UNIVERSITY OF LIVERPOOL

Natural Immunity to Influenza Virus in Humans Following 2009 Pandemic H1N1 Influenza

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Abstract

Influenza is a highly contagious and acute respiratory infection caused by influenza virus in the mucosa of the respiratory tract. Both seasonal and pandemic influenza continue to cause substantial morbidity and mortality in humans. The 2009 pandemic H1N1 (pH1N1) influenza and the potential of a highly pathogenic avian H5N1 (aH5N1) pandemic highlighted the need for effective preventative strategies. Understanding the development of natural immunity following the pH1N1 pandemic may provide important information on host protective immunity in humans, which could inform future more effective vaccination strategies against influenza.

In this thesis, naturally developed mucosal immunity to 2009 pH1N1 virus was studied in children and adults using cells derived from human nasal-associated lymphoid tissue (NALT). Firstly, the frequency of HA-specific memory B cells in human NALT to pH1N1 virus and their ability to produce cross-reactive antibodies were studied. Patients who had serological evidence of previous exposure to pH1N1 virus developed large numbers of IgG memory B cells in NALT that produce cross-reactive neutralizing antibodies against a number of influenza subtypes upon pH1N1 virus antigen stimulation. The presence of such memory B cells in human NALT appears to have primed the host for cross-reactive mucosal memory response against other H1N1 and the highly pathogenic aH5N1 virus strains. These findings may have important implications in future vaccination strategies against influenza.

Secondly, serum specific anti-pH1N1 HA IgG antibodies were analysed using ELISA. HA-specific antibody levels to pH1N1 in adults were significantly higher than that of children. The results may suggest that adults had been exposed to more cross-reactive influenza viruses than children, and developed more cross-reactive memory responses against some influenza viruses than in children. Significantly higher HA-specific IgG antibody titres to pH1N1 HA (measured using ELISA) were found in subjects who had HAI titres$\geq$40 than in those with HAI antibody titre<40. This suggests that following the 2009 pH1N1 pandemics, large numbers of people developed anti-pH1N1 HA antibodies to both the circular head and the stalk regions of HA which may have broader protective immunity.

Thirdly, HA-specific memory $CD4^+$ T cell response to pH1N1 virus was shown in tonsillar cells from children and adults. This suggests that following the 2009 pandemic H1N1 influenza, humans developed memory T cell response to the pH1N1 HA protein antigen at the mucosal level in the nasopharynx. There appeared to be an age-associated increase in this memory response.

Finally, mucosal antibody responses in NALT to HAs of a number of influenza A viruses were investigated following *in vitro* stimulation of adenotonsillar cells with LAIV vaccine which contains a 2009 pandemic H1N1 virus, a seasonal H3N2 and a B influenza strain. Significant antibody responses of all 3 isotypes (IgG, IgA and IgM) to the HA of pandemic H1N1 virus were observed in tonsillar cells following LAIV stimulation. It suggests that the *in vitro* model of human NALT using adenotonsillar cell culture could be used to study the LAIV-induced immune responses which may predict the immunogenicity and efficacy of candidate LAIV vaccines in humans.

Dedication

I dedicate this work to my mother who prays for me constantly and inspires me. A dedication also goes to my brothers and my sons (Abdulrahman,Osama and Omar) and my daughter (Loujain) who were always encouraging and supportive throughout my PhD. Also, special thanks go to my wife for her encouragement and support during this time and for looking after my children.

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List of publications

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Abbreviations

µ: micro

405 nm: the wave length used (nanometre)

ab: antibody

aH5N1: Avian H5N1 influenza

Anti-HA: Anti-haemagglutinin

Anti-NA: Anti-neuraminidase

APCs: Antigen Presenting Cells

ASC: antibody-secreting cell

BSA: bovine serum albumin

CBA: Cytometric Bead Array

CDC: Centres for Disease Control and Prevention

CFSE: Carboxyfluorescein succinimidyl ester

CTL: Cytotoxic T cell

DCs: Dendritic cells

ELISA: Enzyme-linked immunosorbent assay

ELISPOT: Enzyme Linked Immunosorbent Spot

ER: Endoplasmic Reticulum

FCS: Foetal calf serum

FITC: Fluorescein isothiocyanate

FSC: forward scatter

GC: Germinal centre

HA: Haemagglutinin protein

HAI: Haemagglutination Inhibition

HEPA: High Efficiency Particulate Air (filter)

HPA: Health Protection Agency

HSC: Human Specimen Control

i.m.: Intramuscular

IFN: Interferon

IFN-γ: Interferon gamma

Ig: Immunoglobulin

IL: Interleukin

IMAC: Immobilized-metal affinity chromatography

InfA: Influenza A

LAIV: Live Attenuated Influenza Vaccine

M: Matrix protein

M1: Matrix 1 protein

M2: Matrix 2 protein

mAbs: Monoclonal antibodies

MALT: Mucosal-associated lymphoid tissue

MHC I: Major Histocompatibility Complex class I

MHC II: Major Histocompatibility Complex class II

MNC: Adenotonsillar mononuclear cell

mRNA: messenger Ribonucleic acid

NA: Neuraminidase protein

NALT: Nasal-associated lymphoid tissue

NIBSC: National Institute for Biological Standards and Control

NK cells: Natural killer cells

NLRs: Nod-like receptors

NLSs: Nuclear Localization Sequences

NP: Nucleocapsid protein

NS: Non-structural protein

NS1: Non-structural 1 protein

NS2: Non-structural 2 protein

NTC: No template control

OD: Optical density

OSA: Obstructive sleep apnoea

PA: Polymerase A protein

PB1: Polymerase B1 protein

PB2: Polymerase B2 protein

PBMC: Peripheral blood mononuclear cell

PBS: Phosphate buffered saline

PCR: Polymerase chain reaction

PE: Phycoerythrin

pH1N1: pandemic 2009 H1N1 influenza

PNPP: P- Nitrophenyl Phosphate

PTC: positive template controls

PVDF: polyvinylidene difluoride

qRT-PCR: Quantitative Real Time PCR

RBCs: red blood cells

RDE: receptor-destroying enzyme

RIG-I: Retinoic acid Inducible Gene 1

RLRs: RIG-I Like receptors

RLU: firefly relative light unit

RNA: Ribonucleic acid

rpm: Revolution per minute

RT-PCR: Real time PCR

SA: Sialic acids

sH1N1: seasonal H1N1 influenza

sIgA: Secretory IgA

S-OIV: Swine-origin influenza virus

ssc: side scatter

swFluA: Universal swine Influenza A

swH1: Swine H1

Th: T helper cell

Th1: T helper 1 cell

Th17: T helper 17 cell

Th2: T helper 2 cell

TIV: Intramuscular Trivalent Inactivated Influenza Vaccine

TLRs: Toll like receptors

TNF-α: Tumour necrotic factor- α

vRNPs: viral Ribonucleoproteins

WHO: World Health Organization

α: Alpha

β: Beta

γ: Gamma

Chapter 1

General Introduction

1. Introduction

Influenza viruses cause annual epidemics and occasional pandemics that have threatened the lives of millions of people all over the world. The occurrence of new strains of influenza virus constantly creates challenges to global health and scientific communities (Schnitzler and Schnitzler 2009, Horimoto and Kawaoka 2005).

1.1 A brief history of pandemic human Influenza

In 1918, the influenza A viruses of the H1N1 subtype infected human population and caused the 'Spanish Flu' pandemic which killed approximately 50 million people. These viruses circulated in humans up to 1957 when a new influenza pandemic, the 'Asian Flu' started and the influenza A/H2N2 viruses substituted the H1N1 subtype viruses (Kreijtz et al. 2011). A decade later in 1968, the 'Hong Kong' influenza pandemic started which was caused by the influenza A/H3N2 subtype. In 1977 influenza A viruses of the H1N1 subtype resurfaced without causing a major pandemic. In 2009, a new influenza A/H1N1 virus of swine-origin caused the first influenza pandemic of the 21st century according to World Health Organization (WHO) 2009 (Stohr 2002, Simonsen et al. 1998, Johnson and Mueller 2002).

The pandemic H1N1 influenza A virus (2009 H1N1) was identified as the cause of outbreaks of respiratory infection, mainly in younger age groups. The virus spread to over 214 countries, with over 18000 deaths reported worldwide (http://www.who.int/csr/don/2010_04_01/en/index.html). In the United States, an estimated 59 million persons were infected by the 2009 H1N1 virus, resulting in 12000 deaths (Writing Committee of the WHO Consultation on Clinical Aspects of Pandemic Influenza 2010).

1.2 Pre-existing immunity to pH1N1 influenza virus

Several studies have shown that in people aged >75 years, there was a considerably lower percentage of influenza cases compared with the younger people as seen in the 1957 H2N2 influenza pandemic (Simonsen et al. 1998). Moreover, evidence of pre-existing immunity to influenza viruses could also be derived from the age-adjusted mortality. Those persons >75 years had a lesser influenza and pneumonia case mortality rate in 1918 than they had for the pre pandemic era of 1911–1917 (Taubenberger and Morens 2006).

Genetic studies showed that the pandemic H1N1 2009 influenza virus was antigenically similar to 1918 Spanish influenza virus. However, it was quite different from seasonal H1N1 virus that started in 1970 and was maintained in human population (Hancock et al. 2009). Several studies have suggested that these antigenic similarities between the 1918 and the 2009 H1N1 viruses account for the pre-existing immunity to the 2009 H1N1 virus in those born before 1947 (Gras et al. 2010).

1.3 2009 pandemic H1N1 influenza

In April of 2009, a novel swine-origin influenza A (H1N1) virus (S-OIV) appeared in Mexico and the United States. WHO declared it a pandemic influenza since the new influenza H1N1 virus caused human-to human transmission of the virus in at least two countries (Neumann et al. 2009). The first cases of novel swine-origin influenza A/H1N1/2009 appeared in late April 2009. A rapid and uncontrolled international spread of the virus occurred subsequently (Allam 2009). This was the

first influenza pandemic in this century and fourth since the first influenza pandemic was reported during World War I (Szucs et al. 2006, Goel et al. 2011).

The new 2009 pandemic H1N1 influenza virus strain is immunologically different from other influenza viruses, leaving large population groups vulnerable to infection (Garten et al. 2009, Brockwell-Staats et al. 2009, Dawood 2009, Hancock et al. 2009).

1.4 Influenza A virus structure

Influenza viruses belong to the family *Orthomyxoviridae*, they are spherically shaped with an average diameter of 120 nm and enveloped negative-strand RNA viruses with segmented genomes (Fouchier et al. 2005). The virus encodes the following components; Haemagglutinin (HA), Neuraminidase (NA), Polymerase A protein (PA), Polymerase B1 protein (PB1), Polymerase B2 protein (PB2), Matrix protein (M): M1 constructs the matrix, M2, Nucleocapsid protein (NP), Non-structural protein (NS1) and Non-structural protein (NS2) (figure 1.1). Table 1.1 describes brief functions of influenza proteins involved in the virus infectivity to the cell.

Viral proteins	Function
HA (haemagglutinin)	Viral entry into target cell
NA (neuraminidase)	Release of viral particles from target cells and help in dissemination of virion particles throughout respiratory tract
PA (acidic polymerase protein)	Endonuclease activity and help in cap snatching mechanism
PB1 (basic polymerase protein-1)	Viral mRNA transcription by 5' cap snatching mechanism
PB2 (basic polymerase protein-2)	Unprimed replication of viral mRNA
PB1-F2	Apoptosis of host cell
M1 (matrix protein)	Role in attachment of vRNP to cell membrane and provide stability
M2 (ion-channel protein)	Help in releasing of vRNP from endosome to cytoplasm
NP (nucleoprotein particle)	Participate in the nuclear import and export of vRNP and viral replication
NS1 (non-structural protein-1)	Suppress IFN-b and host protein production
NS2 (non-structural protein-2)	Help in nuclear import of vRNP

Table1.1: Properties of influenza virus proteins. The table shows some of the roles exerted by proteins of the influenza virus used in the course of infectivity to the host (Dangi and Jain 2012).

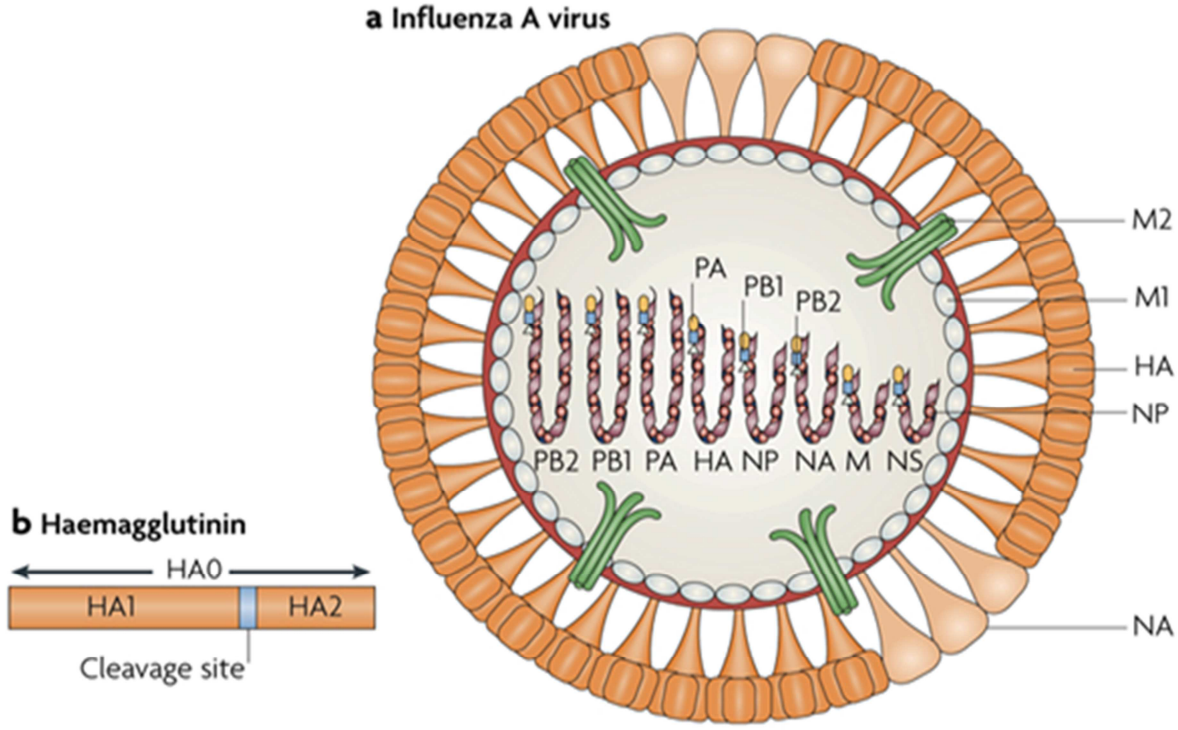

Figure 1.1: Structure of influenza A virus. The influenza A virus particle has a lipid envelope that is derived from the host cell membrane. (**a)** Three envelope proteins haemagglutinin (HA), neuraminidase (NA) and an ion channel protein (matrix protein 2, M2) are embedded in the lipid bilayer of the viral envelope. HA (rod shaped) and NA (mushroom shaped) are the main surface glycoproteins of influenza A viruses. The ratio of HA to NA molecules in the viral envelope usually ranges from 4:1 to 5:1. (**b)** The HA glycoprotein is synthesized as an HA0 molecule that is post-translationally cleaved into HA1 and HA2 subunits; this cleavage is essential for virus infectivity. The HA glycoprotein is responsible for binding of the virus to sialic-acid residues on the host cell surface and for fusion of the viral envelope with the endosomal membrane during virus uncoating. The NA glycoprotein cleaves sialic-acid receptors from the cell membrane and thereby releases new virions from the cell surface. M2 functions as a pH activated ion channel that enables acidification of the interior of the virion, leading to uncoating of the virion. Matrix protein 1 (M1), which is the most abundant protein in the virion, underlies the viral envelope and associates with the ribonucleoprotein (RNP) complex. Inside the M1 inner layer are eight single-stranded RNA molecules of negative sense that are encapsidated with nucleoprotein (NP) and associated with three RNA polymerase proteins polymerase basic protein 1 (PB1), PB2 and polymerase acidic protein (PA) to form the RNP complex. The PB1, PB2 and PA proteins are responsible for the transcription and replication of viral RNA. The virus also encodes a non-structural protein (NS) that is expressed in infected cells and a nuclear export protein (NEP) (Subbarao and Joseph 2007).

1.5 Influenza virus types

There are three types of influenza viruses, A, B viruses and C, and all have many biological properties in common (Fouchier et al. 2005). A key difference between them is their host range: whereas influenza viruses of types B and C are predominantly human pathogens although have sporadically been isolated from seals and pigs (Guo et al. 1983, Osterhaus et al. 2000), influenza A viruses have been isolated from many animal species, including humans, pigs, horses, mink, marine mammals, and a wide range of domestic and wild birds (Webster et al. 1992, Murphy et al. 1996).

Wild birds, predominantly ducks, geese, and shorebirds form the reservoir of influenza A viruses in nature. Avian influenza viruses preferentially infect cells lining the intestinal tract of birds and are found in high concentrations in their feces. While avian influenza viruses are generally non-pathogenic in wild birds, they sometimes cause significant morbidity and mortality upon transmission to other species, including domestic birds and mammals (Webster et al. 1992, Palese 2007).

1.6 Influenza virus subtypes

In wild aquatic birds and poultry around the world, influenza A viruses carrying 16 antigenic subtypes of haemagglutinin (H1-H16) and 9 antigenic subtypes of neuraminidase (N1-N9) have been described (Fouchier et al. 2005).

The HAs are sharing between 40% and 60% amino acid sequence identity (Corti et al. 2010, Lambert and Fauci 2010). The sixteen subtypes are further clustered into two groups based on the molecular relatedness of the HA sequences. Group 1 (H1,

H2, H5, H6, H8, H9, H11, H12, H13 and H16) and group 2 (H3, H4, H7, H10, H14 and H15) (figure 1.2) (Lambert and Fauci 2010, Nabel and Fauci 2010).

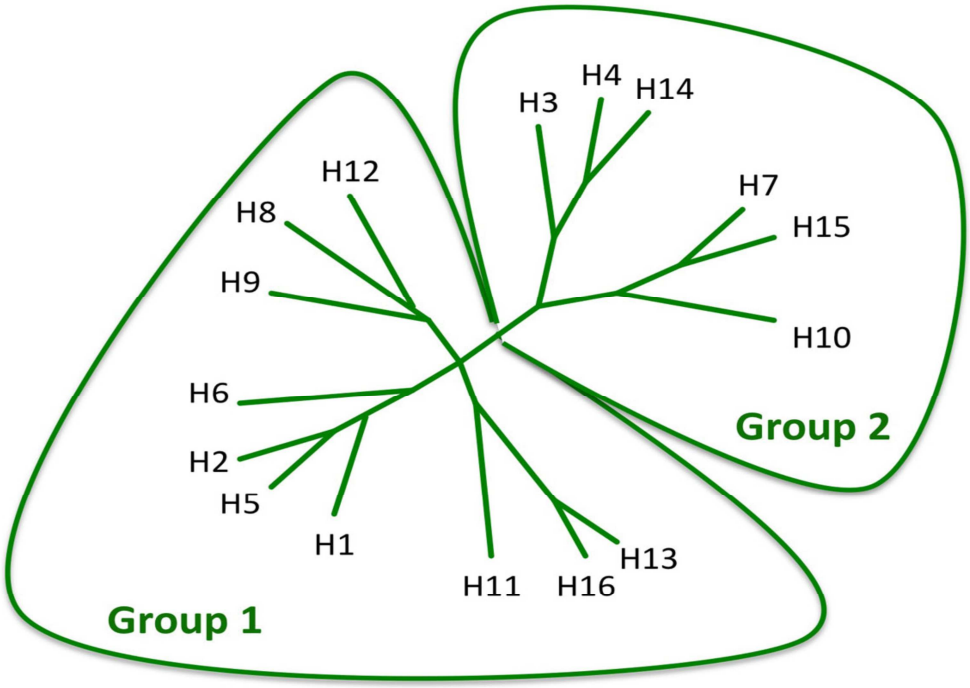

Figure 1.2: Phylogenetic tree showing the relationships between the 16 HA subtypes. The figure shows the 16th HA subtypes classified into two groups. Group 1 includes (H1, H2, H5, H6, H8, H9, H11, H12, H13 and H16) and group 2 includes (H3, H4, H7, H10, H14 and H15) (Nabel and Fauci 2010)

1.7 Influenza viruses evade immune system

The replication of the influenza A virus is an error-prone process. The virus has no system for proof reading of the transcription of its genes. There are a total of approximately 14,000 nucleotides in the influenza genome, giving an average of one point mutation in every 5^{th} virus (Widjaja et al. 2012). The influenza B and C viruses drift, but at a much lower rate. Because of the mutation, annual influenza outbreaks happen, and this is also why the strains of virus included in the influenza vaccine must be assessed and updated every year (Parvin et al. 1986, Stech et al. 1999).

Influenza viruses can evade the immune system. The HA and NA can evade pre-existing immunity via either antigenic drift or antigenic shift, in which the virus gains an HA of a new subtype by genetic reassortment with another influenza A virus (Parvin et al. 1986, Stech et al. 1999).

Both the A and B influenza viruses are constantly mutating due to hosts selection pressure. These mutations can result in antigenic changes in the important surface glycoproteins HA and NA, but also other antigenic viral proteins that are important for the virus to escape the hosts' previously acquired immunity (Rimmelzwaan et al. 2004). If an influenza A virus reassorts either or both of the HA or NA segments, this possibly will be the start of a new pandemic strain to which the population does not have any immunologic memory (Hancock et al. 2009).

1.7.1 Antigenic Drift

Antigenic drift means small and gradual changes that happen through point mutations in the two genes that contain the genetic material to produce

haemagglutinin, and neuraminidase. These point mutations occur unpredictably and result in minor changes to these surface proteins. Antigenic drift produces new virus strains that may not be recognized by antibodies to earlier influenza strains (http://www.cdc.gov/flu/avian/gen-info/flu-viruses.htm).

Continuous genetic changes that alter amino acids (antigenic drift) in the antigenic portions of external glycoproteins could produce selective advantages for viral strains by allocating them to escape pre-existing immunity (Chen and Holmes 2006).

1.7.2 Antigenic Shift

Antigenic shift is an abrupt, major change in the influenza A viruses, resulting in new haemagglutinin and/or neuraminidase proteins in influenza viruses that infect humans. Shift results in a new influenza A subtype or a virus with a haemagglutinin or a haemagglutinin and neuraminidase combination that has emerged from by the exchange or reassortment of gene segments (http://www.cdc.gov/flu/about/viruses/change.htm). It occurs between human and non-human influenza viruses when they co-infect animals or humans and produce a virus that is so different from the same subtype in humans that most people do not have immunity to the new virus (Palese 2007, Schnitzler and Schnitzler 2009). An example of antigenic shift is the pandemic 2009 H1N1 virus which contains a unique combination of swine, avian, and human influenza virus genes (Zeng et al. 2011).

Because swine are vulnerable to infection with both avian and human influenza viruses, novel reassortant influenza viruses can be generated in this mammalian species by reassortment of influenza viral segments leading to the “mixing vessel” theory (Wenjun Ma 2009).

The human influenza HA preferentially binds to sialic acid with galactose in a α2, 6 configuration, while the avian influenza virus prefers a α2, 3 configuration. Pigs have the α2, 3 and the α2, 6 configuration of sialic acid in the upper respiratory tract mucosa, and can therefore be infected with both human and avian influenza viruses. If a pig cell is simultaneously infected with a human and an avian influenza virus, it can act as a mixing vessel for the two viruses, and this can result in a reassorted humanized influenza virus with new avian genes (Oxford 2000). This virus can infect humans that have no pre-existing immunity (antibodies) against the new virus and this can then result in a pandemic (Smith et al. 2004).

1.8 Major virulent proteins of influenza virus

1.8.1 Haemagglutinin protein (HA)

Haemagglutinin (HA) of influenza virus is responsible for virus access into host cells, by binding to the host receptor, internalization of the virus, and later membrane-fusion proceedings. HA is primarily synthesized as an ancestor polypeptide, HA0, that needs proteolytic cleavage into disulfide linked HA1 and HA2 prior to becoming functional and the virus particles being infectious (Yoshida et al. 2009). The main part of HA1 forms the ‘globular head’ region, composed of part of HA1 and is the essential component for binding to the sialic acid receptors. The ‘stem or stalk’ region is principally composed of portions of HA1 and all of HA2 (Hai et al. 2012), which restrains the fusion peptide and membrane attachment domain. The presence of HA-specific antibodies has a vital role in defence against influenza infection in vivo (Puck et al. 1980, Gerhard et al. 1997, Luke and Subbarao 2006). HA-specific antibody responses induced during the influenza virus infection are required to specifically neutralize the virus (Mancini et al. 2011).

1.8.2 Neuraminidase (NA) protein

Antibodies to neuraminidase (NA) protein of influenza virus do not prevent infection, but do decrease the severity of disease by limiting the release of virus from infected host cells (Keynan et al. 2011). The anti-NA antibodies produced are type specific and give little cross-protection towards infections caused by heterotypic or heterosubtypic strains (Jeon et al. 2002)

1.9 Influenza virus life cycle

Structurally, influenza A virus has a lipid bilayer envelope, within which are eight RNA genomic segments, every segment is related with the trimeric viral RNA polymerase (PB1, PB2, PA) and covered by several nucleoproteins (NPs) to protect the viral Ribonucleoproteins (vRNPs). The external layer of the lipid envelope is spiked with many copies of HA, NA as well as a small number of M2, while the M1 molecules have vRNPs attached to the internal layer (Schnitzler and Schnitzler 2009).

After Influenza virus infects the cell, it uses the host cell for producing new viruses and that occurs in several stages. Firstly, the viral surface glycoprotein HA attaches to the host cell-surface sialic acid receptors, and then the virus is transported into the cell in an endocytic vesicle. Due to the low pH of the endosome, it causes a conformational change in the HA protein that consequently leads to fusion of the viral and endosomal membranes. Moreover, the low pH similarly elicits the movement of protons into the virus through the M2 ion channel, therefore detaching the vRNPs from M1 matrix proteins. The vRNPs that are released into the cytoplasm

are transported into the nucleus by recognition of the nuclear localization sequences (NLSs) on nucleoproteins (Wu and Pante 2009).

Secondly, in the nucleus, the viral polymerase starts synthesis of viral mRNA with 5′-capped RNA fragments cleaved from host pre-mRNAs. The PB2 subunit attaches the 5′ cap of host pre-mRNAs (Ulmanen et al. 1981), and then the endonuclease domain in PA subunit cleaves the pre-mRNA (Plotch et al. 1981). Following that, viral mRNA transcription is consequently initiated from the cleaved 3′ end of the capped RNA segment.

Thirdly, viral mRNAs are moved to the cytoplasm for translation into viral proteins. The viral surface proteins HA, NA and M2 are processed within the endoplasmic reticulum followed by glycosylation step in the Golgi apparatus and then transported to the cell membrane. The NS1 protein of influenza A virus plays an important role in suppressing the production of host mRNAs by inhibiting the 3′-end processing of host pre-mRNAs (Nemeroff et al. 1998), therefore blocking the formation of host mRNAs.

Fourthly, the vRNPs cross the cell membrane to be merged into new viruses that are budded out (Nayak et al. 2004). The newly formed HA and NA proteins in new viruses contain terminal sialic acids that would lead the viruses to clump together and then adhere to the cell surface. Finally, the NA of newly synthesized viruses cleaves these sialic acid residues, hence releasing the virus from the host cell (figure 1.3) (Das et al. 2010).

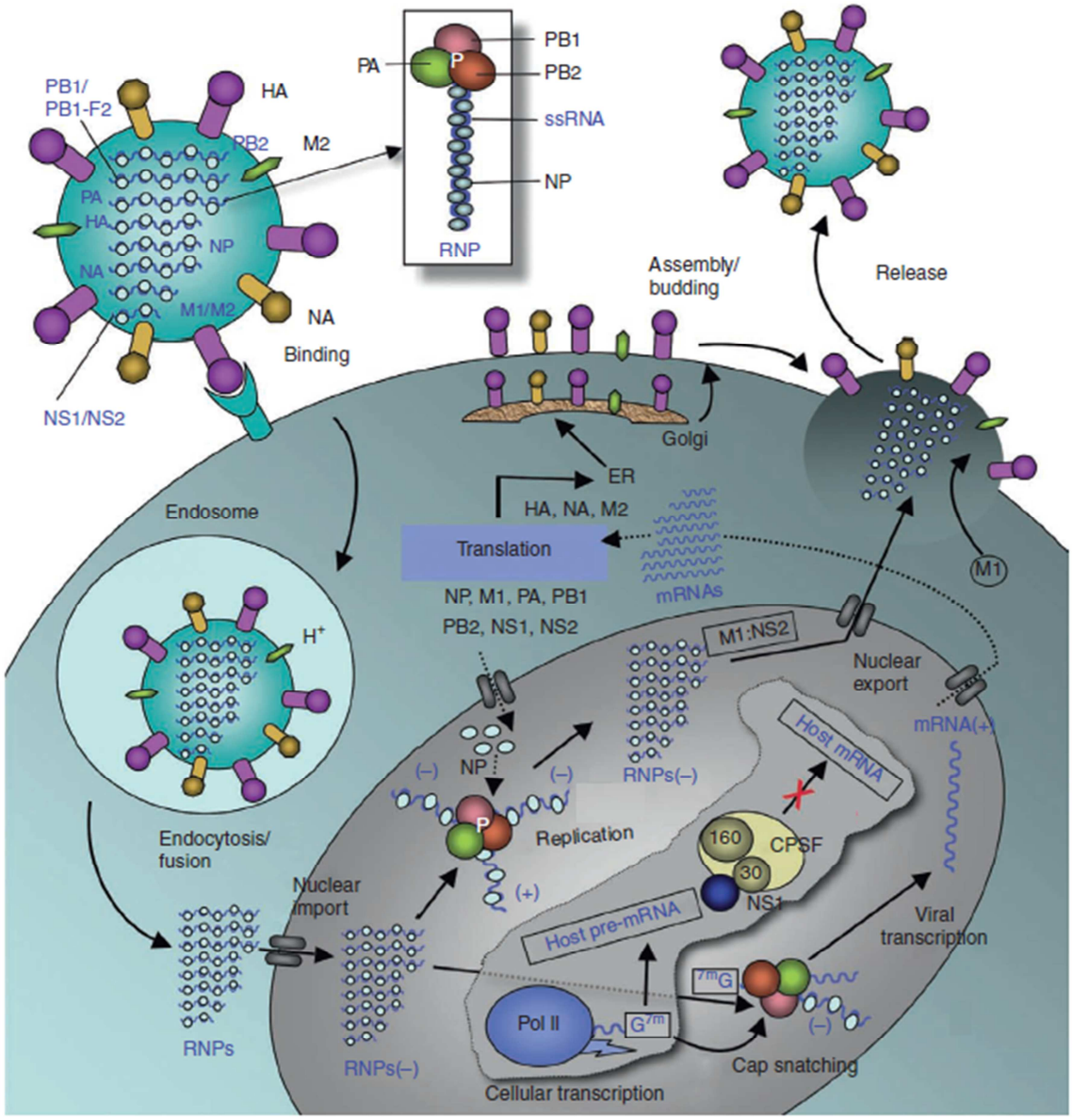

Figure 1.3: Scheme of influenza virus life cycle. After receptor-mediated endocytosis, the viral ribonucleoprotein (vRNP) complexes are released into the cytoplasm and subsequently transported to the nucleus, where replication and transcription take place. Messenger RNAs are exported to the cytoplasm for translation. Early viral proteins, that is, those required for replication and transcription, are transported back to the nucleus. Late in the infection cycle, the M1 and NS2 proteins facilitate the nuclear export of newly synthesized vRNPs. PB1-F2 associates with mitochondria. The assembly and budding of progeny virions occurs at the plasma membrane (Das et al. 2010).

1.10 Immunity to influenza virus

The immune defence against influenza infection is divided into two different systems, the innate (non-specific) and the adaptive (specific) immune system.

1.10.1 Innate Immunity

The innate immune system is fast acting, detecting and destroying influenza viruses within a short period of time (Tamura and Kurata 2004). Innate immunity is a necessary requirement for the adaptive immune response by limiting the initial viral replication and antigen load. Also, the antigen-specific lymphocytes of the adaptive immune response are triggered by co-stimulatory molecules that are provoked on cells of the innate immune system during their interaction with viruses (Jost et al. 2011). The innate immunity against influenza includes type 1 interferon, macrophages, dendritic cells (DCs) and natural killer cells (NK cells).

1.10.1.1 Type 1 interferon

Type 1 interferons have strong antiviral action that they work by protein synthesis inhibition in host cells and prevention of virus replication (Lund et al. 2004, Pang and Iwasaki 2011).

Influenza virus infection triggers mechanisms mediated via all three major families of innate receptors, namely the Toll like receptors (TLRs), Nod-like receptors (NLRs) and RIG-I like receptors (RLRs). TLR7 recognizes influenza ssRNA, activating a transcriptional program that leads to the induction of Type I IFN, IL-12, and IL-6 (Diebold et al. 2004). NOD-like receptor NLRP3 is reported to become activated in response to influenza RNA and lead to Type I interferon production (Allen et al. 2009, Sabbah et al. 2009). Also, the prototypical RLR, RIG-I,

recognizes influenza virus RNA, a process which appears to induce significant IFN-a production by the infected cell (Valkenburg et al. 2011).

1.10.1.2 Macrophages

Macrophages of the alveoli become activated and phagocytose (apoptotic) influenza virus-infected cells and hence limit viral spread (Kim et al. 2008).

1.10.1.3 Dendritic cells (DCs)

Dendritic cells (DCs) are considered specific antigen-presenting cells (APC) in influenza virus infections. Following degrading of viral proteins by the DC, therefore the immuno-peptides (epitopes) are presented by Major Histocompatibility Complex (MHC) class I or class II molecules (GeurtsvanKessel and Lambrecht 2008). For the presentation of MHC class I, the influenza virus-derived peptides are released in the cytosol through proteasomes and then moved to the endoplasmatic reticulum in which they link together with MHC class I molecules. Consequently the MHC I peptide complexes are transported transiently through the Golgi complex to the cell membrane for recognition by specific $CD8^+$ cytotoxic T cells (CTL) (Kreijtz et al. 2011). On the other hand, MHC class II bound to viral proteins are destroyed in endosomes/lysosomes and the major peptides attached with MHC class II molecules. These complexes are then transported to the cell membrane for recognition by $CD4^+$ T helper (Th) cells (GeurtsvanKessel et al. 2009a, GeurtsvanKessel et al. 2009b).

1.10.1.4 Natural killer (NK) cells

Natural killer (NK) cells are an essential factor of the innate immune response against influenza (Ada and Jones 1986). They can identify antibody-bound influenza virus infected cells and lyse these cells, a process called antibody dependent cell cytotoxicity (Mandelboim et al. 2001). The NKp46 receptor, expressed by NK cells, is critical for controlling influenza infections, as influenza-virus-infected cells are eliminated through the recognition of the viral haemagglutinin (HA) protein by NKp46 (Bar-On et al. 2013).

1.10.2 Adaptive Immunity

The adaptive immunity against influenza includes humoral immune response and cellular immune response.

1.10.2.1 The humoral immune response

The humoral immune system includes both the mucosal and systemic arms that play a key role in immunity to influenza virus infection. It primarily provides antibody mediated immunity against influenza virus infection (Cox et al. 2004). A brief background about the components of the humoral immune system is reviewed as below.

1.10.2.1.1 B lymphocytes

The humoral branch of the immune system comprises B-lymphocytes, which after interaction with influenza virus differentiate into antibody-secreting plasma cells (Dorner and Radbruch 2007). Humoral immunity relies on immunological memory provided by memory B cells, which secrete protective antibodies upon antigen

challenge to differentiate into plasma sells (Dorner and Radbruch 2005). B cell memory i.e., production of antibodies that are protective especially over extended periods in the apparent absence of the antigen, provides the host with a first line of defence against reinfection. It is also a helpful biomarker for previous infection to a particular pathogen (Ahmed and Gray 1996, Slifka and Ahmed 1996).

Plasma cells which present in the bone marrow provide immediate protection against reinfection with influenza. Antibody recall responses are mediated by memory B cells that rapidly proliferate and differentiate into plasma cells in response to antigenic stimulation (Dorner and Radbruch 2007, Sallusto et al. 2010). The presence of memory B cells to reinfection with influenza is an important marker in the effectiveness of humoral protection against reinfection (Onodera et al. 2012) (figure 1.4).

