009).

The mutation could also prevent the virus from antibody recognition by steric interference or bury the antigenic epitope by introducing a new oligosaccharide attachment (Bizebard *et al.*, 1995; Fleury *et al.*, 1998; Skehel and Wiley, 2000; Wilson and Cox, 1990). The regular recurrence of influenza endemics or epidemics further addresses the battle between the antibody immunity and the evolving influenza viruses. Therefore, there is always the urgent need to identify the antibodies that broadly neutralise different strains and subtypes of influenza virus. C179 antibody is a traditional mAb derived from hyperimmunized mice, which recognizes the HA2 subunit and neutralises both H1 and H2 influenza A strains by inhibiting the membrane fusion (Okuno *et al.*, 1993). Lately, several human mAb generated from MBC or the phage display library are able to neutralise different subtypes of

influenza virus, including H1, H2, H5 and H9 subtypes (Corti *et al.*, 2010; Ekiert *et al.*, 2009; Sui *et al.*, 2009; Throsby et al, 2008). All these broadly cross-reactive antibodies bound to the HA2 subunit and are shown to block the acid-sensitive membrane fusion. In the present, the protective immunity induced by vaccination or infection is primarily determined as a serum antibody titre $\geq 1:40$ on HI assay (Dowdle *et al.*, 1973). However, the HI assay essentially only measures the antibodies that prevent the attachment of virus and host cells. Therefore, the HI assay used as the standard test for evaluation of the vaccine-induced antibody response could have overlook those broadly neutralising antibodies. An efficient and specific assay to examine the highly cross-reactive antibody to various strains or even various subtypes of influenza A viruses is still lacking, despite considering the currently most used methods, such as enzyme-linked immunosorbent assay (ELISA) and the pseudovirus assay.

A limited number of studies have demonstrated the cross-reactive neutralising antibodies elicited by inactivated influenza vaccination or natural infection in human. Nevertheless, there is much debate about their breadth and magnitude within the elicited antibody repertoire (Corti *et al.*, 2010; Wrammert *et al.*, 2011). More studies should be conducted to explore the development and function of the cross-reactive antibody response at the cellular level, which would be of value to the future design of influenza vaccines.

1.3.2.2.2 The role of ASC

The mechanism of maintaining protective antibody level upon influenza infection or vaccination remains controversial. It is generally believed that

ASC and MBC play a crucial role in the sustained antibody immunity (Bernasconi *et al.*, 2002; Slifka and Ahmed, 1998).

ASC are the primary source of antigen-specific antibodies and there are generally two phases of ASC response upon antigen encounter (Shapiro-Shelef and Calame, 2005). In animal model, antigen-specific ASC are quickly detected in spleen and local lymph nodes one week after viral infection, which would decline within weeks, followed by a significant increase of antigen-specific ASC in bone marrow (Ho *et al.*, 1986; Slifka and Ahmed, 1998; Smith *et al.*, 1997). The expression of chemokine receptors and integrins on the ASC are important for their fate of migration site. Expression of C-X-C chemokine receptor (CXCR) type 4 and 6 are crucial for ASC homing to bone marrow, where stroma cells express the ligand, CXCL type 12 and 16 (Hargreaves *et al.*, 2001; Kunkel and Butcher, 2003). The IgG ASC express $\alpha_4\beta_1$ -integrin, facilitating their migration to bone marrow, where the ligand, vascular cell adhesion molecule 1 (VCAM1), is expressed. The IgA ASC expressing $\alpha_4\beta_7$ -integrin would preferentially stay at mucosal area where the ligand, mucosa vascular addressin cell adhesion molecule 1 (MADCAM1), is expressed (Kunkel and Butcher, 2003). In animal model, it has been demonstrated that the ASC residing in bone marrow niches could survive from months to years upon their activation and continue secreting protective antibodies without repeated exposure to antigen or help of MBC (Ahuja *et al.*, 2008; Manz *et al.*, 1997; Slifka *et al.*, 1995; Slifka and Ahmed, 1998). The molecular mechanism that allows the prolonged survival of ASC remains unclear (Radbruch *et al.*, 2006). It is suggested that B cell-activating factor of the TNF family (BAFF) and a proliferation inducing ligand (APRIL) are not only

important to the development of peripheral B cells, but also crucial to the transduction of survival signals to ASC. The interaction between the B cell maturation receptor (BCMA) and BAFF/APRIL ligands might act as a key survival mechanism for ASC (Benson *et al.*, 2008; Darce *et al.*, 2007; O'Connor *et al.*, 2004). Moreover, the expression of B-lymphocyte induced maturation protein-1 (Blimp-1) is found critical for both the development and survival of ASC (Kallies and Nutt, 2007; Radbruch *et al.*, 2006; Shapiro-Shelef *et al.*, 2005).

Before 1990s, there was an immunological dogma suggesting that ASC are all short lived (days to weeks), which has been continuously challenged by the demonstration of long-lived ASC in the bone marrow and also the life-long protective antibody level elicited by measles vaccination, yellow fever, or smallpox infection. In fact, this previously established paradigm is based on the studies focusing on the early phase of humoral response (Makela and Nossal, 1962; Nossal and Makela, 1962). In animal, these early-phase ASC were found in the local lymph nodes within one week upon vaccination (Nossal and Makela, 1962). In human, the early-phase ASC response could be detected in the peripheral blood, by first week upon many vaccine antigens, including influenza vaccination (Cox *et al.*, 1994; Kelly *et al.*, 2009; Lee *et al.*, 2011; Nieminen *et al.*, 1998; Wu *et al.*, 2011). These ASC are thought to differentiate from memory or naïve B cells activated at the site of vaccination and the draining lymph nodes. Around 6-8 days after vaccination, these ASC leave the lymph nodes and transiently appear in the circulation. It was suggested that these cells would migrate to specific sites and tissues of the body, including the bone marrow niches, as previously described. It

remains unclear if the generation of early-phase ASC undergoes further somatic hypermutation (Frölich *et al.*, 2010), though they could be highly somatic mutated and secrete high-affinity antigen-specific antibodies (Wrammert *et al.*, 2008).

In contrast to the ASC residing in the local tissue or bone marrow, the readout of early-phase ASC is much easier, though the timing of collecting samples is crucial. The peripheral blood mononuclear cells could be utilized to examine the kinetics and magnitude of early-phase ASC by B-cell Enzyme-linked immunosorbent spot (ELISpot) and flow cytometry assay. The early-phase ASC open a window to examine the overall B cell response to vaccination or infection. While these ASC are isolated and cultured *ex vivo*, secreted antibodies would exclusively represent recently induced humoral response without the interference of pre-existing antibody level. It has been reported that the ASC collected on day 7 after influenza vaccination are able to produce neutralising antibodies, presenting the microneutralisation (MN) titre as high as 1:320 (He *et al.*, 2011).

In addition, the isolation of early-phase ASC would allow the analysis of the Ig repertoire of vaccine or infection-activated B cells. A recently developed strategy combining the single cell reverse transcription polymerase chain reaction (RT-PCR) of Ig gene and the expression vector cloning allows the efficient production of human recombinant mAb for functional analysis (Smith *et al.*, 2009; Tiller *et al.*, 2008). A few studies have focused on making a panel of recombinant mAb from early-phase ASC induced by influenza vaccination or infection (Wrammert *et al.*, 2008; Wrammert *et al.*, 2011). Considering the continuing threat of pandemic, the intrinsic limitation of vaccine components,

and the potential need for antibody-based therapy, there should be more effort to explore the characteristics of early-phase ASC, to generate plasma cell-derived mAb, and to push the clinical application of influenza neutralising antibodies.

1.3.2.2.3 The role of memory and other B cell

Based on the cell surface markers, human peripheral B cells could be divided into naïve ($\text{IgD}^{\text{pos}} \text{CD27}^{\text{neg}}$), memory ($\text{IgD}^{\text{neg}} \text{CD27}^{\text{pos}}$), marginal zone ($\text{IgD}^{\text{pos}} \text{IgM}^{\text{pos}} \text{CD27}^{\text{pos}}$), and antibody secreting ($\text{IgD}^{\text{neg}} \text{CD27}^{\text{hi}} \text{CD38}^{\text{hi}}$) cells (Jackson *et al.*, 2008; Klein *et al.*, 1998). In the steady state, less than 0.2~0.5% of peripheral blood mononuclear cells are ASC. The majority of peripheral B cells are naïve cells, which account for ~60% of total B cells. The memory and marginal zone B cells equally account for 15~20% of total B cells (Morbach *et al.*, 2010; Weill *et al.*, 2009).

The involvement of peripheral B cells subsets in the development of influenza-specific humoral immunity is poorly understood. Most studies are conducted in animal model. In mice, upon infection, CD5^{pos} B1 cells expand in the local lymph nodes, secrete influenza-binding IgM antibodies and regulate the production of influenza-specific IgG antibodies (Baumgarth *et al.*, 2000; Choi and Baumgarth, 2008; Waffarn and Baumgarth, 2011). In human, B1 cells could be found in the CD27^{pos} B cells, however, their phenotyping and function remain unclear (Griffin *et al.*, 2011). Marginal zone B cells constitute a major component of circulating CD27^{pos} B cells and are generally thought to provide protection against polysaccharide-encapsulated bacteria, especially in spleen (Weill *et al.*, 2009). Although a substantial proportion of marginal zone

B cells is noted in the periphery in human, the role of these cells in viral infection is poorly understood (Throsby *et al.*, 2008; Lanzavecchia and Sallusto, 2009; Weill *et al.*, 2009; Weller *et al.*, 2004) (figure 1.3).

In human, peripheral MBC expressing IgG or IgA or IgM (few) are generated through germinal centre reaction and isotype switching in the secondary lymphoid organs. It has been shown that influenza-specific IgG and IgA MBC increase in frequency in the peripheral blood following vaccination (Sasaki *et al.*, 2007). These MBC are thought to play a major role in the humoral memory. The longevity of humoral memory following influenza infection or vaccination has not been determined in human, though at least 6 years of humoral memory has been observed with prime-boost regime of H5 vaccine (Galli *et al.*, 2009a).

Upon vaccination or infection, sustained protective antibody level is important for long-term protection. It has been shown that via antigen-independent polyclonal activation, MBC could differentiate into ASC *ex vivo*, indicative of their potential for replenishing ASC (Bernasconi *et al.*, 2002). However, in the steady state, the underlined mechanism that maintains sustained humoral immunity needs to be further explored.

1.3.3 Original antigenic sin

Although annual vaccination plays an important role in the protection against influenza, the occurrence of 'original antigenic sin (OAS)' might potentially weaken the defense shield. This phenomenon is first observed within individuals with sequential influenza A infections producing the antibody response preferentially against the original (previous) strain (Davenport and

Hennessy, 1955). It is also noted that OAS only takes place when the host sequentially encounters two similar influenza A strains. Later on, this phenomenon was demonstrated in ferrets with sequential influenza infections (Webster, 1966). When OAS occurs in the infected host, due to absent or diminished antibody immunity against the latest strain, the infected host may therefore develop severe illness. In mice, OAS leads to the deficit of neutralising antibody, hence previously immunized mice still develop high viral load in lung upon latest viral challenge (Kim *et al.*, 2009). Furthermore, OAS is also noted in the T-cell response to dengue virus and is suggested to be associated with the development of severe dengue infection in human (Mongkolsapaya *et al.*, 2003). Tracing back the history of original antigenic sin, it is mostly observed in the circumstance of live influenza infection. Whether or not this phenomenon exists in human with sequential inactivated influenza vaccination is still much debated (Powers and Belshe, 1994; Wrammert *et al.*, 2008).

1.4 THE TECHNOLOGY OF MAKING MONOCLONAL ANTIBODY

The techniques of producing mAb were developed in 1970s. (Schwaber and Cohen, 1973; Köhler and Milstein, 1975). Initially, the production of mAb, using hybridoma technology, was exclusively from the mice cell line. The application of mouse mAb to human could lead to the induction of human anti-mouse antibodies which would inactivate the mouse antibody and limit their effectiveness, and could cause some side effects such as serum sickness and anaphylaxis. The development of chimeric and humanized mAb, using complementarity-determining region grafting technique, greatly reduces the

mouse proportion of the sequence and promotes the medical use in human (Jones *et al.*, 1986; Morrison *et al.*, 1984; Padlan, 1991; Riechmann *et al.*, 1988). In 1990s, the fully human mAb was successfully achieved by the method of phage display or produced in transgenic mice containing human Ig gene (Green *et al.*, 1994; Green, 1999; Huls *et al.*, 1999; Mendez *et al.*, 1997).

The invention and evolution of methods for producing mAb give rise to a new class of diagnostic tools, laboratory reagents and most importantly, therapeutic drugs. There are several mAb granted approval for treatment of cancer and inflammatory diseases (Feldmann and Maiui, 2001; Stern and Herrmann, 2005). The mAb has also been shown a realizable tool to combat emergent infectious disease. In early 2003, SARS corona virus broke out in human population. Within one year, the cellular receptor for viral S glycoprotein and the viral antigenic epitopes were identified with the help of mAb (Li *et al.*, 2003; Xiao *et al.*, 2003). By early 2004, human neutralising antibodies blocking viral entry had been developed for the emergency prophylaxis and treatment of SARS (Sui *et al.*, 2004). All these demonstrate an efficient bench-to-bedside transition of mAb technology (Saylor *et al.*, 2009).

Though there are several infectious diseases, including influenza, where mAb could play a critical role in prevention or treatment, so far there is only one approved mAb for an infectious disease (palivizumab against respiratory syncytial virus) (Harkensee *et al.*, 2006). The relatively low economic value and high costs of producing and delivering mAb could be the reasons why

fewer efforts on developing mAb in infectious diseases than antimicrobial or antiviral drugs (Saylor *et al.*, 2009; Steinitz, 2009).

In the latest decade, a number of neutralising anti-influenza mAb are made using the phage display library or immortalized memory B cell line (Corti *et al.*, 2010; Kashyap *et al.*, 2008; Simmons *et al.*, 2007; Sui *et al.*, 2009; Throsby *et al.*, 2008; Yu *et al.*, 2008). These antibodies are mostly of human origin and many of them provide a significant protection against influenza virus.

Importantly, several antibodies provide broad immunity to the diverse influenza subtypes, including H5N1 or pandemic H1N1 virus. These findings represent a plausible rationale for antibody-based intervention of severe influenza illness or the future pandemic. Despite the successful identification of neutralising antibodies, processing immortalized B-cell line requires extensive subcloning and this shotgun approach have produced very few useful antibodies even over the extensive periods of time (Simmons *et al.*, 2007). The construction of phage display library and the selection of antibody are also laborious and time-consuming. Moreover, the random pairing of fully human heavy and light chains *in vitro* in the phage display method cannot provide a true evaluation of the antibody repertoire that human generates *in vivo*. Hence, an efficient method of making mAb would be required for the manufacturing of mAb for clinical trials and commercial production.

In the late 2000s, cell-based microarray and microengraving techniques greatly facilitate the screening and detection of secreting antibodies at single cell level (Jin *et al.*, 2009; Love *et al.*, 2006; Ogunniyi *et al.*, 2009). The technique of 'immunospot array assay on a chip' (ISAAC) combining the cell-based microarray and the ELISpot method, allow rapid and efficient detection

of individual antigen-specific antibody-secreting cell in the microwell, with up to 234,000 individual cells being screened in one day. Another microengraving technique combining the protein microarray and ELISA could screen 100,000 antibody-secreting cells in less than 3 days. Importantly, for both techniques, the single cell of interest could be retrieved for further production of mAb. Both techniques provide a robust way for cloning of Ig genes from desired single cell, including hybridoma or primary antibody-secreting cell (An, 2010).

However, since the ELISpot or ELISA assay was applied to screen the secreting antibodies, the detection antigen has to be prepared prior to the experiment. Therefore, for certain emerging infectious pathogen, their application could be limited because the antigen is unavailable yet. As previously mentioned, early-phase ASC could be a valuable source for making mAb to a variety of infectious pathogens, including influenza. Single B cell sorting technique plus following molecular cloning and expression of Ig gene allow the production of large numbers of mAb in one month. Importantly, early-phase ASC induced by infection or vaccination are found mostly antigen-specific, though the variation occurs (Smith *et al.*, 2009; Wrammert *et al.*, 2008). Up to 70% of mAb derived from early-phase ASC have been reported to be antigen-specific (Smith *et al.*, 2009). Therefore, for instance, while the pandemic or an emerging infection occurs, the utilization of ASC would allow the rapid production of a library of human antibodies, which could be useful to characterise the target antigen, and most importantly, protect the host from the target pathogen.

1.5 AIMS

The main objective of the DPhil project is to characterise the properties of antibody and B cells in response to influenza A virus. Despite our understanding of humoral response elicited by influenza vaccination or infection, the role of antibody and humoral memory in the protection against influenza A virus are not completely understood. Very little is known about the B cell response in acute influenza A infection in human. Systemic studies with human subjects have been more difficult than those with mouse because of the difficulties in obtaining blood samples from acutely infected or vaccinated individuals. It would be important to dissect how the influenza-specific humoral immunity develops, maintains and confers protection upon vaccination or during the course of infection in human. The knowledge of viral structure and the characterisation of viral surface proteins pointed out how the human body generates protective immunity against virus and also how the influenza viruses evolve to escape the immune attack. However, the diversity and cross-reactivity of humoral response to influenza vaccination or infection are poorly explored. Although the traditional serological tests provide the fundamental information of induced antibody profile, it becomes increasingly important to dissect the antibody and B cell response at cellular and molecular level. Following the introduction of single B cell antibody technology, it provides the efficient and robust way to discover mAb with potential therapeutic values or repertoire study in basic research. In brief, the specific aims of this DPhil project are as follows:

- i. To determine the specificity, kinetics, breadth, size, and quality of influenza-specific antibody and B cell responses to influenza A virus in human.

- ii. To describe the patterns of influenza-specific antibodies and B cells recognizing across variant subtypes of influenza A virus and the immunodominance hierarchy.
- iii. To examine the roles of antibody and B cell response in the control of acute influenza A infection in human.
- iv. To illustrate the antibody repertoire and the Ig diversification in the influenza-induced B cells at single cell level.
- v. To characterise the specificity and function of anti-influenza mAb derived from early-phase ASC and their potential for clinical use.

To achieve the above aims, one influenza vaccine trial and two influenza human challenge studies were conducted to compare the B cell antibody responses on exposure to live influenza infection or inactivated influenza vaccination. In the pandemic H1N1 vaccine trial, 151 healthy adult volunteers were immunized with pandemic H1N1 vaccine and/or seasonal trivalent influenza vaccine. In the challenge studies, 44 healthy adult volunteers without protective antibody level were nasally challenged by live seasonal H1N1 viruses. The experimental approaches and results are described in the following chapters.

CHAPTER 2

MATERIALS AND METHODS

2.1 COHORTS AND MATERIALS

2.1.1 Influenza vaccine trial

In 2009, a prospective, randomized, observer-blind, parallel-group vaccine trial was conducted in Beijing, China. The goal of this study was to examine the effect of seasonal influenza vaccine on immunogenicity and safety of the pandemic 2009 H1N1 vaccine. The health adults (age 18-60) were enrolled, randomized, and given two-dose regimen of influenza vaccination. Further details about enrolment, group assignment and outcome would be described in following Chapter 3.

This study was conducted in accordance with both Good Clinical Practice guidelines and the Declaration of Helsinki. The protocol was also approved by the ethics review committee. All subjects provided written informed consent.

The vaccines used in the study were from Sinovac Biotech Co., Ltd (Beijing, China). The vaccines were prepared in embryonated chicken eggs using standard techniques used for the production of inactivated vaccine as described previously (Liang *et al.*, 2010). The pandemic 2009 H1N1 vaccine is a monovalent, unadjuvanted, and split vaccine, which was prepared from reassortant vaccine virus NYMC X-179A (New York Medical College) that derived from the A/California/7/09 virus (H1N1 CA07), and contains 15µg/0.5ml HA per vial. The seasonal influenza vaccine was prepared from high growth reassortants of A/Brisbane/59/07 (H1N1 BR59)-like,

A/Uruguay/716/07 {an A/Brisbane/10/07 (H3N2 BR10)-like virus} and B/Brisbane/60/08-like viruses, in accordance with the WHO recommended composition of vaccine virus for use in the 2009-2010 northern hemisphere influenza season.

After an on-site safety observation of 30-min duration, subjects were requested to record underarm body temperature, any injection-site and systemic reactions on diary cards. Any local adverse events at the injection site and systemic adverse events were recorded for three days after each immunization. Investigators determined the diameter of any erythemas, swelling, indurations and rashes. Daily temperatures were recorded by self-reporting in diary cards. All adverse events were graded using standard scale.

20-30ml heparin-containing blood and 3-5ml serum samples were collected for each subject on day 0, 7, 21 after each immunization.

Apart from the study of humoral immune response covered in the study, in parallel, the role of T cells in the cohort was being investigated by Miss Cecilia Chui.

2.1.2 Influenza A H1N1 challenge study

In 2009 and 2010, two prospective, randomized, clinical studies of experimental influenza A infections in human were conducted in Cambridge, UK. The purpose of this study was to evaluate the humoral immune response to an acute influenza infection. A total of 44 healthy, non-pregnant adult volunteers between the ages 18 and 45 were participated. Further details about enrolment and outcome would be described in following Chapter 4.

The studies were in compliance with both Good Clinical Practice guidelines (CPMP/ICH/135/95) and the Declaration of Helsinki. The protocol was also approved by the ethics review committee. All subjects provided written informed consent.

In both studies, the volunteers were nasally infected with the influenza A/Brisbane/59/07 (H1N1 BR59) viruses on day 0 and were quarantined in the study site for 9 days (from day -2 till day 7). The challenged viruses were manufactured by GlaxoSmithKline, UK to a GMP grade. The titre of the stock virus was 10^7 TCID₅₀ infectious dose. The inoculum dose of the virus was diluted as 1:10 to 1:10000 from the original virus stock. On day 7, all volunteers were given Tamiflu and discharged.

50 ml of heparin-containing blood and 3-5ml serum samples were collected on day -2, 3, 7 and 28. Clinical symptoms and signs, including oral temperature, nasal stuffiness, runny nose, sore throat, cough, sneezing, earache/pressure, breathing difficulty, muscle aches, general malaise, headache, feverish feeling, hoarseness, chest discomfort, and overall discomfort were recorded throughout the study period. Nasopharyngeal samples were collected daily until day 7 to determine the viral load.

Apart from the study of humoral immune response covered in the study, in parallel, roles of T cells and NK cells in the cohort were being investigated by Miss Cecilia Chui and Dr. Ling-Pei Ho, respectively.

2.2 CHEMICALS AND REAGENTS

2.2.1 Chemicals

- Phosphate Buffered Saline (PBS) (Invitrogen)
- Tween® 20 (Promega)
- RPMI Medium 1640 (Gibco)
- Heat-inactivated Foetal Calf Serum (FCS), L-glutamine, streptomycin, and penicillin were prepared in Human Immunology Unit, Weatherall Institute of Molecular Medicine, Oxford, and stored at 4°C

2.2.2 Reagents for cellular assay

- ELISpot plate – PVDF membrane, 96-well Multiscreen Filter plate (Millipore, Cat. No. MAIPS4510)
- Human B-cell ELISpot:
 - Goat Anti-human Ig's (Caltag, Cat. No. H17000)
 - Goat Anti-Human IgG, γ -Chain Specific Alkaline Phosphatase Conjugate (Calbiochem, Cat. No. 401442)
 - Goat Anti-Human IgA, α -Chain Specific Alkaline Phosphatase Conjugate (Calbiochem, Cat. No. 401132)
 - Goat Anti-Human IgM, μ -Chain Specific Alkaline Phosphatase Conjugate (Calbiochem, Cat. No. 401902)
 - Alkaline Phosphatase Conjugate Substrate Kit (Bio-Rad, Cat. No. 170-6432)
- Memory B-cell culture:

- Staphylococcus aureus Cowan Strain (SAC) (Calbiochem, Cat. No. 507858)
- CpG-2006 (5' TCG TCG TTT TGT CGT TTT GTC GTT 3') (InvivoGen, Cat. No. tlr-hodnb)
- Pokeweed mitogen (PWM) (Sigma-Aldrich, Cat. No. L9379)

2.2.3 Reagents for serological assay

- Human antisera to H1N1 A/California/07/09, H1N1 A/Brisbane/59/07, H3N2 A/Brisbane/10/07, and B/Brisbane/60/08 (Beijing-based National Influenza Center).
- Receptor destroying enzyme (RDE) (Beijing-based National Influenza Center)
- HI assay:
 - Turkey red blood cells (RBC) in Alsever's Solution (Beijing-based National Influenza Center)
- MN assay:
 - Madin-Darby canine kidney (MDCK) cells (Beijing-based National influenza Center)
 - Trypsin-EDTA (0.05% Trypsin 0.53 mM EDTA) (Invitrogen, Cat. No. 25300-054)

- Dulbecco's Modified Eagle Medium (DMEM) high glucose (1X), liquid, with L-glutamine, without sodium pyruvate (Invitrogen, Cat. No. 11965-092)
- Fetal Bovine Serum (FBS) (Hyclone, Cat. No. SH30070.03)
- Bovine Serum Albumin Fraction V (BSA), protease-free from bovine serum (Roche, Cat. No. 03117332001)
- Hepes buffer solution (1M) (Invitrogen, Cat. No. 15630-080)
- Anti-influenza A NP mouse monoclonal antibody (Millipore, Cat. No. MAB8257)
- Goat Anti-Mouse IgG Horseradish Peroxidase Conjugate (KPL, Cat. No. 074-1802)
- Citrate Buffer (Sigma-Aldrich, Cat. No. P4922)
- σ -Phenylenediamine dihydrochloride (OPD) (Sigma-Aldrich, Cat. No. P8287)

2.2.4 Antibody for phenotyping, intracellular cytokine staining and sorting (table 2.1)

2.2.5 Reagents for phenotyping and intracellular cytokine staining

- Brefeldin A (BFA) (Sigma-Aldrich)
- Phorbol 12-myristate 13-acetate (PMA) (Sigma-Aldrich, Cat. No. P8139)

Table 2.1. Antibody list					
Marker	Fluorescence	Clone	Isotype	Company	Cat. No.
CD3	Pacific Blue (PB)	UCHT1	IgG1 κ	Dako	PB982
CD19	Allophycocyanin (APC)	HIB19	IgG1 κ	BD	555415
CD20	Allophycocyanin-H7 (APC-H7)	L27	IgG1 κ	BD	641396
CD27	Phycoerythrin-Cyanin 7 (PE-Cy7)	M-T271	IgG1 κ	BD	560609
CD38	Phycoerythrin-Cyanin 5 (PE-Cy5)	HIT2	IgG1 κ	BD	555461
Ki67	Fluorescein Isothiocyanate (FITC)	B56	IgG1 κ	BD	51-36524X
CD56	Allophycocyanin (APC)	NCAM16.2	IgG2b	BD	341027
IFN γ	Fluorescein Isothiocyanate (FITC)	25723.11	IgG1 κ	BD	340449
IgM	Phycoerythrin-Cyanin 5 (PE-Cy5)	G20-127	IgG1 κ	BD	551079
IgD	Fluorescein Isothiocyanate (FITC)	IA6-2	IgG2a	BD	555778

- Ionomycin from *Streptomyces conglobatus* (Sigma-Aldrich, Cat. No. I9657)
- FACS Lysing Solution (BD, Cat. No. 347691)
- FACS Permeabilizing Solution (BD, Cat. No. 340457)

2.2.6 Reagents for generation of monoclonal antibodies

- RNasin Plus RNase Inhibitor (Promega, Cat. No. N2611)
- 1M Tris pH 8.0 (Applied Biosystems, Cat. No. AM9855G)
- Cell strainer (BD Falcon, Cat. No. 352235)
- PCR Plates (Bio-Rad, Cat. No. HSP9641)
- Clear Polyolefin Seal (StarLab, Cat. No. E2796-9793)
- OneStep RT-PCR Kit (Qiagen, Cat. No. 210212)
- HotStarTaq DNA polymerase (Qiagen, Cat. No. 203203)
- Primers used for RT-PCR, cloning PCR and sequencing (Sigma)
- RNA extracted from the B cell line (Dr Juthathip Mongkolsapaya, Imperial college)
- QIAquick Gel Extraction Kit (Qiagen, Cat. No. 28706)
- Agel-HF with NEBuffer and BSA (New England Biolabs, Cat. No. R3552L)
- Sall-HF with NEBuffer (New England Biolabs, Cat. No. R3138S)

- BsiwI with NEBuffer (New England Biolabs, Cat. No. R0553S)
- XhoI with NEBuffer and BSA (New England Biolabs, Cat. No. R0146S)
- T4 DNA Ligase with Ligase Reaction Buffer (New England Biolabs, Cat. No. M0202L)
- Competent DH5 α bacteria (New England Biolabs, Cat. No. C2987H)
- QIAprep Spin Miniprep Kit (Qiagen, Cat. No. 27106)
- 293T Cell Line (Dr Juthathip Mongkolsapaya, Imperial college)
- Polyethylenimine (PEI) (Sigma, Cat. No. 408727)
- UltraCHO Serum-free Medium (Lonza, Cat. No. 12-751F)

2.2.7 Reagents for validation of monoclonal antibodies

- ELISA plates (F96 Maxisorp NUNC Immuno plate) (NUNC, Cat No. 442404)
- Mouse monoclonal anti-human IgG (γ -chain specific) (Sigma-Aldrich, Cat No. I5885)
- Carbonate buffer (Sigma-Aldrich, Cat No. C3041)
- Bovine serum albumin (BSA) (ACROS, code 268131000)
- Goat anti-human IgG antibody conjugated with alkaline phosphatase (Fc specific) (Sigma-Aldrich, Cat No. A9544)

- Alkaline phosphatase substrate kit (Bio-Rad, Cat No. 172-1063),
- L Cell Line (Alain Townsend)
- Goat Anti-Mouse IgG Horseradish Peroxidase Conjugate (Alain Townsend)
- Rabbit Anti-Human IgG Horseradish Peroxidase Conjugate (Alain Townsend)
- DAB (3,3'-diaminobenzidine) Substrate Solution (Alain Townsend)
- UltraPure™ TEMED (N,N,N',N'-tetramethylethylenediamine) (Invitrogen, Cat. No. 15524-010)
- ProSieve Color Protein Markers (Lonza, Cat. No. 50550)

2.3 PERIPHERAL BLOOD MONONUCLEAR CELLS SEPRATION

Fresh heparinised human blood samples were layered on Leucosep tubes (Greiner Bio-One) with lymphoprep (Axis-Shield). The peripheral blood mononuclear cell layer was separated from whole blood by density gradient centrifugation for 20 min, 800 g at 20°C. The plasma supernatant was isolated and collected; while peripheral blood mononuclear cells (PBMC) were transferred to a separate tube and diluted up to 50 ul of RPMI medium 1640 (Gibco) (R0). The cells were then pelleted by centrifugation at 720 g for 10 min. The supernatant was then discarded and the pellet was washed again and re-suspended in RPMI medium 1640 (supplemented with 2% FCS (R2) at 400 g for 10min. Finally, the cells were resuspended in RPMI medium 1640

supplemented with 10% FCS, 2 mM L-glutamine, 100 µg/ml streptomycin, 100 U/ml penicillin (R10).

PBMC were counted by ABX Pentra 60 cell counter (Horiba Medical) and each sample was adjusted to 1×10^6 cells/mL in R10 and used immediately.

2.4 EX-VIVO B-CELL ELISPOT

The following influenza vaccine antigen provided by Sinovac Biotech Co., Ltd and recombinant influenza HA provided by Alain Townsend, were used:

- H1N1 A/California/07/09 (CA07)-like
- H1N1 A/Brisbane/59/07 (BR59)-like
- H3N2 A/Brisbane/10/07 (BR10)-like
- Recombinant Eng195 HA

The ELISpot plates were coated with purified influenza vaccine antigen (65µg/ml for H1N1 CA07, 20 µg/ml for H1N1 BR59, and 20 µg/ml for H3N2 BR10 in PBS) overnight at 4°C before use and each antigen was coated in duplicates. The wells coated with PBS were negative controls and those coated with polyvalent anti-human Ig's (10 µg/ml in PBS) were positive controls for detecting all IgG, IgM and IgA ASC.

Prior to adding the PBMC suspension, plates were washed with PBS for three times and blocked using R10 for an hour at 37°C. For antigen-specific and negative control wells, 2×10^5 PBMC were added in the final volume of 100 µl. For positive control wells, 2×10^4 PBMC were added in first well then 2-fold

dilution for second well and so forth. Plates were incubated for 18-24 hours at 37°C, 5% CO₂.

After washing, plates were incubated with alkaline phosphatase conjugates anti-human IgG, IgM or IgA (1:5000 dilution in R10) for 2 hours at room temperature. After washing, plates were developed with alkaline phosphatase substrate kit (4% 25x buffer, 1% color reagent A, and 1% color reagent B in milliQ water) for 2~5 minutes at room temperature. Spot-forming cells were measured and counted with automatic ELISpot reader (AID iSPOT reader) and AID software version 5.0 (Cadama Medical Ltd).

The analysis criteria for each spot were based on the intensity, size and gradient. For antigen-specific wells, ≥ 5 spots for each of duplicate wells were positive. Total antigen-specific ASC per million PBMC were calculated as the following equation: (the mean spot number of antigen wells – the mean spot number of negative control wells) $\times$ 5. For positive control wells, ≥ 5 spots in first well were taken as positive response. Total IgG, IgM or IgA ASC per million PBMC were calculated as the following equation: ($\frac{1}{2}$ spot number of first well + spot number of second well) – mean spot number of negative controls $\times$ 50.

2.5 MEMORY B CELL CULTURE

This method is to measure antigen-specific MBC in human peripheral blood. Briefly, following a 6-day polyclonal stimulation of PBMC, the B-cell ELISpot was performed to detect memory B cells that have differentiated into ASC *in vitro*.

2×10^5 PBMC were plated in the central area of 96-well dishes with the final volume of 100 μ l for each well. Each PBMC well was then cultured with 100 μ l of the optimized mix of polyclonal mitogens: 1/2500 SAC, 5 μ g/ml CpG-2006, and 40 ng/ml PWM. The wells cultured in R10 alone were taken as negative control. Prior to incubation, the surrounding wells were filled with PBS for preventing the over-evaporation of cultured medium. Plates were incubated for 6 days at 37°C, 5% CO₂ incubator. On day 6, cultured cells were pooled, washed and resuspended in R10 for B-cell ELISpot assay.

2.6 HI ASSAY

HI assay is a traditional assay for measuring antibody response to influenza HA protein. The HA protein could agglutinate erythrocytes. The binding between specific antibody and viral HA would interfere with the attachment of the viral HA on the receptors of erythrocytes. This leads to the inhibition of haemagglutination and is the basis for the HI assay.

2.6.1 RDE treatment of serum samples

Human sera could have various sialic acid containing glycans, which might bind the HA protein and compete with influenza-specific anti-HA antibodies. Therefore, the serum samples were pretreated to remove these non-specific inhibitors. Prior to testing in an HI assay, the mix of serum sample and the RDE were incubated for 18~20 hours at 37°C, and heat-inactivated for 30 min at 56°C in water bath.

2.6.2 Preparation of 1% Turkey RBC suspension

The turkey blood was carefully filtered and pipetted into the conical centrifuge tube, gently topped off with cold PBS, gently mixed by inversion and centrifuged for 10 min, 300 g at 4°C. The supernatants were aspirated without disturbing the pellet of RBC. The RBC were washed twice again with cold PBS for 5 min, 300 g at 4°C. Finally, the RBC were resuspended in cold PBS and counted using the hemocytometer. The RBC suspension were adjusted to 1% in cold PBS and may be stored at 4°C for use on the second day in the HI assay. The prepared RBC suspension would be discarded at the end of the second day.

2.6.3 Adsorption of serum samples with RBC to remove nonspecific agglutinins

The presence of nonspecific agglutinins in serum samples would interact with the erythrocytes, which might result in false negative results in the HI assay. Specific anti-HA antibody can inhibit the virus-erythrocyte interaction, but has no impact on the nonspecific agglutinins-erythrocyte interaction. Diluted serum and a solution of erythrocytes were mixed to examine nonspecific agglutinins. If a serum dilution of 1:20 or higher agglutinated with RBC, the adsorption would be performed.

The mix of RDE-treated serum sample and RBC were incubated for 30 minutes at 4°C, then centrifuged for 5 min, 600 g. The adsorbed serum was carefully collected without disturbing the pellet of RBC.

2.6.4 Influenza viral strains

The following influenza viral strains provided by Beijing-based National Influenza Center:

- H1N1 A/California/07/09
- H1N1 A/Brisbane/59/07
- H3N2 A/Brisbane/10/07
- B/Brisbane/60/08

2.6.5 Determination of HA titre of influenza virus

A serial 2-fold dilution of viruses were mixed with the RBC suspension in the round-bottom plates and incubated for 30 min at room temperature. All viral strains were tested in duplicate. The wells without adding viruses were served as the RBC control. The HA titre was read by tilting the plate at 45 to 60° angle. Negative results would present as dots in the center of round-bottom well and form a tear-shape due to gravity. The highest dilution of virus that causes complete haemagglutination was considered as the HA titration end-point. The reciprocal of this dilution of viruses was therefore the HA titre. The working solution of virus was prepared as 8 HA unit/50 µl in cold PBS.

2.6.6 Determination of HI titre of serum samples

Treated serum samples were prepared as 2-fold serial dilution with a starting dilution of 10. All serum samples were tested in duplicate. The working solution of virus of 4 HA unit was mixed with the prepared serum samples and incubated for 30 min at room temperature. The RBC suspension was then

added to the mix of serum-virus and incubated for 30 min at room temperature.

The wells without the serum-virus mix were setup as the RBC controls. The human antisera to the corresponding virus were used as positive controls. The virus back titration of the viral working solution was setup as well.

The HI titre of the serum sample was read by tilting the plate at 45 to 60° angle. The inhibition of agglutination would present as dots in the center of round-bottom well and form a tear-shape due to gravity. The reciprocal of the highest serum dilution that completely inhibit haemagglutination was considered as its HI titre. The HI titre below 1:10 was given a value of 1:5

2.7 MN ASSAY

The MN assay was a highly sensitive and specific way for measuring influenza-neutralising antibodies. In brief, it was performed in two steps. Firstly, the dilutions of serum was mixed with live viruses to allow the reaction between virus and antibody. Secondly, the mix of serum-virus was inoculated into the MDCK cell cultures. The conventional neutralisation test was based on the inhibition of cytopathic effect formation in cell cultures, which usually took 3 to 7 days. The MN assay described in the study utilized an ELISA to detect viral infected cells, which could be done on the second day upon setup.

The following medium and solution were prepared:

- MDCK cell culture maintenance medium (DMEM, 10% FBS, 100 U/ml penicillin, 100 ug/ml streptomycin, and 2 mM L-glutamine)

- Virus Diluent (DMEM, 1% BSA, 100 U/ml penicillin, 100 ug/ml streptomycin, 20 mM Hepes)
- Fixative (cold 80% Acetone in PBS)
- OPD Stop Solution (0.5 M sulfuric acid in dH₂O)
- Antibody Diluent (PBS, 0.3% Tween-20, 5% milk)

2.7.1 Influenza viral strains

The following influenza viral strains provided by Beijing-based National Influenza Center were used:

- H1N1 A/California/07/09
- H1N1 A/Brisbane/59/07

2.7.2 Determination of tissue culture infectious dose of the virus

A standardized amount of virus is required to mix with the serum sample for evaluating neutralising antibodies. Tissue culture infectious dose (TCID) determination is performed to quantify the amount of virus.

The viruses were tested at three different starting dilutions, which were dilutions of 10^{-2} , 10^{-3} , and 10^{-4} in virus diluent (e.g. 100 ul virus + 9.9 ml virus diluent for the 10^{-2} dilution). A serial $\frac{1}{2}$ log₁₀ dilution would be further setup on each start dilution of virus in the microtitre plates. For instance, the dilutions would be 10^{-2} , $10^{-2.5}$, 10^{-3} , $10^{-3.5}$... 10^{-7} , if the starting dilution was 10^{-2} . 1.5×10^4 MDCK cells were then added to each well of diluted virus, and incubated for 18-20 hours at 37°C.

Importantly, the cell control wells containing virus diluent only were also setup.

After washing, the inoculated cells were treated with cold fixative for 10-12 min at room temperature. After decanting fixative and drying the plates, the cell monolayer could be visually checked.

After washing, plates were incubated with anti-influenza A NP antibody for 1 hour at room temperature. After washing, plates were incubated with secondary antibody conjugated with horseradish peroxidase for 1 hour at room temperature.

After washing, plates were developed with OPD substrate (per OPD tablet in 20 ml citrate buffer) in room temperature. After adding stop solution, the absorbance (OD value) of the wells at 490nm was read on a microplate reader.

The OD cutoff for positive viral growth was defined as > 2 times the mean OD of cell control wells. The TCID was calculated by the Reed-Muench method. The virus suspension was then prepared as 200 TCID in 50 μ l for use in the MN assay.

2.7.3 RDE treatment of serum samples

The sera having sialic acid containing glycans could interfere with the binding between influenza-specific anti-HA antibodies and the viruses. The serum samples were pretreated to remove these non-specific inhibitors. Prior to testing in an MN assay, the mix of serum sample and the RDE were incubated for 18~20 hours at 37°C, and heat-inactivated for 30 min at 56°C in water bath.

2.7.4 Determination of MN titre of serum samples

Treated serum samples were prepared as 2-fold serial dilution with a starting dilution of 10. All serum samples were tested in duplicate. The virus suspension of 200 TCID was mixed with the prepared serum samples and incubated for 1 hour at 37°C. 1.5×10^4 MDCK cells were then added to the mix of serum-virus and incubated for 18-20 hours at 37°C.

The wells containing only 200 TCID of virus plus MDCK cells were setup as the virus controls. The wells without the serum-virus mixture were setup as the cell controls. The human antisera and negative serum sample were used as serum controls. The virus back titration of the virus suspension was also setup.

The following procedures for the fixation, the incubation with anti-influenza A NP antibody, the incubation with secondary antibody conjugated with horseradish peroxidase, and the development with the OPD substrate were the same as the TCID determination. The developed MN plates were read on a microplate reader.

The mean OD of cell controls should be less than 0.2 and the mean OD of viral controls should be more than 0.8. The cut-off value to determine a 50% neutralising antibody titre was calculated based on the equation: $\frac{1}{2}$ mean OD of virus controls + $\frac{1}{2}$ mean OD of cell controls. The values of serum sample below or equal to the cut-off value was positive for neutralisation. The reciprocal of the highest serum dilution that meet the neutralisation cut-off was considered as its MN titre.

2.8 PHENOTYPES OF B CELL IN THE PERIPHERAL BLOOD

The following solutions were prepared in the phenotyping and intracellular cytokine staining:

- FACS wash (1% FBS in PBS and 1% Sodium azide, store at 4°C)
- FACS fix (1% paraformaldehyde, store at 4°C)

This method is to detect naïve, marginal zone, isotype switched memory B and plasma cells in the peripheral blood.

Briefly, for phenotyping of B cell subsets, 1×10^6 PBMC were stained with a mix of antibodies of optimal amount specific for surface markers, including PB anti-CD3, FITC anti-CD19, PE-Cy7 anti-CD27, PE-Cy5 anti-IgM, and FITC anti-IgD for 30 minutes in darkness at room temperature.

For phenotyping of plasma cells, another 1×10^6 PBMC were stained with a mix of antibodies of optimal amount specific for surface markers, including PB anti-CD3, FITC anti-CD19, APC-H7 anti-CD20, PE-Cy7 anti-CD27, and PE-Cy5 anti-CD38 for 30 minutes in darkness at room temperature.

After washing, the stained cells were suspended in FACS fix and were analyzed on a CyAn ADP machine (DakoCytomation). At least 20000 B lymphocytes were collected for analysis. Lymphocytes gate was set based on forward scatter (FSC) and side scatter (SSC). Then total, naïve, marginal zone, isotype switched memory B and plasma cells were demonstrated and examined. The collected data were further analyzed using Flowjo 9.0.1 software (Tree Star Inc.).

2.9 PHENOTYPES OF PROLIFERATING CELL IN THE PERIPHERAL BLOOD

The expression of Ki-67 as a proliferation marker was assessed for peripheral B cells by flow cytometry.

1×10^6 PBMC were stained with a mix of surface antibodies, including PB anti-CD3, APC-H7 anti-CD20, PE-Cy7 anti-CD27, and PE-Cy5 anti-CD38 for 30 minutes in darkness at room temperature. After washing, cell permeabilization was carried out by resuspending cells in FACS perm-fix solution for 20 minutes at room temperature. After washing, the permeabilized cells were stained with intracellular antibody FITC anti-Ki67 for 20 minutes in darkness at room temperature. After washing, the stained cells were suspended in FACS fix and were analyzed on a CyAn ADP machine (DakoCytomation).

2.10 WHOLE BLOOD INTRACELLULAR CYTOKINE STAINING ASSAY AND FLOW CYTOMETRY

The whole blood intracellular cytokine staining (ICS) assay was performed to phenotype cytokine-producing lymphocytes upon stimulation. Besides, it allows multiple properties (function and phenotype) of a single cell to be analyzed simultaneously.

200 μ l of whole blood was incubated with peptide pools at final concentration of 2 μ g/ml for each peptide. The negative control (medium alone) and positive control (PMA and Ionomycin) were also setup. After incubating for 1 hour at 37°C, the secretion inhibitor BFA (10 μ g/ml) was added. The incubation was terminated after additional 6 hours.

Whole blood were then stained with a mix of surface antibodies, including PB anti-CD3 and APC anti-CD56 for 30 minutes in darkness at 4°C. After surface staining, RBCs were lysed with FACS lysing solution for 10 minutes at room temperature.

After washing, cell permeablization was performed by resuspending cells in FACS perm-fix solution for 20 minutes at room temperature. After washing, the permeabilized cells were stained with intracellular antibody FITC anti-IFN γ for 20 minutes in darkness at room temperature.

After washing, the stained cells were suspended in FACS fix and were analyzed on a CyAn ADP machine (DakoCytomation). At least 2×10^4 lymphocytes were collected for further analysis with Flowjo 9.0.1 software (Tree Star Inc.).

2.11 GENERATION OF RECOMBINANT MONOCLONAL ANTIBODY

Human mAb were generated as previously described (Smith *et al.*, 2009; Tiller *et al.*, 2008).

2.11.1 Sorting of ASC

The following solutions were prepared:

- Staining buffer (10% FCS in RPMI medium)
- RNase-inhibiting RT-PCR catch buffer (for 5 ml of RNase-free water, add 50 μ l of 1M Tris pH 8.0 and 125 μ l of RNasin in Rnase-free eppendorf or tube)

PBMC were suspended in the staining buffer. A mix of surface antibodies, including PB anti-CD3, FITC anti-CD19, APC-H7 anti-CD20, PE-Cy7 anti-CD27, and PE-Cy5 anti-CD38, were added to the aliquot of PBMC for sorting. After incubation for 30 minutes on ice, the cells were washed with staining buffer and pass through the cell strainer to avoid clogs in the cytometer.

The MoFlo cell sorter (DakoCytomation) was used for sorting. After applying a gate on $CD3^{neg}/CD20^{neg&lo}/CD27^{hi}/CD38^{hi}$ cells, single ASC was sorted in to PCR plates containing 10 μ l of RNase-inhibiting RT-PCR catch buffer. To facilitate the next RT-PCR step, cells were only sorted into half of the plate (Row A, B, C and D) and there were no cells sorted in Row H. Row H containing only catch buffer was served as PCR negative controls. Each plate was immediately sealed with the plate seal, centrifuged at 1000 rpm for 1 minute and placed on dry ice until the cell sorting is finished. The plates were stored in the -80°C freezer.

2.11.2 Singe cell RT-PCR for gamma heavy, lambda and kappa chains

The following master mix (for one reaction) was prepared in one lab using designated bench, pipettes and filter tips:

- 1 μ l forward primer (from stock containing 10 μ l each of 100 μ M L-V_H1, L-V_H3, L-V_H4/6, L-V_H5, L-V_k1/2, L-V_k3, L-V_k4, L-V _{λ} 1, L-V _{λ} 2, L-V _{λ} 3, L-V _{λ} 4/5, L-V _{λ} 6, L-V _{λ} 7, and L-V _{λ} 8)
+ 1 μ l reverse primer (from stock containing 10 μ l each of 100 μ M C _{μ} C_H1, C_k543, C _{λ} plus 70 μ l water)

+ 1 µl dNTP mix + 5 µl OneStep RT-PCR buffer + 6.5 µl water + 0.5 µl
OneStep RT-PCR enzyme mix

The plates were thawed and spun very briefly in centrifuge. 9 µl water was added to the bottom right well (H12) for the positive control. 15 µl master mix was added to the each well of Row A, B, C, D, and H. 1 µl RNA extracted from the B cell line was added to H12 as positive control.

After applying the dome lids to the plates, the following program was used in the OneStep RT-PCR reaction:

- 50°C for 30 minutes for the RT
- 95°C for 15 minutes to deactivate RT and activate thermal *Taq*
- 50 cycles of 94°C for 30 seconds, 58°C for 30 seconds and 72°C for 1 minutes
- 72°C for 10 minutes

2.11.3 Nested PCR for heavy, lambda or kappa chains

The following master mix (for one reaction) was prepared in one lab using designated bench, pipettes and filter tips:

- 1 µl forward primer (for heavy chain, from stock containing 10 µM 5' Agel V_H mix; for lambda chain, from stock containing 10 µM 5' Agel V_λ mix; for kappa chain, from stock containing 10 µM 5' Pan V_κ)
+ 1 µl reverse primer (for heavy chain, from stock containing 10 µM 3' Sall J_H mix; for lambda chain, from stock containing 10 µM 3' XhoI C_λ mix; for kappa chain, from stock containing 10 µM 3' C_κ 494)

+ 0.5 µl dNTP mix + 4 µl HotStarTaq buffer + 30.25 µl water + 0.25 µl
HotStarTaq enzyme

The 37 µl master mix were added to 3 µl of RT-PCR product.

After applying the dome lids to the plates, the following program was used in the nested PCR reaction:

- 95°C for 15 minutes
- 50 cycles of 94°C for 30 seconds, 58°C for 30 seconds and 72°C for 45 seconds
- 72°C for 10 minutes

The nested PCR products were checked by 1% agarose gel electrophoresis. Heavy chain products were approximately 380 bp, lambda chain 405 bp and kappa chain 540 bp, respectively. The nested PCR products were extracted, purified, and eluted in the elution buffer using QIAquick gel extraction kit.

Kappa chain nested PCR product would be sent for sequencing using C_κ 494 primer. The sequence data were analyzed to determine which cloning PCR primer to use from 5' Agel V_κ specific and 3' BsiWI J_κ specific primer sets. After further cloning PCR with gene specific primers, the kappa PCR products were approximately 350 bp and extracted using QIAquick gel extraction kit.

2.11.4 Digestion of heavy, lambda or kappa chain variable gene inserts (PCR product)

The purified PCR products were prepared and digested in the following reaction.

For heavy chain:

- Nested PCR product (up to 43 µl with water) + 0.5 µl BSA + 5 µl NEBuffer 4 + 0.125 µl AgeI-HF + 1 µl SalI-HF

The mix was incubated for 3 hours at 37°C

For lambda chain:

- Nested PCR product (up to 44 µl with water) + 0.5 µl BSA + 5 µl NEBuffer 4 + 0.125 µl AgeI-HF + 0.5 µl XhoI

The mix was incubated for 3 hours at 37°C

For kappa chain:

- Cloning PCR (2nd round) product (up to 45 µl with water) + 5 µl NEBuffer 1 + 0.125 µl AgeI-HF

The mix was incubated for 3 hours at 37°C. Then additional 0.5 µl BsiWI was added and incubated for 3 hours at 55°C

The digested PCR products were checked by 1% agarose gel electrophoresis and were approximately 400 bp. The digested PCR products were extracted, purified, and eluted in the elution buffer using QIAquick gel extraction kit.

2.11.5 Ligation of the expression vector and insert (PCR product)

The Ig_γ, Ig_κ and Ig_λ expression vectors (NCBI GenBank accession numbers: FJ475055, FJ475056 and FJ517647) were originally designed by Patrick

Wilson (Smith *et al.*, 2009) and kindly provided by Dr Juthathip Mongkolsapaya, Imperial college.

The expression vectors were engineered to contain a murine Ig signal peptide sequence and variable gene cloning sites upstream of the appropriate human Ig constant regions followed by an SV40 polyadenylation sequence (Smith *et al.*, 2009). Transcription was driven by the human cytomegalovirus immediate-early promoter. The ampicillin resistance gene was included as a marker gene. Prior to ligation, the expression vector had been digested in the same way as the insert plus a dephosphorylation step with shrimp alkaline phosphatase.

Since the vector was approximately 5700 bp and the insert was 350-400 bp, a 3:1 molar ratio of insert to vector would be used for ligation. The mix of vector and insert DNA was incubated for 5 minutes at 65°C then placed on ice until it become cold. For the mix of 2 µl vector and 6 µl insert DNA, 2 µl ligase buffer and 0.5 µl T4 DNA ligase were added and incubated overnight at 16°C.

2.11.6 Transformation of DH5α bacteria

The LB broth was prepared as follows:

- 10 g NaCl + 10 g Tryptone + 5 g Yeast Extract, make up to 1 litre of water

The mix of ligated DNA and DH5α bacteria was placed for 30 minutes on ice, then placed briefly for 30 seconds at 42°C (heat shock), and immediately placed back on ice for 5 minutes. 1 ml of LB broth was added into the mixture and incubated for 1.5 hours in 37°C shaker, shaking at 225 rpm. The cells were then pelleted by centrifugation at 6000 rpm for 5 minutes. The

supernatant was then discarded and the pellet was re-suspended in the left broth and plated on the LB plate containing 50 µg per ml of ampicillin. The plates were incubated 12~16 hours at 37°C.

Four colonies were chose from the plate to ensure the identification of a consensus variable gene sequence. Each colony was inoculated in one 14-ml round-bottom tube containing 7ml LB broth and 50 µg per ml of ampicillin. The tubes were incubated overnight in 37°C shaker, shaking at 225 rpm.

2.11.7 Miniprep (plasmid DNA isolation)

The bacteria were then pelleted by centrifugation at 3000 rpm for 20 minutes at 4°C. The supernatant was then discarded and the pellet was further processed following the protocol outlined in the QIAprep Spin Miniprep Kit.

