ABSTRACT

The role of T cell immunity in natural influenza A infection in a UK cohort: Flu Watch

Lili Wang

Wolfson College, University of Oxford

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A novel influenza A virus bearing the characteristics of high virulence, the ability to infect humans and transmit from human to human, such as the pandemic influenza of 1918, could lead to millions of deaths. The 2009 influenza pandemic demonstrated that a novel influenza A virus could spread globally in a few months despite the availability of modern comprehensive surveillance systems and systemic prevention and control measurements. Novel pandemic strains that could occur naturally or be created in laboratory, pose a serious threat to public health.

Current available vaccines are capable to induce neutralizing antibodies against the viral surface antigen hemagglutinin (HA), which provide sterilizing immunity by blocking infection. However this antibody protection is serotype specific and therefore offers limited or no protection against a serologically distinct influenza virus. An alternative vaccine approach is the induction of cross-protective T cell immunity, directed at influenza A conserved internal proteins which could potentially offer broad protection against different influenza strains in humans. This approach may complement antibody-inducing vaccines and greatly enhance the protective efficacy of influenza vaccines.

This study builds on previous evidence derived from animal models and human experimental challenge studies, to demonstrate the heterotypic T cell immunity in the context of natural influenza A infection in humans. Pre-existing influenza A specific T cell immunity was quantified by human IFN-γ ELISpot assay and was detectable in over 70% of Flu Watch participants. Nucleoprotein was the most immunodominant viral protein; and the most potent viral protein in eliciting influenza A specific CD8+ T cells. The nucleoprotein specific T cells exhibited high level of cross reactivity to the 2009 pandemic influenza and reduced the occurrence of nasal viral shedding in the absence of antibody immunity, following acquisition of pandemic influenza infections.

This study provides evidence to support the development of a T cell based influenza vaccine, and provides important evidence to empower future studies of this kind.

Declaration

I declare that the work presented in this D.Phil. thesis entitled “The role of T cell immunity in natural influenza A infection in a UK cohort: Flu Watch” is my own work, except where the contributions of my collaborators have been clearly acknowledged, and that it has not been submitted for any other degree.

September 2012

Lili Wang

MRC Human Immunology Unit
Weatherall Institute of Molecular Medicine

Wolfson College
The University of Oxford

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Abbreviations

APC	Antigen presenting cells
CDR	Complementary determing regions
CMV	Cytomegalovirus
CTL	Cytotoxic T lymphocytes
DC	Dendritic cells
β_2m	Beta-2-microglobulin
BCR	B cell receptor
BSA	Bovine serum albumin
DMSO	Dimethyl sulfoxide
ELISpot	Enzyme-linked immuosorbent spot
FACS	Fluorescence activated cell sorting
FCS	Foetal calf serum
HA	Hemagglutinin
HI	Hemagglutinin inhibition assay
HLA	Human leukocyte antigen
ICS	Intracellular cytokine staining
IFN-$\alpha\beta$	Type 1 interferon
IFN-γ	Interferon-gamma
LAIV	Live attenuated vaccine
M	matrix protein
MHC	Major histocompatibility complex
NA	Neuraminidase
NK	Natural Killer cell
NP	nucleoprotein
NS	non-structure protein
PA	polymerase acidic protein
PB2	polymerase basic protein 2
PB1	polymerase basic protein 1
PBMCs	Peripheral blood mononuclear cells
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PE	Phycoerythrin
PHA	Phytohaemagglutinin
PAMP	Pathogen-associated molecular pattern
RNA	Ribonucleic acid
SFC	Spot forming cell
TCR	T cell receptor
T_{EM}	Effector memory T cell
T_{CM}	Central memory T cell
Th	T helper
TIV	Trivalent inactivated influenza vaccine
TLR	Toll-like receptor
TNF	Tumour necrosis factor
WHO	World Health Organisation

Table of Contents

Chapter 1: Introduction 10

1.1 Influenza virus 14
1.1.1 Influenza A virus virion structure 14
1.1.2 Influenza A virus replication cycle 16
1.2 Host range restriction and pathogenicity 19
1.3 Antigenic variation 23
1.3.1 Antigenic drift 23
1.3.2 Antigenic shift 23
1.4 Ecology of influenza A viruses 24
1.5 Influenza epidemiology 25
1.5.1 Modes of transmission 25
1.5.2 Circulating influenza strains in the Flu Watch cohort 27
1.6 Clinical Features 31
1.7 Diagnosis 32
1.8 Influenza vaccines 33
1.9 Host antiviral immune response to influenza infection 37
1.9.1 A brief overview of innate immunity against influenza infection 37
1.9.2 Adaptive immunity to influenza infection 42
1.10 Rationale for the study of T cell immunity in humans against natural influenza A virus infection 50
1.11 Aims of this study 51
Chapter 2: Materials and methods 52

2.1 Study design 53
2.2 Influenza specific definitions applied in the Flu Watch study 55
2.3 Flu Watch cohort characteristics 56
2.4 Clinical and epidemiology data collection 59
2.5 Statistical analysis and power considerations 59
2.6 Laboratory experimental procedures 60
2.6.1 Peptide design 60
2.6.2 Blood specimen collection 61
2.6.3 PBMC isolation, cryopreservation and thawing 62
2.6.4 *Ex vivo* IFN-γ ELISpot assay 63
2.6.5 Intracellular cytokine staining (ICS) of IFN-γ production by cultured influenza specific T cells 64
2.7 Assay standardization 67
2.7.1 Assessing the impact of delayed PBMC isolation on the detection of influenza specific T cell responses measured by human IFN-γ ELISpot Assay 67
2.7.2 Assessing the impact of cryopreservation on the detection of influenza specific T cell responses measured by human IFN-γ ELISpot assay 69
Chapter 3: Characterization of *ex vivo* pre-existing influenza A (H3N2) specific T cells in a UK cohort 71

3.1 Introduction 72
3.2 Results 74
3.2.1 Defining a positive T cell response 74
3.2.2 Assessment of ELISpot intra-replicate variations 75
3.2.3 The breadth and magnitude of *ex vivo* pre-existing influenza A (H3N2) specific T cells 78
3.2.4 NP and M are immunodominant targets of influenza A specific T cells at the population level 83
3.2.5 CD4+ and CD8+ phenotyping of pre-existing influenza A specific T cells 86
3.2.6 T cell epitope mapping and characterization of their CD4+, CD8+ phenotype and HLA restrictions 91
3.3 Discussion 97
Chapter 4: Durability and cross- reactivity of *ex vivo* influenza A (H3N2) specific T cells 103

4.1 Introduction 104
4.2 Results 106

4.2.1 The durability of *ex vivo* influenza A (H3N2) specific T cells in the absence of detectable influenza infection or influenza vaccination 106
4.2.2 Assessing the level of subtype specific T cell immunity and the cross-reactivity of *ex vivo* T cells directed at immunodominant internal proteins of NP and M 111
4.3 Discussion 116
Chapter 5: Assessing if sex and/or age may be confounders or modifiers 122
5.1 Introduction 123
5.2 Results 124
5.2.1 Assessing if there were associations between *ex vivo* pre-existing influenza A (H3N2) specific T cell responses and age, sex 124
5.2.2 Assessing if the age and gender influenced the disease severity if infected with influenza 131
5.3 Discussion 134
Chapter 6: The role of T cell immunity in natural influenza A infection 141
6.1 Introduction 142
6.2 Results 144
6.2.1 The influenza burden in the UK and the specificity of clinical signs and symptoms 144
6.2.2 Investigating the role of *ex vivo* pre-existing influenza A (H3N2) specific T cells in protection against symptoms and nasal viral shedding in natural seasonal and pandemic influenza infection 150
6.2.3 Power of detection 161
6.3 Discussion 164
Chapter 7: General discussion 170
7.1 The importance of the accurate identification of influenza infection 172
7.2 Highly cross-reactive and immunogenic influenza A nucleoprotein (NP) was the most attractive target for a T cell based influenza vaccine 174
7.3 The likely value of this study for the development of a T cell based vaccine 177
7.4 Challenges of this study 180
7.5 Future directions 181
7.6 Conclusions 183
References 185
Appendixes: 204
Appendix 1: Preparation of peripheral blood mononuclear cells (PBMCs) from whole blood 204
Appendix II: *Ex vivo* ELISpot assay for the Flu Watch study 212
Appendix III The amino acid sequence alignment of seasonal H1N1, H3N2 and pandemic H1N1 217

List of Figures

Figure 1.1: The genetic origin of pandemic strain of H1N1 12
Figure 1.2: Electron micrograph of an influenza virus. 14
Figure 1.3: Schematic diagram of the influenza A virus virion 15
Figure 1.4: Replication cycle of an influenza virus 18
Figure 1.5: Schematic demonstration of sialic acid α2,3 linkage and α2,6 linkage to galactose 19
Figure 1.6: Hemagglutinin cleavage sites 21
Figure 1.7: Eras of Human influenza virus 30
Figure 1.8: Adaptive immunity in influenza infection 42
Figure 2.1: Flu Watch Bleeding schedule 54
Figure 2.2: Flu Watch sample selection for T cell study 57
Figure 2.3: The age distribution of Flu Watch cohort in comparison with national statistics 58
Figure 2.4: FACs analysis gating strategy for the characterization of CD4 and CD8 restrictions 66
Figure 2.5: The delayed PBMC isolation did not significantly impact the *ex vivo* frequency of influenza A (H3N2) specific T cells against all influenza antigens 68
Figure 2.6: Cryopreservation did not impact the detection of influenza specific T cells using human IFN-γ ELISpot assay 69
Figure 2.7: Cryopreservation did not impact the performance of PBMCs in human IFN-γ ELISpot assay 70
Figure 3.1: Example of Flu Watch ELISpot output 76
Figure 3.2: The non-specific responses detected in *ex vivo* human IFNγ ELISpot assay were extremely low 76
Figure 3.3: Most participants have influenza A (H3N2) specific T cell responses 80
Figure 3.4: Considerable variations in *ex vivo* pre-existing influenza A (H3N2) specific T cell responses measured by human IFN-γ ELISpot assay 81
Figure 3.5: *Ex vivo* pre-existing influenza A (H3N2) specific T cells were detected in the UK population at low frequency by human IFN-γ ELISpot assay 82
Figure 3.6: The percentage of contributions from H3N2 viral antigen specific T cell responses to the total magnitude of influenza A (H3N2) T cell responses in the Flu Watch cohort 85
Figure 3.7: The breadth of *ex vivo* pre-existing influenza A (H3N2) specific T cell responses stratified by viral antigen specificity 85
Figure 3.8: The relationship between culture explanded and *ex vivo* influenza A (H3N2) specific T cell responses. 89
Figure 3.9:The distribution of CD4+ and CD8+ influenza A (H3N2) specific T cell responses across the whole proteome of influenza A (H3N2) virus 90
Figure 3.10: The distribution of mapped individual epitopes across full proteome of influenza A H3N2 virus 95
Figure 3.11: The distribution of peptides inducing CD4 and/or CD8 T cell responses across the whole proteome of influenza A (H3N2) virus 96
Figure 4.1: *Ex vivo* influenza A (H3N2) specific T cell responses detected at the baseline correlated with their corresponding post season responses (N=190) 107
Figure 4.2: The durability of influenza A (H3N2) specific T cell responses at population level from pre season to post season (N=190) 108
Figure 4.3: The durability of *ex vivo* influenza A (H3N2) specific T cells over a period of 1.5 years. 110
Figure 4.4: H3N2 specific T cell immunity was higher than H1N1 specific T cell immunity in the Flu Watch cohort. 113
Figure 4.5: anti-NP and anti-M T cells were highly cross-reactive against pandemic H1N1 2009 virus 115
Figure 4.6: Strong and significant correlations were observed between influenza specific (both NP specific and M specific) T cell responses against H3N2 and pandemic H1N1 (pH1N1) 115
Figure 5.1: *Ex vivo* pre-existing influenza A (H3N2) specific T cell responses declined after the age of 50 126
Figure 5.2: The age related decline in *ex vivo* influenza A (H3N2) specific T cell responses in participants aged 50-year or over was not antigen specific 127
Figure 5.3: *Ex vivo* pre-existing influenza A (H3N2) specific T cell responses were lower in female participants compared to male participants. 129
Figure 5.4: There was no difference in the baseline influenza A specific antibodies between female and male participants 130