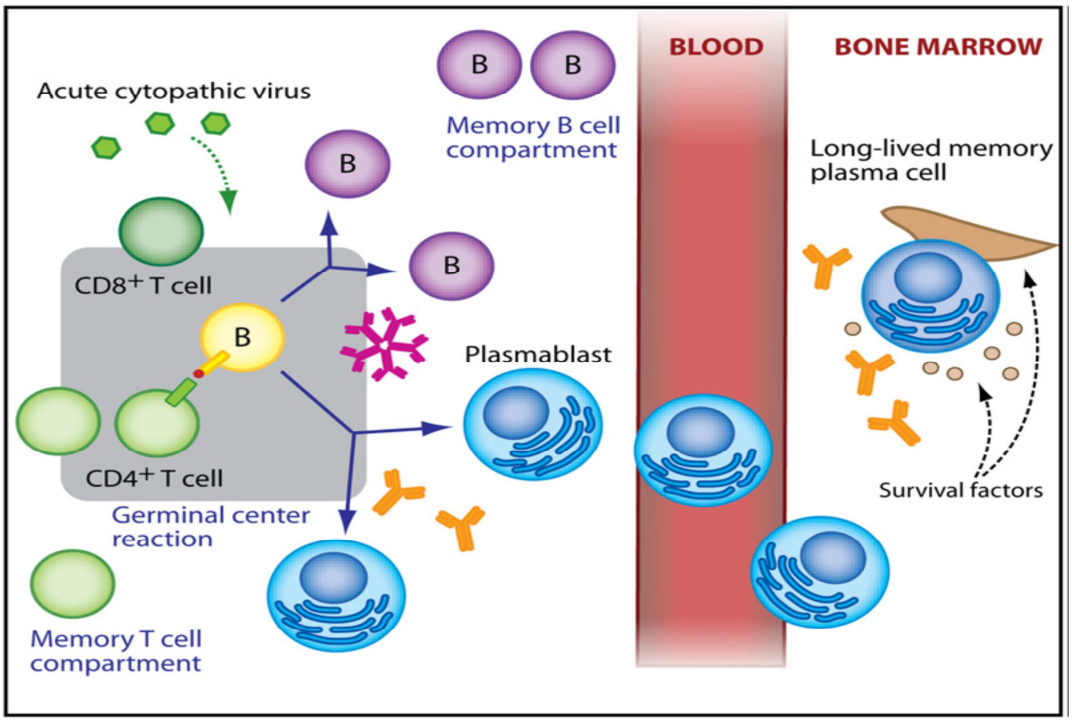

Figure 1.4: Long-Term Antiviral Memory Induction Induced by an Acutely Cytopathic Virus. After vaccination or infection, B cells differentiate into plasmablasts producing antibodies at extrafollicular sites in a T cell-independent manner. Simultaneously, activation of antigen-specific CD8+ and CD4+ T cells occurs. As part of the germinal centre (GC)-reaction, CD4+ T cells provide help to naive B cells (yellow), which eventually become long-lived plasma cells producing high amounts of neutralizing IgG antibodies in the bone marrow. These naive B cells also give rise to long-term memory B cells. Survival niches for these cells are likely within secondary lymphoid organs. The GC reaction is dependent on antigen recognized by specific B cell receptors and the presence of specific T cells. Survival of established long-lived plasma cells residing in the bone marrow and memory B cells is thought to be critically dependent on soluble and insoluble survival factors (as indicated for plasma cells in the bone marrow) but independent of the cognate antigen and T cell help (Dorner and Radbruch 2007).

1.10.2.1.2 Characteristics of B cells

Naive B cells passing into lymph node via high endothelial venules are selected by the antigen in the germinal centre reaction, yielding B cells with high affinity immunoglobulins (Igs) selected to become memory B cells ($CD20^{+}CD19^{+}CD27^{+}CD38^{-}$), and plasmablasts ($CD20^{-}CD19^{+}CD27^{++}CD38^{++}$).

Memory B cells exit into peripheral blood and could live for a short period, except when they are recruited into mucosa or bone marrow niches, contingent on their chemokine receptor expression (Arce et al. 2004, Mei et al. 2009). These niches offer these plasmablasts the elements to persist and become long-living mature plasma cells. These long-lived plasma cells are capable of secreting large amounts of antibodies for a long-period of time (Tarlinton et al. 2008, Batista and Harwood 2009).

1.10.2.1.3 HA-specific antibodies to influenza virus

As mentioned earlier, the HA of influenza virus is composed of two parts, a variable globular head and a conserved stalk/stem. It has been shown that antibodies targeting the stem region are capable of binding HA molecules from different virus subtypes and have broader neutralizing capacity than those that targeting the circular head. Recent studies show that monoclonal antibodies derived from patients infected with the pandemic 2009 H1N1 influenza were cross-reactive, and suggest that the pandemic 2009 H1N1 influenza virus may activate memory B cells primed by previous influenza virus infection. It has been hypothesized that the memory B cells may recognize the conserved regions of HA that are common to most influenza A viruses (Wrammert et al. 2011).

The anti-HA antibodies protect against both disease and infection with homologous influenza viruses and the induction of anti-HA neutralizing antibodies is one of the main goals of immunization with vaccines (Barbey-Martin et al. 2002).

While anti-globular head antibodies are able to neutralize virus by preventing virus binding to the host cell, anti-stalk antibodies have been shown to prevent the fusion stage of virus entry (Sui et al. 2009, Wang et al. 2010, Ekiert et al. 2009). As anti-stalk antibodies are characteristically specific for epitopes that are relatively conserved, these antibodies can be cross-reactive between HAs of distinct subtypes (figure 1.5) (Pica et al. 2012).

A new study by Steel et al in a mouse model has shown that a system expressing an adapted HA molecule which was deficient in the globular head domain but retained the integrity of the stalk region and with both the HA1 and HA2 portions. After vaccination of mice with such a headless HA immunogen, they obtained antisera that were cross-reactive against various subtypes of HA and provided protection against lethal influenza virus challenge. it was suggested that, during optimization of antigen delivery and immunogenicity, a headless HA molecule might offer a broadly protective influenza virus vaccine (Steel et al. 2010).

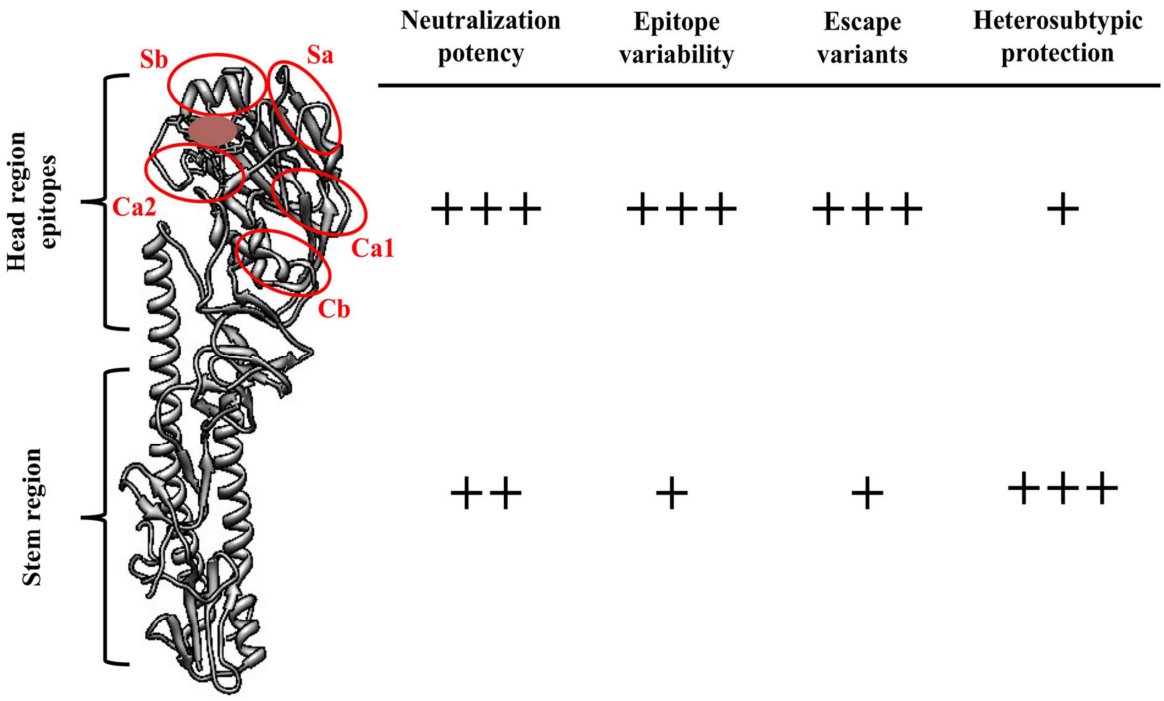

Figure 1.5: Comparisons between the features of influenza HA globular head epitopes and HA stem region epitopes. The figure shows structural view of the H1 HA molecule showing the different globular head epitopes (red circles) and their localization close to the receptor-binding domain (brown). The figure illustrates that antibody produced against the globular head is not cross-reacting with different influenza strain. In contrast, antibody induced from stem region is cross-reacting and would be able to show heterosubtypic immunity. The number of (+) signs was arbitrarily determined (Ellebedy and Ahmed 2012).

1.10.2.1.4 Homotypic and heterosubtypic immunity to influenza A viruses

Until recently, 16 antigenically different HA subtypes of viruses have been recognized. Screening viral escape mutants have shown that anti-HA antibodies mainly target some immunodominant epitopes inside the globular head region of the HA molecule. Normally, these epitopes are positioned around the receptor-binding domain within the HA globular head. Although antibodies targeting such epitopes are neutralizing and protective, they are strain-specific, and therefore do not have the much-desired broad cross- neutralizing activity to different HA virus subtypes (Ellebedy and Ahmed 2012). The immunity resulting from such antibodies is known as homotypic immunity i.e., it helps only to protect against the same strain that initially caused the infection.

On the other hand, several epitopes in the HA stalk region have been identified as possible targets by a group of human monoclonal antibodies (mAbs) (Ekiert et al. 2009,Sui et al. 2009,Wrammert et al. 2011,Throsby et al. 2008). The main benefit of such epitopes is that they are conserved across dissimilar influenza HA virus subtypes, in contrast to the epitopes in the HA circular head. It is suggested that antibodies targeting the HA stalk regions may be broadly neutralizing (Ekiert et al. 2011, Ellebedy and Ahmed 2012). The immunity resulting from such antibodies is known as heterosubtypic immunity i.e., it helps to protect against infection from different influenza strains (Ellebedy and Ahmed 2012).

1.10.2.1.5 Mechanism of HA-specific antibody-mediated neutralization of viral infectivity

There are several mechanisms by which antibodies reduce influenza infectivity. They can do so at different stages in the early life cycle of influenza. For example, direct blocking of the first virus attachment to target cells by anti-HA antibodies capable of neutralizing the virus are consequently interfere with virus receptor interaction (Barbey-Martin et al. 2002). Following the primary attachment, receptor-bound viruses will be taken up into cells via endocytosis. The low pH situation of the endosome causes key conformational alterations in the HA ectodomain, which then triggers the fusion step of the virus with the membrane of the endosome as a result releasing the uncoated viral ribonucleoprotein (RNP) composite into the cytoplasm (Sui et al. 2009).

Additionally, anti-HA antibodies interfere with conformational changes and/or the essential interactions connecting the endosomal membranes and the virus required for fusion. Consequently, inhibition of the necessary primary steps of viral infection can efficiently disrupt viral transmission (Neutra and Kozlowski 2006). The viral RNP is then transported from the cytoplasm into the nucleus in which transcription and replication of viral RNA happens (Huang et al. 2008).

The newly formed viral RNP and protein complexes are consequently gathered into viral elements that bud at the plasma membrane, a process that needs the enzymatic activity of neuraminidase (NA) to release the virions (Huang et al. 2008, Collins et al. 2008).

Anti NA-specific antibodies have been shown to limit replication of the virus by stopping release of descendants from the infected cells, thus reducing the period and

severity of illness (Powers et al. 1996, Webster et al. 1988) however they do not have the ability to stop influenza virus infection. For that reason, focus on virus neutralization occurring by antibodies directed toward HA is the most important aim of vaccine-induced protective immune responses (figure 1.6) (Han and Marasco 2011).

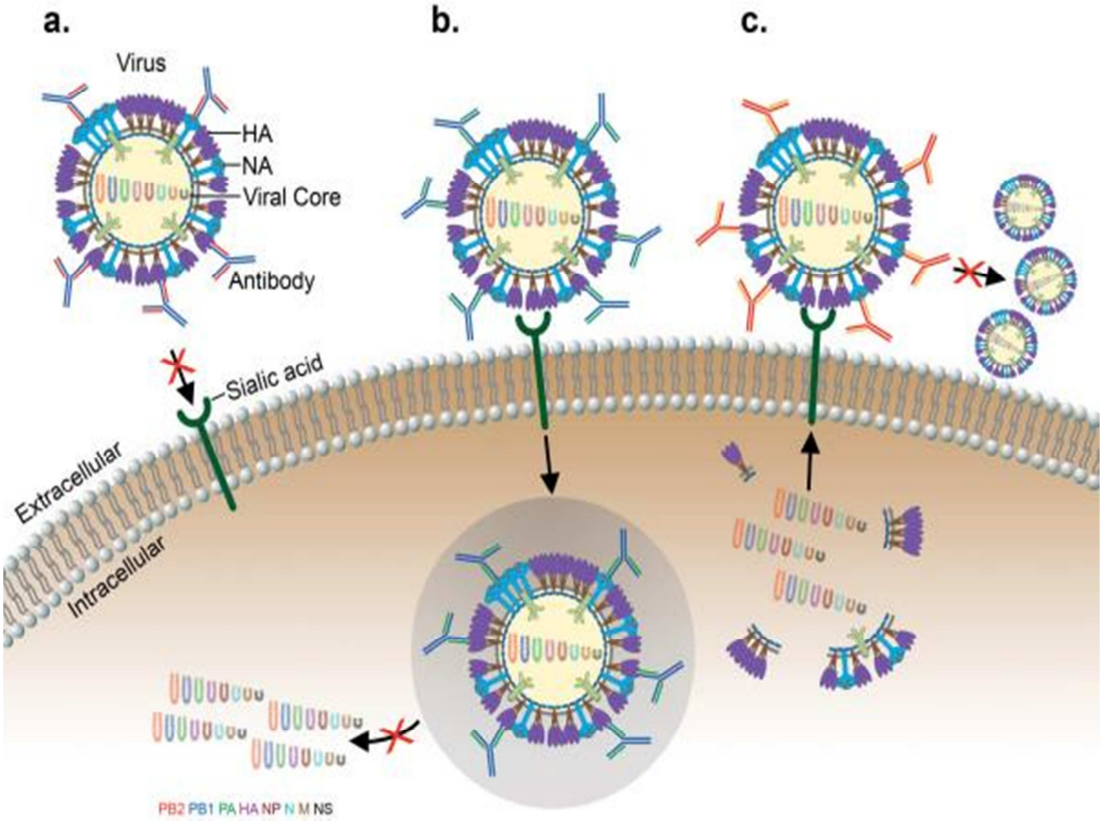

Figure 1.6: Mechanisms of antibody-mediated neutralization of the influenza virus. (a) Antibodies can block influenza HA1 glycoprotein binding to sialic acid residues of receptor proteins on host cells. (b) Antibodies specific to the HA2 glycoprotein of the virus can inhibit its low-pH triggered fusion activity in the endosome at the post-binding/pre-fusion stage, which inhibits replication of the virus. (c) Antibodies to surface neuraminidase can prevent the release of influenza virions from the infected cell surface (Han and Marasco 2011).

1.10.2.2 The cellular immune response

Infection by influenza virus results in the stimulation of cytotoxic T lymphocytes specific for killing virally infected cells (Shortman and Liu 2002, Silver et al. 1992). In addition, the $CD4^+$ subpopulation of human T lymphocytes contains helper/inducer cells that play an essential function in the induction of an immune response (Clement et al. 1988)

Role of CD4 and CD8 T cells in immunity to influenza virus

Naive T cells become activated upon encounter with antigen on the surface of antigen presenting cells in association with MHC molecules and then proliferate and differentiate to become effector cells (Germain 1994). The memory T-cell compartment contains both $CD4^+$ as well as $CD8^+$ T-cells that can rapidly gain effector functions to kill infected cells and/or secrete inflammatory cytokines that inhibit replication of the pathogen. Effector $CD4^+$ T cells also help B-cell responses and enhance $CD8^+$ T-cell development, through the activation of antigen presenting cells (APCs) or secretion of cytokines, such as interleukin-2 (IL-2), IL-4 and IL-5 (Jenkins et al. 2001). In some conditions, protective immunity can be mediated by only one of the branches of the immune system- such as by antibodies or $CD8^+$ T cells - however for optimal control of pathogens, both the humoral and cellular immune responses are essential to be activated (Kaech et al. 2002).

Endogenous antigens from the viral infection of dendritic cells are processed and presented to $CD8^+$ T cells on MHC I molecules. Exogenous antigens are presented via MHC II molecules to $CD4^+$ T lymphocytes. Otherwise, dendritic cells may present antigens they have taken up from infected cells, or move antigen to the adjacent dendritic cells in the lymph node which then start a $CD8^+$ T cell response

through a course termed cross-presentation (Belz 2003, Smith et al. 2004, Schnorrer et al. 2006, Belz et al. 2004). The triggered T cells gain effector cell roles then travel to the location of infection as a consequence they mediate antiviral actions (Belz et al. 2004).

Following an infection, immunological memory normally develops in which the individual is capable to control a new infection by a similar pathogen (Ahmed and Gray 1996). Memory is kept by antigen-specific T cells that persist at increased frequencies, have decreased requirements for co-stimulatory signals in contrast to naïve T cells, also respond rapidly to antigenic restimulation (Woodland and Scott 2005).

CD4 and CD8 memory T cells all contribute to control of an influenza virus re-infection, however in case of primary infection, clearance of the virus depends on CD8 T lymphocytes (Woodland and Dutton 2003). Additionally, CD4 T lymphocytes give help to B lymphocytes to produce specific anti-HA antibodies in a process called the T cell-dependent antibody response. Moreover, there are differences between HA epitopes recognised by CD4 T helper cells and those recognised by anti-HA antibodies. Th cells are further divided into Th1 and Th2 cells, based on the type of cytokines they produce (Treanor et al. 2005).

1.11 The pathogenesis of influenza in humans

Human influenza virus infects the epithelial cells of the upper respiratory tract (Lee 2007). Replication of the virus in these epithelial cells leads to release of pro-inflammatory cytokines, and necrosis of ciliated epithelial cells (Adachi et al. 1997).

Epidemiologic studies have confirmed airborne transmission of influenza (Tang and Li 2007, Beigel 2008). Moreover, the spreading of influenza from person to person is mostly by aerosols as well as droplets. The smaller droplets (1-4μm in diameter), primarily created by sneezing, pass in deeper in the airways and need fewer virus particles to be infectious than larger droplets that are dropped in the nasal cavity (Bridges et al. 2003, Brankston et al. 2007).

Infected individuals may habitually touch mucous membranes prior to interpersonal contact (e.g. hand shaking) or through otherwise indirect contact for example, touching common surfaces (Brankston et al. 2007). Influenza virus has been detected on over 50% of the fomites tested in homes and day care centres during influenza season (Koopmans et al. 2004,Bigl et al. 2002, Beigel and Bray 2008).

The unpredictable evolution of influenza A and B viruses contributes to annual influenza epidemics in humans. Pandemics of influenza are likely formed by influenza A viruses, although influenza B does not cause pandemics as there is no animal reservoir of the virus (Bridges et al. 2003).

Extensive studies performed on the genetic differences among the influenza A viruses and between influenza A and influenza B viruses, which are restricted to humans, could be informative in interpreting the factors that direct mammalian adaptation of influenza A viruses (Beveridge 1991, Cox and Brokstad 1999). RNA-dependent RNA polymerase commonly yield replication errors in the influenza virus synthesis which is usually $1/10^4$ bases per replication cycle; while DNA polymerases produce replication errors in $1/10^9$ bases per replication cycle (Zambon 2001).

Several factors in humans contribute to determining the pathogenicity and severity of the clinical state. For example, age and health condition, e.g. immunocompromised individuals, elderly people (especially those living in close contact in nursing homes), young children with a naïve immune system and people with serious chronic diseases (e.g. heart, lung and metabolic diseases) (el-Madhun et al. 1998, Fujino et al. 2013).

The incubation period of influenza virus infection varies from one to four days and infected adults usually shed virus for three to five days, whereas children and immunocompromised individuals might shed virus for a much longer period (Cox and Subbarao 1999, Cheung and Poon 2007) .

In humans, influenza virus starts replication and consequently causes tissue destruction following the infection of the epithelial cells of the upper respiratory tract. Damage of the epithelial cell barrier in addition to the local inflammatory response result in common symptoms of influenza: coughing, sneezing, sore throat, runny and blocked nose (Kuiken et al. 2012). The systemic symptoms of influenza are related to production of cytokines in the inflammatory course of the infection. Many of these cytokines, such as IL-1, IL-6 and tumour necrosis factor- α (TNF-α), are endogenous pyrogens. When they spread from the hypothalamus through the bloodstream, they stimulate production of prostaglandin E2, causing symptoms like fever, myalgia and headache (Brydon et al. 2005).

1.12 The mucosal immune response

Much of the immunological understanding associated with influenza has been acquired from research in small animals. However, although the mouse model is commonly used, influenza virus infections do not naturally infect mice (Doherty et al. 2006, Suzumoto et al. 2006). Thus, care should be taken in the interpretation of results obtained from animal studies into human use (Perry and Whyte 1998, Haley 2003).

Human adenoids and tonsils are major components of nasal-associated lymphoid tissues (NALT) which are considered to be an important part of the mucosal immune system (Wu et al. 1997a,Kiyono et al. 2004, Bernstein et al. 1999). However, studies have shown there are some major differences between human NALT in the nasopharynx and other mucosal compartments such as Peyer's patches in the intestine. B cells in the former predominantly produce IgG, whereas the majority of B cells in the latter produce IgA (Boyaka et al. 2000a, Nadal et al. 1992a). Studies demonstrated that pneumococcal protein antigens elicited a predominantly IgG memory B cell response in human NALT (Zhang et al. 2002a, Zhang et al. 2010b). The NALT tissues are considered to be important induction sites for both mucosal and systemic immunity against upper respiratory pathogens including influenza (Kiyono et al. 2004, Wiley et al. 2001, Zuercher et al. 2002, Guthrie et al. 2004b).

The induction of immunological memory against influenza virus most likely involves these immunocompetent NALT tissues, where antigen-specific memory B cells are primed. However, limited data exist on the development and function of such memory B cells in humans. These secondary lymphoid constructions are made up

like a lymph node with B- and T-cells structured into follicles. Tonsils are vital structures in the mucosal immune system important for immunomodulation and homing of lymphoid cells (Savage 1977).

The mucosal IgG levels are generally considered to be passively diffused from the systemic compartment, although some studies also suggested local production. Secretory IgA is produced in large quantities locally (Renegar et al. 2004). Both serum and mucosal antibodies are likely to be important in protection (figure1.7) (Treanor et al. 2006).

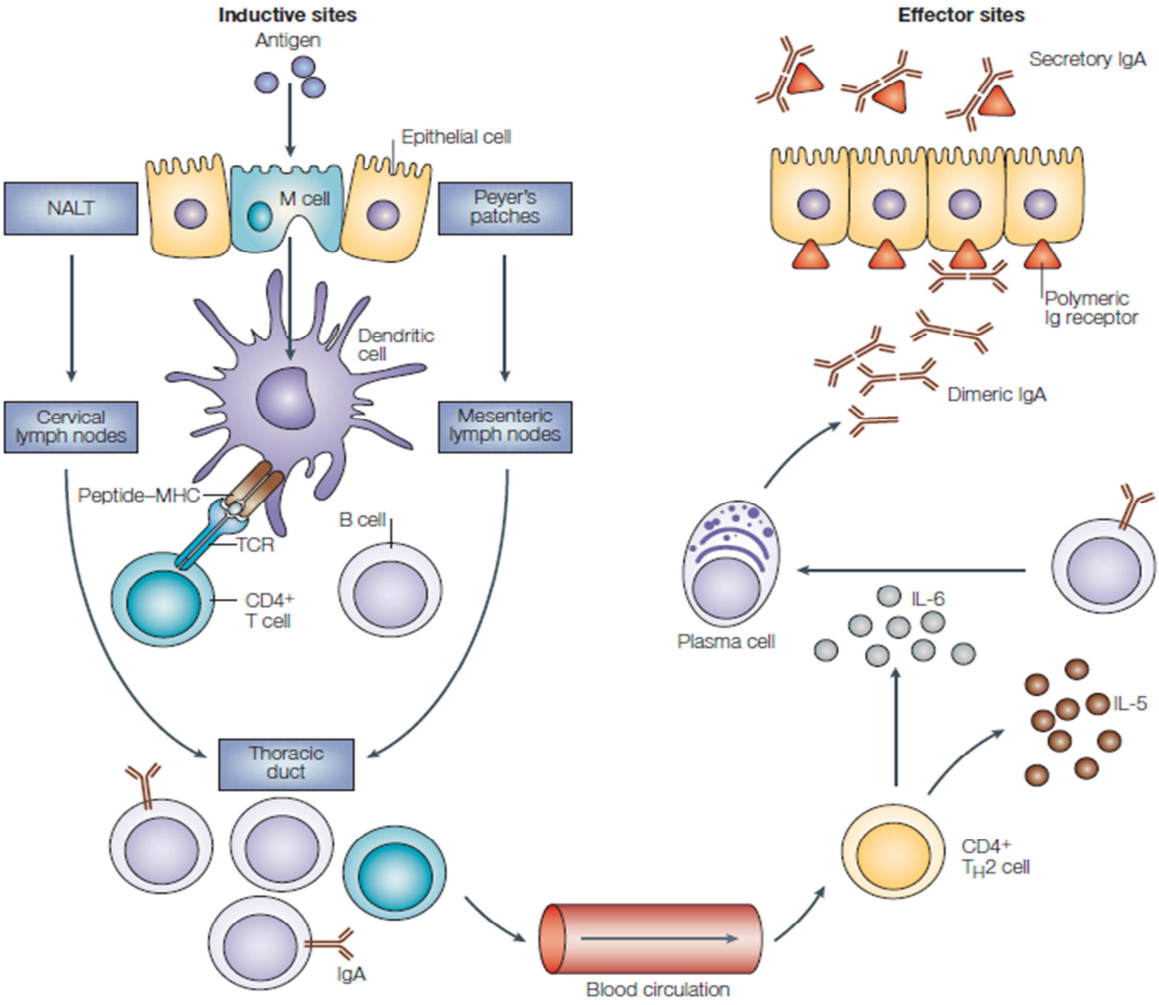

Figure 1.7: The common mucosal immune system. Luminal antigens are transported to the nasopharynx-associated lymphoid tissue (NALT) and Peyer's patches through microfold (M) cells that are present in the epithelium overlying NALT and Peyer's-patch follicles. Dendritic cells process and present antigens to T cells in these lymphoid tissues. CD4+ T cells that are stimulated by dendritic cells then preferentially induce IgA-committed B-cell development in the germinal centre of the lymphoid follicle. After IgA class switching and affinity maturation, B cells rapidly migrate from NALT and Peyer's patches to the regional cervical lymph nodes and mesenteric lymph nodes respectively, through the efferent lymphatics. Finally, antigen-specific CD4+ T cells and IgA+ B cells migrate to effector sites (such as the nasal passage and intestinal lamina propria) through the thoracic duct and blood circulation. IgA+ B cells and plasmablasts then differentiate into IgA-producing plasma cells in the presence of cytokines (such as interleukin-5 (IL-5) and IL-6) that are produced by T helper 2 (TH2) cells, and they subsequently produce dimeric (or polymeric) forms of IgA. These dimeric forms of IgA then become secretory IgA by binding to polymeric Ig receptors (which become the secretory component in the process of secretory IgA formation) that are displayed on the monolayer of epithelial cells lining the mucosa. Secretory IgA is then released into the nasal passage and intestinal tract (Kiyono and Fukuyama 2004).

1.13 Effect of adenotonsillectomy

All samples used in this thesis were obtained from patients undergoing tonsillectomy and/or adenoctomy operations. Removing the tonsils is a fairly common surgical procedure. The indications for these operations are usually hypertrophy of the tonsils initiating respiratory problems for instance obstructive sleep apnoea (OSA) and otitis media (glue ear) or recurrent tonsillitis (Paradise et al. 1984, van Staaij et al. 2004).

It is still debated that whether tonsillectomy and adenoidectomy affects the immune system and particularly the upper airway mucosa immunology. As these operations are some of the commonest operations worldwide, it might appears that removal of a portion of the Mucosal-associated lymphoid tissue (MALT) in the Waldeyer's Ring does not appear to lead to a major immunological disadvantage (Nave et al. 2001). This possibly could be due to other parts of the local MALT substituting the role of the removed tissue (Ogra 1971). The tonsils are essential sites of B-cell production and differentiation, in addition to function as both inductor and effector sites (Brandtzaeg 2003a).

A study performed by Ogra has shown that IgA antibody levels in the nasopharyngeal fluid were reduced after adenotonsillectomy (Ogra 1971), also another observation by Ostergaard has shown that levels of IgA antibodies were decreased after tonsillectomy in both saliva as well as serum after two years from the operation (Ostergaard 1977).

1.14 Nasopharynx-associated lymphoid tissue (NALT)

The oropharynx and nasopharynx are the entrance to the respiratory tract, and is hence susceptible to airborne infectious pathogens such as influenza viruses. The nasal mucosa is usually the port of entry of respiratory viruses where they encounter the immune system of the host (Doherty et al. 2006) .

The beginning of antigen-specific immune responses takes place at particular 'gateways', which incorporate microfold (M) cells located in the epithelium overlying follicles of the mucosa-associated lymphoid tissues (MALT). These contain all of the immunocompetent cells that are required for the generation of an immune response (T cells, B cells and antigen-presenting cells). Peyer's patches, in the gut, and nasopharynx-associated lymphoid tissue (NALT) - two of the main components of MALT - are important inductive tissues for the generation of mucosal immunity through the ingestion and inhalation of antigen in the intestinal and respiratory tracts respectively (Kiyono and Fukuyama 2004) .

The epithelial lining of respiratory tract is ciliated with tight junctions, and this signifies a mechanical barrier to inhaled particles including pathogens. There is furthermore a huge turnover of epithelial cells lining the mucosa (Doyle et al. 2007).

1.15 The immunological components of tonsils and adenoid

The tonsils and adenoids as mentioned previously, are part of the NALT .They are part of Waldeyer's ring and they are beneficially situated to present regional immune antigens because of them being exposed to antigens either airborne or alimentary (Boyaka et al. 2000b). As described by Brandtzaeg, tonsils and adenoids

comprise four specialized lymphoid components contributing to the immune functions of these organs, namely; the reticular crypt epithelium, the extrafollicular area, the mantle zones of lymphoid follicles and the follicular germinal centres (GCs).The GCs are related to: (a) clonal expansion of B cells;(b) somatic hypermutation in B-cell immunoglobulin (Ig) variable (Ig V)-region genes;(c) positive selection of B cells that are able to obtain antigen-specific signals by high affinity;(d) consequent differentiation to B memory cells and plasma cells of diverse isotypes; and (e) production of the J-chain gene in a variable subset of B cells (Brandtzaeg 2003b, Brandtzaeg and Halstensen 1992, Brokstad et al. 2001).

Passa`li et al, have shown that a large percentage of B lymphocytes were present in adenoids and tonsils (Passali et al. 2003). CD4 lymphocytes were major components in adenotonsillar tissues. A low percentage of CD8 cytotoxic lymphocytes were also found in both tissues as well (Bernstein et al. 1999). Mean percentages of B cells, CD4 and CD8 lymphocytes in adenoids and tonsils are shown in table (1.2)

Several studies have found that B cell of the IgG isotype is predominant in adenotonsillar tissues. In contrast, B cells of the IgM and IgA isotypes were relatively lower in tonsil and adenoid tissues than that of IgG isotype (figure 1.8) (Edwards et al. 1986, Passali et al. 2003).

	Adenoid (%)	Tonsil (%)
B lymphocytes	25	28
CD 4 T cells	27	22
CD 8 T cells	7	6

Table 1.2: Mean percentage of lymphocyte populations in adenoids and tonsils. The table shows comparison between the mean percentage of B lymphocytes ,CD4 and CD8 T cells in adenoid and tonsil (Passàli 2003).

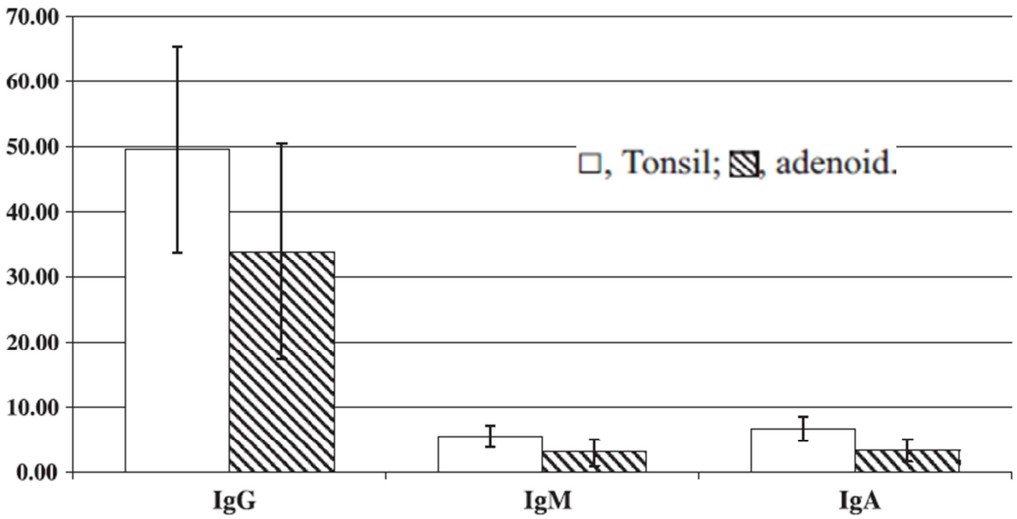

Figure 1.8: Mean IgG, IgM and IgA antibody isotypes of B cells in tonsil and adenoid tissues. The figure shows higher percentage of IgG antibody in adenotonsillar tissues, whereas lower percentage was observed for IgM and IgA antibody isotypes (Passàli 2003).

1.16 Vaccines against influenza

Influenza vaccine development has been begun soon after the influenza virus was discovered. Inactivated influenza vaccines were presented in the 1940s, and are still the major formulation of influenza vaccine. The aim of vaccination is to offer protection against disease through generation of immunological memory responses (Tandale et al. 2013). Protection following natural infection with influenza is primarily mediated by anti-haemagglutinin HA-specific antibodies in serum and mucosa, and T-cell responses associated with reduced disease severity (Brokstad et al. 2001). Because the influenza virus genome is segmented, coinfection of a particular host cell by two or more different influenza is made possible resulting in reassortment of their genetic materials (Plotkin et al. 2002, Lambert and Fauci 2010).

Influenza vaccination is the main method to prevent influenza and severe complications associated to the disease (Cox and Subbarao 1999, Nichol and Treanor 2006). Numerous reports and studies have shown the positive effect of vaccination on decreasing the morbidity and mortality (Jefferson et al. 2010, Smith et al. 2006). Beneficial cost-efficacy relationships have also been shown for influenza vaccination (Rivetti et al. 2006).

Global Influenza Surveillance Network updates the virus strains of the vaccines twice a year by the WHO's, for the northern as well as southern hemisphere. The vaccines are prepared by propagating the influenza viruses in embryonated hen's eggs (Brokstad et al. 2002, Eriksson et al. 2003).

The effectiveness of influenza vaccination is affected by several factors, e.g. type of vaccine, route of vaccination, immunologic status, age and finally matching of vaccine to the circulating influenza strains (Cox et al. 2004). The majority of adults

are likely to have experienced several infections with influenza and have developed memory to the subtypes of the virus in circulation. One dose of the influenza vaccine was shown to offer protective levels of antibodies in 60-90% of cases (Cox et al. 2004, Cowling et al. 2010). Young children who could be immunologically naïve to influenza, may require more than one dose to achieve protective levels of antibody (Eriksson et al. 2003).