The miniprep DNA was eluted in the elution buffer. A restriction digest on the 3 µl miniprep DNA was performed to check the presence of insert. The resulting digest was run by 1% agarose gel electrophoresis. If the inserts were present on the gel, the four miniprep DNAs were sent for sequencing.

The miniprep DNA sequences were analyzed and compared using immunoglobulin sequence software (IMGT/V-QUEST) and DNA sequence alignment software (ClustalW). The consensus miniprep DNA would be chose for transfection.

2.11.8 Transfection of 293T cells

The following medium were prepared:

- 293T cell culture medium (DMEM, 10% FBS, 100 U/ml penicillin, 100 ug/ml streptomycin, and 2 mM L-glutamine)

- UltraCHO serum-free medium (UltraCHO, 100 U/ml penicillin, 100 ug/ml streptomycin, and 2 mM L-glutamine)

293T cells were grown and passaged. When the 293T cells were 50-80% confluent, the cells were spread out in the 12-well tissue culture plate.

The following mix was prepared (for one well of 12-well plate):

- 240 µl cell culture medium + 1.5 µl heavy chain miniprep DNA + 1.5 µl light chain miniprep DNA + 5 µl PEI

After adding PEI, the mix was vortexed immediately and then incubated for 15 minutes at room temperature. The mix was gently added to the 293T cells and incubated for 8~16 hours in 37°C, 5% CO₂ incubator. The mix using pEGFP-N1 plasmid was setup as positive control and its transfection could be checked 8~12 hours upon incubation.

The transfection plates were washed with cell culture medium and added with UltraCHO serum-free medium, then incubated for further 4 days in 37°C, 5% CO₂ incubator.

The medium was collected and was spun for 15 minutes at 3500 rpm. The antibody supernatant was carefully collected and frozen for further testing.

2.12 VALIDATION OF RECOMBINANT MONOCLONAL ANTIBODY

2.12.1 ELISA for determining the yield of mAb

The yield of mAb was determined using standard IgG curve by means of an ELISA assay. The ELISA plates were coated with mouse monoclonal anti-

human IgG at dilution of 1:1000 in carbonate buffer and incubated overnight at 4°C. After washing, the plates were blocked with the solution of PBS with 3% BSA for 1 hour at room temperature on a shaker. After washing, the mAb supernatants of optimal dilution were added and incubated for 1 hour at 37°C. For standard IgG curve, human IgG starting at 100ng/ml was added and incubated as well. All the samples and standards were simultaneously tested in duplicates. After washing, the goat anti-human IgG antibody conjugated with alkaline phosphatase was added and incubated for 1 hour at 37°C. After washing, the plates were developed with pNPP solution at room temperature. pNPP solution was prepared by mixing together 1ml of 5X diethanolamine buffer and 4ml of water for each pNPP tablet (Alkaline phosphatase substrate kit), and should be colorless or pale yellow. The well would turn into yellow color when pNPP solution reacted with alkaline phosphatase-labeled conjugates. The optical density of the yellow color was measured at OD415 on a microplate reader with Magellan ELISA program (Tecan Group Ltd.).

2.12.2 ELISA for characterisation of antibody specificity

The following influenza vaccine antigen provided by Sinovac Biotech Co., Ltd were used:

- H1N1 A/California/07/09 (H1N1 CA07)-like
- H1N1 A/Brisbane/59/07 (H1N1 BR59)-like
- H3N2 A/Brisbane/10/07 (H3N2 BR10)-like
- H3N2 A/Perth/16/09 (H3N2 PR16)-like
- H5N1 A/Vietnam/1194/04 (H5N1 VN1194)-like

The ELISA plates were coated with detection antigen at dilution of 10 µg/ml in carbonate buffer and incubated overnight at 4°C. After washing, the plates were blocked with the solution of PBS with 3% BSA for 1 hour at room temperature on a shaker. After washing, a serial dilution of the mAb supernatants were added and incubated for 1 hour at 37°C. All the antibody supernatants were tested in duplicates. The following incubation with secondary antibody and development with the pNPP solution were the same as the previous description. The optical density of the yellow color was measured at OD415 on a microplate reader with Magellan ELISA program (Tecan Group Ltd.).

2.12.3 Dot blot assay

The antigen-specificity of human mAb was determined by the Dot Blot assay. This assay was similar to the western blot assay but differs in the coated proteins were not separated electrophoretically but were dotted directly onto the nitrocellulose membrane.

The following influenza vaccine antigen provided by Sinovac Biotech Co., Ltd and recombinant influenza HA provided by Alain Townsend, were used:

- H1N1 A/California/07/09 (H1N1 CA07)-like
- H1N1 A/Brisbane/59/07 (H1N1 BR59)-like
- H3N2 A/Brisbane/10/07 (H3N2 BR10)-like
- H3N2 A/Perth/16/09 (H3N2 PR16)-like
- H5N1 A/Vietnam/1194/04 (H5N1 VN1194)-like

- Recombinant Eng195 HA
- Recombinant PR8 HA

After preparing the nitrocellulose membrane, 2µl of the vaccine virus of optimal concentration was slowly dotted onto the membrane at the centre of the region. Incubating the dotted membrane at room temperature would ensure the dots were dry prior to going to the next step. The dotted membrane was then blocked with 5% dry milk in PBS for 1 hour at room temperature. After washing with PBS-T (0.1% Tween 20 in PBS), the dotted membranes were incubated with primary antibody (original mAb supernatant, 1:1000 to 1:100000 dilution for antisera) overnight at 4°C. After washing with PBS-T, the membranes were incubated with secondary antibody conjugated with horseradish peroxidase for 1 hour at room temperature. After washing with PBS-T, the membranes were incubated with ECL reagent for 1 minute at room temperature and covered with Saran-wrap for removing excessive solution from the surface. The developed membranes were then exposed to the film in the dark room.

2.12.4 Immunocytochemistry staining

The immunocytochemistry staining was performed by Judy Bastin and Tim Powell, the Group of Prof. Alain Townsend, Weatherall Institute of Molecular Medicine.

The following influenza viral strains were used:

- H1N1 A/Puerto Rico/8/34
- H1N1 A/California/07/09 (H1N1 CA07)-like

- H1N1 A/Brisbane/59/07 (H1N1 BR59)-like
- H3N2 A/Brisbane/10/07 (H3N2 BR10)-like

The L cells were grown on sterile glass cover slides overnight at 37°C. After washing, the cells were incubated with an aliquot of influenza virus for 2 hours at 37°C. After washing, the infected cells were incubated for further 6 hours at 37°C.

After washing, the infected cells were fixed with cold acetone and allowed to air dry. After washing, the infected cells were incubated with antibody supernatant for 30 minutes at room temperature. After washing, the infected cells were incubated with secondary antibody conjugated with horseradish peroxidase for 30 minutes at room temperature. After washing, the slides were developed using the DAB solution and were observed under a light microscope.

2.12.5 Western blot

The following influenza viral antigens were used:

- H1N1 A/California/07/09 (H1N1 CA07)-like
- H3N2 A/Brisbane/10/07 (H3N2 BR10)-like

The following solution and gel were prepared:

- Polyacrylamide stock solution (30.8%) (crylamide 30 g, Bis-acrylamide 0.8 g, in 100 ml deionized water)
- Resolving gel buffer, pH 8.8 (3.0 M Tris-HCl) (Tris base 36.3 g, 1M HCl 48 ml, in 100 ml deionized water)

- Stacking gel buffer, pH 6.8 (0.5 M Tris-HCl) (Tris base 6 g, 1 M HCl 48 ml, in 100 ml deionized water)
- 10% SDS solution (SDS 10 g in 100 ml deionized water)
- 10% Ammonium persulfate (Ammonium persulfate 1 g in 10 ml deionized water)
- 4X Reducing buffer (mercaptoethanol 4 ml, 0.5M Tris-HCl pH 6.8 8ml, SDS 1.6 g, Glycerol 8 ml, Bromophenol blue 4 mg)
- 10X Running buffer, pH 8.3 (Tris base 30.3 g, Glycine 144 g, SDS 10 g, in 1 litre deionized water)
- Polyacrylamide resolving gel (for 1 page) (other concentration of the acrylamide are achieved by varying the proportion of water and the 30.8% polyacrylamide stock solution in the mixture) (In preparation, 10% ammonium persulfate and TEMED must be added immediately before pouring gel)

	10% gel	12% gel	15% gel
30.8% Polyacrylamide	1.67 ml	2.0 ml	2.5 ml
3.0 M Tris-HCl	0.63 ml	0.63 ml	0.63 ml
10% SDS	0.05 ml	0.05 ml	0.05 ml
10% ammonium persulfate	0.038 ml	0.038 ml	0.038 ml
water	2.63 ml	2.29 ml	1.8 ml
TEMED	0.0025 ml	0.0025 ml	0.0025 ml

- Polyacrylamide stacking gel (for 1 page) (In preparation, 10% ammonium persulfate and TEMED must be added immediately before pouring gel)

30.8% Polyacrylamide	0.25 ml
0.5 M Tris-HCl	0.5 ml
10% SDS	0.02 ml
10% ammonium persulfate	0.015 ml
water	1.22 ml
TEMED	0.0015 ml

- Transfer buffer (Towbin's buffer) (Tris 3 g, Glycine 14.4 g, SDS 1 g, in 800 ml distilled water and 200 ml methanol)

The western blot assay was used to identify and characterise proteins based on their molecular mass and antigenic activity to react with specific mAb.

When the resolving and stacking polyacrylamide gels were ready. The influenza antigens were diluted and boiled in 1X reducing buffer. The comb was removed from the stacking gel and the wells were rinsed with deionized water to discard the debris of the gel. The antigens and the protein marker were load into the wells and the gels were run in 1X running buffer under constant voltage conditions.

The separated antigens/proteins were transferred to nitrocellulose membranes for 1.5~2 hours under the constant current 50 milliamps per 1 unit. After the protein transfer is complete, the membranes were blocked using

5% skim milk for an hour at room temperature. After washing with PBS-T (0.1% Tween 20 in PBS), the membranes were incubated with primary antibody (original mAb supernatant, 1:1000 to 1:100000 dilution for antisera) overnight at 4°C. After washing with PBS-T, the membranes were incubated with secondary antibody conjugated with horseradish peroxidase for 1 hour at room temperature. After washing with PBS-T, the membranes were incubated with ECL reagent for 1 minute at room temperature and covered with Saran-wrap for removing excessive solution from the surface. The developed membranes were then exposed to the film in the dark room.

2.12.6 HI and MN assay

The HI and MN assay were performed by Prof. Alain Townsend to characterise the specificity and neutralising function of mAb.

The similar protocol used for the serum sample was applied except the treated antibody supernatants were prepared as 2-fold serial dilution of original soup. All antibody supernatants were tested in duplicates.

CHAPTER 3

INFLUENZA VACCINE TRIAL

3.1 INTRODUCTION

Early in 2009, a novel swine-origin influenza A (H1N1 CA07) virus emerged, which was a reassortant virus harboring avian, swine and human influenza A genes. It crossed the species barrier and caused a high attack rate among the susceptible human population (SOIA Novel *et al.*, 2009; Zimmer and Burke, 2009). In June 2009, WHO declared the swine flu outbreak a pandemic. Mass vaccination is one important way to generate humoral immunity against influenza and to help control a pandemic. Several pandemic H1N1 vaccine trials had been undertaken and there was a general conclusion that a single dose containing 15 μ g HA without adjuvant induced a protective antibody response sufficient for licensing in the adult population (Greenberg *et al.*, 2009; Liang *et al.*, 2010; Plennevaux *et al.*, 2010; Zhu *et al.*, 2009).

Meanwhile, another seasonal H1N1 virus had been circulating since 2008. This meant that there were two different H1N1 viruses co-circulating in 2009. In general, the annual seasonal vaccination did not induce protective antibody against the pandemic H1N1 virus. Only a proportion of elders over the age of 60 years had detectable pre-existing antibody immunity against pandemic H1N1 virus (Hancock *et al.*, 2009). Most young adults were exposed to the threat of the H1N1 pandemic. It was therefore recommended that annual seasonal vaccine should be given together with pandemic H1N1 vaccine for the susceptible population (CDC, 2009b).

When it is necessary to give two or more inactivated influenza vaccines, the most common immunization strategy is to give the vaccines on the same day, or with any interval between them. This is the consensus for giving two or more doses of inactivated vaccines in a short time. For example, the young children would take the IPV and DPT vaccines at the same time. However, when the vaccines contain antigenic and genetically related components, for instance, there is over 70% of amino acid sequence homology between HA of pandemic H1N1 and seasonal H1N1 virus (Ikonen *et al.*, 2010), it is not known whether the immune responses would interfere with each other when both are given either sequentially or simultaneously, or whether the order of administration is important.

To investigate this question a prospective, randomized, and observer-blind vaccine trial was conducted in healthy adults to compare three possible immunization schedules using a licensed pandemic H1N1 vaccine and a seasonal trivalent influenza vaccine recommended for the 2009–2010 season (refer to Chapter 2). The safety and immunogenicity of both vaccines were evaluated in the vaccine trial.

3.2 STUDY DESIGN

Healthy, non-pregnant adults between the ages of 18 and 60 years were eligible for enrolment. Exclusion criteria included confirmed or suspected pandemic H1N1 infection and history of pandemic H1N1 or seasonal influenza vaccine during the preceding 6 months. At last, a total of 151 eligible subjects were enrolled, randomized and assigned into 3 groups in a 1:1:1 ratio (figure 3.1). Group 1 received pandemic H1N1 vaccine on day 0 and

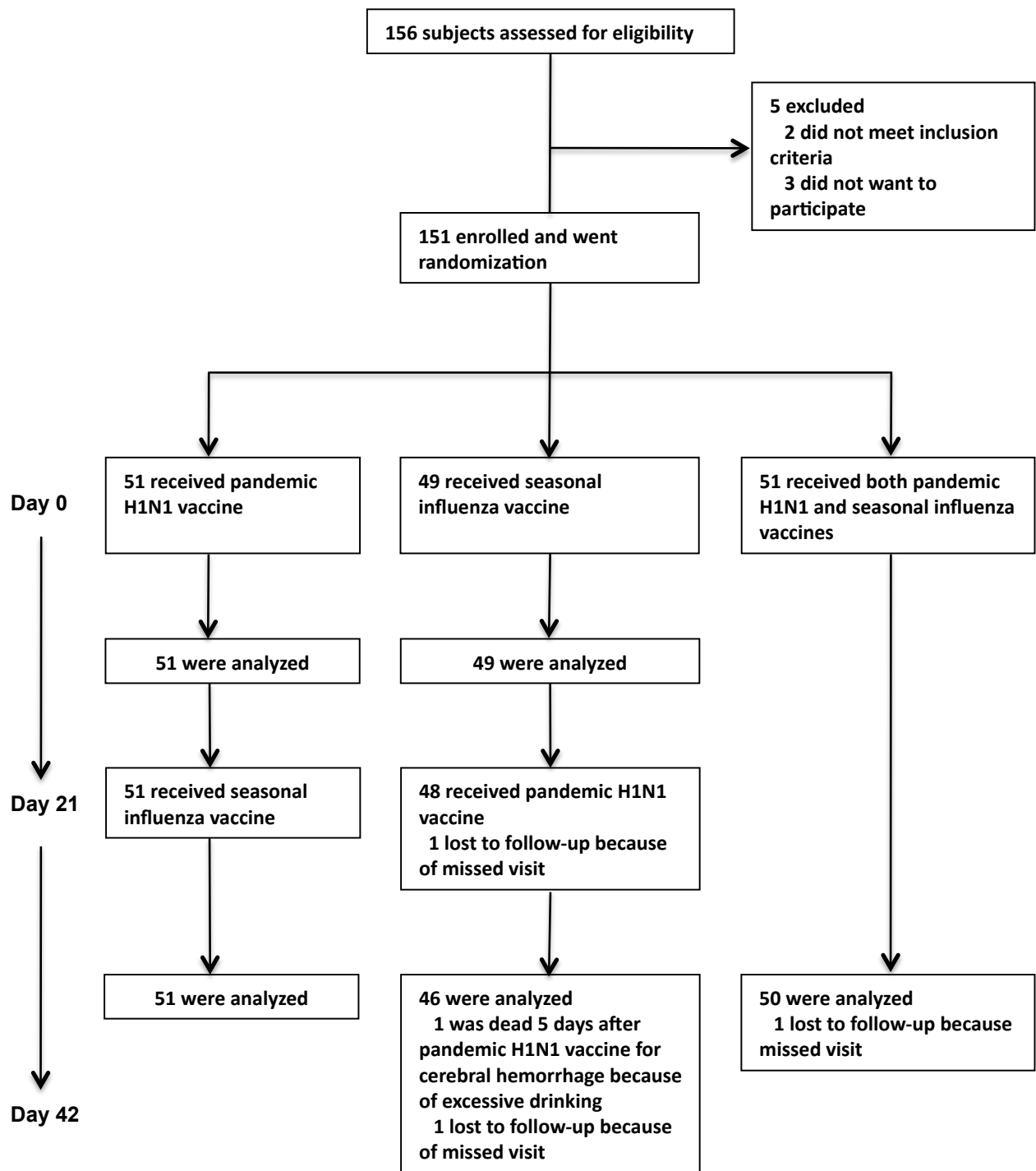


Figure 3.1. Enrolment and outcomes.

seasonal vaccine on day 21. Group 2 received seasonal vaccine on day 0 and pandemic H1N1 vaccine on day 21. Group 3 received both pandemic H1N1 and seasonal vaccines on day 0.

The randomization list was prepared by an independent statistician. The vaccine administrator was provided with the randomization code in a sealed envelope. All subjects and investigators were masked from the assignments. All vaccinations were done by personnel who did not take part in the subsequent assessment of safety and immunogenicity, thus maintaining the masking.

The vaccines used in the study were provided by Sinovac Biotech Co., Ltd (Beijing, China). The pandemic H1N1 vaccine is a monovalent, unadjuvanted, and split vaccine, which was prepared from reassortant vaccine virus NYMC X-179A that derived from the A/California/7/09 virus (H1N1 CA07), and contains 15µg/0.5ml HA per vial. The seasonal influenza vaccine was prepared from high growth reassortants of A/Brisbane/59/07 (H1N1 BR59)-like, A/Brisbane/10/07 (H3N2 BR10)-like, and B/Brisbane/60/08-like viruses.

On day 0 and day 21, each dose of vaccine was administered intramuscularly into the deltoid muscles of either left or right arm. Any local and systemic adverse events were recorded and evaluated. Blood and serum samples were collected on day 0, 7, 21, 28 and 42. PBMC were separated for B-cell ELISpot assay to measure the breadth and magnitude of antigen-specific ASC. Sera were tested with the HI and MN assays. For serology assays, all samples were blinded and assayed in duplicates by independent investigators not involved in the trial and the assay procedures were monitored by the National

Institute for the Control of Pharmaceutical and Biological Products (NICPBP), China.

This vaccine trial was conducted jointly by Xiaoning's Lab (MRC, UK), China CDC and Sinovac Biotech Co., Ltd in 2009. I made use of those fresh blood samples to study the ASC response in China CDC, Beijing then. The serological raw data (provided by Sinovac) were analyzed, interpreted and compared with the B-cell ELISpot result.

In parallel, another experiment was performed with subjects on a small scale. The aim was to study the kinetics of peripheral lymphocyte subsets using the flow cytometry method. Twenty-eight adult subjects from China CDC were enrolled and separated into two groups. One group had one dose of pandemic H1N1 vaccine. Another group had first dose of seasonal vaccine then second dose of pandemic H1N1 vaccine with 21 days interval. Blood and serum samples were collected on day 0, 7, 21, 28 and 42. In addition to the B-cell ELISpot assay and serology test, the flow cytometry staining was performed to identify B cell subsets in the peripheral blood.

3.3 RESULTS

3.3.1 The demographic characteristics of study subjects were analyzed.

The mean age was 34.7-41.4 years in three groups. There was a difference in age among three groups, which might result from the completely randomized stratification. There was no significant difference observed in body height, weight and sex between the groups.

3.3.2 Only mild local adverse reactions were reported following the pandemic H1N1 or seasonal vaccination.

There were no serious adverse events or adverse events of special interest noted in three groups. No significant difference of the frequency of adverse events was noted between the groups ($p=0.375$). The most common adverse reaction was pain at the injection site and most adverse reactions were mild in intensity. The main systemic reactions were headache and fatigue and no difference was noted between the groups.

3.3.3 One dose of inactivated, unadjuvanted, 15 μ g-HA containing pandemic H1N1 vaccination was highly immunogenic in naïve adults.

On day 0, only 10% of subjects had HI titre of 1:40 or more against pandemic H1N1 virus. In contrast, there was much higher percentage of subjects who were seropositive against seasonal H1N1, H3N2 and type B influenza virus (42.4%, 17.9% and 69.5% respectively) (table 3.1).

After one dose of pandemic H1N1 vaccination, there were 70-fold increase in geometric mean titre (GMT), 96% for seroconversion and 98% for seropositive within group 1. Also, pandemic H1N1 vaccine gave high seroconversion and seropositive rates within group 2 and 3. Although the three groups all met the European Union Committee for Medicinal Products for Human Use (EUCHMP)'s requirements, compared with group 1, the GMT was over fifty percent lower within group 2 and 3 ($p=0.003$).

Table 3.1. HI antibody response before and after immunization in three groups*

Day and Antigen	Group 1					Group 2					Group 3				
	GMT (95%CI)	GMT ratio (95%CI)	Seroconversion (95%CI)	Seropositivity (95%CI)	GMT (95%CI)	GMT ratio (95%CI)	Seroconversion (95%CI)	Seropositivity (95%CI)	GMT (95%CI)	GMT ratio (95%CI)	Seroconversion (95%CI)	Seropositivity (95%CI)	GMT (95%CI)	GMT ratio (95%CI)	Seroconversion (95%CI)
Day 0	N=51					N=49					N=51				
Pandemic H1N1	9.1 (7.2–11.5)	/	/	13.7 (6.2–27.0)	8.1 (6.5–10.1)	/	/	10.2 (3.8–23.1)	7.1 (6.0–8.4)	/	/	5.9 (1.5–17.3)	7.1 (6.0–8.4)	/	/
Seasonal H1N1	30.9 (23.6–40.5)	/	/	45.1 (31.4–59.8)	25.4 (19.6–33.0)	/	/	32.7 (20.4–47.8)	28.1 (21.4–36.8)	/	/	49.0 (35.0–63.5)	28.1 (21.4–36.8)	/	/
Seasonal H3N2	12.4 (9.1–17.0)	/	/	25.5 (14.8–40.1)	9.9 (7.5–13.0)	/	/	14.3 (6.4–28.0)	9.9 (7.5–13.0)	/	/	13.7 (6.2–27.0)	9.9 (7.5–13.0)	/	/
Seasonal B	33.5 (28.8–39.0)	/	/	70.6 (56.0–82.5)	36.7 (31.0–43.6)	/	/	71.4 (56.5–83.4)	31.7 (26.9–37.4)	/	/	66.7 (52.0–79.2)	31.7 (26.9–37.4)	/	/
Day 7	N=51					N=49					N=51				
Pandemic H1N1	145.5 (93.6–226.1)	16.0 (10.4–24.6)	76.5 (62.2–87.2)	82.4 (68.6–91.6)	35.7 (26.4–48.3)	4.4 (3.4–5.7)	42.9 (29.1–57.9)	51.0 (36.5–65.6)	112.4 (78.1–161.8)	15.8 (11.1–22.4)	90.2 (77.8–97.0)	90.2 (77.8–97.0)	112.4 (78.1–161.8)	15.8 (11.1–22.4)	90.2 (77.8–97.0)
Seasonal H1N1	54.7 (41.7–71.7)	1.8 (1.4–2.3)	17.6 (8.9–31.5)	74.5 (60.1–85.6)	151.2 (107.1–213.4)	5.9 (4.0–8.9)	57.1 (42.3–71.2)	93.9 (82.1–99.3)	212.8 (143.2–316.3)	7.6 (5.0–11.5)	60.8 (46.1–74.1)	60.8 (46.1–74.1)	212.8 (143.2–316.3)	7.6 (5.0–11.5)	60.8 (46.1–74.1)
Seasonal H3N2	16.3 (11.9–22.4)	1.3 (1.1–1.5)	3.9 (0.7–14.6)	25.5 (14.8–40.1)	87.1 (51.9–146.2)	8.8 (5.3–14.7)	63.3 (48.3–76.5)	73.5 (58.7–85.0)	115.5 (70.7–188.6)	11.7 (7.2–19.0)	64.7 (50.0–77.5)	64.7 (50.0–77.5)	115.5 (70.7–188.6)	11.7 (7.2–19.0)	64.7 (50.0–77.5)
Seasonal B	41.7 (35.9–48.3)	1.2 (1.1–1.4)	7.8 (2.5–19.8)	80.4 (66.5–90.2)	87.1 (67.4–112.5)	2.4 (1.8–3.1)	40.8 (27.3–56.0)	93.9 (82.1–99.3)	92.9 (74.4–116.0)	2.9 (2.3–3.8)	49.0 (35.0–63.5)	49.0 (35.0–63.5)	92.9 (74.4–116.0)	2.9 (2.3–3.8)	49.0 (35.0–63.5)
Day 21	N=51					N=48					N=50				
Pandemic H1N1	640.0 (430.9–950.7)	70.4 (48.8–101.5)	96.1 (85.4–100.0)	98.0 (88.2–100.0)	43.0 (31.0–59.7)	5.4 (4.0–7.4)	52.1 (37.4–66.8)	62.5 (47.3–76.0)	282.5 (198.9–401.2)	39.4 (27.8–55.8)	98.0 (88.0–100.0)	98.0 (88.0–100.0)	282.5 (198.9–401.2)	39.4 (27.8–55.8)	98.0 (88.0–100.0)
Seasonal H1N1	121.9 (90.3–164.5)	3.9 (2.8–5.5)	49.0 (35.0–63.5)	96.1 (85.4–100.0)	668.3 (481.5–927.8)	26.1 (17.9–38.2)	93.8 (81.8–99.3)	100.0 (90.8–100.0)	613.9 (425.9–884.9)	22.6 (14.9–34.3)	94.0 (82.5–99.3)	94.0 (82.5–99.3)	613.9 (425.9–884.9)	22.6 (14.9–34.3)	94.0 (82.5–99.3)
Seasonal H3N2	21.1 (15.2–29.3)	1.7 (1.4–2.1)	11.8 (4.9–24.6)	41.2 (27.9–56.0)	302.0 (179.0–509.7)	30.6 (18.5–50.7)	85.4 (71.6–94.1)	89.6 (76.6–96.8)	278.6 (190.2–408.1)	27.9 (18.5–42.0)	92.0 (79.9–98.2)	92.0 (79.9–98.2)	278.6 (190.2–408.1)	27.9 (18.5–42.0)	92.0 (79.9–98.2)
Seasonal B	37.9 (30.4–47.2)	1.1 (0.9–1.4)	9.8 (3.7–22.3)	68.6 (54.0–80.8)	151.0 (116.2–196.3)	4.1 (3.1–5.5)	66.7 (51.5–79.6)	97.9 (87.5–100.0)	141.2 (109.2–182.6)	4.5 (3.5–5.8)	64.0 (49.1–77.0)	64.0 (49.1–77.0)	141.2 (109.2–182.6)	4.5 (3.5–5.8)	64.0 (49.1–77.0)
Day 28	N=51					N=47					N=50				
Pandemic H1N1	403.2 (272.6–596.2)	44.3 (30.7–64.1)	96.1 (85.4–100.0)	98.0 (88.2–100.0)	70.1 (50.8–96.7)	8.7 (6.2–12.3)	70.2 (54.9–82.6)	78.7 (63.9–89.3)	/	/	/	/	/	/	/
Seasonal H1N1	230.9 (176.6–302.0)	7.5 (5.3–10.6)	78.4 (64.3–88.7)	100.0 (91.3–100.0)	505.5 (363.6–702.7)	19.4 (13.0–28.9)	91.5 (78.7–98.1)	100.0 (90.6–100.0)	/	/	/	/	/	/	/
Seasonal H3N2	132.3 (84.9–206.0)	10.6 (6.8–16.5)	76.5 (62.2–87.2)	84.3 (70.9–93.1)	272.1 (162.9–454.5)	27.2 (16.3–45.3)	83.0 (68.7–92.4)	87.2 (73.6–95.4)	/	/	/	/	/	/	/
Seasonal B	94.2 (72.4–122.5)	2.8 (2.1–3.7)	41.2 (27.9–56.0)	92.2 (80.3–98.2)	140.1 (103.9–188.9)	3.8 (2.8–5.3)	61.7 (46.4–75.5)	97.9 (87.3–100.0)	/	/	/	/	/	/	/
Day 42	N=51					N=46					N=50				
Pandemic H1N1	622.8 (422.6–918.0)	68.5 (47.8–98.2)	96.1 (85.4–100.0)	98.0 (88.2–100.0)	296.8 (217.5–404.9)	36.3 (26.2–51.2)	100.0 (90.4–100.0)	100.0 (90.4–100.0)	220.1 (158.8–305.0)	30.7 (22.0–42.8)	94.1 (82.5–99.3)	94.1 (82.5–99.3)	220.1 (158.8–305.0)	30.7 (22.0–42.8)	94.1 (82.5–99.3)
Seasonal H1N1	606.1 (466.8–787.0)	19.6 (13.3–29.0)	92.2 (80.3–98.2)	100.0 (91.3–100.0)	466.4 (348.9–623.4)	17.5 (12.2–25.2)	93.5 (81.1–99.3)	100.0 (90.4–100.0)	541.9 (380.8–771.1)	20.0 (12.9–30.9)	92.0 (79.9–98.2)	92.0 (79.9–98.2)	541.9 (380.8–771.1)	20.0 (12.9–30.9)	92.0 (79.9–98.2)
Seasonal H3N2	342.5 (223.3–525.2)	27.6 (17.9–42.4)	92.2 (80.3–98.2)	96.1 (85.4–100.0)	263.1 (159.9–432.8)	26.3 (16.3–42.4)	84.8 (70.5–93.8)	89.1 (75.6–96.7)	239.2 (162.2–352.6)	23.9 (15.9–35.9)	86.0 (72.6–94.3)	86.0 (72.6–94.3)	239.2 (162.2–352.6)	23.9 (15.9–35.9)	86.0 (72.6–94.3)
Seasonal B	7 (117.6–184.9)	4.4 (3.4–5.6)	62.7 (48.1–75.8)	100.0 (91.3–100.0)	135.6 (107.2–171.4)	3.7 (2.8–4.8)	65.2 (49.7–78.6)	97.8 (87.0–100.0)	147.2 (119.0–182.2)	4.7 (3.7–6.1)	68.0 (53.2–80.4)	68.0 (53.2–80.4)	147.2 (119.0–182.2)	4.7 (3.7–6.1)	68.0 (53.2–80.4)

* GMT=geometric mean titres. Proportions of subjects are based on the total number of subjects tested at each time point.

Seroconversion was defined as a titre before vaccination of less than 1:10 and a titre after vaccination of 1:40 or more, or a titre before vaccination of 1:10 or more and at least a fourfold increase after vaccination. Seropositive was defined as HI titre >=1:40.

Seasonal influenza vaccine induced robust antibody response within three groups. The seroconversion rate against seasonal H1N1 and H3N2 virus reached ninety percent. All three groups met the EUCHMP's requirements and no significant difference for GMT was observed ($p=0.98$).

As for MN assay, the results were similar to that for HI assay (table 3.2). After one dose of pandemic H1N1 vaccine, a strong neutralising antibody response with 50-fold increase in GMT, 96% for seroconversion and 96% for seropositive were detected within group 1. However, group 2 and group 3 had substantially lower MN titre ($p=0.001$) than group 1. In terms of MN titre of seasonal H1N1 virus, no significant difference was found among three groups ($p=0.84$).

The parallel study gave similar results to vaccine trial. One dose of pandemic H1N1 vaccine elicited strong antibody response and high seroconversion rate. However, pre-vaccination with the seasonal vaccine resulted in much lower HI ($p=0.03$) and MN ($p=0.04$) titre of pandemic H1N1 virus, compared with naïve group.

3.3.4 The frequency of antigen-specific ASC peaked on day 7 after vaccination and the amplitudes of the response varied among three groups.

The frequency of influenza-specific IgG ASC was detected using *ex vivo* B-cell ELISpot. In the B-cell ELISpot, the concentration of coating antigen was crucial to demonstrate antigen-specific spots on the well. Several kinds of coating antigens, including the inactivated virus, live virus and purified split vaccine antigen, were compared. When inactivated virus and live virus were

Table 3.2. MN antibody response against the pandemic H1N1 and seasonal H1N1 virus in three groups*

Day and Immunogenicity	Pandemic H1N1			Seasonal H1N1		
	Group 1	Group 2	Group 3	Group 1	Group 2	Group 3
Day 0	N=51	N=49	N=51	N=51	N=49	N=51
GMT (95%CI)	7.4 (6.0–9.0)	7.4 (6.0–9.2)	6.4 (5.5–7.4)	8.1 (6.6–10.0)	8.2 (6.5–10.3)	8.4 (6.8–10.4)
Seropositivity (95%CI)	3.9 (0.7–14.6)	8.2 (2.6–20.6)	0.0 (0.0–8.7)	2.0 (0.1–11.8)	6.1 (1.6–17.9)	5.9 (1.5–17.3)
Day 21	N=51	N=48	N=50	N=51	N=48	N=50
GMT (95%CI)	369.1 (256.5–530.9)	16.0 (11.1–22.9)	154.5 (107.9–221.3)	20.1 (15.3–26.2)	97.0 (72.2–130.3)	84.3 (61.8–114.9)
GMT ratio (95%CI)	50.1 (34.9–71.9)	2.2 (1.6–2.9)	24.1 (16.2–36.0)	2.5 (1.9–3.2)	11.7 (8.1–17.0)	10.4 (7.2–15.0)
Seroconversion (95%CI)	96.1 (85.4–100.0)	18.8 (9.4–33.2)	86.0 (72.6–94.3)	15.7 (7.5–29.2)	72.9 (57.9–84.7)	72.0 (57.3–83.7)
Seropositivity (95%CI)	96.1 (85.4–100.0)	29.2 (17.4–44.4)	88.0 (75.0–95.7)	27.5 (16.3–42.1)	83.3 (69.2–92.6)	82.0 (68.1–91.5)
Day 42	N=51	N=46	N=50	N=51	N=46	N=50
GMT (95%CI)	303.4 (210.0–438.2)	162.4 (116.5–226.3)	119.2 (82.6–171.9)	90.8 (71.4–115.4)	81.7 (61.4–108.7)	77.7 (57.2–105.7)
GMT ratio (95%CI)	41.2 (29.0–58.5)	21.9 (15.5–30.9)	18.6 (12.5–27.6)	11.2 (8.1–15.5)	9.7 (6.9–13.5)	9.6 (6.7–13.7)
Seroconversion (95%CI)	90.2 (77.8–97.0)	87.0 (73.0–95.3)	78.0 (63.7–88.5)	76.5 (62.2–87.2)	73.9 (58.6–85.7)	68.0 (53.2–80.4)
Seropositivity (95%CI)	90.2 (77.8–97.0)	89.1 (75.6–96.7)	82.0 (68.1–91.5)	86.3 (73.1–94.4)	80.4 (65.6–90.7)	72.0 (57.3–83.7)

* GMT=geometric mean titres. Proportions of subjects are based on the total number of subjects tested at each time point. Seroconversion was defined as a titre before vaccination of less than 1:10 and a titre after vaccination of 1:40 or more, or a titre before vaccination of 1:10 or more and at least a fourfold increase after vaccination. Seropositive was defined as MN titre $\geq$ 1:40.

used as coating antigen, the antigen-specific spots were vague and hard to define (figure 3.2). Therefore, the purified split vaccine antigen was chosen to detect antigen-specific ASC. The titration curve of coating antigen was also tested to find the saturated concentration for the best detection of ASC (figure 3.3).

The kinetics of antigen-specific IgG ASC in the peripheral blood was then measured by *ex-vivo* B-cell ELISpot. The response could only be detected on day 7 after vaccination but not day 0 or other time-points (figure 3.4 and 3.5). On day 7, the influenza-specific IgG ASC accounted for $53.9 \pm 3.4\%$ of total IgG ASC in the peripheral blood.

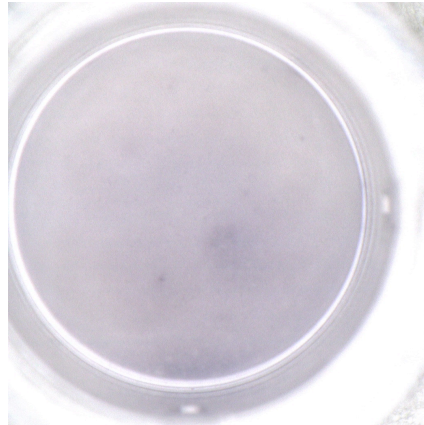
100% of vaccinated subject had detectable ASC response on day 7 by ELISpot. The average frequency of pandemic H1N1-specific IgG ASC was over 250 per million PBMC within group 1, which was significantly higher than group 2 and 3 ($p < 0.0001$). Nevertheless, for seasonal H1N1 and H3N2, the ASC response was alike among three groups ($p = 0.08$) (figure 3.6).

The parallel study showed a similar result. Pre-vaccination with the seasonal influenza vaccine resulted in a reduced ASC response to pandemic H1N1 virus ($p = 0.03$).

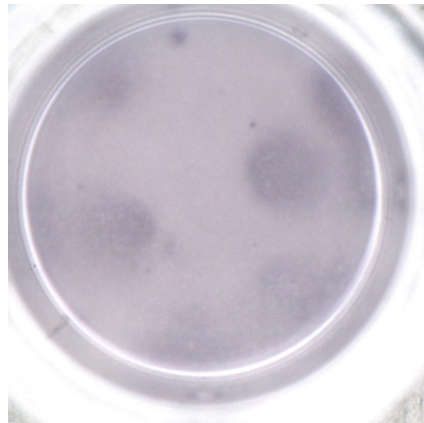
3.3.5 The frequency of antigen-specific ASC correlated with the antibody response, as measured by HI, MN and Anti-HA IgG titre.

After log transforming the frequency of antigen-specific IgG ASC on day 7 and the circulating antibody titre on day 21 after vaccination, the data were then

Live virus with HA
titre of 128 (1:4
dilution)



Inactivate whole
virus with HA
titre of 128 (1:4
dilution)



Purified split
vaccine antigen
(65 µg/ml)

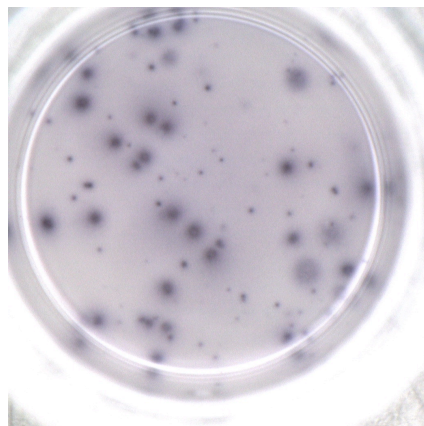


Figure 3.2. Comparison of pandemic H1N1-specific ASC with different coated antigens by B-Cell ELISpot. The forming spots using live virus or inactivated whole virus as coated antigen are blurred and hard to define, compared with the firm and well-defined spots on wells with purified vaccine antigen.

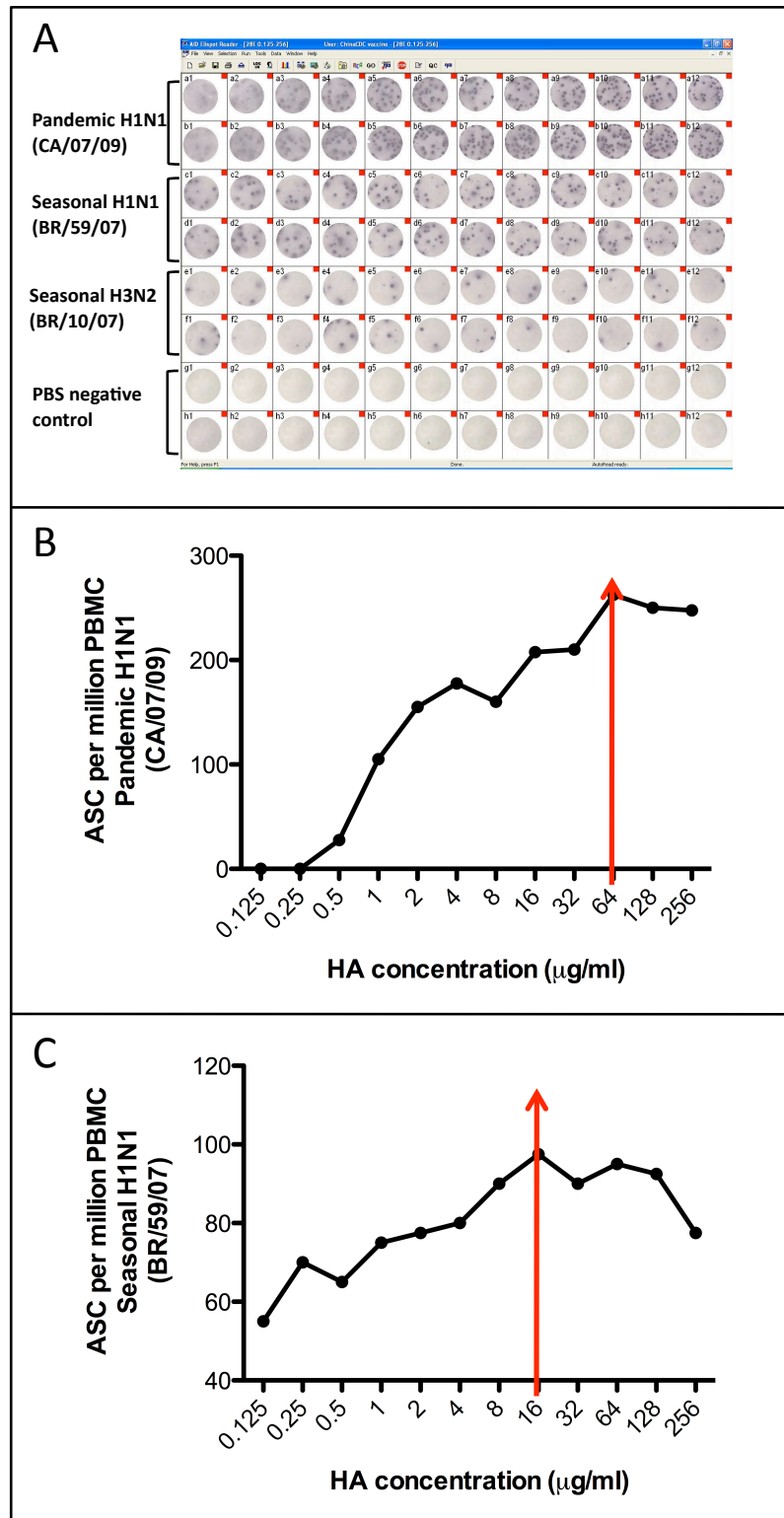


Figure 3.3. A. Picture of B-cell ELISpot using purified vaccine antigens with different concentration. The HA concentration of coated antigen was 0.125 to 256 $\mu\text{g/ml}$, from left to right column. B. The titration curve of pandemic H1N1 antigen for B-cell ELISpot. The vaccine antigen of HA 64 $\mu\text{g/ml}$ reaches the saturated concentration to detect antigen-specific ASC. C. The titration curve of seasonal H1N1 antigen for B-cell ELISpot. The vaccine antigen of HA 16 $\mu\text{g/ml}$ reaches the saturated concentration to detect antigen-specific ASC.

pV: pandemic H1N1 vaccinee
sV: seasonal influenza vaccinee

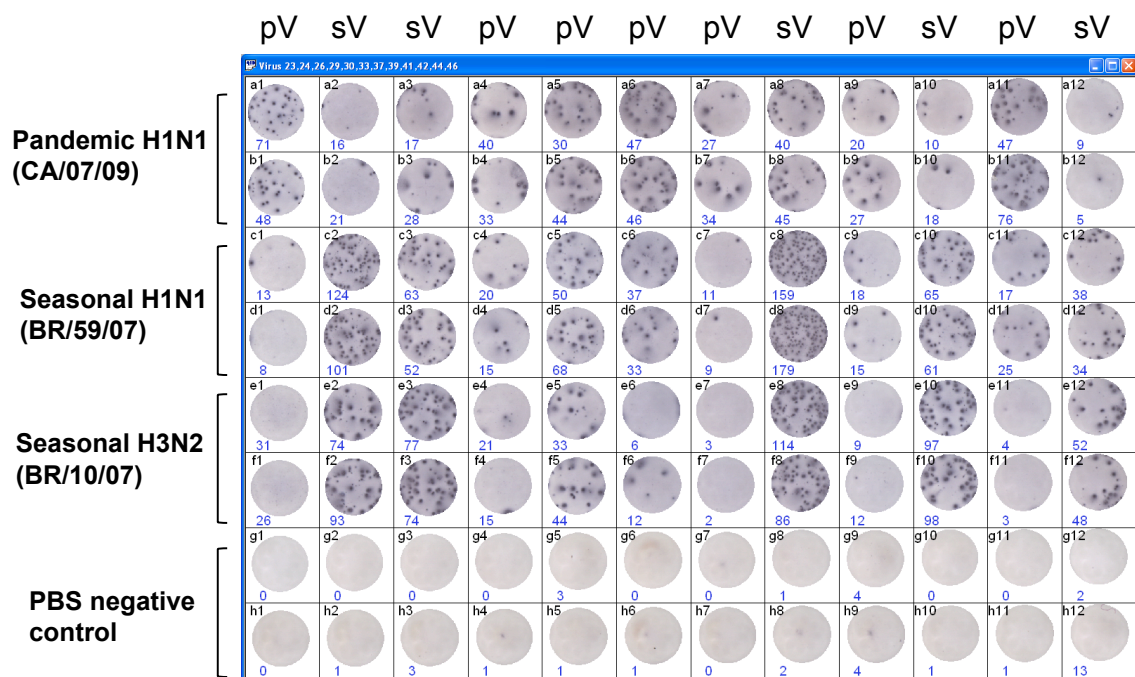


Figure 3.4. Antigen-specific ASC were detected by ex-vivo B-cell ELISpot assay on day 7 after vaccination. Each column represents one subject (left to right, Lab ID. 23, 24, 26, 29, 30, 33, 37, 39, 41, 42, 44, and 46). Lab ID. 23, 29, 30, 33, 37, 41 and 44 received pandemic H1N1 vaccine. Lab ID. 24, 26, 39, 42 and 46 received seasonal influenza vaccine.

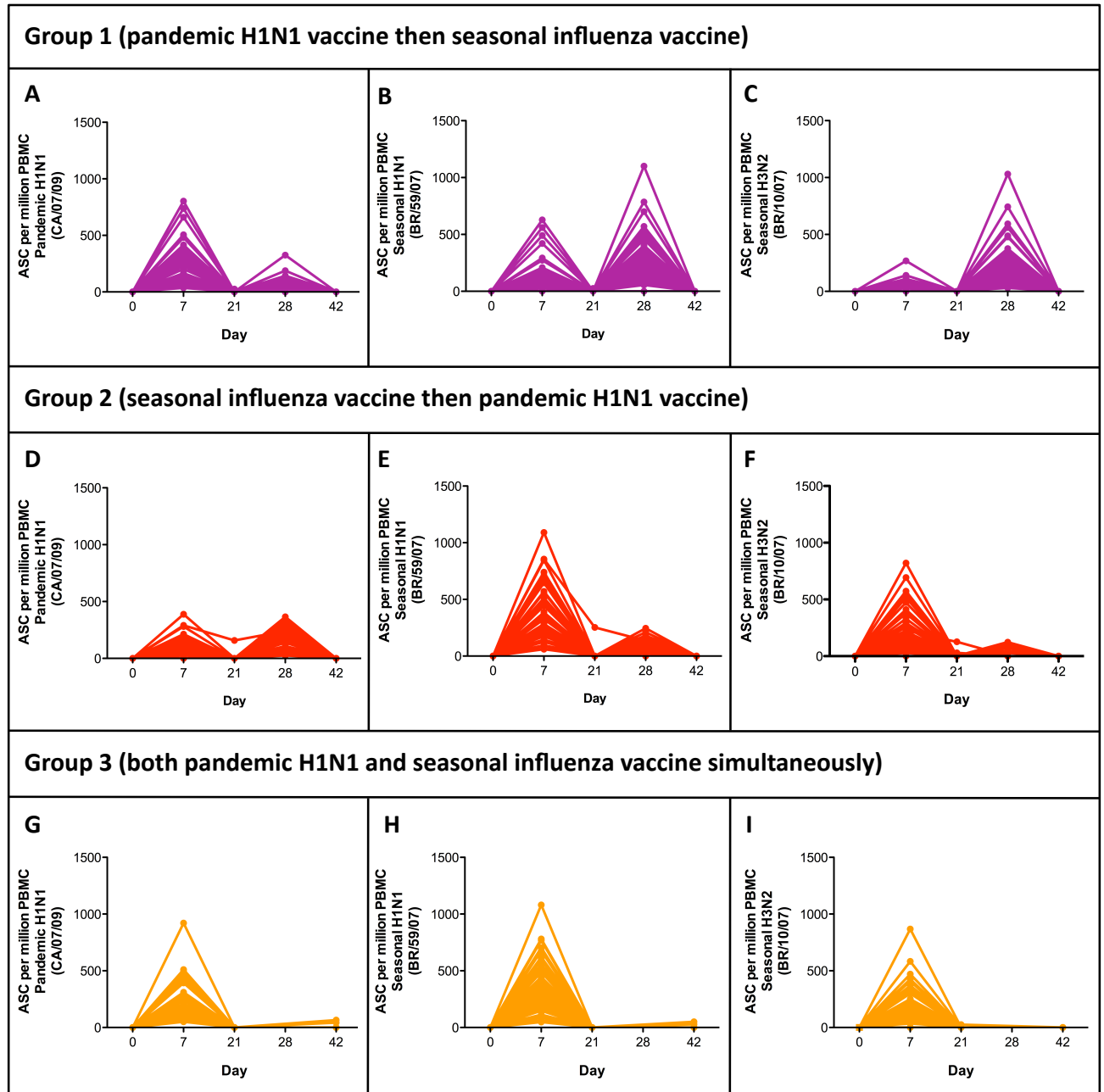


Figure 3.5. Kinetics and frequency of influenza-specific ASC in the peripheral blood, as measured by B cell ELISpot coated with A/California/7/09 H1N1-like (Panel A, D and G), A/Brisbane/59/07 H1N1-like (Panel B, E and H), and A/Brisbane/10/2007 H3N2-like vaccine antigen (Panel C, F and I, respectively). Shown are numbers of ASC per 10^6 PBMC.

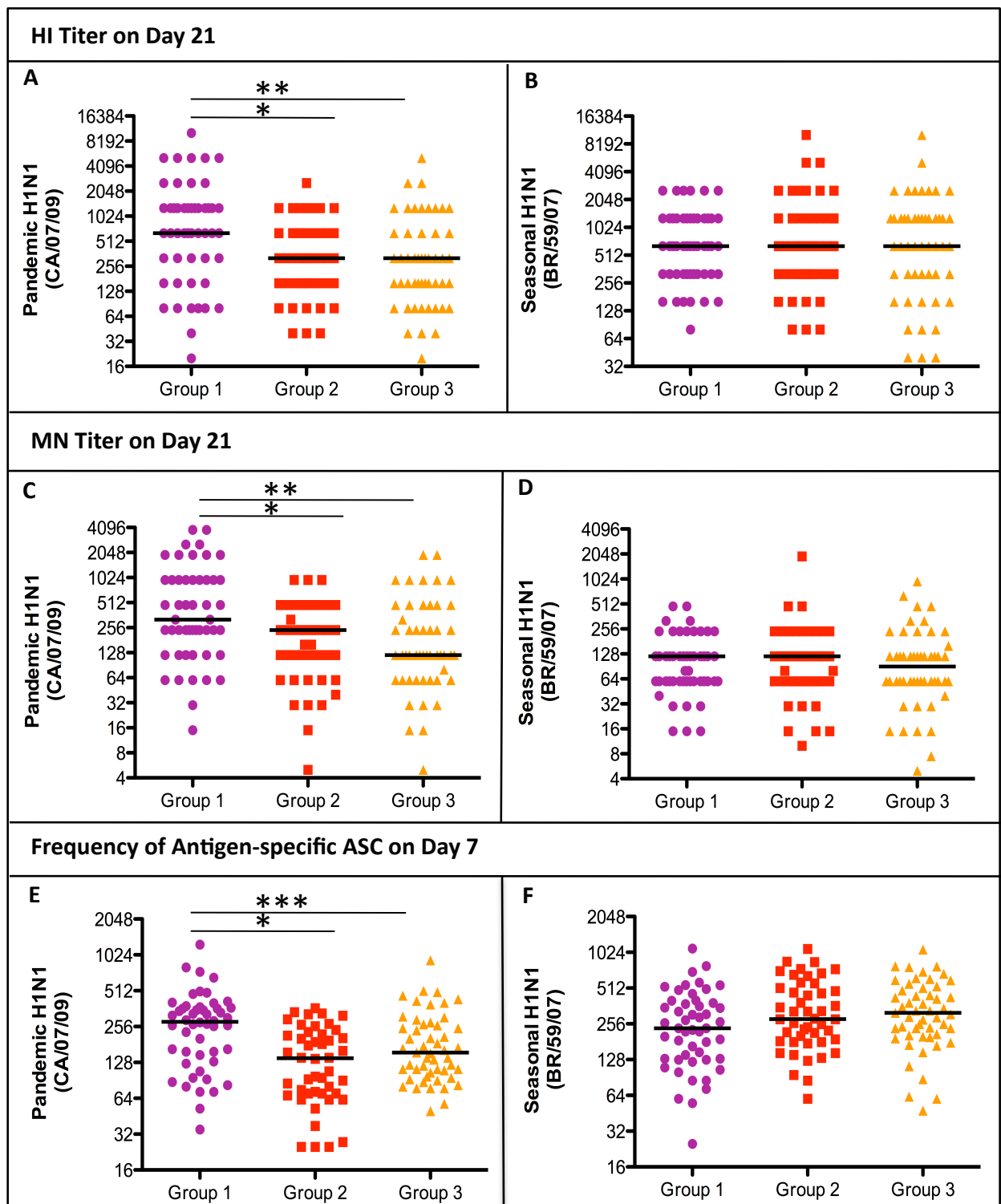


Figure 3.6. The magnitude of antibody response 21 days after immunization. Shown are immune response associated with the pandemic H1N1 and seasonal H1N1 virus using HI assay (Panel A and B) and MN assay (Panel C and D), as well as B-cell ELISpot (Panels E and F, respectively). Titers are expressed as reciprocal of the dilution and are given on a $\log_2$ scale. Differences between groups were tested using non-parametric Kruskal-Wallis test with post hoc Dunn's test for multiple comparisons. A p value of less than 0.05 was considered significant (* $p < 0.05$; ** $p < 0.01$; *** $p < 0.0001$).

interpreted in linear regression. The significant correlation between the day 7 ASC response and the day 21 neutralising and HI titre was observed both in the vaccine trial and parallel study (figure 3.7). In contrast, the correlation between the ASC response and antibody titre on day 7 was not statistically significant.