Figure 6.1: Over 80% of participants infected were seronegative.....145
Figure 6.2: Influenza infection rates in seasonal and pandemic influenza and the disease severity....148

List of Tables

Table 1.1: Predominant circulating strains over the period of the Flu Watch study 30
Table 1.2: Influenza Diagnostic Methods 32
Table 2.1: The recruitment of each influenza season in the Flu Watch study 56
Table 3.1: The distribution of variance to median ratios from triplicate experimental wells in the Flu Watch human IFN-γ ELSIpot Assay 77
Table 3.2: List of epitopes identified in Flu Watch cohort 93
Table 4.1: The amino acid sequence alignment of seasonal influenza H3N2, H1N1and pandemic H1N1 (pH1N1) 112
Table 5.1: The influenza infection rates did not differ between females and males 130
Table 5.2: Clinical outcomes of seasonal influenza infection in the absence of serotype specific antibodies stratified by age and sex 132
Table 5.3: There was little evidence that age and sex associated with the disease severity if infected with seasonal influenza virus 133
Table 6.1: Flu Watch participants infected with more than one subtypes 145
Table 6.2: Influenza like illness with confirmed fever was associated with natural influenza infection 149
Table 6.3: Immune correlate of protection conferred by total *ex vivo* pre-existing H3N2 specific T cells against pandemic H1N1 2009 152
Table 6.4: Immune correlate of protection conferred by *ex vivo* pre-existing H3N2 NP specific T cells against pandemic H1N1 2009 153
Table 6.5: Immune correlate of protection conferred by *ex vivo* pre-existing cross-reactive anti-NP T cells against pandemic H1N1 2009 154
Table 6.6: Immune correlate of protection conferred by *ex vivo* pre-existing H3N2 M specific T cells against pandemic H1N1 2009 155
Table 6.7: Immune correlate of protection conferred by *ex vivo* pre-existing cross reactive anti-M T cells against pandemic H1N1 2009 156
Table 6.8: Total *ex vivo* pre-existing H3N2 specific T cells did not protect against either influenza like illness or nasal viral shedding if infected with natural seasonal influenza H3N2 and/or H1N1 . 158
Table 6.9: *Ex vivo* pre-existing H3N2 NP specific T cells did not protect either influenza like illness or nasal viral shedding if infected with natural seasonal influenza H3N2 and/or H1N1 159
Table 6.10: *Ex vivo* pre-existing H3N2 M specific T cells did not protect either influenza like illness or nasal viral shedding if infected with natural seasonal influenza H3N2 and/or H1N1 160
Table 6.11: The Flu Watch study was underpowered to detect the protection against nasal viral shedding conferred by *ex vivo* pre-existing cross-reactive NP specific T cells 163
Table 6.12: Infected individuals that would be need to power a study in detecting heterotypic immunity against nasal viral shedding 163

Chapter 1: Introduction

Influenza infection is a highly contagious viral infection leading to acute respiratory disease and substantial mortality and morbidity. It is estimated that there are approximately 600 million cases of influenza infection with 3 million cases of severe illness and 250,000 to 500,000 deaths in a non-pandemic year globally[1]; The majority (approximately three quarters) of influenza infections are caused by type A influenza infections. A pandemic influenza could result in millions of deaths worldwide [2] with a devastating economic and social impact.

Influenza A pandemics occur when an influenza virus with a hemagglutinin (HA) from animal reservoir emerged and spreads within human populations that have little or no immunity against the newly introduced HA. The "Spanish flu" (H1N1) pandemic in 1918 is the most devastating influenza pandemic in history, causing the deaths of approximately 40 million people, which is several times higher than other pandemic influenza. It is not fully understood the cause of the high mortality rate of 1918 pandemic. It also remains uncertain whether 1918 pandemic H1N1 influenza A virus was the result of adaptation of an influenza virus from animal reservoir or was a reassortment between animal and human influenza viruses[3-5]. Three less severe pandemics followed, which were products of reassortment. The 1957 pandemic influenza (also known as "Asian influenza" H2N2) and the 1968 influenza (also known as "Hongkong Influenza" (H3N2)) were both the results of reassortments between avian and human influenza A viruses. In the 1957 pandemic, both the surface antigens of the influenza virus, hemagglutinin (HA) and neuraminidase (NA), and one internal protein, polymerase basic protein 1 (PB1) were of avian origin [6, 7]. In the 1968 pandemic, the HA and PB1 of the influenza virus were of avian origin[7, 8]. The global death tolls in 1957 and 1968 were estimated to be 2 million and 1 million,

respectively[9]. The swine-like pandemic influenza in 2009 (H1N1) was the product of triple reassortment (Figure 1.1) [10] and It is estimated that, in the first 12 months of its circulation, approximately 300,000 deaths were associated with the 2009 pandemic globally[11].

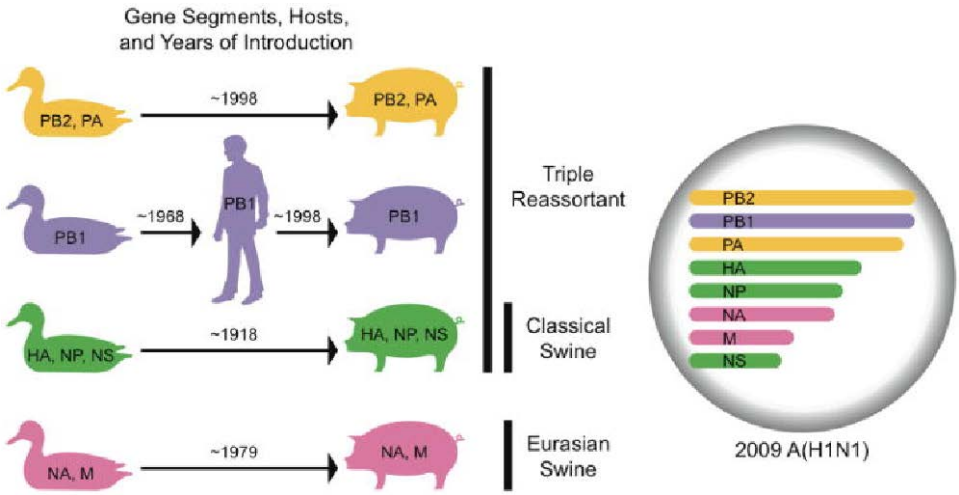


Figure 1.1: The genetic origin of pandemic strain of H1N1
PB2 polymerase basic 2, PB1 polymerase basic 1, PA polymerase acidic, HA hemagglutinin, NP nucleoprotein, NA neuraminidase, M matrix gene, NS nonstructural gene. Colour of gene segment in circle indicates host. Lineages are determined by phylogenetic study. Adopted from illustration by Garten, R.J., et al., Science, 2009. **325**(5937): p. 197-201.

Annual vaccination is the most effective way to prevent influenza A infection and severe clinical outcomes from the illness. Neutralizing antibodies can provide sterilizing immunity to a homologous influenza strain by preventing the establishment of infection. Current influenza vaccines available to the public largely target the surface glycoprotein of HA. Tremendous effort is made by Center of Disease Control,

USA (CDC) to monitor antigenic drift and shift through its annual surveillance programme, which consists of World Health Organization (WHO) collaborating laboratories, public health laboratories, academic medical Centers and hospital laboratories. The annual review of vaccine composition aims to combat the progressive loss of humoral immunity via antigenic drift. However it is impossible to predict antigenic shift via re-assortment or adaptation, which could result in a pandemic strain. The H1N1 pandemic in 2009 highlighted the unpredictable nature of a pandemic influenza. Similarly, the emergence of the highly pathogenic Avian Influenza virus H5N1 in 1997 and the rapid spread of this extremely virulent influenza A virus in birds has raised serious concerns about a new highly pathogenic human Influenza A pandemic. It would take approximately 6 months to formulate a new vaccine and deliver them to health care providers [12]. The vaccine for pandemic H1N1 2009 first became available in early October 2009, which was indeed approximately 6 months after it first emerged in April 2009[13]. Whilst this process is seemingly rapid, in the event of a pandemic this time frame actually poses a serious public health risk. In a simulation model of pandemic influenza, it was predicated that it would take around 90 to 100 days for the UK to reach the peak attack rate of a pandemic influenza after the pandemic virus first emerged elsewhere in the world and approximately 15% to 30% of population would be infected. This study also predicted that vaccination started after 120 days (4 months) would have no effect in reducing attack rate[14]. While antibody-based vaccines continue to be the main immunization strategy, alternative approaches towards generating cross protective immunity that could be used in combination with, or engineered into, antibody-based vaccines are needed to achieve protection against a novel pandemic influenza.

1.1 Influenza virus

1.1.1 Influenza A virus virion structure

Influenza A virus is a single-stranded, negative-sense RNA virus of the Orthomyxoviridae family (Figure 1.2). The influenza genome consists of 8 segments encoding 11 viral proteins: HA (hemagglutinin), NA (neuraminidase), NP (nucleoprotein), M1 (Matrix protein 1), NS1 (non-structure protein 1), PB2 (polymerase basic unit 2), PB1 (polymerase basic unit 1), PA (polymerase acidic unit), and M2 (matrix protein 2), NS2 (non-structure protein 2), and PB1-F2. M2 and NS2 are encoded by a second RNA of M and NS genes as the result of mRNA splicing. PB1-F2 is the product of an alternative open reading frame near the 5' end of the PB1 gene.

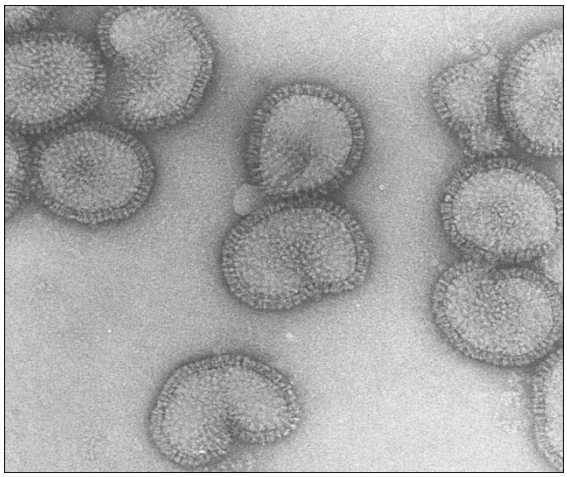


Figure 1.2: Electron micrograph of an influenza virus.
Credits: Rob Ruigrok/the Unit of Virus Host-Cell Interaction.
http://www.esrf.eu/news/general-old/general-2009/flu/

The two surface glycoproteins, HA and NA, and the matrix protein 2 (M2) are inserted into the viral lipid envelope and project from the virus surface. Approximately 80% of the surface projections are haemagglutinin and the remaining 20% is neuraminidase [15, 16]. Matrix protein 1 (M1) forms an inner protein layer beneath the viral lipid envelope, enclosing the nuclear export protein NEP, also known as nonstructural protein 2 (NS2), and the ribonucleoprotein complex (RNP)- which consists of the viral RNA segments coated with nucleoprotein (NP). The RNPs are associated with RNA-dependent RNA polymerase complex that consists of 3 subunits: two polymerase basic units (PB2, PB1) and one polymerase acidic unit (PA) (Figure 1.3).