1.16.1 Influenza vaccine types

1.16.1.1 Trivalent Inactivated Influenza Vaccine (TIV; intramuscular)

An inactivated vaccine contains three strains of influenza viruses: one influenza A (H3N2) virus, one influenza A (H1N1) virus, and one influenza B virus (WHO). It is given as an injection subcutaneously or intramuscularly. In primed individuals it will induce a quick systemic humoral immune response in the blood. In serum, the influenza specific IgG antibody response is predominant after vaccination (El-Madhun et al. 1999). Therefore, the antibody secreting cells produced in blood are similarly mostly IgG positive, with minor IgA and IgM positive cells (Wrammert et al. 2008).

1.16.1.2 Live Attenuated Influenza Vaccine (LAIV; intranasal)

LAIV delivered intranasally induce a strong immune mucosal response, although a weaker systemic response (De Filette et al. 2006). Also, LAIV vaccines may stimulate stronger cellular immune response by inducing influenza specific T memory cells (Vajdy et al. 2007).

Both live attenuated vaccine and inactivated vaccines are currently in use. The protection they offer differs generally, depends on the antigenic matching between the viruses contained in the vaccine and those that are circulating during influenza season also on the age recipients and them health status (Fiore et al. 2010).

Seasonal influenza vaccination is the most important way of preventing seasonal influenza virus infections and potentially severe complications (Kilbourne 2006, Chen et al. 2010). Seasonal influenza vaccination reduces the possibility of becoming ill with influenza or transmitting influenza to others (CDC, http://www.cdc.gov/flu/professionals/vaccination/vaccine_safety.htm).

At present, the trivalent inactivated influenza virus vaccine (TIV), is certified globally and recommended for various populations, including children 6 months and older, individuals with a diversity of chronic illnesses and health care workers (Kroger et al. 2006). However, LAIV is administered as an intranasal vaccine and replicates in the nose. It can be used for healthy people 2-49 years of age who are not pregnant (Vellozzi et al. 2009). The inactivated influenza virus vaccine, used since 1945, has been commonly well accepted and accounted to provoke considerable intensities of protection, ranging from70 to 90% once the vaccine as well as circulating wild-type strains are antigenically analogous (Beyer et al. 2002, Block 2004).

Intramuscular vaccinations with TIV usually induce serum haemagglutination inhibition (HAI) antibody responses, mainly in older children and adults who have significant immunological memory to influenza virus. In contrast, intranasal administration of LAIV induces both serum and mucosal antibody responses, particularly in young, non-immune children; whereas the level of influenza virus

specific antibody responses in the serum of older children and adults is generally lower compared to TIV (Belshe et al. 2000, Beyer et al. 2002, Edwards et al. 1994).

Serum neutralizing antibodies and the mucosal surfaces are the preferential protective measure against influenza infection. Viral surface glycoproteins are the major targets of protective antibodies against the infection. Binding to these glycoproteins prevents initial viral attachment and the subsequent infection, therefore allowing “sterilizing protection” (Gerhard 2001).

Intramuscular administration of inactivated vaccine is different to natural infections, in that it induces serum antibodies, but not mucosal immune responses (Boyce et al. 2000,Clements and Murphy 1986, Muszkat et al. 2000). Protection of the lower respiratory tract is assumed to be throughout the circulating antibodies that transude into the lungs (Muszkat et al. 2003, Chen et al. 2001). Table 1.3 contains licensed influenza vaccines in humans.

Licensed vaccines	**Comments**
Trivalent Inactivated Influenza Vaccine (TIV)	
Inactivated whole virus	Safe and immunogenic in humans
Inactivated split virus	Safe and immunogenic in humans
Inactivated subunit virus	Safe and immunogenic in humans
Live attenuated influenza vaccine (LAIV)	
Cold-adapted (licensed 2003 in USA)	Safe and immunogenic in humans

Table 1.3: Currently available influenza vaccines (Cox et al. 2004).

1.16.2 Development of mucosal vaccines

As mentioned previously, NALT contains all of the immunocompetent cells that are necessary for the generation of antigen-specific immune responses. Intranasal vaccination may critically depend on such immune tissues. Nasal vaccination has been demonstrated to be a successful regime for stimulation of respiratory immune system (Chin-ichi Tamura 2004). Moreover, this course of mucosal immunization can induce both humoral and cell mediated antigen-specific immune responses (Yanagita et al. 1999). Another advantage of nasal immunization is that it requires a lower quantity of antigen than that used by other routes of vaccinations for the induction of antigen-specific mucosal and systemic immune responses (Gwinn et al. 2010).

Intranasal administration of killed vaccine antigen alone has failed to fully stimulate NALT (Yuki and Kiyono 2003). When non-replicating antigens (proteins, peptides, polysaccharides) are used for nasal immunization, adjuvants or otherwise live attenuated virus must be used to maximize the induction of antigen-specific immune responses since nasal immunization in the absence of adjuvant may not induce the desired immune response and may induce antigen specific tolerance (Miller et al. 2007).

1.17 Antiviral drugs

Two groups of antiviral drugs against influenza are available. The M2-channel inhibitors group, amantadine and rimantadine, and they are only effective against influenza A. The other group of antiviral drugs against influenza is the neuraminidase inhibitors, zanamivir (Relenza®) and oseltamivir (Tamiflu®). The second group is currently recommended for the treatment of influenza in the UK.

During influenza seasons, Oseltamivir and zanamivir are only recommended as treatment for influenza in those considered to be " at risk" of developing more serious complications from influenza infection, such as the elderly or those with underlying conditions like asthma or heart disease. It is recommended for "at risk" patients who present and who can start treatment within 48 hours of the onset of symptoms of influenza-like illness .These drugs are only recommended for use in this way during the period when influenza is known to be circulating (table 1.4). http://www.hpa.org.uk/web/HPAweb&HPAwebStandard/HPAweb_C/1195733852084

Drugs	Suitable for the treatment of children "at risk"	Suitable for the treatment of adults "at risk"	Suitable for short- term protection of those "at risk" who have been exposed to influenza
Zanamivir	YES (>5 years old)	YES	YES (>5 years old)
Oseltamivir	YES (>1 year old*)	YES	YES (>1 year old*)

Table1.4: Summary of the current guidance for the antiviral drugs used for influenza virus infection. *in exceptional circumstances oseltamivir can be used for the treatment or post exposure prophylaxis of influenza in children under one year of age.

1.18 Aims of the thesis

1. To investigate the frequency of HA-specific memory B cells in human NALT to influenza viruses following 2009 H1N1 pandemic influenza and their ability to produce cross-reactive antibodies.
2. To study HA-specific antibodies to influenza viruses in serum samples from children and adults following 2009 H1N1 pandemic.
3. To analyze HA-specific memory $CD4^+$ T cell response to the 2009 pH1N1 virus in human NALT.
4. To study the mucosal antibody responses in an *in vitro* model of NALT following *in vitro* stimulation with a LAIV vaccine.

Chapter 2

Materials and Methods

2.1 Patients and samples

Adenoids and tonsils were obtained from children and adults (1–37years) undergoing adenoidectomy and/or tonsillectomy. The samples were kept in fridge at 4°C in HANKS transport medium (Sigma-Aldrich) while being transported to the laboratory for further separation of MNC. HANKS medium was already prepared and the following reagents were added; glutamine (2mM), penicillin (50u/ml), and streptomycin (50µg/ml) (Sigma-Aldrich).

A venous blood sample was obtained as well and kept with heparin to avoid blood clotting. Patients previously vaccinated against influenza or who were immunocompromised in any way were excluded. Saliva from patients was collected using specialised transport sponge bud container. The study was approved by the local ethics committee (Liverpool Paediatric Research Ethics Committee) and written, informed consent obtained from each patient/parent as appropriate.

2.2 Cell counting and culture

10 µl of mononuclear cell suspension was transferred to a haemocytometer and the cells counted using a light microscope with 40x objective magnification. All the cells in the $1mm^2$ centre square of the grid were counted. RPMI 1640 medium with HEPES supplemented with 10% fetal bovine serum (FBS), glutamine (2mM), penicillin (50u/ml), and streptomycin (50µg/ml) (Sigma-Aldrich) was used to culture the cells (culture medium). From now on this medium will be called (RPMI complete). All the cells to be cultured were placed in an incubator in a humidified 5% CO2 atmosphere at 37°C.

2.3 Mononuclear cell Isolation

2.3.1 Peripheral blood mononuclear cells (PBMC)

All separation procedures were performed in fully sterilized conditions inside a pre-sterilized hood fitted with HEPA filters and under a negative pressure situation to avoid any probable contamination. The blood was taken into preservative heparinised tube (30µ/ml of blood) and peripheral blood mononuclear cells (PBMC) were isolated by Ficoll gradient centrifugation (Ficoll-Paque™ PREMIUM GE Healthcare, United Kingdom). Blood was gently layered onto an equal volume of Ficoll-Paque ™ and centrifuged at 400g (1800 rpm) for 30 min at room temperature. Then, PBMC were harvested from the interface and washed twice with phosphate-buffered isotonic saline (PBS; Sigma) and spun down at 400g for 10 min. After that, cell pellets were resuspended in culture medium and adjusted to 4 x10 ^{6} cells/ml concentrations.

2.3.2 Adenotonsillar mononuclear cells (MNC)

The adenoids and/or tonsils were transported to the laboratory at room temperature in minimum essential medium (HANKS) (Sigma-Aldrich) supplemented with glutamine and antibiotics (penicillin, 100 U/ml; streptomycin, 100 µg/ml).The Adenotonsillar tissues were transferred to an 8 cm-diameter sterile Petri dish and checked grossly. Each sample was minced using a sterile scalpel to release cells into the medium. The cell suspension was allowed to sediment for 5 min and was then passed through a nylon mesh (70-µm pore size).

Mononuclear cells were isolated using Ficoll-Paque™ gradient centrifugation (400 x*g* for 30 minutes). The cells were washed twice in sterile phosphate-buffered saline (PBS) and resuspended in 5 ml of RPMI complete medium (Sigma-Aldrich) to

culture the cells (culture medium). Each cell suspension was adjusted to contain 4 x10 ^{6} cells/ml concentrations.

2.4 Influenza virus antigens and proteins

2.4.1 Influenza virus antigens

Influenza antigens used for cell stimulation experiments were β-propiolactone inactivated, partially purified whole virus antigens from National Institute for Biological Standards and Control (NIBSC, UK). The pH1N1, sH1N1, sH3N2 and aH5N1 virus antigens were derived from A/California/04/2009, A/Brisbane/59/2007, A/Brisbane/10/2007 and A/Vietnam/1203/2004 strains respectively. Intranasal LAIV (FluMist) vaccine included A/H1N1/2009; A/H3N2 and B influenza strains (BEI resources ATCC).

2.4.2 Recombinant HAs

The recombinant HAs of pH1N1 and sH1N1 contain a C-terminal histidine tag and produced in High Five™ insect cells using a baculovirus expression vector system (Stevens et al.2004). The HAs were purified from cell culture supernatant by Immobilized-metal affinity chromatography (IMAC) and contain a trimerizing (foldon) domain (Stevens et al. 2004). The recombinant HAs of sH3N2 and aH5N1, H2N2 and H7N3 viruses were full length glycosylated HA that were produced in Sf9 insect cells using a baculovirus expression vector system, and membrane-extracted from infected cells and purified under native conditions by affinity chromatography that preserve their biological activity and tertiary structure. The purified HA forms trimers (Gale et al. 1998).

Purified recombinant HA proteins of pH1N1 (A/California/04/2009), sH1N1 (A/Brisbane/59/2007), sH3N2 (A/Brisbane/10/2007), aH5N1 (A/Vietnam/1203/2004), H2N2 (A/Singapore/1/57), and H7N3 (A/Canada/RV444/04) were from Biodefence and Emerging Infections Research Resources Repository, ATCC (Manassas VA, USA).

2.5 Cell culture and stimulation by influenza virus antigens for antibody production

Adenotonsillar MNC were isolated and then resuspended and the concentration was adjusted to 4 x 10^6/ml in RPMI complete medium. Cells were cultured in 96-well flat bottom culture plates and then 250 µl/well of cells added (Corning Inc, Corning, USA) in the presence or absence of different stimulants. The influenza virus antigens used were; pH1N1, sH1N1, sH3N2 and aH5N1 virus antigens were derived from A/California/04/2009, A/Brisbane/59/2007, A/Brisbane/10/2007 and A/Vietnam/1203/2004 strains respectively all were from National Institute for Biological Standards and Control (NIBSC, UK). Cells then were cultured for up to 10 days in 5% CO_2 at 37°C. After that cell culture supernatants were collected and stored at –70°C until assay.

2.6 Enzyme-linked immunosorbent assay (ELISA) for measurement of HA specific antibodies

ELISA is widely used at a very broad spectrum of fields, such as, experimental, diagnostic, serologic surveillance and other purposes (Lequin 2005). The high sensitivity of this test permits the examination of very small volume of sample

without pre-treatment step (Bishai et al. 1981). During the past few years, ELISA has been advanced for the detection of antibodies to a variety of viruses.

The reliability of any immunological assay depends on the stable standardization of all reagents and measures used (Turner et al. 1982).

ELISA assay in this work was designed and developed to detect antibodies present in serum, cell culture supernatants and saliva samples directed against influenza A haemagglutinin glycoproteins. 96-well ELISA Plates (Costar) were coated after reconstitution in PBS with 100 µl/well at concentration of (2µg/ml) of Haemagglutinin (HA) recombinant purified proteins (ATTC) of the previously mentioned influenza virus strains. All HA proteins were tested and optimized to reach optimal coating concentration. Following that, plates were covered by adhesive seal and then incubated overnight at 4°C. Plates were washed 5 times with PBS containing 0.05% Tween-20 (Sigma-Aldrich) before that plates contents were discarded into a bucket containing 1% virkon and then plates were blotted on a paper towel. After that the plates were blocked with150 µl/well of blocking buffer PBS containing 10% Foetal calf serum (FCS) (Sigma-Aldrich) for one hour and half at room temperature. Samples and controls were diluted (1:100 and 1:400 respectively) using blocking buffer and then 50 µl/well were added in duplicate and plates were incubated for 1.5 hour at room temperature. Plates were washed 5 times with PBS containing 0.05% Tween-20 and then blotted on a paper towel. Addition of 50µl/well alkaline phosphatase conjugated goat anti-human IgG (Sigma-Aldrich) after dilution to the concentration of (1:1000 in blocking buffer) and then incubated at room temperature for 1.5 hour. Plates were washed 5 times with PBS containing 0.05% Tween-20 and then blotted on a paper towel. Just before the end of the last washing

cycle, the substrate was prepared by dissolving the 5mg tablet in 5ml of P-Nitrophenyl Phosphate (PNPP) substrate buffer and then 50µl substrate were added per well. The plates then were kept in the dark away from direct light until the colour developed. Optical densities (OD) at 405 nm then was measured using ELISA (Opsys MR Microplate Reader Data) and then analysed using DeltaSoft software (BioMetallics Inc, NJ). Sandoglobulin (Sandoz, UK) which contains high antibody IgG titres to sH1N1 and sH3N2 HA was used as a reference standard for measurement of antibodies to sH1N1 and sH3N2. A human convalescent serum from a subject with confirmed pH1N1 infection (BEI Resources, ATCC) was used as a standard for measurement of anti-pH1N1 HA antibodies. Both reference standards were assigned with an antibody titre of 5000 Units/ ml arbitrary.

2.7 Inhibition ELISA

ELISA plates were coated with 100 µl/well (2µg/ml) HA proteins of pH1N1 (A/California/04/2009), sH1N1 (A/Brisbane/59/2007), and aH5N1 (A/Vietnam/1203/2004), overnight at 4°C. Plates were washed 5 times with PBS containing 0.05% Tween-20 (Sigma-Aldrich) before that plate contents were discarded into a bucket containing 1% virkon and plates were blotted on a paper towel. Plates were blocked with150 µl/well blocking buffer PBS containing 10% FCS (Sigma-Aldrich) for one hour and half at room temperature. During the blocking step four serum samples with high antibody concentrations were co-incubated after dilution in the buffer PBS containing 10% FCS at 1:100, for inhibition step for an hour at room temperature using the corresponding HA protein. For example, HA of sH1N1 was used to inhibit (adsorb) anti-sH1N1 antibody in serum and all other HAs were tested as well. In contrast, heterologous HAs were used to adsorb antibody in

serum. For example, aH5N1 HA antigen was used to adsorb HA-specific to pH1N1 and so on. Different adsorbents concentrations including 10µg/ml, 5µg/ml, 0.1µg/ml and 0.0µg/ml, were used respectively. After incubation, the blocking buffer was discarded and the plates were blotted on a paper towels, 50µl/well adsorbed samples were added in duplicate and then the plates were incubated for one hour and half at room temperature. Plates were then washed 5 times with PBS containing 0.05% Tween-20 and then plates were blotted on a paper towel. Addition of 50µl/well alkaline phosphatase conjugated goat anti-human IgG (Sigma-Aldrich) (1:1000) and then incubated at room temperature for an hour. Plates were then washed 5 times with PBS containing 0.05% Tween-20 and plates were blotted on a paper towel. Finally, 50µl per well of substrate PNPP (1mg/ml) dissolved in substrate buffer was added. Optical density (OD) at 405 nm then was measured using ELISA plate reader (Opsys MR Microplate Reader Data).

2.8 Enzyme Linked Immunosorbent Spot (ELISpot) Assay

Although there are several assays established to measure antibody specificity and reactivity (e.g. ELISA and immunoblot), few of them focus directly on the antibody-secreting cell (ASC). One of these is the B-cell ELISpot, first designated in 1983 (Czerkinsky et al. 1983, Sedgwick and Holt 1983). A very sensitive method also at the cellular level of this system one can detect and count both the total number of ASC in addition to those secreting antibodies to a specific antigen. By performing B-cell ELISpot one can get information not simply accessible using other techniques. Moreover it has, for example, been performed to show the existence also frequencies of long-term memory B cells in the blood (Crotty et al. 2003).

The B-cell ELISpot is a useful method to analyse different features of the antibody immune response. It can be mostly appropriate in situations where a great degree of sensitivity is required otherwise when the response is best considered at the cellular level (Arlen et al. 2000). Principally, two main applications have been in the detection of B-cell responses to natural infections in addition to those provoked by vaccination (Kelly et al. 2006, Mamani-Matsuda et al. 2008).

In this thesis ELISpot assay was used to measure the frequencies of memory B cell to influenza viruses after stimulation of freshly isolated tonsillar MNC. The assay sensitivity is increased by using plates lined with polyvinylidene difluoride (PVDF) membrane which is the major step in making the assay to be the robust and high resolution assay nowadays. Additionally, PVDF membrane has several advantages. Firstly, it consists of dense uniform pore structure to support antibody binding also to increase sensitivity for better, sharper spot definition. Secondly, it allows the computerized analysis of the image of the formed spots (Zhang et al. 2009).

Following isolation of fresh adenotonsillar mononuclear cells (MNC), they were co-cultured with individual influenza virus antigens at 2µg/ml for all antigen stimulations. The influenza antigens included: pH1N1, sH1N1, sH3N2 and aH5N1 virus antigens which were derived from A/California/04/2009, A/Brisbane/59/2007, A/Brisbane/10/2007 and A/Vietnam/1203/2004 strains respectively (National Institute for Biological Standards and Control, NIBSC, UK). Tonsillar MNC were cultured in RPMI complete medium and incubated for five days in 5% CO_2 at 37°C. Cell culture supernatants were collected and kept at -70°C for further antibody production measurement by ELISA. Cells were harvested after washing by 0.05% BSA (Sigma-Aldrich) and then suspended in RPMI complete medium. 96-well

ELISPOT filter plates (Millipore) were coated and kept overnight at 4°C with pH1N1 (A/California/04/2009), sH1N1 (A/Brisbane/59/2007), sH3N2 (A/Brisbane/10/2007), aH5N1 (A/Vietnam/1203/2004) (Biodefence and Emerging Infections Research Resources Repository, ATCC (Manassas VA, USA)). The contents were discarded into 1% virkon and the plates were washed 3 times with PBS containing 0.05% Tween20. Following that the plates were blocked using RPMI complete medium for an hour. Blocking medium were then discarded and stimulated adenotonsillar MNC were added in triplicate with each well containing $1x10^5$ cells. The cells were incubated overnight at 37°C. Plates were washed three times with PBS containing 0.05% Tween-20, and then incubated with (50µl) of biotinylated anti-human IgG (γ) antibody (Invitrogen) for 30 minutes at room temperature. After washing three times by PBS containing 0.05% Tween-20, the plates were incubated with (50µl) an avidin-D-HRP conjugate (Vector Laboratories). Substrate was added (50µl) using AEC substrate (3 amino-9 ethylcarbazole (Sigma-Aldrich). Developed spots were scanned and analyzed using an automated ELISPOT counter (AID, Autoimmune Diagnostika GmbH,Germany).

2.9 Haemagglutination inhibition (HAI) assay

Haemagglutination inhibition (HAI) is the greatest broadly used assay for detection of antibodies to influenza viruses. The test was first developed by Hirst who incidentally discovered the capability of influenza viruses to agglutinate chicken red blood cells (RBCs) (Knossow and Skehel 2006).

The assay is complicated by the fact that all sera tested should be pre-treated with receptor-destroying enzyme (RDE) overnight to eliminate the various sialic acid-

containing glycans in sera which could bind to the virus HA and mimic the binding of influenza-specific anti- HA antibodies causing false positive results (Ito et al. 1997). Treatment with RDE is followed by heat inactivation of the RDE and serum complement. Furthermore, particular sera should also be adsorbed with RBCs to eliminate nonspecific agglutinins of RBCs to avoid a false-negative result (Subbarao et al. 1992, Katz et al. 2011).

In this work haemagglutination inhibition assays were performed following standard methods at the Microbiology Services-Colindale, Health Protection Agency (London, UK). The virus strains used included the following. Pandemic H1N1: NIBRG122 is a reassortant prepared from A/England/195/2009(H1N1v), the prototype UK isolate antigenically and genetically closely related to A/California/4/2009. Seasonal H1N1: A/H1N1/Brisbane/59/2007; seasonal H3N2: A/H3N2/Brisbane/10/2007; avian H5N1: NIBRG-14 is a reassortant prepared from A/H5N1/Vietnam/1194/2004 virus.

2.10 Influenza pseudotype virus production and neutralization assay

Virus neutralization is a very sensitive as well as specific technique for identifying strain-specific antibodies that inhibit virus entry or block virus replication, including HA-mediated fusion of the viral envelope and the endosomal membrane (Kida et al. 1985, Skehel and Wiley 2000).

As a substitute to using live virus in neutralization assays, retroviral vectors pseudotyped with influenza HA have been established. Pseudotyped virus particles could bind to cells expressing terminal sialic acid glycoproteins that work as

receptors, undergo the stages of viral entry, and subsequent reverse transcription and combination express a reporter gene such as luciferase (Nefkens et al. 2007, Temperton et al. 2007).

Unlike haemagglutination inhibition and neutralization assay, pseudotype neutralization assay does not need live virus as the HA gene can be produced for cloning into the expression plasmid (Stephenson et al. 2009). Haemagglutinin-pseudotyped particles are formed using retroviral vectors in a two or three-plasmid system (Hassantoufighi et al. 2010, Katz et al. 2011).

Influenza neutralization assay to screen serum samples for anti-aH5N1 virus was performed in Dr Temperton's laboratory in Kent University. The construction of lentiviral pseudotypes with an HA envelope glycoprotein derived from the highly pathogenic avian influenza virus H5N1 (A/Viet Nam/1194/04) (has been described by Temperton et al.2007). H1N1 HA expressing plasmids were constructed for A/Brisbane/59/2007 (H1N1) and A/South Carolina/1/18 (H1N1) using analogous methodologies. Pseudotype viruses were produced by co-transfection of HEK293T/17 cells with the respective HA plasmids, the HIV gag-pol plasmid p8.91, and the reporter plasmid pCSFLW (expressing firefly luciferase) using the Fugene-6 transfection reagent (Roche, UK). For the production of the H1N1 pseudotypes, protease expressing plasmid was additionally added to the transfection mixture (Corti et al. 2011).

The HA content was normalized via a surrogate readout of the firefly relative light units (RLU)/ml of each virus. For the virus neutralization assays, cell culture supernatant samples were two-fold serially diluted in culture medium and mixed with each pseudotype virus ($1x10^6$ RLU firefly luciferase input) at a 1:1 v/v ratio. After

incubation at 37°C for 1 hour, $1x10^4$ HEK293T cells were added to each well of a white 96-well flat-bottomed tissue culture plate. Firefly RLU was evaluated 72 hour later by luminometry using the Bright-Glo assay system (Promega, UK).

2.11 Naïve T cell depletion from MNC

2.11.1 Principle of naïve T cell depletion

First, the $CD45RA^+$ cells were magnetically labeled with CD45RA Microbeads. Then, the cell suspension was loaded onto a MACS® Column, which is placed in the magnetic field of a MACS Separator. The magnetically labeled $CD45RA^+$ cells were retained within the column. The unlabeled cells run through; this cell fraction is thus depleted of $CD45RA^+$ cells. After removing the column from the magnetic field, the magnetically retained $CD45RA^+$ cells can be eluted as the positively selected cell fraction. $CD45RA^+/RO^-$ cells were irradiated and used as feeder/accessory cells.

2.11.2 Reagent and instrument requirements

2.11.2.1 Buffer

Preparation of a solution containing phosphate-buffered saline (PBS), pH 7.2, 0.5% bovine serum albumin (BSA, Sigma Aldrich) was firstly applied.

2.11.2.2 MACS Columns and MACS Separators

$CD45RA^+$ cells were enriched by using LD Columns and using of Midi MACS, Quadro MACS.

2.11.2.3 Magnetic labeling

After adenotonsillar mononuclear cells (MNCs) were separated and washed with sterile PBS they were kept cold by placing them in ice (buffer containing 0.5% BSA, and using pre-cooled solutions as well). This will prevent capping of antibodies on the cell surface and non-specific cell labeling. Following that cell number was

determined. After that, cell suspension was centrifuged at 300×g for 10 minutes. And then aspiration of supernatant completely was applied. And then cell pellet was resuspended in 80 µl of buffer per 10^6 total cells. Adding of 20 µl of CD45RA Microbeads per 10^6 total cells was performed, then mixed well and incubated for 15 minutes in the refrigerator (2−8 °C). After that cells were washed by adding 1−2 ml of buffer per 10^6cells and centrifuged at 300×g for 10 minutes. Then the supernatant was aspirated and discarded. Finally, cells were resuspended up to 10^6cells in 500 µl of buffer and then proceed to magnetic separation.

2.11.2.4 Magnetic cell separations

After the tonsillar MNCs were resuspended, LD column was placed in the magnetic field of MACS Separator. Column was prepared by rinsing with 3 ml of buffer. Cell suspension was then applied onto the column. New sterile tube was used to collect unlabeled ($CD45RA^-$) cells that passed through and then the column was washed with the appropriate amount of buffer. The total effluent was then collected. Washing steps were performed by adding buffer three times. The column was removed from the separator and placed on a suitable collection tube. Following that appropriate amount of buffer was pipetted onto the column. The magnetically labeled cells were flushed out by firmly pushing the plunger into the column and the $CD45RA^+$ cells were collected for checking the purity of cell preparations.

2.12 Memory T cell depletion from MNC

2.12.1 Principle of memory T cell depletion

First the $CD45RO^+$ cells are magnetically labeled with CD45RO Microbeads. Then the cell suspension is loaded onto a MACS® Column which is placed in the magnetic field of a MACS Separator. The magnetically labeled $CD45RO^+$ cells are

retained on the column. The unlabeled cells run through the column which contains the cell fraction depleted of $CD45RO^+$ cells. After removal of the column from the magnetic field, the magnetically retained $CD45RO^+$ cells can be pushed out in the buffer using the plunger as the positively selected cell fraction

2.12.2 Reagent and instrument requirements

2.12.2.1 Buffer

Preparation of a solution containing phosphate-buffered saline (PBS), pH 7.2, 0.5% bovine serum albumin (BSA, Sigma Aldrich) was firstly applied.

2.12.2.2 MACS Columns and MACS Separator

$CD45RO^+$ cells were enriched by using LD Columns and using of Midi MACS, Quadro MACS.

2.12.3 Magnetic labeling

After adenotonsillar mononuclear cells (MNCs) were separated and washed with sterile PBS they were kept cold by placing them in ice (buffer containing 0.5% BSA, and using pre-cooled solutions as well). This will prevent capping of antibodies on the cell surface and non-specific cell labeling. Following cell number counting cell suspension was centrifuged at 300×g for 10 minutes. Cell pellet was resuspended in 80 μl of buffer per 10^6 total cells. 20 μl of CD45RO Microbeads per 10^6 total cells was added, mixed and incubated for 15 minutes in the refrigerator (2−8 °C). Cells were washed and then centrifuged at 300×g for 10 minutes. Cells were then resuspended up to 10^6 cells in 500 μl of buffer and proceed to magnetic separation.

2.12.4 Magnetic cell separation with LD Columns

After the tonsillar MNCs were resuspended, LD column was placed in the magnetic field of MACS Separator. Column was prepared by rinsing with 3ml of buffer. The cell suspension was applied onto the column. A sterile tube was used to collect

unlabeled naive ($CD45RO^-$) cells that passed through and then the column was washed with the appropriate amount of buffer. The column was removed from the separator and placed on a suitable collection tube. Fresh buffer was added onto the column and the magnetically labeled cells were flushed out by firmly pushing the plunger into the column. These $CD45RO^+$ cells were collected for staining purposes to check the efficiency of cell separation.

2.13 Flow cytometric analysis of cell surface antigens

Flow cytometry is an emerging technology that has numerous applications in immunology. The use and development of high-speed single-cell laser-based assays permits quantisation of a variety of cell populations such as lymphocytes, monocytes, etc.(Burchiel et al. 1999).

Forward scatter (FSC) is usually used as an approximate indicator of cell size, which permits, for example, distinction of erythrocytes from lymphocytes and monocytes of tested samples. Using a combination of FSC and side scatter (SSC) allows recognizing subsets of cells present in complex cell populations, such as blood, bone marrow, spleen, thymus, and lymph node single-cell preparations. The FSC is used to discriminate cell subsets based on size and is generally used to distinguish viable cells from dead cells, which also usually demonstrate low FSC (Benoist and Hacohen 2011).

Flow cytometry has proven to be advantageous from a clinical as well as diagnostic purpose and its use has considerably expanded. Moreover, immunophenotyping and intracellular flow cytometry have proven to be valuable added methods in immunology fields (Oliveira et al. 2008).

All analyses in this study were carried out on a BD FACScalibur flow cytometer (BD Biosciences). Multiple parameters were used to define the lymphocytes, including gating, based on FSC and SSC. Fluorescence emission was measured at different wavelengths: FL1: 519 nm (FITC), FL2: 578 nm (PE) and FL3: 695 nm (PerCP-Cy5.5). CellQuest software was used for flow cytometer data acquisition. Data analysis was performed using WinMDI 2.9 software (http://en.bio-soft.net/other/WinMDI.html).

2.14 Measurement of cell proliferation by CFSE staining and flow cytometry

To examine T cell proliferation, cells were stimulated with different antigens following CFSE staining of tonsillar MNC. After stimulation, cells were harvested in 0.02% BSA-PBS buffer (Sigma-Aldrich). Cells were washed and centrifuged at 400xg for 8 minutes. The supernatants were discarded into 1% virkon and the cell pellet was resuspended in 50 µl 0.02% BSA for surface staining. 5 µl of mouse anti-human CD4-PE-Cy5 and 5 µl of mouse anti-human CD8-PE (BD) were added to stain $CD4^+$ or $CD8^+$ T cells. The antibodies were incubated with the cells at 4°C for 30 minutes. Then the cells were washed twice with 500 µl 0.02% BSA to remove any unbound antibody and centrifuged for 8 minutes at 400g at 4°C. The cells were resuspended in 300 µl 0.02% BSA and then transferred to FACS tube (Falcon) before analysing them on the flow cytometer.

2.15 Carboxyfluorescein succinimidyl ester (CFSE) staining

Adenotonsillar mononuclear cells were prepared and resuspended in sterile PBS in 45 ml sterile tube (Sterling). CFSE was diluted from a stock solution of 5mM (stored -20°C) just before use by adding 5 µL stock to 10 ml sterile PBS. After that 3 ml of the preparation were added to the cells then kept into the incubator with 5% CO_2 at 37°C for 8 minutes. Addition of 10 ml ice cooled RPMI 1640 complete medium (Sigma-Aldrich) and then centrifuged at 400 x g for 10 minutes. The pellet was resuspended into 2 ml RPMI and cell number was counted by a haemocytometer. After adjusting cells concentration to 4 x10^{6} cells/ml, cells were ready for stimulation with different flu antigens and then incubated with 5% CO_2 at 37°C for four days. After incubation, cell culture supernatants were collected for cytokine analysis and cells harvested for T cell proliferation assay by flow cytometer (FACScan; Becton Dickinson).

2.16 Cytometric Bead Array (CBA) for cytokine measurement

The presentation of flow cytometric bead-based technology has added a novel method for investigators to measure several analytes in biological and environmental samples in a simultaneous condition. This innovative technology has several advantages. First, one can assess many analytes in one sample. Second, minimal sample volumes are required to acquire data. Third, it shows high reproducibility and a rapid evaluation of various samples in one platform (Morgan et al. 2004).

The CBA system uses the wide dynamic array of fluorescence detection afforded by flow cytometry and antibody-coated beads to proficiently capture analytes. Each bead in the array has distinctive fluorescence intensity therefore those beads could be

mixed and analysis performed instantaneously in a single tube. This method considerably lessens sample requirements as well as time to results in comparison with traditional techniques (Vignali 2000).

The CBA was used in this work to measure multiple cytokines. Tonsillar MNC were stimulated with different influenza A viruses and then T cell effector responses were measured in the cell culture supernatants for the presence of Th1, Th2, Th17 and proinflammatory cytokines responses. Each capture bead in a BD^{TM} CBA kit has been conjugated with a specific antibody. The detection reagent provided in the kit is a mixture of phycoerythrin (PE)-conjugated antibodies, which provides a fluorescent signal in proportion to the amount of bound analyte.

2.16.1 Principle of the assay

The BD™ CBA BD CBA Human Th1/Th2/Th17 Cytokine Kit can be used to measure Interleukin-2 (IL-2), Interleukin-4 (IL-4), Interleukin-6 (IL-6), Interleukin-10 (IL-10), Tumor Necrosis Factor (TNF), Interferon-γ (IFN-γ), and Interleukin-17A (IL-17A) levels in a single sample. The kit performance has been optimized for analysis of physiologically relevant concentrations (pg/mL levels) of specific cytokine proteins in tissue culture supernatants, EDTA plasma, and serum samples.

When the capture beads and detector reagent are incubated with an unknown sample containing recognized analytes, sandwich complexes (capture bead + analyte + detection reagent) are formed. These complexes can be measured using flow cytometry to identify particles with fluorescence characteristics of both the bead and the detector.

Step	Description
1	Preparation of Th1/Th2/Th17 Cytokine Standards.
2	Mixing Human Th1/Th2/Th17 Cytokine Capture Beads.
3	Diluting samples.
4	Performing instrument setup with Cytometer Setup Beads.
5	Performing the Th1/Th2/Th17 Assay.
6	Acquiring samples.
7	Data analysis.

Table 2.1: **The overall workflow steps for CBA assay.** The table shows the steps for performing the CBA assay as recommended from the manufacturer.

Procedure	Incubation time
Preparing standards	15 minutes
Preparing Capture Beads	30 minutes
Preparing Cytometer Setup Beads	30 minutes
Performing the assay	3 hours

Table 2.2: Summary of the incubation time needs for performing the CBA assay. The table shows the incubation time summary list of the protocol.

2.16.2 CBA Procedure

After determining the number of assay tubes (including standards and controls), Capture Bead suspensions were vigorously vortexed for a few seconds before mixing them with the cell culture supernatants. Addition of a 50-µL aliquot of each Capture Bead, for each assay tube to be analyzed, into a single tube labelled "mixed Capture Beads" (e.g., 10 µl of IL-2 Capture Beads) and then the tubes containing specific capture beads were vortexed thoroughly. Following that, 50 µl of controls and samples were transferred to the tubes and then addition of 50 µl of the Human Th1/Th2 - II PE Detection Reagent to all assay tubes. All tubes were incubated for 3 hours at room temperature, protected from light. One mL of Wash Buffer to each assay tube was added and all tubes were centrifuged at 200xg for 5 minutes. Carefully aspiration and discarding of the supernatant from each assay tube was followed by the addition of 300 µl of Wash Buffer to each assay tube to resuspend the bead pellet. Finally, sample acquisition was applied using Becton Dickinson FACScalibur flow cytometer and then data were analysed using the BD^{TM} CBA Analysis Software.