Here, in the parallel study, the IgG titre of pandemic H1N1 in serum was measured by indirect ELISA assay (performed by Yuan-Yuan Yao, Institute for viral Disease Control and Prevention, Beijing, China). The purified baculovirus-expressed HA of pandemic H1N1 was used as detection antigen. The pre-coating with HA of different influenza subtypes and pre-absorption of serum samples with vaccine antigen of different influenza subtypes largely eliminates the background, which is the common drawback to the ELISA assay. The totality of ninety samples was tested and the coincidence rate between positive ELISA titre and HI titre of pandemic H1N1 was 77%. A linear regression analysis of the ELISA titre, either with the ASC response, the HI or with the MN antibody titre, showed the significant correlation (figure 3.8).

3.3.6 Moderate homotypic response was found after pandemic H1N1 vaccination and seasonal influenza vaccination (table 3.1 and 3.2).

Firstly, within group 1, the cross-reactive antibody response to seasonal H1N1 was examined after pandemic H1N1 vaccination. On day 21, with HI assay, there was 4-fold increase in GMT of seasonal H1N1 and over 90% of seropositive rate to seasonal H1N1 virus. With MN assay, there were 2.5-fold increase in GMT and the seropositive rate rose from 2% on day 0 to 27% on

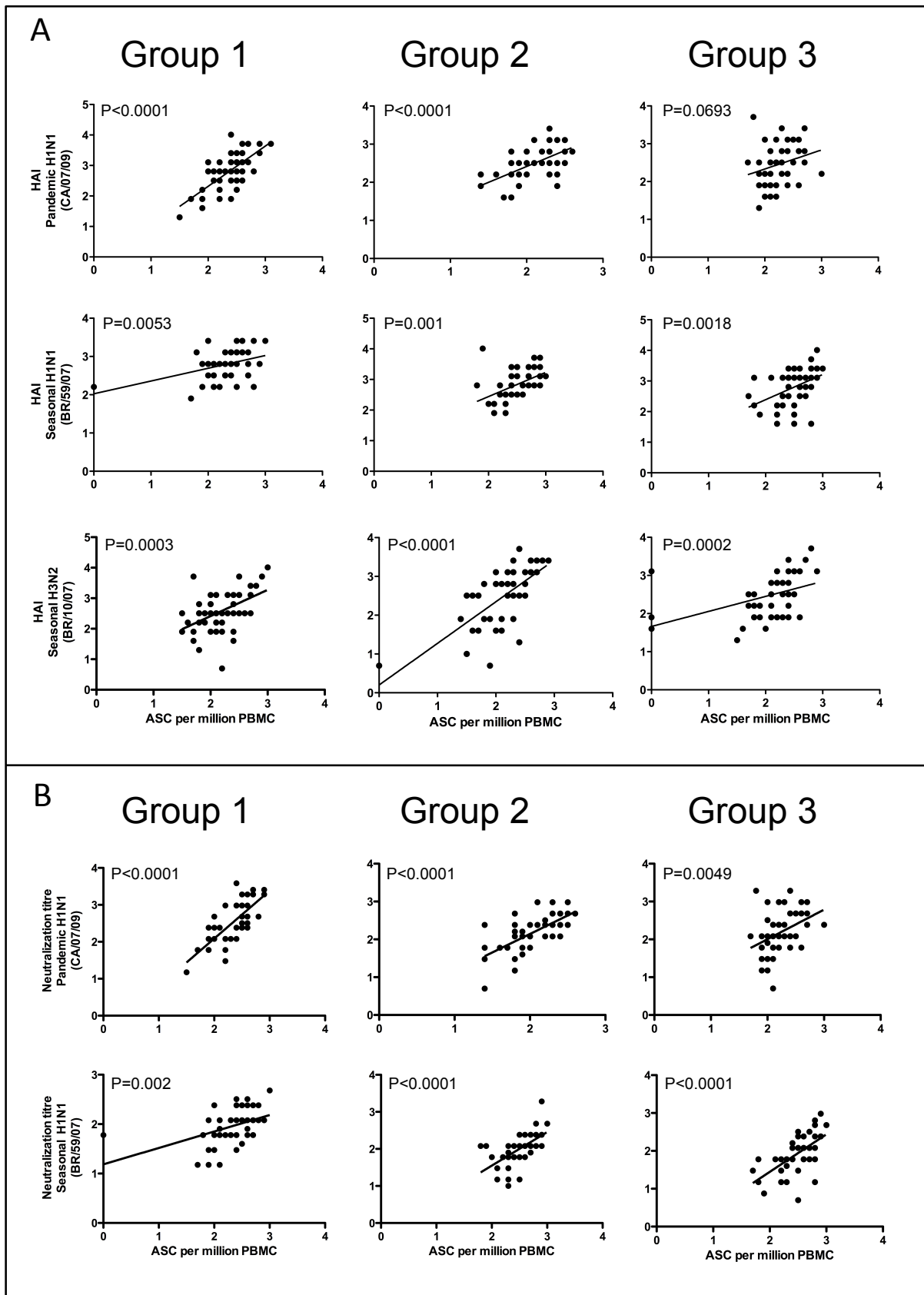


Figure 3.7. Correlation of influenza-specific ASC number, HI titre (Panel A) and MN titre (Panel B) on day 21 after immunization, according to vaccination groups. Following logarithmic transformations of both ASC number and titers, linear regression was performed to examine their association.

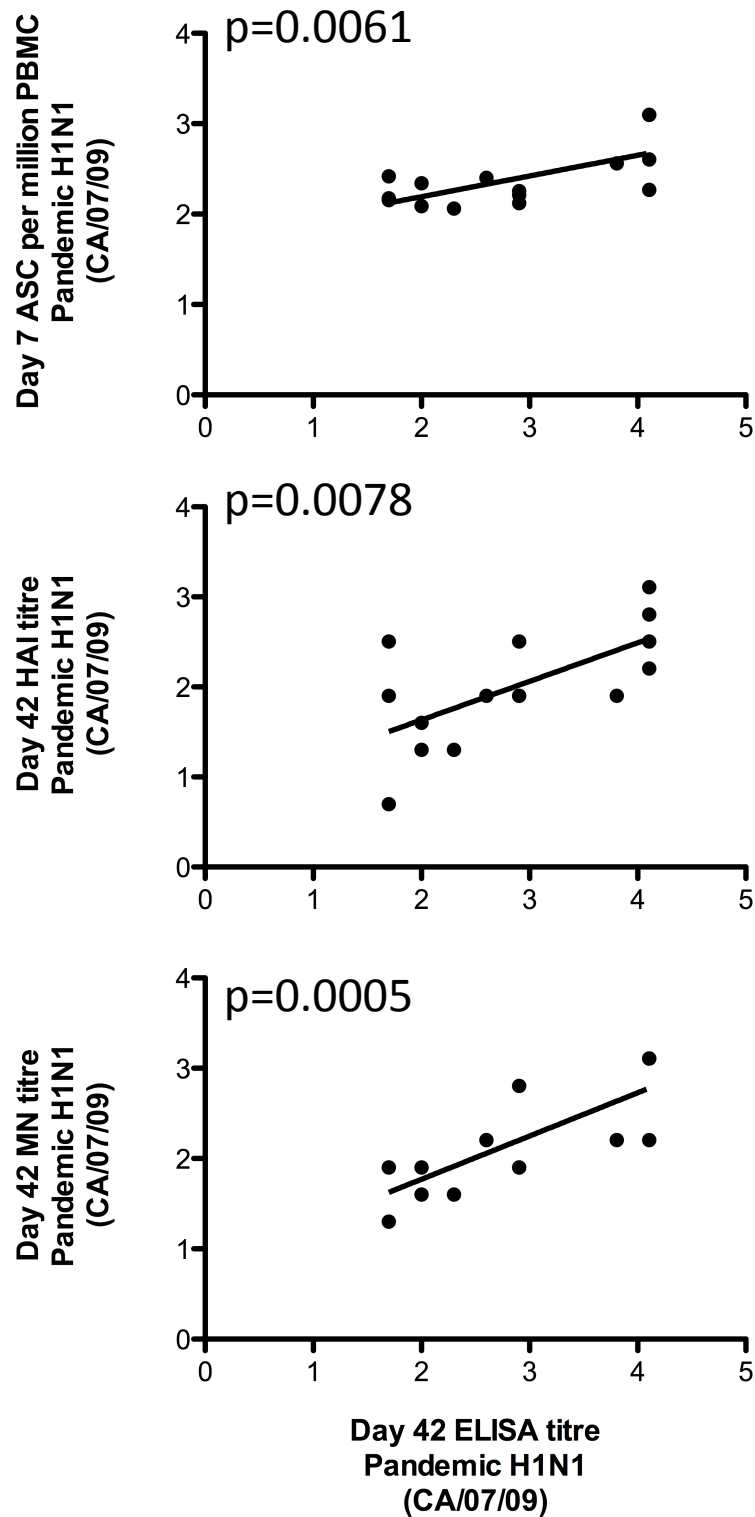


Figure 3.8. Correlation of HA-specific ELISA titre with day 7 ASC number, HI titre or MN titre on day 42 after pandemic H1N1 immunization. Following logarithmic transformations of both ASC number and titers, linear regression was performed to examine their association.

day 21.

Secondly, within group 2, they were immunized with seasonal vaccine first. Thus the cross-reactive antibody response to pandemic H1N1 virus was examined on day 21. With HI assay, 50% percent of seroconversion rate to pandemic H1N1 was detected. With MN assay, there was 2.2-fold increase in GMT and the seropositive rate rose from 8% on day 0 to 29% on day 21. Therefore, compared with group 1, it was observed that pandemic H1N1 vaccine induced stronger cross-reactive response than seasonal vaccine.

Thirdly, in view of day 7 ASC response, considerable cross-reactive ASC response was detected. It had to be pointed out that the interpretation of B-cell ELISpot result greatly depended on the nature of coating antigen. In the study, the coating antigen is the purified split vaccine antigen. Due to the application of sucrose-gradient purification, ideally, the end product should only contained highly concentrated HA protein of the vaccine strain. Despite this, minimal contaminating of NA, matrix or nucleoprotein from master strain seems inevitable. However, given the significant correlation of the ASC response with the HI or anti-HA ELISA titre, it could be estimated that the majority of influenza-specific ASC should be HA-specific. However, since the measurement of HA-specific ASC response was lacking, the level of cross-reactive response related to internal protein remained to be further determined.

Taken together, these results indicated that homotypic immunity did exist in human immunized with inactivated influenza vaccines, but the magnitude of response differed with immunized antigens.

3.3.7 Marginal heterotypic response was noted against seasonal H3N2 virus after pandemic H1N1 vaccination (table 3.1 and 3.2).

Group 1 had pandemic H1N1 vaccine first, therefore, the heterotypic response to H3N2 virus could be examined on day 21. With HI assay, the seropositive rate against H3N2 was quite low. A few of H3N2-specific ASC were detected in B-cell ELIspot assay as well. Compared with the obvious homotypic response to seasonal H1N1 virus, the cross-reactive response to H3N2 virus was minimal and could have been the result of contaminating conserved core proteins.

3.3.8 Minimal OAS was induced upon subjects immunized sequentially with seasonal influenza (day 0) then pandemic H1N1 vaccine (day 21).

Since group 2 had the seasonal influenza vaccine first, the antibody response to 2nd dose of pandemic H1N1 virus could be examined whether the original antigenic sin presented. Meanwhile, the antibody response to the first dose of pandemic H1N1 vaccine within group 1 could be considered as the comparable control (figure 3.9A).

Following pandemic H1N1 vaccination, despite observing lower HI titre of pandemic H1N1 than group 1, a robust antibody response ($p < 0.0001$) and increased seropositivity rate were induced. Meanwhile, the antibody level of previous vaccine virus (seasonal H1N1) did not increase but gradually declined (figure 3.9A).

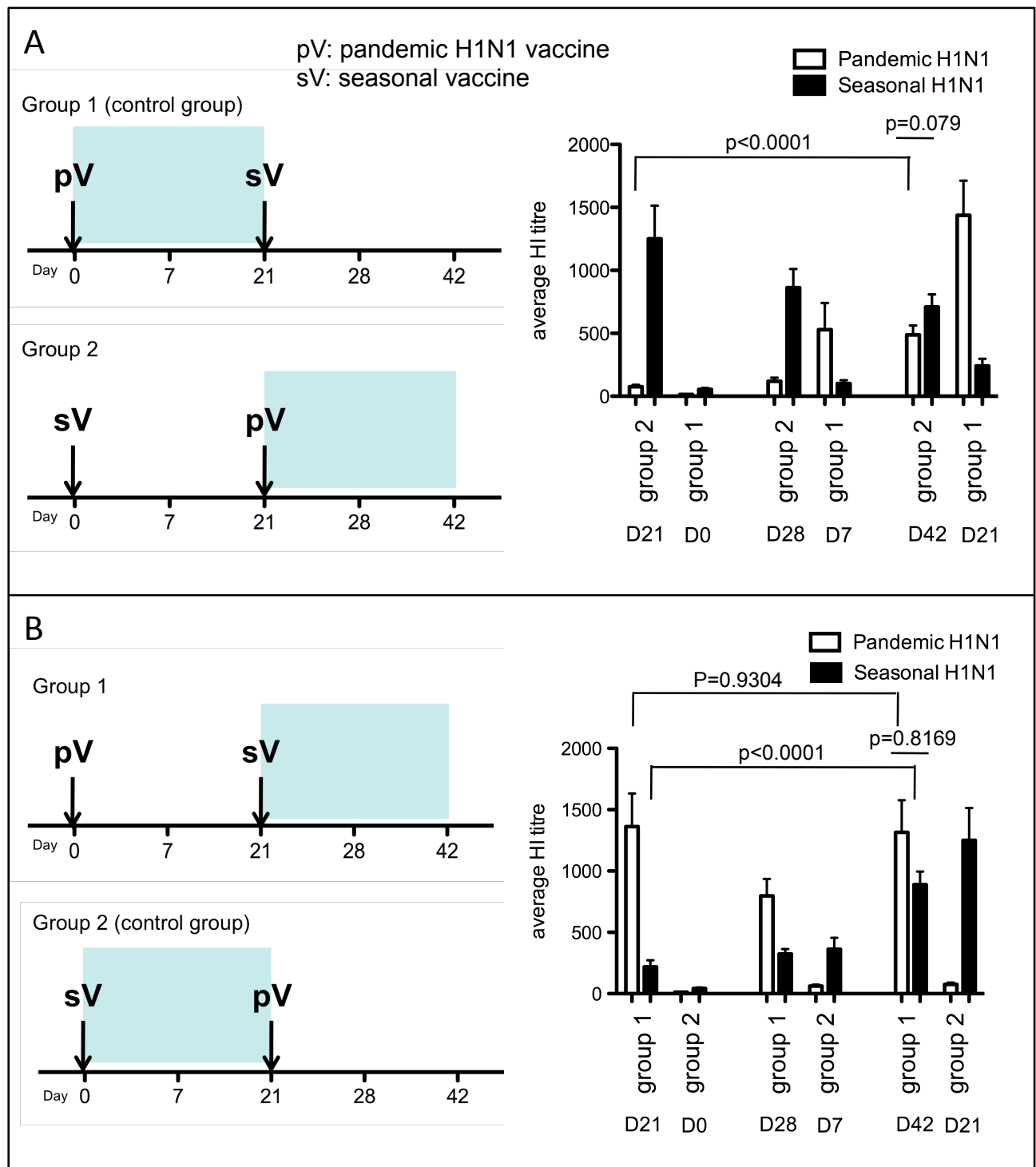


Figure 3.9. A. Although pre-immunization with seasonal H1N1 virus indeed leads to a diminished response to pandemic H1N1 vaccine, group 2 still produced predominant antibody response against pandemic H1N1 virus. B. Upon immunization with seasonal influenza vaccine, no significant change of antibody response against pandemic H1N1 was noted on day 28 and day 42 ($p=0.09$, 0.9304). On day 42, there was no significant difference of average HI titre between against pandemic H1N1 and seasonal H1N1 virus ($p=0.8169$).

With regard to neutralising antibody response, following pandemic H1N1 vaccination, significantly increased MN titre was induced against pandemic H1N1 virus ($p=0.002$). In contrast, the MN titre of seasonal H1N1 virus gradually declines, which is similar as the HI result.

In conclusion, after 2nd dose of pandemic H1N1 vaccination, group 2 still produced predominant antibody response against latest vaccine virus.

3.3.9 Minimal OAS was induced upon subjects immunized sequentially with pandemic H1N1 (day 0) then seasonal influenza vaccines (day 21).

Since group 1 had the pandemic influenza vaccine first, the antibody response to subsequent seasonal vaccination could be examined for original antigenic sin. Meanwhile, the antibody response to the first dose of seasonal H1N1 virus within group 2 could be considered as the comparable control (figure 3.9B).

Following seasonal vaccination, the HI antibody response against previous vaccine virus (pandemic H1N1) had no significant change ($p=0.9304$). In contrast, the HI titre of seasonal H1N1 rose significantly ($p<0.0001$). On day 42, there was no significant difference of average HI titre between pandemic H1N1 and seasonal H1N1 virus ($p=0.8169$) (figure 3.9B).

In terms of neutralising antibody response, following seasonal vaccination, the average MN titre against seasonal H1N1 is lower than previous vaccine virus (pandemic H1N1) ($p<0.0001$) on day 42. However, there is no significant

change seen in MN titre of pandemic H1N1 virus upon seasonal vaccination (p=0.61).

Taken together, in the study, the effect of OAS is barely seen in human immunized sequentially with pandemic H1N1 and seasonal split vaccines.

3.3.10 The frequency of B-cell subsets was steady in the peripheral blood upon vaccination.

The frequency of B-cell subsets was examined using frozen PBMC on day 0 and day 7 following vaccination. The gating strategy for total B cell, naïve B cell and memory B cell is shown in table 3.3 and figure 3.10A. Fluorescence Minus One (FMO) controls were included to determine the positive staining of given surface markers.

CD27^{pos} and CD27^{neg} cells accounted for 42% and 56% of total B cells on average. The majority of CD27^{pos} B cells were isotype switched cells (CD27^{pos} IgD^{neg} phenotype), followed by marginal zone cells (CD27^{pos} IgM^{pos} IgD^{pos} phenotype). The majority of CD27^{neg} B cell were naïve cells (CD27^{neg} IgM^{pos} IgD^{pos} phenotype), followed by isotype switched cells (CD27^{neg} IgD^{neg} phenotype).

The frequency of total B cells and the subsets for each subject were presented as the percentage of total lymphocytes (figure 3.10B). It was noted that the average frequency is similar between day 0 and day 7 after vaccination.

3.4 DISCUSSION

Table 3.3. Gating strategy for B cell subsets					
Cell type and surface marker	CD3	CD19	CD27	IgM	IgD
Total B cell	neg	pos			
Naïve B cell	neg	pos	neg	pos	pos
Memory B cell					
CD27 ^{pos} isotype switched	neg	pos	pos	neg	neg
CD27 ^{neg} isotype switched	neg	pos	neg	neg	neg
Marginal zone B	neg	pos	pos	pos	pos

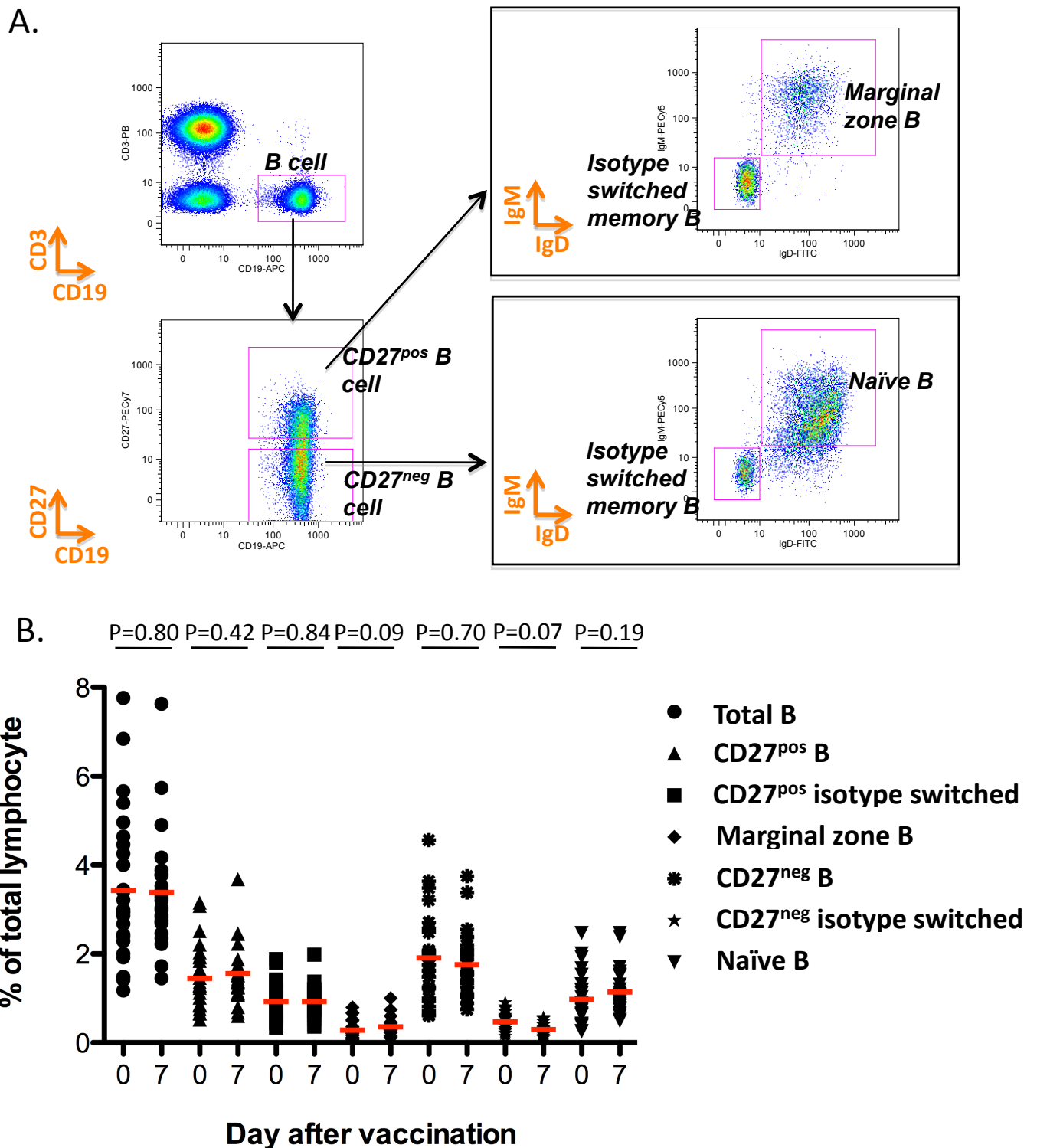


Figure 3.10. A. Gating strategy of B cell subsets. B. The frequency of B cell subsets in the peripheral blood, on day 0 and 7 after influenza vaccination. The frequency was presented as percentage of total lymphocytes, measured by FACS staining. Differences between groups were tested using student's t-test.

In the influenza vaccine trial, one dose of 15µg HA of pandemic H1N1 vaccine induced a significant humoral immune response since it fulfilled all international licensing criteria. The previous experience on influenza H5N1 virus showed that a booster dose of HA or an adjuvanted H5N1 vaccine is necessary for the protective level of antibody response among naïve population (Ehrlich *et al.*, 2008; Lin *et al.*, 2006). Nevertheless, the vaccine trial demonstrated the satisfactory immunogenicity of single dose pandemic H1N1 vaccine. Similar immunogenicity was observed in other pandemic H1N1 vaccine trials (Greenberg *et al.*, 2009; Liang *et al.*, 2010; Nolan *et al.*, 2010; Plennevaux *et al.*, 2010; Zhu *et al.*, 2009).

All three vaccination strategies were able to induce protective antibody response that meet the licensing criteria. However, a significant decrease, by up to 50%, in the antibody response of pandemic H1N1 was noted, when the pandemic H1N1 vaccine was administrated after or alongside the seasonal vaccine. In another vaccine trial, participants with simultaneous administration of pandemic and seasonal vaccines also had similarly lower antibody response to pandemic H1N1 (Vajo *et al.*, 2010). The mechanism of reduced antibody response to pandemic H1N1 virus is unclear, but it recalls the concept of OAS (Virelizier *et al.*, 1974; Webster, 1966). The antibody and ASC response were used to examine if OAS exists in this setting. It was found that subjects generally produced predominant antibody response against the latest vaccine virus but not the previous one. Thus, no obvious OAS effect was noted within subjects who receive two consecutive influenza split vaccines. The previous study also showed the minimal impact of OAS after influenza vaccination, by demonstrating that the vaccination-induced antibody

bound to the latest vaccine strain with high affinity but not to the previously encountered strain (Wrammert *et al.*, 2008). The OAS effect could be shown in mice infected with live influenza virus and could impair the ability of clearing current infection (Kim *et al.*, 2009). Nevertheless, it remains unclear if OAS will present in human vaccinated with live attenuated virus.

Although all three vaccination strategies could result in over 90% of seropositive rate, the diminished antibody response of pandemic H1N1 raises the consideration whether it will affect the clinical protection by pandemic H1N1 vaccine. Compared with the seropositive rate, the mean antibody titre could better represent the efficacy of vaccination (Nauta *et al.*, 2009). In view of the public health and scientific awareness, further analysis of post-pandemic epidemiology and the efficacy of different vaccine strategies should be more vigorous.

In the trial we observed cross-reactive HI response to seasonal H1N1 after pandemic H1N1 vaccination. However, the neutralising antibody response measured by MN assay was rather low. The dominant neutralising epitopes in pandemic H1N1 are quite different from those of recent seasonal strain. It is therefore suggested that those cross-reactive antibodies might recognize the conserved non-neutralising epitopes shared by influenza H1N1 strains. This means the cross-reactive antibodies induced by pandemic vaccine might not confer effective protection against seasonal H1N1 viruses. The efficacy of the pandemic vaccine against seasonal H1N1 infection remains uncertain (Vajo *et al.*, 2010). Hence, in 2009-2010 influenza season, annual influenza vaccine is still recommended for high-risk population to gain effective protection against seasonal influenza viruses.

This trial showed that inactivated seasonal vaccine generates weak cross-reactive response against pandemic H1N1, which could not meet the licensing criteria. The similar result was observed in other vaccine trials as well (CDC, 2009a). The majority of adult population could have encountered seasonal H1N1 virus, but did not have significant neutralising or HI antibodies against pandemic H1N1 (Hancock *et al.*, 2009). Despite these, whether or not the seasonal vaccine confers partial protection against pandemic H1N1 is still debatable (Chen *et al.*, 2011; Echevarría-Zuno *et al.*, 2009; Garcia-Garcia *et al.*, 2009; CDC, 2009b; Del Giudice *et al.*, 2009; Kobinger *et al.*, 2010; Xing and Cardona, 2009). With low level of protective antibody, it remains unclear whether pre-existing cross-reactive T cells play a role in the protection against new influenza infection. Besides, some argue that we are not sure about the incidence of subclinical influenza infection and it is therefore hard to evaluate the effect of seasonal vaccine on reducing the clinical severity of pandemic H1N1 infections. More detailed serological survey and post-pandemic epidemiology information are needed to elucidate the correlation of antibody level and clinical severity. Interestingly, in a previous study, cross-reactive monoclonal antibodies recognizing the stem region of HA were generated from seasonal influenza vaccinees. These antibodies could neutralise pandemic H1N1 virus and limit the viral replication in challenge mice (Corti *et al.*, 2010). Although influenza vaccination in human may not respond in the same way as high dose of monoclonal antibody given in mice does, it provided insight into the flexibility of protection elicited from inactivated influenza vaccine.

This trial demonstrated a robust ASC response induced by inactivated influenza vaccination. This ASC response could be observed in the peripheral blood upon many vaccine antigens (Kelly *et al.*, 2009; Lee *et al.*, 2011; Nieminen *et al.*, 1999; Wrammert *et al.*, 2008). In general, the ASC response peaks on day 6-8 upon antigen exposure, and would disappear by day 14. In the study, the peak IgG ASC response significantly correlated with day 21 serum antibody level, in accordance with another inactivated vaccine study (Halliley *et al.*, 2010). A limited number of studies have explored the relationship of the ASC response with antibody level, the result varies in the type of antigen and the site of detection. For live-attenuated influenza vaccination, no correlation was found between the IgG ASC response and serum neutralising antibody level (Sasaki *et al.*, 2007). After pneumococcal vaccination, the IgG ASC response did not correlated with serum IgG antibody level, but a significant correlation with mucosal IgG antibody level was noted (Nieminen *et al.*, 1998). Thus, this indicates that the ASC response induced by different type of antigens could be very diverse and its role in the humoral immunity needs to be further studied.

In a ferret model, a higher antibody response to the adjuvanted pandemic H1N1 vaccination was noted in the group with previous seasonal vaccination, compared with the naïve group (Del Giudice *et al.*, 2009). This boosting effect might result from the activation of pre-existing MBC. It remains undermined whether the pre-existing immune memory contributes to the robust immunogenicity to a single dose of pandemic 2009 H1N1 vaccine (Chen *et al.*, 2011). It is important to explore the role of MBC in the development of humoral immunity against influenza.

In the study, the frequency of peripheral B cell subsets was examined. It was expected to observe the expansion of MBC induced by vaccination, since isotype-switched MBC would have been generated after antigen exposure and would constitute the major part of antigen-specific immune memory. Without the antigen-specific staining, there was no significant change of B cell subsets detected by FACS staining. Hence, the future study will aim to examine the kinetics and frequency of influenza-specific MBC and its correlation with the humoral immune response to influenza infection or vaccination.

In conclusion, the vaccine trial revealed that one dose of unadjuvanted split pandemic H1N1 vaccine was well tolerated and generated a potent antibody and B cell response. However, Pre- or co-vaccination with the seasonal flu vaccine led to a significant reduction by 50% in HI antibody response to pandemic H1N1 virus. This effect was also noted in the neutralising antibody and the ASC response. When seasonal and pandemic H1N1 vaccines were given sequentially, the antibody response was predominantly against current H1N1 virus, but not previous (original) vaccine virus. Besides, a significantly homotypic cross-reactive response was found after the inactivated influenza vaccination. Of note, the influenza vaccine trial demonstrated the humoral response to influenza antigens from serological and cellular aspects. The early-phase ASC response significantly correlated with serum HI and neutralisation antibody level three weeks later. The ready detection of early-phase ASC response indicated a promising way of further analyzing the role of antigen-specific B cell in the field of influenza immunology.

CHAPTER 4

INFLUENZA A H1N1 CHALLENGE STUDY

4.1 INTRODUCTION

Influenza is an acute respiratory viral infection and has pandemic potential. A rough estimate by WHO suggests that seasonal epidemics of influenza cause a global disease burden from 1 billion infections, to 3 million to 5 million cases of severe disease, and between 300,000 and 500,000 deaths annually (Lambert and Fauci, 2010). In most mild to moderate cases, it is a self-limiting infection. However, in severe cases, prolonged virus shedding and pronounced disease severity result in complications and deaths. Disease and severe illness could be effectively prevented by vaccination. In the protective immunity in human, neutralising antibodies against surface antigens play a central role in preventing infection and serve as a goal of successful vaccine (Dormitzer *et al.*, 2011). Neutralising antibodies are directly produced by the ASC, which could appear transiently in large number in the periphery on the first week after antigen exposure (refer to Chapter 3; Cox *et al.*, 1994; Sasaki *et al.*, 2007; Wrammert *et al.*, 2008; Wu *et al.*, 2011). Over 50% of the vaccination-induced ASC could be antigen-specific. The size of ASC response strongly correlates with the level of neutralising antibodies upon inactivated influenza vaccination (refer to Chapter 3). However, it is unclear how the host responds to natural infection on the size of ASC response and the quality of antibody production.

Sustained serum antibody level is maintained by long-lived ASC and MBC (Bernasconi *et al.*, 2002; Crotty and Ahmed, 2004; Slifka and Ahmed, 1998;

Slifka *et al.*, 1998). Long-lived ASC reside in the bone marrow and are barely detected in the peripheral blood (Slifka *et al.*, 1995). MBC circulate in the peripheral blood and local lymph nodes. MBC, which are remarkably long-lived, are believed to replenish long-lived ASC to maintain long-term antibody levels in the absence of antigen (Bernasconi *et al.*, 2002; Slifka *et al.*, 1998). MBC are responsible for driving the rapidly anamnestic antibody response upon re-exposure to antigen. In H5N1 vaccination, cross-reactive MBC could be induced by priming with MF59-adjuvanted mismatched H5N3 vaccine and were readily expanded to elicit neutralising and long-lasting cross-reactive antibodies upon boosting with the H5N1 vaccine (Galli *et al.*, 2009a). Although MBC are important for long-term humoral immunity, it is unclear how well MBC are generated after infection, how large the antigen-specific pools of MBC are, and how long they are maintained without re-exposing to a cognate antigen. It is also unclear whether MBC correlate with serum antibody level for most antigens, and it is therefore important to track MBC as an independent parameter of antigen-specific immune memory.

A number of laboratory animal models have been developed to explore the influenza pathogenesis and the induced immune response (Barnard, 2009). Some of them were setup to evaluate the efficacy of new vaccine or therapeutic agents. Mice model is most widely used. In mice challenge model, infection-induced B cells and antibody response make a significant contribution to the resolution of infection (Baumgarth *et al.*, 2000; Choi and Baumgarth, 2008; Mozdzanowska *et al.*, 2005; Waffarn and Baumgarth, 2011). However, use of animal models has limited applications on humans. Mice are not readily susceptible to newly isolated human influenza strain.

Adapted viruses are therefore required to setup the challenge model.

Challenged mice do not have some symptoms presented on human, such as fever and cough (Barnard, 2009). Most importantly, laboratory mice are usually naïve to flu, but the majority of adult human population has immune memory to flu. Naturally infected patients could be the optimal resource to study influenza-induced immunity though, but clinical samples are difficult to obtain and pre-infection samples are often lacking. In human challenge model, the enrolled participants, the inoculum dose, the type of challenge virus, the time of infection and sampling could be tightly controlled. Influenza challenge models in human are therefore setup by Retroscreen Virology Ltd, London, UK in year 2009 and 2010. Here, I made use of the samples from two challenge studies to examine the context of humoral immunity following an acute influenza A infection (refer to Chapter 2).

4.2 STUDY DESIGN

In the study, healthy, non-pregnant adult volunteers between the ages of 18 and 45 years were eligible for enrolment. The exclusion criteria included confirmed or suspected seasonal H1N1 infection and history of seasonal influenza vaccines during the preceding 6 months. All volunteers had HI titre less than 1:40 to A/Brisbane/59/07 (H1N1 BR59) virus, the challenged viral strain in the studies. At last, 24 and 20 healthy adult volunteers were enrolled and randomized in 2009 and 2010, respectively (figure 4.1).

Enrolled volunteers were inoculated nasally with the GMP grade of influenza A virus, egg grown H1N1 A/Brisbane/59/07 virus. After challenging, all were quarantined for 7 days until truncated by Tamiflu medications. Then all had

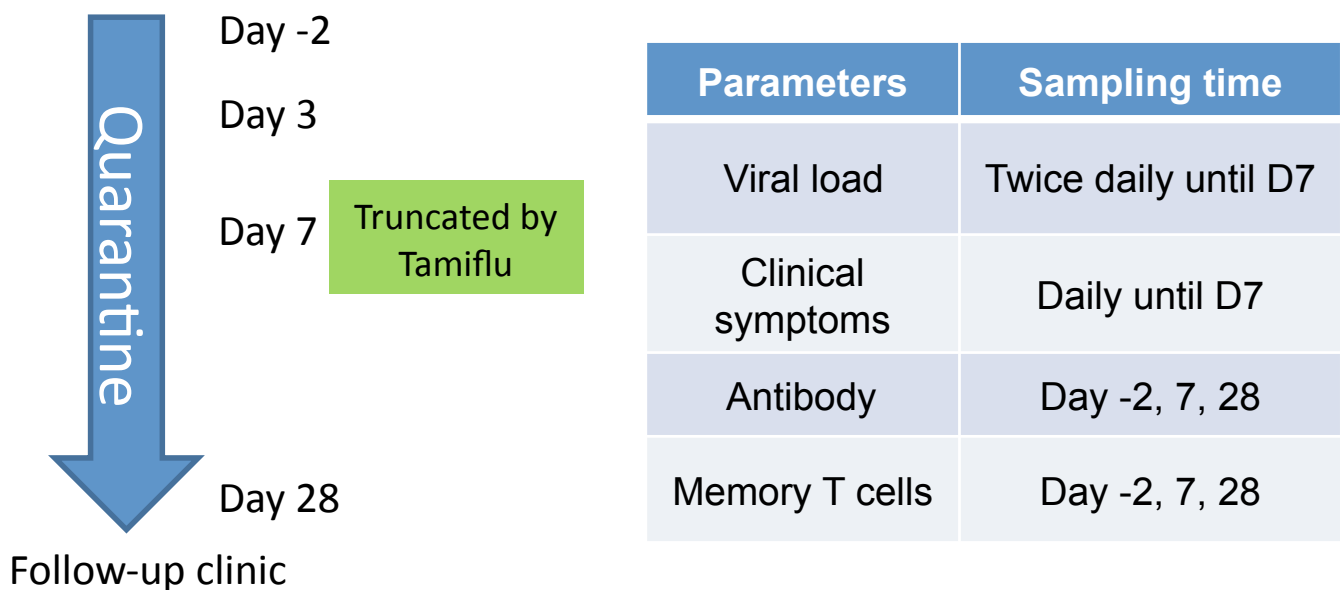
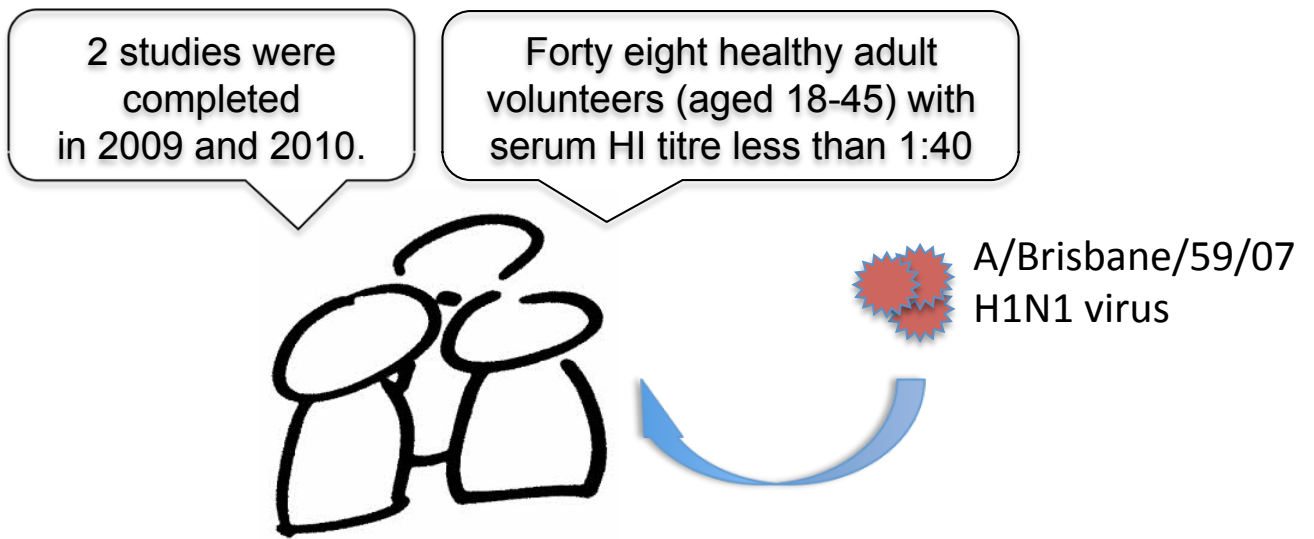


Figure 4.1. The setup of experimental human influenza infection.

continued clinical follow-up on day 28 (figure 4.1). No medications were administered, except acetaminophen for treatment of severe symptoms.

Clinical symptoms (cough, sore throat, running nose, general malaise, muscle soreness, etc.) and oral temperature were recorded throughout the study period. Nasopharyngeal swabs were collected daily during the quarantine period. Fresh whole blood samples were taken on day -2, 3, 7 and day 28 after challenge (figure 4.2).

4.3 RESULTS

4.3.1 The setup of influenza challenge model provided the platform for assessing the clinical response and measuring the kinetics of immune response.

Combining two challenge studies, a total of 44 healthy adult volunteers aged between 18 and 45 were inoculated intra-nasally with 10^6 TCID₅₀ of influenza H1N1 BR59 viruses. Among volunteers, 15 out of 44 (34%) were female and the median age was 24 yr (range 19~35).

Prior to influenza virus challenge, none of volunteers has protective antibody titre against H1N1 BR59 virus (HI titre equal to or above 40). Among volunteers, 36 out of 44 (82%) were HI seronegative, 3 had a HI titre of 1:10, 4 had a titre of 1:20 and 1 had a titre of 1:28 to H1N1 BR59 virus. All volunteers were influenza virus negative by the nasopharyngeal swab and the PCR assay. After challenge, all were then longitudinally followed in symptoms/signs development, viral shedding and serum antibody response. In this study, the establishment of infection was defined as the development of

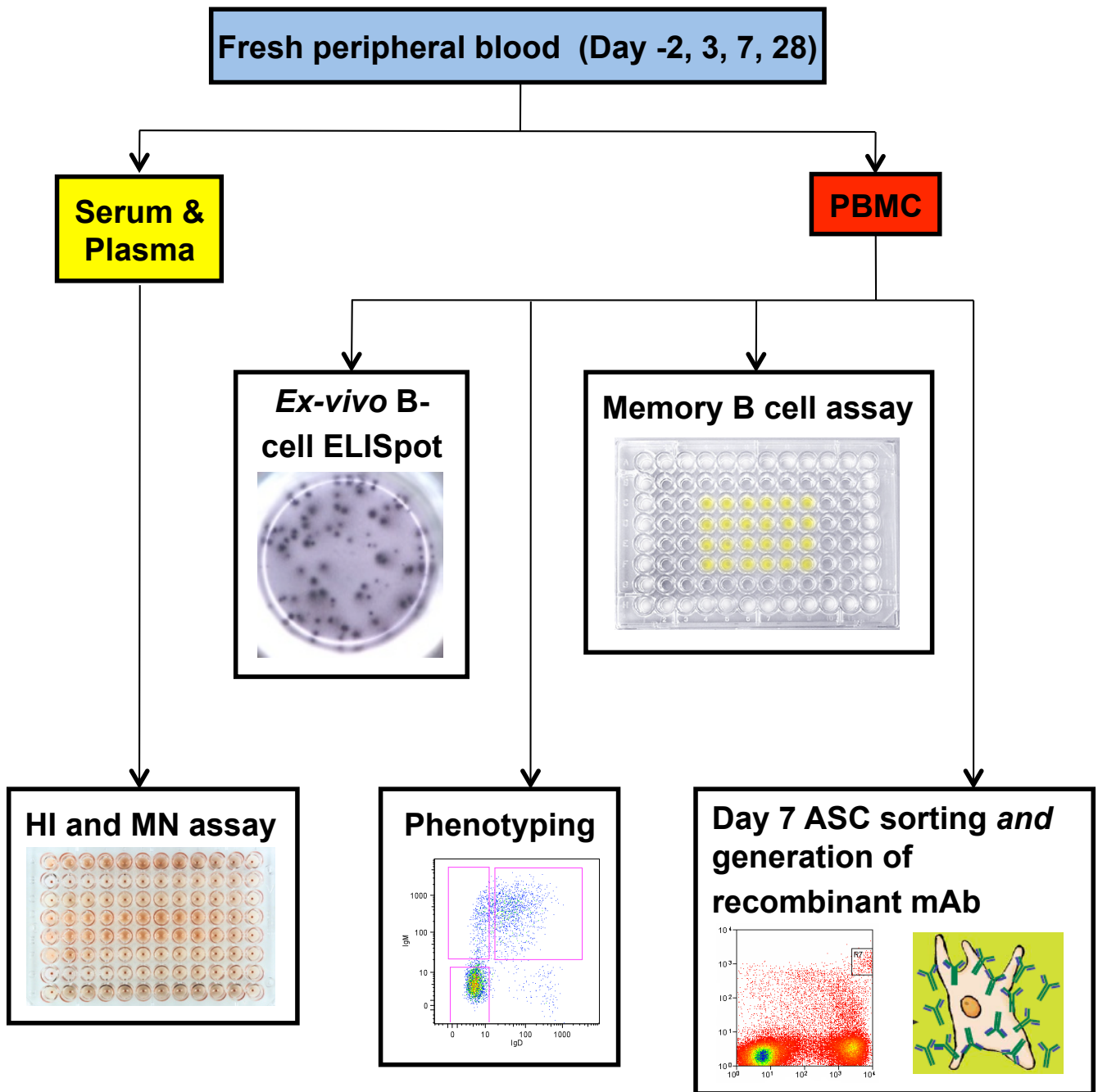


Figure 4.2. Approaches for H1N1 BR59 challenge studies. Fresh whole blood samples were taken from challenged volunteers on day -2, 3, 7 and 28 after challenge. To examine the kinetics and frequency of influenza-specific B cell response, PBMC were separated and used to setup *ex-vivo* B-cell ELISpot and memory B cell culture assay. Lymphocyte phenotyping was setup to examine the kinetics and frequency of peripheral B cell subsets. On day 7, peripheral ASC were stained and sorted for making human recombinant mAb. Besides, serum and plasma were collected to setup HI and MN assays to examine the influenza-specific antibody response.

influenza-like illness and/or virus shedding and/or seroconversion by day 28 (table 4.1).

The overall infection rate was 12 out of 44 (27%) enrolled volunteers. Among infected volunteers, 12 of 12 (100%) develop influenza-like illness, presenting cough, sore throat, headache, rhinorrhea, myalgia, or general malaise with or without a documented fever; 10 of 12 (83%) had either positive viral load or seroconversion on day 28 (table 4.1).

In the study, the mean duration of viral shedding is 2.9 (range 1~6) days.

Some infected individuals had prolonged virus shedding for as long as 6 days, but most of them cleared the virus completely in 5 days.

4.3.2 Influenza-specific ASC were detected in the peripheral blood and moderately correlated with viral load after H1N1 BR59 infection.

In the study, the frequency of total and influenza-specific IgG ASC were measured by *ex-vivo* B-cell ELISpot on day -2, 3, 7 and 28.

On day -2, the frequency of total IgG ASC was found at 158 ± 20 per million PBMC (0.0158% of PBMC) on average. There were no detectable influenza-specific ASC within all volunteers on day -2.

On day 3 after challenge, the frequency of total IgG ASC was similar to day -2 ($p=0.9077$). No influenza-specific ASC were detected either.

During the first week after challenge, 4 volunteers (Lab ID 2, 8, 9 and 16) dropped out. Only nasopharyngeal swabs and clinical manifestations were continually collected but no blood samples were collected for these 4

Table 4.1. Summary of H1N1 BR59 challenge studies.

Subject No	Lab ID	Flu-like illness*	Fever [#]	Viral Load (Log ₁₀ TCID ₅₀)							HI titre		BR59 IgG ASC per 10 ⁶ PBMC	
				D1	D2	D3	D4	D5	D6	D7	D-2	D28	D7	D7
Challenge study in July 2009 ^{&}														
B001	3	-	-	-	-	-	-	-	-	-	NDA	NDA	0	0
B002	1	-	-	-	-	-	-	-	-	-	NDA	NDA	0	0
B003	18	-	-	-	-	-	-	-	-	-	NDA	10	0	0
B004	19	-	-	-	-	-	-	-	-	-	NDA	20	0	0
B005	15	Yes	-	-	-	-	-	2.50	-	-	NDA	40	56	189
B006	14	Yes	-	-	-	-	-	-	-	-	NDA	40	10	0
B007	24	-	-	-	-	-	-	-	-	-	NDA	10	32	25
B008	23	Yes	-	2.25	-	-	-	-	-	-	NDA	160	0	0
B009	20	Yes	-	-	1.75	-	-	-	-	-	NDA	10	25	0
B010	21	-	-	-	-	-	-	-	-	-	NDA	NDA	0	0
B011	17	-	-	-	-	-	-	-	-	-	NDA	20	173	277
B012	16	Yes	Yes	2.25	-	-	-	-	-	-	NDA	40	0	0
B013	10	Yes	Yes	-	-	-	-	-	-	-	NDA	NDA	277	0
B014	6	-	-	-	-	-	-	-	-	-	NDA	NDA	80	0
B015	7	Yes	-	-	2.50	-	-	2.25	3.00	-	NDA	20	0	30
B016	28	-	-	-	-	-	-	-	-	-	NDA	NDA	0	0
B017	2	Yes	-	-	-	-	-	-	-	-	NDA	40	0	0
B018	29	-	-	-	-	-	-	-	-	-	NDA	NDA	0	0
B019	13	-	-	-	-	-	-	-	-	-	NDA	NDA	0	0
B020	12	-	-	-	-	-	-	-	-	-	NDA	NDA	0	0
B021	8	Yes	-	-	-	-	-	-	-	-	NDA	160	30	0
B022	9	-	-	-	-	-	-	-	-	-	NDA	NDA	0	0

volunteers on day 7. On day 7 after challenge, 12 out of 40 (30%) volunteers demonstrated a significant increase of the frequency of total IgG ASC, which was 717 ± 218 per million PBMC v.s. 154 ± 44 on day -2 ($p=0.0007$) (figure 4.3 B). In parallel to the burst of total ASC response, H1N1 BR59-specific ASC was firstly detected on ELISpot. The frequency of H1N1 BR59-specific IgG ASC was 166 ± 56 per million PBMC on average (figure 4.3 A). The percentage of H1N1 BR59-specific IgG ASC averaged 23.3 % (range 5~50.4%) of total IgG ASC (figure 4.3 C).

It was noted that all 12 infected volunteers had positive ASC response on day 7. The lineal regression analysis revealed that the ASC response measured by ELISpot moderately correlated with the viral load ($r^2=0.6582$, $p=0.0268$) and the duration of viral shedding ($r^2=0.5703$, $p=0.0497$). However, the ASC response did not correlate with serum HI titre on day 28 (figure 4.4).

In the 2010 challenge study, the IgM and IgA ASC response upon influenza challenge were measured by ELISpot.

On day -2, the circulating total ASC were IgA predominant, followed by IgG and IgM ($p<0.0001$) (figure 4.5). No influenza-specific IgM or IgA ASC were detected.

Similar to the IgG ASC response, on day 7, those infected volunteers presented the H1N1 BR59-specific IgM and IgA ASC response, which are 37 ± 12 and 93 ± 58 per million PBMC, respectively (figure 4.6). It is interesting to observe that the IgG ASC response seemed comes in the strongest, followed by IgA, and IgM ASC response after infection. However, it should be noticed

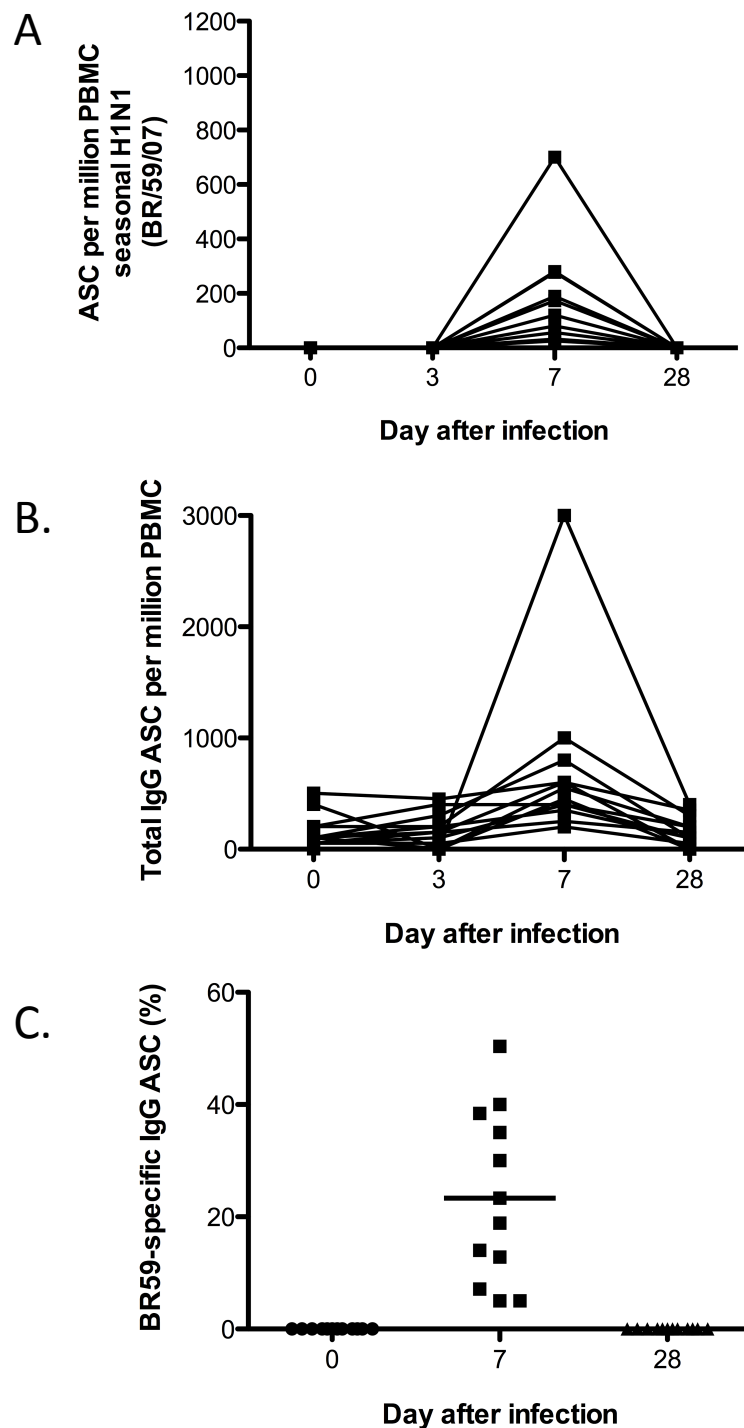


Figure 4.3. Analysis of BR59-specific and total ASC of twelve infected volunteers in the 2009 & 2010 H1N1 BR59 challenge studies. A. The kinetics of BR59-specific ASC was measured by B cell ELISpot against vaccine antigen of A/Brisbane/59/09 H1N1-like virus. The data were presented as the number of IgG BR59-specific ASC per million PBMC. B. The kinetics of total IgG ASC was measured by B cell ELISpot. The data were presented as the number of total IgG ASC per million PBMC. C. The percentage of BR59-specific IgG ASC in total circulating IgG ASC was calculated based on the result of B cell ELISpot.