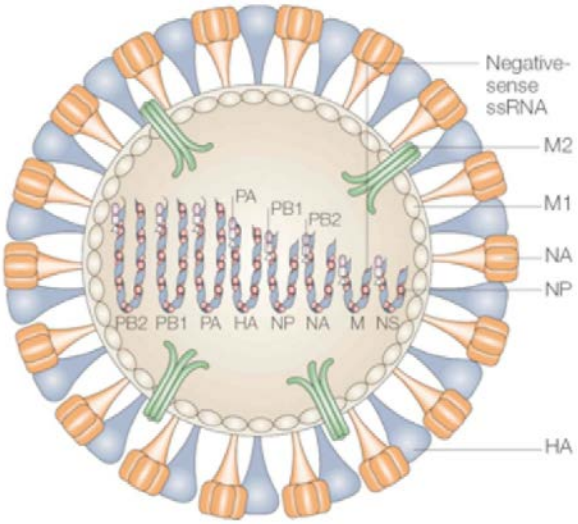


Figure 1.3: Schematic diagram of the influenza A virus virion
Influenza virus consists of eight single stranded negative-sense RNA segments coated with nucleoprotein (NP). Matrix protein 1(M1) forms an inner protein layer underneath the lipid envelope and Matrix protein 2 (M2) inserted into the lipid layer. Two glycoproteins are expressed on the virus surface: hemagglutinin (HA) and neuraminidase (NA). PB2: Polymerase basic protein 2; PB1: Polymerase basic protein 1; PA: Polymerase acidic protein (PA); NS: non-structure protein.
Copyright 2005 Nature Publishing Group, Horimoto, T., et. al., Influenza: Lessons from past pandemics, warnings from current incidents, Nature Reviews Microbiology 3, 591-600.

1.1.2 Influenza A virus replication cycle

The influenza virus replication cycle is summarized in Figure 1.4 and the details are shown below.

The virus replication cycles consists of the following steps:

a) The globular surface subunit of HA known as HA1, binds to the sialic acid (N-acetyl-neuraminic acid) receptors expressed on the surface of the epithelial cells lining the sinuses and respiratory tract [17, 18].

b) The virion is then internalized by receptor–mediated endocytosis via both clathrin-mediated [19, 20] and clathrin and cavelolin- independent pathways that lead to successful infection [19, 21].

c) In the endosomal vesicles, host proteases cleave HA into HA1 and HA2 [22, 23] and the influx of protons through the M2 ion channel reduces the pH levels in the endosome. HA2 undergoes conformational changes at low pH (5-6) and translocates the relatively hydrophobic region, referred to as the fusion peptide, at the N-terminal towards the lipid bilayer of the target membrane. This ultimately leads to the fusion of the viral and cell membranes. Viral RNPs are then released into cytoplasm [24].

d) The viral RNPs separate from M1 protein and are imported into the host nucleus through nuclear pores mediated by nuclear localization signals on NPs[25, 26].

e) In the nucleus of their host cells, transcription and synthesis of viral RNAs takes place. The viral RNPs serve as templates for the production of positive-sense viral messenger RNA (mRNA) and complementary RNA (cRNA)[27]

f) Viral mRNAs are exported from the nucleus to the cytoplasm for translation. The viral cRNAs remain in the nucleus to serve as a template for the production of negative-senses viral genomic RNA (vRNA)

g) Viral mRNAs are translated into viral proteins by cytoplasmic ribosomes

h) Many newly synthesized viral proteins (polymerases, NP, NS1 and NS2) are imported back into the nucleus[28, 29] for the vRNP assembly and for exporting vRNPs out of the nucleus[30].

i) vRNPs are assembled in the nucleus then are exported out of the nucleus

j) vRNPs form progeny virions and bud from the plasma membrane[31]

k) NA cleaves sialic acid off the cell surface and progeny virions are released.

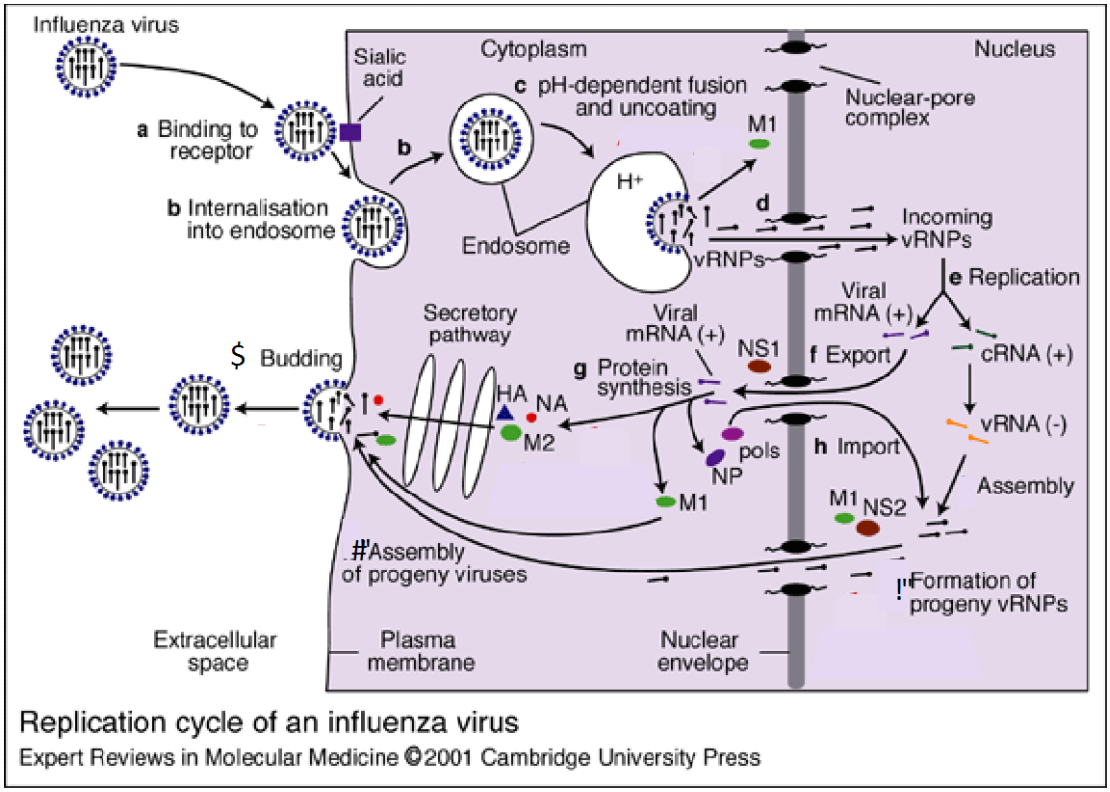


Figure 1.4: Replication cycle of an influenza virus
(a) The virus binds to receptors on the surface of the host cell (b) virus is internalized by endocytosis (c) virus fusion and uncoating events (d) the release of the viral genome (e) virus replication (f) mRNA are exported out of the nucleus into the cytoplasm (g) viral protein sythesis (h) some of newly synthesized viral proteins are imported back into the nucleus (i) vRNPs assembly (j) progeny viruses assembly (k) virions bud from plasma membrane then released from the host cell. Abbreviations used: mRNA: messenger RNA; vRNP: viral ribonucleoprotein complex. Modified from illustration by Gary R. Whittaker (2001) [32]

1.2 Host range restriction and pathogenicity

Sialic acid is a generic term for a nine-carbon, acidic amino sugar. Influenza hemagglutinin (HA) binds to sialic acid linked to galactose. Human influenza viruses preferentially bind to the sialic acid linked to galactose by α2,6-linkage (SAα2,6Gal), while avian influenza viruses prefer the α2,3-linkage (SAα2,3Gal) (**Figure 1.5**) [33, 34]. SAα2,6Gal is predominantly found in the epithelial cells of human nasal mucosa, paranasal sinuses, pharynx, trachea, bronchi, but is also expressed in the epithelial cells of the terminal and respiratory bronchioles [35]. SAα2,3Gal is found in lower respiratory tracts on non-ciliated cuboidal bronchiolar cells at the junction between the respiratory bronchiole and alveolus as well as on cells lining the alveolar wall [35, 36]. Therefore, although H5N1 virus preferentially binding to SAα2,3Gal can be transmitted from birds to humans, the efficient virus replication is restricted to lower respiratory tract, where SAα2,3Gal receptors are available. Such restriction may limit transmission through sneezing and coughing, therefore, provide an explanation for the inefficient human-to-human transmission so far[35]. Avian HAs could bind to the SAα2,6Gal with low affinity and mutations in human HAs were associated with the adaptation of the receptor bind specificity of the avian HA to SAα2,6Gal by increasing binding affinity[37].

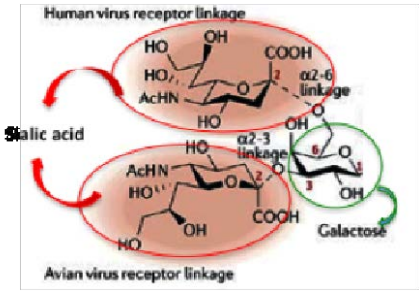


Figure 1.5: Schematic demonstration of sialic acid α2,3 linkage and α2,6 linkage to galactose
Sialic acids are circled in red and the galactose is circled in green. Numbers in red indicate the carbon position of sialic acid.

Influenza hemagglutinin is synthesized as a precursor form (HA0); HA0 is cleaved by host protease into two subunits, the globular surface unit HA1 and the stalk-like transmembrane unit HA2 (Figure 1.6). This cleavage step is essential for virus infectivity as it liberates the fusion peptide, the hydrophobic N-terminus of HA2, and acquires structural changes in HA molecule that allow the subsequent molecular rearrangements for fusion[38]. Human influenza virus and low pathogenic avian influenza virus (LPAIV) contains a single arginine (or rarely a single lysine) [39, 40] at the cleavage site and are cleaved by trypsine and trypsin-like host proteases extracellularly [22, 23]. This limits the spread of virus in hosts to tissues where the appropriate proteases are encountered. Highly pathogenic avian influenza A viruses (HPAIV) contain multiple basic amino acid at the cleavage site that can be cleaved by ubiquitous subtilisin-like proteases such as furin[41, 42] and PC6[43]. Furin is a cellular endoprotease in nearly all cell types and accumulates in the *trans*-Golgi network, which is also the cellular compartment where this HA type is cleaved

Highly pathogenic influenza virus HA can be cleaved intracellularly in the TGN/biosynthetic pathway leading to systemic infections [23, 44].

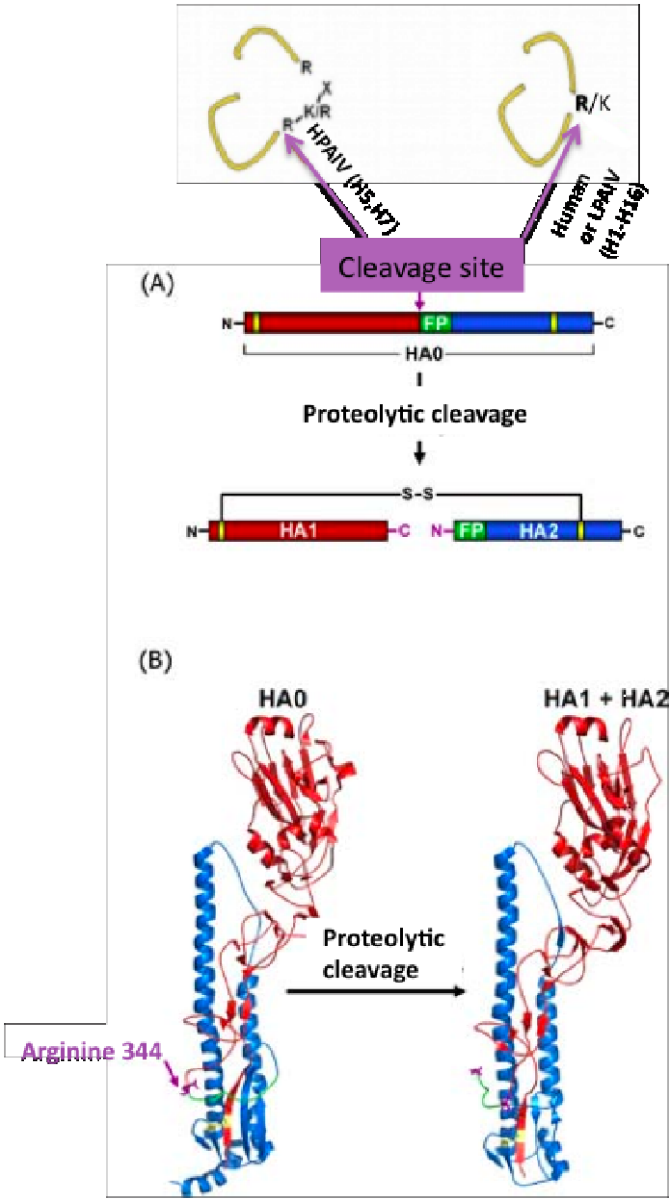


Figure 1.6: Hemagglutinin cleavage sites
Multibasic cleavage sites harbour multiple arginines and/or lysines and are found in the HA-proteins of high pathogenic avian influenza viruses (HPAIV), with a consensus motif of R-K/R-X-R. Monobasic cleavage sites consist of a single arginine (Arg 344 (purple) in case of the 1918 influenza virus or lysine and are found in the HA-proteins of human influenza viruses and low pathogenic avian influenza virus (LPAIV). The HA precursor HA0 is cleaved into two subunits HA1 (red) and HA2 (blue) by host proteases which recognize either a multibasic or monobasic cleavage site located in a loop between HA1 and HA2. The subunits HA1 and HA2 remain covalently linked by a single disulfide bond (yellow), the newly formed C-terminus of HA1 and N-terminus of HA2 are labeled in purple. (A) Schematic representation of HA cleave and amino acid sequence cleavage site (B) Structural changes associated with cleavage of the 1918 influenza HA (A/South Carolina/1/18 (H1N1), Accession-No: ADD17229, HA0 (1RD8.pdb), HA1 + HA2 (1RUZ.pdb). Modified from illustration by Bertram, S., et al., Rev Med Virol, 2010. 20(5): p. 298-310. [45]

Polymerase basic protein 2 (PB2) is a component of influenza virus polymerase complex, which is essential for viral replication. A glutamic acid to lysine mutation at position 627 of the PB2 protein has been reported to be associated with the efficient replication of avian influenza viruses in humans[46-49].