2.17 RNA extraction

Nasal swabs were kept at -80°C after taking the sample from the patients. Extraction of nasal swab was carried out using QIAamp Viral RNA Mini Kit (QIAGEG) the swab samples were thawed after being kept at - 80°C. To set up the procedure, 560µl of prepared AVL containing carrier RNA was added into each 1.5 ml microcentrifuge tube (Starlab) and mixed with 140µl from nasal swab samples. After that the samples were pulse-vortexed for 15 seconds. After vortexing the tubes were

placed for 10 minutes at room temperature and then briefly centrifuged to remove drops from the inside of the lid. Following the incubation, 560µl of ethanol (96-100%) was added to each sample followed by pulse-vortexing for 15 seconds and then they were briefly centrifuged. Following that 630 µl of the preparation was applied to the QIAamp Mini column and centrifuged at 6,000 x g (8000 rpm) for 1 minute. After that, the QIAamp Mini column was transferred to a clean 2 ml collection tube. The previous step was repeated with the remaining volume of the preparation. Following that, 500 µl of buffer AW1 was added with new 2 ml clean tubes and centrifuged again at 6,000 x g (8000 rpm) for a minute. Following that 500 µl of buffer AW2 was added with new 2 ml clean tubes and centrifuged another time at 20,000 x g (8000 rpm) for three minutes. After that, the QIAamp Mini column was placed in a clean 1.5ml sterile Eppendorf tube after discarding the old collection tube containing the filtrate. The volume of 60 µl buffer AVE was added to each tube then incubated for 1 minute at room temperature. Finally, all the tubes were centrifuged at 6,000 x g (8,000 rpm) for 1 minute and the extracted RNA samples were stored at -80 °C for the next RT-PCR steps.

2.18 One step Quantitative Real Time PCR (qRT-PCR)

After the extraction of viral RNA, the qRT-PCR was applied using (BEI resources kit) for detection of seasonal H1N1, swine influenza H1N1 and seasonal H3N2 viruses.

These protocols were optimized using quantitative one-step probe RT-PCR (Invitrogen SuperScript™III Platinum® One-Step Quantitative Kit) that have been

shown to produce comparable results on 96-well format thermocycler systems Applied Biosystems ™ real-time PCR systems 7300.

Because of the sensitivity of fluorogenic 5' nuclease assays, special precautions have been taken to avoid false positive amplifications such as maintaining separate areas for assay setup and handling of nucleic acids. Also, wearing a clean lab coat and powder-free disposable gloves (not previously worn) when setting up assays, changing gloves between samples and whenever suspected they may be contaminated and keeping reagent and reaction tubes capped or covered as much as possible.

2.18.1 Primers and probes

According to WHO slandered RT-PCR assay was applied (http://www.who.int/csr/resources/publications/swineflu/WHO_Diagnostic_RecommendationsH1N1_20090521.pdf). Frozen aliquots of primer and probes (BEI resources) were thawed (thawed aliquots of probes may be stored in the dark up to 3 months at 2-8°C) ,and then vortexed, briefly centrifuged and placed in a cold rack. Table (2.3) contains all primers and probes used during the RT-PCR assay. Amplification and detection was performed using an ABI Biosystems 7500 machine.

2.18.2 Real time RT-PCR reagents

Master Mix and enzyme were placed in a cold rack, the 2X Reaction Mix vial was thawed, mixed by inversion and that and the enzyme was briefly centrifuging and placed in a cold rack.

Primers and Probes	Sequence (5'-3')	Working Concentration
InfA Forward	GAC CRA TCC TGT CAC CTC TGA C	40 µM
InfA Reverse	AGG GCA TTY TGG ACA AAK CGT CTA	40 µM
InfA Probe[1]	TGC AGT CCT CGC TCA CTG GGC ACG	10 µM
SW InfA Forward	GCA CGG TCA GCA CTT ATY CTR AG	40 µM
SW InfA Reverse	GTG RGC TGG GTT TTC ATT TGG TC	40 µM
SW InfA Probe[2]	CYA CTG CAA GCC CA"T" ACA CAC AAG CAG GCA	10 µM
SW H1 Forward	GTG CTA TAA ACA CCA GCC TYC CA	40 µM
SW H1 Reverse	CGG GAT ATT CCT TAA TCC TGT RGC	40 µM
SW H1 Probe[2]	CA GAA TAT ACA "T"CC RGT CAC AAT TGG ARA A	10 µM
RnaseP Forward	AGA TTT GGA CCT GCG AGC G	40 µM
RnaseP Reverse	GAG CGG CTG TCT CCA CAA GT	40 µM
RnaseP Probe[1]	TTC TGA CCT GAA GGC TCT GCG CG	10 µM

Table 2.3: Primers and probes were used during performing the RT-PCR.

1: TaqMan® probes are labeled at the 5'-end with the reporter molecule 6-carboxyfluorescein (FAM) and with the quencher, Blackhole Quencher 1 (BHQ1) (Biosearch Technologies, Inc., Novato, CA) at the 3'-end. 2: Taqman® probes are labeled at the 5'-end with the reporter molecule 6-carboxyfluorescein (FAM) and quenched internally at a modified "T" residue with BHQ1, with a modified 3'- end to prevent probe extension by Taq polymerase (WHO).

2.18.3 Tests for each RT-PCR run

Each sample RNA extract was tested by separate primer/probe sets: InfA, Universal swine (swFluA), Swine H1 (swH1) and RNaseP (RP). The RNaseP primer and probe set targets the human RNase P gene and thus serves as an internal positive control for human nucleic acid. The No template controls (NTC) and positive template controls (PTC) for all primer/probe sets were included in each run. The Human Specimen Control (HSC) provides a secondary negative control that validates the nucleic extraction procedure and reagent integrity was applied as well.

2.18.4 Reaction setup

Reaction assay mixtures were made as a cocktail and dispensed into the 96-well reaction plate. Water and extracted nucleic acid or positive template controls were then added to the appropriate test reactions and controls. Labelling of one 1.5 ml microcentrifuge tube for each primer/probe set was applied. Determining of the number of reactions (N) to set up per assay was taken into account, it is necessary to make excess reaction cocktail to allow for the NTC, PTC, HSC reactions and pipetting error. After that the master Mix was prepared as well as calculating of the amount of each reagent to be added for each primer/probe set reaction master mix. The calculations are as shown in the table (2.4)

After addition of the water, the reaction mixtures were mixed by pipetting up and down. After that centrifuge for 5 second to collect contents at bottom of the tube, and then the tubes were placed in a cold rack. And then setting up plates in 96-well (Applied Biosystems) cooler rack dispenses 20µl of each master mix into each well.

Reagents	**Volume of reagents added per reaction**
Nuclease-free water	Number of samples X 5.5 µl
Forward primer	Number of samples X 0.5 µl
Reverse primer	Number of samples X 0.5 µl
Probe	Number of samples X 0.5 µl
SuperScript™ III RT/Platinum® *Taq* Mix	Number of samples X 0.5 µl
2X PCR Master Mix	Number of samples X 12.5 µl
Total volume	Number of samples X 20 µl

Table 2.4: The calculations of the master mix and reagents used to perform the RT-PCR. The table shows the calculation of the master mix should be used to perform the RT-PCR according to the manufacturer.

Before moving the plate to the nucleic acid handling area, setting up the NTC reactions for column 1 in the assay set-up area was applied. After that samples were added by column by pipetting of 5 µl of nuclease free water into the NTC wells, and then the reaction plate was covered and moved to the nucleic acid handling area. After that the tubes containing the samples were vortexed for 5 second and then centrifuged for 5 second. After setting up the extracted nucleic acid samples in the cold rack, samples were added by column. Pipetting 5 µl of the first sample into all the wells labelled for that sample. All samples were added in quadruplicate. Addition of 5 µl of HSC extracted sample to the HSC wells (column 11). Finally, pipetting of 5 µl of positive template control RNA into all PTC wells was performed. After that the plates were centrifuged at 500 x g for 30 seconds at 4°C and returned to the cold rack.

	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**
A	NTC	S1	S3	S5	S7	S9	S11	S13	S15	S17	S19	PTC
B	NTC	S1	S3	S5	S7	S9	S11	S13	S15	S17	S19	PTC
C	NTC	S1	S3	S5	S7	S9	S11	S13	S15	S17	S19	PTC
D	NTC	S1	S3	S5	S7	S9	S11	S13	S15	S17	S19	PTC
E		S2	S4	S6	S8	S10	S12	S14	S16	S18	HSC	
F		S2	S4	S6	S8	S10	S12	S14	S16	S18	HSC	
G		S2	S4	S6	S8	S10	S12	S14	S16	S18	HSC	
H		S2	S4	S6	S8	S10	S12	S14	S16	S18	HSC	

Table 2.5: Layout of samples and controls used to perform the qRT-PCR. The table shows the template used for running the samples and controls to perform the qRT-PCR as suggested from the manufacturer.

2.18.5 RT-PCR amplification conditions

The reaction volume was 25µl for all samples and controls. The 7300 Applied Biosystems ™ real-time PCR system was programmed as shown table (2.6)

Reverse Transcriptase	50°C for 30 minutes
Taq inhibitor activation	95°C for 2 minutes
PCR amplification (45 cycles)	95°C for 15 second 55°C 30 seconds*

Table 2.6: The program was used in performing the RT-PCR. The table shows the program should be used before performing the test according the manufacturer. ***** Fluorescence data (FAM) should be collected during the 55°C incubation step.

Chapter 3

HA-Specific Memory B cell Responses to Influenza Viruses following the 2009 H1N1 Pandemic

3.1 Introduction

Influenza is a highly contagious and acute respiratory infection caused by influenza virus in the mucosa of the respiratory tract (Murphy et al. 1996). Both seasonal and pandemic influenza continue to cause substantial morbidity and mortality in humans. The 2009 pandemic H1N1 (pH1N1) influenza and the potential of a highly pathogenic avian H5N1 (aH5N1) pandemic highlighted the need for effective preventative strategies. Understanding the development of natural immunity following the pH1N1 pandemic may provide important information on host protective immunity in humans, which could inform future vaccination strategies against influenza.

The pH1N1 virus was antigenically different from seasonal H1N1 (sH1N1) viruses, and affected large population groups who were immunologically naïve to the virus (Brockwell-Staats et al. 2009,Dawood et al. 2009, Hancock et al. 2009). Little is known on the development of immunological memory following the pH1N1 infection, how it interacts with other influenza viruses, and whether this memory provides any protective immunity against aH5N1 virus, a pathogen with considerable potential to cause future pandemic.

Surface haemagglutinin (HA) is a major virulence factor crucial for virus binding to host cell membrane, and essential in the induction of host protective immunity. HA-specific antibodies play a key role in protection against influenza (Puck et al. 1980, Simmons et al. 2007). During the 2009 pH1N1 pandemic, older people (>65 years) were protected because they had existing anti-HA antibodies induced by previous exposure to antigenically related H1N1 strains e.g. pandemic A/H1N1 1918 virus or

strains circulating before 1957 (Hancock et al. 2009, Ichinohe et al. 2009, Miller et al. 2010).

Structurally, HA consists of two domains: a globular head, composed of part of HA1, and a stalk structure, composed of portions of HA1 and all of HA2 (Hai et al. 2012). The globular head contains the variable region of HA and is the major target for neutralizing antibodies that inhibit virus binding to target cells. These neutralizing antibodies are traditionally detected by haemagglutination inhibition assay (HAI). The stalk domain is more conserved. Recent studies have suggested that antibodies targeting the stalk region may also have neutralizing activity and may contribute to the cross-reactive immunity to different influenza viruses induced by either infection or vaccination (Corti et al. 2010, Brokstad et al. 1995, Pica et al. 2012, Wrammert et al. 2011). There are 16 different influenza subtypes of HA and they are clustered into two groups based on the molecular relatedness of the HA sequences. group 1 (H1, H2, H5, H6, H8, H9, H11, H12,H13 and H16) and group 2 (H3, H4, H7, H10, H14 and H15) (Air 1981).

Influenza virus is transmitted through airborne droplets and infects human nasopharyngeal mucosa. Human adenoids and tonsils are major components of nasal-associated lymphoid tissues (NALT) which are considered to be an important part of the mucosal immune system (Bernstein,Gorfien, and Brandtzaeg. 1999,Kiyono and Fukuyama 2004, Wu and Russell 1997).

However, studies have shown there are some major differences between human NALT in the nasopharynx and other mucosal compartments such as Peyer’s patches in the intestine. B cells in the former predominantly produce IgG, whereas the majority of B cells in the latter produce IgA (Boyaka et al. 2000b, Nurkka et al.

2000). It has been demonstrated previously that pneumococcal protein antigens elicited a predominantly IgG memory B cell response in human NALT (Zhang et al. 2010a, Zhang et al. 2002b). The NALT tissues are considered to be important induction sites for both mucosal and systemic immunity against upper respiratory pathogens including influenza (Guthrie et al. 2004a, Kiyono and Fukuyama 2004, Wiley et al. 2001,Zuercher et al. 2002). The induction of immunological memory against influenza virus most likely involves these immunocompetent NALT tissues, where antigen-specific memory B cells are primed.

However, limited data exist on the development and function of such memory B cells in humans. Recent studies using monoclonal antibodies from B cells isolated from patients infected with either the 1918 or 2009 pandemic H1N1 viruses suggest the presence of memory B cells (Krause et al. 2010, Xu et al. 2010, Yu et al. 2008). It was also reported that some HA-specific monoclonal antibodies isolated from these patients were cross-reactive with the stalk regions of HAs of a number of different influenza strains (Li et al. 2012, Wrammert et al. 2011).

In this study, we investigated the HA-specific memory B cell responses in human NALT to pH1N1, sH1N1, sH3N2 and aH5N1 viruses. We demonstrate that patients who had serological evidence of previous exposure to pH1N1 virus showed memory B cell response in NALT that produce cross-reactive neutralizing antibodies against a number of influenza subtypes upon pH1N1 virus antigen stimulation. The result suggests the 2009 pH1N1 infection primed human host with cross-reactive mucosal memory response against other H1N1 and the highly pathogenic aH5N1 virus strains. These findings may have important implications in future vaccination strategies against influenza.

3.2 AIMS OF STUDY

To investigate the frequency of HA-specific memory B cells in human NALT to influenza viruses following 2009 H1N1 pandemic influenza and their ability to produce cross-reactive antibodies.

3.3 EXPERIMENTAL DESIGN

Adenotonsillar MNCs were stimulated with different influenza antigens. HA-specific memory B cell responses were measured using ELISpot and cell culture supernatants were analysed for antibody production by ELISA. Additionally, HA-specific antibodies were further analysed using HAI and influenza virus neutralization assays.

3.3.1 Patients and samples

Adenoids and tonsils were obtained from children and adults (3–30 years) undergoing adenoidectomy and/or tonsillectomy between Mar 2011-Mar 2012. A venous blood sample was obtained. Patients previously vaccinated against influenza or who were immunocompromised in any way were excluded. The study was approved by the local ethics committee (Liverpool Paediatric Research Ethics Committee) and written, informed consent obtained from each patient/parent as appropriate.

3.3.2 Influenza virus antigens

Influenza antigens used for cell stimulation experiments were β-propiolactone inactivated, partially purified whole virus antigens from National Institute for Biological Standards and Control (NIBSC, UK) following a standard procedure as described previously (Wood et al. 1977). The pH1N1, sH1N1, sH3N2 and aH5N1

virus antigens were derived from A/California/04/2009, A/Brisbane/59/2007, A/Brisbane/10/2007 and A/Vietnam/1203/2004 strains respectively.

3.3.3 Recombinant HAs

Purified recombinant HA proteins of pH1N1 (A/California/04/2009), sH1N1 (A/Brisbane/59/2007), sH3N2 (A/Brisbane/10/2007), aH5N1 (A/Vietnam/1203/2004), H2N2 (A/Singapore/1/57), and H7N3 (A/Canada/RV444/04) were from Biodefence and Emerging Infections Research Resources Repository, ATCC (Manassas VA, USA). The recombinant HAs of pH1N1 and sH1N1 contain a C-terminal histidine tag and were produced in High Five™ insect cells using a baculovirus expression vector system (Stevens et al. 2004). The HAs were purified from cell culture supernatant by Immobilized-metal affinity chromatography (IMAC) and contain a trimerizing (foldon) domain (Stevens et al. 2004). The recombinant HAs of sH3N2 and aH5N1, H2N2 and H7N3 viruses were full length glycosylated HA that were produced in Sf9 insect cells using a baculovirus expression vector system, and membrane-extracted from infected cells and purified under native conditions by affinity chromatography that preserve their biological activity and tertiary structure. The purified HA forms trimers (Smith et al. 1988).

3.3.4 Cell separation

Adenoidal and tonsillar tissues were transported to the laboratory in Hanks buffered salt solution supplemented with glutamine and antibiotics (penicillin 100 U/ml and streptomycin 100 μg/ml). Mononuclear cells (MNC) from adenoids and tonsils were isolated using Ficoll density centrifugation following methods described previously (Zhang et al. 2010a, Zhang et al. 2011).

3.3.5 Cell culture and stimulation by influenza virus antigens

Adenotonsillar MNC were cultured at 4 x 10^6/ml in RPMI medium containing glutamine, penicillin, streptomycin and 10% fetal bovine serum (FBS), with and without a predetermined optimal concentration of influenza virus antigens. For enumerating antibody secreting cells (ASC) by enzyme-linked immunospot (ELISpot) assay, adenotonsillar MNC were cultured for 5 days before being transferred to ELISpot plates. Cell culture supernatants were collected at day 7 and stored at –70°C until assay for measuring antibodies by ELISA. Paired experiments in adenoidal and tonsillar MNC revealed no difference in memory B cell responses activated by influenza virus antigens.

3.3.6 Measurement of memory B cell response by ELISpot assay

HA-specific memory B cell responses following individual virus antigen stimulations were analyzed using ELISpot assay to enumerate HA-specific ASC as described previously (Crotty et al. 2003). Briefly, ELISpot plates (Millipore, UK) were coated overnight with optimized concentrations of recombinant HAs in PBS. Plates were washed and blocked by incubation with RPMI containing 10% FBS at 37°C for 2 hours. Antigen-stimulated MNC were added to the plates and incubated overnight at 37°C. Plates were washed and incubated with biotinylated anti-human IgG/IgA antibody (Invitrogen, UK) for 30 minutes at room temperature. After washing, avidin-D-HRP conjugate (Vector Laboratories) was added and incubated. Coloured spots were developed with the addition of substrate (3-amino-9-ethylcarbazole, Sigma) and counted using an automated ELISpot reader (AID, Autoimmune Diagnostika GmbH, Germany). The ELISpot assay shows the predominance of HA-

specific IgG memory B cell responses following stimulation by influenza virus antigens, so only IgG ASC results are shown.

3.3.7 Measurement of HA-specific antibodies by ELISA

HA-specific IgG antibodies were analyzed following a standard ELISA procedure as previously described (Zhang et al. 2006). In brief, ELISA plates were coated with recombinant HAs and incubated overnight at 4°C. After washing, plates were blocked with 10% FBS followed by incubation of cell culture supernatants at predetermined optimized dilutions for 1.5 hour. Alkaline phosphatase conjugated anti-human IgG (Sigma) was then incubated for 1.5 hour. After washing, p-nitrophenyl phosphate substrate was applied. Optical density was measured at 405 nm and data were analyzed using DeltaSoft microplate analysis software (BioMetallics Inc, NJ).

Sandoglobulin (Sandoz, UK) which contains high antibody titres to sH1N1 and sH3N2 HA was used as a reference standard for measurement of antibodies to sH1N1 and sH3N2. A human convalescent serum from a subject with confirmed pH1N1 infection (BEI Resources, ATCC) was used as a standard for measurement of anti-pH1N1 HA antibodies. Both reference standards were assigned with an antibody titre of 5000 Units/ ml arbitrarily.

3.3.8 Haemagglutination inhibition (HAI) assay

Haemagglutination inhibition assays were performed following standard methods (Miller et al. 2010) at the Microbiology Services Colindale, Health Protection Agency (London, UK). The virus strains used included the following. Pandemic H1N1: NIBRG122 is a reassortant prepared from A/England/195/2009(H1N1v), the prototype UK isolate antigenically and genetically closely related to

A/California/4/2009. Seasonal H1N1: A/H1N1/Brisbane/59/2007; seasonal H3N2: A/H3N2/Brisbane/10/2007; avian H5N1: NIBRG-14 is a reassortant prepared from A/H5N1/Vietnam/1194/2004 virus**.**

3.3.9 Influenza pseudotype virus production and neutralization assay

The construction of lentiviral pseudotypes with an HA envelope glycoprotein derived from the highly pathogenic avian influenza virus H5N1 (A/Viet Nam/1194/04) has been described previously (Temperton et al. 2007). H1N1 HA expressing plasmids were constructed for A/Brisbane/59/2007 (H1N1) and A/South Carolina/1/18 (H1N1) using analogous methodologies.

Pseudotype viruses were produced by co-transfection of HEK293T/17 cells with the respective HA plasmids, the HIV gag-pol plasmid p8.91, and the reporter plasmid pCSFLW (expressing firefly luciferase) using the Fugene-6 transfection reagent (Roche, UK). For the production of the H1N1 pseudotypes, protease expressing plasmid was additionally added to the transfection mixture. The HA content was normalized via a surrogate readout of the firefly relative light units (RLU)/ml of each virus. For the virus neutralization assays, cell culture supernatant samples were two-fold serially diluted in culture medium and mixed with each pseudotype virus ($1x10^6$ RLU firefly luciferase input) at a 1:1 v/v ratio. After incubation at 37°C for 1 hour, $1x10^4$ HEK293T cells were added to each well of a white 96-well flat-bottomed tissue culture plate. Fireflies RLU were evaluated 72 hour later by luminometry using the Bright-Glo assay system (Promega, UK).

3.3.10 Statistical analysis

Differences in memory response or antibody titres between different groups were analyzed by analysis of variance and Student's t-test. Association between two factors was analyzed by Pearson’s correlation. A p value of <0.05 was considered statistically significant. Statistical analysis was performed using SPSS software (version 16).

3.4 Results

3.4.1 2009 pH1N1 virus induces memory B cell responses that cross-react with sH1N1 and aH5N1 viruses

To analyze pH1N1 HA-specific memory B cell response in tonsillar MNC, ELISpot assay was performed to enumerate numbers of antigen-specific ASC to HA after stimulation with pH1N1 virus antigen. Large numbers (mean ASC/10^6 MNC: 50.0) of HA-specific IgG ASC to pH1N1 were found after pH1N1 virus antigen stimulation in subjects with serum anti-pH1N1 HAI titre ≥40, whereas minimal numbers (5.5) of ASC were seen in those with low HAI titres (<40) (figure 3.4.1a,b, $p<0.01$). In contrast, when the subjects were divided into two groups with serum HAI titres<40 and ≥40 against either sH1N1 or sH3N2 viruses, there was no difference in the numbers of pH1N1 HA specific IgG ASC after pH1N1 antigen stimulation between the two groups (figure 3.4.1f, $p>0.05$). To determine whether this pH1N1 HA-specific memory B cell response cross-reacted with other influenza A subtypes, memory B cell responses to sH1N1, sH3N2 and aH5N1 HAs were also analyzed following tonsillar MNC stimulation with pH1N1 virus antigen. Numbers of HA specific IgG ASC to sH1N1 and aH5N1 after the antigen stimulation in subjects with serum anti pH1N1 HAI titre ≥40 were significantly higher than in those who had anti-pH1N1 HAI titre <40 (36.2 vs. 8.7 and 35.0 vs. 6.1 for anti-sH1N1 and aH5N1 ASC respectively) (figure 3.4.1c,d, $p<0.01$). Further analysis revealed a good correlation ($r=0.73$, $p<0.001$) between the number of HA-specific ASC to pH1N1 and that to sH1N1 virus (Figure 3.4.1e). However, no difference was found in the number of specific IgG ASC to sH3N2 HA after pH1N1 virus antigen stimulation between subjects with anti-pH1N1 HAI titre ≥40 and those with HAI titre <40 (Figure 3.4.1c).

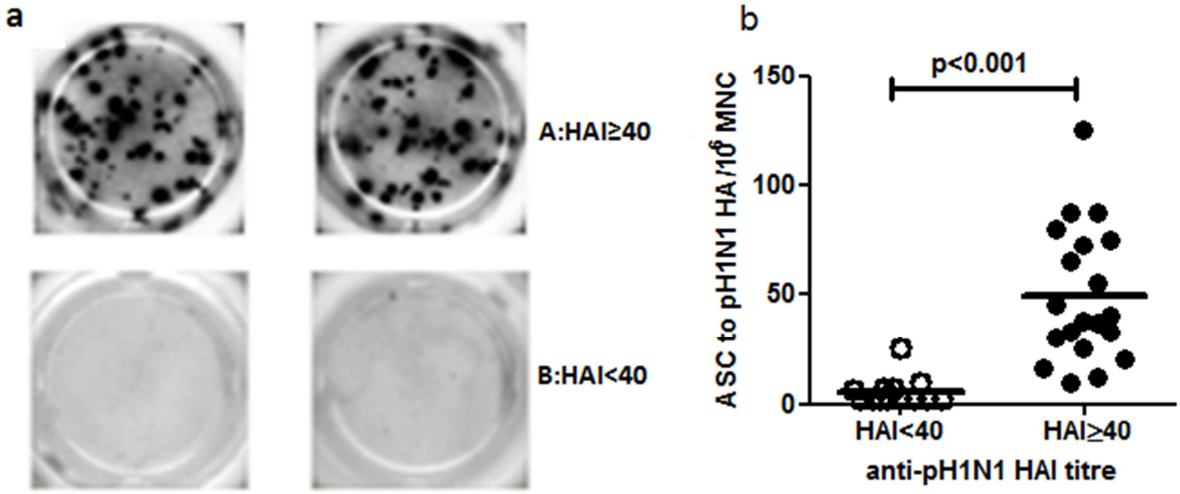

Figure 3.4.1(a, b):pH1N1 virus antigen induces a strong HA-specific memory B cell response. Numbers of HA-specific IgG ASC in tonsillar MNC enumerated by ELISpot assay after stimulation by pH1N1 virus antigen in subjects with serum anti-pH1N1 HAI titre ≥40 (n=20) and low HAI titres (<40, n=14) (a+b). 1a, A and B: representative samples from patients with HAI≥40 and HAI<40 respectively. 1b: lines represent the mean of number of HA-specific ASC ($p<0.001$ compared with those with HAI<40).

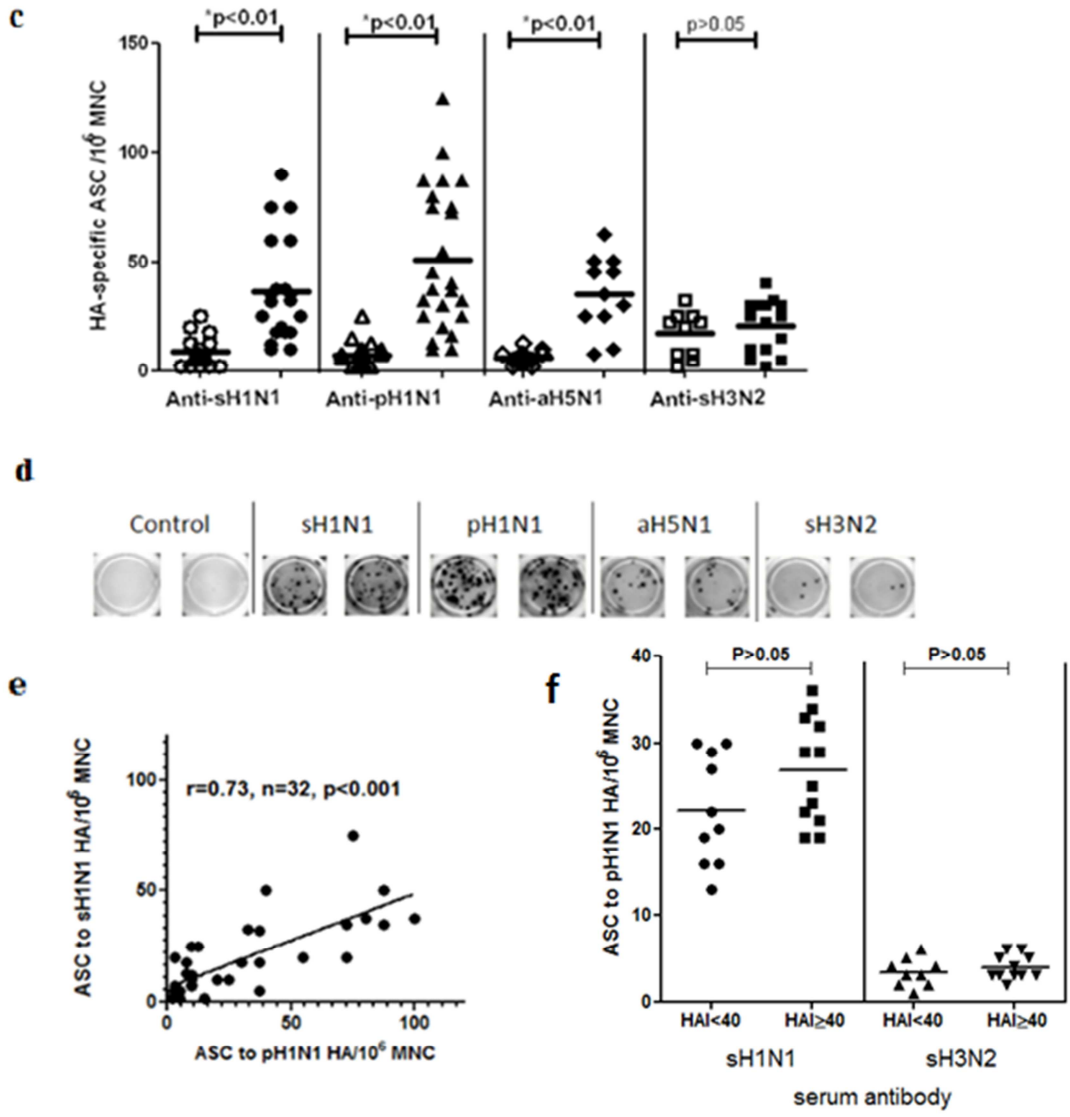

Figure 3.4.1(c,d,e and f): pH1N1 virus antigen elicits memory B cell responses that cross-react with sH1N1 and avian H5N1 viruses. The magnitude of HA-specific IgG memory B cell responses in tonsillar MNC to sH1N1 (o) pH1N1 (Δ), aH5N1 (♢) and sH3N2 (□) were analyzed after pH1N1 virus antigen stimulation, and compared between subjects with serum anti-pH1N1 HAI titre ≥40 (filled symbols) and those with HAI<40 (open symbols) (c, *p<0.01). ELISpot images of HA specific ASC to sH1N1, pH1N1, aH5N1 and sH3N2 in tonsillar MNC from one representative patient after pH1N1 antigen stimulation; control: negative control with no specific HA antigen coating in ELISpot assay (d). There was a good correlation between numbers of HA-specific ASC to pH1N1 and that to sH1N1, after pH1N1 antigen stimulation (e, r=0.73, p<0.001). When the subjects were divided into two groups with serum HAI titres<40 and ≥40 against sH1N1 and sH3N2 viruses, there was no difference in the numbers of pH1N1 HA specific IgG ASC after pH1N1 antigen stimulation between the two groups (f, p>0.05).

3.4.2 pH1N1 virus elicits stronger cross-reactive memory B cell responses than sH1N1 and sH3N2 virus antigens

To compare pandemic and seasonal influenza A induced memory B cell responses and their cross-reactivity, HA-specific memory B cell responses in tonsillar MNC following stimulation with sH1N1 and sH3N2 virus antigens were analyzed. Stimulation with the sH1N1 virus antigen (A/Brisbane/59/2007) elicited a modest increase in the number of HA-specific ASC to sH1N1 (mean ASC/10^6 MNC: 24.8) and pH1N1 (26.5), but no increase in the number of specific ASC to sH3N2 and aH5N1 HAs (Figure 3.4.2a), neither in patients with anti-sH1N1 HAI≥40 nor in those with anti-sH1N1 HAI<40 (data not shown). This was in contrast to the stronger cross-reactive memory B cell responses elicited by pH1N1 antigen stimulation, not only to pH1N1 (50.8), but also to sH1N1(38.0) and aH5N1 (32.7) viruses in patients with anti-pH1N1 HAI titre ≥40 (Figure 3.4.2a). Nevertheless, there was a positive correlation between pH1N1 HA-specific memory B cell response activated by the pH1N1 antigen and sH1N1 HA-specific memory response elicited by the sH1N1 antigen stimulation (Figure 3.4.2b, $r=0.88$, $p<0.001$). In comparison, stimulation with the sH3N2 antigen did not induce an increase in the number of HA-specific ASC to the sH1N1, pH1N1and aH5N1, although it did induce a strong increase in the number of ASC to sH3N2 HA (49.6) (figure 3.4.2c).

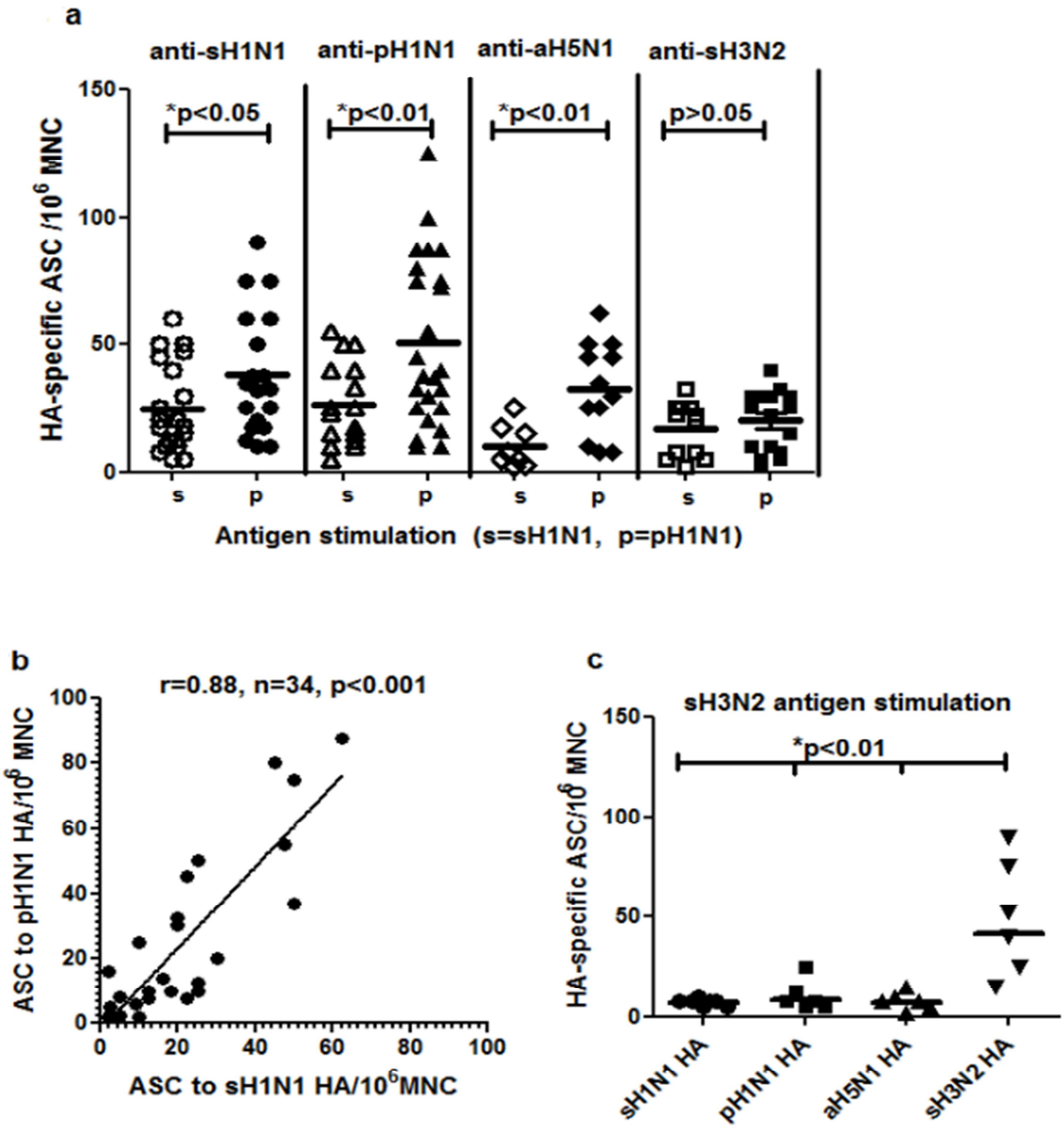

Figure 3.4.2: pH1N1 virus elicits a stronger cross-reactive memory B cell response than seasonal H1N1 virus antigen. HA-specific IgG memory B cell responses to sH1N1 (o) pH1N1 (Δ), aH5N1 (♢) and sH3N2 (□) in tonsillar MNC was analysed and compared between sH1N1 antigen (open symbols) and pH1N1 antigen-induced response (filled symbols) in subjects with serum anti-pH1N1 HAI titre ≥40 (a). A good correlation was shown between HA-specific memory B cell responses to pH1N1 and sH1N1 virus induced by pH1N1 and sH1N1 antigen stimulation respectively (b). sH3N2 virus antigen stimulation induced HA-specific memory B cell response to H3N2 but not H1N1 and H5N1 viruses (c, n=6). Horizontal bars represent the mean number of HA-specific ASC.