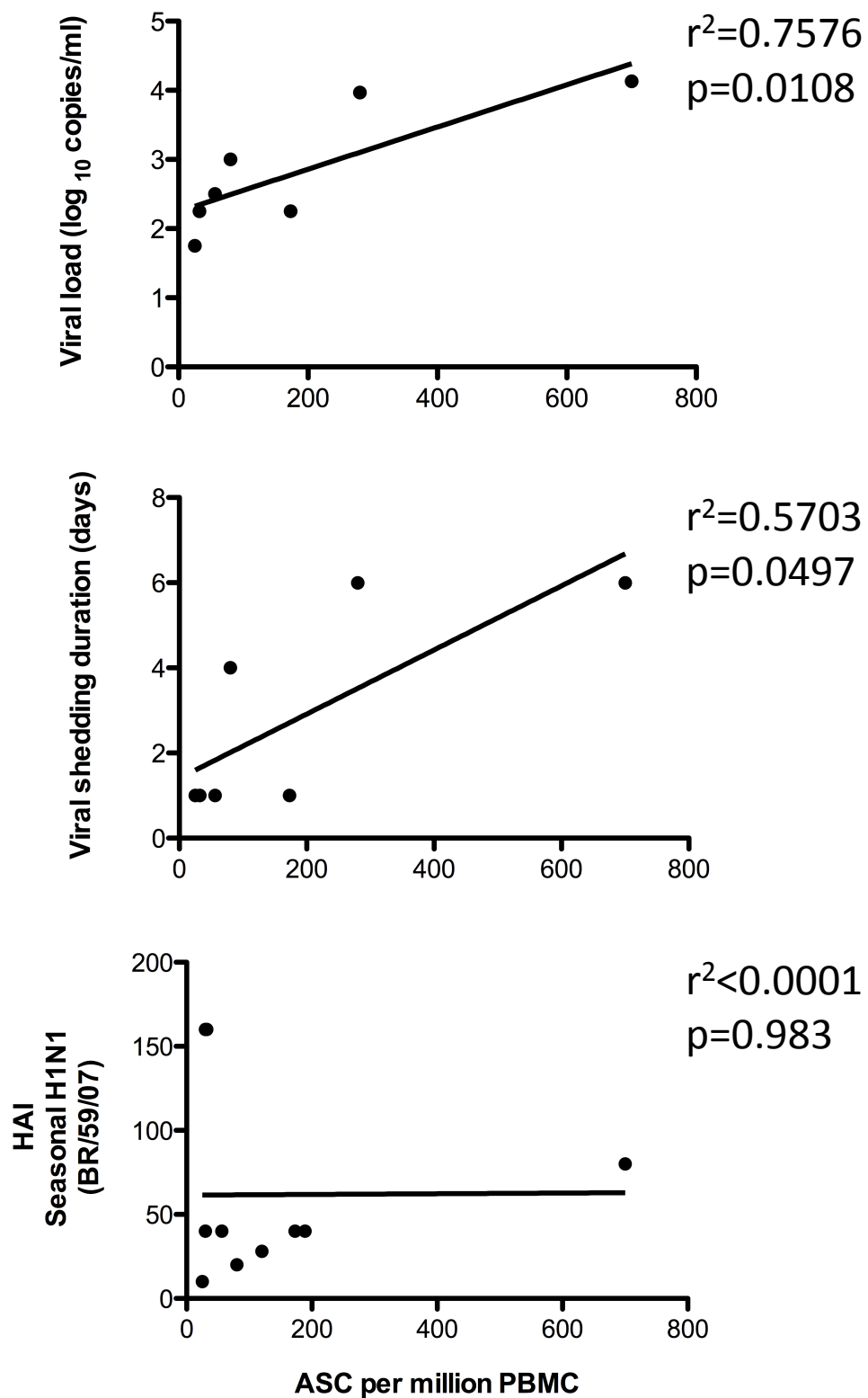


Figure 4.4. Analysis of day 7 BR59-specific ASC response with viral load, viral shedding duration and day 28 HI titre of 12 infected volunteers. Linear regression analysis was performed to examine their association by GraphPad Prism.

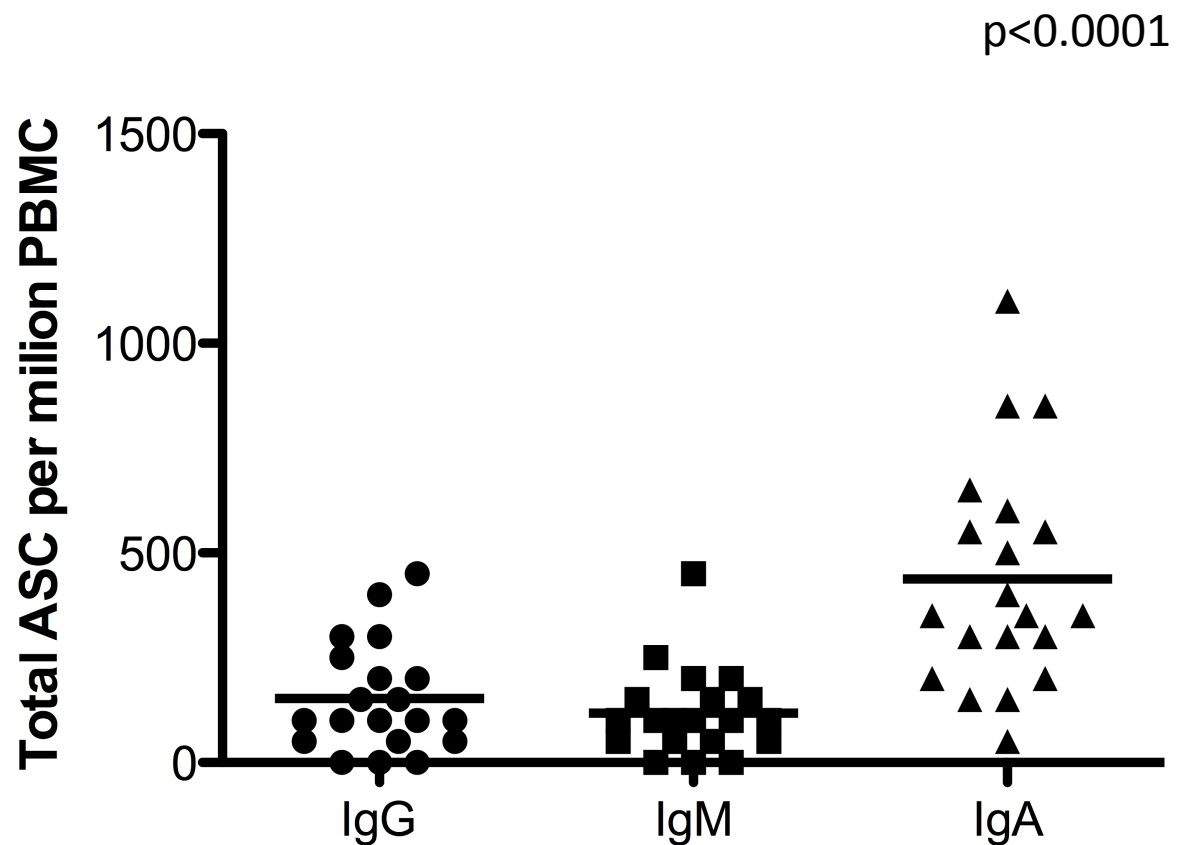


Figure 4.5. The frequency of total IgG, IgM and IgA ASC on day -2, as measured by ELISpot. The result from twenty subjects in the 2010 challenge study were shown in the figure. Kruskal-Wallis test was performed to compare the groups of data by GraphPad Prism.

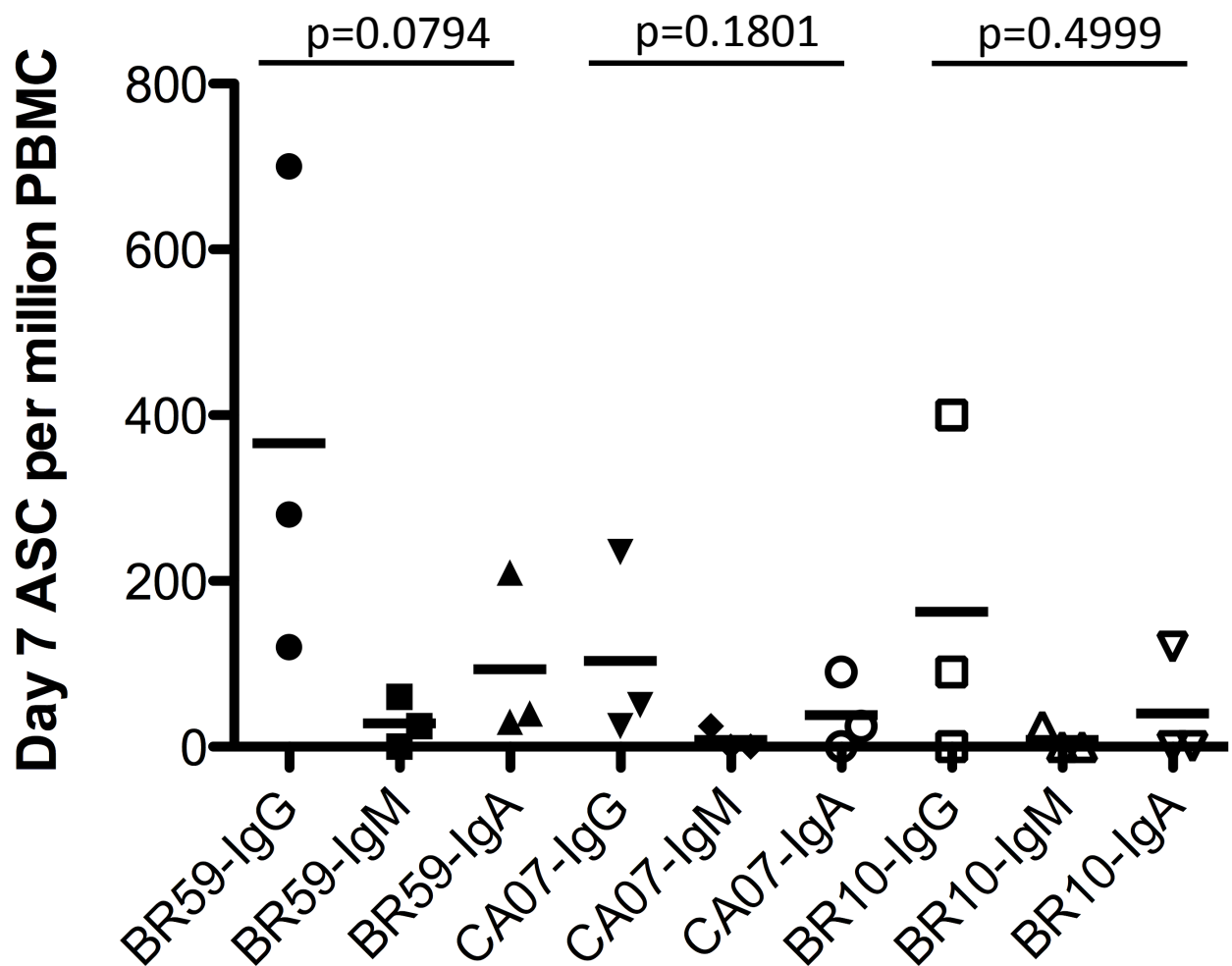


Figure 4.6. The frequency of IgG, IgM and IgA influenza-specific ASC on day 7 after H1N1 BR59 infection was measured by B cell ELISpot. The result from three infected volunteers was shown in the figure. Kruskal-Wallis test was performed to compare the groups of data by GraphPad Prism.

that the number of infected volunteer was so few in the 2010 challenge study that the difference cannot reach the statistical significance ($p=0.0794$).

By coating the ELISpot with other strains or subtypes of influenza vaccine virus, e.g. pandemic H1N1 A/California/59/07 or H3N2 A/Brisbane/10/07-like virus, cross-reactive ASC response after influenza infection can be detected. In the 2010 challenge study, on day 7, those H1N1 BR59-infected volunteers presented H1N1 CA07-specific and H3N2 BR10-specific IgG ASC response, which are 103 ± 66 and 163 ± 121 per million PBMC, respectively (figure 4.6). However, the H1N1 BR59-specific ASC response was predominantly induced upon infection. Based on the ASC response in a limited number of infected cases, no OAS was noted in the study.

The duration of influenza-specific ASC response is uncertain due to the lack of sampling between day 7 and 28. However, on day 28 after challenge, no influenza-specific ASC were detected in the peripheral blood. The frequency of total IgG ASC was back to pre-infection level ($p=0.7465$).

Taken together, a transient but significant ASC response was detected in the peripheral blood one week after infection. The percentage of influenza-specific ASC varied among infected volunteers and could be up to 50% per total ASC. These results indicated the infection induced the selective expansion of influenza-specific ASC over time.

4.3.3 The kinetics of influenza-specific MBC in the peripheral blood was detected after H1N1 BR59 infection.

In the study, the frequency of influenza-specific MBC was determined by memory B cell culture assay plus B-cell ELISpot on day -2, 3, 7 and 28.

As opposed to antibody secreting cells, MBC do not secrete immunoglobulins that are necessary to form spots in the ELISpot. Therefore, *in vitro* pre-activation of MBC into ASC is the prerequisite for their detection with the ELISpot assay (figure 4.7A). In the study, the culture medium, CpG/SAC/PWM cocktail, was used to activate all peripheral B cells into ASCs. After then, the ELISpot was performed to measure antigen-specific B cells. Some PBMC would die through the activation process and the efficiency of B cell activation would differ within subjects. After collecting cultured cells, the frequency of influenza-specific B cell was presented as the percentage of the total peripheral B cell measured by ELISpot (figure 4.7B).

On day -2, 80% of volunteers had detectable H1N1 BR59-specific IgG cells. The frequency was $0.485\% \pm 0.055\%$ of total IgG cells (figure 4.8). The similar result has been noted in the previous study, that the frequency of influenza-specific B cell was about 0.2~0.5% (Sasaki *et al.*, 2007). It has been reported that over 70% of adults tested had detectable influenza-specific IgG cells (Sasaki *et al.*, 2007; Yarchoan *et al.*, 1983).

After challenge, a significant increase of H1N1 BR59-specific IgG cells was observed within infected volunteers. On day 3, its frequency averaged $0.699\% \pm 0.0167\%$; on day 7, it averaged $1.008\% \pm 0.170\%$; while on day 28, it averaged $1.954\% \pm 0.055\%$ of total IgG cells ($p < 0.0001$ for all time point comparisons) (figure 4.9A). Neither day 7 nor day 28 H1N1 BR59-specific IgG response correlated with day 28 serum HI titre ($r^2 = 0.2388$, $p = 0.1518$). The

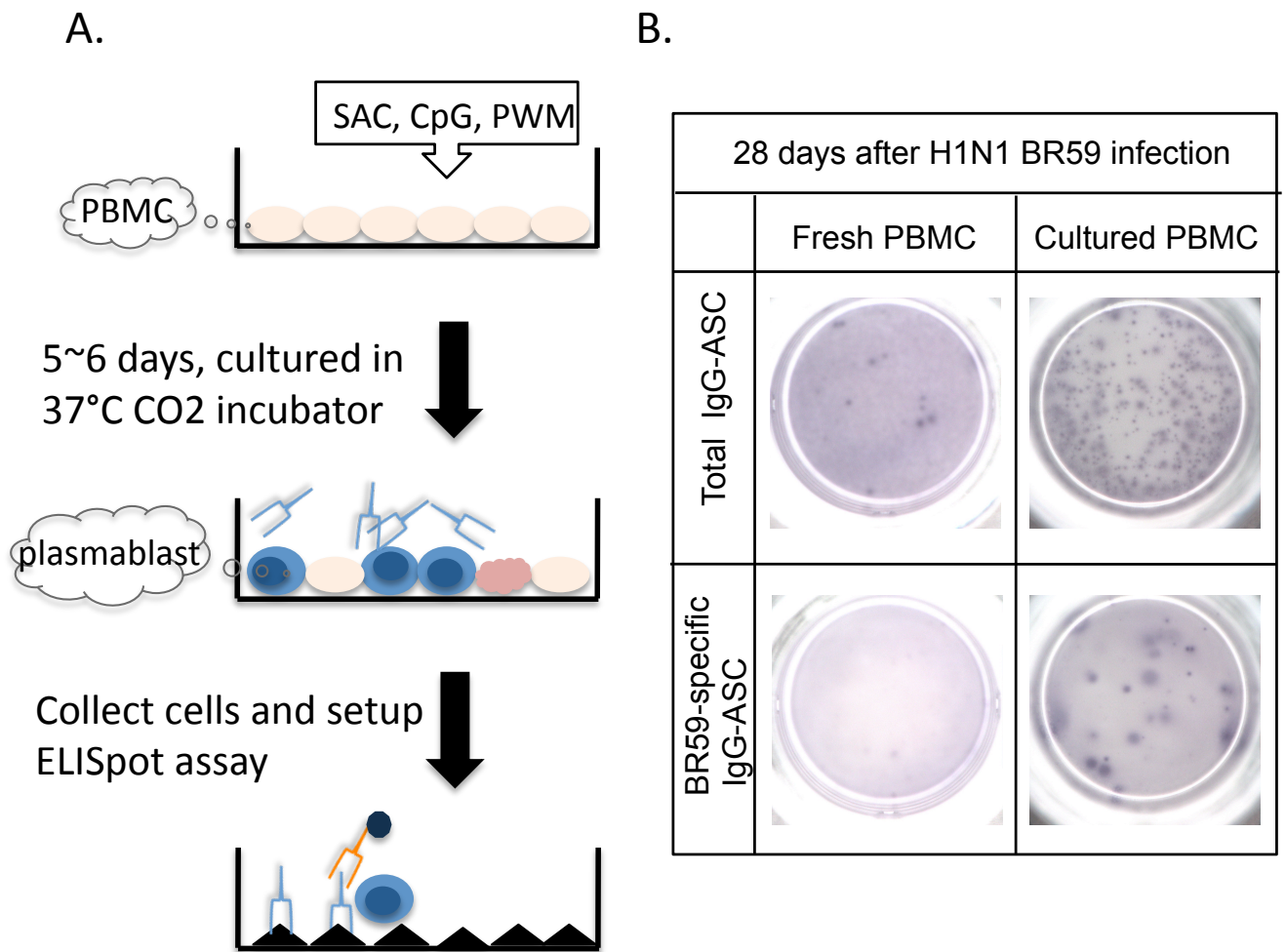


Figure 4.7. A. The diagram of memory B cell culture assay. B. After culture, plasmablasts differentiated from MBC would secrete antibody and are able to be measured by B cell ELISpot assay.

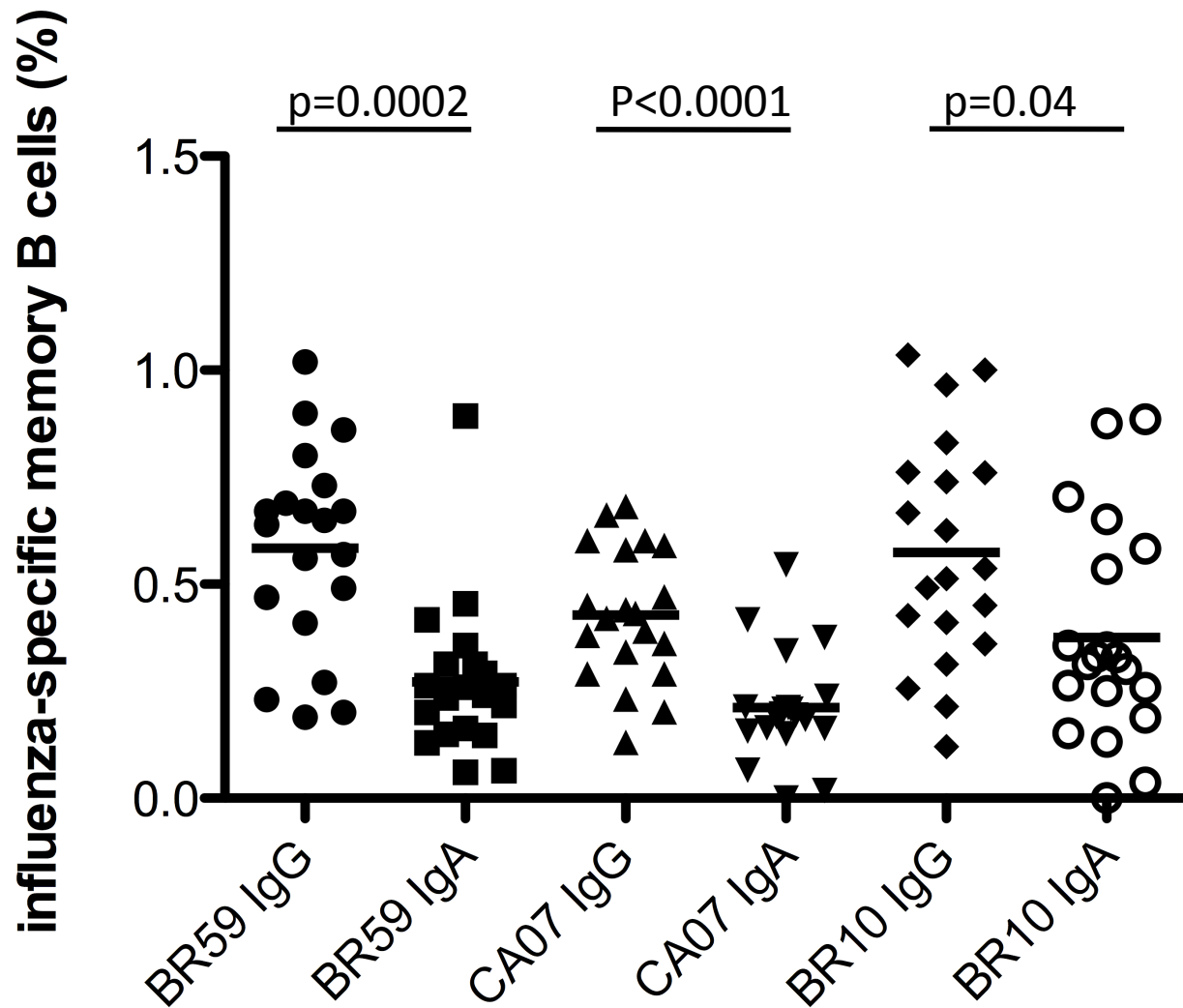


Figure 4.8. The frequency of pre-existing influenza-specific MBC among the 2010 challenge volunteers. Data are presented as the percentage of total IgG or IgA B cells. The student's t-test was performed to compare two groups of data by GraphPad Prism.

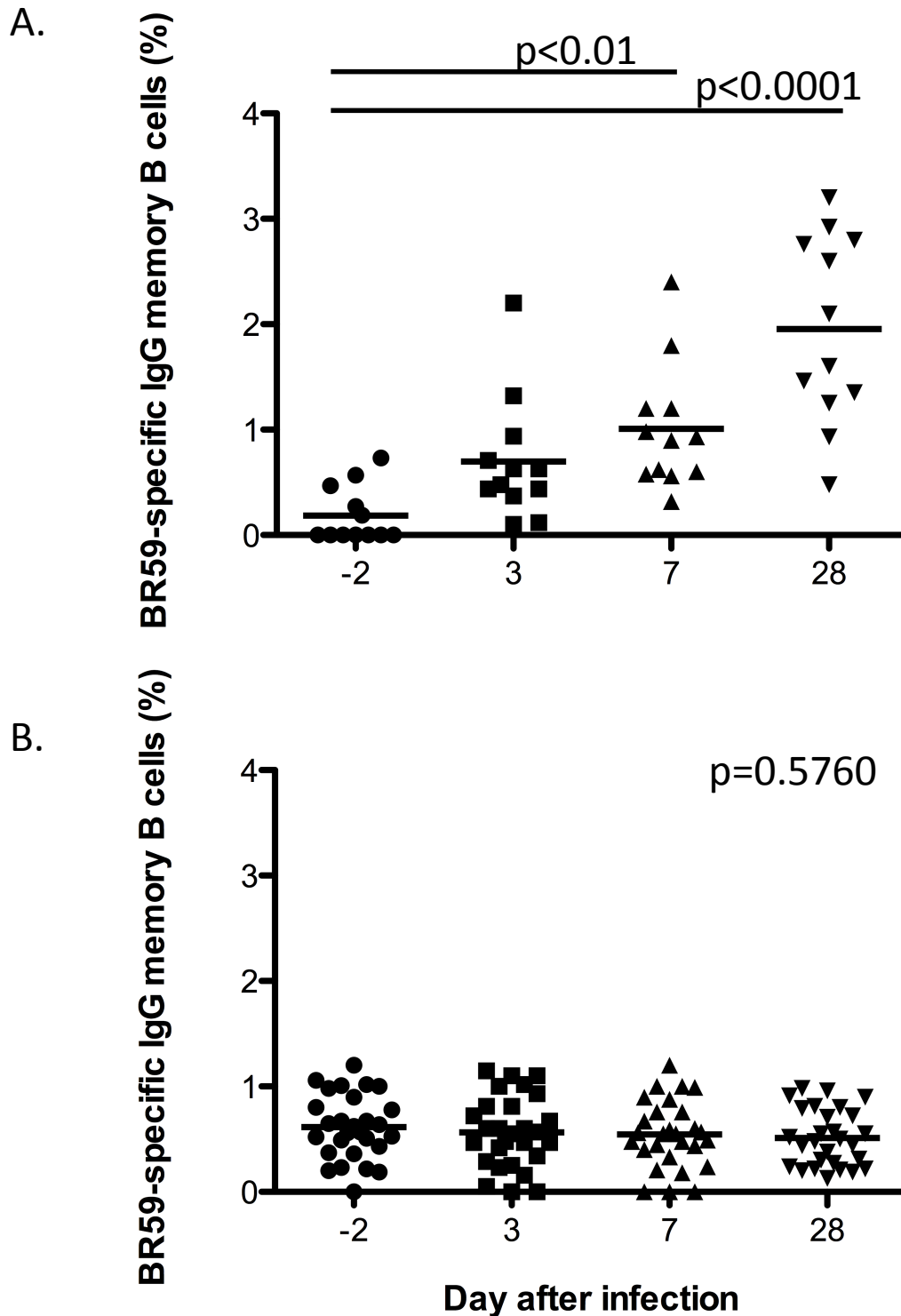


Figure 4.9. A. The kinetics of BR59-specific IgG MBC for 12 infected individuals in the 2009 & 2010 challenge studies was shown in the figure. Differences between groups were tested using Kruskal-Wallis test with post hoc Dunn's test for multiple comparisons. B. The kinetics of BR59-specific IgG MBC for 28 uninfected individuals in the 2009 & 2010 challenge studies was shown in the figure. Kruskal-Wallis test was performed by GraphPad Prism.

H1N1 BR59-specific IgG response of uninfected volunteers was low and similar across the study period ($p=0.5760$) (figure 4.9B).

IgA cells were also measured in the 2010 challenge study. On day -2, compared with influenza-specific IgG cells, there was lower percentage of influenza-specific IgA cells, which was $0.272\% \pm 0.040\%$ of total IgA cells ($p=0.0002$) (figure 4.8). The similar result has been noted in the previous study, that the frequency of pre-existing influenza-specific IgG cells increased gradually with age and could outnumber IgA cells in adults (Sasaki *et al.*, 2007). After infection, similar as the H1N1 BR59-specific IgG cells, there observed a rising trend of H1N1 BR59-specific IgA cells. However, it has to be mentioned that the number of infected volunteers in the 2010 challenge study was too few to reach the statistical significance (figure 4.10).

In the 2010 challenge study, H1N1 CA07-specific and H3N2 BR10-specific B cells were measured as well. Since the H1N1 CA07 pandemic occurred in 2009, It was not surprised to observe pre-existing CA07-specific IgG and IgA cells at $0.427\% \pm 0.035\%$ of total IgG and $0.212\% \pm 0.029\%$ of total IgA cells, respectively (figure 4.8). Within H1N1 CA07-specific IgG cells, an average of $49\% \pm 7\%$ was HA-specific, as measured by ELISpot (figure 4.11). Pre-existing H3N2 BR10-specific IgG and IgA cells were also detected at $0.574\% \pm 0.059\%$ and $0.375\% \pm 0.057\%$, respectively. After infection, similar to H1N1 BR59-specific B cells, there observed a rising trend of H1N1 CA07 and H3N2 BR10-specific B cells, although it has to be mentioned again that the number of infected volunteers here was too few to reach the statistical significance (figure 4.10).

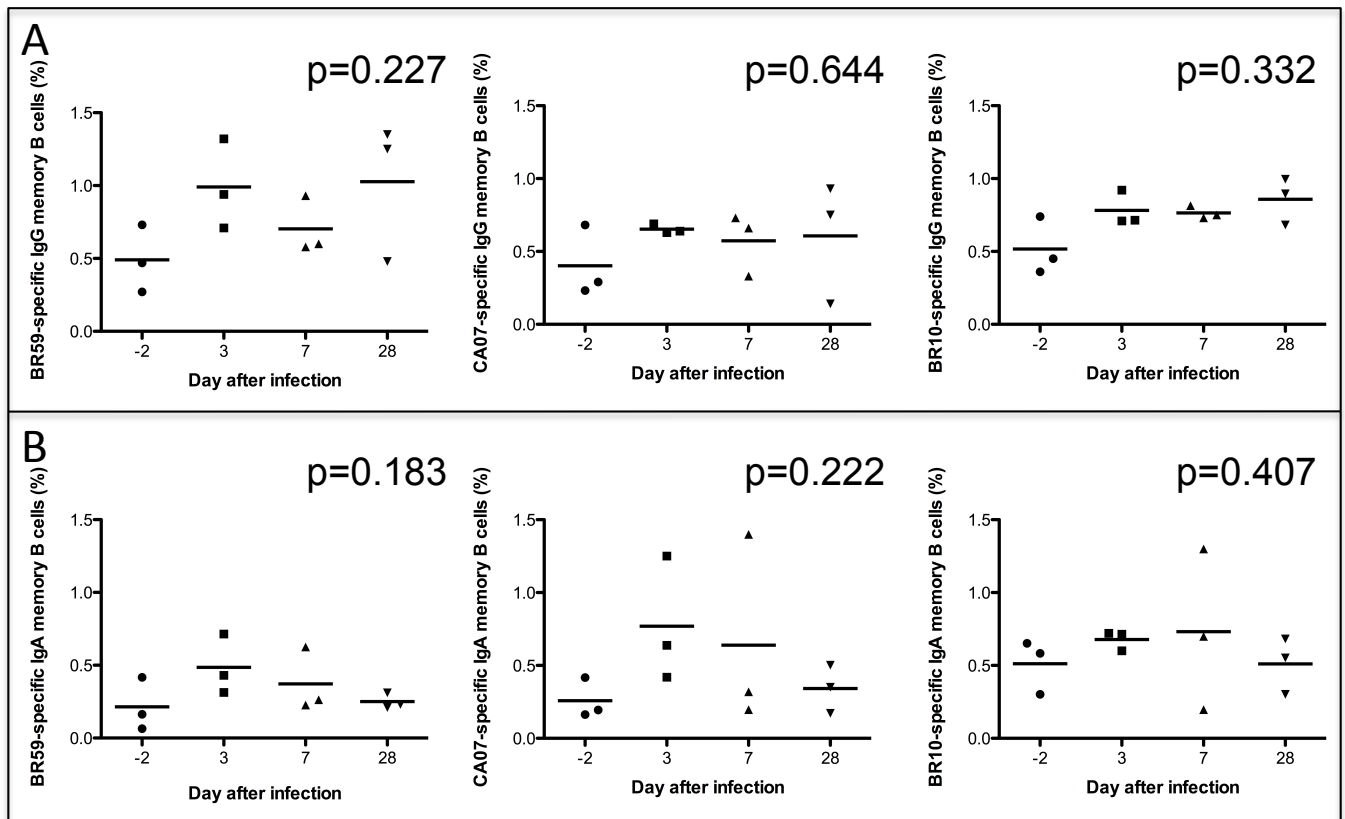


Figure 4.10. A. The kinetics of IgG MBC for H1N1 BR59, pH1N1 CA07, and H3N2 BR10. B. The kinetics of IgA MBC for H1N1 BR59, pH1N1 CA07, and H3N2 BR10. The result from infected volunteers (Lab ID. 1, 14 and 18) in the 2010 challenge study was shown in the figure. Kruskal-Wallis test was performed to compare the groups of data by GraphPad Prism.

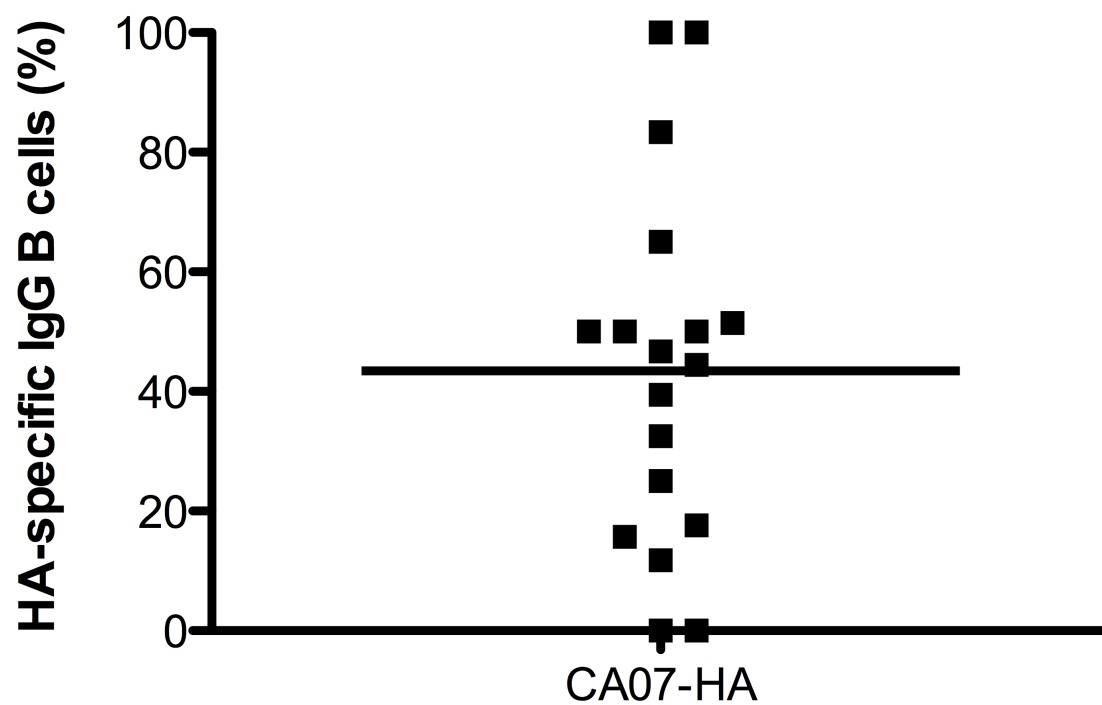


Figure 4.11. The percentage of HA-specific cells within H1N1 CA07-specific IgG MBC. The result was collected on day -2 in the 2010 challenge study.

When observing the significant expansion of H1N1 BR59-specific B cells after infection, a question about which B cells subset these influenza-specific cells belong to was raised. Therefore, naïve, marginal zone and isotype switched B cells were sorted from one infected volunteer (No. 4046 in the 2010 challenge study) on day 28 (figure 4.12). Sorted cells were co-cultured with heterologous irradiated PBMC in the memory B cell assay. Then, cultured cells were collected and put into ELISpot to identify influenza-specific cells. For that infected individual, the result demonstrated that most influenza-specific IgG cells come from CD27^{pos} IgM^{neg} IgD^{neg} and CD27^{neg} IgM^{neg} IgD^{neg} isotype switched B cell subsets (figure 4.13). There were no detectable influenza-specific IgG cells within the marginal zone and naïve B cell subsets. This result is compatible with the current knowledge of the development of T-cell dependent humoral immunity. It is widely believed that antigen-specific MBC would be generated through hypermutation and isotype-switching in the germinal center upon antigen exposure. With regard to the distribution of influenza-specific IgG cells, H1N1 BR59-specific cells account for 0.74% of CD27^{pos} IgM^{neg} IgD^{neg} and 0.28% of CD27^{neg} IgM^{neg} IgD^{neg} B cell pool; while H1N1 CA07-specific cells account for 0.28% of CD27^{pos} IgM^{neg} IgD^{neg} and 0.07% of CD27-IgM-IgD- B cell pools. It seemed that there is higher frequency of influenza-specific cells in the CD27^{pos} IgM^{neg} IgD^{neg} than CD27^{neg} IgM^{neg} IgD^{neg} B cell pool. However, it has to be pointed out that the case number is too low to draw any concrete conclusion.

In the study, memory B cell culture assay plus B-cell ELISpot had been tried to determine influenza-specific IgM B cells but the result was difficult to interpret and validate due to their high background. Since the culture medium

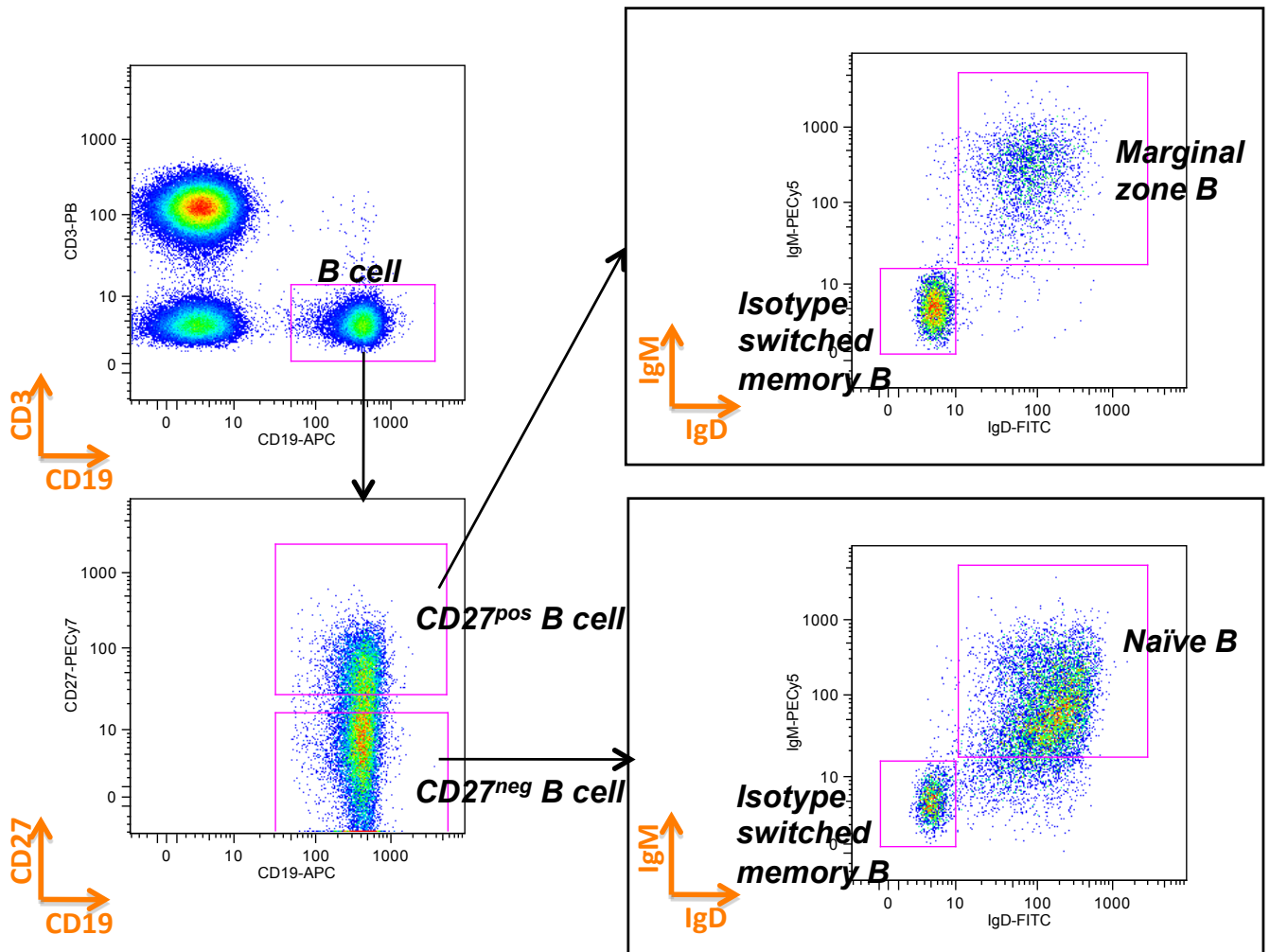


Figure 4.12. The panel for peripheral B cell staining and sorting. PB anti-CD3, APC anti-CD19, PECy7 anti-CD27, PECy5 anti-IgM, and FITC anti-IgD from BD Biosciences were used to stain PBMC. The data were analyzed with FlowJo software.

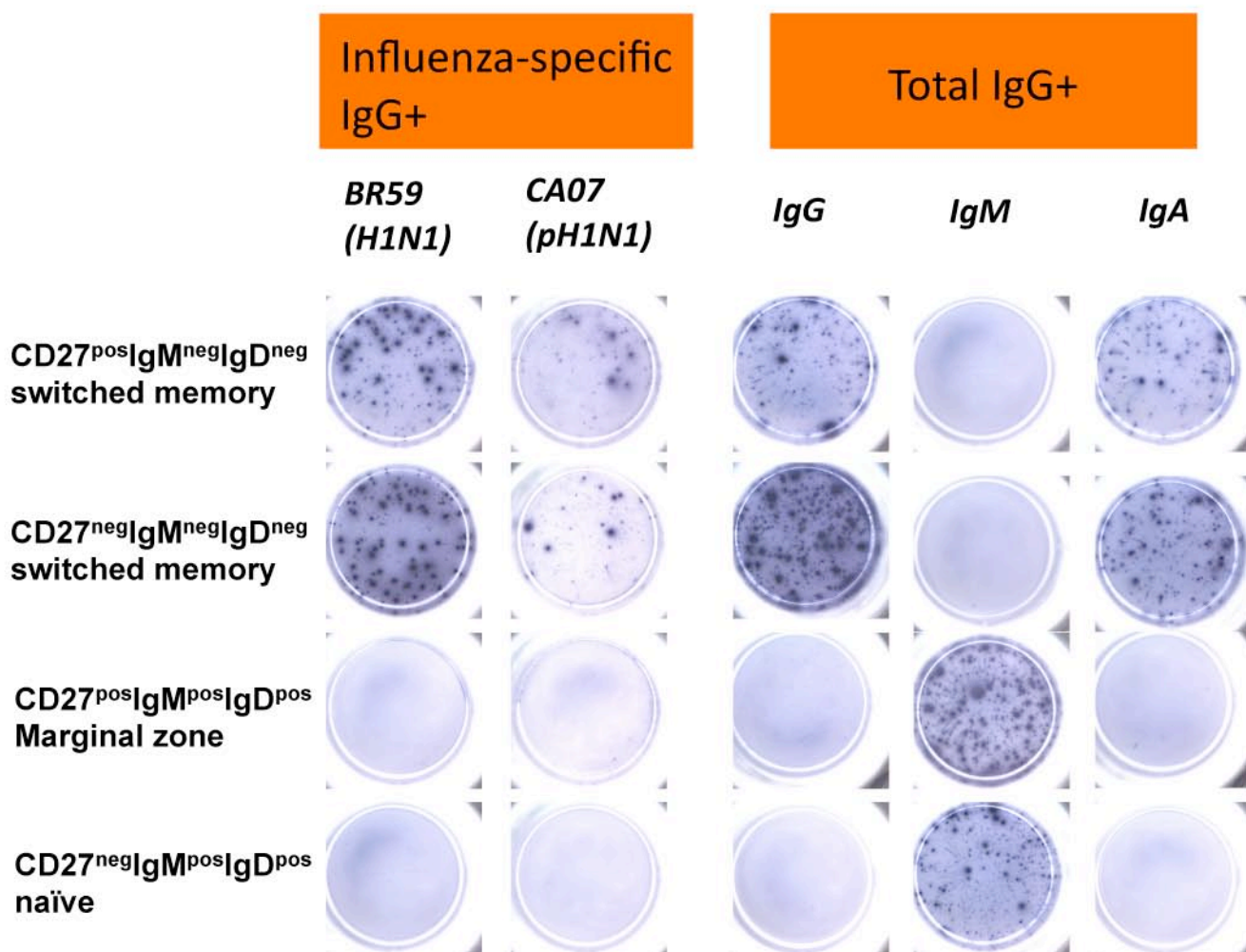


Figure 4.13. The influenza-specific IgG B cells reside within the population of IgM^{neg}IgD^{neg} isotype switched MBC. Sorted B cell subsets were cultured then determined with B-cell ELISpot assay.

used in the study, CpG/SAC/PWM cocktail, activated not only MBC but also naïve B cells into ASC, the majority of naïve and marginal zone B cells were expected to differentiate into IgM-producing ASC. Hence, a large number of IgM ASC with unknown specificity might result in the high background in the ELISpot.

Taken together, during the study period, the frequency of influenza-specific IgG B cell increased significantly over time after the H1N1 BR59 infection, indicative of the generation of influenza-specific MBC upon antigen exposure.

4.3.4 The frequency of ASC in the peripheral blood increased significantly on day 7 by FACS staining and correlated with the ASC response measured by ELISpot.

In the study, lymphocyte subsets in the peripheral blood were measured by *ex-vivo* FACS staining. The staining panels were shown in the figure 4.12 and figure 4.14. The frequency of lymphocyte subset was presented as the percentage of total lymphocytes gated by the FSC/SSC singlets.

On day -2 and 3 after challenge, the average frequency of ASC was $0.0125\% \pm 0.0012\%$, and $0.0121\% \pm 0.0013\%$ of lymphocytes respectively. The ASC population was barely visible on the FACS. On day 7, a significant increase of ASC was observed within infected volunteers and the ASC appear as a distinct population on the FACS (figure 4.15). The day 7 ASC frequency was $0.0996\% \pm 0.029\%$ of lymphocytes ($p < 0.0001$ for all time point comparisons) (figure 4.17A). Using Ki-67 as a proliferation marker, the majority of day 7 ASC expressed this protein, indicating that they have been recently proliferated (figure 4.16). The linear regression analysis showed that the ASC

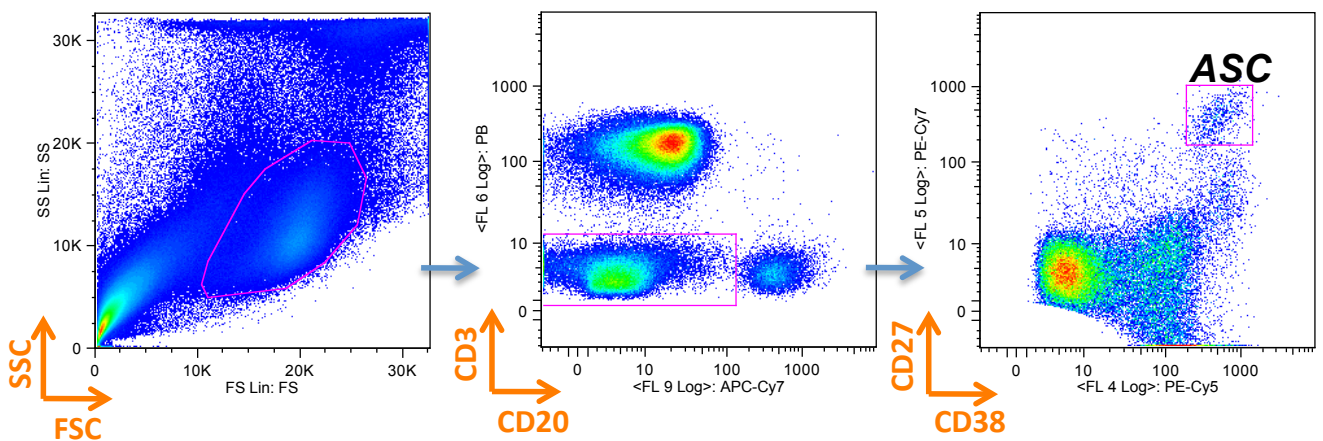


Figure 4.14. The staining panel for ASC. PB anti-CD3, APC anti-CD19, APC-Cy7 anti-CD20, PECy7 anti-CD27, and PECy5 anti-CD38 from BD Biosciences were used to stain PBMC. The data were analyzed with FlowJo software.

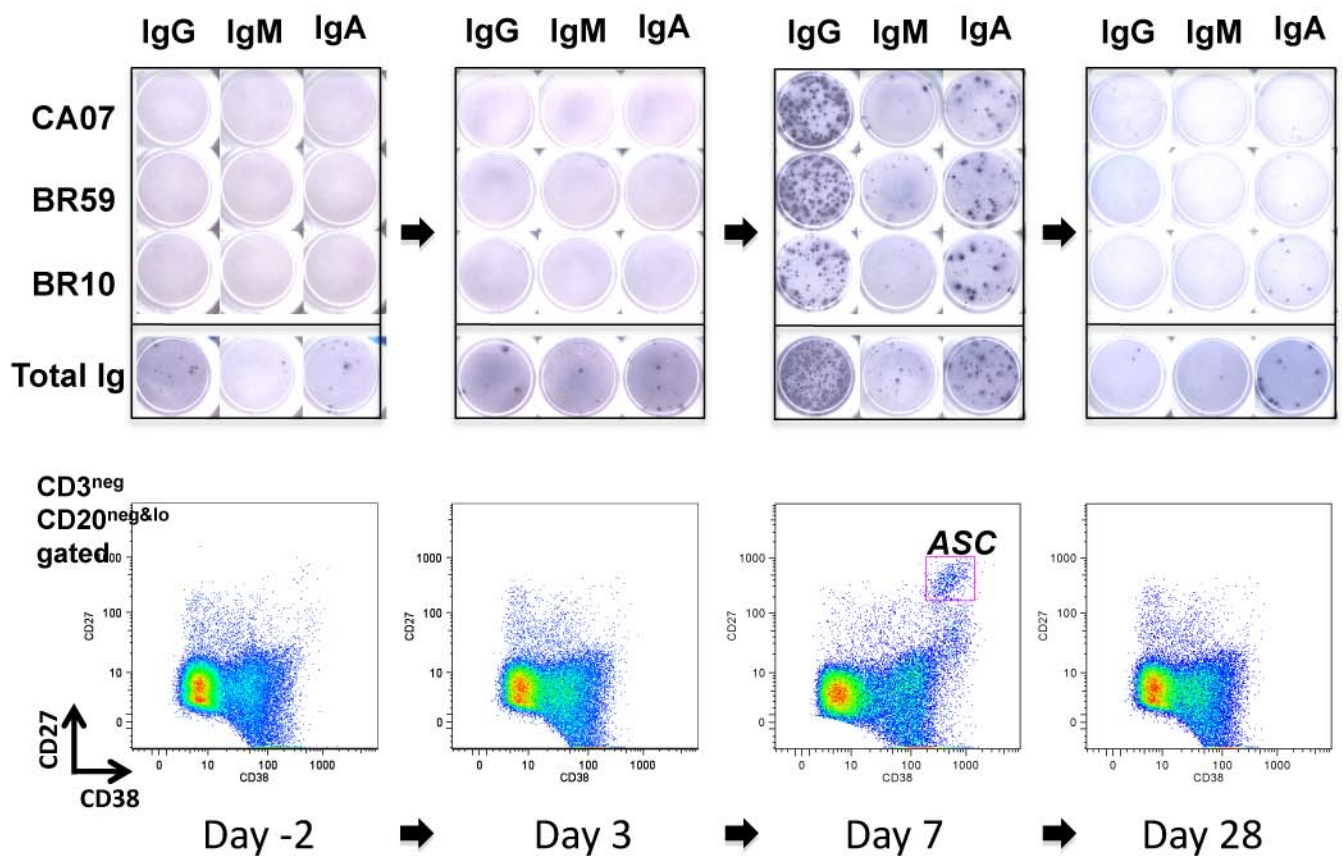


Figure 4.15. Induction of day 7 ASC response after H1N1 BR59 infection. By B cell ELISpot, an expansion of influenza-specific and total ASC was observed. A distinct population of ASC was identified simultaneously by FACS staining. The ASC were shown as CD3^{neg}, CD20^{neg&lo}, CD27^{hi}, CD38^{hi} cells.