NS1 protein is an antagonist to interferons (IFNs) [50]. Type I IFNs (IFNα and IFNβ) are the most important anti-viral cytokines. The antiviral effects of Type I IFNs are discussed in details in section 1.9.1 of this thesis. NS1 protein inhibits interferon IFNβ production and the activation of interferon induced antiviral genes, thus allow efficient viral replication [50-54]. The NS1 gene of highly pathogenic influenza virus may be more efficient at counteracting the host immune response than those less pathogenic influenza viruses as in human cells. The NS1 gene of the 1918 Spanish flu more efficiently blocked the expression of IFN-regulated genes than that of the A/PR/8/34 (H1N1) virus [55]. Recombinant viruses containing the NS1 gene of the highly pathogenic H5N1 1997 human isolates were resistant to antiviral treatment of IFN and TNF-α. This resistance is associated with glutamic acid at position 92 of the NS1 protein[56, 57]. Taken together, the NS1 protein of highly pathogenic influenza viruses likely contributes to the extreme pathogenicity in humans.

1.3 Antigenic variation

1.3.1 Antigenic drift

Antigenic drift is the result of random mutations introduced by the lack of proofreading of viral RNA polymerase. Under selection pressure, random mutations that result in non-synonymous changes that allow escape from humoral immunity become predominant circulating strain. The loss of humoral immunity from antigenic drift is a cumulative and gradual process that can lead to an epidemic.

1.3.2 Antigenic shift

Antigenic shift is the result of re-assortment of RNA segments and / or introduction of a new strain from an animal reservoir (adaptation). Both sequence database studies and *in vitro* co-infection studies confirmed that influenza viral RNAs could re-assort between different human viral stains or between human and animal influenza virus[58-60]. These re-assortments can produce virus strains with novel surface glycoproteins against which there is no pre-existing humoral immunity in the host population thus leading to a pandemic.

1.4 Ecology of influenza A viruses

There are 17 subtypes of hemagglutinin (H1-17) and 10 subtypes of neuraminidase (N1-10). Phylogenetic studies of influenza A viruses has suggested that aquatic birds are natural hosts to all influenza virus [61, 62]. All subtypes of influenza A virus could be isolated from avian species [63, 64]. The recently identified H17N10 was only found in fruit bats [65, 66]. Influenza A viruses have been isolated from a wide range of mammalian hosts, including humans, horses, pigs, seals and whales [61]. Only 3 HA (H1, H2 and H3) and 2 NA (N1, N2) subtypes have been established in humans with rare infections caused by H5, H7 and H9 subtypes[67].

1.5 Influenza epidemiology

1.5.1 Modes of transmission

Influenza is mainly transmitted via large droplets (>5μm in diameter) expelled from sneezing and coughing by an infected individual. This mode of transmission requires close contact between the index case and recipient as large droplets travel only short distances of approximately 6 feet or less. Contact transmission can also occur. It has been shown that influenza virus could be recovered by culture or HA testing from hard nonporous surfaces (steel, plastic) up to 48 hours after deposition and from porous surfaces of tissue, cloth and paper less than 8-12 hours following deposition. Influenza virus which was transferred from these surfaces to hands survived a further 5 minutes [68]. The possibility of aerosol transmission was also demonstrated in animal and human studies. Animal studies showed that artificially aerosolized influenza from liquid suspension of different influenza strains (<5μm in diameter) could infect various animals including mice[69, 70], ferrets [71, 72] and guinea-pigs [73]. In a human infection study, the volunteers were infected by inhaling carefully titrated aerosolized influenza virus suspensions with particle sizes of 1μm-3μm through a facemask [74]. Infections in this study were confirmed by seroconversion or by recovery of infectious virus from throat swabs taken daily after exposure. Recent studies provided evidence that airborne influenza viruses are detectable in public premises[75-78], human exhaled breath[79] and coughs[80]. However, it is difficult to elicit the exact contribution from each mode of transmission in natural human influenza infection, as it is difficult to separate out the contribution from each mode of transmission. Study of the mode of influenza transmission in humans is further complicated by pre-existing host immunity, host genetic determinants and

their susceptibility to different strains of influenza virus and antigenic drift of circulating influenza virus. In summary, the exact contribution of each mode of influenza transmission is not fully understood.

1.5.2 Circulating influenza strains in the Flu Watch cohort

During the first 3 years of our Flu Watch study (October 2006 onwards), the predominant circulating virus alternated between H3N2 and H1N1 subtypes (Table 1.1). In 2009, the swine-like pandemic H1N1 (referred to as pandemic H1N1 2009 in this report) became the predominant subtype. As shown in Figure 1.7, H1N1 was first entered in humans in 1918, which resulted in the most severe influenza pandemic-also known as the "Spanish flu"[81]. The 1918 H1N1 influenza A virus continued to circulate until the A (H2N2) influenza pandemic of 1957. During this period, human H1N1 influenza viruses acquired substantial antigenic drift away from the 1918 H1N1[82]. Then the H1N1 from the 1950's reemerged in humans in 1977[61] and circulated until 2009. From 1977 to 2009, further substantial antigenic evolutions of the human H1N1 led to eight updates of the H1 component of the influenza virus vaccine[83]. By 1998, the antigenic drift of H1N1 had created a substantial antigenic divergence from the classical swine H1N1 and thus the swine virus became the potential reservoir for a pandemic H1N1[10]. H3N2 was first entered in humans in 1968, which led to influenza pandemic- also known as the "Hongkong Flu"[84]. H3N2 and H1N1 had been co-circulating from 1977 to 2009 (Figure 1.7). The swine-like pandemic H1N1 first reached the UK in May 2009 after its early outbreaks were reported in North America in April 2009. Studies done by Centre of Disease Control (CDC, USA) suggested that the pandemic H1N1 2009 was a triple reassortant of H1N1, H1N2 and H3N2 swine influenza viruses [10, 85]; After the 2009 H1N1 pandemic, H3N2 returned to circulation and co-existed with pandemic 2009 H1N1 in humans[86]. Genetic analysis of pandemic H1N1 2009 showed it originated from animals and was unrelated to human seasonal H1N1[87], thus vaccination with recent (2005-2009) seasonal influenza vaccine was unlikely to provide sufficient protection

against this novel pandemic H1N1 virus [88, 89]. But a recent systematic review and meta-analyses suggested that seasonal influenza vaccine provided moderate (34-38%) cross protection against laboratory confirmed 2009 pandemic influenza illness.

Pre-existing cross-reactive antibodies to pandemic H1N1 at the required protective level (HI>40) were rarely detectable (less than 9%) in the population under 64 years of age in the United State; in contrast, about a third of its population aged over 60 years had protective levels of cross-reactive antibody [88, 89]. The level of pre-existing cross-reactive antibodies to pandemic H1N1 2009 in England was investigated by Health Protection Agency; about 80% of population under 65 years of age were seronegative (microneutralisation titer <10) and around 30% of population aged 65 years or over had protective levels of antibody (microneutralisation titer >40)[90]. As the vaccination with current inactivated seasonal influenza vaccine in human did not result in the increase in cross-reactive antibody against pandemic H1N1 2009 in those age 60 or older[88, 89], the protective level of cross-reactive antibody against pandemic H1N1 may be induced by previous exposure or vaccination with influenza viruses that were closely related to pandemic H1N1 2009 in this age group. Study of historical sera suggested that the pandemic H1N1 2009 virus was antigenically related to 1918 H1N1 pandemic virus and a single dose of H1N1vaccine in 1976 could boost antibody primed through pre exposure that cross reacted to pandemic H1N1 2009 [10]. Serological study of over 1,000 samples taken in the UK before the pandemic H1N1 2009 suggested that those were born before 1957 may have been exposed to a H1 strain that was closely related to pandemic H1N1[91].

Around 80% of the Flu Watch cohort had no pre-existing antibody response against pandemic H1N1 2009 (reported in chapter 6, Figure 6.1). This is in agreement with a separate population study of 587 Italian serum samples, in which, 16% of people aged 65 or under had pre-existing HI titers >10, suggesting 84% of population under 65 years were seronegative (HI<10) against pandemic H1N1 2009 [92]. Therefore the pandemic H1N1 2009 provided an excellent opportunity for us to investigate cross-protection mediated by pre-existing influenza specific T cell immunity in the absence of antibody protection.

Table 1.1: Predominant circulating strains over the period of the Flu Watch study

Year	06-07		07-08		08-09		09-10	
Predominant circulating strains	H3N2		H1N1		H3N2		Pandemic H1N1	
Influenza type (subtype)	A (H1)	A (H3)	A (H1)	A (H3)	A (H1)	A (H3)	A (H1 pandemic 2009)	A (Non-pandemic 2009)
Cumulative total	33 (4%)	847 (96%)	551 (93%)	43 (7%)	105 (6%)	1644 (94%)	2983 (97.2%)	76 (2.5%)

Above data were collected by Health Protection Agency, UK (www.hpa.org.uk)

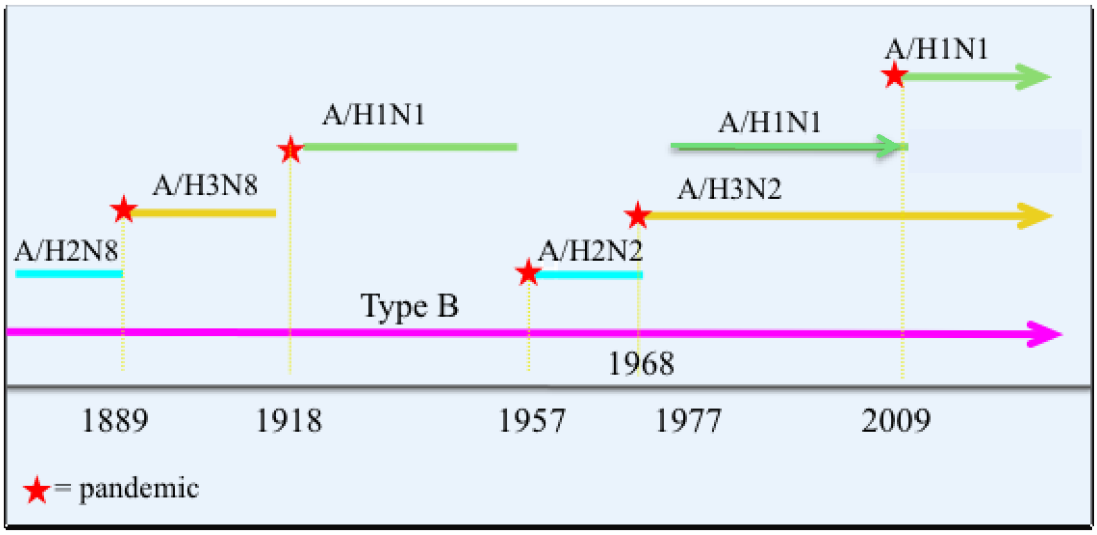


Figure 1.7: Eras of Human influenza virus
H1N1 first emerged in humans in 1918, and then reemerged in 1977. In 2009, a swine like H1N1 emerged in humans. H3N2 first emerged in 1968 then H3N2 and H1N1 has been co-circulating in humans since 1970's. Modified from illustration of Alan W Hampson&John S Mackenzie [93].