3.4.3 Avian H5N1 virus antigen elicits a similar cross-reactive memory B cell response as pH1N1 antigen

We reasoned if pH1N1 infection in patients induced memory B cells cross-reactive to aH5N1, these cells should mount a memory response upon an antigenic challenge by aH5N1 virus. Tonsillar MNC were stimulated with aH5N1 virus antigen followed by analysis of HA-specific ASC. Indeed, this stimulation elicited memory B cell responses with mean numbers of IgG ASC to sH1N1 (20.8) and pH1N1 HAs (50.8), similar to that induced by pH1N1 antigen, in patients who had an anti-pH1N1 HAI titre $\geq$40 (Figure 3.4.3a). A moderate response to aH5N1 (14.6) but not sH3N2 HA was also observed in these patients (Figure 3.4.3a). Figure 3.4.3b shows that the numbers of pH1N1 HA-specific ASC elicited by aH5N1 antigen stimulation correlated well with that elicited by pH1N1 antigen stimulation ($r=0.85$, $p<0.01$). No significant ASC response to HA of any of the four viruses was found after aH5N1 antigen stimulation in subjects with an anti-pH1N1 HAI titre<40.

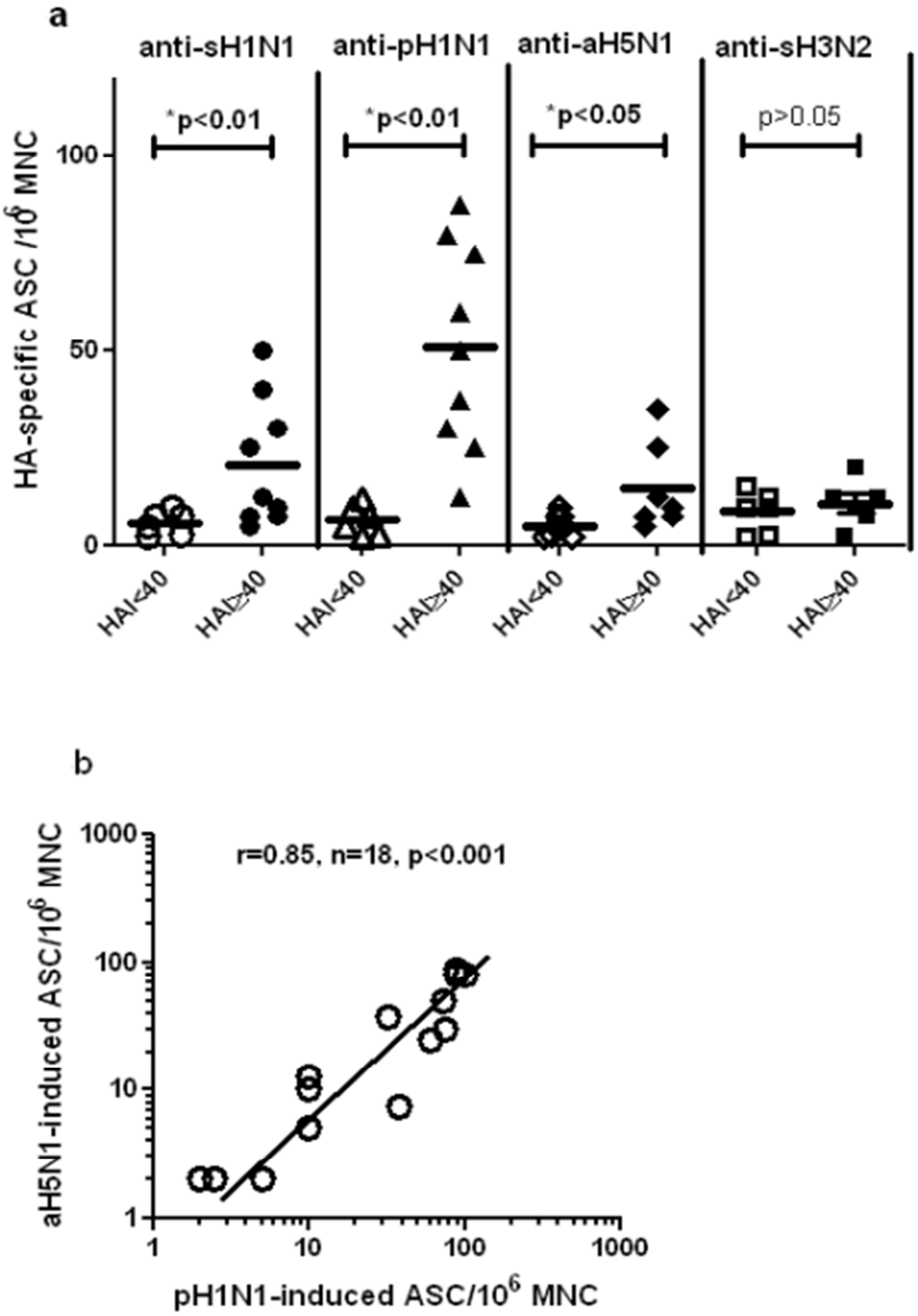

Figure 3.4.3: Avian H5N1 virus antigen elicits cross-reactive memory B cell responses. HA specific IgG memory B cell responses in tonsillar MNC to HAs of sH1N1 (o) pH1N1 (Δ), aH5N1 (◊) and sH3N2 (□) viruses after stimulation with avian H5N1 virus antigen were analyzed and compared between subjects with serum anti-pH1N1 HAI titre ≥40 (filled symbols, n=9) and those with HAI <40 (open symbols) (a, n=9). There was a good correlation between numbers of anti-pH1N1 HA-specific ASC induced by pH1N1 and that induced by avian H5N1 antigens (b, r=0.85, p<0.001).

3.4.4 pH1N1 virus activates memory B cell responses that produce cross-reactive neutralizing antibodies

As expected, there was a good correlation between the numbers of pH1N1 HA-specific IgG ASC in tonsillar MNC and anti-HA IgG antibody titres in cell culture supernatants after pH1N1 antigen stimulation (Figure 3.4.4a, $r=0.78$, $p<0.001$). To determine whether pH1N1 virus antigen activated memory B cells produce cross-reactive neutralizing antibodies, cell culture supernatants were analyzed for virus neutralizing activity. In subjects from whom a memory B cell response to pH1N1 HA was detected, high levels of neutralizing antibodies against sH1N1 (A/Brisbane/59/2007) and 1918 H1N1 (A/South Carolina/1/18) pseudotype viruses were detected after stimulation with both pH1N1 and aH5N1 virus antigens, but only a low level of the neutralizing activity was induced by sH1N1 virus antigen (Figure 3.4.4b, $p<0.001$). Similarly, neutralizing activity against the aH5N1 pseudotype virus was also detected in cell culture supernatants after stimulation by pH1N1 or aH5N1 virus antigen (Figure 3.4.4c, $p<0.001$), whereas no neutralizing activity against aH5N1 virus was detected in cell culture supernatants after stimulation by sH1N1 virus antigen (Figure 3.4.4c). No neutralizing activity against sH1N1, 1918 H1N1 and aH5N1 viruses was detectable in subjects in whom no memory B cell response to pH1N1 HA was detected (data not shown).

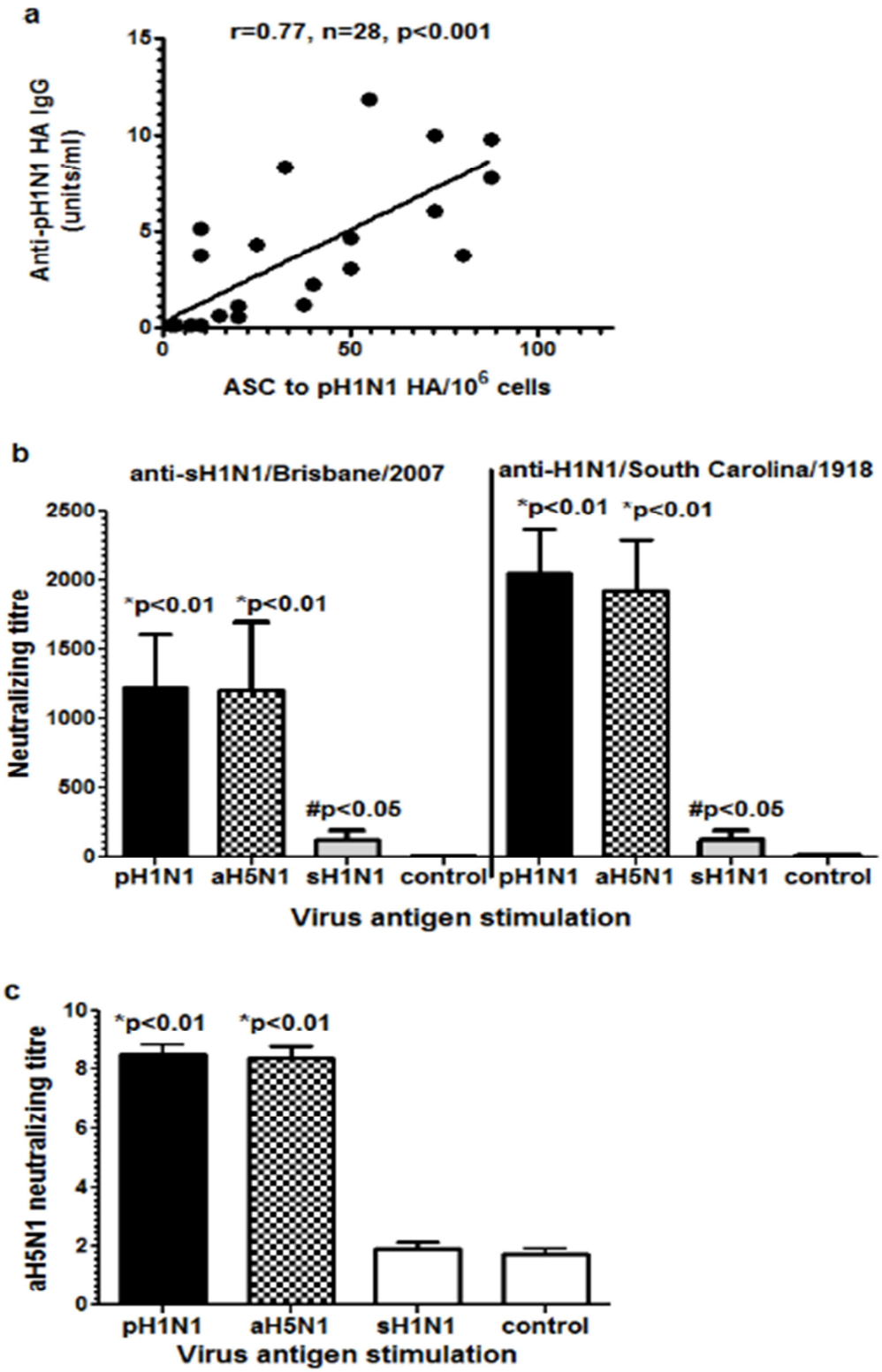

Figure 3.4.4: pH1N1 virus antigen activates a memory B cell response that produces cross reactive neutralizing antibodies. Correlation between numbers of HA specific IgG ASC after stimulation by pH1N1 virus antigen and anti-pH1N1 HA IgG antibody titres in tonsillar cell culture supernatants (a, r=0.77, p<0.001). Virus neutralization activities against sH1N1 and 1918 H1N1 (b) and aH5N1 (c) pseudotype viruses in tonsillar cell culture supernatants after cell stimulation by pH1N1, aH5N1 and sH1N1 virus antigens respectively (*p<0.01 compared with sH1N1 stimulation, #p<0.05 compared with unstimulated control, n=6).

3.5 DISCUSSION AND CONCLUSION

The 2009 pH1N1 virus caused a global pandemic in 2009, infected an estimated 11-21% of the world population and resulted in considerable morbidity and mortality (Kelly et al. 2011). It remains unclear whether the pH1N1 virus infection induced mucosal B cell memory in the infected population and whether this memory provides cross-protective immunity against different types of influenza viruses.

In this study, significant HA-specific memory B cell response to pH1N1 virus was shown in tonsillar cells from individuals with serological evidence of prior exposure to pH1N1virus (serum HAI ≥40), whereas no such memory response was found in those with serum HAI< 40. It was also shown that stimulation with pH1N1 virus antigen activated an IgG memory B cell response with production of HA-specific antibodies against not only pH1N1, but also sH1N1 and aH5N1 viruses. In addition, abundant anti-H2N2 HA IgG antibody production was also elicited in tonsillar cell culture supernatants after pH1N1 antigen stimulation in these subjects (with a mean titre (units/ml) of 4.5 compared to 0.8 in those with HAI< 40). This suggests that 2009 pH1N1 infection primed or activated cross-reactive memory B cells in human NALT to HAs of different influenza viruses. There was a good correlation between the numbers of HA-specific ASC to pH1N1 and that to sH1N1, as shown after stimulation with pH1N1 and sH1N1 antigens respectively. This suggests these NALT memory B cells were likely to be primed by the same antigenic epitopes derived from both pH1N1 and sH1N1 viruses.

The finding that the pH1N1 virus antigen-activated memory B cell response was cross-reactive to sH1N1 and aH5N1 HAs, but not sH3N2 HA , is consistent with

previous studies evaluating the cross-reactivity of serum antibodies in patients infected with pH1N1 virus (Pica et al. 2012). This is likely due to the structural similarities between the group 1 HAs, including H1, H2 and H5 subtypes, which are phylogenetically different from group 2 HAs including H3 and H7 subtypes. Indeed, no detectable levels of anti-H7N3 HA IgG antibodies were observed in the tonsillar cell culture supernatants after stimulation with pH1N1, nor did we find any significant production of the antibody following stimulation by sH3N2 virus antigen in this study.

This study is the first report to demonstrate a significant memory B cell response to pH1N1 virus in human NALT 1-2 years after the 2009 pH1N1 pandemic. Upon pH1N1 antigen stimulation the memory B cell response produces cross-reactive antibodies against HAs of a number of different influenza virus strains. These results are consistent with the presence of plasmablasts secreting cross-reactive neutralizing antibodies in patients infected with pH1N1 (Corti et al. 2011, Ekiert et al. 2011, Krause et al. 2010, Manicassamy et al. 2010, Sui et al. 2009, Xu et al. 2010), and are in agreement with the hypothesis that pH1N1 infection may activate pre-existing memory B cells targeting conserved regions of HA molecule (Pica et al. 2012, Wrammert et al. 2011).

It could be argued that if previous infection with seasonal viruses (e.g. sH1N1) had induced cross-reactive B cell memory through repeated exposure, then most individuals would have had developed immunity against pH1N1 virus before the pandemic. The results show here that there is a significant difference in the capacity to activate cross-reactive memory B cell responses and to produce neutralizing

antibodies between pH1N1 and sH1N1 virus antigens. The former (pH1N1) activated a cross-reactive memory response and neutralizing antibodies whereas the latter (sH1N1) elicited only a moderate memory response and a low level of cross-reactive neutralizing antibodies. This relative inability of sH1N1 virus antigen to activate memory B cells to produce cross-reactive neutralizing antibodies may help explain the failure of previous sH1N1 infections to induce immunological protection against the pH1N1 infection (Ellebedy and Ahmed 2012). The reasons for the difference in the ability to activate cross-reactive memory B cells between pH1N1 and sH1N1 viruses are not clear. It is likely due to the difference in the host immunogenicity of the two viruses, including innate immunity.

Recent studies showed that cross-reactive anti-HA stalk antibodies were boosted following both 2009 pH1N1 infection and the pH1N1 influenza virus vaccination in humans (Miller et al. 2013, Wrammert et al. 2011). It has been postulated that cross-reactive memory B cells specific for conserved regions of the HA stalk of sH1N1 virus were selectively boosted by pH1N1, whereas repeated seasonal H1N1 infection tended to stimulate memory B cells that target the head of HA which were less cross-reactive (Pica et al. 2012).

The cross-reactive memory response to aH5N1 HA in individuals with previous exposure to pH1N1 virus is of particular interest and may have important implications given that aH5N1 is a highly pathogenic virus and potential cause of future influenza pandemics. It remains to be seen whether this cross-reactive memory induced by natural infection alone offers any protection against aH5N1 infection, as the neutralization activity of the memory B cell response against aH5N1 appears to

be modest compared to its high neutralizing activity against H1N1 strains. However, it is plausible to enhance such cross-reactive B cell memory by vaccination, e.g. intranasal mucosal immunization to boost this natural immunity. The ability of pH1N1 virus antigen to elicit a strong HA-specific memory B cell response and cross-reactive neutralizing antibodies suggests it may be possible to utilize pH1N1 HA or conserved HA regions in an influenza vaccine to induce cross-reactive immunity against influenza viruses including aH5N1.

Considering none of the subjects in this study had been exposed to aH5N1 virus, it is intriguing that aH5N1 virus antigen could induce a memory B cell response to pH1N1 and sH1N1 HAs. The finding that this memory response was detected only in those who had previous pH1N1 exposure suggests that pH1N1 infection primed the host for cross-reactive memory against different virus strains including aH5N1. It was reported previously that serum antibodies in an aH5N1 infected patient bind to a variety of conserved peptides in the stem region of HA (Khurana et al. 2009), so it is possible that there are cross-reactive epitopes in the HAs of pH1N1 and aH5N1 viruses.

The pH1N1 virus caused an influenza pandemic which spread rapidly worldwide in 2009. The predominant virus circulating in the subsequent 2010-2011 influenza season was pH1N1, which essentially replaced the previously circulating sH1N1 viruses (Pica et al. 2012), Health Protection Agency (HPA) website at www.hpa.org.uk/webc/HPAwebFile/HPAweb_C/1296687414154. This phenomenon is similar to that described following previous influenza pandemics in 1957 and 1968 when circulating virus strains disappeared after the emergence of the pandemic

strains (Palese and Wang 2011). It has been hypothesized that the induction of cross-reactive antibodies may contribute to the disappearance of the circulating strains (Palese and Wang 2011, Pica et al. 2012). The cross-reactive memory B cell response activated by pH1N1 virus as described in this study may contribute to the reduction of sH1N1 and help explain the low influenza activity in the 2011/12 influenza season in the UK (HPA Weekly National Influenza Report. Summary of UK surveillance of influenza and other seasonal respiratory illnesses.16 August 2012.http://www.hpa.org.uk/webc/HPAwebFile/HPAweb_C/ 1317135659994).

It is generally considered that IgA antibodies are predominant at the mucosal level. However, the question of whether mucosal IgA memory can be induced in humans either through natural infection or vaccination is being debated. A number of studies have shown that antigen-specific mucosal IgA responses are short-lived and that reimmunization does not reliably induce memory type IgA responses (Korkeila et al. 2000, Nurkka et al. 2000). Although IgA ASC were reported to increase in tonsillar cells after influenza vaccination, they were likely to represent mainly a primary rather than memory IgA response (Brokstad et al. 1995). The predominance of antigen-specific IgG memory B cells to influenza HA in tonsillar tissues shown in this study is concordant with previous studies demonstrating the predominance of IgG memory B cell responses to protein antigens in human NALT (Boyaka et al. 2000b, Nadal et al. 1992b, Zhang et al. 2002b).

Taken together, the results present evidence that pH1N1 infection in humans primed the host with cross reactive memory B cells in NALT tissues that can respond strongly to stimulation by both pH1N1 and aH5N1 virus antigens to produce cross-

reactive neutralizing antibodies. These findings may have important implications to future vaccination strategies against influenza. It will be important to induce and/or enhance such cross-protective mucosal memory B cells. The ability of pH1N1 and aH5N1 virus antigens to stimulate cross-reactive memory B cell responses in human NALT warrants efforts to explore the conserved regions of these HA as components of future vaccines, for example, in intranasal mucosal vaccination to induce broad immunity against influenza.

Chapter 4

HA-specific Antibody Levels in Children and Adults Following 2009 Pandemic H1N1 infection

4.1 INTRODUCTION

Influenza virus is an important cause of respiratory tract infection and responsible for 3–5 million clinical infections and 250,000–500,000 fatal cases annually worldwide (Stohr 2002). Infection with influenza virus induces host immune responses that help to reduce virus replication and prevent further spread. Immunological memory resulted from the infection is usually protective against subsequent influenza virus infection of the same serotype, but offers limited protection against other subtypes (Kreijtz et al. 2011).

Antibody response plays a critical role in the protection against pathogens including influenza virus (Ichinohe and Iwasaki 2009).The surface haemagglutinin (HA) glycoprotein of influenza virus is a major target for an antiviral activity as the immune response to HA offer neutralizing antibodies following vaccination or natural infection (Tan et al. 2012). As HA is a major virulence factor crucial for virus binding to host cells, HA-specific antibodies are important to prevent the attachment of the virus to host cell thus prevent infection (Wang et al. 2011).

Structurally, the HA protein consists of two parts; a globular head which mediates the attachment of the virus to the host cells, and a stem (stalk), which mediates the fusion of the virus to the host cell membrane, enabling the viral genome to enter the cells (Thomson et al. 2012).

There are 16 different influenza subtypes of HA and they are clustered into two groups based on the molecular relatedness of the HA sequences; group 1 includes (H1, H2, H5, H6, H8, H9, H11, H12, H13 and H16) and group 2 includes (H3, H4, H7, H10, H14 and H15) (Air 1981).

Typical anti-HA neutralizing antibodies sterically block viral attachment to its cellular ligand by binding in or around the receptor-binding site of the globular head. Although highly effective, they are mostly strain specific and have little or no broad spectrum activity (Smith et al. 2004, Krammer et al. 2012).

A number of recent studies show broadly neutralizing antibodies targeting the stalk region of HA (Ekiert et al. 2009, Sui et al. 2009, Corti et al. 2011), such antibodies are able to cross-protect against influenza virus of a different subtypes following pH1N1 infection and /or vaccination (Krause et al. 2010, Xu et al. 2010, Manicassamy et al. 2010, Li et al. 2012).

More recently, several HA2 heterosubtypic neutralizing monoclonal antibodies against H1 and H5 subtype (Group 1) influenza viruses have been isolated experimentally (Wang et al. 2011). Several studies have shown that the stalk domain is highly conserved; antibodies directed against the stalk are more likely to be cross-reactive, even between subtypes (Ekiert et al. 2009, Corti et al. 2011, Sui et al. 2009, Krammer et al. 2012).

Serum IgG antibodies may leak to the respiratory tract and be involved in local protection. Mucosal IgA antibodies are produced locally in the respiratory tract are important in local mucosal protection. IgA antibodies are able to neutralize influenza virus intracellularly (Kreijtz et al. 2011). Beyer and colleagues reported that virus-specific local IgA antibodies can suppress viral shedding (Beyer et al. 2002).

4.2 AIMS OF STUDY

To study HA-specific antibodies to influenza viruses in serum samples from children and adults following the 2009 H1N1 pandemic.

4.3 EXPERIMENTAL DESIGN

1. To set up an ELISA for measuring HA-specific antibody levels to influenza viruses.
2. To determine the specificity of the ELISA for HA-specific antibodies to pH1N1, sH1N1 and aH5N1 viruses.
3. Use the established ELISA to measure antibodies in serum samples in children and adults.
4. To find out if serum pH1N1 antibodies increased following the pandemic and whether there are any cross reactive antibodies with sH1N1 and aH5N1 viruses.

4.3.1 Recombinant HAs

Purified recombinant HA proteins of pH1N1 (A/California/04/2009), sH1N1 (A/Brisbane/59/2007) and aH5N1 (A/Vietnam/1203/2004), were from Biodefence and Emerging Infections Research Resources Repository, ATCC (Manassas VA, USA).

4.3.2 ELISA assay

ELISA assay was performed as described in methods (chapter 2) with some modifications. In brief, ELISA plates were coated with individual recombinant HAs and incubated overnight at 4°C. After washing, plates were blocked with 10% FBS followed by incubation of cell culture supernatants at predetermined optimized

dilutions for 1.5 hour. Alkaline phosphatase conjugated anti-human IgG (Sigma) was then added and incubated for 1.5 hour. After washing, p-nitrophenyl phosphate substrate was applied. Optical density was measured at 405 nm and data were analyzed using DeltaSoft microplate analysis software (BioMetallics Inc, NJ) (for details see materials and methods chapter 2).

4.3.3 Haemagglutinin Inhibition (HAI) assay

Haemagglutinin Inhibition assay (HAI) was carried out at the Microbiology Services-Colindale, Health Protection Agency (London, UK) briefly, (for details see chapter 2 materials and methods). HAI assay was performed following standard methods (Miller et al. 2010). The virus strain used included the pandemic H1N1: NIBRG122 that is a reassortant prepared from A/England/195/2009(H1N1v), the prototype UK isolate antigenically and genetically closely related to A/California/4/2009.

4.3.4 Real-time PCR

Real-time PCR was performed using the standard protocol from WHO following extraction of viral RNA from nasal swabs (for more details see chapter 2 materials and methods). The aim of performing this assay was to investigate the possible influenza colonization of nasopharynx (for more details see chapter 2 materials and methods)http://www.who.int/csr/resources/publications/swineflu/WHO_Diagnostic_RecommendationsH1N1_20090521.pdf accessed on 13.02.13

4.4 Results

4.4.1 Establishment of ELISA for measuring HA-specific antibodies

ELISA assay was designed and established to measure HA-specific antibodies to influenza viruses. For each set of experiment, optimal antigen coating concentration, sample dilution and conjugate antibody concentration were established. A standard curve was created for each HA-specific antibody to get antibody titres of individual samples based on a reference standard (for details of the reference standards see materials and methods in chapter 2). As shown in figure (4.4.1) standard curve was obtained to calculate concentration of HA-specific antibody levels for each individual virus. For accurate and optimal measurement of antibody titres, the optical densities (OD) of all samples should fall within the standard curve. Otherwise the sample analysis would be repeated, e.g. after more sample dilution.

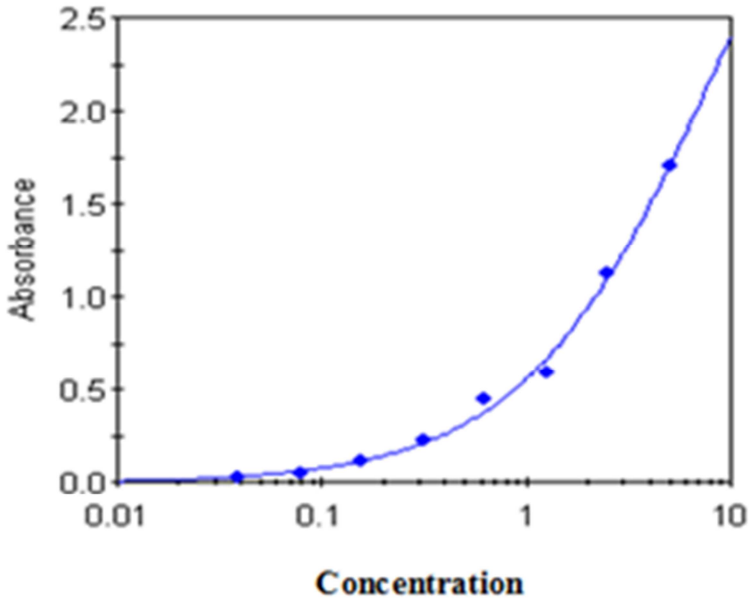

Figure 4.4.1: Establishment of standard curve after performing ELISA. Following the analysis of samples using ELISA assay, standard curve was established after optical density (OD at 405 nm) obtained from the ELISA plate reader. The standard curve was plotted following the analysis of the data using DeltaSoft microplate analysis software (BioMetallics Inc, NJ).

4.4.2 Determination of the specificity of ELISA assays for measuring HA-specific antibodies to sH1N1, pH1N1 and aH5N1 viruses

4.4.2.1 Specificity of ELISA assay for detection of HA- specific antibody- to sH1N1 virus

The inhibition ELISA assay was adopted from Zhang et al. 2006 with some modifications (details see chapter 2 materials and methods). To assess the specificity of ELISA assay in detecting sH1N1 HA-specific IgG antibody, different concentrations of the recombinant purified HA of sH1N1 were used. A number of four serum samples with high antibody titres were used and prepared at a dilution of 1:100. The following HA antigen concentrations 10, 1, 0.1 and 0.0 (without antigen) µg/ml was co-incubated with the serum samples to adsorb the antibodies present in the serum. The percentage of inhibition was 90% at 10ug/ ml of antigen, and was 23% at 0.1 µg/ ml of HA antigen. The mean concentration of the HA antigen needed for 50% inhibition of antibody activity for the samples was 1 µg/ml.

As shown in figure (4.4.2.1a), the inhibition curve with increasing HA concentrations confirms the specificity of ELISA assay to detect HA-specific anti-sH1N1 antibody. The specificity of the assay was also supported by the relative failure of inhibition by absorption with heterologous HAs including pH1N1 (figure 4.4.2.1b) and aH5N1 (figure 4.4.2.1c).

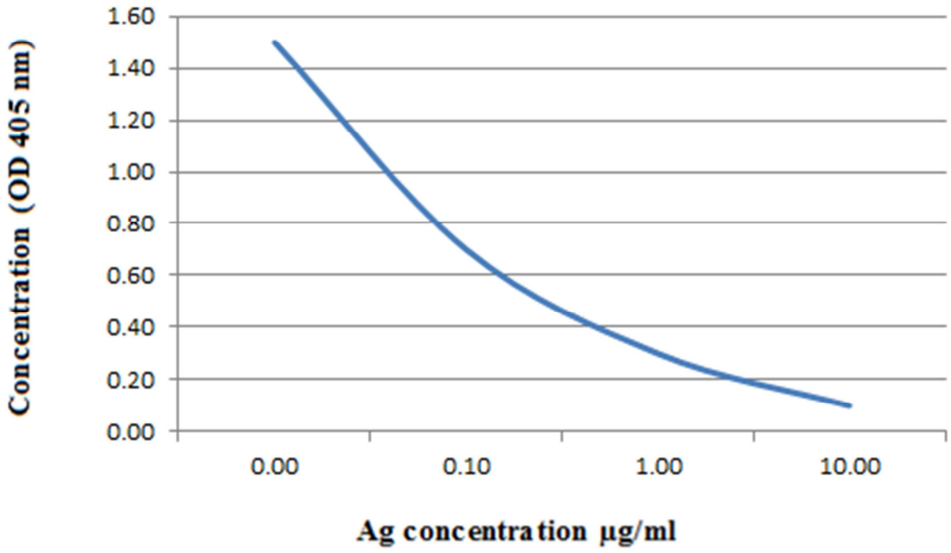

Figure 4.4.2.1a: Specificity assay shows the correlation between antigen (Ag) concentrations and antibody level to sH1N1 in serum samples. Antibody titre in the serum decreased with the increase of concentration of the adsorbent sH1N1 HA antigen in a dose dependent manner which supports the specificity of the ELISA assay. (One of four representative samples was shown).

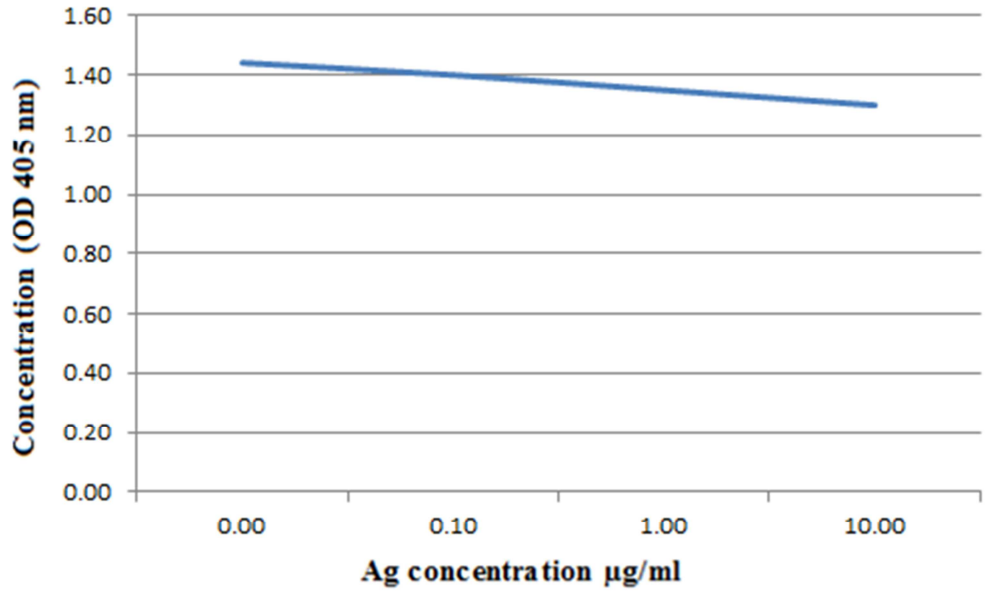

Figure 4.4.2.1b: Inhibition ELISA for HA-specific IgG antibody to sH1N1 by pH1N1 HA. Serum samples were adsorbed by pH1N1 HA antigen using different concentrations. The figure shows pH1N1 HA only weakly adsorbs the anti-sH1N1 antibody in the samples. (One of four representative samples was shown).

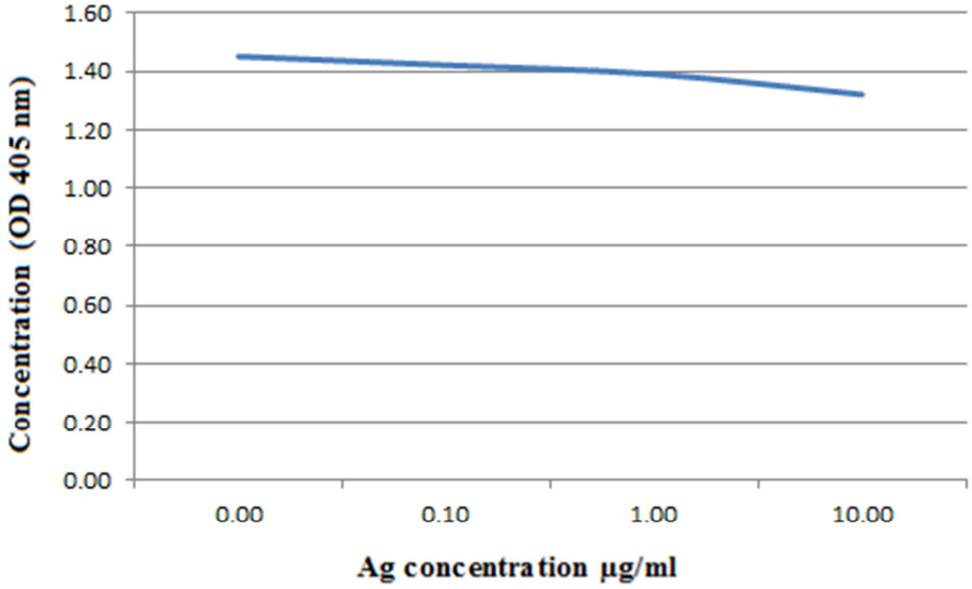

Figure 4.4.2.1c: Inhibition ELISA for HA-specific IgG antibody to sH1N1 by aH5N1 HA. Serum samples were adsorbed by aH5N1 HA antigen using different concentrations. The figure shows aH5N1 HA only weakly adsorbs the anti-sH1N1 antibody in the samples. (One of four representative samples was shown).

4.4.2.2 Specificity of ELISA assay for the detection of specific anti-pH1N1 HA

The specificity of the ELISA assay to detect HA-specific anti-pH1N1 antibody was determined as above for anti-sH1N1 antibody. Firstly, purified pH1N1 HA was used to adsorb specific antibodies. As shown in figure 4.4.2.2a, the percentage of inhibition was 86% at 10µg/ ml of antigen and was 35% at 0.1 µg/ ml antigen. The concentrations of HA needed for 50% inhibition of antibody activity was 1 µg/ml.