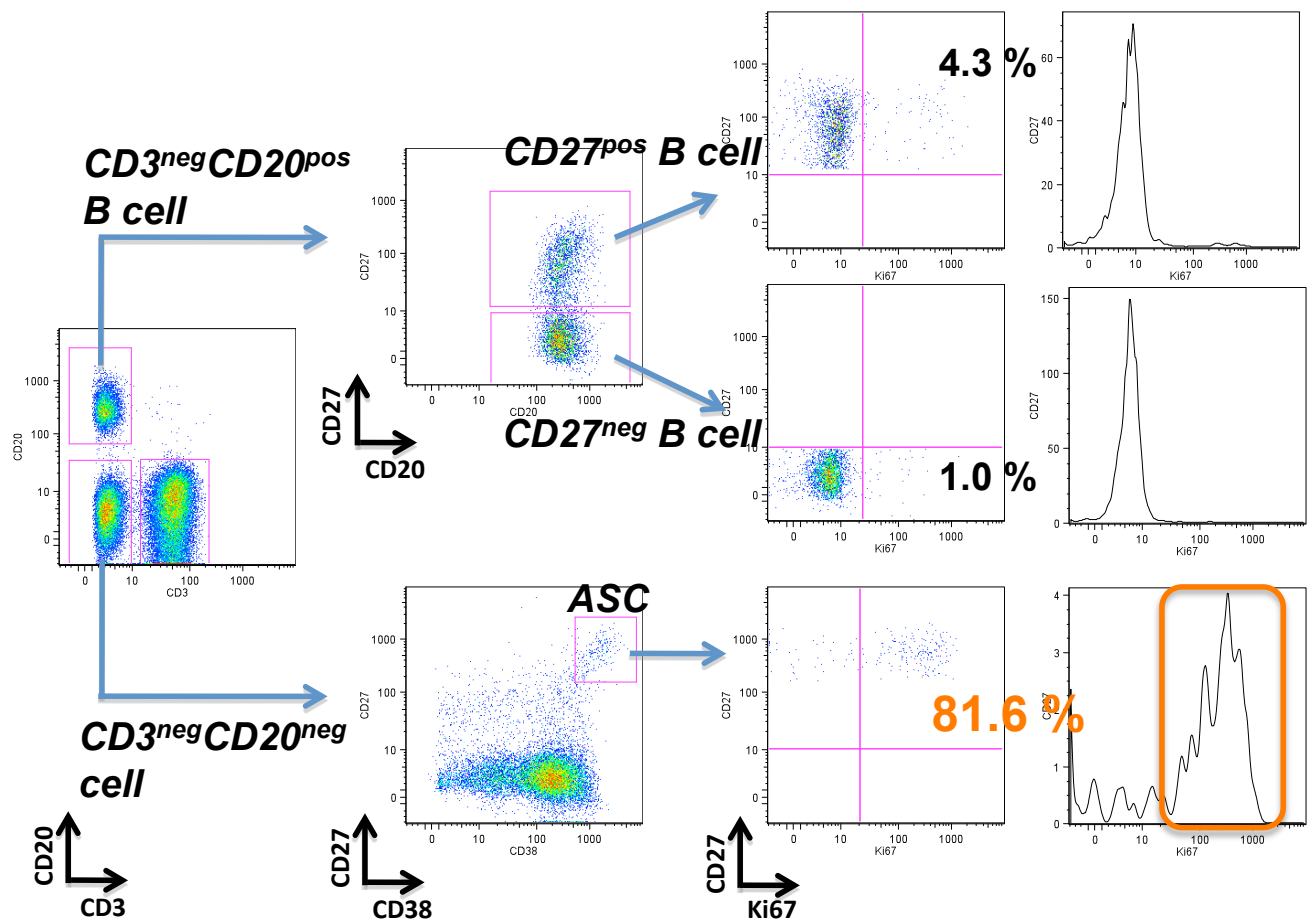


Figure 4.16. Intracellular expression of Ki-67 by day 7 ASC, naïve or memory B cells. Recent proliferation of day 7 ASC was shown by FACS staining.

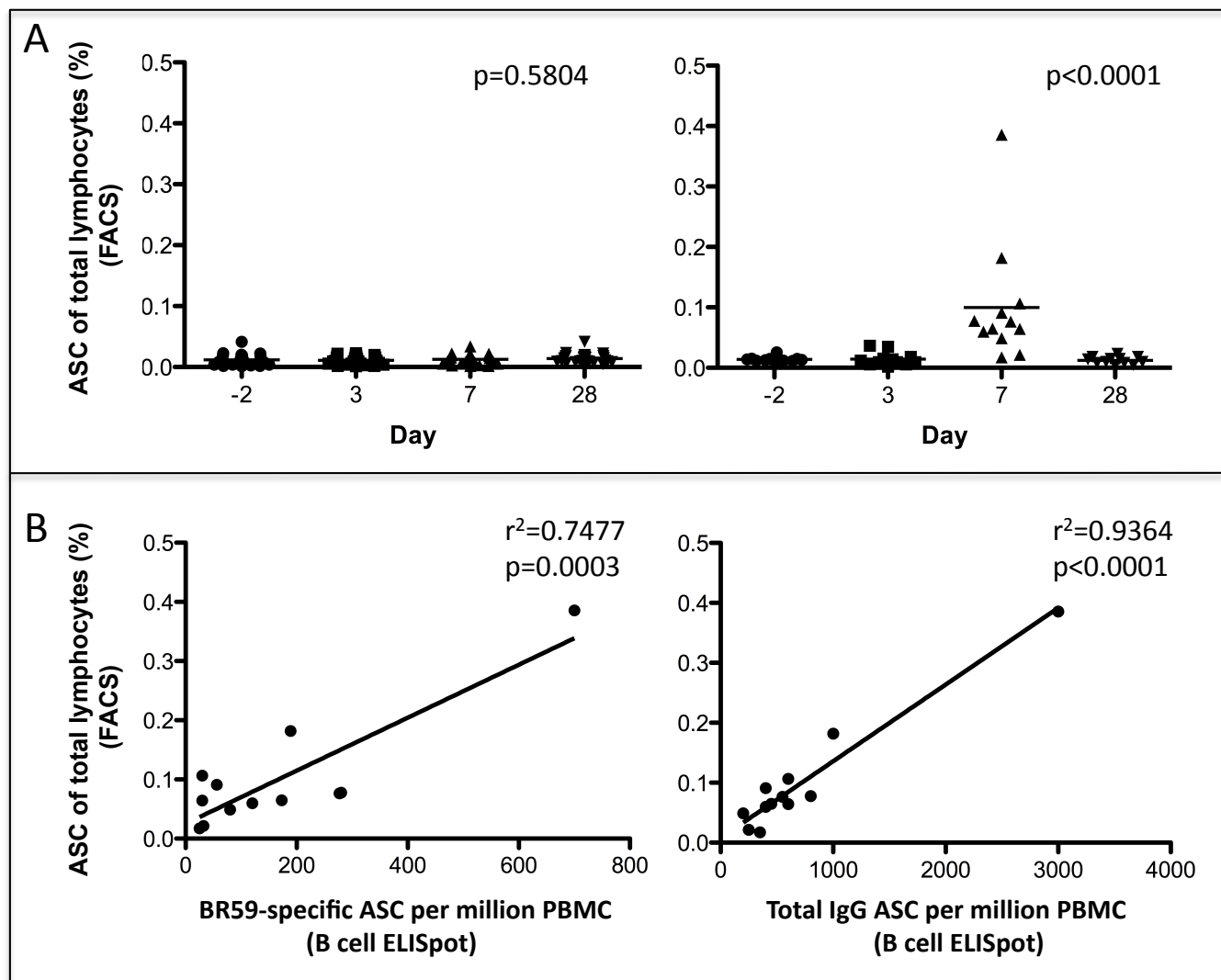


Figure 4.17. A. The kinetics of circulating ASC within uninfected (left) and infected (right) volunteers, measured by FACS staining. Kruskal-Wallis test was performed to compare the groups of data by GraphPad Prism. B. The day 7 ASC frequency measured by FACS is correlated with either H1N1 BR59-specific (left) or total IgG ASC (right) measured by ELISpot. Linear regression analysis was performed to examine their correlation by GraphPad Prism.

frequency measured by FACS significantly correlates with the H1N1 BR59-specific IgG ASC ($r^2=0.7483$, $p=0.0003$) and total IgG ASC ($r^2=0.9351$, $p<0.0001$) measured by ELISpot (figure 4.17B).

On day 28 after challenge, the ASC frequency on the FACS went back to the pre-infection level (figure 4.17A).

FACS phenotyping revealed that T and B cells constitute the majority of peripheral lymphocytes, which accounts for 64% and 13% of lymphocytes, respectively (figure 4.18A). For B cell population, CD27 cell surface marker is generally used to differentiate CD27^{pos} memory B cells from other B cells. In the study, CD27^{pos} and CD27⁻ cells accounts for 28% and 72% of B cells on average, respectively. Across the study period, the frequency of T cells, B cells, CD27^{pos} B cells, and CD27⁻ B cells did not change significantly, no matter among infected or uninfected volunteers (figure 4.19).

Within B cell population, CD27^{pos} B cells can be divided into two major subsets, which are marginal zone cells and isotype switched cells. It was noted that CD27^{pos} marginal zone cells express IgD and account for 10% of B cells; while CD27^{pos} isotype switched cells lack IgD and account for 14% of B cells. CD27⁻ B cells could be divided into two major subsets as well, which are naïve cells and isotype switched cells. CD27⁻ native cells express IgD and account for 55% of B cells; while CD27⁻ isotype switched cells lack IgD and accounts for 7% of B cells (figure 4.18B). During the study period, the frequency of B cell subsets had fluctuated but had not changed significantly, although it had to be mentioned that the number of infected volunteers in the

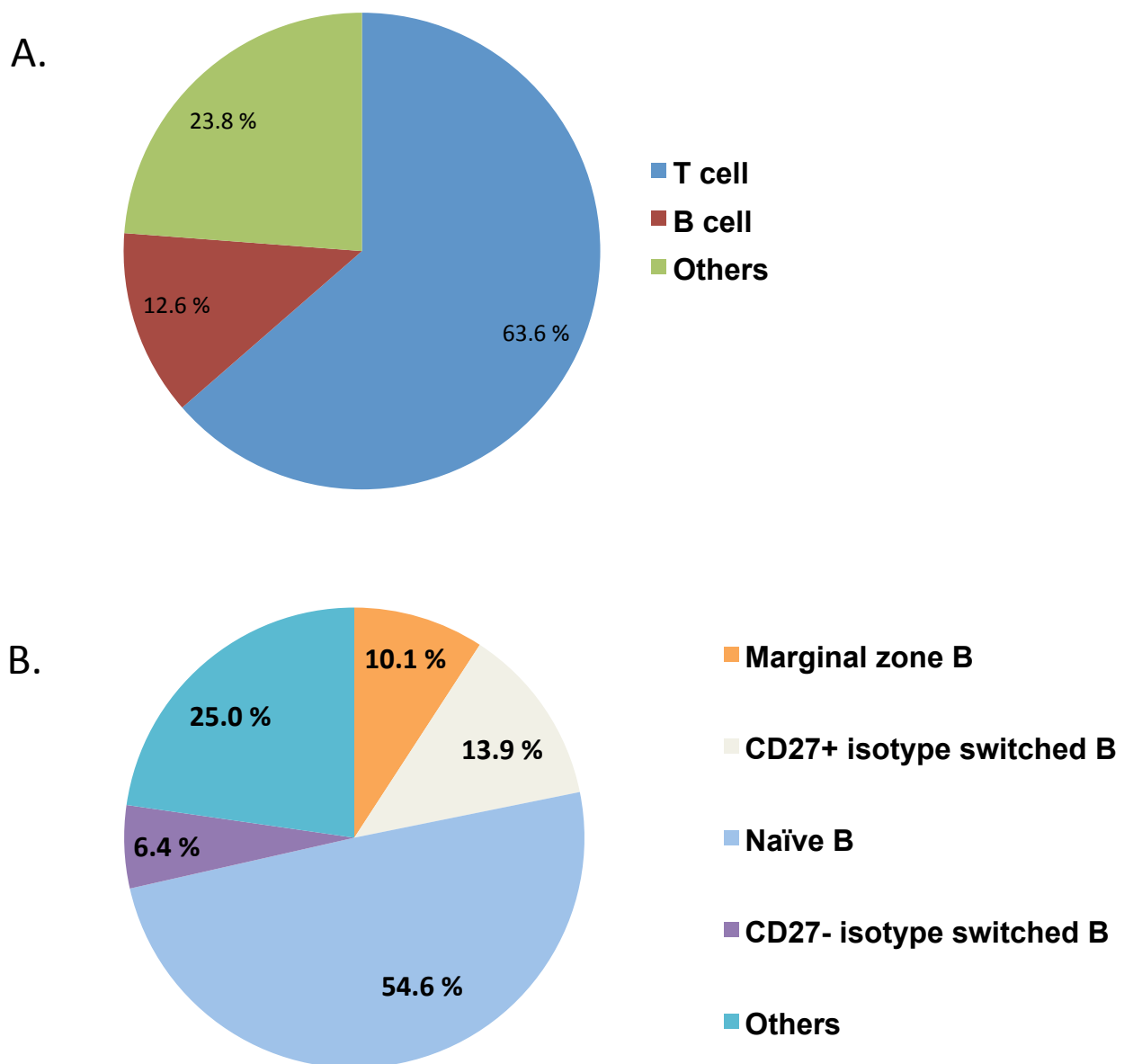


Figure 4.18. A. The percentage of T and B cells in total lymphocytes, as measured by FACS staining. The lymphocytes were defined and gated based on forward scatter and side scatter. B. The percentage of B cell subpopulations in total B cells. The data are collected in the 2010 challenge study, as measured by FACS staining.

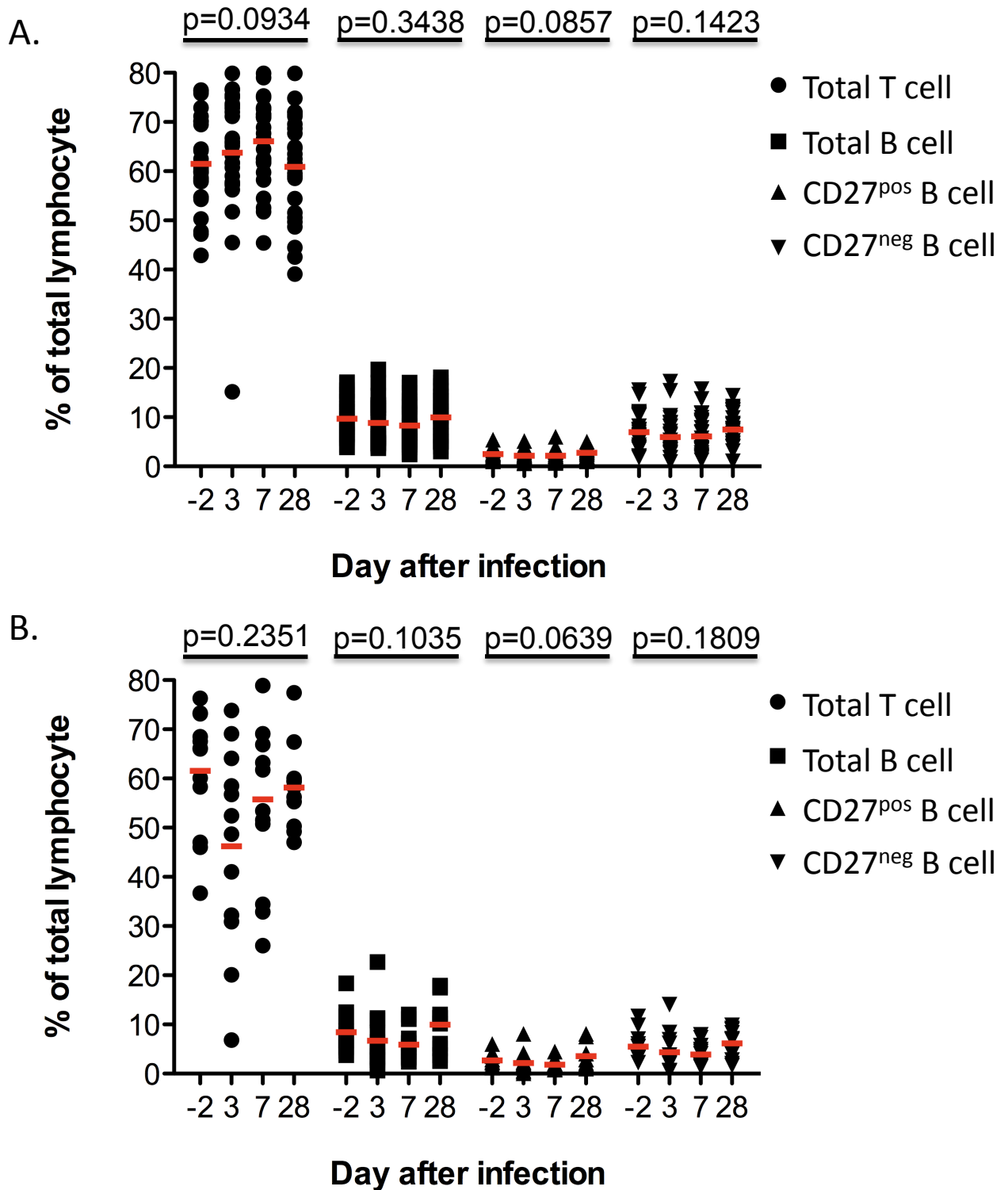


Figure 4.19. The frequency of lymphocyte subpopulations among uninfected volunteers (A) and infected volunteers (B) for 2009 & 2010 challenge studies. The frequency was determined as the percentage in lymphocytes, as measured by FACS staining. Lab ID. 2, 8, 9, and 16 in 2010 study were excluded due to drop out in the middle of experiment. Kruskal-Wallis test was performed to compare the groups of data by GraphPad Prism.

2010 challenge study was too few to reach statistical significance (figure 4.20).

4.3.5 Influenza-specific IFN γ expression of non-CD3 lymphocytes was barely detected in the whole blood ICS after H1N1 BR59 infection.

It is generally believed that influenza-specific antibodies are important to protect against influenza virus. In addition to neutralising virus, it is unclear if influenza-specific antibodies conduct the antibody-dependent cell-mediated cytotoxicity (ADCC) response, which relies on the cooperation of humoral and cellular effector elements. Effector cells mediate the lysis of target cells while Fc receptors of effector cells attach to Fc portion of antibodies bound to the epitopes of target cells. However, so far, ADCC has not been universally observed in all kind of viral infection. This phenomenon is limitedly described on some chronic viral infection or parasite infection (Nattermann *et al.*, 2005; Voltarelli *et al.*, 1983). In the past, to assay the ADCC response was thought to be difficult: it is time-consuming as the traditional killing assay required the preparation of labeled target cell lines; also mapping ADCC-epitopes by using pulsed whole protein or virus is difficult (Chung *et al.*, 2008). Lately, the whole blood ICS assay pulsed with peptide pool was introduced to study ADCC in human immunodeficiency virus (HIV) infection. Only a small amount of blood is needed, no radiolabelled cell lines are required, and the assay is ready to map epitopes (Chung *et al.*, 2009; Stratov *et al.*, 2008). Using whole blood ICS assay, the ADCC response has been shown in some linear epitopes for Env, Nef and Vpu surface proteins in HIV-infected patients. NK cells play the major role of interacting with HIV-specific antibody and of conducting the

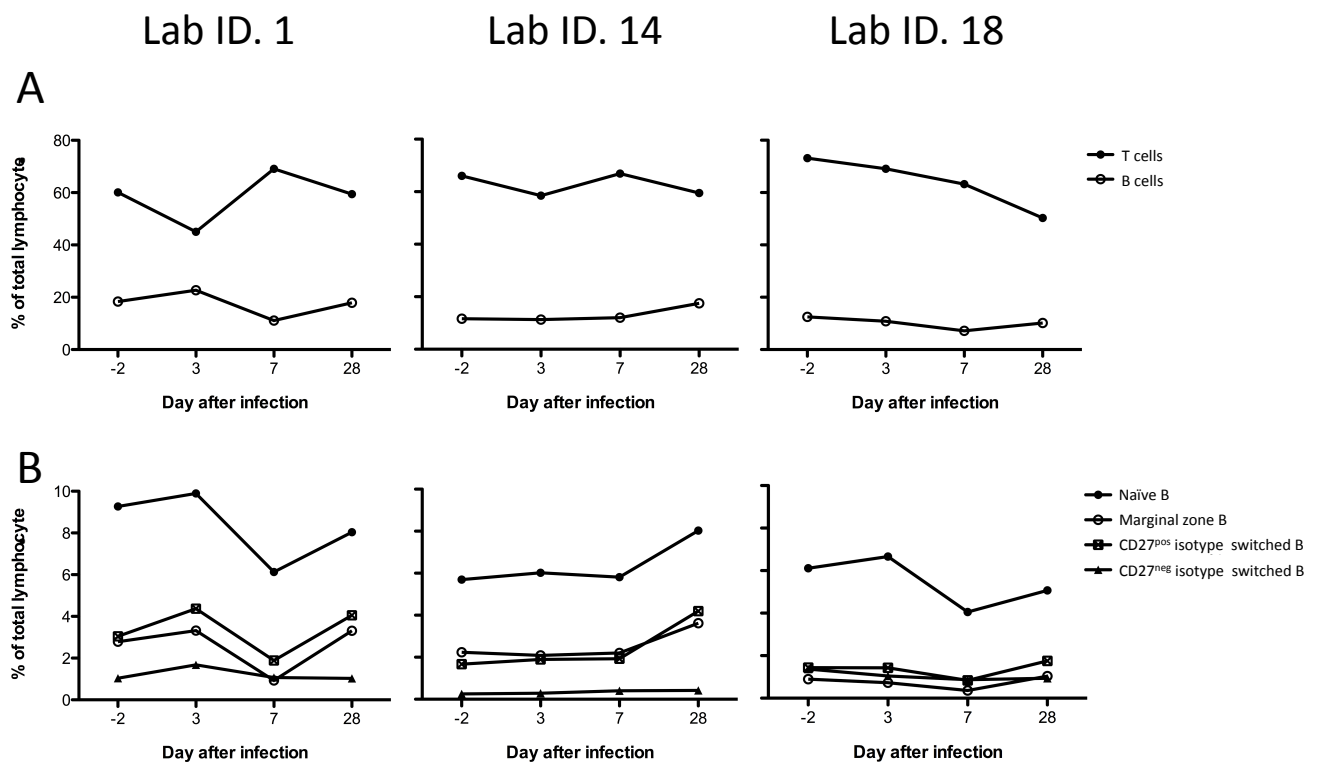


Figure 4.20. The kinetics of lymphocyte (A) and B cell (B) subsets from three infected volunteers in the 2010 challenge study, as measured by FACS staining.

response. In the present, measuring the IFN γ expression of peptide-stimulated CD3^{neg} lymphocytes and NK cells in the whole blood ICS assay has been an established and sensitive method to screen whether the ADCC response potentially exists in the HIV patients. However, little is known if this assay can be demonstrated in acute influenza infection. Therefore, I made use of the blood samples from the 2009 challenge study to examine the CD3^{neg} lymphocytes/NK cells response by IFN γ intracellular cytokine staining of whole blood pulsed by overlapping influenza peptide pools.

Prior to the study, the HIV patient sample was utilized to optimize the whole blood ICS assay. The HIV-specific expression of IFN γ by CD3^{neg} lymphocytes was seen and the majority of the CD3^{neg} effector cells were NK lymphocytes in the assay (figure 4.21). NK lymphocytes could be further divided into CD56(dim) and CD56(bright) subsets. In general, CD56(dim) cells accounts for 90% of NK cells and were believed to possess more cytotoxic ability than CD56(bright) NK cells. In optimizing the assay, the majority of IFN γ -expressing NK cells come from CD56(dim) cells, indicative of the cytotoxic potential of effector cells (figure 4.21). This result is in accordance with other previous studies in HIV infection (Chung *et al.*, 2009; Stratov *et al.*, 2008).

In the 2009 challenge study, the frequency of IFN γ -expression lymphocytes following the incubation with influenza peptide pools was measured by whole blood ICS assay. To identify influenza-specific lymphocyte response, overlapping synthetic peptides (18-mer peptides overlapping by 10 amino acid residues) spanning the whole proteome of the H1N1 A/Brisbane/59/07 virus were used. The total numbers of peptide used in detecting antigen-specific

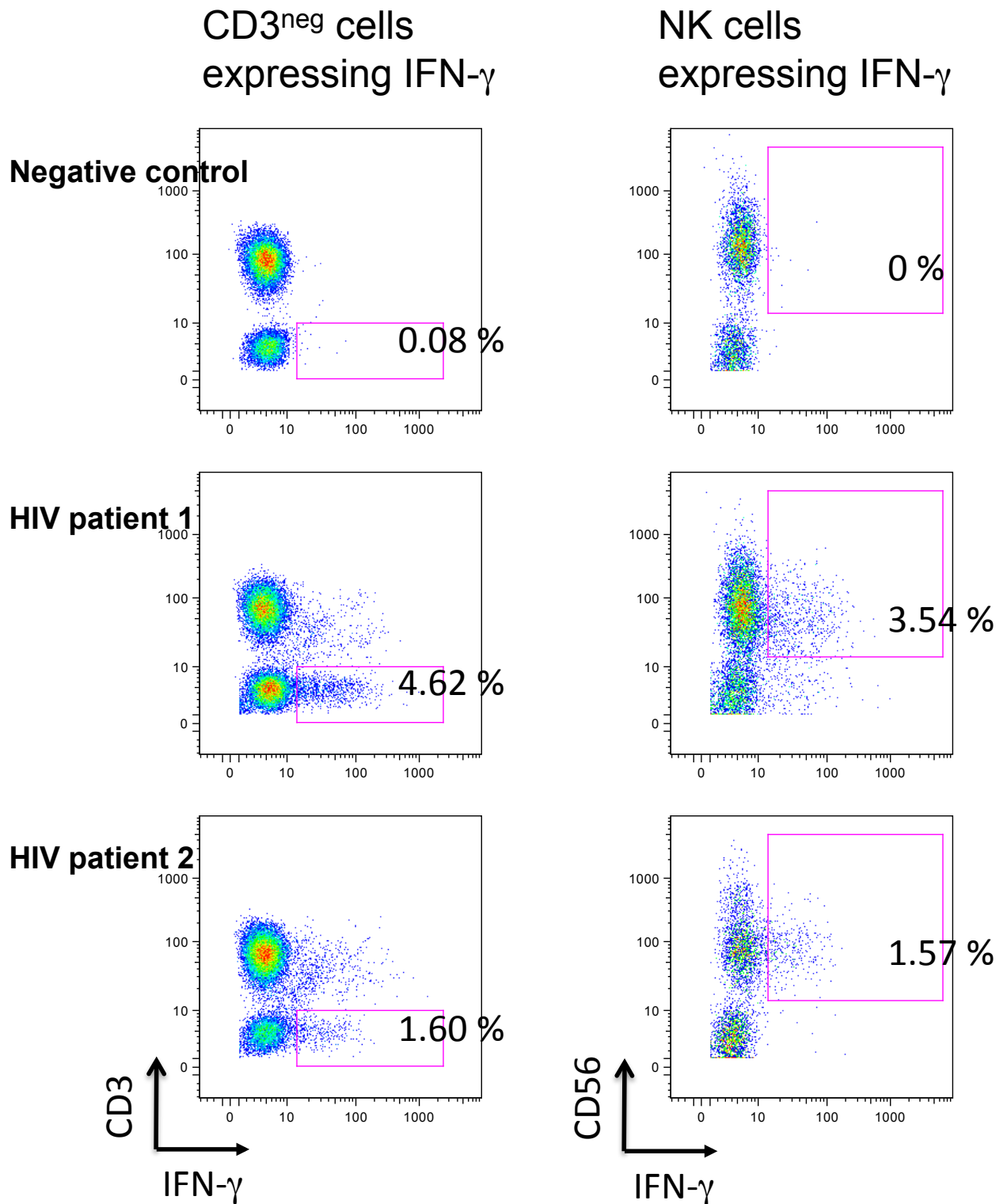


Figure 4.21. CD3^{neg} lymphocytes (left) and NK cells (right) expressing IFN-γ in response to HIV peptides. HIV Env/Pol/Vpu peptide pool (clade B consensus pool, 18 mers) with final concentration of 1 μg/ml was used to setup whole blood ICS assay. The frequency of IFN-γ expressing cells was presented as the percentage within total lymphocytes. Over 18000 lymphocytes were acquired.

responses to BR59 strain were 554. Peptides for HA, NA and M2 proteins were combined into one pool, as well for NP and M1 proteins, and for NS, PB1, PB2 and PA proteins. Whole blood from challenged volunteers was incubated with three separate peptide pools. The positive control was PMA/Ionomycin stimulated cells. The frequency of IFN γ -expressing lymphocytes was presented as the percentage of total lymphocytes measured by FACS (table 4.2). The positive response was defined as more than threefold the background response.

On day -2, 7 out of 24 volunteers had detectable IFN γ -expression of CD3^{neg} lymphocytes, which was quite low at 0.56 ± 0.30 of total lymphocytes on average. None of them was noted to have increased response on day 28. There were 9 infected volunteers in 2009 and 6 of them had HI seroconversion. Within infected volunteers, the IFN γ -expression of CD3^{neg} lymphocytes and NK cells were compared between day -2 and day 28. The positive CD3^{neg} lymphocytes and NK cell response seemed to increase on day 28, but the difference did not reach statistical significance. Moreover, compared with HIV infection (Stratov *et al.*, 2008), the positive CD3^{neg} lymphocytes and NK cell response related to influenza infection were pretty low, even within seroconverted volunteers.

4.4 DISCUSSION

The goal of this study was to explore the kinetics and magnitude of the ASC and MBC response to H1N1 BR59 influenza infection. This longitudinal study demonstrated that there is a significant ASC response in the peripheral blood on day 7 after influenza infection. However, the response observed was

Table 4.2. IFN- γ expression of CD3^{neg} cells in response to influenza peptides.

Lab ID*	Non-CD3 cell response						NK cell response					
	Baseline			Day 28			Baseline			Day 28		
	HA/ NA/ M2	NP/ M1	NS/ PB1/ PB2/ PA	total	HA/ NA/ M2	NP/ M1	NS/ PB1/ PB2/ PA	total	HA/ NA/ M2	NP/ M1	NS/ PB1/ PB2/ PA	total
1	-	-	-	-	-	-	-	-	-	-	-	-
2	-	-	-	-	0.40	-	-	-	-	-	-	0.25
3	-	-	-	-	-	-	-	-	-	-	-	-
4	-	0.41	0.35	0.76	-	-	0.31	0.30	-	-	-	-
5	-	-	-	-	-	-	-	-	-	-	-	-
6	-	-	-	-	-	-	-	-	-	-	-	-
7	-	-	-	-	-	-	-	-	-	-	-	-
8	0.26	-	-	0.26	0.28	-	-	0.11	0.23	-	-	0.23
9	-	-	-	-	-	-	-	-	-	-	-	-
10	-	-	-	-	0.06	-	-	0.06	-	-	-	0.04
11	-	2.31	-	2.31	-	1.61	-	1.61	-	2.10	-	1.61
12	-	-	-	-	-	-	-	-	-	-	-	-
13	-	-	-	-	-	-	-	-	-	-	-	-
14	0.10	0.11	-	0.21	-	-	-	0.09	0.03	0.03	-	0.12
15	-	0.12	-	0.12	-	-	-	0.03	-	-	-	0.03
16	-	-	-	-	0.63	-	-	0.48	-	-	-	0.48
17	-	-	-	-	-	-	-	-	-	-	-	-
18	-	-	-	-	-	-	-	-	-	-	-	-
19	0.12	-	-	0.12	-	-	-	0.08	-	-	-	-
20	-	-	-	-	0.22	-	-	0.22	-	-	-	0.08
21	-	-	-	-	-	-	-	-	-	-	-	-
22	-	-	-	-	-	-	-	-	-	-	-	-
23	0.18	-	-	0.18	-	-	-	0.14	-	-	-	0.14
24	-	-	-	-	-	-	-	-	-	-	-	-
Mean	0.16	0.73	0.35	0.56	0.31	1.61	0.51	0.11	0.23	1.61	0.45	0.46
$\pm$ SD	± 0.04	± 0.53		± 0.30	± 0.09		± 0.23	± 0.01	± 0.07		± 0.28	± 0.24

#: The lab ID of nine infected volunteers was indicated in red capital number.
 -: negative response (less than threefold above background response for non-CD3 and NK cell responses)

significantly lower than that previously described (Wrammert *et al.*, 2011). An average of 950 ± 152 flu-specific IgG ASC per million PBMC was detected after natural infection in adult patients (Wrammert *et al.*, 2011). In the study, there is only 166 ± 55 flu-specific IgG ASC per million PBMC detected after nasal experimental infection in adults. This discrepancy could be related to the clinical severity. In the study, most infected volunteers presented mild illness and only 25% (3/12) of them had onset of fever. In contrast, in Wrammert's study, all six patients had onset of fever and two of them were admitted due to severe illness. In the present study, those with fever were also found to have higher ASC response than those without fever. It was also noted the size of ASC response significantly correlates with the viral load and the duration of viral shedding upon infection. In Wrammert's study, the patient with severe illness had persistent viral shedding over several weeks. Nearly all the ASC response were detected between day 9 to 18 after infection, and the ASC response could have been persistently detected in the peripheral for several weeks in severely ill patients. These findings indicated that the ongoing and severe influenza infection might continuously induce the generation and accumulation of influenza-specific ASCs and result in the detection of stronger and prolonged ASC response.

In the previous studies, the IgG ASC response was similar between vaccinated subjects and naturally infected patients (Wrammert *et al.*, 2008; Wrammert *et al.*, 2011). In the present study, the experimental infection induced significantly lower IgG ASC response, while comparing with the response induced by inactivated influenza vaccination (group 2 in the vaccine trial, refer to Chapter 3) (figure 4.22). Milder clinical presentations of BR59-

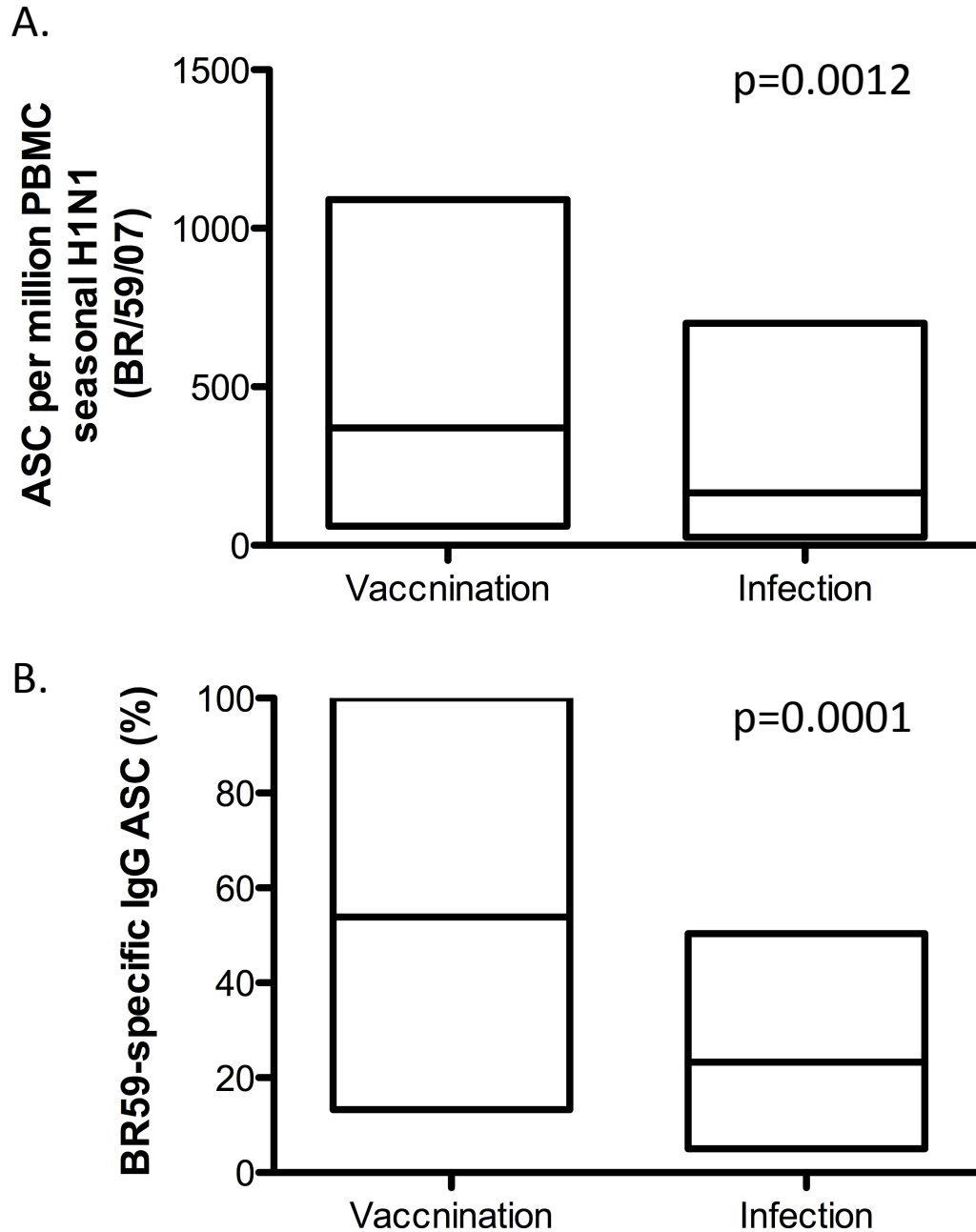


Figure 4.22. A. Comparison of the frequency of influenza-specific ASC on day 7 between influenza vaccinated and infected volunteers. B. The percentage of influenza-specific ASC in total circulating ASC was calculated based on the result of B cell ELISpot. The vaccine group included 46 volunteers receiving 2009-2010 seasonal influenza vaccine. The infection group included 12 infected volunteers challenged with H1N1 BR59 virus. The ASC response was determined by B cell ELISpot. The student's t-test was performed to compare two groups of data by GraphPad Prism.

infected volunteers might explain their weaker ASC response. Besides, since this early-induced ASC response could origin from the pre-existing memory cells in the local lymph nodes, the level of pre-existing humoral memory might also affect the ASC response upon antigen exposure (Frölich *et al.*, 2010; Wrammert *et al.*, 2008; Wrammert *et al.*, 2011).

The ASC response had also been compared between TIV and CAIV immunization (Sasaki et al, 2007). It was found that the CAIV induced significantly lower IgG ASC response, but had similar IgA ASC response as TIV (Sasaki et al, 2007). This pointed out the possibility that the mucosal infection might induce predominantly local ASC response in nature, rather than circulating IgG ASC response. Hence, in order to provide a more comprehensive view of the ASC response, the future study may look at the B cell response in nasal secretion and tonsils and comparing this with the response in the circulation (Sasaki et al, 2007).

In the study, the influenza-specific ASC response did not correlated with the serum antibody titre on day 28, as measured by HI assay. Moreover, upon influenza infection, lower than 60% of the ASC responders had positive serum antibody response on day 28. The question is therefore raised about if these early-induced influenza-specific ASCs play a role in the protection against infection. It has to be pointed out that the destination of day 7 ASC upon infection remains unclear in human (Slifka *et al.*, 1995). The cell population mainly responsible for the sustained antibody level is also undetermined in human (Joo *et al.*, 2010). The present study provided here that there is, at least transiently, the detection of circulating anti-HA ASC response upon infection, since the HA-purified split vaccine antigen is coated to measure the

ASC response on ELISpot. In the previous study, polyclonal antibodies derived from sorted day 7 ASCs induced by influenza vaccination could present HAI and neutralising activities, indicative of the protective potential of day 7 ASC (He *et al.*, 2011). Exploring the breadth and diversity of antibodies produced by day 7 ASC will allow further understanding of their role in response to infection or vaccination. It is important to know whether infection-induced ASC produce antibodies that could neutralise virus, or that could not neutralise but inhibit viral replication, or that just simply bind the virus without subsequent function. Further analysis aimed at the mAb generated from individually sorted ASC will help to explore their intrinsic function at the single-cell level.

By FACS staining, this study demonstrated that an average of 20% of circulation B cells were MBC. Isotype switched MBC could be considered as the pool of antigen-specific B cells, since they are generated through germinal center reaction upon antigen exposure. In order to delineate the role of MBC in humoral immunity, a number of studies focused on the relationship between the circulating antibody level and the frequency of MBC at two time points. Firstly, at the steady state, which means without antigen exposure for a while, it is interesting to know if antigen-specific MBC contribute to the maintenance of circulating antibody level. Their correlations have been reported strong (Bernasconi *et al.*, 2002), equivocal (Amanna *et al.*, 2007; Buisman *et al.*, 2009) or negative (Blanchard-Rohner *et al.*, 2010; Galli *et al.*, 2009a). In the present study, the frequency of pre-existing influenza-specific MBC was detected around 0.5% in the peripheral blood, and was found no correlation with the day -2 antibody titre, similar to those previously described (Pinna *et*

al., 2009; Sasaki *et al.*, 2007). To my knowledge, no correlation has been found between baseline frequency of influenza-specific MBC and serum antibody level. It is believed that the level of circulating influenza-specific MBC would remain constant over time (Sasaki *et al.*, 2007). In contrast, anti-influenza antibody titre would fade away in about one year after vaccination. These results seemed to indicate that in response to influenza virus, MBC and antibody immunity may represent two independent components of long-term protection in human. Secondly, the relationship of induced MBC response with the convalescent antibody level following infection or vaccination might serve as a predictive marker for the long-term immunity. In the present study, influenza-specific MBC response significantly increased after infection (rising from 0.19 on day -2 to 1.96 % of total IgG cells on day 28), but no correlate was found with day 28 serum antibody level. The strong correlation has been previously reported in the several studies of inactivated influenza vaccination (Galli *et al.*, 2009a; Pinna *et al.*, 2009). However, in those studies, the vaccination-induced MBC response averaged 12~20% of total IgG cells, which is much higher than that detected in the present study. Firstly, this discrepancy could be related to the component of influenza exposure. A booster dose of adjuvanted influenza vaccine could induce a significantly higher MBC response up to 20% of total IgG cells on day 21 in those primed with adjuvanted vaccine, compared with those unprimed or primed with plain vaccine (Galli *et al.*, 2009a). It seemed that the combination of vaccine-boosting and adjuvant effect enhanced the elicitation of MBC response, although the underlined mechanism remained to be determined. Secondly, the discrepancy could be related to the route of influenza exposure. In human,

it remains to be determined if the level of IgG memory response differ with the route of antigen exposure and the site of detection (Sasaki *et al.*, 2007). It had been reported that in mice, the IgG MBC response was highly regulated at respiratory tissues, but rarely in the blood, upon nasal influenza infection (Joo *et al.*, 2010). Since most studies about human MBC had been done using the peripheral blood, this might overlook the MBC response induced at other tissue sites and its relationship with the maintenance of humoral immunity.

Currently, there are two known methods for determining the frequency of influenza-specific MBC, which are the limiting dilution assay (Slifka and Ahmed, 1996), and the memory B cell culture assay (Crotty *et al.*, 2004). The latter was successfully used to enumerate MBC specific for influenza during the course of infection. However, these methods both require a large number of PBMC, which might preclude the study of multiple antigen-specific MBC from small clinical samples. Secondly, these methods require *in vitro* polyclonal stimulation of B cells into ASC, which might preclude the study of phenotype and intrinsic function of antigen-specific B cells. Lately, the flow cytometry assay was developed and useful for accurate quantification of rare MBC population for various antigens in human and mice (Amanna and Slifka, 2006). Importantly, the flow cytometry assay allows the *ex-vivo* analysis of the phenotype of antigen-specific MBC and their function at single cell level (Amanna and Slifka, 2006). It has been demonstrated that staining with purified and biotinylated influenza HA allow the antigen-specific B cells to be identified in the local lymph nodes of immunized mice (Doucett *et al.*, 2005). However, in human, direct staining of fluorescence-labelled virus or recombinant HA usually results in a non-specific staining of circulatory B cells

(figure 4.23). This background staining probably results from the non-specific binding of HA antigens to sialic acids on the cell surface. A novel flow cytometry assay using fewer PBMC to detect antigen specific MBC *ex vivo* has been developed (personal communication with Drs. Monia Bardelli and Grazia Galli). An optimal staining of influenza-specific MBC has been greatly improved by blocking the non-specific binding with irrelevant HA of a different subtype before antigen specific staining with specific HA. This results in a very pure population of MBC for clonotyping despite the loss of cross-reactive cells. The antigen specificity of influenza-specific B cells could have been confirmed by a sorting process and the following memory B cell assay. This technique had been used in experimental study on influenza vaccine research at Novartis Vaccines (personal communication with Drs. Monia Bardelli and Grazia Galli, Novartis Vaccines and Diagnostics Research Center, Siena). Therefore, in the future, this flow cytometry assay would be useful for analyzing the influenza-specific B cell response both in the laboratory as well as in the clinical settings. The diversity and function of influenza-specific B cells could also be explored at single cell level upon infection or vaccination.

In the H1N1 BR59 challenge study, the possibility of ADCC response induced by acute influenza infection was examined by whole blood ICS assay.

Influenza-specific IFN γ expression of non-CD3 lymphocytes was barely detectable after infection. The ADCC reactivity to influenza virus had been previously demonstrated in human sera collected after natural infection or vaccination and could have been mediated mainly by anti-HA and anti-NA antibodies (Greenberg *et al.*, 1979; Hashimoto *et al.*, 1983; Vella *et al.*, 1980).

Another study had showed in mice that the M2-specific antibody induced by

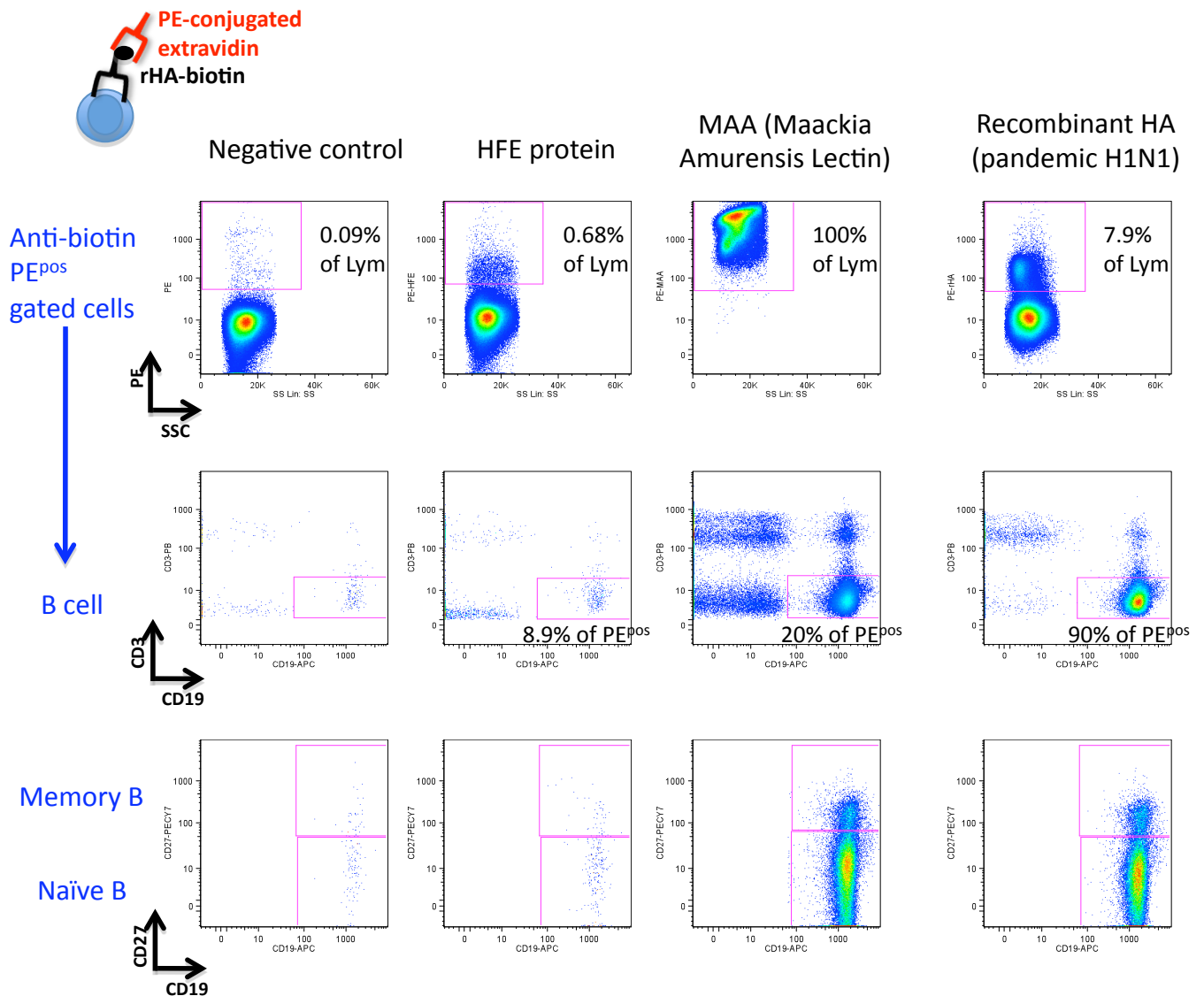


Figure 4.23. The surface staining of peripheral B cells with biotinylated recombinant HA of pandemic H1N1 Eng195 strain. MAA bound to sialic acid of cell surface was used as positive control. HFE protein and the absence of first antibody were used as negative control. It was noted over 90% of B cells were bound to influenza rHA, highly suggestive of non-specific binding of influenza antigen.

vaccination also played some role in the ADCC response and pointed out the mediated antibody may not necessarily confer neutralising function (Jegerlehner *et al.*, 2004). In the present study, the weak antibody response and the lack of ADCC-related antibody might result in the feeble ADCC response within infected volunteers. Furthermore, most previous studies that were able to demonstrate anti-influenza ADCC response used influenza virus-infected cells as target cells in the ^{51}Cr release assay. Since whole blood ICS assay could only measure those antibodies recognizing linear influenza epitopes, it might overlook the majority of influenza-specific antibodies that recognize conformational epitopes. In the future, further study comparing the efficiency between whole blood ICS assay and other traditional assays could be necessary prior to assessing the existence and nature of ADCC response to influenza.

In conclusion, influenza H1N1 BR59 challenge studies demonstrated the kinetics and magnitude of influenza-specific B cells upon acute influenza A infection. A significant ASC response was detected on day 7 and the expansion of influenza-specific MBC was observed after infection. The ASC response correlated with the viral load and the duration of viral shedding. Of note, the ASC population identified by the FACS staining associated with the ASC response measured by B-cell ELISpot. Further studies aiming to explore the repertoire and function of the influenza-specific B cells at single cell level are crucial to delineate the role of these cells in the development of humoral immunity against influenza virus.

CHAPTER 5

THE PRODUCTION AND CHARACTERISTICS OF RECOMBINANT HUMAN MONOCLONAL ANTIBODIES

5.1 INTRODUCTION

The vaccine trial and influenza challenge study demonstrated the expansion of not only ASC but also MBC upon influenza exposure, indicative of the involvement of these effector cells in the development of humoral response (refer to Chapter 3 and 4). Importantly, at the early phase of the humoral response, an average of 20~50 % of circulating IgG ASC were found influenza-specific. The influenza-specific ASC response significantly correlated with the HI and neutralising antibody level after vaccination. However, the role of the ASC and MBC response in controlling influenza infection remained unclear in human. It is important to further characterise the antibody repertoire within the ASC and MBC induced upon influenza exposure. The illustration of the Ig diversification and gene segment usage within ASC and MBC would broaden our understanding of the development of humoral immunity in human and also provide an alternative approach to evaluate the immune response to vaccine antigens in the near future.

To date, Ig gene analysis mostly comes from the cDNA library of bulked B cell subsets or from the single cell of defined origin. The accumulated information helps to determine the molecular characteristics of Ig genes in the different development stage of B cells (Huang and Stollar, 1991; Shiokawa *et al.*, 1999). However, without the identification of antibody specificity, the sequence

data alone provided limited information about the molecular fingerprint of antigen-specific B cells.

In human, Isotype-switched and hypermutated MBC constitute the majority of antigen-specific B cell pool in the circulation. Molecular cloning of Ig genes and producing mAb from the EBV-immortalized MBC line or from the combinatorial display library of MBC allow the analysis of antigen-specific clones at single cell level (Simmons *et al.*, 2007; Throsby *et al.*, 2008). However, without the optimization of staining of antigen-specific cells, time-consuming selective procedures would be required. Moreover, the uncertainty of EBV transformation rate or the low efficiency of making stable antibody-secreting clones would limit the application of the above methods in the field of molecular immunology (Doria-Rose *et al.*, 2009; Laffly and Sodoyer, 2005; Martinez *et al.*, 2009).

Currently, an integrated method by combining the isolation of ASC and the molecular cloning technique promote the efficient yield of Ig gene profiles and human mAb (Smith *et al.*, 2009; Tiller *et al.*, 2008). Nowadays, when tracking the kinetics of influenza-specific B cells during the course of vaccination or infection in human, the circulating ASC at the early phase could be sorted for molecular analysis. Ig variable regions of both heavy and light chain derived from single B cell could be rapidly cloned to produce human mAb (Smith *et al.*, 2009).

The variable region, the crucial area for antigen binding, is made of variable (V), diversity (D) and joining (J) gene segments in the heavy chain, and of V and J only in the kappa and lambda chain, respectively. In human, there are

about 50 functional V_H gene segments grouped into 7 families, 40 V_k into 6 families, 30 V_λ into 10 families, and 25 D_H into 6 families; while there are 6 J_H , 5 J_k and 4 J_λ gene segments, respectively.

The Ig gene segments are randomly rearranged during B cell development in the bone marrow, which results in highly diverse combinations of variable region (Janeway *et al.*, 2008). The RAG endonucleases delete the DNA between rearranged V(D)J gene segments and the non-homologous end-joining machinery completes the joining of gene segments (Chaudhuri *et al.*, 2007). At the junctions between segments, terminal deoxynucleotidyl transferase randomly adds non-templated nucleotides to the ends of DNA chain, which contribute to the diversity of sequences. In the end, DNA polymerase adds complementary nucleotides to make two ends compatible for joining (Gellert, 2002).