1.6 Clinical Features

The clinical symptoms of influenza infection include any or all of those listed below:

- Fever (can last 3-5 days)
- Muscle aches (can last 3-5 days)
- Headache
- Lack of energy (may last 2 or more weeks)
- Dry cough (may last 2 or more weeks)
- Sore throat
- Runny nose

The initial symptoms of influenza infection listed above can be similar to those caused by other infectious agents such as: adenovirus, respiratory syncytial virus, rhinovirus and parainfluenza virus. Therefore, it is difficult to diagnose influenza infection based on clinical symptoms alone. However studies have shown that the presence of cough and high fever is likely to be associated with influenza infection: In a clinical trial of an anti-influenza drug in which 3,744 unvaccinated adults and adolescents with influenza-like symptoms were studied, the positive predictive value was 79% amongst patients presenting with cough and fever (>37.8°C) [94]. In a separate study of general practioners in three different clinics, the positive predictive value was 86.8% amongst patients presenting with fever (>38°C) and cough [95].

1.7 Diagnosis

A number of laboratory diagnostic tests are available to identify true influenza infection; the tests are listed in the Table 1.2. Serology testing requires paired acute and convalescent sera, it is not suitable for routine diagnostic testing, but is valuable for research purpose and epidemiological studies.

Table 1.2: Influenza Diagnostic Methods

Procedure	Influenza Virus Types Detected	Acceptable Specimens	Test Time
Viral culture	A and B	NP[2] swab/aspirate, nasal swab/aspirate/wash, throat swab, bronchioalveolar lavage	3-10 days[3]
Immunofluorescence [Direct Fluorescent Antibody (DFA) or Indirect Fluorescent Antibody (IFA) Staining	A and B	NP[2] swab/aspirate, nasal swab/aspirate/wash, throat swab	2-4 hours
RT-PCR[5]	A and B	NP[2] swab/aspirate, nasal swab/aspirate/wash, throat swab, bronchioalveolar lavage sputum	2-4 hours
Serology*	A and B	Paired acute and convalescent serum samples[6]	2 weeks or more
(EIA)	A and B	NP[2] swab/aspirate, nasal swab/aspirate/wash, throat swab	2 hours

Adopted form Centers for Disease Control and Prevention (http://www.cdc.gov/flu/professionals/diagnosis/rapidclin.htm#table)

1.8 Influenza vaccines

The currently licensed influenza vaccines are inactivated vaccines and live attenuated vaccines; these are egg-based vaccines[96]. There are various candidate vaccines including cell-based vaccines, vectored and non-vectored vaccine strategies are in development. These were reviewed by Gambotto, A., et al. [97] and will not be discussed in detail in this thesis.

Inactivated influenza vaccines are licensed worldwide and contain the hemagglutinins of influenza strains that are likely to circulate in human populations. Current seasonal influenza vaccines include two type A influenza viruses (H1N1 and H3N2) and one influenza B, thus are also known as trivalent inactivated vaccines (TIV). There are three types of licensed inactivated influenza vaccine; they are whole virus, split virion and subunit surface antigen vaccines. The inactivated influenza vaccines contain killed virus, as they induce antibodies against surface antigen, they need to be reformulated each year due to the antigenic changes in HA. The quantification of TIV relies on the HA content quantification, NA is not standardized and therefore is variable.

Live attenuated influenza vaccines are only licensed for use in Russia, UK and in USA; they are cold adapted live virus vaccines (LAIV) containing influenza viruses that only replicate in the upper respiratory tract where temperatures do not exceed 32–33 °C[98, 99]. Cold-adapted (LAIV) influenza vaccines offers superior efficacy over TIV vaccines in children [100-107]. There are limited and insufficient data to identify whether live attenuated vaccines may offer a clear advantage over inactivated

vaccines in healthy adults[106, 107], possible advantages may include the potential to induce a broad mucosal and systemic immune response[108-110] and its non-invasive administration[111-113].

All but one of influenza vaccines available in the UK are inactivated vaccines (split virion and subunit surface antigen vaccines). The Fluenz®, manufactured by AstraZeneca, is the only cold adapted live virus influenza vaccine that is available in the UK[112]. The inactivated influenza vaccine contains no live virus, but most of the inactivated influenza vaccines are administrated by intramuscular injection, the exception being Intanza®) which is administrated by intradermal injection. Live attenuated influenza vaccine is administrated by nasal spray[112].

Influenza vaccine target groups

Trivalent inactivated influenza vaccine (TIV) can be used for any person aged≥ 6 months with no contraindications (such as egg allergy) to vaccination. Live attenuated influenza vaccine (LAIV) may be used for healthy nonpregnant persons aged 2 to 49 years[114], but is only available to persons aged 2 to less than 18 years in the UK. TIV vaccine is recommended for persons at increased risk of health complications in the UK as listed below [112]:

- All people 65 years of age and older.
- All people 6 months of age and older, with the following conditions
    - Chronic respiratory disease, including asthma.
    - Chronic heart disease.
    - Chronic kidney disease.
    - Chronic liver disease.

- Chronic neurological disease.
- Diabetes.
- Immunosuppression.
- All pregnant women.

- Health and social care staff directly involved in the care of their patients or clients, and carers in receipts of a carer's allowance
- People living in long-stay residential and nursing homes or other long-stay care facilities (this does not include prisons, young offender's institutions, or university halls of residence).

The efficacy and effectiveness of influenza vaccine

The efficacy of a vaccine is a measure of the prevention of illness among vaccinated persons in controlled trials and the effectiveness is a measure of the prevention of illness in vaccinated populations. The efficacy and effectiveness of influenza vaccines depends in part on age, immunocompetence of vaccine recipient and the degree of similarity between vaccination strain and circulating strain. Outcome measures in influenza vaccine efficacy and effectiveness studies included the prevention of medically attended acute respiratory illness (MAARI), laboratory-confirmed influenza virus illness, influenza or pneumonia-associated hospitalizations or deaths, or seroconversion. Less specific outcomes such as MAARI and pneumonia-associated hospitalizations or deaths will undermine influenza efficacy due to infections caused by other agents that are not expected to be prevented by influenza vaccine. Randomized controlled trials that measure laboratory-confirmed influenza virus infections as the outcome are the most persuasive evidence of vaccine efficacy. However these cannot be conducted ethically among groups recommended to be

vaccinated annually. A systematic review and meta analysis using laboratory-confirmed [107] influenza infection as a measured outcome (by RT-PCR or culture) found that influenza efficacy was at 59% (95% CI 51-67) for TIV in adults aged 18 to 65 years and 83% (95% CI 69-91) for LAIV in children aged 6 months to 7 years. In this systematic review, 35% (6 out of 17) of studies showed significant effectiveness (mainly TIV vaccines) against laboratory-confirmed influenza infection in the outpatient and inpatient settings, and the effectiveness ranged from 47% to 79%. In a separate systematic review, in healthy adults aged between 16 to 65 years old, it was suggested that the effectiveness of TIV vaccines against laboratory-confirmed influenza illness was higher (70%-90%) when vaccine and circulating viruses were antigenically similar than when they were antigenically dissimilar (47%-77%) [115]. These studies provided evidence that the currently licensed vaccines can provide moderate protection against laboratory-confirmed influenza infection. New vaccines with improved efficacy and effectiveness are needed to further reduce influenza related illness.

1.9 Host antiviral immune response to influenza infection

1.9.1 A brief overview of innate immunity against influenza infection

Physical and chemical barriers

The invading influenza virions are first cleared by a non-specific defence system, the epithelial barriers in respiratory tract. These epithelial barriers include physical barriers such as the mucin layer, the ciliary action of the ciliated epithedial cells and chemical barriers, such as antimicrobial proteins [117] and a range of antimicrobial peptides produced by the epithelial cells[118].

Innate recognition of influenza virus

Influenza viruses, that successfully penetrated these physical barriers and survived the chemical barriers, infect respiratory epithethial cells and subsequent viral replication leads to host cell apoptosis and lysis. Progeny viruses released from respiratory epithelial cells infect alveolar marcrophages [119] and dendritic cells (DCs) [120]. In influenza virus infected cells, innate recognition receptors, such as the cytosolic sensor RIG-I which recognizes 5′-triphospharates on ssRNA[121], and endosomal sensor Toll like receptor 7 (TLR7 of plasmacytoid dendritic cells and B cells) [122, 123] and Toll like receptor 3 (TLR3 of lung epithelial cells [124, 125]) which recognize ssRNA and dsRNA respectively. In addition, the nucleotide oligomerization domain (NOD) like receptor NLRP3, recognizes influenza M2 protein (intracellular M2 Ion channel in primed macrophages and dendritic cells) [126]. The detection of influenza virus by innate recognition receptors triggers

intracellular signaling cascades. TLR7 signaling occurs via adaptor molecule MyD88 (myeloid differentiation factor 88) to ultimately activate NF-kB (nuclear factor-kappaB) and the production and secretion of proinflammatory cytokines such as IL-12, IL-6 and tumor necrosis factor (TNF-α)[122, 123]. TLR3 signaling pathway is independent of MyD88, but occurs via the adaptor protein of TRIF (Toll/interleukin-1 receptor *(*TIR*)*-domain-containing adapter-inducing interferon-β), which activates NF-kB and IRF3 (interferon regulatory factor 3) and ultimately leads to the production of proinflammatory cytokines such as IL-8, IL-6, IFN-β and chemokine RANTES [125] [124] and antiviral cytokines, type 1 IFNs[125, 127]. The RIG-I signaling pathway is independent of MyD88 and TRIF. The RIG-I binds to IPS-1 (interferon-beta promoter stimulator 1) and activates IRF3, IRF7 and NF-kB, [128], leading to the production of type I IFNs [129, 130]. Influenza virus M2 (matrix protein 2), a proton selective ion channel, on trans-Gogi-network (TGN) membrane equilibrates the pH of TGN with host cell cytoplasm. This prevents premature the conformational rearrangement of newly synthesized haemagglutinin during transport to the cell surface and leads to disturbances in intracellular ionic concentrations of host cells [131]. NLRP3 detects influenza virus infection by sensing the disturbances in intracellular ionic concentrations and forms multimolecular complexes known as inflammasome. This inflammasome recruits ASC (apoptosis-associated speck-like protein), which contains a caspase-recruitment domain that in turn recruits pro-caspase-1 ultimately activating caspase-1 and the proteolytic processing of pro-IL-1β and pro-IL-18 to release active cytokines IL-1β and IL-18 [132, 133].

Cytokine and chemokine antiviral effects

Pro-inflammatory cytokines and chemokines have several downstream effects [134]: Chemokine signaling induces adhesion molecules on vascular endothelium, which facilitate further recruitment of inflammatory cells and amplifies the production of cytokines and chemokines [135]; IL-6 and TNF-α levels correlates with fever during early infection (2-4 days post infection) [136]; type I IFNs (IFNα and IFNβ) are important anti-viral cytokines. Type 1 IFNs induce the expression of Mx genes, which interfere with the transcription by influenza PB2 [137, 138] and form a complex with influenza nucleoprotein that may inhibit the elongation step of viral RNA synthesis [139]. IL-1, IL-6 and TNF-α induce hepatocytes to produce acute phase response proteins [134]. Amongst these acute phase proteins, Mannose binding lectin (MBL), another pattern recognition receptor (PRR) [140], bind to PAMPs (pathogen associated molecular patterns), activating MBL associated protease 2 (MASP 2). Activated MASP 2 cleaves Component C2 and C4, whose resulting fragments cleave C3 and initiates the Complement cascade. The Complement cascade is a system of sequentially interacting plasma proteins, that act to enhance inflammation, aid phagocytosis through opsonising of micro-organisms and directly mediate lysis via formation of Memberane Attack Complex (MAC), which forms transmembrane pores, leading to cell lysis and death [134]. Mannose-binding lectin (MBL) binding to influenza hemagglutinin mediates complement dependent influenza virus neutralization [141], and lysis of influenza virus infected cells [142].