As shown in figure (4.4.2.2a), the inhibition curve with increasing HA concentrations confirms the specificity of ELISA assay to detect HA-specific anti-pH1N1 antibody. The specificity of the assay was also supported by the relative failure of inhibition by absorption with heterologous HAs including sH1N1 (figure 4.4.2.2b) and aH5N1 (figure 4.4.2.2c).

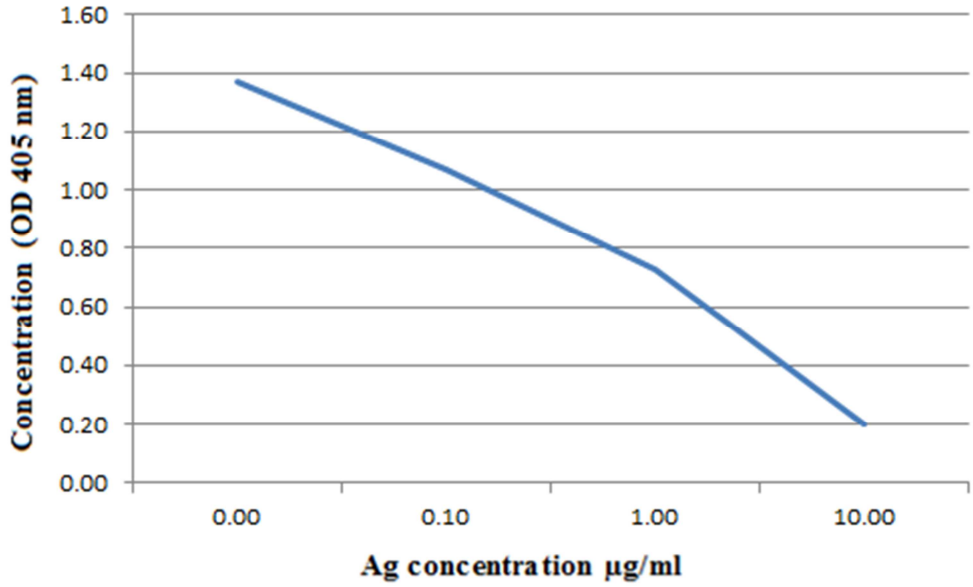

Figure 4.4.2.2a: Specificity ELISA assay for HA-specific IgG antibody to pH1N1 by pH1N1 HA. The figure shows that antibody titre in the serum decreased with the increase of concentration of the adsorbent pH1N1 HA antigen which supports the specificity of the ELISA assay. (One of four representative samples was shown).

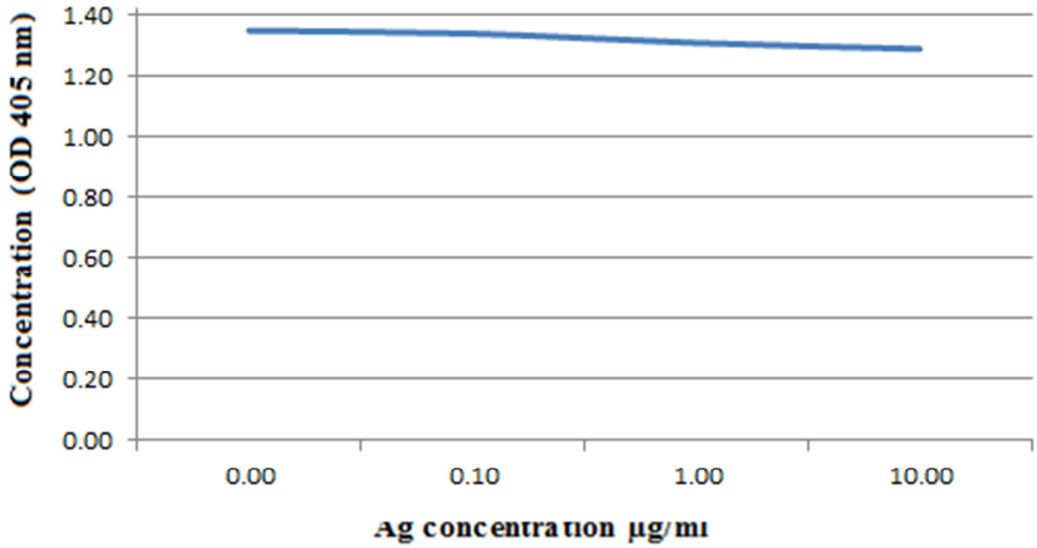

Figure 4.4.2.2b: Inhibition ELISA for HA-specific IgG antibody to pH1N1 by sH1N1 HA. Serum samples were adsorbed by sH1N1 HA antigen using different concentrations. The figure shows sH1N1 HA only weakly absorbs the anti-pH1N1 antibody in the samples. (One of four representative samples was shown).

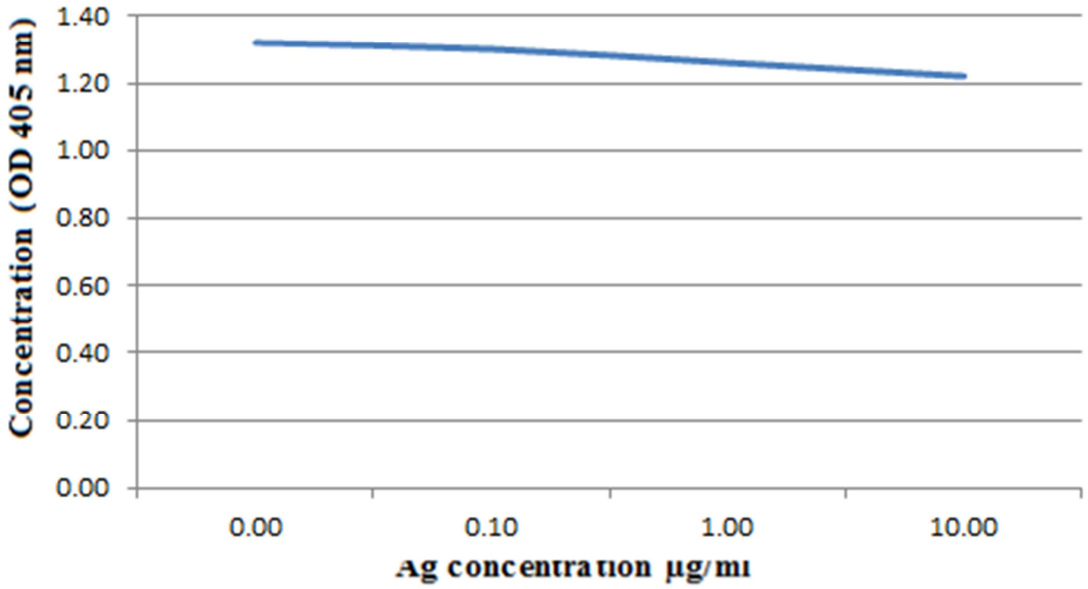

Figure 4.4.2.2c: Inhibition ELISA for HA-specific IgG antibody to pH1N1 by aH5N1 HA. Serum samples were adsorbed by aH5N1 HA antigen using different concentrations. The figure shows aH5N1 HA antigen only weakly absorbs the anti-pH1N1 antibody in the samples. (One of four representative samples was shown).

4.4.2.3 Specificity of ELISA assay for the detection of specific anti-aH5N1 HA

The specificity of the ELISA assay to detect HA-specific anti- aH5N1 antibody was determined as above for anti-sH1N1 as well as anti-pH1N1 antibodies. Firstly, purified aH5N1 HA was used to adsorb specific antibodies. As shown in figure 4.4.2.2a, the percentage of inhibition was 92% at 10ug/ ml of antigen and was 70% at 0.1 µg/ ml antigen. The concentrations of HA needed for 50% inhibition of antibody activity was 0.1 µg/ml.

As shown in figure (4.4.2.3a), the inhibition curve with increasing HA concentrations confirms the specificity of ELISA assay to detect HA -specific anti- aH5N1 antibody. The specificity of the assay was also supported by the relative failure of inhibition by absorption with heterologous HAs including pH1N1 (figure 4.4.2.3b) and sH1N1 (figure 4.4.2.3c).

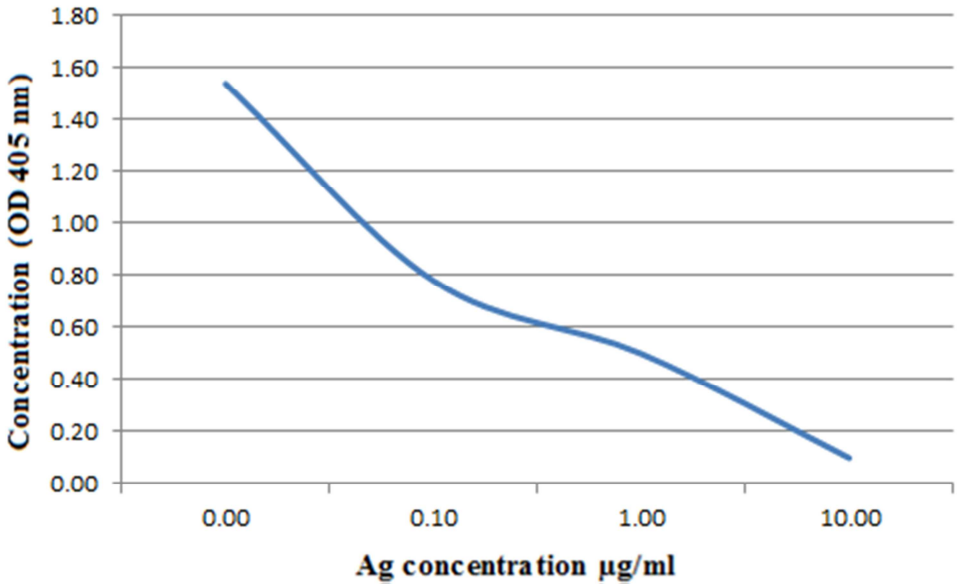

Figure 4.4.2.3a: Specificity ELISA for HA-specific IgG antibody to aH5N1 by aH5N1 HA. Antibody titre in serum samples was inhibited by the addition of aH5N1 HA antigen in a dose-dependent manner. (One of four representative samples was shown).

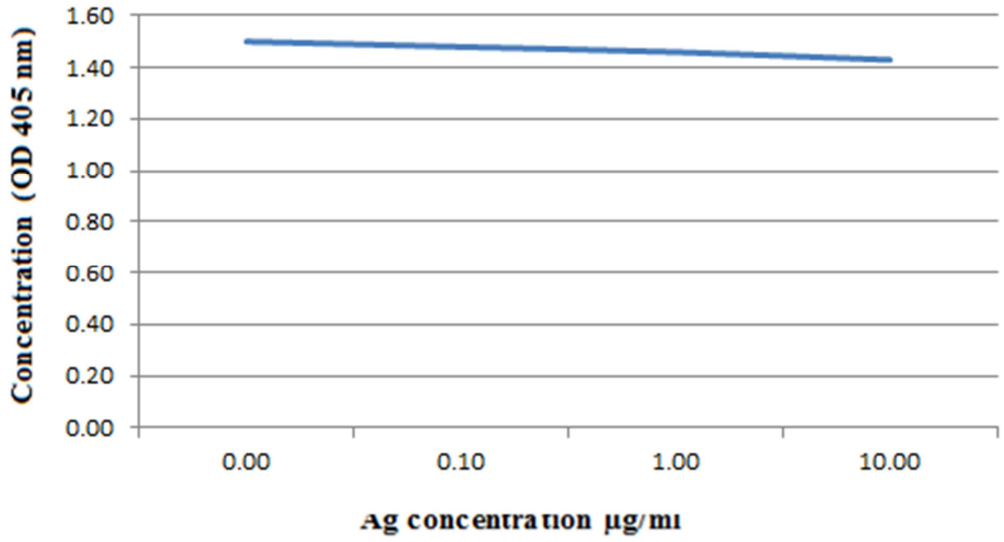

Figure 4.4.2.3b: Inhibition ELISA for HA-specific IgG antibody to aH5N1 by sH1N1 HA. Serum samples were adsorbed by sH1N1 HA antigen using different concentrations. The figure shows sH1N1 HA antigen only weakly adsorbs the anti-aH5N1 antibody in the samples. (One of four representative samples was shown).

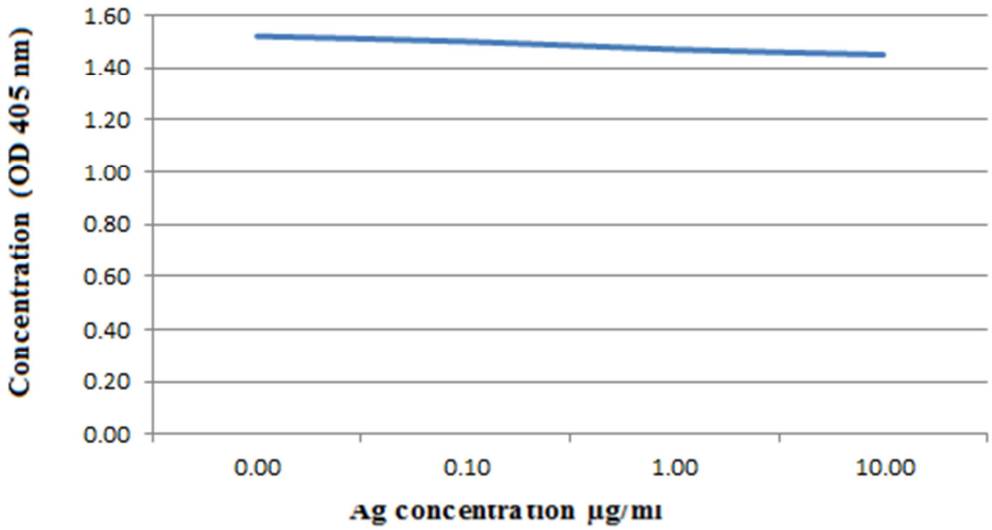

Figure 4.4.2.3c: Inhibition ELISA for HA-specific IgG antibody to aH5N1 by pH1N1 HA. Serum samples were adsorbed by pH1N1 HA antigen using different concentrations. The figure shows pH1N1 HA only weakly adsorbs the anti-aH5N1 antibody in the samples. (One of four representative samples was shown).

4.4.3 HA-specific IgG antibody to pH1N1 in serum in children and adults

ELISA was used for measuring HA-specific antibodies in serum samples to pH1N1 virus, and the antibody levels in those subjects with HAI titre ≥40 were compared with those with HAI titre <40. As described in chapter 3, anti-pH1N1 HAI titre ≥40 was used as a serological marker of exposure to pH1N1 virus in this study. A shown in figure 4.4.3, subjects with serum anti-pH1N1 HAI titre ≥40 were found to have high serum anti-pH1N1 HA antibody titres (measured by ELISA) and were significantly higher than those who had anti-pH1N1 HAI titre <40 (figure 4.4.3, n=230 (children = 140 and adults= 90), p<0.001).

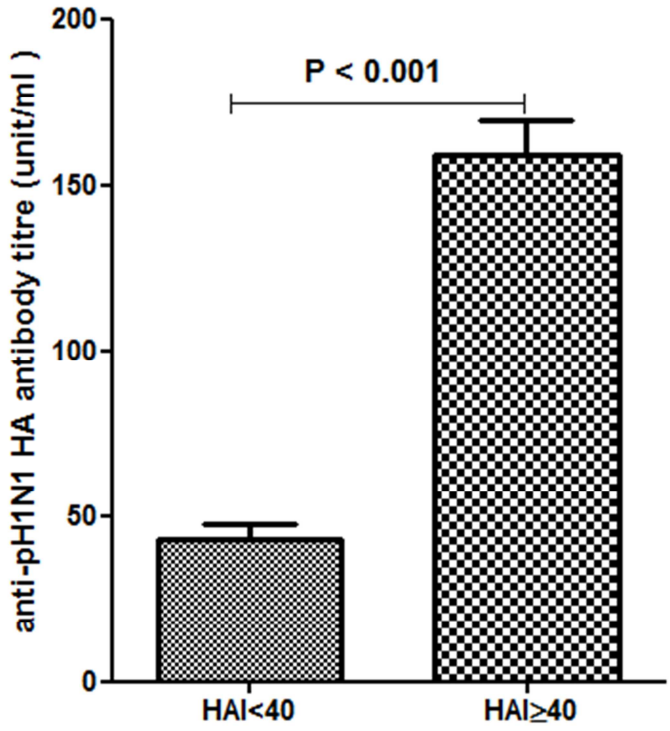

Figure 4.4.3: Comparison of serum HA-specific IgG antibody titre to pH1N1 between subjects with or without serological evidence of previous exposure to pH1N1 virus. Serum antibody levels determined using ELISA in subjects with serum anti-pH1N1 HAI titre ≥40 were significantly higher than that in those who had anti-pH1N1 HAI titre <40. Mean antibody titres and standard errors are shown (unpaired t-test, n=230, p<0.001).

4.4.4 Association between HA-specific IgG antibody levels to pH1N1with age

To investigate if there is any association between HA-specific IgG antibody levels to pH1N1 and age, serum samples from children and adults were measured using ELISA and analysed in association with age. Figure 4.4.4 shows that there is an age-associated increase in the HA-specific IgG antibody to pH1N1 virus.

The finding in this part is consistent with that by Miller and colleagues which has referred the age association to the frequency of exposing to the influenza virus, in older children and adults were higher than those younger ones (Miller et al.2010). This might be explained by that adults possibly exposed more frequently than children, so that they developed stronger immunological memory than children.

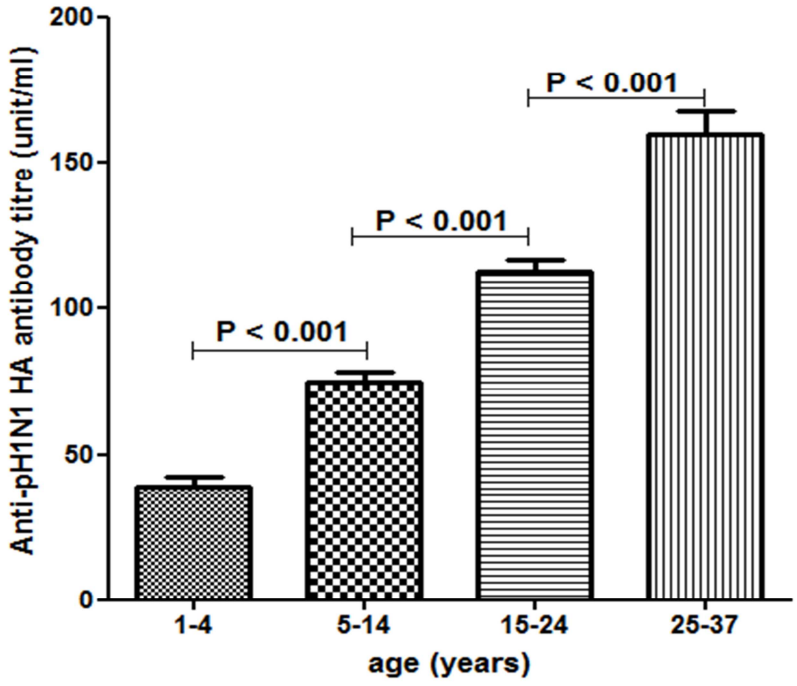

Figure 4.4.4: Relationship between specific anti-HA IgG antibody titres to pH1N1and age. Serum samples from children and adults were analysed for anti-pH1N1 HA antibody using ELISA and further analysed in association with age. Anti-pH1N1 HA antibody levels were compared between age groups 1-4, 5-14, 15-24 and 25-37 years. An age-related increase in antibody titres was clearly seen ($p<0.001$). (Mean antibody titres and standard errors are shown, n=230).

4.4.5 Serum antibody level to pH1N1 HA before, during and after 2009 H1N1 pandemic in children

Following the pandemic H1N1 virus infection in 2009, it is possible many people in the population would have developed specific immunity to the virus. In this study, HA-specific anti-pH1N1 IgG antibody levels in serum samples collected at different time points representing before, during and after pH1N1 infection were analysed by ELISA.

The serum HA-specific anti-pH1N1 antibody levels in samples collected from children before the pandemic were very low (figure 4.4.5). The specific antibody levels in the year of the pandemic (2009) were significantly higher than that of the pre pandemic period ($p<0.001$). The antibody level in the 2009-2010 flu season (Nov-Mar) were significantly higher than that observed in the non-flu season 2009-2010 (May-Oct) ($p<0.01$), and a gradual increase in the antibody levels was shown. One year after the pandemic, during the flu season 2010-2011 (Nov-Mar), the antibody levels were shown to be significantly higher than that observed in the previous flu season 2009-2010 (Nov-Mar) ($p<0.01$). Two years past the pandemic, during the flu season 2011-2012 (Nov-Mar), the HA-specific anti-pH1N1 antibodies were shown to be higher ($p<0.01$) than the previous flu season 2010-2011 (Nov-Mar) (figure 4.4.5).

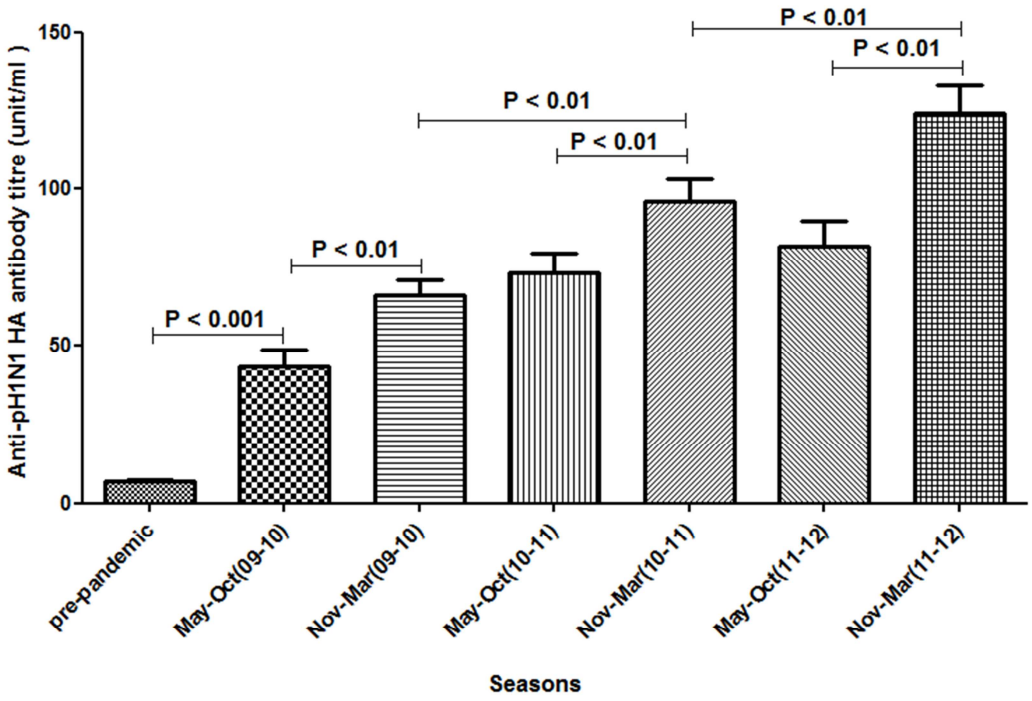

Figure 4.4.5: Anti-pH1N1 HA antibody levels in serum samples from children collected from pre, during and post pandemic period. Serum specific anti-pH1N1 HA IgG antibodies in children were analysed by ELISA. The figure shows that antibody levels increased gradually with time, during and post the pH1N1pandemic. Pre-pandemic serum samples showed very low antibody levels. Mean antibody titres and standard errors are shown, n= 140).

4.4.6 Cross-reactivity between anti-pH1N1 HA antibody with other influenza viruses in serum

To find out if there is any relationship between the serum specific antibody levels to the different influenza A virus strains measured by ELISA assay, Pearson correlation analysis was performed using Graph Pad Prism 5 software. There was a positive correlation ($r = 0.76$) between specific anti-sH1N1 and anti-pH1N1 HA antibody titre (figure 4.4.6a). This result is consistent with the findings on cross-reactivity between sH1N1 and pH1N1 shown in chapter 3.

However the anti-aH5N1 HA antibody levels were much lower than that to pH1N1, but it was detectable in some samples when measured by ELISA assay. Moreover, further analyses of serum anti-aH5N1 HA IgG was performed. The subjects were divided into two groups; with or without serological evidence of previous exposure to pH1N1 virus. The result show that anti-aH5N1 HA IgG antibody titre (analysed by ELISA) in subjects with serum anti-pH1N1 HAI titre ≥ 40 were significantly higher levels to aH5N1 HA than that in those who had anti-pH1N1 HAI titre <40 (figure 4.4.6b). This result suggests that positive correlation between antibodies to these influenza A viruses and cross-reactivity among them and again the cross-reactivity was seen in chapter 3 as well.

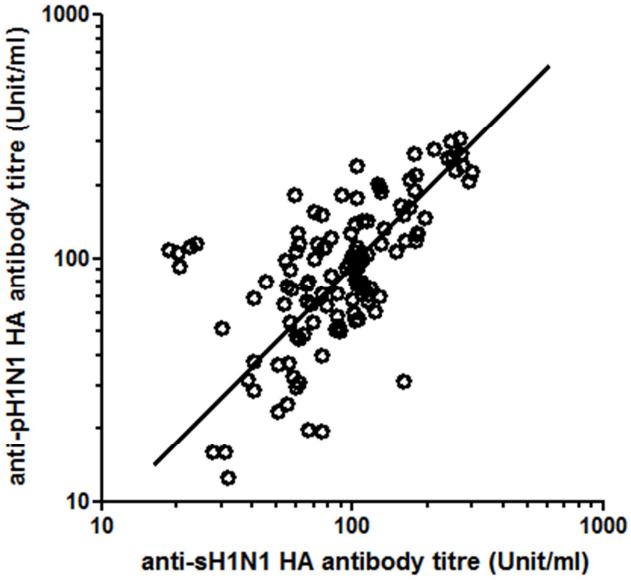

Figure 4.4.6a: Correlation between serum specific antibody level (IgG) to sH1N1 and pH1N1 HA. After the serum specific anti-HA to sH1N1 and pH1N1 antibodies titres were analysed by ELISA assay, a positive correlation was found between them. (Pearson correlation = 0.76 (P< 0.001, n=230).

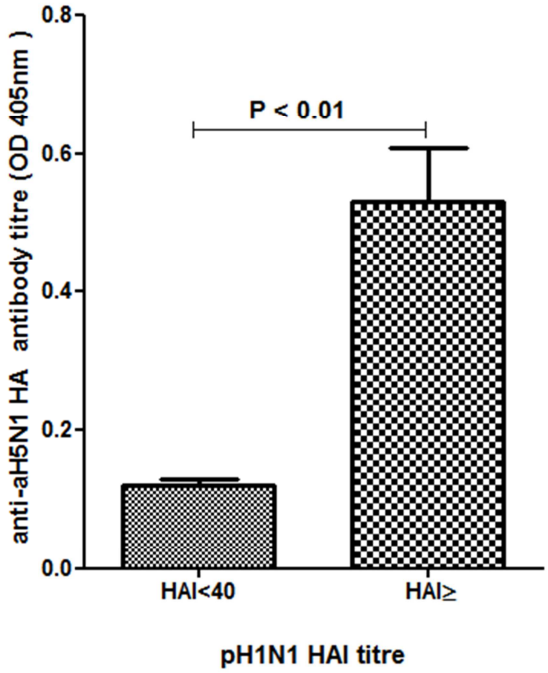

Figure 4.4.6b: Comparison of serum HA-specific IgG antibody titre to aH5N1 between subjects with or without serological evidence of previous exposure to pH1N1 virus. Serum antibody levels to aH5N1 HA determined using ELISA. In subjects with serum anti-pH1N1 HAI titre ≥40 were significantly higher levels to aH5N1 HA than that in those who had anti-pH1N1 HAI titre <40. Mean optical density and standard errors are shown (unpaired t-test, n=25, p<0.01).

4.4.7 HA-specific serum IgG antibody and salivary IgA to pH1N1 HA

ELISA was used for measuring HA-specific antibodies in saliva samples to pH1N1 virus, and the antibody levels in subjects with serum IgG HAI titre ≥40 were compared with those with HAI titre <40. As described previously, anti-pH1N1 HAI titre ≥40 was used as a serological marker of exposure to pH1N1 virus in this study. A shown in (figure 4.4.7), subjects with serum anti-pH1N1 HAI titre ≥40 were found to have high salivary anti-pH1N1 HA IgA antibody titres (measured by ELISA) and were significantly higher than those who had serum anti-pH1N1 HAI titre <40. It suggests that pH1N1 infection induced both systemic and local antibodies and collectively, the serum IgG as well as salivary IgA antibodies may provide protection against influenza infection.

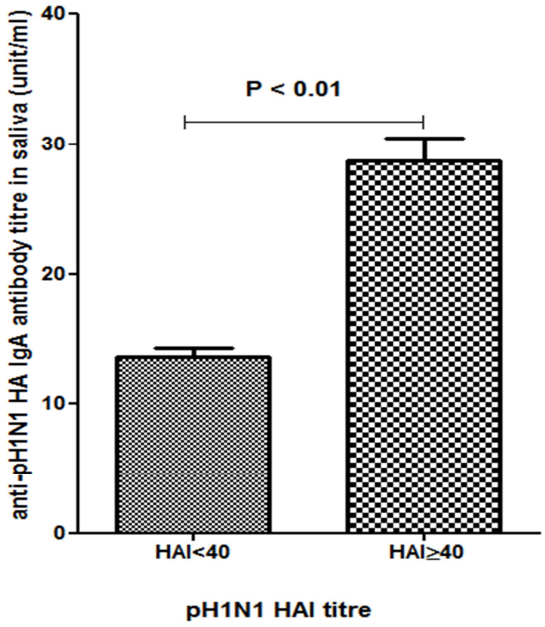

Figure 4.4.7: Relationship between serum HA-specific IgG antibody titre and salivary IgA to pH1N1 between subjects with or without serological evidence of previous exposure to pH1N1 virus. Serum IgG and salivary IgA antibodies to pH1N1 HA were analysed by ELISA assay. In subjects with serum anti-pH1N1 HAI titre ≥40 were significantly higher levels of salivary IgA to pH1N1 HA (measured by ELISA) than that in those who had anti-pH1N1 HAI titre <40. Mean antibody titres and standard errors are shown (unpaired t-test, n=60, p<0.01).

4.5 DISCUSSION AND CONCLUSION

Influenza, with recurring global epidemics, causes a major public health problem. It affects hundreds of millions of people every year, with high morbidity and mortality in high-risk populations (Katz et al. 2011, Chen et al. 2001).

Antibody response is critical in the protection against pathogens including influenza virus (Ichinohe et al. 2009).The surface haemagglutinin (HA) glycoprotein of influenza virus is a major target for an antiviral activity as the immune response to HA offers neutralizing antibodies against the virus following either vaccination or natural infection (Tan et al. 2012). Antibodies to the conserved stem (stalk) region of HA block membrane fusion and also prevent productive infection by different influenza viruses (Lingwood et al. 2012). According to the genetic relatedness of the 16 different HAs, there are two broad groups of influenza viruses. Each group appears to share a region of sequence conservation in the stem region that can serve as a target of broadly neutralizing antibodies focussed to this region (Nabel and Fauci 2010).

The 2009 pandemic H1N1 infection affected large numbers of people worldwide. Because of its novel genetic structure, the majority of younger adults and children were naïve to it when it first emerged, whereas the elderly were less affected population due to the pre-existing antibody protecting against infection (Miller et al. 2010).

ELISA assay is widely used in measurement of serum antibodies for diagnostic or/and experimental and research purposes. Well-established ELISAs are commonly used as a rapid, sensitive, specific and cost effective laboratory method to detect antibodies to different pathogens including the influenza viruses (Kim et al. 2011).

In this study, an ELISA was set up to measure HA-specific antibodies to several different influenza viruses. Inhibition ELISA was performed using HAs of homologous and heterologous virus strains to confirm the specificities of each assay in detecting HA-specific anti-influenza antibodies.

The results obtained by analysing serum specific anti-pH1N1 HA IgG antibody titre using ELISA assay show HA-specific antibody levels to pH1N1 in adults were significantly higher than that of children. The results may suggest that adults had been exposed to more cross-reactive influenza viruses than children, and developed more cross-reactive memory responses against some influenza viruses than in children.

The results show that there is an association between specific anti-pH1N1 HA IgG antibody titre detected by ELISA assay and the age of studied subjects. This result is consistent with the previous finding by Miller and colleagues that there was an age association to the frequency of exposure to the influenza virus, in older children and adults were higher than in younger ones (Miller et al. 2010).

In this study, the influenza season was defined as period from November to March, and non- influenza season as the period from May to October. The results show that higher titres of specific anti-pH1N1 HA IgG antibodies were detected in the influenza seasons whereas the lower titres were in the non-influenza seasons. Additionally, the results also show that during the 2011-2012 influenza seasons there was the highest level of anti-pH1N1 HA IgG antibodies. According to the HPA, the overall GP consultation rates for influenza-like illness in England and Wales, in 2011/2012 flu season was the lowest on record (http://www.hpa.org.uk/NewsCentre/NationalPressReleases/

2012PressReleases/120621winterflulow/) (accessed on 05.10.2012). This may support the hypothesis that following the pandemic pH1N1 infection, cross-reactive immunity was enhanced which protected against a number of antigenically related viruses such as seasonal H1N1 virus. Several studies have shown that antibodies derived from natural infection were associated with protection (Ohmit et al. 2011).

Influenza HA consists of two domains, a circular head and a stalk region. The circular head contains the variable region of HA and is the major target for antibodies that inhibit virus binding to target cells. These antibodies are traditionally detected by haemagglutination inhibition assay (HAI) which is specific to a particular virus strain. The stalk region is more conserved. The ELISA assay based on the whole HA molecule would likely detect antibodies against both the circular head and the stalk domains of HA. HAI titres ≥40 are generally considered to be protective against influenza virus (Bright et al. 2007,Miller et al. 2010, Kreijtz et al. 2011). Using HAI titres ≥40 as a serological evidence of exposure to the 2009 pH1N1 virus infection, antibody levels measured by ELISA were compared in individuals with HAI≥40 and those with HAI<40. Significantly higher HA-specific IgG antibody titres to pH1N1 HA (measured using ELISA) were found in subjects who had HAI titres≥40 than in those with HAI antibody titre<40. This suggests that following the 2009 pH1N1 pandemics, large numbers of people developed anti-pH1N1 HA antibodies to both the circular head and the stalk regions of HA which may have broader protective immunity.

The results show that anti-pH1N1 HA antibody levels increase every influenza season after the first emergence of the pandemic. Additionally, the results show that antibodies to pH1N1 increased with time. It is possible to infer that frequent

exposure to the virus develops immunological memory which helps in protection against future infection.

Considering none of the subjects in this study had been exposed to aH5N1 virus, it is intriguing that specific antibody to aH5N1 virus was detected at some levels in the serum samples. This study showed positive correlations between antibodies to sH1N1and pH1N1 as well as pH1N1and aH5N1, which suggest cross-reactive immunity between these virus strains.

Both circulating and mucosal antibodies are considered to be protective against infection by influenza virus in humans and animals (Chen et al. 2001). The results show that subjects with serum anti-pH1N1 HAI titre ≥40 were found to have high salivary anti-pH1N1 HA antibody titres (measured by ELISA) and were significantly higher than those who had serum anti-pH1N1 HAI titre <40. It suggests that pH1N1 infection induced both systemic and local antibodies and collectively, the serum IgG as well as salivary IgA antibodies may provide protection against influenza infection. The presence of salivary antibodies may have significant neutralizing activity against influenza A virus as supported by previous studies based on haemagglutination inhibition and neutralization assays (White et al. 2009).

Nasal swabs (n=80) were extracted for possible detection of viral RNA to pH1N1 influenza viruses using standard protocol. Subsequently, Real-time PCR of all extracted samples was performed using standard protocol available on the WHO website. None of the subjects were found to have influenza viruses in the nasal samples (data not shown). It suggests that no current infection was detected at the time of obtaining the samples.

Chapter 5

HA-specific Memory $CD4^+$ T cell Response To Pandemic H1N1 2009 Influenza Virus in Children and Adults

5.1 INTRODUCTION

Influenza virus infects via the mucosal surface of the upper respiratory tract. The local mucosal immune system plays an important role in protection and clearance of influenza infection (Clements and Murphy 1986).

Human tonsils (secondary lymphoid organs located in the oro- and nasopharyngeal cavity) are major organs of the nasal-associated lymphoid tissue (NALT) which are part of the common mucosal immune system. Tonsillar tissues are rich in $CD4^+$ T cells (Passali et al. 2003, Bernstein, Gorfien, and Brandtzaeg. 1999), which are likely to be an important reservoir of memory and immune competent cells serving the respiratory tract. The tonsils may function as an induction and effector site for immune responses against respiratory pathogens.