Following the development in the bone marrow, mature B cells enter the circulation and peripheral lymphoid organs. Upon antigen exposure, mature B cells are activated in the germinal centers of peripheral lymph organs, which involve the differentiation and selection of antigen-specific B cells (McHeyzer-Williams and McHeyzer-Williams, 2005). In the germinal centre, activated B cells undergo the process of somatic hypermutation, which allows the rearranged variable region to be further diversified. Meanwhile, this process also underpins the affinity maturation of Ig. Activation-induced deaminase initiates the somatic hypermutation in the variable region by deaminating cytosine to uracil. Normally, the U:G mispair would be restored through the base-excision repair pathway. After the uracil base was removed and the

abasic site was generated by uracil-DNA glycosylase, an endonuclease would cleave the abasic site, which left a gap filled by the DNA polymerase. During somatic hypermutation process, not high-fidelity DNA polymerase (e.g., pol β) but rather low-fidelity DNA polymerase (e.g., Pol η) would participate the gap-filling process then mutations would be introduced into the DNA of variable region (Seki *et al.*, 2005; Teng and Papavasiliou, 2007; Weill and Reynaud, 2008). With the presence of these mutations, an array of slightly different Ig coded by the variable region would have been transcribed and translated.

Somatic mutations have been shown to cluster within complementarity determining regions (CDRs), which comprise so-called antigen-binding sites. This could result from the process of affinity maturation in the germinal centre. It involves the clonal selection of antigen-specific B cell, which would pick out mutations leading to the high-affinity antibody-antigen binding. Hotspots for deamination and mutation can occur in the framework regions (FRs) as well. It has been noted that the selection favors amino acid replacement mutations (R) over silent mutations (S) in CDRs (Zheng *et al.*, 2005). In the FRs where the conserved domain is needed to maintain the overall antibody structure, silent mutations are preferentially selected over replacement mutations (Gonzalez-Fernandez *et al.*, 1994; Ohlin and Zouali, 2003; Rajewsky, 1996; Wagner and Neuberger, 1996; Zheng *et al.*, 2005). The ratio of replacement to silent mutations (R/S ratio) in the CDRs and FRs could be compared to examine the rate of somatic hypermutation.

5.2 STUDY DESIGN

In the study, day 7 ASC were sorted from three adults either infected with H1N1 BR59 virus or vaccinated with the 2010-2011 seasonal TIV (Sanofi-Pateur) which was composed of inactivated, split H1N1 A/California/07/09 (H1N1 CA07)-like, H3N2 A/Perth/16/09 (H3N2 PR16)-like, and B/Brisbane/60/08-like viruses. The Ig variable regions for heavy chain and light chain were amplified through PCR. The amplified variable regions were inserted into vectors that bring C_γ1 and either C_κ or C_λ. Vectors were transfected into the mammalian cells, where heavy and light chains were expressed and joined by disulphide bonds (Smith et al., 2009). Full-length IgG1 human mAb were therefore produced *in vitro* (figure 5.1). The role of early-phase ASC was examined by identifying the properties of mAb with respect to the antigen specificity, cross-reactivity among strains and subtypes and the neutralising function.

During the process of making mAb, the amplified and sequenced variable regions serve as the valuable information for analyzing the Ig genes derived from early-phase plasma cells. The comparable germline sequence of variable region could be acquired either by V-Base database developed at the MRC centre for Protein Engineering or by IMGT created by Marie-Paule Lefranc for global reference in immunogenetics and immunoinformatics (<http://vbase.mrc-cpe.cam.ac.uk/>; Lefranc *et al.*, 2009). All the sequences were analyzed with regard to their gene segment usage, clonality, and the frequency and distribution of somatic hypermutation.

5.3 RESULTS

5.3.1 The production and characterisation of mAb

Source: PBMC from vaccinated or infected volunteers (Day 7)

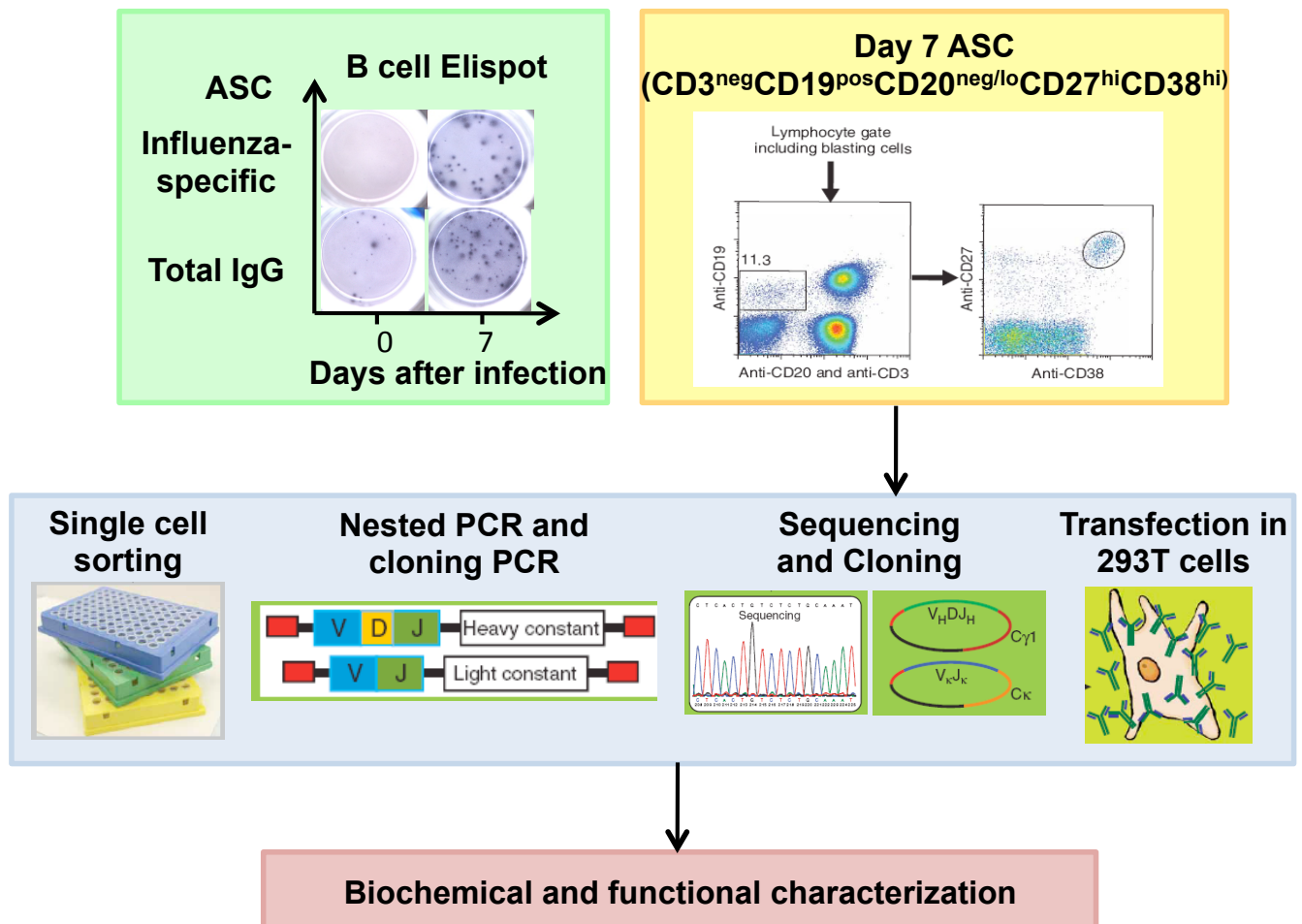


Figure 5.1. The flow chart of making recombinant mAb from day 7 ASC. [adapted from Tiller *et al.*, 2008; Smith *et al.*, 2009).

5.3.1.1 ASC were isolated from influenza vaccinated and infected donors.

Prior to sorting peripheral ASC, *ex-vivo* B cell ELISpot was performed to measure influenza-specific ASC response within three donors. Donor 14C and 1C were infected with H1N1 BR59 virus and donor CL was vaccinated with 2010-2011 seasonal TIV (Sanofi-Pateur). The ASC response peaked on day 7. The frequency of influenza-specific ASC averaged 301 ± 199 per million PBMC on day 7 (table 5.1). Importantly, the majority of ASC were IgG-positive, with minor of IgA or IgM-positive cells. Moreover, almost all ASC expressed the Ki67 proteins, indicating their recent proliferation. These findings suggested that these ASC were most possibly differentiated from isotype-switched MBC compartment recently recruited in the local lymph node upon infection or vaccination.

The aim of sorting ASC after influenza vaccination or infection was for making recombinant mAb. Day 7 after vaccination or infection, the separated PBMCs were stained with CD3-PB, CD19-FITC, CD20-APCH7, CD27-PECy7, and CD38-PECy5. In accordance with the previous study (Wrammert *et al.*, 2008), lymphocytes were first gated on FSC and SSC, then ASC population could be seen as a distinct subset of CD3^{neg} CD20^{neg&lo} cells that had high CD27 and CD38 expression. The majority of ASC express CD19, which is a typical B cell surface marker (figure 5.2).

It was noted that soon after PBMC separation, ASC couldn't last long, even PBMC were kept in 37°C and 5% CO₂ incubator. For infected donors (14C and 1C), PBMC were separated one day prior to sorting, as a result, the ASC

Table 5.1. Summary of day 7 ASC response of three donors.

Lab ID.	1C	14C	CL
Flu-specific and total IgG ASCs per million PBMC			
BR59	700	120	85
CA07	235	25	105
BR10	400	0	70
Total IgG	3000	400	890

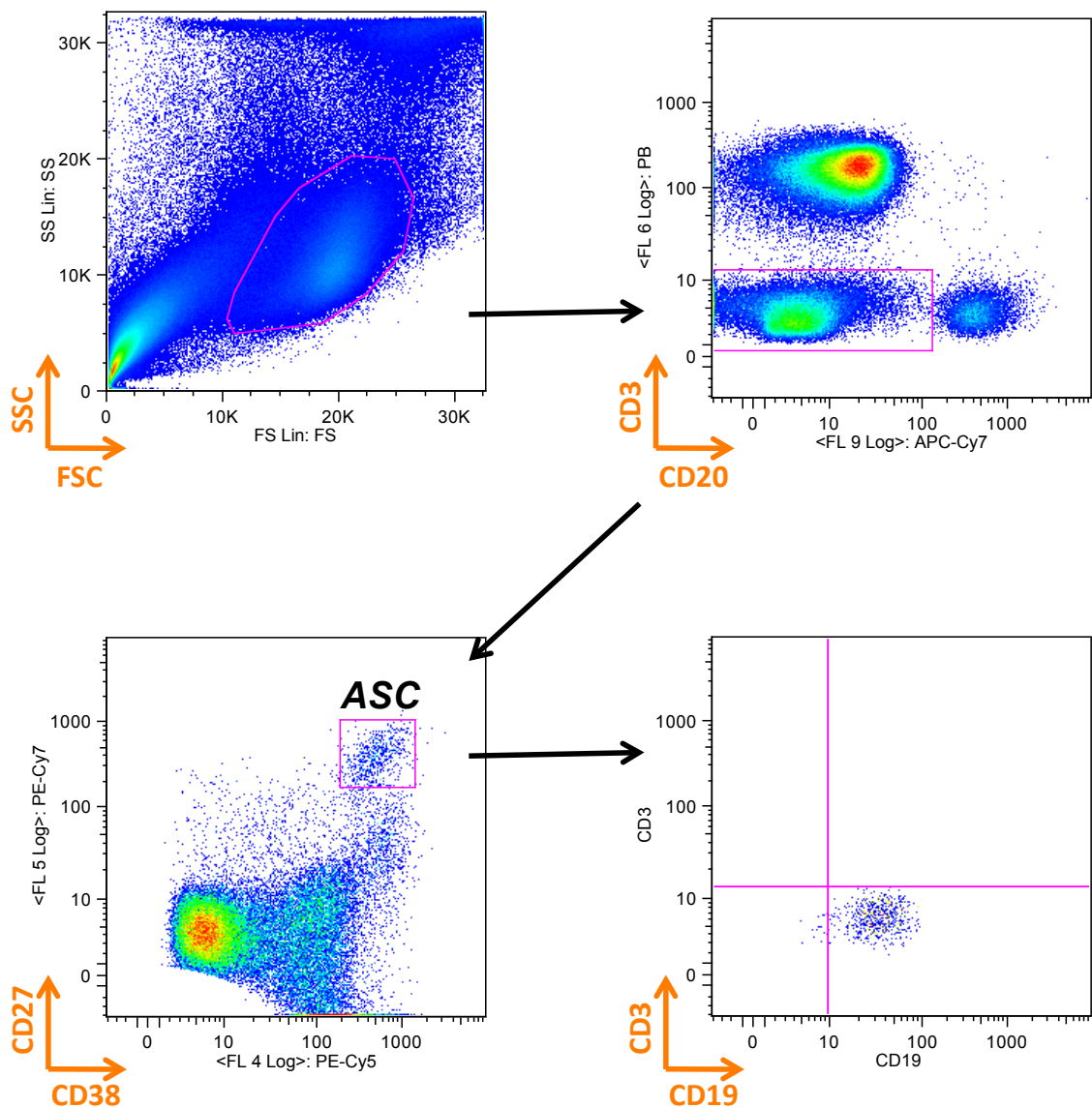


Figure 5.2. The staining and sorting panel for day 7 ASC. PB anti-CD3, APC anti-CD19, APC-Cy7 anti-CD20, PECy7 anti-CD27, and PECy5 anti-CD38 from BD Biosciences were used to stain PBMC. The data were analyzed with FlowJo software.

population shrunk more than 70% if comparing with the previous day's size on FACS. In the end, there were finally 96 ASC sorted from donor 1C and 27 ASC sorted from donor 14C, respectively. For the vaccinated donor (CL), ASC were sorted as soon as PBMC were separated, and there were 144 ASC sorted for PCR amplification of Ig gene and making recombinant mAb.

5.3.1.2 The heavy and light chain variable regions were amplified and sequenced.

A total of 267 ASC were sorted as single cell into 96 well plates and frozen in the RNase-inhibiting RT-PCR buffer. PCR was performed on a total of 6 plates of cells from 3 donors. Because cells would have been lysed through the freeze-thaw process, the RT-PCR mix was added directly to the wells. The cell RNA was reverse-transcribed and amplified by one-step RT-PCR.

PCR amplification of heavy chain leader, variable, diversity and joining gene segments and part of constant regions were performed with previously designed primers (Tiller *et al.*, 2008). A mix of primers specific for each of different heavy chain leader regions and a primer specific for the Igγ constant region were used (figure 5.3). Since this first PCR product based on single cell RNA was scanty, the following nested PCR was necessary. Nested PCR would amplify variable, diversity and joining gene segments and insert the restriction sites (figure 5.3). This nested PCR used a pool of primers that could attach all varieties of variable and joining gene segments.

Light chain PCR amplifications were performed in a similar way to the heavy chain PCR (figure 5.4). Kappa and lambda chain were performed independently. First PCR was carried out using a pool of primers specific for

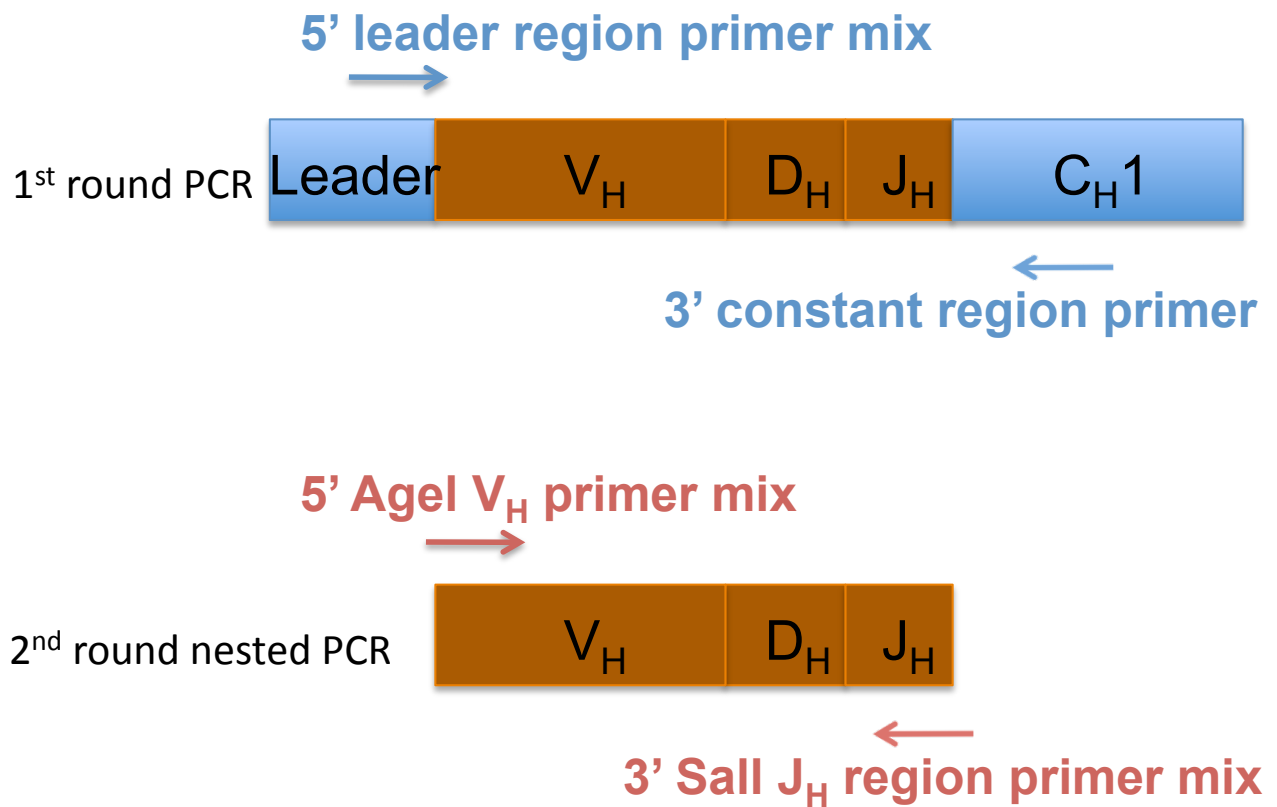


Figure 5.3. PCR amplification of heavy chain variable region. V_H , D_H , and J_H were variable, diversity and joining gene segments of heavy chain, respectively.

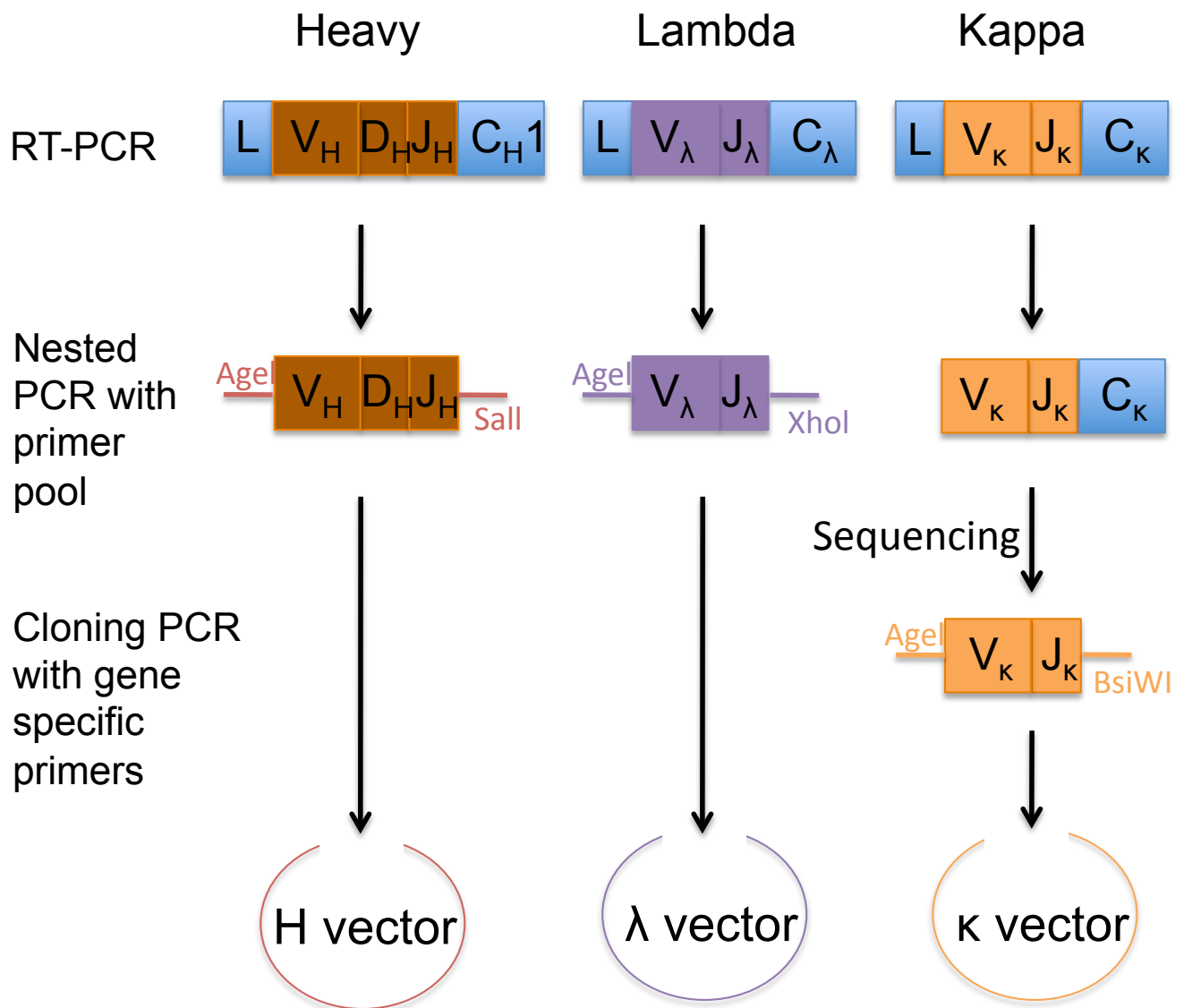


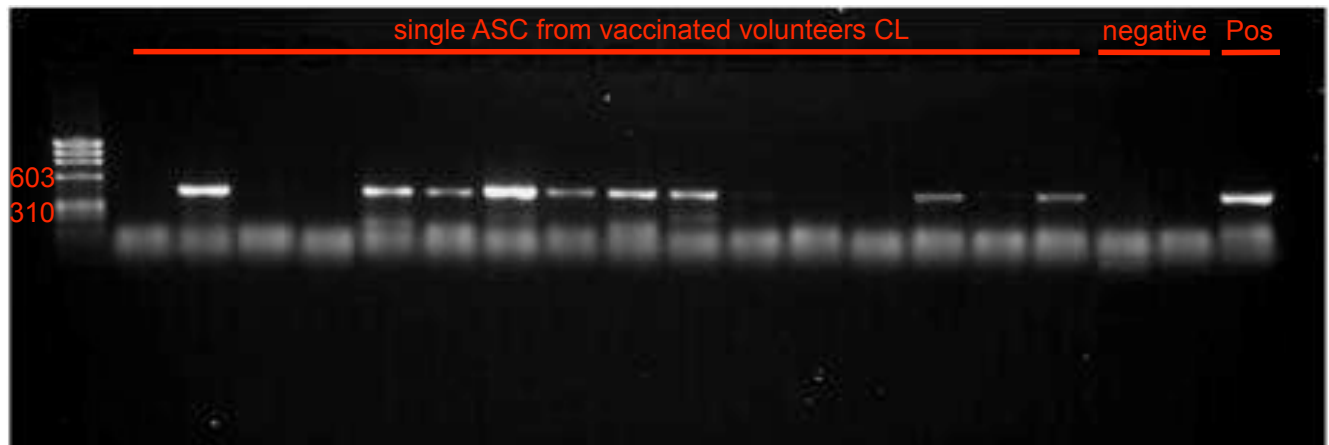
Figure 5.4. The flow chart for amplification and cloning of Ig variable regions. PCR amplification of leader (L), variable (V), diversity (D) and joining (J) gene segments and part of the constant (C) regions were performed with mixed primers specific for all families of genes. First PCR product was used in nested PCR. The nested PCR product of heavy and lambda chains contained restriction site and were directly cloned into expression vectors. Kappa chain nested PCR product was sent to sequence first to identify the gene family. Then the RT-PCR product was amplified again with gene-specific primers.

all leader regions and a primer for Igk or Igλ constant region. Then, nested PCR was performed using a pool of primers specific for the array of variable gene segments and an inner primer for the constant region (figure 5.4).

In the study, the RT-PCR product was used first in nested PCR of heavy chain. Then, the nested PCR product was checked by agarose gel electrophoresis for visualization (figure 5.5A). The number of positive wells and the density of the PCR product band varied among plates. In the study, there are total 91 wells having positive heavy chain. The positive rate of heavy chain averaged 31~41% of cells per plate. Although the cells were sorted in the RNase-inhibiting buffer, the degradation of cells and RNA might result in the negative findings in the study. Moreover, since only the gamma specific heavy chain was amplified in the study, the negative well might contain other isotypes of antibody. However, when there was no band detected in the heavy chain nested PCR, the PCR wouldn't be repeated due to time constraints. If the positive band of low density is noted, the higher concentration of DNA will be used in the stage of cloning.

When those wells containing positive heavy chain DNA were confirmed, the light chain nested PCR followed. The nested PCR for kappa and lambda chain was done at the same time for each positive well to identify if the well contains both light chains (figure 5.5B). It was noted that the occurrence of this existed in approximate 17% of positive wells. When two types of light chain were detected in the one well, there might be more than one cell sorted in this well. In the previous studies, this also could occur when a cell might have two functionally rearranged transcripts (Tiller *et al.*, 2008).

A.



B.

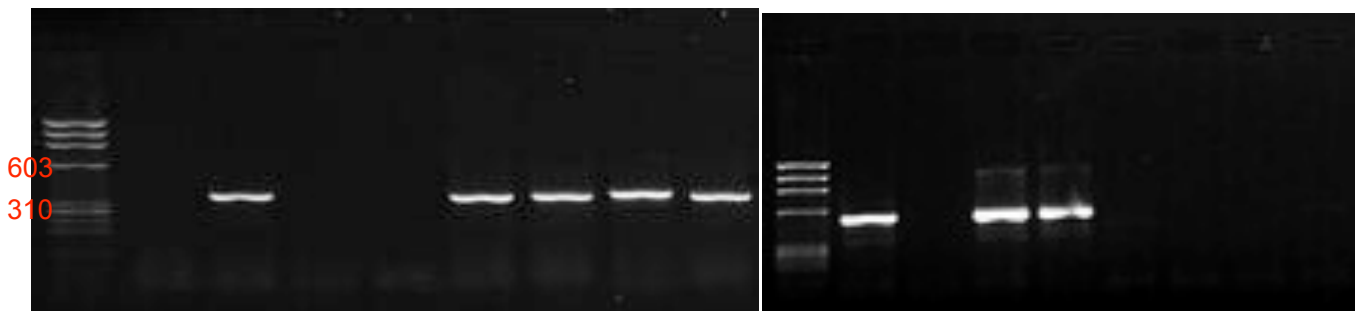


Figure 5.5. A. Nested PCR product of heavy chain variable region; B. Nested PCR product of lambda (left) and kappa (right) chain variable region. The nested PCR product was checked on 1% agarose gel. The DNA ladder showed sizes in base pairs. The heavy chain nested PCR product was seen as a band of approximately 380bp. In the figure, 9 out of 16 wells were heavy chain positive. Negative controls came from the wells where no cells had been sorted and RT-PCR and nested PCR were performed. The positive control came from the well with nested PCR product originating from RNA of a memory B cell clone. Light chain nested PCR were performed for those wells having positive heavy chain. Lambda and kappa nested PCR product were approximately 400bp and 540bp on the agarose gel, respectively. In the figure, 5 out of 8 wells were lambda chain positive, and the other 3 wells were kappa chain positive.

In the study, nested PCR products of heavy and lambda chains were directly cloned without sequencing, as performed in the original methods (Tiller *et al.*, 2008) (figure 5.4). This direct cloning had been justified by comparing sequences of heavy/light chain PCR product using pooled primers with those using gene-specific primers (personal communication with Dr Juthathip Mongkolsapaya, Imperial college). Rare amino acid differences but one amino acid difference in the V_H 5' primer region might occur. This position of amino acid difference is in the framework region, which was presumed to have little impact on the function of mAb.

The nested PCR of kappa chain did not incorporate restriction sites thus it would require the sequencing step and additional cloning PCR (figure 5.4). Kappa chain sequence data were then analyzed on the IMGT to identify which specific primers should be used for cloning PCR. For instance, when kappa chain sequence align with V_k gene family 1-9 and J_k gene family 3, the corresponding primers 5' Agel V_k 1-9 and 3' BsiWI J_k 3 will be used to amplify kappa chain for vector cloning (table 5.2).

5.3.1.3 Recombinant antibodies were expressed following the transfection of single cell heavy and light chain DNA.

PCR products of heavy and light chain were individually cloned into the Ig_γ1, Ig_κ and Ig_λ expression vectors (Smith *et al.*, 2009). Vectors were transformed into competent *E. coli* bacteria to increase copy number. After mini-preparation, isolated plasmid DNA was mini-digested and the insert band was checked by agarose gel electrophoresis (figure 5.6). Plasmids were also sent for sequencing and then the cloned insert could be analyzed using IMGT/V-

Table 5.2. Single cell PCR primer sequences. Restriction sites are shown in bold.						
	Sense	Primer sequence (5'-3')	AA sequence	Antisense	Primer sequence (5'-3')-expt	Primer sequence (3'-5')-reverse complement
Heavy chain Ig_H						
First PCR	5' L _V ,1	ACAGTGCCCACTCCAGGTGCAG	TGAHSQVQ	3' C _Y C _H ,1	GGGAATTCTCACAGGAGACGA	TCGTCTCCTGTGAGAAATCCC
	5' L _V ,3	AAGGTGTCCAGTGTGARGTGCAG	GVQCEVQ			VSCENS
	5' L _V ,4/6	CCAGATGGTCTCTGCCAGGTGCAG	PRWLSQVQ			
	5' L _V ,5	CAAGGAGTCTGTCCGAGGTGCAG	QGVCSQVQ			
Second PCR	5' Agel V _H ,1/5	CTGCA ACCGGT GTACATTCGAGGTGCAGTGTGCGAG	ATGVHSEVQLVQ	3' Sall J _H ,1/2	TGCCAA GTCCGAC GCCTGAGGAGACGGGTGACCAG	CTGGTCAACCGTCTCTCTCAGCG GTCCGAC TTCCGA
	5' Agel V _H ,3	CTGCA ACCGGT GTACATTCGAGGTGCAGTGTGCGAG	ATGVHSEVQLVE	3' Sall J _H ,3	TGCCAA GTCCGAC GCCTGAGGAGACGGGTGACCATTTG	CAATGGTCAACCGTCTCTCTCAGCG GTCCGAC TTCCGA
	5' Agel V _H ,4	CTGCA ACCGGT GTACATTCGAGGTGCAGTGTGCGAG	ATGVHSDVQLQOE	3' Sall J _H ,4/5	TGCCAA GTCCGAC GCCTGAGGAGACGGGTGACCAG	CTGGTCAACCGTCTCTCTCAGCG GTCCGAC TTCCGA
	5' Agel V _H ,3-23	CTGCA ACCGGT GTACATTCGAGGTGCAGTGTGCGAG	ATGVHSEVQLLE	3' Sall J _H ,6	TGCCAA GTCCGAC GCCTGAGGAGACGGGTGACCAGTGTG	CACGGTCAACCGTCTCTCTCAGCG GTCCGAC TTCCGA
	5' Agel V _H ,4-34	CTGCA ACCGGT GTACATTCGAGGTGCAGTGTGCGAGTGTG	ATGVHSDVQLQ			
Light chain Ig_K						
First PCR	5' L _V ,1	GGTCTGGGCCAGTCTGTGCTG	SWAQSVL	3' C _K	CACCAAGTGTGGCCTTGTGGCTTG	CAAGCCAAAGGCCACACACTGGTG
	5' L _V ,2	GGTCTGGGCCAGTCTGCCCTG	SWAQSA			QANKATLV
	5' L _V ,3	GCCTGTGACCTCCTATGAGCTG	SVTSYEL			
	5' L _V ,4/5	GGTCTCTCSCAGCTGTGCTG	SLSQP/LVL			
	5' L _V ,6	GTTCTGGGCCAATTTATGCTG	SWANFML			
	5' L _V ,7	GGTCAATTCYAGGCTGTGGTG	SNSQA/TVV			
	5' L _V ,8	GAGTGGATTCTCAGACTGTGGTG	VDSQTVV			
Second PCR	5' Agel V _L ,1	CTGCT ACCGGT TCCTGGGCCAGTCTGTGCTGACKCAG	ATGSWAQSVLT	3' XhoI C _K	CTCCTCA CTCCGAG GGYGGGAACAGAGTG	CACCTCTGTTCCCRCC CTCCGAGT GTAGGAG
	5' Agel V _L ,2	CTGCT ACCGGT TCCTGGGCCAGTCTGCCCTGACTCAG	ATGSWAQSA/TQ			
	5' Agel V _L ,3	CTGCT ACCGGT TCCTGACCTCCTATGAGCTGACWCAG	ATGSVTSYELT			
	5' Agel V _L ,4/5	CTGCT ACCGGT TCCTCTCSCAGCTGTGCTGACTCA	ATGSLSQL/PVLT			
	5' Agel V _L ,6	CTGCT ACCGGT TCCTGGGCCAATTTATGCTGACTCAG	ATGSWANFMTQ			
	5' Agel V _L ,7/8	CTGCT ACCGGT TCCAATTCYAGRGTGTGGTGACYCAG	ATGSNSQT/AVK/TQ			
Light chain Ig_κ						
First PCR	5' L _V ,1/2	ATGAGGSTCCYGCCTCAGCTGCTGG	MRVPAQLL	3' C _K , 543	GTTTCTCGTAGTCTGCTTTGCTCA	TGAGCAAAGCAGACTACGAGAAAC
	5' L _V ,3	CTCTCTCTCTGCTACTCTGGCTCCAG	LFLLLLWLP			SKADYEK
	5' L _V ,4	ATTCTCTGTGCTCTGGATCTCTG	ISLLLLWS			
Second PCR	5' Pan V _K	ATGACCCAGWCTCCABYCWCCCTG		3' C _K , 494	GTGCTGTCTTGTGCTGCTCTGCT	AGCAGGACAGCAAGGACAGCAC
Specific PCR	5' Agel V _L ,1-5	CTGCA ACCGGT GTACATTCGACATTCAGATGACCCAGTC	ATGVHSDIQTQS	3' BsiWI J _L ,1/2/4	GCCAC CGTACCG TTTGATATCCACCTTGGTC	GACCAAAGTGGARATCAAA ACGTACCG GTGGC
	5' Agel V _L ,1-9	TTGTGCTGCA ACCGGT GTACATTCAGACATCCAGTTGACCCAGTCT	CAATGVHSDIQLTQS	3' BsiWI J _L ,3	GCCAC CGTACCG TTTGATATCCACTTTGGTC	GACCAAAGTGGATATCAAA CGTACCG GTGGC
	5' Agel V _L ,1D-43	CTGCA ACCGGT GTACATTCGACATTCAGATGACCCAGTC	ATGVHCAIRMTQ	3' BsiWI J _L ,5	GCCAC CGTACCG TTTATCTCCAGTCTGCTC	GACACGACTGGAGATTAA CGTACCG GTGGC
	5' Agel V _L ,2-24	CTGCA ACCGGT GTACATGGGGATATTGTGATGACCCAGAC	ATGVHGDVMTQ			
	5' Agel V _L ,2-28	CTGCA ACCGGT GTACATGGGGATATTGTGATGACTCAGTC	ATGVHGDVMTQ			
	5' Age V _L ,3-11	TTGTGCTGCA ACCGGT GTACATTCAGAAATTTGTGTTGACACAGTC	CAATGVHSEI			
	5' Age V _L ,3-15	CTGCA ACCGGT GTACATTCAGAAATGTGATGACCCAGTC	ATGVHSEIVMTQ			
	5' Age V _L ,3-20	TTGTGCTGCA ACCGGT GTACATTCAGAAATTTGTGTTGACCCAGTCT	CAATGVHSEIVLTSQ			
	5' Age V _L ,4-1	CTGCA ACCGGT GTACATTCGACATCGATGACCCAGTC	ATGVHSDVMTQ			

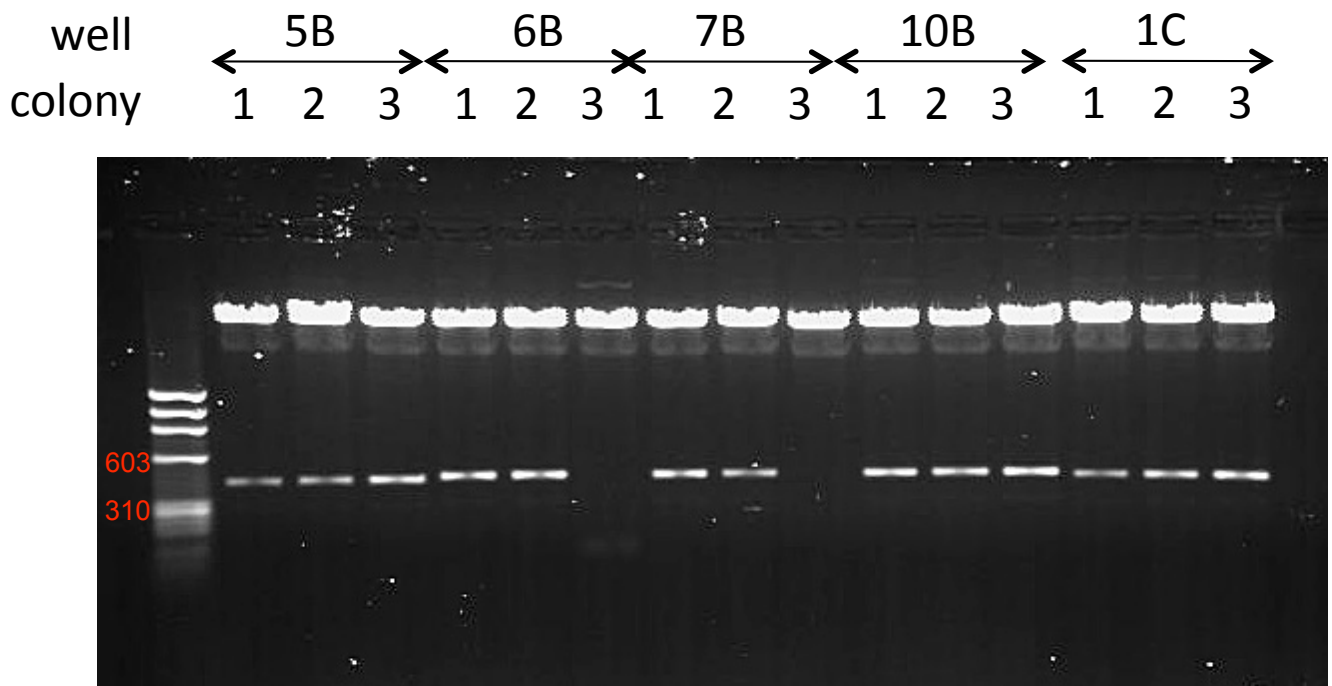


Figure 5.6. Agarose gel analysis of mini-preps of bacterial plasmids containing heavy chain inserts. After mini-preps, plasmid DNA was digested and then checked on the 1% agarose gel. The DNA ladder showed sizes in base pairs. The heavy chain insert was seen as a band of approximately 400bp. In the figure, 15 plasmids were digested and checked on agarose gel. 13 out of 15 were heavy chain insert-positive.

QUEST alignment tool. In the study, ligation of PCR product was over 96% successful. Cloned plasmids of heavy and light chain were then transfected into 293T cells for expression.

After antibody supernatants were harvested from cells, the yield of antibody was determined by IgG-ELISA. The IgG of known concentration was prepared in serial dilution to establish the standard curve. The standard curve would be used to calculate the concentration of antibody supernatants based on their OD₄₀₅ value (figure 5.7). In the study, around 2 ml of each antibody supernatant volume were collected from 6-well tissue culture plate, and the antibody yield ranged from 19 µg/ml to 92 µg/ml.

5.3.1.4 Recombinant antibodies were analyzed by influenza dot blot, ELISA and immunocytochemistry.

In the study, transfections of heavy and light chain DNA expressed 107 recombinant antibodies in total. To determine if these antibodies were specific to influenza virus, they were examined on dot blot, immunocytochemistry (ICH) and ELISA assays.

In the dot blot, vaccine split viruses were titrated to find the optimal dotting concentration (figure 5.8). Nitrocellulose strips were dotted with vaccine split virus of optimal concentration and incubated with antibody supernatant. Upon incubation with the secondary antibody, HRP-conjugated goat anti-human IgG, ECL substrate was used to develop the dotted strip.

In the ELISA assay, the plates were coated with vaccine split virus of 10µg/ml then incubated with antibody supernatant. Upon incubation with the

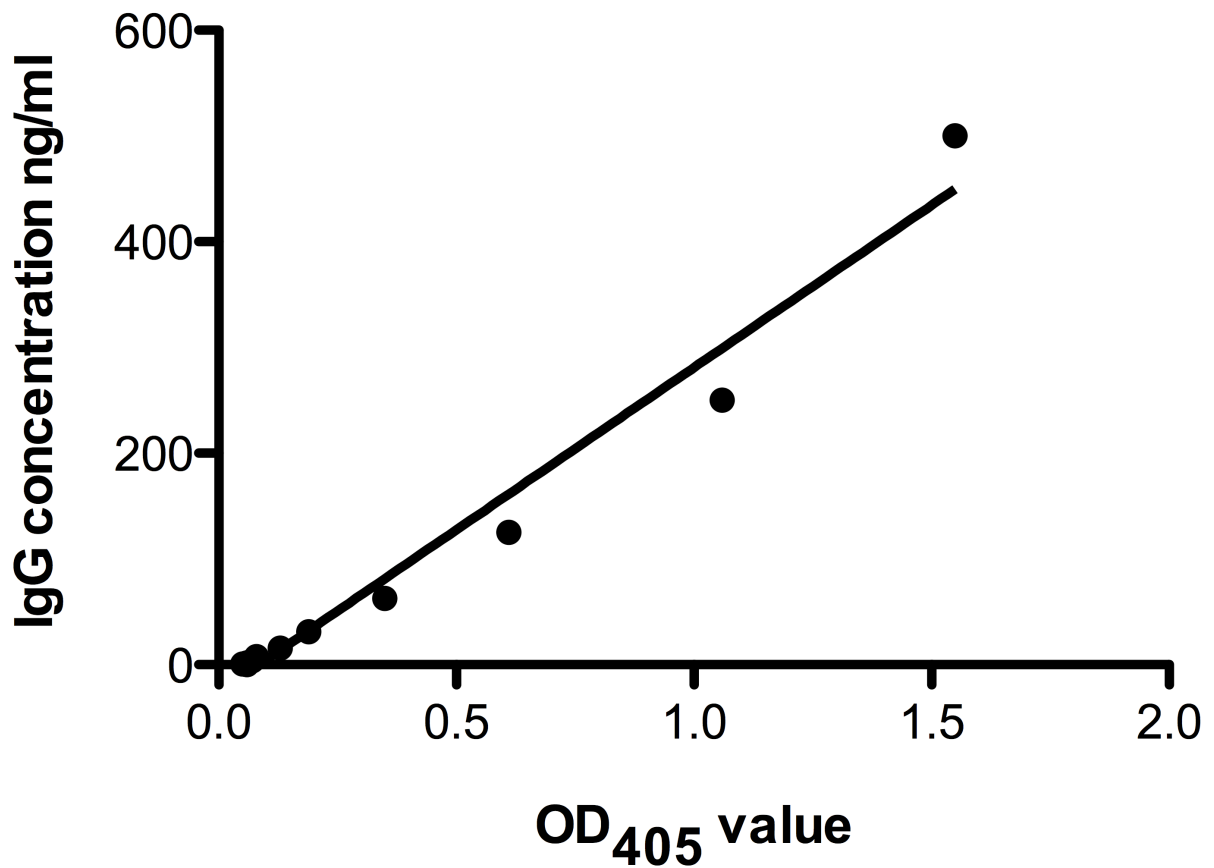


Figure 5.7. IgG standard curve. The anti-human IgG was pre-coated on ELISA plates to capture IgG of known concentration. After incubation, secondary anti-human IgG (Fc) conjugated with alkaline phosphatase and substrate was added to develop yellow color. The OD₄₀₅ value would be read. Thus, the OD₄₀₅ value and the IgG concentration on the corresponding ELISA wells could be plotted to create a standard equation. This equation would be applied to calculate the concentration of recombinant mAbs based on their OD₄₀₅ value.

Negative serum

Vaccinee serum

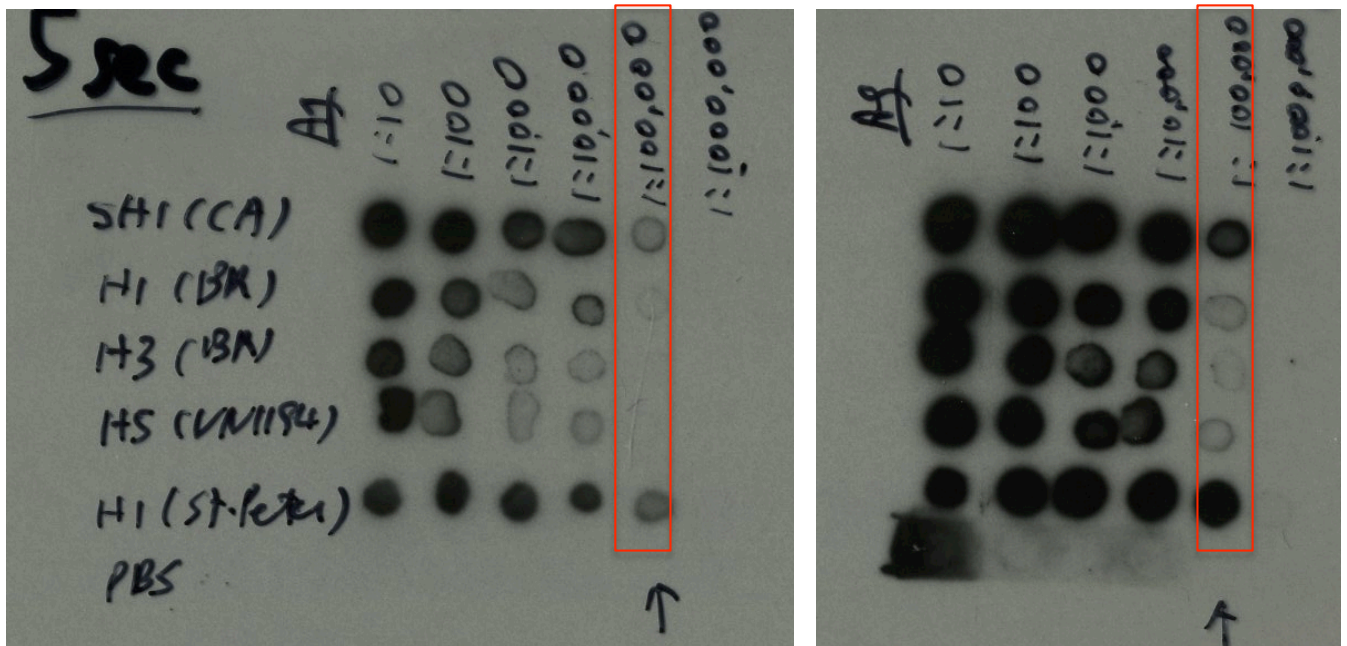


Figure 5.8. Titration analysis of vaccine antigen used in the dot blot assay. Pandemic H1N1 (CA07), seasonal H1N1 (BR59), seasonal H3N2 (BR10), avian H5N1 (VN1194) and inactivated pandemic H1N1 (St. peterburg stain) were titrated from 1:10 to 1:10⁶ dilution in testing serum. Negative serum was collected in 2010 from one donor without previous influenza vaccination history. Positive serum was collected from one pandemic H1N1 vaccinated donor in 2010. The concentration of 1:10⁵ dilution is optimal to determine the presence of influenza-specific antibody in the serum.

secondary antibody, ALP-conjugated goat anti-human IgG, pNPP substrate was used to develop the ELISA plate.

With regard to ICH assay (performed by Judy Bastin and Tim Powell, the Group of Prof. Alain Townsend), the live vaccine pandemic H1N1 (CA07, NYMCX-179A), seasonal H1N1 (BR59, IVR-148), H3N2 (BR10, NYMCX-187) and live PR8 virus were used to infect L cells. The infected L cells were fixed and permeabilized with acetone then incubated with antibody supernatants and other mAb controls. Upon incubation with secondary antibodies, HRP-conjugated rat anti-human or goat anti-mouse IgG, DAB substrate was used to develop the stained cells (figure 5.9).

The convalescent serum from influenza-infected donor was used as positive control and dengue mAb were used as negative control in the dot blot, ELISA and ICH assay. Of the 107 antibodies tested, 8 were all positive on three independent assays. There were no antibodies bound to the mock-infected supernatant.

Of the 8 anti-influenza antibodies, 4 were broadly cross-reactive against influenza serotypes including H1N1 CA07, H1N1 BR59, H3N2 BR10, H3N2 PR16 and H5N1 VN1194 influenza virus. These cross-reactive antibodies were 2-8B H-L, 2-8C H-L, P4-4B H-L and P2-8B H-L. There were 3 antibodies specific to H1N1 and 1 antibody specific to H3N2 influenza virus, which were 2-12C H-L, 2-6C H-K, 2-12C H-K and 2-9C H-L antibodies, respectively (table 5.3, figure 5.10 and figure 5.11). It is interesting to see only cross-reactive antibodies were made from infected donors, however, the number is too few to draw any concrete conclusion.

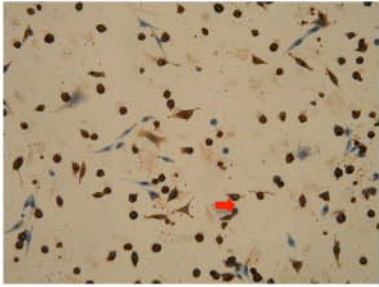
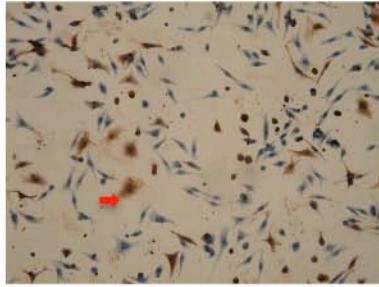
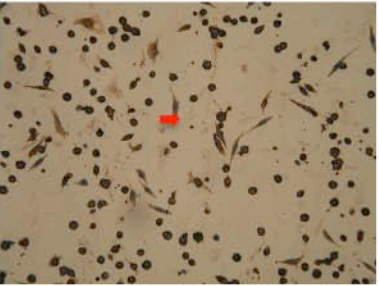
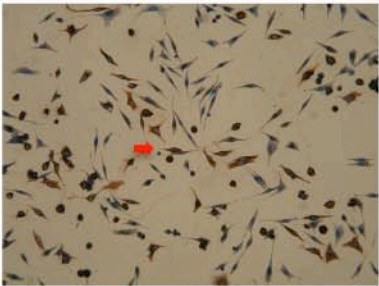
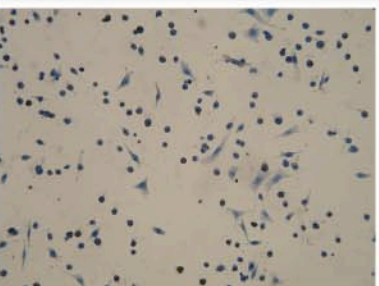
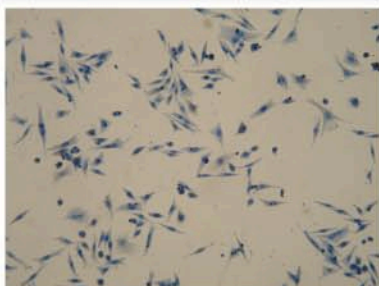
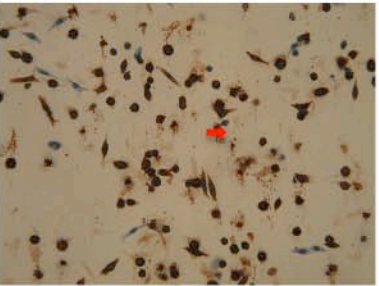
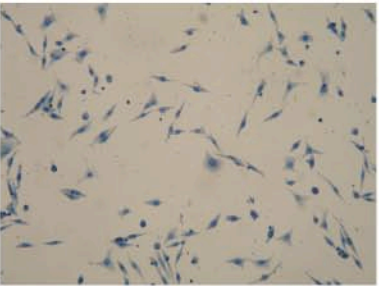
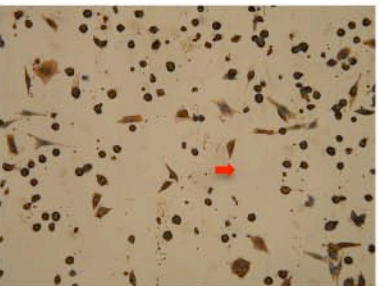
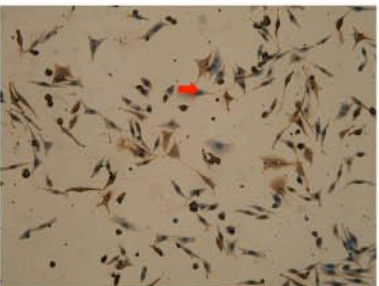
	H1N1 PR8-infected	H1N1 BR59-infected
C179 (anti-HA2)		
P2-8B H-L (anti-NP)		
P2-3C H-L		
H28-E23 (anti-PR8 HA1)		
MCA400 (anti-NP)		

Figure 5.9. Immunocytochemistry staining with mAb (performed by Judy Bastin and Tim Powell, the Group of Prof. Alain Townsend). Red arrow indicated positive stained cells presenting dark-brown color. The negative cells would present contrast blue color. Monoclonal antibodies C179, H28-E23, and MCA400 were incubated with infected L cells as positive controls.

Table 5.3. Binding specificity of anti-influenza mAb.