Elimination of influenza virus infected cells by innate cells

IL-15 induces natural killer cell proliferation. IL-2, IL-12, IL-18 and type 1 interferons activate Natural Killer (NK) cells to produce IFN-γ [143]. This IFN-γ production contributes to the NK-mediated antiviral defense in a cytotoxic activity independent manner [144, 145]. Activated NK cells kill virus infected cells via perforin-mediated cytotoxic activity [145] and cause induction of apoptosis of virus infected cells via tumor necrosis factor (TNF) superfamily member receptor pathways such the Fas-FasL [146] and TRAIL-TRAIL receptor interactions[147]. The sialic acid of NK cell activating receptor (NKp46) binds to the hemaggultinin of influenza virus, leading to the sailylation-mediated lysis of influenza virus infected cells [148]. Alterations (glycoslation modifications) in the receptor binding sites of H3N2 influenza isolates was shown to reduced NK lysis of cells infected with influenza virus carrying these glycoslation modifications, and deletion of these glycoslation modifications led to increased NK lysis activity [149]. Influenza infection was lethal in mice lacking natural killer cell activating receptor, NCr1 (NCr 1-/-) (NKp46 in humans) [150].

In addition to proinflammatory cytokine and chemokine mediated antiviral defense, macrophages and neutrophils also contribute to the clearance of influenza virus infected apoptotic cells [151-153] by phagocytosis [154-156].

Antigen presentation and induction of T cell response

Immature DCs expressing an abundance of receptors including Fc receptors, DC-SIGN, and macrophage mannose receptors that make then ideally adapted to capture antigens by phagocytosis, endocytosis. DC maturation by inflammatory cytokines or TLR ligation is essential for the augmentation of the antigen presentation and costimulation of T cells. Proinflammatory cytokines and chemokines, induced by TLR ligand engagement, such as CC-chemokine ligand 2 (CCL2), CCL5, CCL20 and CCL 21, activate and mobilize respiratory DCs [157, 158]. During influenza infection, respiratory DCs acquire viral antigens, mature then migrate from the lungs to lymph nodes in a transient manner (within 24 to 48 hours) [159] where they activate influenza specific naïve [160] [159, 161] [153] as well as antigen specific memory T cells[160]. After initial activation, newly recruited DCs in the lung provide cell contact-, influenza peptide-, and MHC class I -dependent interactions with influenza specific CD8+ T cells that are essential for the protective function of influenza specific CD8+ T cells[162].

1.9.2 Adaptive immunity to influenza infection

The adaptive immunity to influenza infection is divided into two branches: humoral (antibody) immunity and cell mediated immunity, which principally consists of CD4+ and CD8+ T cells (Figure 1.8)[163].

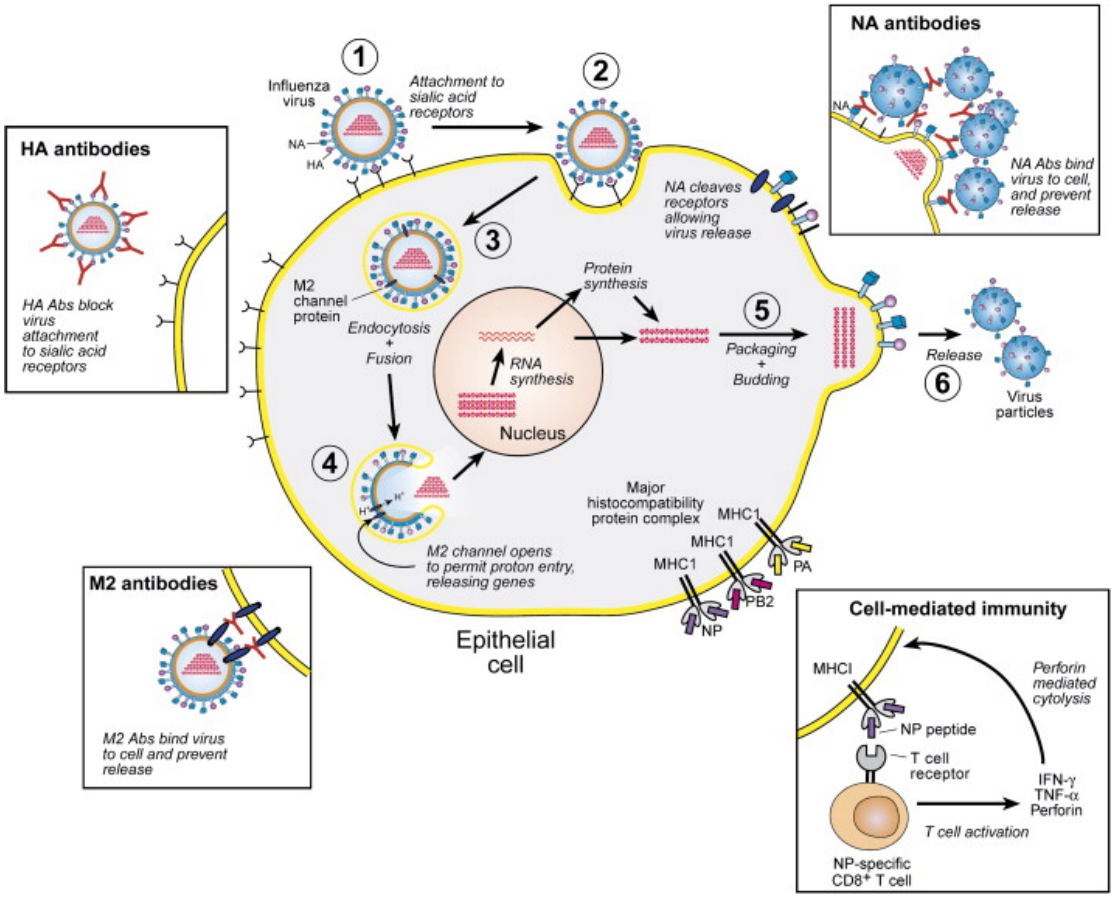


Figure 1.8: Adaptive immunity in influenza infection
The adaptive immunity branch into humoral immunity and cellular immunity, which consists of antibody and T cell mediated immune responses to influenza infection. Numbers indicate the steps in influenza virus replication cycle. Mechanisms of antibody and T cell mediated immunity are indicated in square box. Adapted from Subbarao K et al. Immunity 24, 5-9 (2006)

Ferrets are susceptible to infection with human influenza viruses without prior adaptation and develop some of the symptoms of influenza that are seen in humans such as fever [164, 165], nasal secretion and sneezing [166]. Ferrets can also transmit influenza infection to susceptible ferrets [167] and the influenza virus attachment in respiratory tract of ferret closely mirrored those in human respiratory tract[168]. Therefore ferrets are widely believed to be the ideal small animal model for influenza research and have been used to study influenza transmission [167, 169, 170], pathogenicity[171], the preclinical evaluation of candidate influenza vaccines [172, 173] and the overall fitness of antiviral drug resistant mutant influenza viruses[174, 175]. However, a lack of ferret-specific reagents and incomplete genome sequencing of this species has limited the use of ferrets in studies of influenza immune responses. Although mice are not natural hosts of influenza A virus, laboratory adapted influenza virus strains can infect mice and substantial virus titers can be achieved in multiple organs including the lung. The ready availability, relative low cost, the variety of genetic backgrounds and mice with targeted gene defects, together with the availability of immunologic reagents have made mouse the most used animal model in influenza research. Mice have been used for influenza vaccine research for decades and various mouse models have been adopted as useful tools to enhance our understanding of immune responses in influenza A infection.

1.9.2.1 Humoral immunity to influenza

Neutralizing antibodies that bind to the HA surface protein block influenza virus attachment to the host cells either by binding to the receptor binding sites [176, 177] or binding elsewhere to exert steric interference [177], thus preventing the establishment of influenza infection and provide sterilizing immunity. Passive transfer of HA specific antibodies conferred full protection from infection against a closely related strain with no influenza virus detectable in the lungs and brain in a mouse experimental challenge study [178]. Neuramanidase (NA) specific antibodies induced by immunization with purified NA protein did not prevent infection, but reduced pulmonary virus titer upon infection [179]. *In vitro,* neuraminidase antiserum from rabbits could depress the yield of influenza virus in chick-embryo tissue culture suggesting that the primary role of anti-NA antibody was related to the release of the influenza virus from infected cells [180]. Passive transfer of immunoglobulin A (IgA) [181] and immunoglobulin G (IgG) [178] demonstrated that both IgA and IgG contributed to antibody mediated immunity. Competitive virus binding assays using Fab fragments of defined monoclonal antibodies revealed a broad repertoire of neutralizing antibodies induced by repeated exposure to various subtypes of influenza virus[182].

In addition to the serotype specific neutralizing antibodies that bind to HA, cross-reactive HA specific monoclonal antibodies has also been identified from memory B cells of vaccinees that received trivalent seasonal influenza vaccines. These cross-reactive monoclonal antibodies could neutralize multiple subtypes from group 1 influenza A virus including H1, H5, H9 and some H2s [183], or group 2 influenza A

virus including wide spectrum of H3 influenza viruses as well as H7 and H10 viruses [184]. Other cross-reactive monoclonal antibodies derived from subjects either shortly after natural infection or who had received seasonal trivalent vaccine, could bind to all 16 subtypes of HA and neutralize subtypes from both group 1 (H1 and H5) and group 2 (H3 and H7) influenza viruses[185]; but multiple somatic mutations seemed to be required for some of the cross reactivity [185]. These HA specific cross-reactive neutralizing antibodies are valuable in the development of antibody therapies, but further study is needed to determine whether these antibodies can be induced by vaccination and to a level that is sufficient to protect from natural infection. In addition to these HA specific cross-reactive neutralizing antibodies, cross-reactive, but non-neutralizing antibodies targeted at conserved matrix protein 2 (M2) may confer partial protection as demonstrated in a mouse challenge study: the immunization with inactivated virus vaccines complemented by Virus like particles containing M2 protein could aid the inactivated vaccines in promoting virus clearance and reducing disease severity [186]. Further investigation to understand the importance of these cross-reactive antibody epitopes in influenza virus survival would indicate the likelihood of immune escape from protection by these cross-reactive antibodies.

1.9.2.2 T cell immunity in influenza A infection

CD8+ T cells

It is particularly challenging to study the activation and migration of naïve CD8+ T cells due to the low frequency of influenza specific CD8+ T cells in naïve hosts. To over come this problem, TCR transgenic mouse models with enhanced antigen specific T cells were used. In a mouse model, in response to *in vivo* stimulation with intranasal inoculation of live influenza virus, adoptively transferred HA-specific TCR transgenic CD8+ T cells were activated and proliferated exclusively within the draining meldiastinal lymph nodes during the first 3 days of post infection. These activated HA-specific CD8+ T cells acquire CTL effector functions, including the production of IFN-γ and granzyme B and then migrate to the infected lungs as well as other secondary lymphoid tissues such as the spleens and non draining peripheral lymph nodes [187]. The protective effects conferred by HA-specific TCR transgenic CD8+T cells were largely determined by their ability to migrate to the site of infection [188]. The protective effects of CD8+ T cells in influenza infection have been studied using various mice models. Gene knock out mice have been employed to study the antiviral effects of CD8+ T cells. Influenza specific CD8+ T cells eliminated influenza virus infected cells via perforin-and granzyme-mediated lysis and Fas or TRAIL mediated apoptosis[189, 190]. The CD8+ T cell responses in primary and secondary influenza infection have been quantified using MHC-peptide tetramer staining of IFN-γ producing CD8+ T cells in response to *in vitro* peptide stimulation. Compared to primary infection, in secondary infection, CD8+ T cells are detected (2 days) earlier in bronchoalveolar lavage with increased frequency and rapid production of antiviral cytokines [191]. Adoptive transfer of splenic influenza A

specific CTLs to MHC class I matched naïve recipient mice protected against lethal challenge with homologous influenza virus and significantly reduced lung virus titers after challenge with sub lethal dose [192, 193]. Adoptive transfer of splenic influenza specific CD8+ T cells protected mice devoid of mature B cells (mice with a targeted mutation in the membrane exon of the immunoglobulin μ chain gene, known as μKO mice) from an otherwise lethal challenge[194]. Delayed influenza virus clearance in the lungs was observed in CD8+ T cell deficient mice (transgenic mice for homozygous β_2-M gene disruption, known as β_2-M (-/-) mice) after challenge with nonlethal influenza virus[195].