One previous study examined histological tissue sections from palatine tonsils for influenza specific antibody secreting cells (ASC) and T cells and found an increase in the number of influenza specific ASC, but a decrease in $CD4^+$T cells in the tonsils of subjects who were vaccinated with influenza vaccines (Eriksson et al. 2003). This suggests that memory $CD4^+$T cells in the tonsils may be activated by influenza vaccination to become effector T cells and migrate to the peripheral mucosa. It also suggests that $CD4^+$ T cells may be actively involved in immune defence against influenza virus. Upon specific antigen re-stimulation, memory $CD4^+$ T cells were found to be activated to become effector $CD4^+$ T cells that migrate in large numbers to the infected lung of mice infected with Influenza virus, suggesting that it may have a direct role in viral clearance in addition to the conventional function of $CD4^+$ T cells in B cell differentiation to antibody-secreting cells (Swain et al. 2004).

There are limited data available as regards to T-cell responses to influenza haemagglutinin (HA) in humans. $CD4^+$ T cells are a subset of T cells also named helper T cells that play an essential role in the induction of an adaptive immune response (Clement et al. 1988). It is generally considered that the influenza virus-specific IgG antibody production in response to the virus infection is $CD4^+$ T cell dependent (Lee et al. 2005).

CD4 T cells are important in helping B cells to generate protective antibody response. It has been shown that $CD4^+$ T-cell-deficient mice had low levels of virus-specific antibodies and were not protected against influenza virus infection (Nguyen et al. 2001). It is known that protein antigen-specific antibody production is generally $CD4^+$ T cell dependent. Zhang et al have shown that depletion of T-cells from adenotonsillar MNC diminished the antibody production to pneumococcal protein antigens (Zhang et al. 2006).

The 2009 pH1N1 virus caused a global pandemic in 2009 which infected an estimated 11-21% of the world population and resulted in considerable morbidity and mortality (Kelly et al. 2011). Little is known about the development of T cell memory following the pH1N1 infection and how it interacts with other influenza viruses.

5.2 AIMS OF STUDY

To analyze HA-specific memory $CD4^+$ T cell response to the 2009 pH1N1 virus in human NALT.

5.3 EXPERIMENTAL DESIGN

Tonsillar MNC were stimulated with purified recombinant HA proteins. T cell proliferation is analyzed by CFSE staining followed by flow cytometry. Memory T and naive T cell responses were analyzed following naive T cell depletion and memory T cell depletion respectively from tonsillar MNC using magnetic (MACS) cell sorting as below.

5.3.1 Recombinant HA

Purified recombinant HA proteins of pH1N1 (A/California/04/2009) and sH1N1 (A/Brisbane/59/2007) were from Biodefence and Emerging Infections Research Resources Repository, ATCC (Manassas VA, USA).

5.3.2 Influenza virus antigen

Influenza antigen (pH1N1) used for naïve $CD4^+$ T cell stimulation experiments was β-propiolactone inactivated, partially purified whole virus antigens from National Institute for Biological Standards and Control (NIBSC, UK). The pH1N1 virus antigen was derived from A/California/04/2009 strain.

5.3.3 Memory $CD4^+$ T cell preparation

To study memory T cell response to HA, tonsillar MNC depleted of $CD45RA^+$ cells were stimulated with individual HA of influenza viruses and analysed for $CD4^+$ T cell proliferation by CFSE staining technique. Briefly, following separation of tonsillar MNC, negative selection of memory T cells ($CD45RO^+/RA^-$) was applied using magnetic microbeads which retained naive T cells to the magnetic column (for details see chapter 2 materials and methods).

5.3.4 Naive $CD4^+$ T cell preparation

To study naive T cell response to HA, tonsillar MNC depleted of $CD45RO^+$ cells were stimulated with specific HA and analysed for $CD4^+$ T cell proliferation by CFSE staining technique. Briefly, following separation of tonsillar MNC, negative selection of naive T cells ($CD45RA^+/RO^-$) was performed using magnetic microbeads which retained memory T cells to the magnetic column. All depletion procedures were performed using Miltenyi MACS kits column (for details see chapter 2 materials and methods).

5.3.5 Analysis of $CD4^+$ T cell proliferation index

Analysis of T cell proliferation by CFSE staining was performed following depletion steps to measure the T cell proliferation index after stimulation of tonsillar MNC with individual recombinant HAs including that from sH1N1 and pH1N1 viruses. In brief, naive or memory T cell-depleted tonsillar MNC were stained with CFSE and then blocked with ice cold media. After washing cells were resuspended in complete RPMI 1640 media. Following adjustment of tonsillar MNC concentration to $4X\ 10^6$ / ml. MNC were stimulated with individual recombinant HAs in 5% CO2 at 37°C for four days. To analyze T cell proliferation, stimulated cells were harvested, washed and then resuspended in PBS for surface staining. T cell proliferation index was

measured using flow cytometry (FACScalibur; Becton Dickinson, for more details see chapter 2 materials and methods).

$CD4^+$ T cell proliferation index was used to quantify the $CD4^+$ T cell response following individual antigen stimulations. Lymphocyte and $CD4^+$ T cell gates were used in this analysis during flow cytometry. Lymphocytes were gated on the basis of their forward scatter and side scatter properties, i.e. based on their size and granularity. As shown in figure (5.3.3a) the lymphocytes were gated on region 1 (R1). $CD4^+$ T cell gate was then set as region 2 (R2) as in (figure 5.3.3b). Finally, R2-gated $CD4^+$ T cells were shown in a new window as in (figure c) with CD4-PE on y axis against CFSE on x axis. The $CD4^+$ T cell proliferative index was defined as the percentage of proliferating $CD4^+$ T cell of total CD4 T cells = A/A+B, A= upper left quadrant, B=upper right quadrant). Example of proliferative index of memory $CD4^+$ T cell following stimulation with pH1N1 HA (figure 5.2.3d) was shown as compared to unstimulated negative control (5.3.3c). The data was analysed using WinMDI software version2.9.

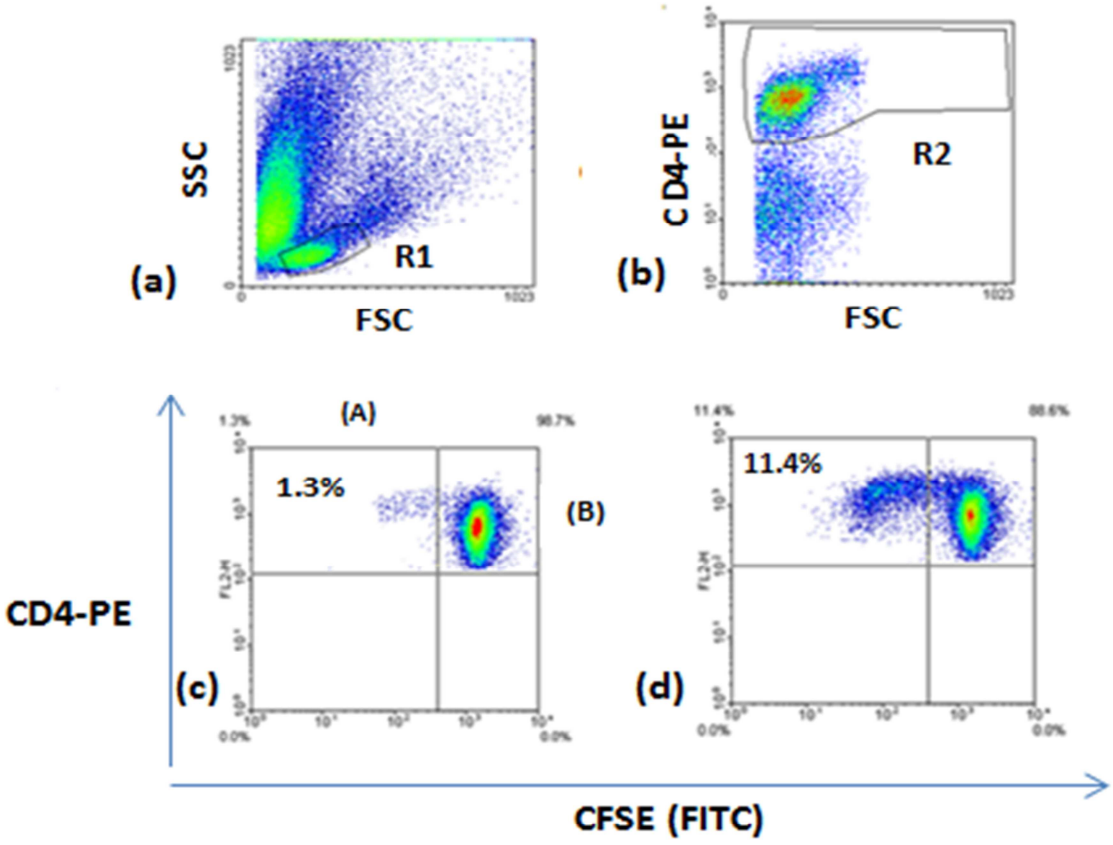

Figure 5.3.3: Gating strategy for determination of $CD4^+$ T cell proliferation index.

5.4 RESULTS

5.4.1 HA-specific memory $CD4^+$ T cell response to pH1N1 HA in tonsillar MNC and PBMC

Memory $CD4^+$ T cell proliferative response in naive T cell-depleted tonsillar MNC was analysed by CFSE staining assay. Serological antibodies–to pH1N1 were assessed by Haemagglutination inhibition (HAI) assay and an HAI titre ≥ 40 was considered as positive.

Significantly higher memory HA-specific $CD4^+$ T cell response to pH1N1 was found after stimulation of tonsillar MNC in subjects with serum anti-pH1N1 HAI titre ≥ 40 than in those with HAI< 40 (P=0.01) figure (5.4.1a). Similarly, HA-specific $CD4^+$ T cell response in PBMC following stimulation by pH1N1 HA was also higher in subjects with serum HAI titre ≥ 40 than in those with HAI< 40 (P<0.001) (figure 5.4.1b). The memory HA-specific $CD4^+$ T cell response in tonsillar MNC following stimulation appeared greater than that in PBMC.

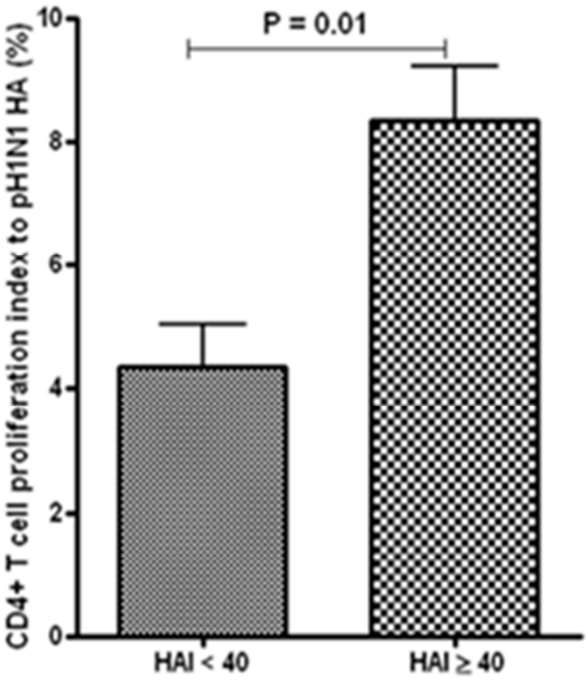

Figure 5.4.1a: 2009 pH1N1 HA induces memory CD4$^+$ T cell response in tonsillar MNC. Tonsillar MNCs were stimulated by pH1N1 HA followed by analysis using CFSE and then flow cytometry. The figure shows that subjects with serum anti-pH1N1 HAI ≥ 40 had higher memory HA-specific CD4$^+$ T cell proliferation index than in those with HAI< 40 (Student's t-test, n= 25).

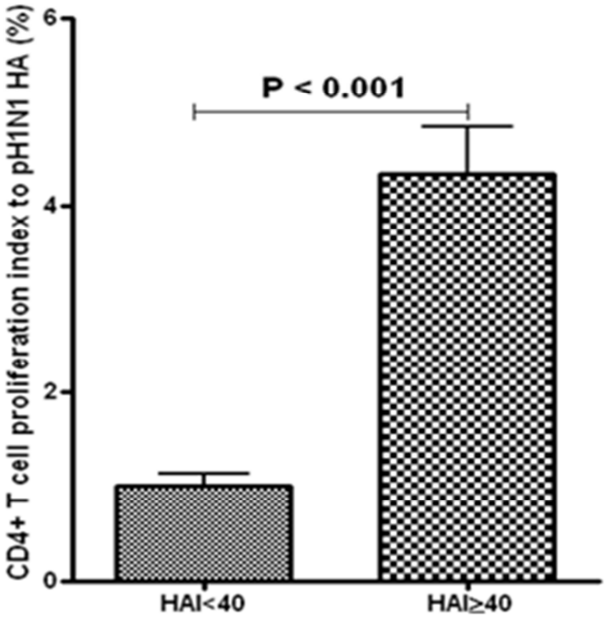

Figure 5.4.1b: pH1N1 HA induces memory CD4$^+$ T cell response in PBMC. Following stimulation of PBMC with pH1N1 HA, CFSE and flow cytometry were performed. Memory HA-specific CD4$^+$ T cell proliferative index was calculated. The figure shows that subjects with serum anti-pH1N1 HAI ≥ 40 had higher memory HA-specific CD4$^+$ T cell proliferation index than in those with HAI< 40 (Student's t-test, n= 26).

5.4.2 Correlation between memory $CD4^+$ T cell responses to sH1N1 and pH1N1 HA in tonsillar MNC

To determine whether there is any relationship between memory $CD4^+$ T cell responses to sH1N1 and pH1N1 HAs, $CD4^+$ T cell proliferation was measured following stimulation of tonsillar MNC with respective HAs. There was a positive correlation (r=0.70) between the memory $CD4^+$ T cell proliferation indices to sH1N1 and pH1N1 HAs (figure 5.4.2). These results may suggest that there are cross-reactive epitopes targeting $CD4^+$ T cells between the HAs of sH1N1 and pH1N1 viruses.

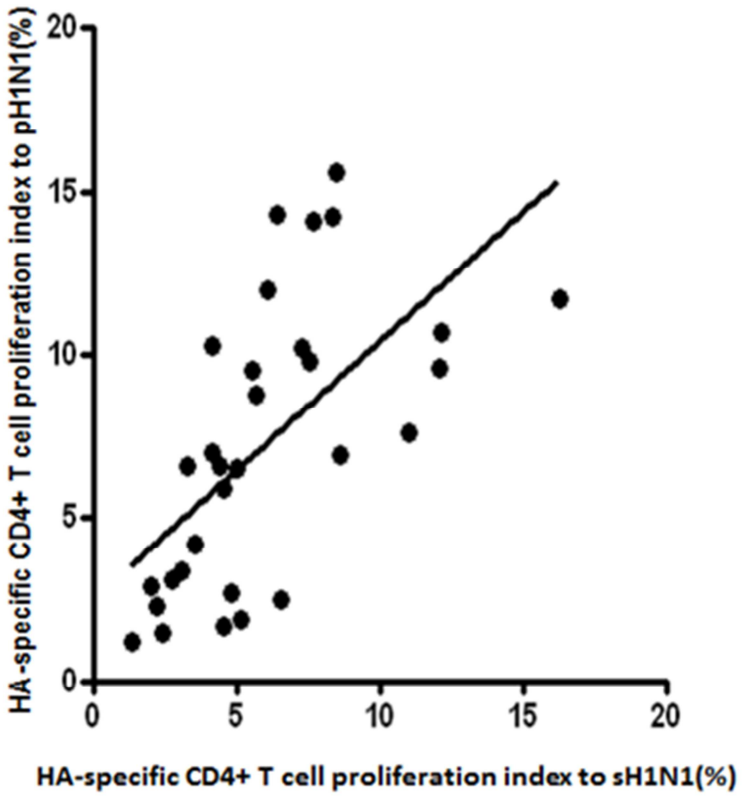

Figure 5.4.2: Correlation between memory $CD4^+$ T cell responses to sH1N1 and pH1N1 HAs. Following stimulation of tonsillar MNC with sH1N1 and pH1N1 HAs, CD4+ T cell proliferation index to both HAs were analyzed by flow-cytometry. There was a good correlation between the memory $CD4^+$ T cell responses to sH1N1 HA and that to pH1N1 HA ($r=0.70$, $p<0.001$, $n=31$).

5.4.3 Association between memory CD4$^+$ T cell response to pH1N1 HA and age

To determine whether there is any correlation between memory CD4+ T cell responses to pH1N1 and ages of patients, CD4$^+$ T cell proliferative response in memory T cell-containing tonsillar MNC was measured following pH1N1 HA stimulation, and analysed in association with patients' ages. As shown in figure 5.4.3, there was a good correlation between memory CD4$^+$ T cell responses to pH1N1 HA and the ages of patients examined (r=0.67). This may suggest that cross-reactive natural immunity to influenza virus develops overtime, i.e. the cross-reactive memory that develops due to repeated exposure to pH1N1 viruses.

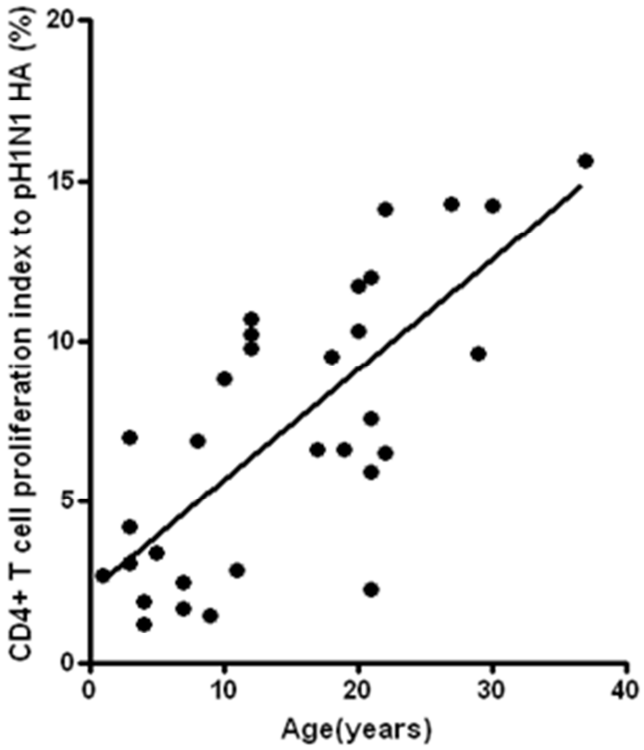

Figure 5.4.3: Association between HA-specific memory CD4$^+$ T cell response to pH1N1 HA and age. Following stimulation of tonsillar MNC with pH1N1 HA, CD4+ T cell proliferation index was analyzed with CFSE assay and flow cytometry. A positive correlation was found between HA-specific memory CD4$^+$ T cell responses and the ages of patients studied (r=0.67, n= 68, p<0.001).

5.4.4 Naive $CD4^+$ T cell response to pH1N1 antigens in tonsillar MNC

Naive $CD4^+$ T cell response to pH1N1 virus was analyzed in memory T cell-depleted tonsillar MNC (CD45RO-) following stimulation by recombinant purified HA or whole virus antigens. As shown in figure 5.4.4a, there was no significant difference in naive $CD4^+$ T cell proliferation index in pH1N1 HA-stimulated MNC when compared to unstimulated negative control (P>0.05). However, naive $CD4^+$ T cell response was detected when $CD45RO^-$ of tonsillar MNC were stimulated with the whole pH1N1 virus antigen (NIBSC) (figure 5.4.4b, p= 0.012). These results suggest that whole virus antigens are stronger immunogen that can induce primary $CD4^+$ T response than the purified HA proteins.

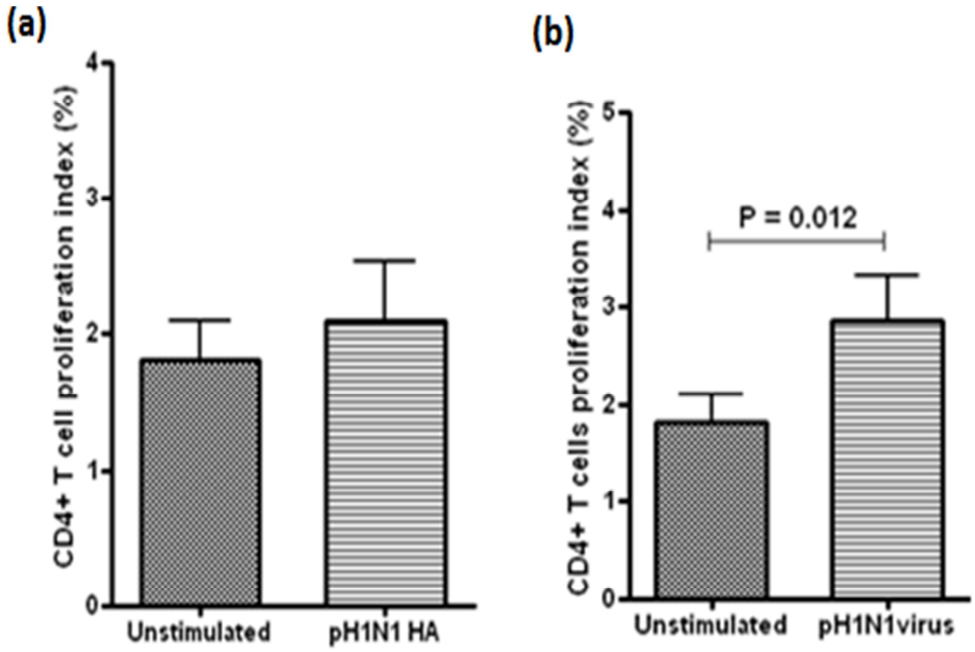

Figure 5.4.4: Naive $CD4^+$ T cell response to pH1N1 virus following stimulation with recombinant HA (a) and whole influenza virus (b). Recombinant HA did not induce naive $CD4^+$ T cell response following stimulation of memory T cell-depleted tonsillar MNC (a, p>0.05, n=15, paired t-test), whereas whole pH1N1 virus antigen induced significant naive $CD4^+$ T cell response. Mean $CD4^+$ T cell proliferation index and standard errors are shown (b, p=0.012, n=20).

5.4.5 HA-specific memory $CD8^+$ T cell response to pH1N1 in tonsillar MNC

To determine whether there is a HA-specific memory $CD8^+$ T cell response to pH1N1 virus in tonsillar MNC, CFSE assay was performed. A detectable $CD8^+$ T cell response was seen following stimulation of tonsillar MNC with pH1N1 HA compared to unstimulated negative control (figure 4.4.5, p=0.025). However this $CD8^+$ T cell response to pH1N1 HA was lower compared to the memory $CD4^+$ T cell response described earlier. It suggests that the number of HA-specific memory $CD8^+$ T cells in human NALT is lower than memory $CD4^+$ T cells which are consistent with the fact that the proportion of $CD8^+$ T cells is much lower than $CD4^+$ T cells in tonsils (Passàli et al 2003, Brandtzaeg 2003, Bernstein 1999). Nevertheless, these $CD8^+$ T cells may still be important in mucosal immunity against influenza.

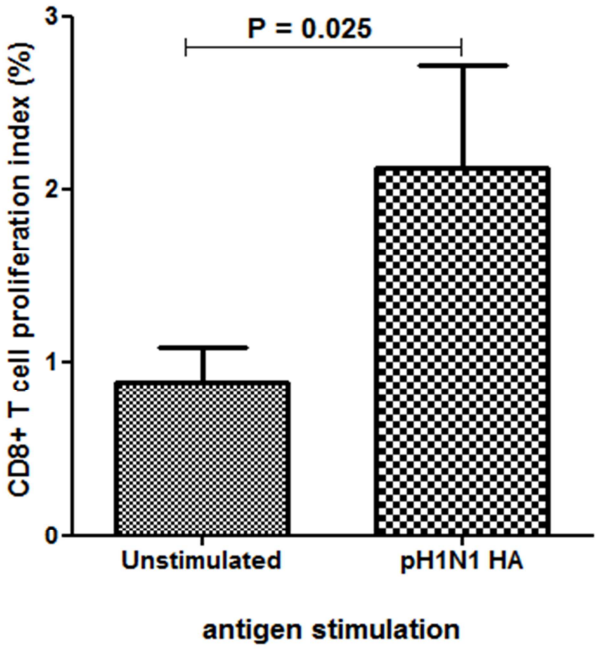

Figure 5.4.5: HA-specific $CD8^+$ T cell response to pH1N1 in tonsillar MNC. CFSE assay was performed to measure the HA-specific $CD8^+$ T cell proliferation index in tonsillar MNC. $CD8^+$ T cell response was detected after stimulation of tonsillar MNC with pH1N1 HA, as compared to unstimulated control. Mean $CD8^+$ T cell proliferation index and standard errors are shown (paired t-test, p=0.025, n= 18).

5.4.6 Cytokine responses to pH1N1 virus in tonsillar MNC

Cytokine bead array (CBA) assay was performed to measure cytokine responses in culture supernatants following tonsillar MNC stimulation with pH1N1 influenza virus antigen (NIBSC). Six cytokines were analysed including IL-17(Th17), IFN-γ, TNF-α, IL-2 (Th1), IL-4, (Th2) and IL-10.

Stimulation with pH1N1 virus antigen induced increased production of IL-17, IFN-γ, and IL-10 in tonsillar MNC (figure 5.4.6, with p values p<0.01, <0.001 and <0.001 respectively) compared to unstimulated controls. No significant levels of TNF-α, IL-4 and IL-2 were detected (figure 5.4.6). The cytokine profile suggests that pH1N1 virus antigen induces a Th1 and Th17 predominant cytokine production. Significant mucosal IFN-γ production in NALT may play a role in protection against influenza infection.

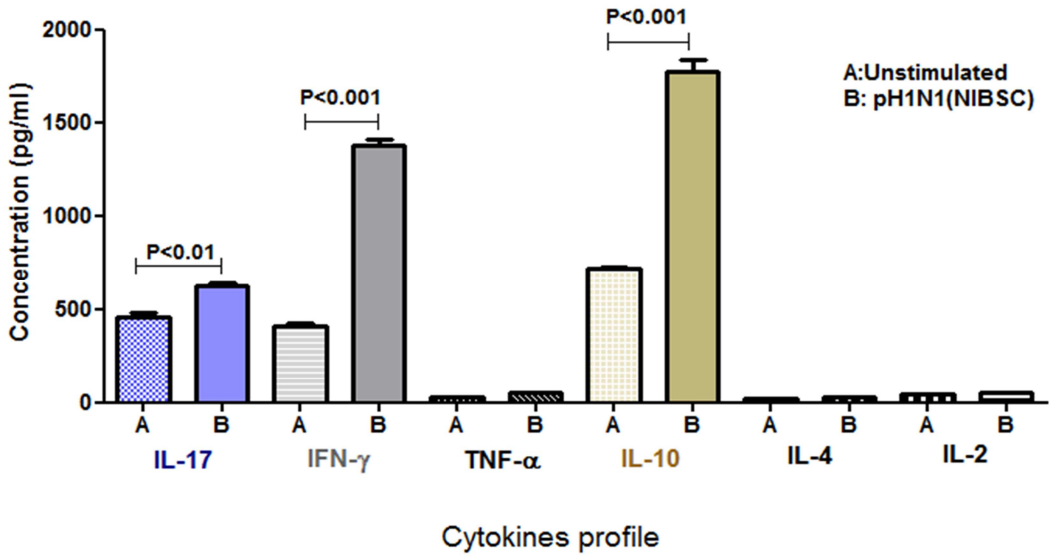

Figure 5.4.6: Cytokine responses to 2009 pH1N1 virus antigen stimulation in tonsillar MNC. Cell culture supernatants were collected following tonsillar MNC stimulation with pH1N1 influenza virus antigen and cytokines were measured using CBA assay. Increased production of IL-17, IFN-γ and IL-10 were detected compared to unstimulated negative control. Mean and standard errors are shown (p<0.01, p<0.001and p<0.001 respectively, n= 14).

5.5 DISCUSSION AND CONCLUSION

Human NALT tissues comprise a large army of immune competent cells that may play an important role in immunity to some respiratory microbes. They are the induction sites for local mucosal immune response to a number of important human pathogens such as influenza virus. Tonsils are rich in lymphocytes and may be an important reservoir of immunological memory cells serving the respiratory tract (Eriksson et al. 2003).

In this study, significant HA-specific memory $CD4^+$ T cell response to pH1N1 virus was shown in tonsillar MNC from children and adults. This suggests that following the 2009 pandemic H1N1 influenza, humans developed memory T cell response to the pH1N1 HA protein antigen at the mucosal level in the nasopharynx. HA-specific memory $CD4^+$ T cell response to pH1N1 virus was also shown in PBMC, although moderate compared to that in tonsillar MNC. This may reflect the relative lower number of memory $CD4^+$ T cells in PBMC than in tonsillar tissue.

A positive correlation (r=0.70) was seen between the HA-specific memory $CD4^+$ T cell responses to sH1N1 and pH1N1 HA in the tonsillar MNC from paired samples. This suggests that there may be cross-reactive epitopes in the HAs of pH1N1 and sH1N1 viruses that target $CD4^+$ T cells. A growing number of recent studies support the hypothesis that 2009 H1N1 virus infection induced cross-reactive anti-HA antibody responses to a range of different subtypes of influenza viruses (Ellebedy and Ahmed 2012, Ekiert et al. 2009, Sui et al. 2009, Wrammert et al. 2011). Our own data on memory B cell responses in NALT to pH1N1 HA as described in chapter 3 also support that the pandemic H1N1 infection in humans primed for strong cross-reactive memory B cell responses (Mahallawi et al. 2013).

It is known that memory $CD4^+$ T cells are crucial in memory B cell responses. It has been shown that memory $CD4^+$ T cells from sH1N1 virus infected individuals could cross-react to peptides from pH1N1 HA protein, despite several amino acid differences between the two influenza strains (Wilkinson et al. 2012). Influenza virus-specific memory CD4 T cells have been shown previously following natural infection and vaccination that persist long-term and recognize determinants in seasonal and pandemic influenza virus strains (Teijaro et al. 2010).

HAI titres of 40 are associated with a 50% reduction in the risk of infection or disease with influenza viruses in human populations (Jiang et al. 2010), so HAI titres ≥40 are usually taken as the cut-off between serum antibody-positive and -negative. In this study we used anti-pH1N1 HAI titres ≥40 as a serological evidence of previous exposure to the 2009 H1N1 virus. Subjects with anti-pH1N1 HAI ≥ 40 showed stronger memory $CD4^+$ T cell response in tonsillar MNC against pH1N1 than those subjects with HAI< 40. It suggests that the 2009 pandemic H1N1 influenza virus primed or activated the HA-specific memory $CD4^+$ T cell response in NALT. The suggested presence of cross-reactive memory CD4+ T cells to pH1N1 and sH1N1 HAs by this study would be in agreement with the hypothesis that previous seasonal H1N1 virus infection or exposure primed for cross-reactive B cell immunity to conserved regions of HA (Wrammert et al. 2011, Pica et al. 2012).

As the aim of vaccination is to develop broad and effective protection, understanding the full potential of memory $CD4^+$ T cells to have an impact on immunity is of great importance. The results from this study may support that pre-exposure to sH1N1 virus potentiated the subsequent antibody response to pH1N1 virus by priming cross-

reactive $CD4^+$ T cells that could be recalled by the pH1N1 infection (Alam and Sant 2011).

Memory HA-specific $CD4^+$ T cell response to pH1N1 virus was observed in both children and adults. However, there appeared to be an age-associated increase in this memory response. This result would also support that the development and enhancement of this memory $CD4^+$ T cell response occur with time, i.e. with previous exposure to cross-reactive HA antigens (such as sH1N1 virus).

Naïve $CD4^+$ T cell response was observed following stimulation by the whole pH1N1 virus antigen but not by the purified recombinant HA antigen in tonsillar cells. This is in agreement with the concept that a primary immune response needs activation of innate immune signals such as toll-like receptors (TLR) on antigen-presenting cells (e.g. dendritic cells) by adjuvant-like properties that exist in the whole virus antigen but not in the recombinant HA antigen.

A moderate memory $CD8^+$ T cell response to pH1N1 HA was observed in tonsillar MNC, which was smaller than the observed memory $CD4^+$ T cell response. This may suggest a low frequency of memory $CD8^+$ T cells to HA in NALT tissues. Memory $CD4^+$ T cells were more abundant than memory $CD8^+$ T cells and were shown to target a wide range of influenza proteins, whereas $CD8^+$ T cell responses were shown to target mostly internal proteins (Fonteneau et al. 2003). A previous study also observed that detected responses to the HA were mostly $CD4^+$ T cell dependent (Lee et al. 2008).

It has been shown that central memory $CD4^+$ T cells in the tonsils were activated by influenza vaccination and/or infection and become effector memory $CD4^+$ T cells which-were considered as primary source of IFN-γ (Sallusto et al. 1999, Kang et al.

2004). It was suggested that these effector memory T cells may migrate to the peripheral mucosa in response to infection and secrete IFN-γ through which they may be actively involved in immune defence against influenza virus.

It was shown in this study that pH1N1 virus antigen stimulation of tonsillar MNC induces significant IFN-γ production. It is possible that the central memory $CD4^+$ T cells in the tonsils were activated after influenza virus antigen stimulation and became effector $CD4^+$ T cells which secreted IFN-γ. This suggests that memory $CD4^+$ T cells may be actively involved in immune defence through IFN-γ production against influenza virus.

After activation, naive $CD4^+$ T cells differentiate into functional subsets called T helper type 1 (Th1) and T helper type 2 (Th2) cells, based on their production of cytokine interferon (IFN)-γ and interleukin (IL)-4, respectively. Th1 cells are essential for protection against a variety of intracellular infections, whereas Th2 responses are protective against certain extracellular infections (Seder and Ahmed 2003). Th1 immune responses are characterized by the release of high levels of IFN-γ and low levels of interleukin IL-4 (Moran 1998). $CD4^+$ T helper cells therefore may respond in an antigen-specific manner and orchestrate the effector T cell response.

IFN-γ is a proinflammatory cytokine that has been suggested to be important in protection against influenza, especially at the mucosal level (McKinstry et al. 2010). Guthrie and colleagues have shown that high levels of IFN-γ were induced in tonsillar MNC cultures following stimulation with influenza antigens which were about 3-fold higher than those in PBMC cultures. This may suggest an important role of IFN-γ in the mucosal protection against influenza virus in the upper respiratory

tract. In the same study they also observed that IFN-γ (Th1) dominated over Th2 cytokines in the mucosal immune response to influenza antigens in this compartment (Guthrie et al. 2004a).

No significant production of IL-4 (Th2 cytokine) in tonsillar MNC was observed after stimulation by pH1N1 virus antigen. This is in contrast to the significant production of IFN-γ. This suggests that the pH1N1 virus induces a predominant Th1 cytokine response.

IL-10 acts as a major immunomodulatory cytokine. It functions in concert to clear pathogens and regulates cellular immune responses which are critical for the host to protect against lethal influenza, and at the same time, to prevent excessive tissue inflammation (Kingsley et al. 2002).

In conclusion, 2009 pH1N1 HA induces a significant $CD4^+$T cell memory response in human NALT cells that suggests the previous infection by pH1N1 virus primed or activated HA-specific memory $CD4^+$ T cells. The finding that the HA-specific $CD4^+$ T cell responses to pH1N1 correlated well with that to sH1N1 virus suggests the presence of cross-reactive T cell epitopes targeting the HAs of both viruses.

Human NALT is an important compartment of the mucosal immune system which may have multiple functions in protection against influenza virus infection. Apart from the memory B cell antibody response discussed in chapter 3 (memory B cell responses to influenza HA), NALT also mounts a memory $CD4^+$ T cell response to pH1N1 virus that may not only offer specific B cell help for antibody production, but also have a more direct anti-virus effect through cytokines such as IFN-γ.

Vaccine strategies against mucosal pathogens including influenza viruses should aim to induce long-term memory at the suitable immunological sites. Whereas the intramuscular (im) delivery of influenza vaccine possibly will efficiently enhance systemic T cell responses, it may not be optimal for inducing mucosal responses. The cross-reactivity of the mucosal T cell response induced by the pH1N1 virus may have important implications to future vaccination strategies to effectively boost local cross-reactive T cell memory and enhance the antibody response to the virus.

Chapter 6

An *in vitro* Cell Culture Model of Human NALT to Evaluate B Cell Response to a Live Attenuated Influenza Vaccine (LAIV)

6.1 INTRODUCTION

Influenza is a highly contagious and acute respiratory illness caused by influenza virus, which infect the mucosa of host respiratory tract. The virus infects host epithelia cells by binding of surface glycoprotein haemagglutinin (HA) to sialic acid receptor on the cell surface (Barbey-Martin et al. 2002).