Donor	mAb	Dot Blot							Immunocytochemistry							EILSA				
		H1N1 BR59	H1N1 CA07	H3N2 Wisconsin67	H3N2 BR10	H3N2 PR16	H5N1 VN1194	H1N1 Eng195 (HA)	H1N1 PR8 (HA)	H1N1 CA07	H1N1 BR59	H3N2 PR16	H1N1 BR59	H1N1 CA07	H3N2 BR10	H3N2 PR16	H5N1 VN1194			
from vaccinated volunteer																				
CL	2-8B H-L	±	-	-	±	±	±	-	-	-	+	+	-	+	+	+	+			
CL	2-8C H-L	+	+	-	+	+	+	-	+	+	+	+	+	+	+	+	+			
CL	2-9C H-L	-	-	-	+	+	-	-	±	-	+	-	-	+	+	+	-			
CL	2-12C H-L	-	+	-	-	-	-	+	+	-	-	-	+	-	-	-	-			
CL	2-6C H-K	+	+	-	-	-	-	+	+	+	-	+	+	-	-	-	-			
CL	2-12C H-K	-	+	-	-	-	-	±	+	-	-	-	+	-	-	-	-			
from infected volunteer																				
14C	P4-4B H-L	+	+	-	+	+	+	+	+	+	+	+	+	+	+	+	-			
1C	P2-8B H-L	+	+	-	+	+	+	-	+	+	+	+	+	+	+	+	+			

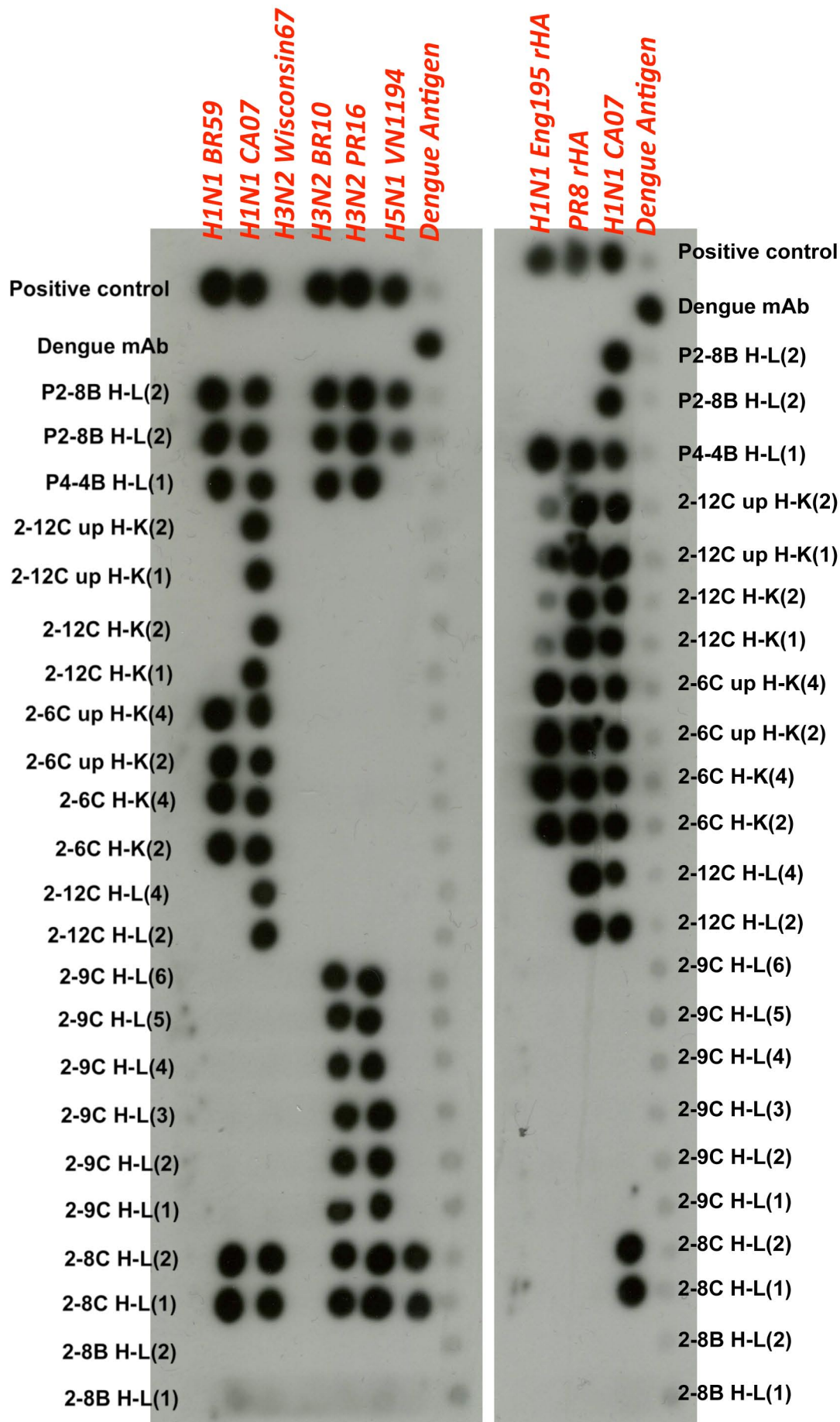


Figure 5.10. Dot Blot analysis for anti-influenza mAb. Nitrocellulose strips were dotted with influenza vaccine antigens (upper) and influenza recombinant HA (lower), and then probed with anti-influenza mAb supernatants. The dengue mAb was used as negative control. Serum from influenza patients was used as positive control.

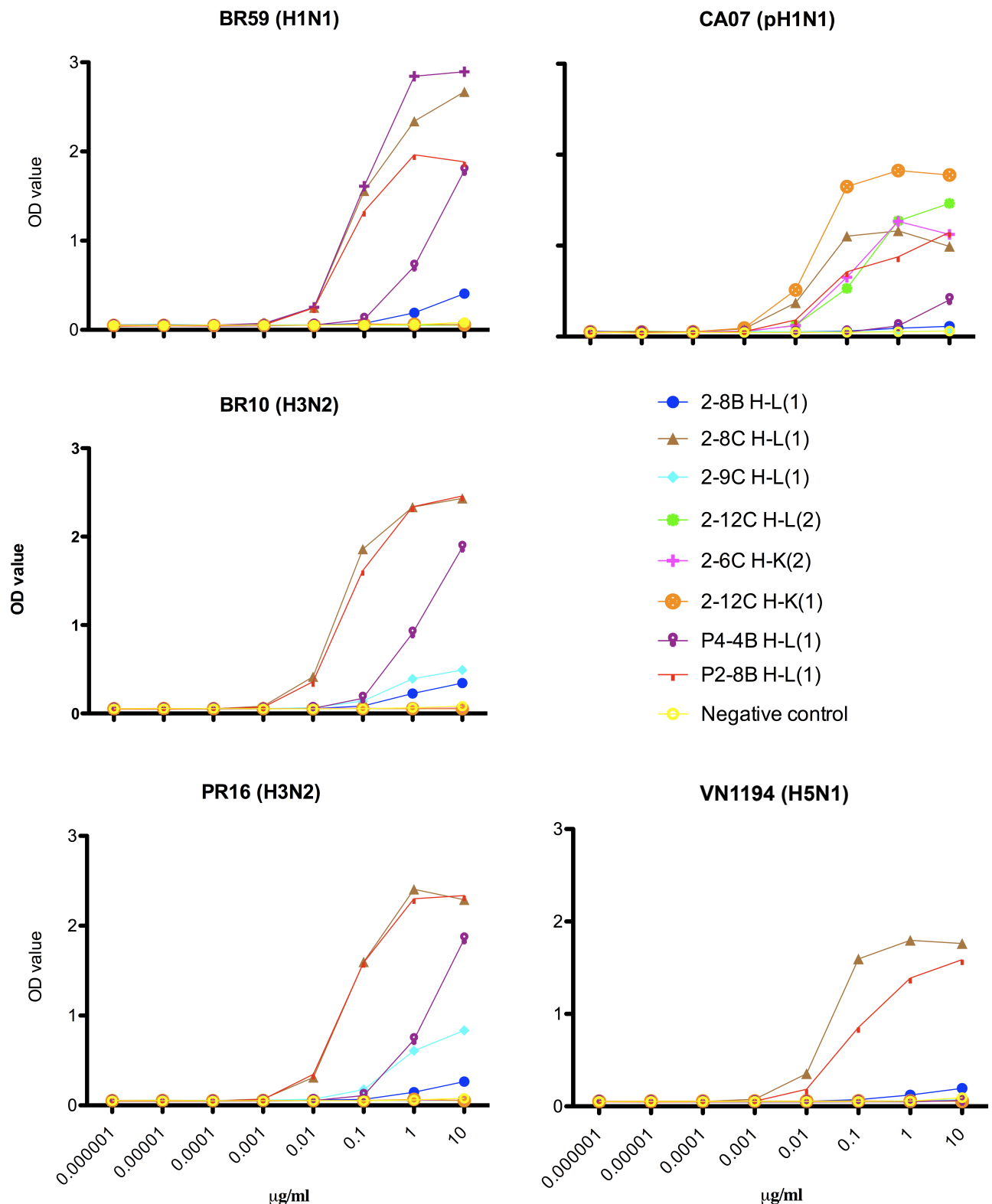


Figure 5.11. ELISA analysis of anti-influenza mAb. BR59, CA07, BR10, PR16 and VN1194 vaccine antigen from sinovac company were used for coating antigen. Antibodies were determined as positive of given antigen, if they bound at least two standard deviations greater than the mean absorbance of negative control antibodies at 10 µg/ml.

The dissociation scores of anti-influenza antibodies were calculated by nonlinear regression analysis of curves from eight dilutions of antibody (10 to 1e-06 ug/ml) in the ELISA assay, as previously described (Warmmert *et al.*, 2008). At least two replicates of each antibody were tested in the ELISA. The lower dissociation scores represent the stronger binding activities. Of the anti-influenza antibodies, the dissociation scores ranged from 3.3e-08 to 1.6e-10 (table 5.4). There were no significant difference of binding activities between cross-reactive and type-specific antibodies, except the P4-4B antibody has a relatively weaker binding ability. The following western blot analysis revealed P4-4B could be an anti-HA2 antibody. The acid-treatment of influenza antigen could enhance the binding activity of P4-4B in the ELISA assay.

Although the vaccinated donor had previous seasonal vaccine history, for instance, 2009-2010 seasonal vaccination containing H1N1 BR59 and H3N2 BR10 strains, those H1N1-specific antibodies generated from the vaccinated donor bound to the current H1N1 CA07 vaccine strain with high binding ability. One antibody (2-6C antibody) bound to previous H1N1 BR59 vaccine strain as well, nevertheless, the binding ability was similar between H1N1 CA07 and H1N1 BR59 strain. Regarding the H3N2-specific antibody, 2-9C antibody also had similar binding scores between H3N2 BR10 and H3N2 PR16 vaccine strains (table 5.4). Though the case number is few and the influenza-specific antibody pool is small, OAS did not seem to occur in this donor with previous vaccination history.

5.3.1.5 The specificity of anti-influenza antibodies was analyzed by western blot.

Table 5.4. Characterization of anti-influenza mAb.

Donor	mAb	Dissociation scores (ELISA)							HI	MN	Western Blot	
		H1N1 BR59	H1N1 CA07	H3N2 BR10	H3N2 PR16	H5N1 VN1194	H1N1 CA07	H1N1 CA07			H1N1 CA07	
from vaccinated volunteer												
CL	2-8B H-L	1.2E-08	-	4.2E-09	6.3E-09	1.3E-09	None	None	None	None	NP	NP
CL	2-8C H-L	7.3E-10	1.6E-10	3.8E-10	5.3E-10	2.8E-10	None	None	None	None	NP	NP
CL	2-9C H-L	-	-	2.5E-09	4.0E-09	-	None	None	None	None	NP	NP
CL	2-12C H-L	-	1.7E-09	-	-	-	None	None	None	None	HA1	HA1
CL	2-6C H-K	8.6E-10	8.1E-10	-	-	-	None	None	None	None	HA1	HA1
CL	2-12C H-K	-	2.1E-10	-	-	-	>1:256	1:512	1:512	1:512	HA1	HA1
from Infected volunteer												
14C	P4-4B H-L	2.0E-08	3.3E-08	1.3E-08	2.0E-08	-	None	None	None	None	HA2	HA2
1C	P2-8B H-L	5.2E-10	5.0E-10	5.4E-10	5.1E-10	8.3E-10	None	None	None	None	NP	NP

Antibodies were tested on western blot to identify the specific protein they bound. H1N1 BR59, H1N1 CA07 and H3N2 PR16 vaccine virus was run on SDS-PAGE and transferred to a nitrocellulose membrane. The choice of vaccine antigen depended on the results from the Dot blot/ELISA/ICH analysis. Membranes were cut into strips and antibodies were incubated with them. The anti-NP mAb and convalescent serum were used as positive control. The dengue-specific mAb and dot blot-negative mAb were used as negative control. The size marker would reveal the corresponding size of any bands.

Clear bands were shown on the western blot of anti-influenza antibodies (figure 5.12 and table 5.4). Of the 4 cross-reactive anti-influenza mAb, 3 bound to NP and 1 bound to HA2 protein. It could be suggested that cross-reactive antibodies bound to the highly conserved area of NP and HA2 proteins. The H3N2-specific mAb bound to NP protein. The other H1N1-specific mAb all bound to HA1 protein.

5.3.1.6 The HA2-binding antibody was analyzed in the acid-treatment environment.

While the mAb were tested by ICH staining, P4-4B antibody stained the PR8-infected L cells showing the punctate pattern, indicative of the intra-vesical binding of antibody (figure 5.13) (Bächi *et al.*, 1985; Yewdell *et al.*, 1988). The HA2 protein band of 25kDa was also shown on western blot. All this supported that P4-4B could be anti-HA2 antibody. The previous study has shown that acid-induced conformational changes would expose the fusion domain, the hydrophobic N-terminal segment of HA2. Low pH environment

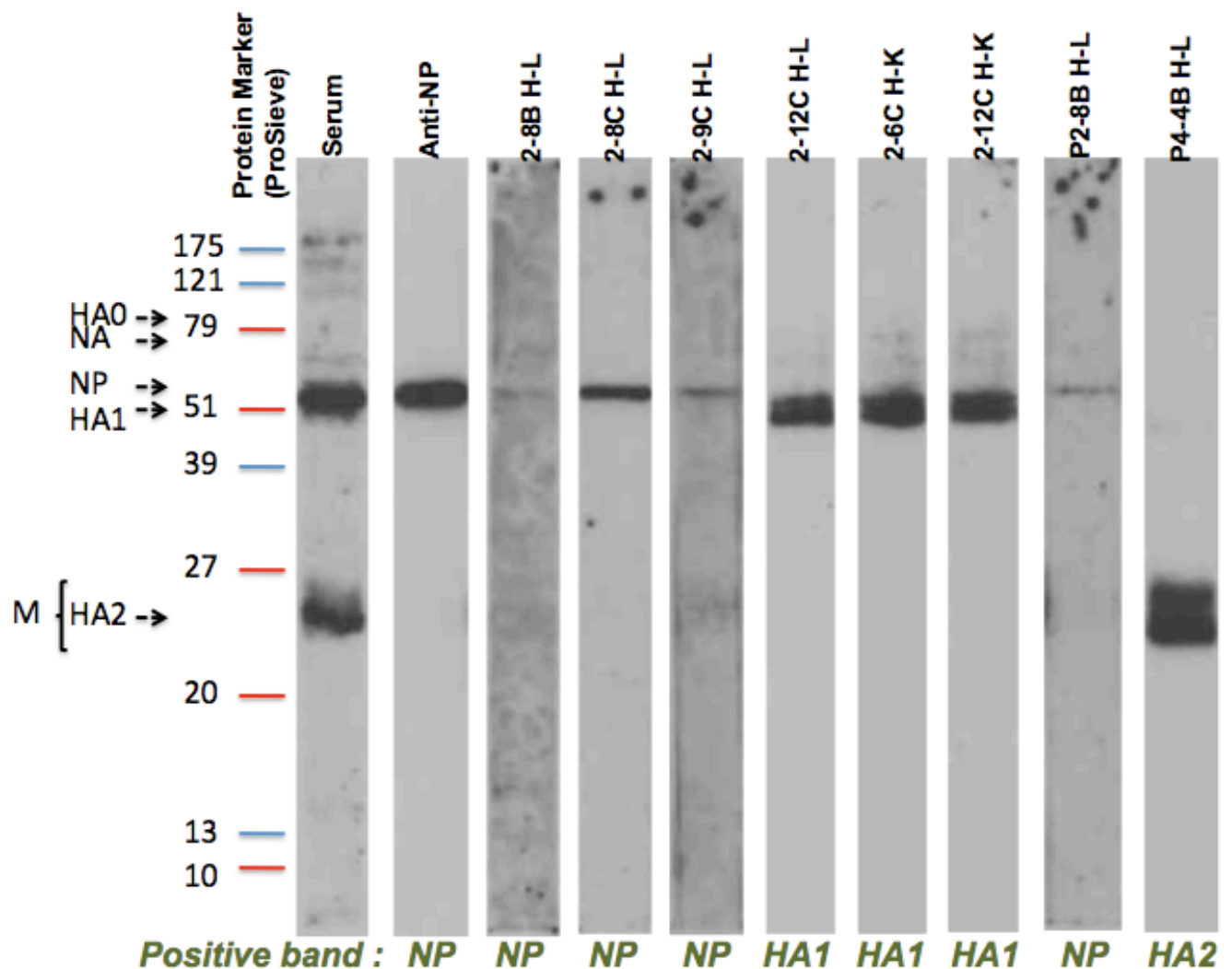


Figure 5.12. The protein band bound by anti-influenza antibodies were shown on western blot assay using heated and reduced H1N1 CA07 vaccine antigen. The serum from pandemic H1N1 vaccinated individual and MCA400 anti-NP monoclonal antibody were used as positive control. Dengue mAb was used as negative control. The protein ladder showed sizes in kDa.

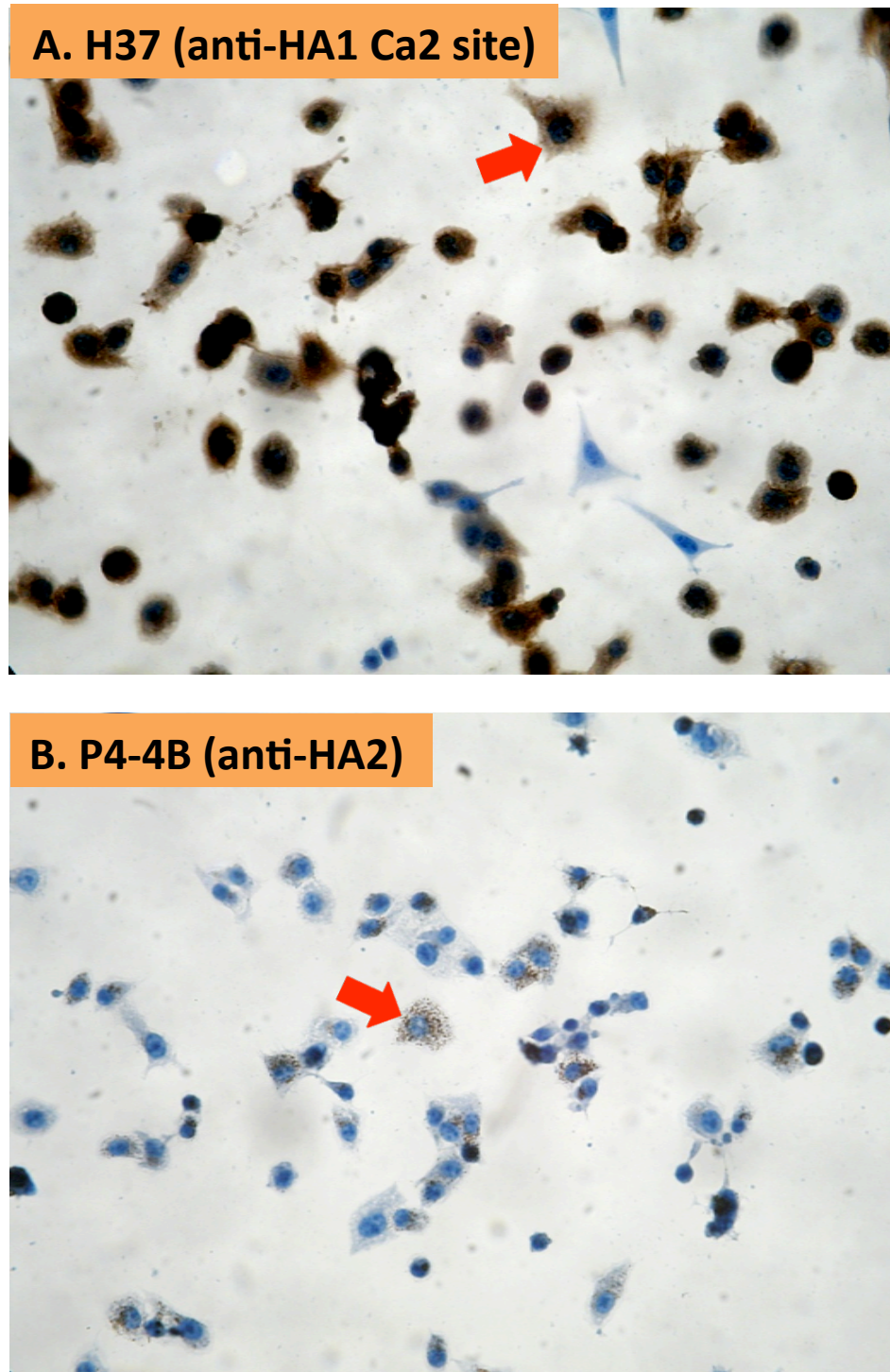


Figure 5.13. The staining pattern of anti-HA1 H37 and anti-HA2 P4-4B mAb. A punctate pattern of anti-HA2 mAb (red arrow, figure B) was shown on L cells infected with PR8 virus, compared to homogenous staining of anti-HA1 mAb (red arrow, figure A). The ICH staining was performed by Judy Bastin and Tim Powell, the Group of Prof. Alain Townsend.

was therefore created *in vitro* to test if this affected the binding activity of P4-4B antibody.

The transfection of MDCK SIAT-II cells with plasmid carrying PR8-HA allowed the expression of HA on MDCK cell membrane. Transfected cells were treated with pH5.0 citrate buffer before incubation with P4-4B antibody. Transfected cells without acid treatment was also incubated with antibody as control. It was noted that acid treatment enhanced the binding of P4-4B antibody on the transfected cell membrane (performed by Judy Bastin, the Group of Prof. Alain Townsend) (figure 5.14).

In the ELISA assay, prior to incubation, the coated influenza antigen was treated with pH5.0 citrate buffer. The wells without acid treatment were taken as control. It was noted that the binding activity of P4-4B antibody increased on acid-treated wells (table 5.5 and figure 5.15). In contrast, the acid treatment didn't affect the binding pattern of anti-NP or anti-HA1 antibodies.

5.3.1.7 The function of anti-influenza antibodies was analyzed.

In the study, anti-influenza antibodies were examined in HI and MN assays (performed by Prof. Alain Townsend).

HI assay was based on mixing virus with antibody of serial dilutions and adding the mix to mammalian RBC. This assay could examine the ability of antibody to inhibit agglutination of RBC by a standardized dose of virus. The level of inhibition was expressed as a diluted titre of antibody. The higher diluted titre of antibody represented the stronger ability to inhibit

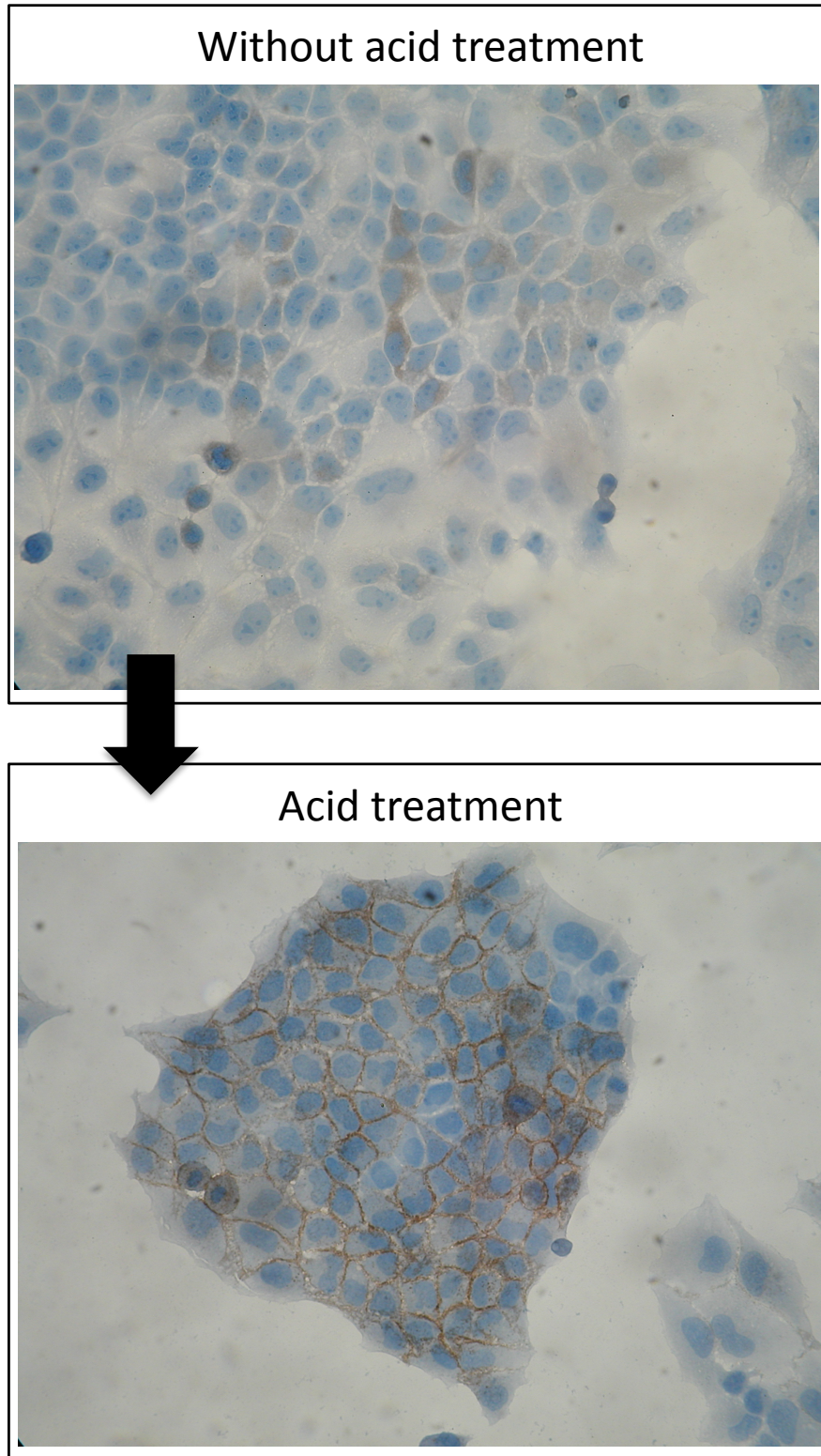


Figure 5.14. Acid treatment enhances the anti-HA2 P4-4B antibody binding on PR8/HA-transfected MDCK-SIAT II cells. The acid-treatment ICH staining was performed by Judy Bastin, the Group of Prof. Alain Townsend.

Table 5.5. The effect of acid treatment on anti-HA2 P4-4B binding activity.

mAb	Binding scores (ELISA)									
	H1N1 BR59		H1N1 CA07		H3N2 PR16		H5N1 VN1194			
	W/O Acid	Acid Tx	W/O Acid	Acid Tx	W/O Acid	Acid Tx	W/O Acid	Acid Tx	W/O Acid	Acid Tx
anti-HA2 P4-4B	1.4E-08	7.2E-09	1.9E-08	2.9E-08	2.3E-08	4.3E-09	-	-	-	-
anti-NP P2-8B	4.0E-10	4.0E-10	1.3E-10	1.6E-10	2.5E-10	2.3E-10	2.3E-10	2.4E-10		
anti-HA1 2-12C	-	-	1.6E-09	1.8E-09	-	-	-	-	-	-

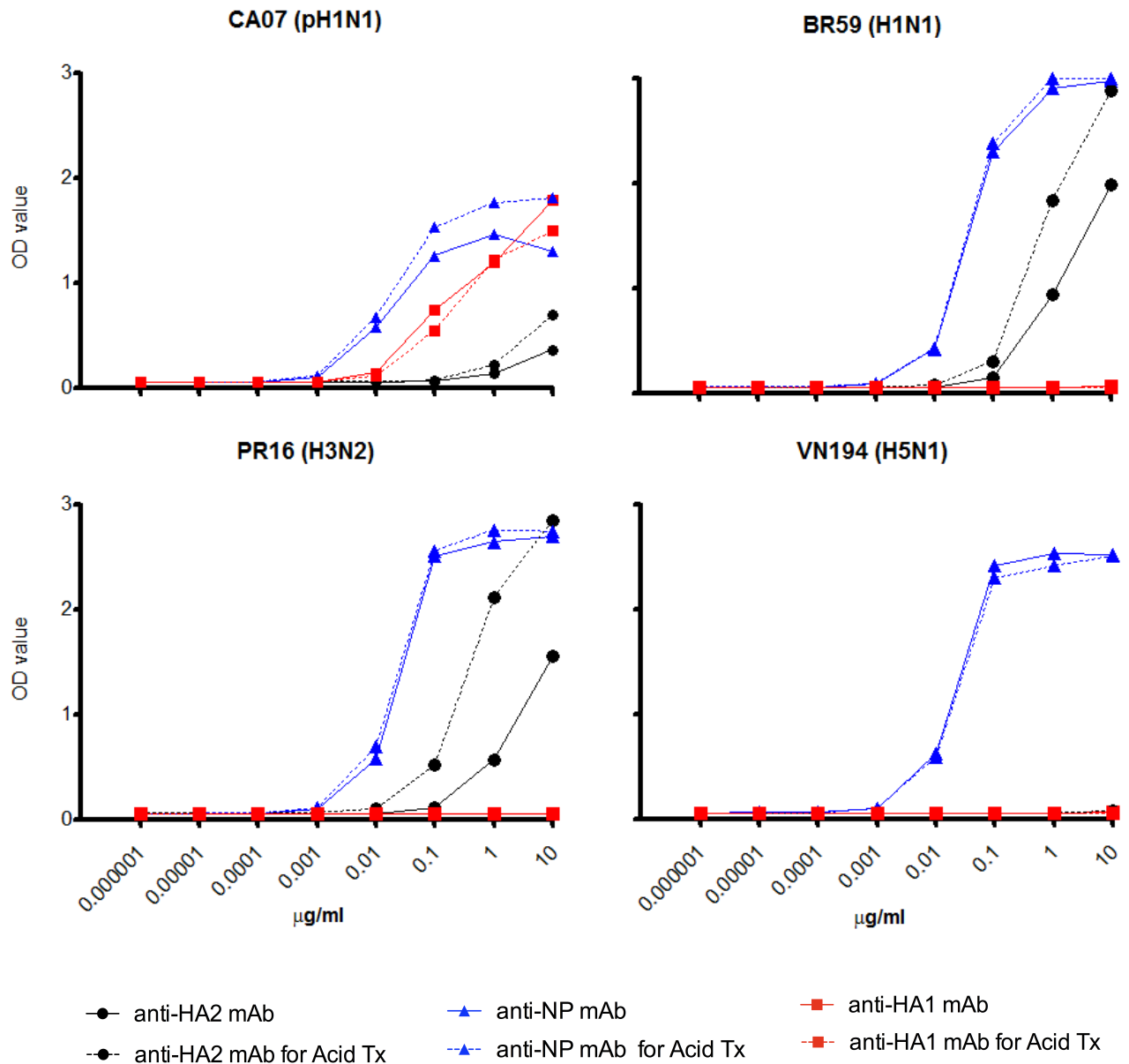


Figure 5.15. Acid treatment enhanced the binding activity of anti-HA2 P4-4B mAb on the H1N1 BR59 and H3N2 PR16 virus, as measured by the ELISA assay. Left shift of binding curve of anti-HA2 mAb was observed upon acid treatment. No change of binding activity of anti-HA1 2-12C and anti-NP P2-8B mAb was shown. Solid line represent the binding curve for usual condition of coated influenza antigen, and dashed line represent the binding curve for acid-treated influenza antigen coated in the ELISA assay.

haemagglutination. MN assay was performed by mixing virus with antibody of serial dilutions and then adding the MDCK cells to the mix. After incubation overnight, infected cells were determined by ELISA using anti-NP antibody as the detection antibody. Since no trypsin was provided during the incubation, the ability of antibody to inhibit virus replication cannot be evaluated in this single-cycle assay. The ability of antibody to neutralise the infectivity of given virus was examined and the level of neutralisation was expressed as a diluted titre of antibody.

Of the eight anti-influenza antibodies, one was positive in both HI and MN assay (table 5.4). The 2-12C H-K antibody was HA1-specific and had over 1:256 HI and MN titre against pandemic H1N1. The other two HA1-specific antibodies couldn't confer HI and neutralisation activities.

In general, anti-HA2 antibody doesn't confer HI activity. In the study, P4-4B antibody is neither HI nor MN positive. Since P4-4B antibody didn't have neutralising activity, it was suggested that this anti-HA2 mAb might not block the membrane fusion.

In the study, it made sense that anti-NP antibodies were all HI-negative, since HA binding is required to inhibit haemagglutination. The MN-negative result indicated that these anti-NP antibodies were unable to inhibit viral transcription, however, the direct assay to measure the function of anti-NP antibodies is lacking.

5.3.2 The characterisation of Ig sequences

5.3.2.1 Antibody gene sequences from ASC upon influenza vaccination/infection were analyzed and compared with previous studies.

In the study, single cell PCR produced 107 variable region sequences for IgG heavy chain, 47 and 60 corresponding kappa and lambda chain sequences from ASC of three donors. The usage of kappa and lambda light chain in ASC repertoire is 50.4 % (range 36.1~63.6%) and 49.6% (range 36.4~63.9%) on average, respectively (table 5.6). All the sequences were analyzed with regard to their gene segment usage, CDR3 length, clonality, as well as the frequency and distribution of somatic hypermutation.

In order to determine the individual gene segments employed by V(D)J rearrangements, the sequences were aligned with germline gene segments using the IMGT alignment tools. The distribution of gene segment usage of the variable region on heavy and light chains were analyzed (figure 5.16, table 5.7, and table 5.8). It was noted that the V, D and J segments are not usually used in equal ratios and a natural dominance towards particular families is present. Similar distribution and dominance involving certain families of variable region had also been observed in infection-induced ASC (Wrammert *et al.*, 2011), memory IgG⁺ B cells (de Wildt *et al.*, 2000), naive IgM⁺ B cells (Poulsen *et al.*, 2007) or randomly selected B cells from healthy adults (Williams *et al.*, 2009) (figure 5.16).

In the study, the most frequent V_H family was V_H3 (51%, 55/107), followed by V_H4 (36%, 38/107) and V_H1 (7%, 7/107). The analysis of V segment usage of light chains revealed that V_k1 (64%, 30/47) was used most frequently for

Table 5.6. Summary of ELISpot result and Ig cloning data.

DONOR ID.	STATUS	ASC RESPONSE (%)	SORTED CELLS (#)	HEAVY CHAIN SEQUENCES (#)	KAPPA CHAIN SEQUENCES (#)	LAMBDA CHAIN SEQUENCES (#)	RECOMBINANT MONOCLONAL ANTIBODY (#)	CLONALITY (%)	ANTI-FLU MONOCLONAL ANTIBODY (#)
14C	BR59 infection	30	27	11	7	4	11	18	1
1C	BR59 infection	23	96	35	18	17	35	17	1
CL	Annual vaccination	10	144	61	22	39	61	18	6

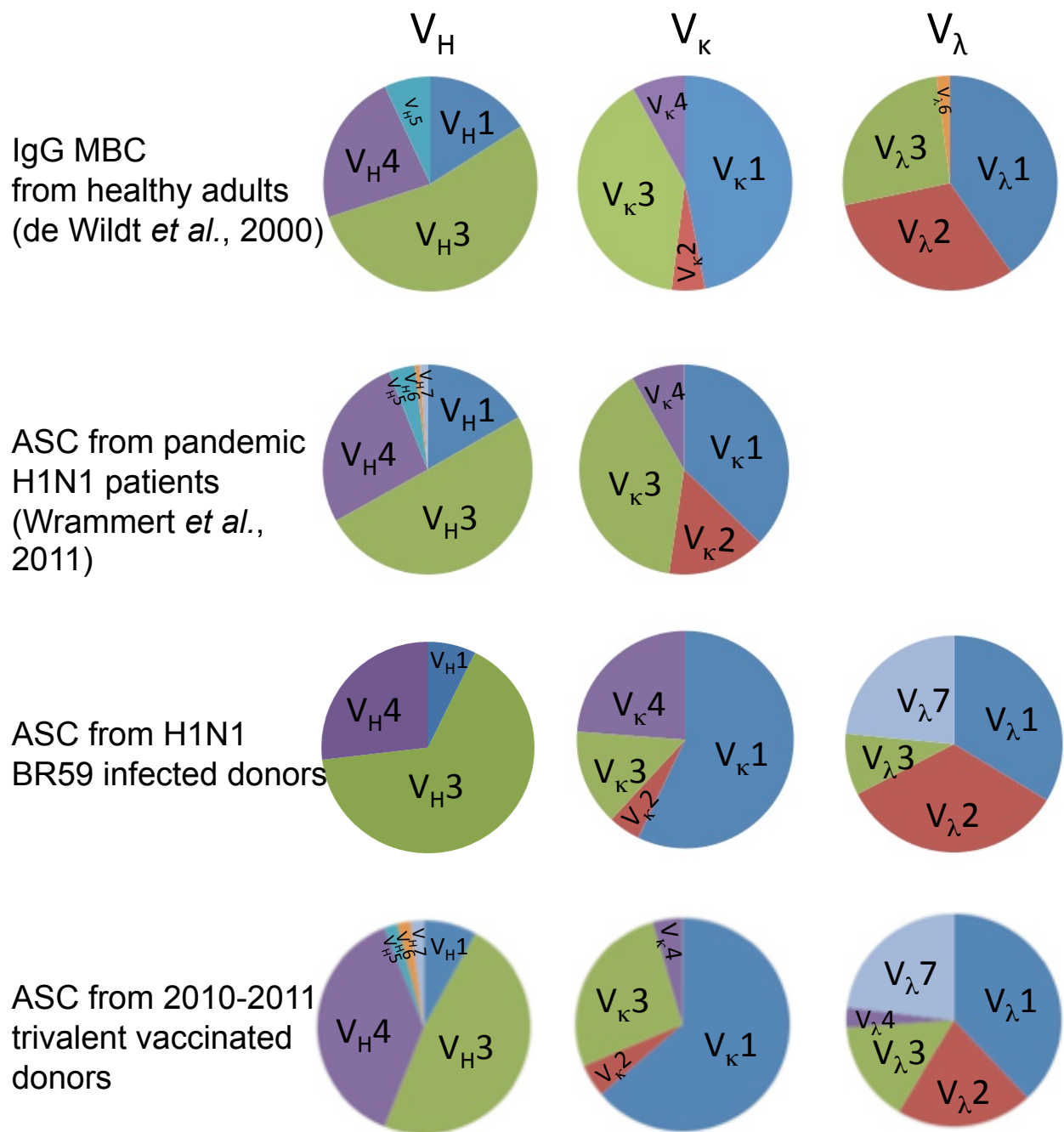


Figure 5.16. The distribution of gene segment usage of heavy and light chain variable region.

Table 5.7. Gene usage of mAb from infected donors.

DONOR	MONOCLONAL ANTIBODY*	HEAVY CHAIN										LAMBDA CHAIN		KAPPA CHAIN	
		V	MUTATIONS	IDENTITY	J	MUTATIONS	IDENTITY	D	FR-LENGTHS	CDR-LENGTHS	AA JUNCTION	V	J	V	J
14C	P4-9A H-L (C1)	3-30-3	12	96	3	0	100	2-2	25.17.38.11	8.8.17	CACIVLPATIRGDAFDIW	2-14	3		
14C	P4-10A H-L	3-30-3	12	96	3	0	100	2-2	25.17.38.11	8.8.17	CACIVLPATIRGDAFDIW	1-40	2 or 3		
14C	P4-3B H-L (C1)	3-30-3	15	95	3	0	100	2-2	25.17.38.11	8.8.17	CACIVLPATIRGDAFDIW	2-14	1		
14C	P4-4B H-L	1-2	18	93	4	6	88	7-27	25.17.38.11	8.8.12	CARILRGTEGLDSW	2-14	3		
1C	P2-6A H-L (C1)	3-30-3	14	95	3	0	100	2-2	25.17.38.11	8.8.17	CACIVLPATIRGDAFDIW	7-46	1		
1C	P2-9A H-L	3-30-3	14	95	3	0	100	2-2	25.17.38.11	8.8.17	CACIVLPATIRGDAFDIW	1-40	1		
1C	P2-10A H-L	3-30-3	13	95	3	0	100	2-2	25.17.38.11	8.8.17	CACIVLPATIRGDAFDIW	2-11	1		
1C	P2-8B H-L	4-39	16	95	4	9	81	3-16	25.17.38.11	10.7.12	CARREFDRISPYW	1-40	2 or 3		
1C	P2-3C H-L	3-30-3	15	95	3	0	100	2-2	25.17.38.11	8.8.17	CACIVLPATIRGDAFDIW	1-51	2 or 3		
1C	P2-5C H-L (C2)	3-30-3	14	95	3	0	100	2-2	25.17.38.11	8.8.17	CACIVLPATIRGDAFDIW	1-44	3		
1C	P2-8C H-L	4-61	37	82	6	11	83	3-3	25.17.38.11	8.6.14	CARVGTIFGNYMDVW	2-8	1		
1C	P2-9D H-L (C2)	3-30-3	14	95	3	0	100	2-2	25.17.38.11	8.8.17	CACIVLPATIRGDAFDIW	1-44	1		
1C	P2-11D H-L	4-39	24	92	6	12	81	3-22	25.17.38.11	10.7.13	CARLSSQYNTMDVW	3-21	3		
1C	P3-11A H-L	3-30-3	14	95	3	0	100	2-2	25.17.38.11	8.8.17	CACIVLPATIRGDAFDIW	2-8	2 or 3		
1C	P3-3B H-L	4-61	37	82	6	11	82	3-3	25.17.38.11	8.8.14	CARVGTIFGNYMDVW	7-46	3		
1C	P3-2C H-L	4-59	31	89	6	11	82	3-3	25.17.38.11	8.6.14	CARVGTIFGNYMDVW	2-8	2 or 3		
1C	P3-5C H-L	4-59	14	95	5	4	92	6-13	25.17.38.11	8.7.14	CASRIAAAGTSWFDPW	7-46	1		
1C	P3-6C H-L	1-2	21	93	6	8	87	1-1	25.17.38.11	8.8.18	CAGRSYNWNIDIFYKYMDVW	3-1	1		
1C	P3-8C H-L	3-23	22	92	6	8	87	5-24	25.17.38.11	8.8.15	CVKFRGPLDHYMDVW	1-51	3		
1C	P3-7D H-L	3-48	18	94	6	7	89	6-6	25.17.38.11	8.8.19	CARDSFTGSSWDFDSYMDVW	7-46	1		
1C	P3-9D H-L (C1)	3-30-3	14	95	3	1	98	2-2	25.17.38.11	8.8.17	CACIVLPATIRGDAFDIW	7-46	1		
14C	P4-6A H-K	3-30-3	13	95	3	0	100	2-2	25.17.38.11	8.8.17	CACIVLPATIRGDAFDIW			1-33	4
14C	P4-7A H-K	3-30-3	15	94	3	0	100	2-2	25.17.38.11	8.8.17	CACIVLPATIRGDAFDIW			4-1	2
14C	P4-12A H-K	3-30-3	14	95	3	0	100	2-2	25.17.38.11	8.8.17	CACIVLPATIRGDAFDIW			1-9	3
14C	P4-6B H-K	3-30-3	14	95	3	0	100	2-2	25.17.38.11	8.8.17	CACIVLPATIRGDAFDIW			1-5	5
14C	P4-7B H-K	1-2	17	94	6	9	85	1-1	25.17.38.11	8.8.18	CACIVLPATIRGDAFDIW			1-39	4
14C	P4-10B H-K	3-30-3	15	95	3	0	100	2-2	25.17.38.11	8.8.17	CAGRSYNWNIDIFYKYMDVW			2-28	2
14C	P4-1C H-K	3-21	49	85	6	11	81	6-19	25.17.38.11	8.8.17	CACIVLPATIRGDAFDIW			1-39	4
1C	P2-2A H-K	3-30-3	13	95	3	0	100	2-2	25.17.38.11	8.8.11	CTRDSSGWYMDVW			1-33	2
1C	P2-11A H-K	4-59	28	90	5	10	80	3-10	25.17.38.11	8.7.13	CARRVWFGESPFDLW			1-9	3
1C	P2-3B H-K	4-39	22	92	5	6	88	2-2	25.17.38.11	10.7.22	CVRPHRYCSSTHCFLTLRFPDW			1-16	1
1C	P2-7B H-K (C3)	3-30-3	14	95	3	0	100	2-2	25.17.38.11	8.8.17	CACIVLPATIRGDAFDIW			1-39	3
1C	P2-3C H-K (C3)	3-30-3	14	95	3	0	100	2-2	25.17.38.11	8.8.17	CACIVLPATIRGDAFDIW			1-39	2
1C	P2-6C H-K	4-61	37	82	6	11	83	3-3	25.17.38.11	8.6.14	CARVGTIFGNYMDVW			4-1	1
1C	P2-8C H-K	4-61	37	82	6	11	83	3-3	25.17.38.11	8.6.14	CARVGTIFGNYMDVW			1-39	1
1C	P2-1D H-K	3-30-3	13	95	3	0	100	2-2	25.17.38.11	8.8.17	CACIVLPATIRGDAFDIW			1-39	1
1C	P3-6A H-K	3-33	15	95	4	5	90	2-8	25.17.38.11	8.8.15	CARDGPLGAQNSPLDYW			4-1	1
1C	P3-12A H-K	4-59	21	93	3	3	94	2-8	25.17.38.11	8.7.12	CARKLLQTDADFDFW			3-20	2
1C	P3-3B H-K	4-61	37	82	6	11	82	3-3	25.17.38.11	8.8.14	CARVGTIFGNYMDVW			1-39	N/A
1C	P3-4B H-K	3-64	19	93	6	15	76	5-12	25.17.38.11	8.8.14	CARGQGGYDGGWFDVW			3D-20	2
1C	P3-9B H-K	4-39	26	91	4	2	96	1-14	25.17.38.11	10.7.12	CGRVSPGNYFEHW			1-39	2
1C	P3-2C H-K	4-59	31	89	6	11	82	3-3	25.17.38.11	8.6.14	CARVGTIFGNYMDVW			4-1	4
1C	P3-7C H-K	3-11	47	84	4	6	88	6-13	25.17.38.11	8.8.16	CSRGPPVNVGAAGDFDSW			1-9	4
1C	P3-5D H-K	3-72	16	95	3	8	84	2-15	25.17.38.11	8.10.18	CGRGLSTCSGGGCTLVDIW			3-20	4
1C	P3-6D H-K	1-69	49	83	4	4	92	2-21	25.17.38.11	8.8.12	CACQGGAWYFPDYW			4-1	2
1C	P3-7D H-K	3-48	18	94	6	7	89	6-6	25.17.38.11	8.8.19	CARDSFTGSSWDFDSYMDVW			1-5	1

* Clonal sequences are denoted by clonal pool number. For instance, clonal pool 1 from a particular donor is "(C1)".

Table 5.8. Gene usage of mAb from vaccinated donor.

DONOR	MONOCLONAL ANTIBODY*	HEAVY CHAIN										LAMBDA CHAIN									
		V	MUTATIONS	IDENTITY	J	MUTATIONS	IDENTITY	D	FR-LENGTHS	CDR-LENGTHS	AA JUNCTION	V	J	V	J	V	J	V	J	V	J
CL	1-10A H-L	3-7	32	89	4	5	90	1-26	25.17.38.11	8.8.8	CTIGHYSNYA	7-46	1								
CL	1-4B H-L	3-23	9	97	4	9	81	6-13	25.17.38.11	8.8.14	CAKVRIAAAGSMDVW	1-51	1								
CL	1-8B H-L	4-59	29	90	4	5	90	3-22	25.17.38.11	8.7.17	CARGSYFDTSGYQPLDSW	1-44	1								
CL	1-10B H-L	4-31	19	93	4	9	81	3-16	25.17.38.11	10.7.18	CARGPRDYVWGTRDAIYW	4-60	3								
CL	1-11B H-L	4-31	16	95	4	5	90	3-10	25.17.38.11	10.7.22	CARVYVYSGNYNAQVGYFDW	1-51	2 or 3								
CL	1-2C H-L	4-34	18	94	6	11	82	3-9	25.17.38.11	8.7.20	CARVYDITLTPYDHYGMDVW	1-47	2 or 3								
CL	1-3C H-L	3-23	34	88	4	5	90	3-10	25.17.38.11	8.8.18	CAKGNFFVSGRYFFFNWSW	1-44	1								
CL	1-5C H-L (C2)	4-59	36	87	5	9	82	6-13	25.17.38.11	8.7.12	CVRGMSLDQQFVLW	7-46	1								
CL	1-9C H-L	3-23	14	95	4	4	91	2-2	25.17.38.11	8.8.6	CAKQPDYW	1-51	2 or 3								
CL	1-2D H-L	7-4-1	15	95	3	0	100	3-3	25.17.38.11	8.8.22	CVRNGPADSITIFGVTPDAFDIW	2-23	2 or 3								
CL	1-6D H-L	3-23	10	97	4	1	98	3-22	25.17.38.11	8.8.19	CAKDPVMTYYDSGYFFDYW	3-10	1								
CL	1-7D H-L	3-30	13	95	6	4	94	5-5	25.17.38.11	8.8.17	CARGHQIQVYYHYGMDVW	7-46	3								
CL	1-8D H-L	3-13	4	99	4	4	92	3-3	25.17.38.11	8.7.14	CARASRFLEWLSLDYW	2-14	2 or 3								
CL	1-11D H-L (C3)	4-34	31	89	6	7	89	3-3	25.17.38.11	8.7.20	CARGKYDTLSGPYDFYGLDTW	3-9	2 or 3								
CL	2-4A H-L	3-48	7	98	4	6	88	5-12	25.17.38.11	8.7.10	CARDSAMNVDYW	7-46	1								
CL	2-12A H-L (C3)	4-34	16	94	6	7	89	3-3	25.17.38.11	8.7.20	CARVYYDTLSGPYYHYGMDVW	3-9	2 or 3								
CL	2-1B H-L	3-21	13	95	5	5	90	1-1	25.17.38.11	8.8.15	CAKDDSTATLSGWFDPW	7-46	1								
CL	2-8B H-L	4-34	24	92	6	8	87	3-9	25.17.38.11	8.7.20	CARVYYDTLSGPYDHYGMDVW	3-9	2 or 3								
CL	2-1C H-L	3-53	15	95	6	3	95	2-21	25.17.38.11	8.7.21	CARDRRFCNCECYHYHYGMDVW	1-40	2 or 3								
CL	2-8C H-L	3-33	25	91	3	3	94	4-17	25.17.38.11	8.8.13	CASLPGTPTDHFYW	1-51	2 or 3								
CL	2-9C H-L	6-1	14	95	5	15	71	3-10	25.17.38.11	10.7.18	CADLIYFTSGSYSNRPDPW	1-51	3								
CL	2-11C H-L (C5)	1-2	20	93	5	10	84	6-19	25.17.38.11	10.9.20	CARDPLTPVAGKYYFFGMDVW	2-23	2 or 3								
CL	2-12C H-L	5-51	14	95	3	3	94	3-10	25.17.38.11	8.8.15	CARGLFSGKDWFDPW	1-40	2 or 3								
CL	2-1D H-L	4-39	31	89	5	2	96	5-5	25.17.38.11	8.8.18	CARLGDVETAMVGQDAFHIW	1-47	2 or 3								
CL	2-3D H-L	3-33	24	92	4	2	96	6-19	25.17.38.11	10.7.13	LCETGRWFGMGYNWFDPW	2-14	2 or 3								
CL	2-6D H-L	3-15	17	94	4	12	75	3-22	25.17.38.11	8.10.14	CARPPSGVYSSGWGYYFDYW	3-21	2 or 3								
CL	2-7D H-L (C2)	4-59	24	92	5	10	80	2-15	25.17.38.11	8.7.21	CCTATSSIRVVEEYW	4-60	2 or 3								
CL	2-8D H-L	4-34	17	94	6	13	79	3-3	25.17.38.11	8.7.20	CARVWPLEFCNGGTCNGPDSW	7-46	1								
CL	3-2A H-L	4-39	15	95	4	4	92	3-3	25.17.38.11	10.7.13	CARGYYDVLSPGYFYFGLDVW	7-46	1								
CL	3-1B H-L (C5)	1-2	16	92	5	2	96	3-10	25.17.38.11	8.8.15	CARHVQWVLYYFDYW	2-8	2 or 3								
CL	3-3B H-L	4-34	16	92	2	2	96	3-10	25.17.38.11	8.8.15	CARIGLFSKDWFDPW	1-40	2 or 3								
CL	3-6B H-L	3-30	43	85	3	4	92	1-1	25.17.38.11	8.7.14	CAVRRDASGYWYFDLW	1-47	2 or 3								
CL	3-10B H-L	3-72	13	96	4	7	85	4-23	25.17.38.11	8.8.18	CAKEVNSYAHGNVGAFDVW	1-47	2 or 3								
CL	3-11B H-L	3-74	25	91	NA	NA	85	NA	NA	8.10.16	CARENGLETTVTVPDLW	3-10	2 or 3								
CL	3-4C H-L	3-11	17	94	4	5	90	3-10	25.17.38.11	NA	NA	2-14	2 or 3								
CL	3-6C H-L	4-59	45	84	5	4	92	3-16	25.17.38.11	8.8.19	CVRGOGGLWFGDLRERFDYW	2-8	NA								
CL	3-6D H-L	4-59	11	96	6	3	95	3-22	25.17.38.11	8.7.15	CARIGGYFLSKWFDW	2-8	2 or 3								
CL	3-9D H-L	3-23	15	95	4	6	88	1-1	25.17.38.11	8.8.15	CARSPRGVDYDSGGYAYYYYMDVW	7-46	1								
CL	1-10A H-K	3-7	34	88	4	5	90	1-26	25.17.38.11	8.8.8	CAKGYKNIITSSFFDQW	1-47	2 or 3								
CL	1-2C H-K (C1)	4-34	17	94	6	11	82	3-9	25.17.38.11	8.7.20	CARVYYDTLTPYDHYHYGMDVW										1
CL	1-9C H-K	3-23	14	95	4	4	91	2-2	25.17.38.11	8.8.6	CAKQPDYW										4
CL	1-2D H-K	7-4-1	16	94	3	0	100	3-3	25.17.38.11	8.8.22	CVRNGPADSITIFGVTPDAFDIW										2
CL	1-10D H-K	3-13	5	98	4	4	92	3-3	25.17.38.11	8.7.14	CARASRFLEWLSLDYW										3

CL	1-11D H-K (C4)	4-34	34	88	6	7	89	3-3	25.17.38.11	8.7.20	CARGKYDTLSGPYDFYYGLDTW	1-33	4
CL	2-1A H-K	3-9	11	96	4	2	96	3-3	25.17.38.11	8.8.13	CAKGISAPIDPFYDW	3-20	3
CL	2-12A H-K (C4)	4-34	15	95	6	7	89	3-3	25.17.38.11	8.7.20	CARVYDTLSGPYYHYHGMVDW	1-33	4
CL	2-4B H-K (C1)	4-34	23	92	6	8	87	3-9	25.17.38.11	8.7.20	CARVYDTLSGPYDYHYHGMVDW	1-33	4
CL	2-8B H-K	3-53	16	94	6	3	95	2-21	25.17.38.11	8.7.21	CARDRFCNGECYHYHYHGMVDW	1-9	3
CL	2-1C H-K	3-33	25	91	3	3	94	4-17	25.17.38.11	8.8.13	CASLPGTPHDNFYIW	3-15	4
CL	2-2C H-K	1-69	27	91	4	5	64	NA	NA	NA	CASLPGTPHDNFYIW	4-1	1
CL	2-6C H-K	1-69	18	94	4	7	85	1-26	25.17.38.11	8.8.12	CASDKSGGHPYFVW	3-15	4
CL	2-12C H-K	5-51	12	96	3	2	96	5-5	25.17.38.11	8.8.18	CARLGDVETAMVGQDAFIW	3-20	4
CL	2-2D H-K	3-15	37	87	4	4	92	2-21	25.17.38.11	8.10.14	CTADTFASDRYSHDYW	1-33	4
CL	2-7D H-K	4-59	25	91	5	10	80	2-15	25.17.38.11	8.7.21	CARVWPLEFCNGGTCNGPDSW	1-33	4
CL	3-7A H-K	3-30	14	95	4	1	98	2-15	25.17.38.11	8.8.19	CAKYCSGSWCPCQRSYYFDHW	1-16	4
CL	3-11A H-K	3-33	30	90	6	11	81	2-8	25.17.38.11	8.8.11	CARDAHTNYLDVW	3D-15	4
CL	3-12A H-K	4-39	32	89	6	15	76	3-22	25.17.38.11	10.7.20	CASGNYDSSGFPGLLYGMDVW	2-28	3
CL	3-8B H-K	4-34	13	95	6	9	85	3-9	25.17.38.11	8.7.20	CARVYDTLTGPYNYHYHGMVDW	1-9	3
CL	3-2C H-K	3-30	33	89	3	5	90	3-10	25.17.38.11	8.8.10	CAREGEGAFDIW	1-39	2
CL	3-9C H-K	4-39	18	93	4	2	96	4-17	25.17.38.11	10.7.10	CAKFYGNFYDSW	1-12	2

* Clonal sequences are denoted by clonal pool number. For instance, clonal pool 1 from a particular donor is "(C1)."

kappa chain, followed by V_k3 (19%, 9/47), and V_k4 (13%, 6/47); V_λ1 (37%, 22/60) was found most often in the lambda chain, followed by V_λ2 (23%, 14/60), V_λ7 (23%, 14/60), and V_λ3 (13%, 8/60), respectively (figure 5.16). With regard to J segment usage, J_H4 was used most frequently (30%, 32/107) for heavy chain, followed by J_H6 (29%, 31/107), and J_H3 (28%, 30/107). The most prominent light chain J segments were J_k4 (39%, 18/46), J_k2 (24%, 11/46) and J_λ2/3 (64%, 38/59) in the study. Some variations of gene usage seemed to exist within donors. For instance, more V_H3 was found within vaccinated donors, or less V_k1 was found within pandemic H1N1 patients. However, these differences were not statistically significant. Thus, in the case of variable region gene usage, no major differences were found among peripheral B cell repertoires.