CD4+ T cells

Comparatively less is known about influenza A specific CD4+ T cells. This is primarily due to technology limitations and the high heterogeneity of CD4+ T cells (Th1, Th2, Th17, follicular helper T cell and regulatory T cells). The role of CD4+ T cells in influenza infections has been studied in various knock out, depletion and adoptive transfer mice models in an effort to understand their role in primary, secondary and memory responses to influenza infection. Influenza A specific CD4+ T cells alone could not effectively clear influenza virus in the lung in CD8-depleted, B cell deficient mice (mice with a targeted mutation in the membrane exon of the immunoglobulin μ chain gene, known as μMT mice) [196, 197]. But CD4+ T cells appear to be important in the generation and maintenance of CD8+ memory T cells in both primary and secondary infections. There is evidence that expansion and recruitment of influenza A specific CD8+ T cells to infected lungs appeared to be impaired by lacking CD4+ T cells as diminished primary and secondary influenza virus –specific tetramer positive CD8+ T cells were observed in CD4 depleted Ig-/-

mice[198] and diminished tetramer positive CD8+ influenza specific memory T cell responses were observed in CD4+ deficient mice (*I-A*$^{b-/-}$ C57BL/6J mice)[199]. Influenza A specific CD4+ T cells in mice also have been shown to have direct effector functions. Adoptive transfer of *in vitro* Th1 (T helper type 1) polarized influenza A specific memory CD4+ T cells prior to sub-lethal challenge significantly lowered lung viral titers at day 3 and 4 in naïve recipients [200]. Likewise, Th1 influenza A specific CD4+ T cells were cytolytic *in vitro* and their adoptive transfer protected recipient mice against lethal influenza A challenge [201]. Taken together, CD8+ and Th1 CD4+ influenza specific T cells play a protective role in influenza infection. However, adoptive transfer of splenic influenza specific Th2 CD4+ T cell led to massive pulmonary eosinophilia and elevated virus titers as compared to control mice [194], suggesting the pathogenic role of Th2 CD4+ T cells in influenza infection.

T cell mediated heterotypic immunity

Unlike antibodies, T cell mediated immunity targets highly conserved influenza internal viral proteins as well as surface antigens. *In vivo*, T cell immunity conferred full protection against lethal challenge with heterologous virus in antibody deficient mice (μMT mice) [202] and double-primed mice (H3N2-H1N1) achieved more rapid virus clearance (by day 3) in the lung and brain against sub lethal H7N7 virus challenge in contrast to naïve mice (by day 10) [203]. *In vitro*, the cross-reactive cytolytic function of nucleoprotein (NP) specific CD8+ T cells was demonstrated in mouse models in which *in vitro* stimulated murine splenocytes derived from mice primed with H2N2 or H3N2 subtypes could lyse the vaccinia infected NP expressing cells in a MHC class I restricted manner [204]. In humans, T cell responses against

internal viral proteins were shown to cross react with different subtypes of influenza A virus [205-210], Similarly, cross-reactive influenza A specific CTLs and CD4+ T cells correlated inversely with nasal viral shedding in human influenza nasal challenge studies [211, 212]. It appeared that T cell mediated immunity against influenza A viral infection did not prevent the establishment of infection but promoted viral clearance and prevented mortality. Potentially, this type of immunity could reduce the morbidity and mortality rates in the face of a novel pandemic influenza A virus. However, further investigations are needed to prove the importance of heterotypic immunity against natural influenza virus infection in man.

1.10 Rationale for the study of T cell immunity in humans against natural influenza A virus infection

Whilst most of our knowledge about T cell immunity against influenza A infection has been derived from the mouse model, mice are not natural hosts of influenza A virus. A human challenge study offers more comprehensive information on the kinetics of influenza specific T cells and the role of T cell immunity in protection, but it is difficult both to perform and to achieve statistical power. Human challenge studies would require a large number of volunteers but only healthy adults are permitted to participate in these types of studies. This leaves the high-risk groups of influenza infection including children, the elderly and people with pulmonary conditions unchecked. It is also not ethically possible to challenge humans with an influenza virus that has not previously circulated in humans, even though this would enable the examination of the role of T cell immunity in the absence of pre existing antibody immunity. To address this problem, Flu Watch recruited a large number of participants of different ages (from 5 years old up to 93 years old) prior to the influenza season, and then monitored their health for evidence of influenza infection. Volunteers included people with different underlying health conditions including a few pulmonary conditions. The H1N1 pandemic influenza 2009 gave us the opportunity to study cross-reactive T cell immunity with the absence of pre-existing antibody. Knowledge on the T cell immunity against natural influenza A virus infection acquired from this study would provide useful information for the development of a T cell based vaccine for humans.

1.11 Aims of this study

1. Assess the pre-existing influenza specific T cell immunity in our community and characterize the CD4 and CD8 restrictions of these pre-existing T cells.
2. Identify cross-reactive and immunogenic T cell targets.
3. Assess the maintenance of influenza specific T cells.
4. Identify influenza T cell epitopes and elicit their HLA restrictions.
5. Investigate the effects of the pre-existing T cell immunity on the disease outcome if infected with seasonal and pandemic influenza and the existence of heterotypic immunity in natural influenza infection in humans.

Chapter 2: Materials and methods

2.1 Study design

Participants were recruited over 4 years through the Medical Research Council (MRC) General Practice Research Framework (GPRF) led by Dr. Andrew Hayward's team at the Department of Primary Care and Population sciences, University College of London. Participants were randomly selected from households on the General Practice register located within a 150-mile radius of Oxford. Blood samples were taken at participants' homes then couriered overnight to Oxford. Blood specimens were taken outside of school and public holidays to maximize the enrolment rate. Blood specimens were taken from week 40 to week 50 of each year (October to mid December) to capture the pre-influenza season window. Participants were bled again the following year from week 18 to week 28 after influenza season (May to mid July). Most of the pre-seasonal samples were collected before the annual increase in influenza like illness recorded by The Royal College of General Practitioners Research and Surveillance Centre (RSC), which is a good indicator of the start of the influenza season (Figure 2.1). Influenza like symptoms and illness episodes during the period that influenza A was known in circulation were monitored. A positive PCR detection from nasal swab or/and a 4-fold rise in antibody (HI) titres was defined as infection. Influenza infections with or without symptoms were used to examine the role of pre-existing T cell immunity in protection against symptoms.

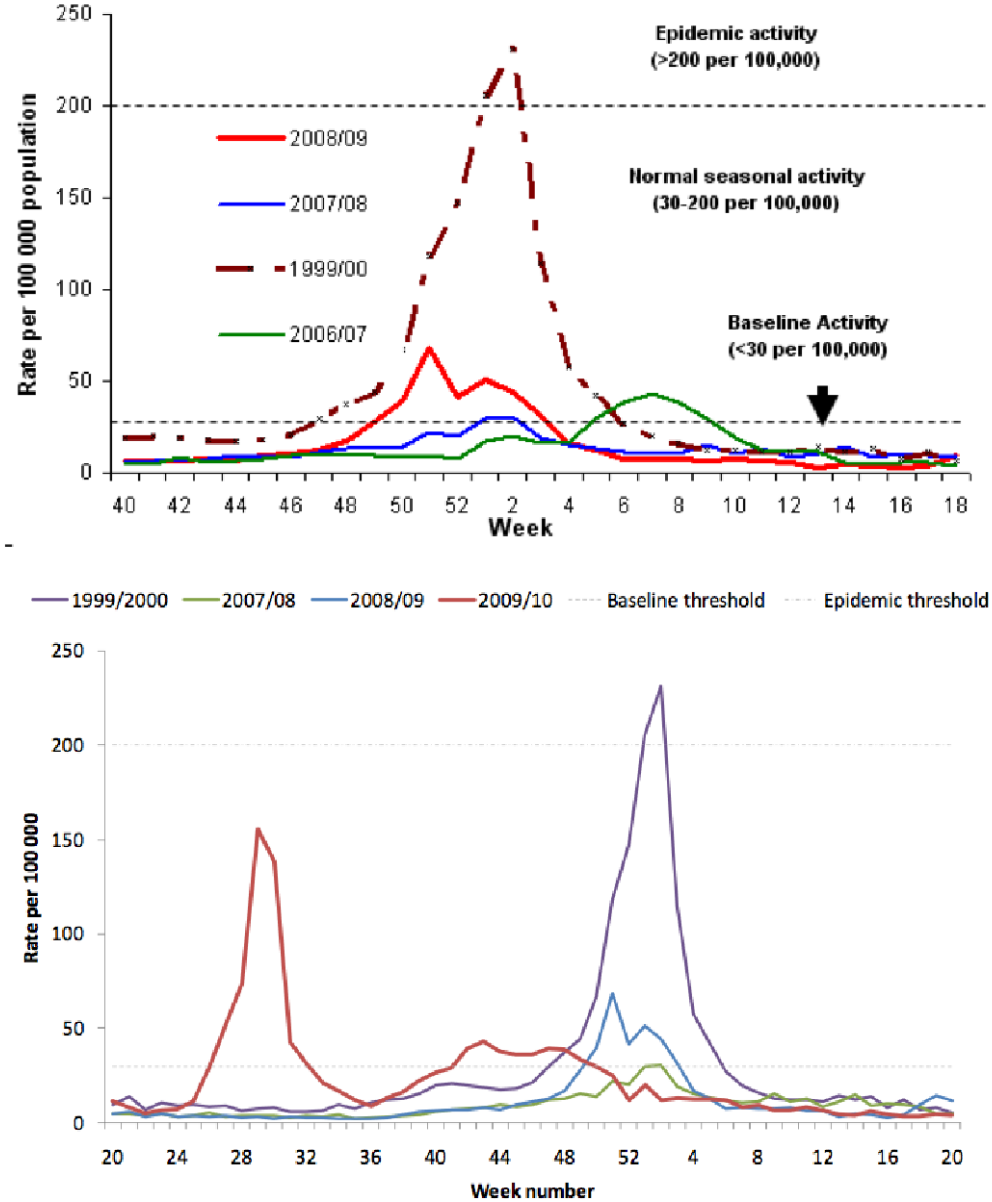


Figure 2.1: Flu Watch Bleeding schedule

The majority of Flu Watch pre-seasonal bleeds were completed before the start of the influenza season between week 40 to 50: October to mid December. The post-seasonal bleed was taken between week 18 to 28: May to mid July. The pandemic H1N1 2009 GP consultation rate in UK is indicated in red in the bottom graph. Blood specimens taken between week 18 to 28 in 2009 were treated as pre-pandemic first wave bleed and blood specimens taken between week 40 to week 50 were treated as pre-pandemic second wave bleed, then the post seasonal bleed for pandemic 2009 was taken between week 18 to week 28 in 2010. Graphs were taken from Health Potection Agency report and the GP consultation rate source data was from The Royal College of General Practitioners Research and Surveillance Centre (RSC).