Influenza virus is transmitted through airborne droplets and via the nasal mucosa. Intranasal immunisation has been proposed to provide a more effective and biologically relevant way of vaccination against respiratory infections such as influenza. Intranasal vaccination critically relies on the local mucosal immune tissue. Human adenoids and tonsils are major components of local mucosal immune organs, namely nasal-associated lymphoid tissue (NALT) in humans and are known to be important induction sites for both mucosal and systemic immunity against upper respiratory tract pathogens (Wu and Russell 1997, Kiyono and Fukuyama 2004, Zuercher et al. 2002). NALT has been shown to be important in immune defence against influenza infection (Tamura and Kurata 2004).

Adenotonsillar tissues contain predominantly B cells (~ 65%) and $CD3^+$ T cells (~ 30%), with macrophages (~ 5%). The T cells were primarily of the $CD4^+$ subset (~ 80%) (Boyaka et al. 2000b).

Intramuscularly (i.m) injected vaccines against influenza have been in use for a long time. The main protective mechanism is through induction of systemic antibodies, mainly of IgG isotype, which prevent systemic spread of the virus. Serum IgG antibodies may also leak to the local mucosa, exert a local protective effect at the mucosal surfaces of lower respiratory tract (Belshe et al. 2004). However, Seasonal H1N1 vaccination by i.m of inactivated influenza vaccines normally offer only HA

serotype-specific protection and induces limited cross-reactive antibody responses to swine origin virus (Manicassamy et al. 2010).

Intranasal vaccination may offer certain advantages over i.m vaccination in that it may induce significant local immunity preventing local spread and transmission of the virus. To date, there is one licensed and commercially available intra-nasal vaccine and this is directed against influenza virus infection. It is based on live attenuated influenza virus strains (LAIVs). The intranasal influenza vaccine consistently showed highest efficacy in young children when compared to the trivalent inactivated vaccine (TIV) delivered by i.m (Osterholm et al. 2012).

Influenza is a mucosal infection in the respiratory tract which is transmitted through the nasal mucosa. Intranasal vaccination with LAIV has been used successfully in several countries with good efficacy. It has been licensed recently in Europe and may be used in the UK in the near future (Belshe 2004). Despite having been shown to be safe and effective in humans, little research has been done in terms of the local mucosal immunity induced by the intranasal vaccine. As it is a live attenuated vaccine containing live viruses and administered via nasal mucosa mimicking natural infection, it may induce immune response resembling natural immunity, e.g. cross-reactive immunity.

It is not known however, whether the LAIV induces cross-reactive immune response, and if it does how extensive it would be. Nasal associated lymphoid tissue (NALT) including adenotonsillar tissues are local mucosal immune organs in the upper respiratory tract, intranasal vaccines are likely to depend on these immune tissues to induce specific immune responses.

In this study, an *in vitro* cell culture model was used to characterize the B cell immune responses induced by a LAIV intranasal vaccine which contains the 2009 pandemic H1N1 virus (A/H1N1/09), an A/H3N2 strain and a B strain.

6.2 AIMS OF STUDY

To study the mucosal antibody responses in an *in vitro* model of NALT following *in vitro* stimulation with a LAIV vaccine.

6.3 EXPERIMENTAL METHODS

6.3.1 Patients and samples

Adenoids and tonsils were obtained from patients (1–37years) undergoing adenoidectomy and/or tonsillectomy. Patients previously vaccinated against influenza or who were immunocompromised in any way were excluded.

6.3.2 Intranasal LAIV (FluMist formula 2010-11) vaccine included

A/H1N1/2009; A/H3N2 and B influenza strains (BEI resources ATCC).

6.3.3 Influenza virus antigens

Influenza antigens used for cell stimulation experiments were β-propiolactone inactivated, partially purified whole virus antigens inactivated (split-virion influenza) from National Institute for Biological Standards and Control (NIBSC, UK). The pH1N1, sH1N1, sH3N2 and aH5N1 virus antigens were derived from A/California/04/2009, A/Brisbane/59/2007, A/Brisbane/10/2007 and A/Vietnam/1203/2004 strains respectively.

6.3.4 Recombinant HAs

Purified recombinant HA proteins of pH1N1 (A/California/04/2009), sH1N1 (A/Brisbane/59/2007), sH3N2 (A/Brisbane/10/2007), aH5N1 (A/Vietnam/1203/2004), H2N2 (A/Singapore/1/57), and H7N3

(A/Canada/RV444/04) were from Biodefense and Emerging Infections Research Resources Repository, ATCC (Manassas VA, USA).

6.3.5 Adenotonsillar MNC separation

Adenoidal and tonsillar tissues were transported to the laboratory in HANKS buffered salt solution supplemented with glutamine and antibiotics (penicillin and streptomycin). Mononuclear cells (MNC) from adenotonsillar tissues were isolated using Ficoll density centrifugation following methods described previously in chapter 2 materials and method.

6.3.6 Cell culture and stimulation

Adenoidal and tonsillar MNC were cultured in RPMI complete medium in the presence of different influenza antigens and LAIV. Unstimulated cells were used as negative controls. Cell culture supernatants were collected at day 10 and assayed for measuring HA-specific antibodies by ELISA.

6.3.7 Measurement of HA-specific antibody levels by ELISA

HA-specific IgG antibodies were analyzed following the ELISA procedure as described in chapter 2. In brief, ELISA plates were coated with individual recombinant HAs and incubated overnight at 4°C. After washing, plates were blocked with 10% FBS followed by incubation of cell culture supernatants at 1:4 dilutions for 1.5 hour. Alkaline phosphatase conjugated anti-human IgG (Sigma) was then incubated for 1.5 hour. After washing, p-nitrophenyl phosphate (PNPP) substrate was applied. Optical density was measured at 405 nm. For more details see materials and methods chapter 2.

6.4 RESULTS

6.4.1 Induction of anti-pH1N1 HA antibodies in adenotonsillar cells by LAIV

Adenotonsillar MNC were isolated and co-cultured with LAIV for up to 10 days. Cell culture supernatants were collected and analyzed by ELISA for the detection of pH1N1 HA-specific specific antibodies.

LAIV stimulation of adenotonsillar MNC induced IgG, IgA and IgM antibodies to pH1N1 HA. Significant anti-pH1N1 IgG antibody titres were detected in the MNC culture supernatants after stimulation (figure 6.4.1a, (mean ±SE:1.35±0.12 compared with unstimulated negative control, $p < 0.01$). LAIV stimulation also induced IgA antibody production to pH1N1 HA in adenotonsillar MNC (figure 6.4.1b, mean ± SE: 0.35±0.06 compared with unstimulated medium control, $p<0.01$). IgM antibodies to pH1N1 HA were also induced in cell culture supernatants (6.4.1c, mean ± SE: 0.58±0.10 compared with unstimulated medium control, $p < 0.01$)

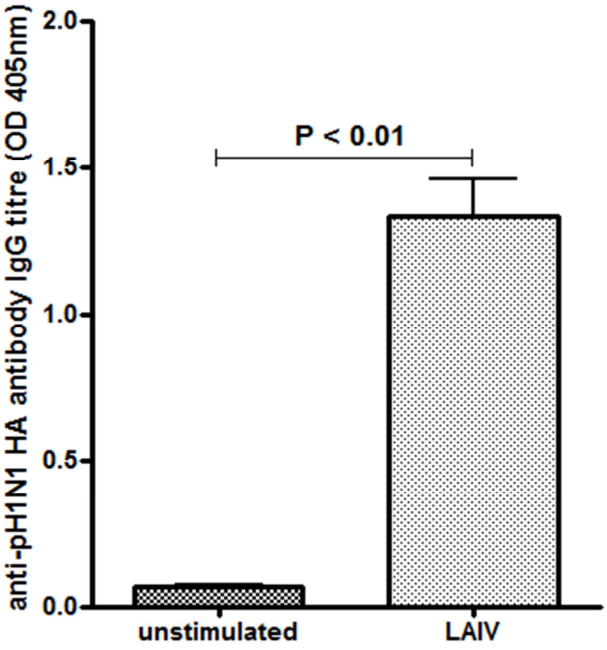

Figure 6.4.1a: LAIV induces specific IgG anti-pH1N1 HA. Tonsillar MNC were stimulated with LAIV. Cell culture supernatants were collected and analysed for anti-pH1N1 HA IgG antibody using ELISA. LAIV induced high level of HA-specific IgG antibody to pH1N1 virus, which was significantly high compared with unstimulated control, paired t-test (n=15,p<0.01). Means and standard errors are shown.

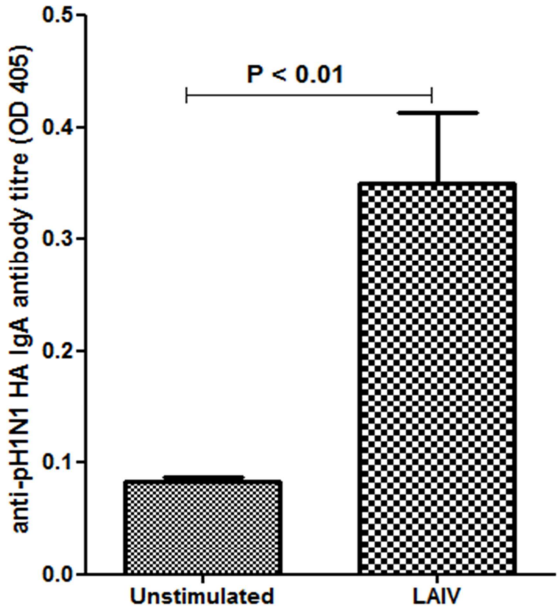

Figure 6.4.1b: LAIV induces HA-specific IgA antibody to pH1N1. Tonsillar MNC were stimulated with LAIV. Cell culture supernatants were collected and analysed for anti-pH1N1 HA IgA antibody production using ELISA. LAIV induced HA-specific IgA antibody production to pH1N1 virus (n=15, p<0.01, compared with unstimulated control, paired t-test). Means and standard errors are shown.

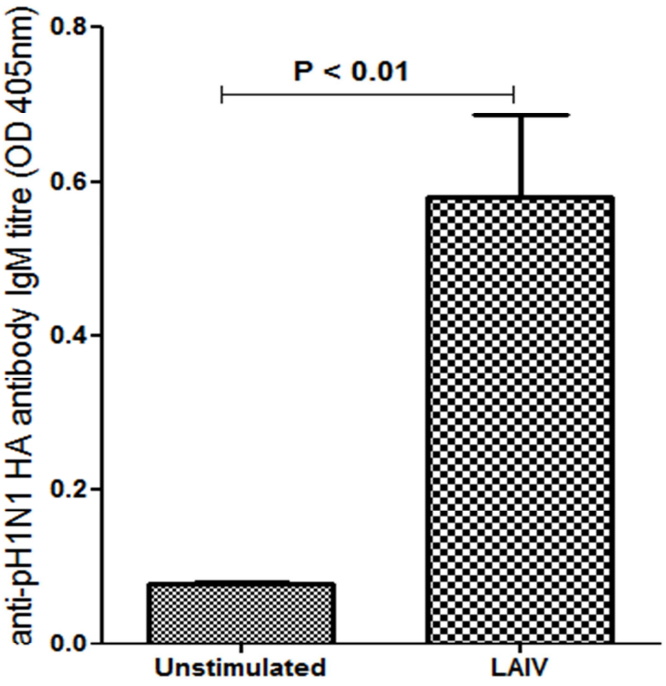

Figure 6.4.1c: LAIV induces HA-specific IgM antibody to pH1N1. Tonsillar MNC were stimulated with LAIV. Cell culture supernatants were collected and analysed for anti-pH1N1 HA IgM antibody production using ELISA. LAIV induced higher level of HA-specific IgM antibody production to pH1N1 virus (n=15, $p<0.01$, compared with unstimulated control, paired t-test). Means and standard errors are shown.

6.4.2 LAIV induces mucosal cross-reactive antibodies

To determine whether LAIV induces cross-reactive antibody responses to HAs of other subtypes of influenza viruses in tonsillar MNC, ELISA assay was performed to measure the HA-specific antibody levels to sH1N1, H2N2, sH3N2 H5N1 and H7N2 influenza A viruses in tonsillar MNC culture supernatants. Significant anti-sH1N1 IgG antibody titres were detected in cell culture supernatants after stimulation (figure 6.4.2a, mean ± SE: 0.43±0.09 compared with unstimulated medium control, $p < 0.01$). In addition, LAIV was shown to induce IgA antibody to sH1N1 HA as well after tonsillar MNC stimulation (figure 6.4.2b mean ± SE: 0.23±0.04 compared with unstimulated medium control, $p < 0.01$). IgM antibodies to sH1N1 HA were also induced in cell culture supernatants (figure 6.4.2c, mean ± SE: 0.47±0.05 compared with unstimulated medium control, $p < 0.01$).

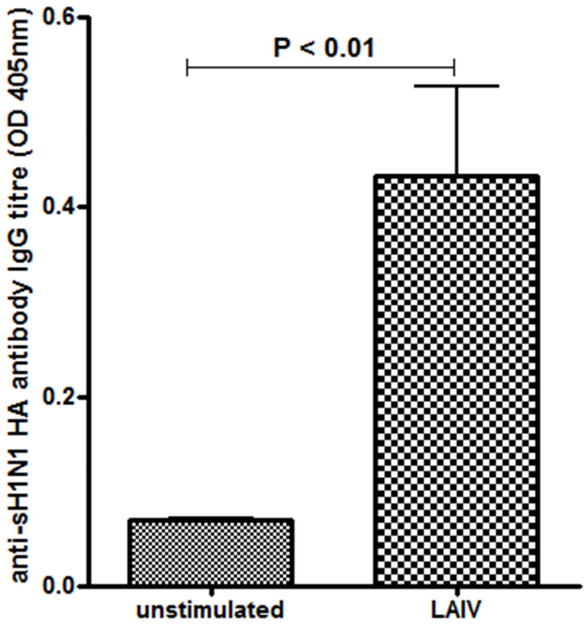

Figure 6.4.2a: LAIV induces HA-specific anti-sH1N1 IgG. Tonsillar MNC were stimulated with LAIV. Cell culture supernatants were collected and analysed for anti-sH1N1 HA IgG antibody using ELISA. LAIV induced significantly higher levels of HA-specific IgG antibody to sH1N1 compared with unstimulated control, paired t-test ($n=15$, $p<0.01$). Means and standard errors are shown.

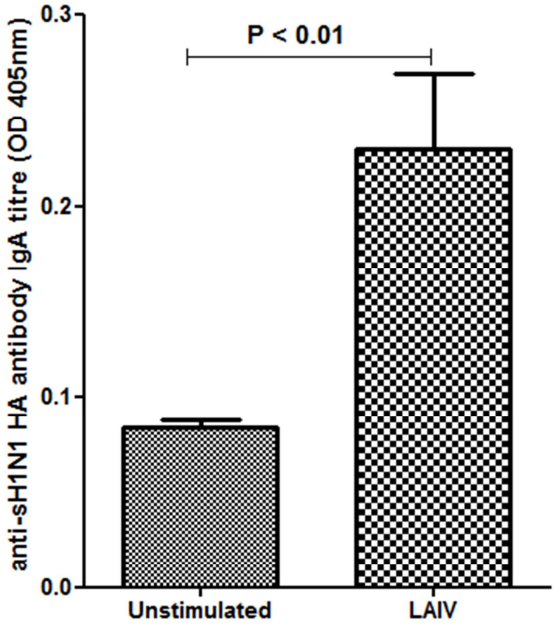

Figure 6.4.2b: LAIV induces HA-specific IgA antibody to sH1N1. Tonsillar MNC were stimulated with LAIV. Cell culture supernatants were collected and analysed for anti-sH1N1 HA IgA antibody production using ELISA. LAIV induced HA-specific IgA antibody production to sH1N1 virus (n=15, p<0.01, compared with unstimulated control, paired t-test). Means and standard errors are shown.

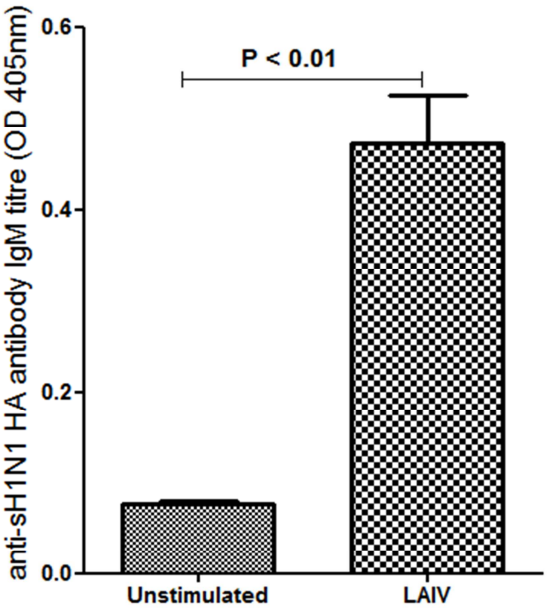

Figure 6.4.2c: LAIV induces HA-specific IgM antibody to sH1N1. Tonsillar MNC were stimulated with LAIV. Cell culture supernatants were collected and analysed for anti-sH1N1 HA IgM antibody production using ELISA. LAIV induced HA-specific IgM antibody production to sH1N1 virus (n=15, p<0.01, compared with unstimulated control, paired t-test). Means and standard errors are shown.

HA-specific antibodies to aH5N1 virus were also measured after LAIV stimulation of tonsillar MNC. LAIV was shown to induce cross-reactive anti-aH5N1 HA antibodies. As shown in figure 6.4.2d, LAIV stimulation induced high level of specific anti-HA IgG antibodies (mean ± SE: 0.84±0.20, $p < 0.01$) to aH5N1 compared with unstimulated control).

Anti-H2N2 HA IgG antibodies were also detected in the cell culture supernatants after LAIV stimulation (figure 6.4.2e, mean ± SE: 0.93±0.23 compared with unstimulated control, $p < 0.01$).

LAIV contains sH3N2 virus. As expected, high level of anti-sH3N2 HA IgG antibodies was detected after LAIV stimulation of tonsillar MNC. High levels of anti-sH3N2 HA IgG antibodies were induced in cell culture supernatants with mean ± SE: 1.2±0.23 ($p< 0.01$, compared with unstimulated medium control figure 6.4.2f).

Anti-H7N3 HA antibodies in cell culture supernatant were also measured using ELISA following tonsillar MNC stimulation with LAIV. There was no significant levels of specific anti-HA IgG antibody to H7N3 detected (figure 6.4.2g, $p>0.05$ compared to unstimulated control).

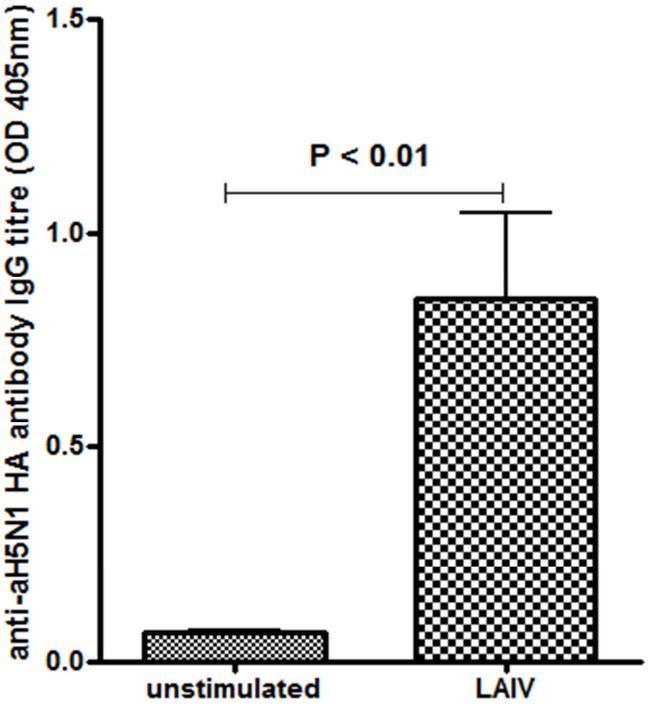

Figure 6.4.2d: LAIV induces anti-aH5N1 HA antibody. Tonsillar MNC were stimulated with LAIV, and anti-aH5N1 HA antibody in cell culture supernatants were analysed using ELISA. LAIV induced significantly higher level of specific anti-HA IgG antibody to aH5N1 compared to unstimulated negative control (n=13, p<0.01, paired t-test). Means and standard errors are shown.

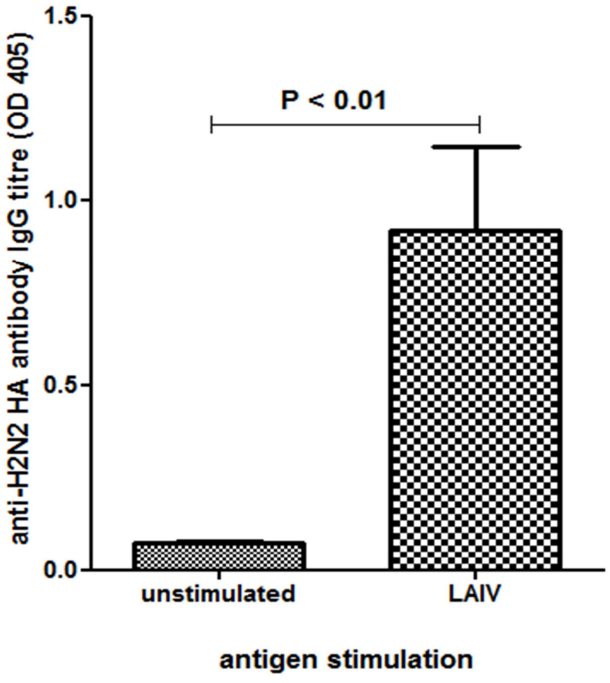

Figure 6.4.2e: LAIV induces anti-H2N2 HA antibody. Tonsillar MNC were stimulated with LAIV, and then cell culture supernatants were analysed using ELISA. LAIV induced significantly higher specific anti-HA IgG antibody to H2N2 compared to unstimulated negative control (n=13, p<0.001, paired t-test). Means and standard errors are shown.

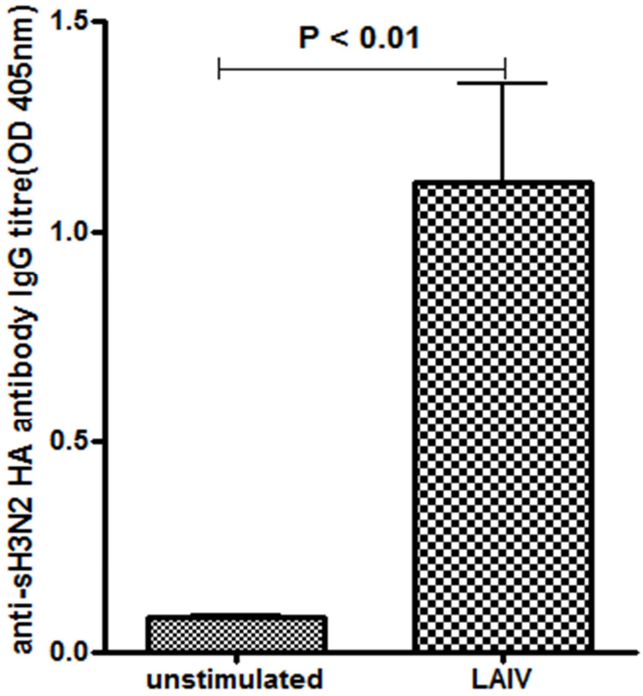

Figure 6.4.2f: LAIV induces anti-sH3N2 HA antibody. Tonsillar MNC were stimulated with sH3N2 influenza antigen and LAIV, and then cell culture supernatants were collected and analysed for anti-sH3N2 HA antibody using ELISA. LAIV induced higher level of anti-sH3N2 HA IgG antibody compared to unstimulated negative control (n=13, p<0.01, paired t-test). Means and standard errors are shown.

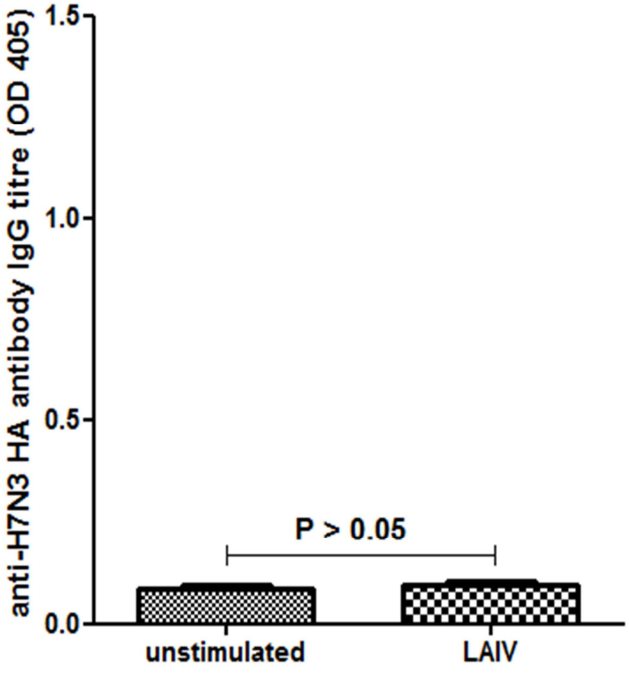

Figure 6.4.2g: LAIV did not induce anti-H7N3 HA IgG antibody production. Tonsillar MNC were stimulated with LAIV followed by analysis of anti-H7N3 HA IgG antibody by ELISA. No significant level of specific anti-HA IgG antibody to H7N3 was detected compared to unstimulated negative control (n=13, p>0.05 paired t-test). Means and standard errors are shown.

6.5 DISCUSSION AND CONCLUSION

In this study, mucosal antibody responses in NALT to HAs of a number of influenza A viruses were investigated following in vitro stimulation of adenotonsillar cells with LAIV vaccine which contains a 2009 pandemic H1N1 virus, a seasonal H3N2 and a B influenza strain. Significant antibody responses of all 3 isotypes (IgG, IgA and IgM) to the HA of pandemic H1N1 virus were observed in tonsillar cells following LAIV stimulation. This suggests human NALT tissues are likely to be a major induction site of immune response against influenza following LAIV immunization. It also suggests that the *in vitro* model of human NALT using adenotonsillar cell culture could be used to study the LAIV-induced immune responses which may predict the immunogenicity and efficacy of candidate LAIV vaccines in humans.

It has been shown previously that LAIV intranasal vaccination induces an immune response that more closely resembles natural immunity than that elicited by injectable inactivated vaccine (Cox et al. 2004), and more likely to induce broader immunity.

LAIV contains pH1N1 (A/H1N1 California 2009). The results in this study support that this LAIV vaccine induces cross-reactive IgG antibody responses to a number of influenza A viruses including seasonal H1N1, H2N2 and to some extent, to avian H5N1. The cross-reactive immunity is likely to be due to immunological memory, i.e. memory B cells, as discussed in chapter 3.

It was shown in this study that LAIV not only induces IgG but also significant levels of IgM and IgA antibodies. This suggests the vaccine induces a strong primary immune response as well as a secondary memory response.

There are 16 different influenza subtypes of HA and they are further clustered into two groups based on the molecular relatedness of the HA sequences. Namely, group 1 (H1, H2, H5, H6, H8, H9, H11, H12, H13 and H16) and group 2 (H3, H4, H7, H10, H14 and H15) (Air 1981).

The LAIV vaccine stimulation elicited cross-reactive antibodies against HAs of a number of different influenza strains including sH1N1, H2N2 and aH5N1 but not H7N3. This is consistent with the results presented in chapter 3 in that tonsillar MNC were stimulated to produce HA-specific IgG ASC to sH1N1, H2N2 and aH5N1 by pH1N1 virus antigen. This is likely to be due to the antigenic relatedness of the same influenza A group 1 viruses. However, when tonsillar MNC were stimulated with the LAIV which contains H3N2 virus (group 2), it did not seem to induce anti-HA antibody to H7N3 virus that belongs to group 2. So we did not find evidence of cross-reactive B cell antibody response between group 2 influenza viruses in this study.

It is known that both serum antibody and mucosal IgA antibody correlated with protection against influenza infection as indicated by prevention of viral shedding (Belshe et al. 2000). It has been shown previously that live attenuated vaccine induced mucosal antibodies that correlated well with protection (Clements and Murphy 1986).

Although all three isotypes, including IgA, IgG and IgM antibodies were induced by LAIV, the highest level shown was of IgG isotype. This is consistent with the results presented in chapter 3 on memory B cells. It is likely that IgG antibody level represented mainly memory B cell response; whereas IgA and IgM antibody levels mainly represent a primary response. It is generally considered that IgA antibodies

are predominant at the mucosal level. However, the question of whether mucosal IgA memory can be induced in humans either through natural infection or vaccination is being debated. A number of studies have shown that antigen-specific mucosal IgA responses are short-lived and that re-immunization does not reliably induce memory-type IgA responses (Nurkka et al. 2000, Korkeila et al. 2000). The predominance of antigen-specific IgG memory B cells to influenza HA in tonsillar tissues shown in chapter three is consistent with previous studies demonstrating the predominance of IgG memory B cell responses to protein antigens in human NALT (Boyaka et al. 2000b, Nadal and Ogra 1992, Zhang et al. 2000).

The route of vaccination is important in influencing immune responses at the initial site of pathogen invasion where protection is most effective. Immune responses required for mucosal protection can differ vastly depending on the individual pathogen. Systemic delivery of inactivated influenza vaccines has been proven to be effective in providing protection against the specific influenza subtypes included in the vaccine. These systemically delivered vaccines are capable of eliciting subtype-specific neutralizing antibodies that prevent disease but may not be sufficient to prevent infection at mucosal surfaces (Berzofsky et al. 2004, Ahlers et al. 2001).

LAIV has been shown to provide protection against both matched and mismatched influenza strains in children and adults. The broader immune protection by LAIV vaccination is likely to be provided by the cross-reactive antibodies shown in this study (Vesikari et al. 2006, Ashkenazi et al. 2006).

This pilot study using *in vitro* cell culture model of human NALT to assess B responses to live attenuated influenza vaccine (LAIV) has provided proof of principle that it could be used as a tool to study intranasal vaccines. Understanding mucosal immunity to respiratory tract infections such as influenza viruses may aid significantly to the development of effective intranasal vaccines against respiratory infections.

Chapter 7

General Conclusion

The 2009 pH1N1 virus caused global pandemic in 2009 which infected an estimated 11-21% of the world population and resulted in considerable morbidity and mortality (Kelly et al. 2011). It remains unclear whether the pH1N1 virus infection induced mucosal B cell memory in the infected population and whether this memory provides cross-protective immunity against different types of influenza viruses.

Little is known on the development of immunological memory following the pH1N1 infection and how it interacts with other influenza viruses. B-cell memory is crucial

to prolonged protection against infection and is dependent on the magnitude of the innate immune response that enhances adaptive cellular responses (Castellino et al. 2009). However, the generalization holds that antibodies prevent infection whereas cellular responses control infection once replication has been established (Plotkin 2010).

Human adenotonsillar tissues are major components of nasal-associated lymphoid tissues (NALT) which are considered to be an important part of the mucosal immune system (Wu et al. 1997b). The induction of immunological memory against influenza virus most likely involves these immunocompetent NALT tissues, where antigen-specific memory B cells are primed. B cells in NALT in the nasopharynx mostly produce IgG antibody, whereas, other mucosal compartments such as Peyer's patches in the intestine, the majority of B cells produce IgA (Boyaka et al. 2000b, Nadal et al. 1992b).

Use of the human NALT in this study to investigate the immunological memory to pH1N1 virus was itself a novel method which could be used in future as a human model to study other respiratory virus infections. The immunological memory to pH1N1 was different from that of the previous seasonal influenza (sH1N1) i.e., pH1N1 virus appeared to induce stronger cross-reactive antibodies than sH1N1 virus. Several studies have reported that the predominant virus circulating in the subsequent 2010-2011 influenza season was pH1N1, which essentially replaced the previously circulating sH1N1 viruses (Pica et al. 2012), Surveillance report of influenza and other respiratory viruses in the UK, 2010-2011 by HPA). The significant finding in this work was that the 2009 pH1N1 infection primed or activated cross-reactive memory B cells in human NALT to haemagglutinin of different influenza viruses.

In this study, we showed that stimulation with pH1N1 virus antigen activated a strong IgG memory B cell response with production of HA-specific antibodies against not only pH1N1, but also sH1N1 and aH5N1 viruses. This finding is consistent with previous studies evaluating the cross-reactivity of serum antibodies in patients infected with pH1N1 virus (Pica et al. 2012). This is likely due to the structural similarities between the group 1 HAs, including H1, H2 and H5 subtypes, which are phylogenetically different from group 2 HAs including H3 and H7 subtypes. Also These results are consistent with the presence of plasmablasts secreting cross-reactive neutralizing antibodies in patients infected with pH1N1 (Corti et al. 2011, Krause et al. 2010, Ekiert et al. 2009), and moreover are in agreement with the hypothesis that pH1N1 infection may activate pre-existing memory B cells targeting conserved regions of HA molecule (Wrammert et al. 2011, Pica et al. 2012).

The functional characteristics of antibodies, as well as quantity, are important and crucial in protecting from infections (Plotkin 2010). This study we also showed a cross-reactive memory response to aH5N1 HA in individuals with previous exposure to pH1N1 virus. This finding may have important implications in future influenza vaccination strategies. It is plausible to enhance such cross-reactive B cell memory by vaccination, e.g. intranasal mucosal immunization to boost this natural immunity.

The ability of pH1N1 virus antigen to elicit a strong HA-specific memory B cell response and cross-reactive neutralizing antibodies suggests it may be possible to utilize pH1N1 HA or conserved HA regions in an influenza vaccine to induce cross-reactive immunity against influenza viruses including aH5N1. It also suggests a

future exposure to aH5N1 virus in individuals who were previously exposed to pH1N1 virus may trigger a memory response against aH5N1 virus. It was reported previously that serum antibodies in an aH5N1 infected patient bind to a variety of conserved peptides in the stem region of HA (Khurana et al. 2009), so it is possible that there are closely related cross-reactive epitopes in the HAs of pH1N1 and aH5N1 viruses.

The results obtained from analysis of serum specific anti-pH1N1 HA IgG using ELISA showed that HA-specific antibody levels to pH1N1 in adults were significantly higher than that of children. It may be an indicator suggesting that adults had been exposed to more influenza viruses than children and developed more cross-reactive memory responses against some influenza viruses. Also, significantly higher HA-specific IgG antibody titres to pH1N1 HA (measured using ELISA) were found in subjects who had HAI titres ≥40 to pH1N1 than in those with HAI antibody titre <40 of the same virus. This suggests that following the 2009 pH1N1 pandemics, large numbers of people developed anti-pH1N1 HA antibodies to both the circular head and the stalk regions of HA which may have broader protective immunity.

$CD4^+$ responses, key to B-cell help and cytokine production, sometimes correlate with protection against influenza virus infection (Plotkin 2010). The results in this thesis demonstrated HA-specific memory $CD4^+$ T cell response to pH1N1 virus in tonsillar cells from children and adults. This suggests that following the 2009 pandemic H1N1 influenza, humans developed memory T cell response to the pH1N1 HA protein antigen at the mucosal level in the nasopharynx. Additionally, there appeared to be an age-associated increase in this memory response.

Furthermore, the study also investigated the possibility to use NALT as a human model to explore LAIV activity to induce antibody response. To establish that, mucosal antibody responses in NALT to HAs of a number of influenza A viruses were investigated following *in vitro* stimulation of adenotonsillar cells with LAIV vaccine that contains a 2009 pandemic H1N1 virus, a seasonal H3N2 and a B influenza strain. Significant antibody responses of all 3 isotypes (IgG, IgA and IgM) to the HA of pandemic H1N1 virus were observed in tonsillar cells following LAIV stimulation. Additionally, LAIV was shown to induce broadly cross-reactive antibodies to HAs of the included strains in the vaccine as well as to other HAs that were not included in the vaccine such as, H2N2, sH1N1and aH5N1. It suggests that the *in vitro* model of human NALT using adenotonsillar cell culture could be used to study the LAIV-induced immune responses which may predict the immunogenicity and efficacy of candidate LAIV vaccines in humans.

In conclusion, these findings add significant information to our understanding on the natural immunity to influenza viruses following the pandemic H1N1 infection. Also, the use of human NALT cells was shown to be a good model to test and explore the mucosal immunity to influenza viruses. Using this novel model to assist and examine future formulation of vaccines could also help the development of vaccines against other respiratory pathogens.

Chapter 8

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