As well as the random assortment of particular gene segments, the structure of Ig could vary according to the characteristics of the CDR3. Heavy chain CDR3 (HCDR3) is required for the formation of the antigen-binding surface and therefore the diverse HCDR3 length and sequence could be critical factors in antigen recognition and specificity. There is usually 10-14 amino acids length of HCDR3 in naïve B cells (Tiller *et al.*, 2007; Wardemann *et al.*, 2003). Of ASC from three donors, the average length of HCDR3 was 16.0±3.5 (range 6~23), with a length of 17 amino acids occurring most frequently (figure 5.17, table 5.7, and table 5.8). Similar result was observed in ASC induced by pandemic H1N1 infection, which had the HCDR3 of 15.7±3.7 amino acid length (Wrammert *et al.*, 2011).

The observation about the robust expansion of ASC upon influenza exposure implied that the response could be highly clonal in nature. Clonally expanded

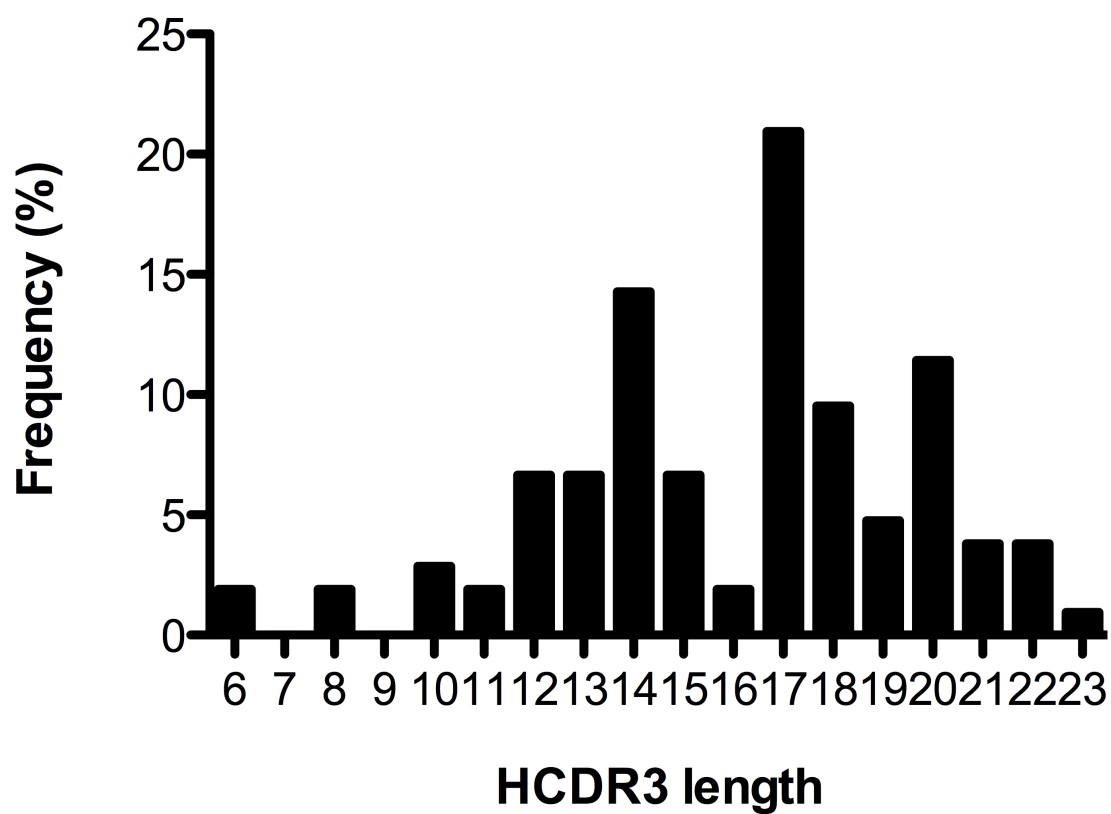


Figure 5.17. The distribution of the amino acid length of HCDR3 from day 7 ASC of three donors. Total 107 Ig genes were analyzed.

ASC had been observed after tetanus vaccination (Poulsen *et al.*, 2007; Frölich *et al.*, 2010). Here, the clonal relationship was therefore analyzed using heavy and light chain sequence of ASC from three donors. Upon aligning sequences against each other, Ig sharing the same gene rearrangements for both their heavy and light chain variable regions could be identified as clones. This means that the individual clone of ASC (or antibodies) might differ in somatic mutations but share identical gene segment usage and junction sequence. In the study, clonal expansion accounted for average 17.6% (range 17~18%) of ASC from three donors (figure 5.18, table 5.7 and table 5.8). This is similar to ASC described previously, which was 16.5 % clonal upon influenza natural infection (Wrammert *et al.*, 2011). The clonal expansion at high as 43% of ASC had been detected upon influenza vaccination (Wrammert *et al.*, 2008). In contrast, naïve, marginal zone and IgG MBC were rarely clonal (Tian *et al.*, 2007; Wrammert *et al.*, 2008), based on V_H region sequences in a comparable fashion. It is interesting to see the clonality of peripheral B cells significantly increased upon antigen exposure, indicative of the clonal selection of antigen-specific B cells over time.

Somatic hypermutation accumulated mutations on Ig variable region and allow the adaptation of activated B cells to exposed antigen. The frequency of somatic mutation was therefore analyzed and compared with other studies to explore the origin of the ASC response. In the study, ASC showed high number of mutations, which averaged 20.1 ± 1.3 V_H gene mutations per donor (figure 5.19, table 5.7 and table 5.8). Similar somatic mutations were observed in ASC induced by natural infection or vaccination (over 19 per donor; Wrammert *et al.*, 2008; Wrammert *et al.*, 2011). Importantly, the

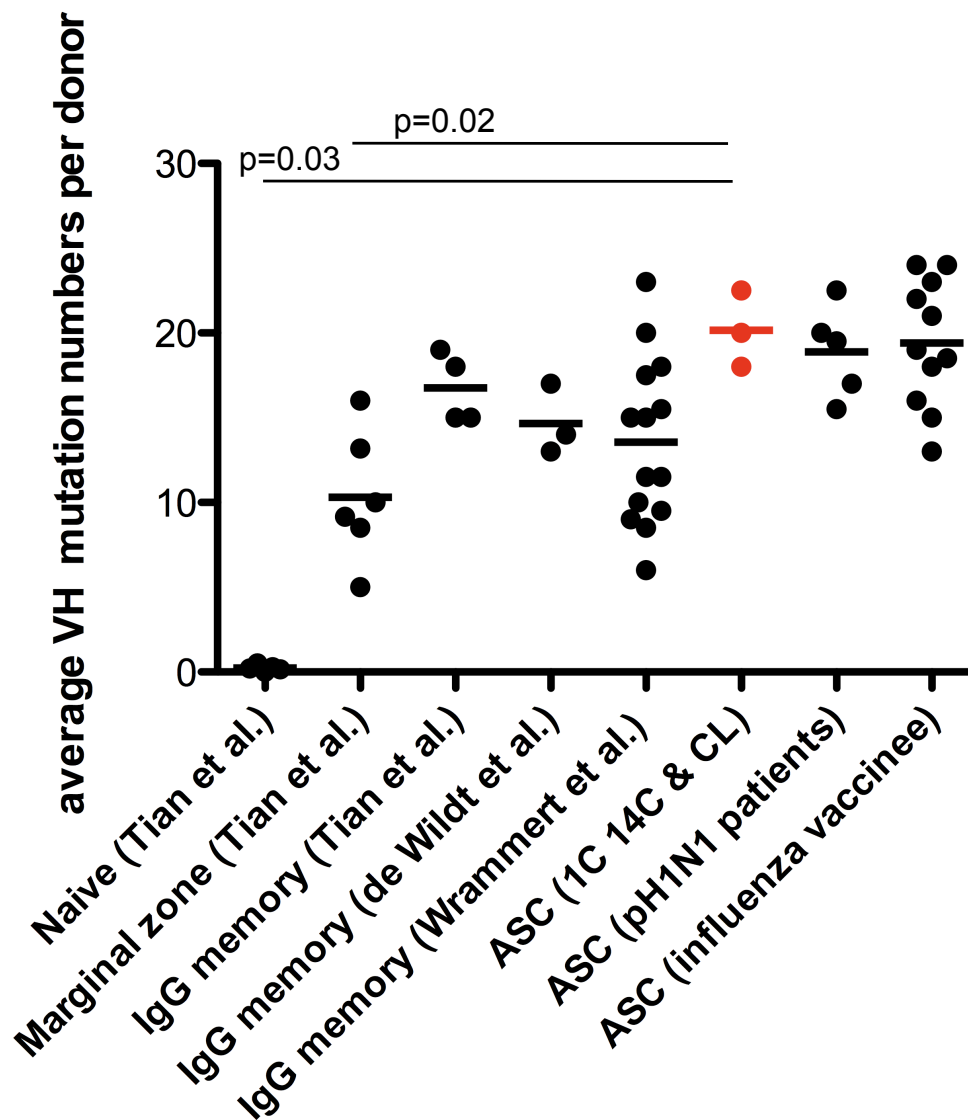


Figure 5.19. Comparison of the average frequency of somatic mutations of V_H from each donor. On average, the ASC had significantly more mutations than either the naïve or marginal zone B cells. The student's t-test was performed to compare the two groups of data. The red dot represent the average somatic mutations of three donors in the study. The data of IgG MBC and ASC from pandemic H1N1 patients and seasonal influenza vaccinee were retrieved from the result published previously (Wrammert *et al.*, 2008; Wrammert *et al.*, 2011). The data of naïve, marginal zone and IgG memory B cells were retrieved from the result published previously (Tian *et al.*, 2007).

accumulated mutations of ASC were significantly higher than that of marginal zone and naïve B cells published by previous studies (Student's t test $P < 0.05$; Tian *et al.*, 2007; de Wildt *et al.*, 2000; Wrammert *et al.*, 2008; Wrammert *et al.*, 2011). While comparing somatic mutations between ASC and IgG MBC, the result is controversial. For these three donors, influenza-induced ASC had similar number of somatic mutations as well as IgG memory cells published by previous studies (Student's t test $P > 0.05$; de Wildt *et al.*, 2000; Tian *et al.*, 2007; Wrammert *et al.*, 2008). Another study also showed that expanded ASC had similar mutation frequency as MBC after boosted tetanus vaccination. Nevertheless, higher somatic mutations in ASC than MBC had been reported upon influenza exposure (Wrammert *et al.*, 2008; Wrammert *et al.*, 2011). These results indicated that, as well as MBC, antigen-induced ASC have substantial mutations accumulated on the Ig variable region, although whether or not the process of generating these ASC involves further somatic hypermutation remains undetermined (Frölich *et al.*, 2010; Wrammert *et al.*, 2008; Wrammert *et al.*, 2011).

Somatic hypermutation presents in the form of single base substitutions affecting the variable region of Ig gene. The nucleotide change could be silent or could result in amino acid replacement. The R/S ratio between CDRs and FRs was comparable in all repertoires thus allowed the rough measure of somatic hypermutation. It has been shown that the heavy chain is more mutated than light chain; therefore, the heavy chain was focused in the study. In CDRs as well as FRs, replacement mutations on average happen more often than silent mutations (table 5.9 and table 5.10). The highest R/S ratio occurred in CDR2 (4.3:1), followed by CDR1 (3.8:1), FR1 (2.6:1), FR2 (1.5:1)

Table 5.9. Variable gene mutation numbers of mAb from infected donors.

DONOR	mAb	CDR1		CDR2		CDR		FR1		FR2		FR3		FR	
		R	S	R	S	R	S	R	S	R	S	R	S	R	S
14C	P4-9A H-L	2	1	2	0	4	1	0	0	0	0	4	3	4	3
14C	P4-10A H-L	2	1	2	0	4	1	0	0	0	0	4	3	4	3
14C	P4-3B H-L	2	2	2	0	4	2	2	0	0	0	4	3	6	3
14C	P4-4B H-L	3	0	5	0	8	0	0	0	2	0	6	2	8	2
1C	P2-6A H-L	2	1	2	0	4	1	0	0	0	0	4	3	4	3
1C	P2-9A H-L	2	1	2	0	4	1	0	0	0	0	4	3	4	3
1C	P2-10A H-L	2	1	2	0	4	1	0	0	0	0	4	3	4	3
1C	P2-8B H-L	5	0	6	1	11	1	1	0	0	0	1	2	2	2
1C	P2-3C H-L	2	1	2	0	4	1	0	0	0	0	4	3	4	3
1C	P2-5C H-L	2	1	2	0	4	1	2	0	0	0	4	3	6	3
1C	P2-8C H-L	10	0	5	1	15	1	4	0	4	4	3	2	11	6
1C	P2-9D H-L	2	1	2	0	4	1	2	0	0	0	4	3	6	3
1C	P2-11D H-L	4	1	4	1	8	2	2	2	3	1	3	3	8	6
1C	P3-11A H-L	2	1	2	0	4	1	2	0	0	0	4	3	6	3
1C	P3-3B H-L	6	1	8	1	14	2	3	1	4	4	5	2	12	7
1C	P3-2C H-L	4	2	7	1	11	3	3	3	3	3	3	2	9	8
1C	P3-5C H-L	2	0	2	1	4	1	5	0	0	2	0	0	5	2
1C	P3-6C H-L	1	1	1	2	2	3	3	1	2	0	7	3	12	4
1C	P3-8C H-L	2	0	4	0	6	0	3	3	2	1	4	3	9	7
1C	P3-7D H-L	0	0	5	3	5	3	1	2	0	1	4	2	5	5
1C	P3-9D H-L	2	1	2	0	4	1	2	0	0	0	4	3	6	3
14C	P4-6A H-K	2	1	2	0	4	1	0	0	0	0	5	3	5	3
14C	P4-7A H-K	2	2	2	0	4	2	0	0	1	0	4	3	5	3
14C	P4-12A H-K	2	1	2	0	4	1	2	0	0	0	4	3	6	3
14C	P4-6B H-K	2	1	2	0	4	1	0	0	0	0	4	3	4	3
14C	P4-7B H-K	1	1	1	2	2	3	0	1	2	0	7	2	9	3
14C	P4-10B H-K	2	1	2	0	4	1	0	0	0	0	4	3	4	3
14C	P4-1C H-K	1	0	3	1	4	1	0	1	1	0	1	1	2	2
1C	P2-2A H-K	2	1	2	0	4	1	0	0	0	0	4	3	4	3
1C	P2-11A H-K	3	0	5	0	8	0	0	2	1	3	8	6	9	11
1C	P2-3B H-K	6	1	3	3	9	4	1	0	2	1	2	2	5	3
1C	P2-7B H-K	2	1	2	0	4	1	0	0	0	0	4	3	4	3
1C	P2-3C H-K	2	1	2	0	4	1	0	0	0	0	4	3	4	3
1C	P2-6C H-K	10	0	7	1	17	1	3	1	3	3	3	2	9	6
1C	P2-8C H-K	10	0	5	1	15	1	3	1	4	4	3	2	10	7
1C	P2-1D H-K	2	1	2	0	4	1	1	0	0	0	4	3	5	3
1C	P3-6A H-K	1	0	2	0	3	0	4	1	2	1	3	1	9	3
1C	P3-12A H-K	3	1	2	0	5	1	0	0	3	1	7	4	10	5
1C	P3-3B H-K	6	1	8	1	14	2	3	1	4	4	5	2	12	7
1C	P3-4B H-K	4	0	2	0	6	0	1	1	0	1	6	4	7	6
1C	P3-9B H-K	2	0	2	0	4	0	5	0	3	3	9	2	17	5
1C	P3-2C H-K	4	2	7	1	11	3	3	3	3	3	3	2	9	8
1C	P3-7C H-K	5	0	5	3	10	3	7	2	3	3	12	7	22	12
1C	P3-5D H-K	3	0	2	1	5	1	1	2	0	0	4	3	5	5
1C	P3-6D H-K	7	1	4	2	11	3	4	1	5	1	19	5	28	7
1C	P3-7D H-K	0	0	5	3	5	3	1	2	0	1	4	2	5	5
	Sum	143	34	150	30	293	64	74	31	57	45	213	128	344	204

Table 5.10. Variable gene mutation numbers of mAb from vaccinated donor.

DONOR	mAb	CDR1		CDR2		CDR		FR1		FR2		FR3		FR	
		R	S	R	S	R	S	R	S	R	S	R	S	R	S
CL	1-10A H-L	2	0	3	2	5	2	1	1	2	0	5	4	8	5
CL	1-4B H-L	1	0	2	0	3	0	0	0	1	1	1	3	2	4
CL	1-8B H-L	3	2	3	0	6	2	8	0	2	3	6	2	16	5
CL	1-10B H-L	2	1	4	0	6	1	2	1	2	0	5	2	9	3
CL	1-11B H-L	3	1	1	0	4	1	0	1	2	0	4	4	6	5
CL	1-2C H-L	1	1	1	1	2	2	5	1	1	0	2	4	8	5
CL	1-3C H-L	3	0	4	1	7	1	5	2	0	2	13	4	18	8
CL	1-5C H-L	0	1	10	0	10	1	4	1	4	3	9	4	17	8
CL	1-9C H-L	1	0	6	1	7	1	2	0	0	1	3	0	5	1
CL	1-2D H-L	1	1	0	1	1	2	4	1	1	0	4	2	9	3
CL	1-6D H-L	3	1	3	0	6	1	1	0	1	0	1	0	3	0
CL	1-7D H-L	2	0	3	0	5	0	0	1	2	0	4	1	6	2
CL	1-8D H-L	0	0	0	0	0	0	0	0	2	0	2	0	4	0
CL	1-11D H-L	2	1	4	0	6	1	6	3	2	1	10	2	18	6
CL	2-4A H-L	0	0	6	0	6	0	0	0	0	0	3	1	3	1
CL	2-12A H-L	1	1	1	1	2	2	4	1	1	1	2	3	7	5
CL	2-1B H-L	1	4	3	0	4	4	1	1	0	1	2	0	3	2
CL	2-4B H-L	3	3	1	0	4	3	5	2	1	0	2	7	8	9
CL	2-8B H-L	3	0	0	1	3	1	2	0	1	0	2	6	5	6
CL	2-1C H-L	4	2	2	2	6	4	3	0	2	0	2	7	7	7
CL	2-8C H-L	4	1	3	1	7	2	6	1	2	4	10	12	18	17
CL	2-9C H-L	0	1	1	0	1	1	5	2	1	0	2	2	8	4
CL	2-11C H-L	10	1	3	2	13	3	0	3	0	1	0	0	0	4
CL	2-12C H-L	3	0	1	1	4	1	0	0	1	0	3	3	4	3
CL	2-1D H-L	2	0	4	0	6	0	1	0	0	0	10	14	11	14
CL	2-3D H-L	4	0	2	2	6	2	4	0	0	1	4	7	8	8
CL	2-6D H-L	3	0	2	2	5	2	1	1	1	0	4	4	6	5
CL	2-7D H-L	2	2	4	0	6	2	6	2	5	2	1	5	12	9
CL	2-8D H-L	3	0	3	2	6	2	1	0	0	2	3	3	4	5
CL	3-2A H-L	2	0	3	0	5	0	1	0	0	2	5	2	6	4
CL	3-1B H-L	1	1	3	2	4	3	0	0	1	1	4	3	5	4
CL	3-3B H-L	2	0	4	0	6	0	3	3	3	0	5	4	11	7
CL	3-6B H-L	4	2	13	0	17	2	2	3	3	0	12	4	17	7
CL	3-10B H-L	0	0	5	0	5	0	0	1	0	0	6	1	6	2
CL	3-11B H-L	0	0	0	0	0	0	0	0	0	0	0	0	0	0
CL	3-4C H-L	2	0	2	3	4	3	1	0	4	1	1	2	6	3
CL	3-6C H-L	8	0	5	1	13	1	4	0	3	0	18	6	25	6
CL	3-6D H-L	2	0	3	0	5	0	0	0	1	2	3	0	4	2
CL	3-9D H-L	1	0	1	2	2	2	1	1	1	0	4	4	6	5
CL	1-10A H-K	2	0	3	2	5	2	1	1	2	0	5	4	8	5
CL	1-2C H-K	1	1	1	1	2	2	5	1	1	0	2	4	8	5
CL	1-9C H-K	1	0	6	1	7	1	2	0	0	1	3	0	5	1
CL	1-2D H-K	1	1	0	1	1	2	4	1	1	0	4	2	9	3
CL	1-10D H-K	0	0	0	0	0	0	0	0	2	0	2	0	4	0
CL	1-11D H-K	2	1	4	0	6	1	6	3	2	1	10	2	18	6
CL	2-1A H-K	2	0	2	0	4	0	1	1	0	1	2	2	3	4
CL	2-12A H-K	1	1	1	1	2	2	4	1	1	1	2	3	7	5
CL	2-4B H-K	3	3	1	0	4	3	5	2	1	0	2	7	8	9
CL	2-8B H-K	3	0	0	1	3	1	2	0	1	0	2	6	5	6
CL	2-1C H-K	4	2	2	2	6	4	4	0	2	0	2	7	8	7
CL	2-2C H-K	4	0	7	0	11	0	3	1	4	0	4	4	11	5
CL	2-6C H-K	2	0	3	0	5	0	2	1	1	0	8	1	11	2
CL	2-12C H-K	3	0	1	1	4	1	0	0	1	0	3	3	4	3
CL	2-2D H-K	3	0	8	2	11	2	2	2	1	2	14	3	17	7
CL	2-7D H-K	2	2	4	0	6	2	6	2	5	2	1	4	12	8
CL	3-7A H-K	1	1	1	0	2	1	3	1	1	0	5	1	9	2

CL	3-11A H-K	6	0	5	1	11	1	1	3	0	3	7	4	8	10
CL	3-12A H-K	5	0	6	1	11	1	5	0	1	0	5	3	11	3
CL	3-8B H-K	2	1	1	1	3	2	0	1	1	0	3	3	4	4
CL	3-2C H-K	2	0	2	3	4	3	5	1	0	3	10	7	15	11
CL	3-9C H-K	3	1	4	1	7	2	1	0	0	0	3	5	4	5
Sum		142	41	181	47	323	88	151	55	81	43	276	207	508	305

and FR3 (1.4:1). The similar R/S ratio had also been reported in ASC induced by boosted tetanus immunization (Jiménez-Gómez *et al.*, 2010). The mutation number in CDR3 is lower but does not take into account N and P nucleotides induced diversity, which introduces amino acid differences from the germline; therefore, the CDR3 is still highly diversified. In the study, the average R/S ratio was 4.0 within CDRs and 1.7 in FRs. The similar result had been published for the repertoire of influenza-induced ASC, which was 3.7 for CDRs and 1.8 for FRs (Wrammert *et al.*, 2008). The preference for replacement mutations in CDRs indicated that somatic hypermutation drives the selection of high affinity Ig within ASC.

Taken together, though based on a limited number of donors, the analysis of variable region sequences indicated that influenza-induced ASC harbored highly clonal and mutated Ig variable genes. These supported the hypothesis that pre-existing somatically mutated MBC were most likely the origin of ASC in response to influenza exposure.

5.3.2.2 Gene sequences of anti-influenza antibodies were analyzed and compared with previous studies.

8 anti-influenza antibodies were produced from day 7 ASC. 6 antibodies were from the vaccinated donor and another 2 were from infected donors. If dividing by antigen specificity, 4 antibodies were broadly cross reactive to influenza strains, and another 4 antibodies were type-specific to pH1N1 or H3N2. Their sequences represented the ASC repertoire with known specificity. In order to characterise the trend among the influenza-specific ASC

response, these sequences were used for analysis of gene usage, CDR3 length and somatic hypermutation (table 5.11).

The usage of V_H3 , 4 and 1 families accounted for 5 of 8 (63%) of anti-influenza antibodies. It is interesting to observe that there are 3 type-specific antibodies using V_H5 or V_H6 family, which is relatively less seen in the ASC repertoire. The usage of V_k3 family was noted in kappa chain anti-influenza antibodies. The $V_\lambda1$ family was found most often in the lambda chain anti-influenza antibodies, followed by $V_\lambda2$. The distribution of usage of light chain variable gene was similar as the ASC repertoire previously described and in the present study (Wrammert *et al.*, 2011). The J_k4 and $J_\lambda2/3$ family were predominantly used in the anti-influenza antibodies. These two families were also most commonly seen in the light chain joining gene of whole ASC repertoire. Therefore, of anti-influenza antibodies, similar distribution and dominance involving certain families of variable region were observed as the whole ASC or other B cell repertoires.

The length of HCDR3 ranges from 12 to 21 amino acids long. The average length of anti-influenza antibodies is 16 amino acids, which is similar as the ASC repertoire describes before (Wrammert *et al.*, 2011). It was noted that all anti-influenza antibodies had N nucleotides additions within the HCDR3 and some had P nucleotides as well (table 5.11). These were added at the stage of somatic recombination in B cell development. This indicated that the increased diversity compared with germline sequence allow the development of antigen-specific B cells. In the future, further knowledge of precise epitope specificity might help to correlate these CD3 properties with antibody function.

Table 5.11. Sequence analysis of anti-influenza mAb

Donor	mAb	V	Mutations	J	Mutations	D	CDR3-lengths	N	P	AA Junction
CL	2-8B H-L Heavy	IGHV3-53*01	15	IGHJ6*02	3	IGHD2-21*01	21	9	1	CARDRRCNGECYHYHYGMDVW
	2-8B H-L Lambda	IGLV1-40*01	17	IGLJ2*01 or 3*01	0		12	0	2	CQSYDRSLSGHVF
CL	2-8C H-L Heavy	IGHV4-31*03	43	IGHJ5*02	8	IGHD3-10*01	18	25	0	CADLIYFYTSGSYSNRLDPW
	2-8C H-L Lambda	IGLV1-51*01	29	IGLJ3*02	4		10	12	0	CGTWANTQLALF
CL	2-9C H-L Heavy	IGHV6-1*01	14	IGHJ6*02	2	IGHD6-19*01	20	15	0	CARDPLTPVAGKSYHYHYGMDVW
	2-9C H-L Lambda	IGLV2-23*02	11	IGLJ2*01 or 3*01	2		11	10	0	CCSYAGSSSRVLF
CL	2-12C H-L Heavy	IGHV5-51*01	12	IGHJ3*02	2	IGHD5-5*01	18	14	2	CARLGDVETAMVGQDAFIHW
	2-12C H-L Lambda	IGLV1-47*02	13	IGLJ2*01 or 3*01	1		11	0	0	CSAWDDSLGLVF
CL	2-6C H-K Heavy	IGHV1-69*01	18	IGHJ4*02	7	IGHD1-26*01	12	11	1	CASDKSGGIHPYFW
	2-6C H-K Kappa	IGKV3-15*01	15	IGKJ4*01	2		10	0	0	CQQYDNWPPLSF
CL	2-12C H-K Heavy	IGHV5-51*01	12	IGHJ3*02	2	IGHD5-5*01	18	14	2	CARLGDVETAMVGQDAFIHW
	2-12C H-K Kappat	IGKV3-20*01	6	IGKJ4*01	1		9	1	0	CQQYDSSPFTF
14C	P4-4B H-L Heavy	IGHV1-2*04	21	IGHJ4*02	7	IGHD7-27*01	12	14	0	CARILRGTTGGLDSW
	P4-4B H-L Lambda	IGLV2-14*01	15	IGLJ3*02	2		10	0	1	CSSYIGSSNWMF
1C	P2-8B H-L Heavy	IGHV4-39*01	16	IGHJ4*02	9	IGHD3-16*02	12	13	0	CARREFDRISPYW
	P2-8B H-L Lambda	IGLV1-40*01	7	IGLJ2*01 or 3*01	1		12	2	1	COSYDSSLSGYVVF

The mutation numbers of V_H gene averaged 23.8±4.3, which is similar as anti-influenza antibodies induced by natural infection (Wrammert *et al.*, 2011).

Cross-reactive antibodies had been reported to accumulate more somatic mutations than type-specific ones induced by natural influenza infection, indicative of further affinity maturation in the peripheral lymph nodes (Wrammert *et al.*, 2011). In the study, it was noted that cross-reactive antibodies had relatively higher V_H mutations than type-specific antibodies, however, the number of antibodies is too few to reach statistically significant (p=0.08, figure 5.20 and table 5.11). In CDRs as well as FRs, the number of replacement mutations was on average higher than silent mutations. Nevertheless CDRs had higher R/S ratio than FRs, indicative of the SHM-driven selection of high affinity antibodies. It was noted that the CDR R/S ratio relative to the FR ratio was similar between cross-reactive and type-specific antibodies, which support the finding of similar binding activities between two groups.

Taken all together, anti-influenza antibodies shared common characteristics, such as gene segment usage, CDR3 length, and somatic mutation frequency, as influenza-induced ASC. The correlation of sequence analysis with antigen specificity of anti-influenza antibodies did not reveal significant pattern.

5.4 DISCUSSION

To evaluate the origin and development of influenza-induced ASC repertoire, the Ig gene was cloned from day 7 ASC isolated from adult donors challenged with BR59 (H1N1) or vaccinated with 2010-11 seasonal TIV (Sanofi-Pasteur), expressed the recombinant mAb and characterised their binding specificity

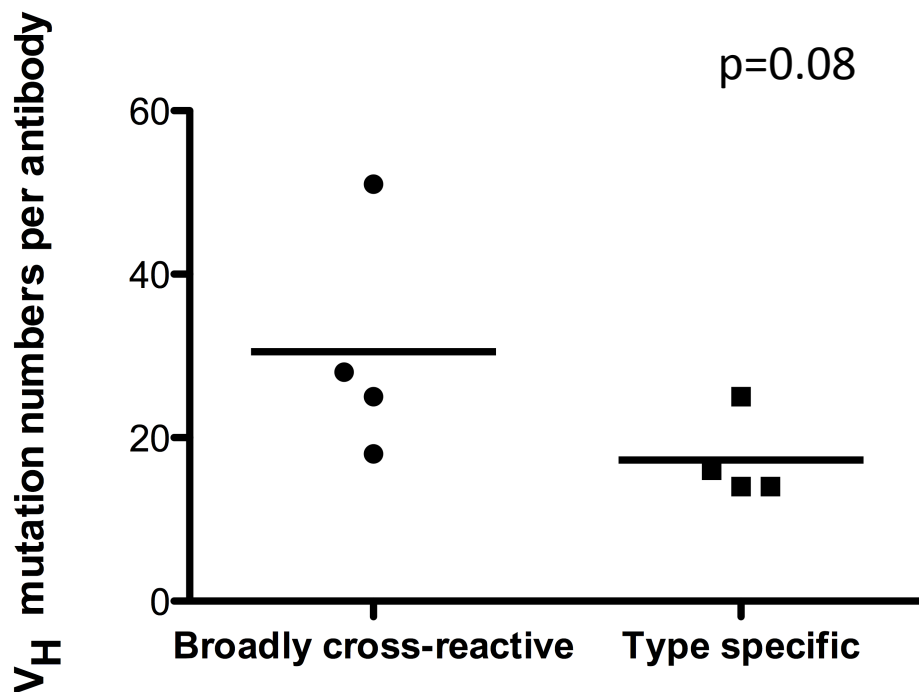


Figure 5.20. Anti-influenza mAb could be divided into two groups according to antigen-specificity. One group included broadly cross-reactive 2-8B H-L, 2-8C H-L, P4-4B H-L, and P2-8B H-L. Another group included type-specific 2-9C H-L, 2-12C H-L, 2-6C H-K, and 2-12C H-K. Broadly cross-reactive antibodies had higher V_H mutation numbers than type-specific one. However, the number of anti-influenza antibodies is too few to reach statistical significance. The student's t-test was performed to compare the two groups of data.

and neutralising function. Eight anti-influenza antibodies were made and could be classified by specificity and function. One is broadly cross-reactive antibodies without neutralising activity, another is H3N2-specific without neutralising activity, and the others are H1N1 specific antibodies with strong neutralising activity, indicative of the broad diversity of ASC repertoire induced by influenza exposure.

This study demonstrated that by using single cell PCR of variable region gene, mAb could be efficiently produced from day 7 ASC but the production rate varied case by case. In the study, the mAb yield rate averaged 40% (range 37~42%) of sorted ASC and anti-influenza mAb accounted for 7% (range 2.9~9.8%) of total mAb. The mAb yield rate had been reported as 30% (range 23~42%) from influenza-vaccinated donors (Smith *et al.*, 2009), or 19% (range 4~27%) from influenza-infected donors (Wrammert *et al.*, 2011). Since Smith and Wrammert only focused on kappa mAb, it made sense their mAb yield rates were relative lower than the present study. Smith had recommended that only kappa mAb were worth producing, because 70% of ASC would be kappa positive. However, in this study, it was noted that there were almost equal percentage of kappa and lambda mAb produced and several anti-influenza antibodies were lambda mAb. The similar light chain usage had also been reported in randomly selected B cells (Williams *et al.*, 2009). Although the number of donors was few in the study, this indicated that the variation existed in the kappa/lambda ratio of peripheral ASC and production of both kappa and lambda mAb might better interpret the whole ASC repertoire. In the study the positive rate of anti-influenza mAb was lower than the data previously published (28~73% of total mAb; Wrammert *et al.*,

2008; Smith *et al.*, 2009; Wrammert *et al.*, 2011). Since the same protocol as previous studies was used and the similar or even better mAb yield rate was achieved in the present study, the discrepancy of positive rate could be explained by the following reasons. Firstly, ELISpot provided the percentage of influenza-specific cell within day 7 IgG ASC. A calculation using the ELISpot positive rate and the number of total mAb made would allow the estimate of anti-influenza mAb. Principally, ELISpot provided a rough indication of which sample would be best for sorting and making the most influenza-specific antibodies. In the study, only these 3 donors were available to give day 7 ASC for sorting. Vaccinated donor CL had pretty low ASC positive rate, and therefore had corresponding low yield of anti-influenza antibodies (table 5.6). Secondly, for infected donors, based on ELISpot result, it was expected to produce 3 anti-influenza antibodies from donor 14C (derived from a calculation: $30\% \times 11 \approx 3$) and 8 anti-influenza antibodies from donor 1C (derived from a calculation: $23\% \times 35 \approx 8$) (table 5.6). However, prior to sorting procedure, their PBMC were left overnight in the incubator, which probably resulted in the massive loss of antigen-specific ASC. The loss of ASC population was also seen by FACS staining. This indicated that PBMC used for sorting must be fresh. If the sorting cannot begin soon after blood collection, store as whole blood at 4°C rather than PBMC was recommended (Smith *et al.*, 2009).

Combination of antibody characteristics and sequences analysis provides insight into the human ASC responses to influenza virus.

Firstly, day 7 ASC is recently differentiated and proliferated upon influenza exposure. The Ig sequences were helpful to analysis if these early-induced ASC were clonally related. Upon aligning with germline variable region to identify the usage of gene segment families, sequences that share identical V and J gene segment and AA junction would be allocated into clonal group. Of 107 Ig sequenced from single ASC, 88 were unique sequence and the remaining sequences were clones (18% clonality). This demonstrated a ready detection of clonal expansion of day 7 ASC induced by influenza infection or vaccination. ASC response to influenza antigen ranged from 16% to 43% clonal (Wrammert *et al.*, 2008; Jin *et al.*, 2009; Wrammert *et al.*, 2011). ASC response to other antigens has also been described as clonal: hepatitis B virus surface protein (41% clonal; Jin *et al.*, 2009), respiratory syncytial virus (14% clonal; Williams *et al.*, 2009), and dengue virus (22% clonal; personal communication with Dr Juthathip Mongkolsapaya, Imperial college). In contrast, naïve, marginal zone and IgG memory B cells were rarely clonal (Tian *et al.*, 2007; Wrammert *et al.*, 2008).

Secondly, in a recent study, broadly cross-reactive antibodies were generated from highly mutated ASC upon pandemic H1N1 infection in humans (Wrammert *et al.*, 2011). In the present study, mAb generated from day 7 ASC bound to influenza vaccine strains with strong binding ability. Mutation analysis of day 7 ASC showed all sequence differ from germline sequences. The ratios of replacement mutations to silent mutations in CDR and FR indicated the rate of somatic hypermutation. Day 7 ASC accumulated high level of somatic mutations on variable region and affinity maturation, as well as anti-influenza antibodies. This was also observed in the ASC induced by

boosted tetanus vaccination (Frölich *et al.*, 2010; Jiménez-Gómez *et al.*, 2010), but rarely seen in the ASC induced by primary immunization of many other vaccines (Wrammert *et al.*, 2011). This indicates that further antigen exposure stimulate B cell clones to undergo maturation, mutating the CDRs to improve affinity. It had been suggested that the pre-existing MBC could have contributed to this ASC response that carry highly mutated Ig gene and produce high-affinity antibodies. Here, the similarity between these values and of the influenza-induced ASC mutations also supports the recall of memory.

Thirdly, preference for a particular V, D, or J segment in the heavy or light chains might indicate a structural link between antibodies. Hence, the distributions of V gene segments and J gene segments for the variable genes of day 7 ASC and anti-influenza antibodies were analyzed. Both repertoires showed extensive diversity in their variable gene segment usage. However, compared with other B cell repertoire (naïve or memory B cell), the most prevalent segment families in the variable region were almost the same with minor differences. In a similar manner, the dominant usage of certain families was also observed in ASC induced by tetanus vaccination (Poulsen *et al.*, 2007). The immunological significance of this remains unclear, but at least the distribution of gene segment usage were not over-presented in the present study. HCDR3 length and composition could be crucial for antigen-binding site topology. In the present study, there was a range in HCDR3 lengths within day 7 ASC and anti-influenza antibodies as well. The longer length of HCDR3 of ASC than naïve B cells suggests that certain range in length is required for recognizing complex antigen surfaces. Longer HCDR3 length would allow more contact points with antigen and might improve affinity (Poulsen *et al.*,

2007). Moreover, N and P additions on CDR3 of anti-influenza antibodies implied that a degree of diversification is required for antigen recognition. Despite this, there was no observable correlation of particular HCDR3 length with antigen specificity of anti-influenza antibodies. Taken together, there was no major bias towards certain gene segment or HCDR3 length for anti-influenza antibodies, although it has to be mentioned that the number of antibodies is too few to draw concrete conclusion. Again in the future the further epitope mapping of antibodies might be helpful to explore the critical structure characteristics of HCDR3 region.

In conclusion, the method of production of human mAb from ASC was successfully deployed and led to production of antigen-specific antibodies from infected and vaccinated donors. The influenza-induced ASC repertoire was genetically diverse. A variety of Ig sequences from ASC as well as anti-influenza antibodies were analyzed. Anti-influenza antibodies did not show preferential gene usage or HCDR3 characteristics. Quantifying the rate of somatic hypermutation in ASC and anti-influenza antibodies showed that they are highly mutated. This could be consistently related to their binding or neutralisation abilities. Importantly, the successful generation of highly neutralising antibody from day 7 ASC supported their protective potential against influenza. Moreover, the functional antibodies could be useful in therapeutic areas in the near future. More donors and antibodies are required to substantiate the findings presented here.

CHAPTER 6

GENERAL DISCUSSION

This study aimed to investigate the antibody and B cell response to influenza A virus in human. The annual influenza epidemics and recurring pandemics, causing severe illness and death worldwide, implicate the escape of viral mutants from the existing humoral immunity in human. The focus of this work was the role of antibodies in influenza vaccination and infection and hence it forms a prelude to treatment of severe patient and even prevention of future outbreaks.

6.1 ANTIBODY

In the study, the inactivated pandemic H1N1 and trivalent seasonal influenza vaccine induced robust antibody response in adults, achieving over 90% of seroconversion and seropositive rate. The elicited neutralising antibodies would provide the primary protection against the given influenza strains. The neutralising antibody titre correlated well with both HI and HA ELISA titre, indicative of the role of anti-HA specific antibodies in humoral immunity. The anti-HA1 virus-neutralising mAb, 2-12C H-K, was derived from day 7 ASC, representing the typical protective antibody induced by influenza vaccination.

In the study, there observed substantial cross-reactive antibody response upon vaccination or infection, as measured by HI, MN and/or B cell ELISpot assay, despite a varied response noted in different antigens. Two kinds of antibody could have contributed to the cross-reactive response in human. One is the antibody recognizing conserved epitopes or proteins of influenza A

viruses, particularly, HA2, NP, or M proteins. Another is the antibody recognizing the common epitopes of HA1 shared by various strains within a specific subtype, in spite of the continual antigenic changes of HA1 subunit. The latter usually displays HI activity with or without neutralising function. These anti-HA1 antibodies generally don't cross-react to other subtypes of influenza A virus and only few has been found in human (Wrammert *et al.*, 2011). In contrast, the first kind of antibody is thought to be the main component of cross-reactivity against influenza A strains and subtypes. These antibodies have no HI activity but could be detected on binding assays, such as the ELISA and B-cell ELISpot. The anti-influenza mAb (2-8B, 2-8C, P4-4B and P2-8B) from day 7 ASC bound either HA2 or NP of various influenza A subtypes, suggested to be the typical cross-reactive antibodies in human. Although the epitope on the HA2 subunit is less reachable for interaction with antibodies than that on the HA1 in native virus, anti-HA2 cross-neutralising antibodies had been detected in sera after seasonal vaccination (Wang *et al.*, 2011). In the recent past, several anti-HA2 mAb capable of neutralizing multiple subtypes across group 1 or group 2 have also been isolated from human MBC and ASC upon vaccination or infection (Corti *et al.*, 2010; Sui *et al.*, 2009; Throsby *et al.*, 2008; Wilson and Cox, 1990; Wrammert *et al.*, 2011). In addition to anti-HA2 antibody, anti-NP antibody could also be one of the potent elements of cross-reactive immunity against influenza A viruses. Although anti-NP antibodies are generally unable to neutralise viral infectivity, they provide anti-viral activity by promoting viral clearance in animal model. In mice, the passive transfer of anti-NP mAb was effective to reduce mortality and morbidity caused by influenza A virus of distinct subtypes (Carragher *et*

al., 2008; LaMere *et al.*, 2011a; LaMere *et al.*, 2011b). In the near future, cross-reactive antibodies may be utilized for passive immunotherapy in human and to give essence to vaccine design because of their broad specificity and anti-influenza potency.

6.2 ANTIBODY SECRETING PLASMA CELL

In the study, the burst of day 7 ASC was captured after influenza vaccination or infection. The size of ASC response was associated with the 3-week postvaccination antibody titre and might reflect the viral load and the duration of viral shedding in the acute stage of infection. A distinct population of day 7 ASC was detected on FACS and served as a readily accessible source of human mAb. The successful production of human mAb in the early stage of antigen exposure would provide an alternative approach against the outbreak of new influenza viruses.

The early-phase ASC are generally enriched and mainly specific for offending or immunizing antigen. However, it was noted a considerable proportion, ranging from 10 to 80% of all circulating ASC, might not produce antibody against the given antigen (refer to Chapter 5; Wrammert *et al.*, 2008). While making mAb from sorted ASC, additional selection based on the staining of antigen-specific cells could have increased the yield of target mAb. It was reported that the combination of cell surface and intracytoplasmic staining with biotinylated HA allowed the identification of influenza-specific B cells in immunized mice (Doucett *et al.*, 2005). Since the ASC have no or reduced surface expression of B cell receptors for binding of antigen, it might require further work to examine if the intracytoplasmic staining alone could identify the

influenza-specific ASC in human. Besides, based on the previous experience of staining of influenza-specific B cells, the stickiness of HA to the cell surface would lead to high non-specific binding. The pre-blocking of cell surface with distinct types of influenza virus might also help with a high-quality selection of antigen-specific ASC *ex vivo*.

The Ig cDNA retrieved from day 7 ASC provided useful starting material for the repertoire analysis. In addition to the IgG sequence and mAb in the present study, other isotypes of Ig could be further isolated. The role of natural IgM antibody in the protection against influenza virus is poorly understood in human, despite the previously successful selection of broad-neutralising IgM mAb from phage libraries (Throsby *et al.*, 2008). Day 7 ASC, as an actual *in vivo* responding population, would be a suitable source to explore the IgM repertoire in the early stage of antigen exposure. Secondly, the IgA-mediated mucosal immunity was shown to associate with the protection against experimental influenza challenge in human (Clements *et al.*, 1986). The passive transfer of anti-influenza IgA mAb protected mice from infection (Mazanec *et al.*, 1992). However, it remains unclear about the diversity and function of IgA mAb derived from early-phase ASC. Full characterizations of other isotypes of day 7 ASC would help further understanding of the breadth of B cell response to influenza A viruses (Throsby *et al.*, 2008; Lanzavecchia and Sallusto, 2009).

The mAb characterisations and Ig sequence of day 7 ASC bring about the investigation of the origin of B cells. In a recent study, the ASC response was broadly cross-reactive in novel H1N1 infection (Wrammert *et al.*, 2011). The mAb made from the ASC of acute H1N1 patients were highly cross-reactive to

the consensus region of HA of multiple influenza subtypes. Moreover, these antibodies had displayed a high level of somatic mutation and affinity maturation in the variable region of heavy chain, which was very different from primary exposure of many antigens. All these suggest that early-phase ASC of pandemic H1N1 infection might derive from MBC of previous influenza subtype. In the present study, upon influenza vaccination or infection, there also observed accumulated somatic mutations in the day 7 ASC and those strong-binding cross-reactive mAb, which indicates their nature of secondary activation. To prove the direct link between antigen-specific ASC and MBC, a clonality analysis of a large number of influenza-specific ASC and MBC in the same cohort would be required in future studies.

6.3 MEMORY B CELL

In the study, a certain level of pre-existing MBC followed by a significant expansion of MBC response was observed in the periphery upon seasonal H1N1 infection. Moreover, it was also noted that a single pandemic H1N1 immunization is sufficient to induce high level of protective neutralising antibody in both children and adults, suggesting that pre-existing MBC may be present to accelerate the antibody response (refer to Chapter 3; Wu *et al.*, 2011; Plennevaux *et al.*, 2010).

The longitudinal samples of the H1N1 BR59 challenge study will be useful to define clearly the development of influenza-specific MBC response in relation to appearance of antibody and relationship with other immune cell types during the course of infection. The new method to detect influenza-specific MBC by flow cytometry over the classical methods would allow direct

measurement of influenza-specific MBC *ex vivo*. The data could be compared with the data on MBC response by memory B cell culture assay. In the present study, it has been showed that 2 % of peripheral IgG cells are H1N1-specific but they did cross react with split vaccine antigen from pandemic H1N1 (~0.5%). Apart from tracking the kinetics of influenza-specific MBC during the course of infection, these cells could be sorted at single cell level for clonality analysis. Moreover, the immunoglobulin variable regions of both heavy and light chain from single memory B cell could be rapidly cloned to produce high-affinity influenza-specific human mAb. The sequence data will provide important information on the size of B cell repertoire and clonal expansion of MBC during the course of influenza infection. A previous study on B cell repertoire and clonal expansion in influenza infection and vaccination was performed by limiting dilution assay (Pinna *et al.*, 2009). The use of longitudinal samples in human challenge infection and the *ex vivo* staining of antigen-specific MBC will clearly define the B cell repertoire at cellular and molecular level. A good understanding of the antibody and MBC response will be pivotal in future generation of vaccine development and validation.

6.4 THE INTERFERENCE BETWEEN VACCINES

In the vaccine trial, prior seasonal vaccination is associated with reduced antibody response to pandemic H1N1 vaccine, compared with the pandemic H1N1 vaccination alone. The observation brings out the concern regarding the immunogenicity of repeated influenza vaccination. In the study, the minimal impact of original antigenic sin was found here, although the underlined mechanism remained unclear. It is still debatable whether the

annual influenza immunization would cause decreased antibody response to the latest strain (Bernstein *et al.*, 2003; Gardner *et al.*, 2001; Sasaki *et al.*, 2008). Since the annual immunization would continue to be the mainstay of influenza prevention policy, further study should be undertaken in those with reduced antibody response to examine if any significant difference exists in the breadth of antibody or B cell repertoire and the subsequent vaccine efficacy.

6.5 CONCLUSIONS

These findings have demonstrated the properties of human antibody and B cell responses to influenza A virus at serological, cellular and sequence level. For influenza virus, the inactivated vaccination induced protective levels of antibody against the circulating strains. A rapid and robust influenza-specific ASC response peaked approximately day 7 after infection or vaccination. The highly mutated Ig variable region of ASC and virus-neutralising/cross-reactive mAb derived from ASC provide a strong evidence of the development of humoral immunity.

The current results will form part of ongoing experiments of further characterisation of anti-influenza mAb, recruitment of more subjects, and identification of antigen-specific MBC *ex vivo*. Ultimately human anti-influenza mAb which could neutralise and react across influenza A subtypes may be possible to limit the threat of future pandemics. The way of evaluating ASC and MBC response at single cell level could be used to validate the response to influenza vaccine or to contribute to their design.

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Publications

1. Wu, J., Zhong, X., Li, C.K., Zhou, J.F., Lu, M., **Huang, K.Y.**, Dong, M., Liu, Y., Luo, F.J., Du, N., Chui, C., Liu, L.Q., Smith, N.M., Li, B., Shi, N.M., Song, L.F., Gao, Y., Wang, D.Y., Wang, X., Zhu, W.F., Yan, Y., Li, Z., Chen, J.T., McMichael, A.J., Yin, W.D., Xu, X.N., and Shu, Y. (2011) Optimal vaccination strategies for 2009 pandemic H1N1 and seasonal influenza vaccines in humans. *Vaccine* 29, 1009-1016.
2. Wilkinson, T., Li, C., Chui, C., **Huang, K.Y.**, Perkins, M., Liebner, Lambkin-Williams, R., Gilbert, A., Oxford, J., and Doek, D.. 2011. Pre-existing influenza-specific CD4+ T cells correlate with disease protection against influenza challenge in humans. *Nature Medicine*, (in press).
3. **Huang, K.Y.**, Li, C.K., Clutterbuck, E., Wongwiwat, W., Jumnainsong, A., Bastin, J., Dejnirattisai, W., Mongkolsapaya, J., Pollard, A., Screaton, G., et al. 2011. Human B cell responses in experimental influenza A infection. (in prep.)