2.2 Influenza specific definitions applied in the Flu Watch study

- Infection: infection is defined by a 4-fold rise in HI titer comparing the sera titer before and after influenza season
- Influenza like illness: CDC influenza like illness (ILI) definition is applied to our cohort which is sudden onset of fever >38°C and cough and/or sore throat in the absence of a known cause other than influenza
- Level of antibody: seronegative (HI<8); Protective level of antibody (HI>32)
- Seasonal influenza baseline collection: Autumn 2006, Autumn 2007, Autumn 2008
- Seasonal influenza post-seasonal collection: Spring 2007, Spring 2008, Spring 2009
- Pandemic I cohort: Spring 2009
- Pandemic II cohort: Autumn 2009

2.3 Flu Watch cohort characteristics

In total, 2,467 participants were recruited over 4 years (**Table 2.1**). The mean number of individuals per household was 2.4, which is equivalent to the national average [213]. 2,395 participants had their corresponding clinical record and the measurement of their antibody titres available. Data from these infected individuals were used to study the role of T cell immunity in protection from illness (Figure 2.2). In general, the Flu Watch T cell study cohort under-recruited the younger population of 29 years old or younger, but over recruited the older population of 45 to 64 years old. The most under recruited age group was 20 to 29 year old, possibly because a proportion of this group were in higher education and missing from the household. A considerable proportion (59.9%) of the participants who were recruited from age groups of 65 to 74 and 75 to 84 year were excluded from the T cell study because subjects were vaccinated before or between baseline and follow up bleed (Figure 2.3).

Table 2.1: The recruitment of each influenza season in the Flu Watch study

	Pre seasonal	**Sample size**	**Post seasonal**	**Sample size**
Seasonal influenza	Autumn 2006	N=488 [1](481)	Spring 2007	N=449
	Autumn 2007	N=621 (573)	Spring 2008	N=526
	Autumn 2008	N=497 (484)	Spring 2009	N=505
Pandemic influenza (N=818)	Spring 2009 (Pandemic I)	N=299	Not taken for T cell study	
	Autumn 2009 (Pandemic II)	N=562	Not taken for T cell study	

[1]The figures in brackets are the number of participants who also had corresponding clinical record documented any respiratory and/or influenza like illness during influenza season and influenza A specific sera antibody titres measured by Haemaglutination Inhibition (HI) Assay.

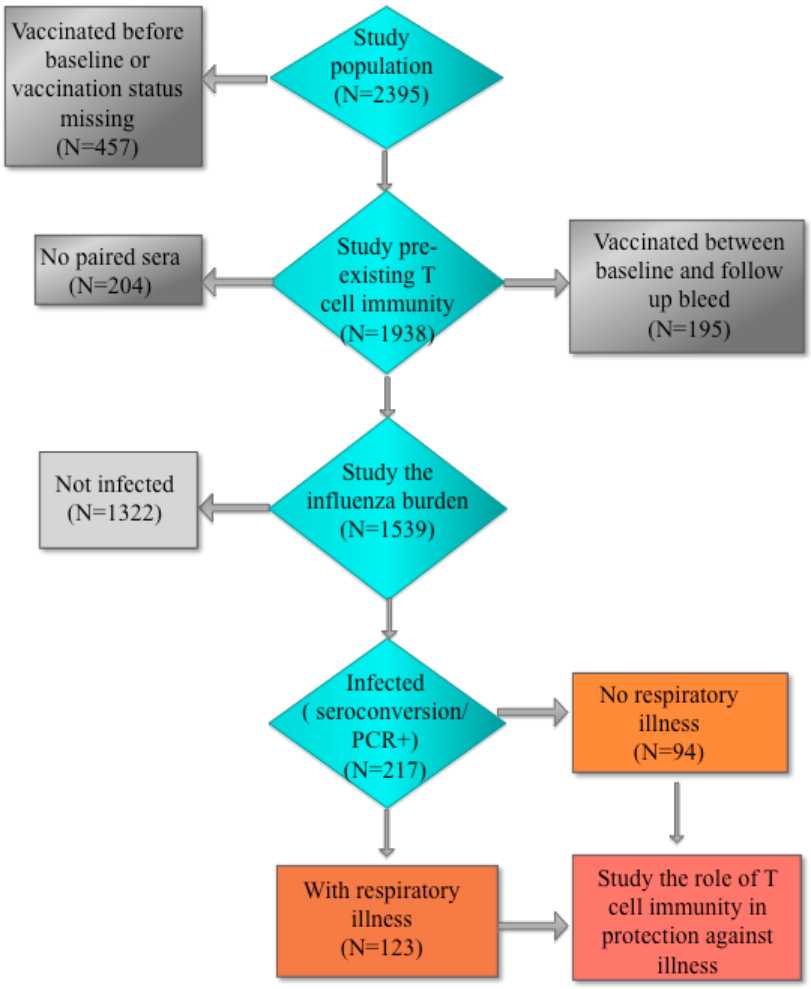


Figure 2.2: Flu Watch sample selection for T cell study
In total, 2395 volunteers were studied for their T cell immunity against influenza A (H3N2) infection with the support of their clinical record and influenza A specific serum HI titres. 231 were infected with influenza and those without pre-existing influenza specific antibodies were entered into the study of T cell immunity in protection against illness. Approximately 80% of infected individuals had no pre-existing influenza serotype specific antibodies

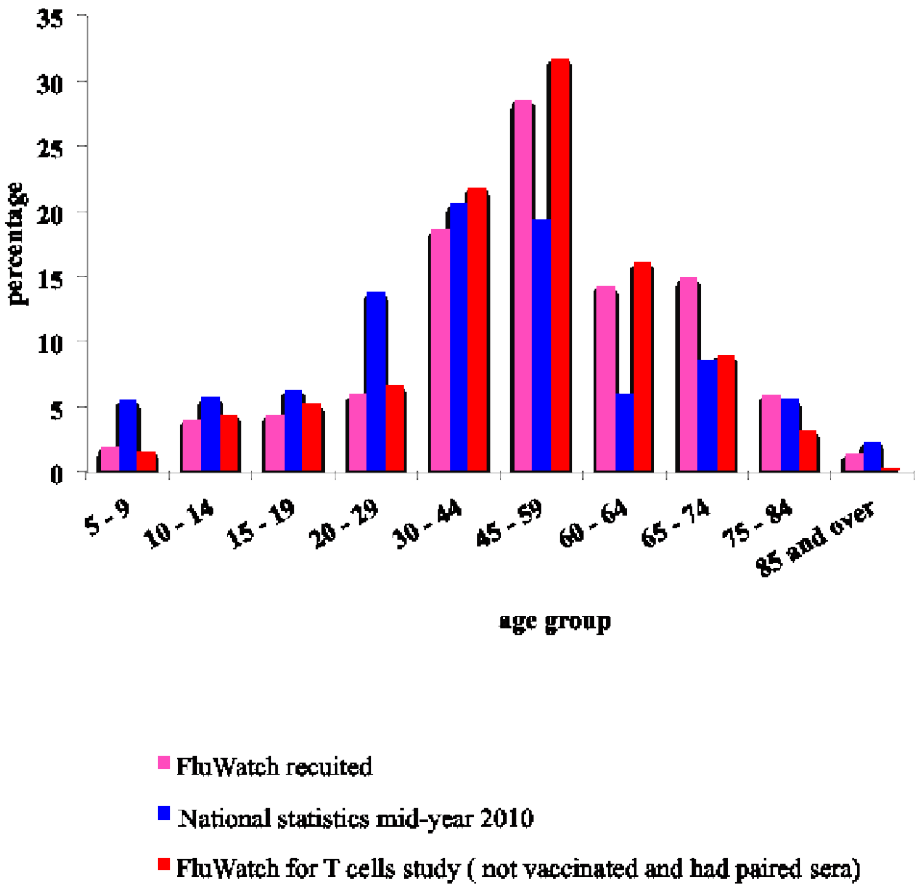


Figure 2.3: The age distribution of Flu Watch cohort in comparison with national statistics
Flu Watch T cell study cohort under recruited the younger population of 29 years old or younger, but over recruited the older population of 45 to 64 years old. The most under recruited age group was 20 to 29 year old, which may have been contributed by the population in higher education, who were missing from household. Considerable proportion of the participants were recruited but excluded in T cell study from age group of 65 to 74 and 75 to 84 year, 59.9% of participants in these two groups were vaccinated before or between baseline and follow up bleed. UK population statistics was obtained from the National Statistics at http://www.ons.gov.uk/ons/publications/re-reference tables.html?edition=tcm%3A77-222649 (from chapter 15 Population tab 15.3)

2.4 Clinical and epidemiology data collection

Research nurses collected basic demographic, social and medical data including age, gender, chronic illness, smoking, number of rooms, occupation, influenza immunization while taking baseline blood. Members of households were also trained at this visit in how to complete symptom diaries for each episode of respiratory illness and how to complete contact diaries outlining the frequency, duration and setting of contact with others. A self-nominated lead member of each household was also trained in how to take temperature, nasal swabs from household members and respond to weekly-automated telephone calls that would record acute illness and symptoms of family members from first visit to the end of April.

2.5 Statistical analysis and power considerations

Based on data from previous studies of influenza in the community and the expected age structure of the recruited population, approximately 400 serologically confirmed cases of influenza were expected from a cohort of 2000 participants. Fifty percent of infected cases were expected to be symptomatic. Fifty percent of volunteers were expected to display a positive T cell response. Based on the above predictions, The Flu Watch study was estimated to have 80% power at 5% significant level to detect a 45% protective efficacy of pre-existing T cell immunity against symptoms in infected cases; to detect a 17% difference in positive pre-existing T cell response between 200 infected cases and equal number of uninfected but exposed household controls. Statistical analysis was performed using STATA and Prism.

2.6 Laboratory experimental procedures

2.6.1 Peptide design

Peptides used in the Flu Watch study are 18mers overlapping by 10 amino acids spanning HA, NA of H3N2 (A/New York 388/2005); internal proteins of H3N2 (A/New York 232/2004); HA and NA of H1N1 (A/Brisbane/59/2007(H1N1)); HA, NA, NP, M of Swine H1N1 (A/California/04/2009(H1N1))]. A two dimensional (2D) peptide matrix was used for the 1st year study (2006-2007), and the first dimension of the matrix was designed to separate the peptides from different viral antigens into different pools. Each peptide appeared twice in the 2D matrix, which allowed us to identify which peptides were giving a T cell response. However, this 2D peptide matrix system required large numbers of cells and we could not afford to generate replicates to assess our assay variation. The following three years' studies had been done using pooled peptides; each pool contained all peptides from one viral antigen and mostly (84% of participants) were done in three replicates for each peptide pool that was tested; about 10% of participants were tested in duplicate and the remaining 6% of participants were tested with no replicates due to insufficient PBMCs. The amino acid sequence alignment of seasonal H1N1, H3N2 and pandemic 2009 H1N1 viruses was in appendix III

2.6.2 Blood specimen collection

Bloods were taken from all family members at participants' homes by research nurses, vacutainers spray coated with 17 IU/ml sodium heparin on the inner wall (BD, cat no. 368480) were used to collect blood for T cell immunity study, 30ml blood was taken from each adult and 15ml and 5ml was taken from children aged 10-18 and 5-10 respectively. Another 5ml blood sample was taken from each family member in vacutainers with no additives for serological study. Blood specimens were taken in the evening between 5-10pm, then were collected from research nurses' homes by courier and delivered to John Radcliffe Hospital, Oxford overnight, laboratory work at Oxford started at 5:30am, PBMCs were isolated within 15 hours of blood collection and blood was kept at room temperature before PBMCs isolation as suggested by previous publications[214, 215].

2.6.3 PBMC isolation, cryopreservation and thawing

PBMCs were isolated by Lymphoprep™ (Axis-shield PoC AS, cat no: 1114547) density gradient separation and Leucosep tubes (Jencons, cat no: 504-219) were used to separate PBMCs in order to achieve the best yield and viability without compromising the function of the T cells [216, 217] and minimize staff training. Whole blood was poured into pre-filled Leucosep tubes, then centrifuged at 800 x g for 20 min. Isolated PBMCs were poured into a fresh 50ml Falcon tube then topped up with R0 to 45ml and centrifuged at 700 x g for 10 min. PBMCs were treated with 5ml DNAseI solution for 1 min then added another 40ml of R0 with 2% FCS (R2) and centrifuged for 10 min at 210 x g. PBMCs were re-suspended in 10ml of R2 for cell counting. Remaining PBMCs (4-10 x 10^6 cells/per vial) were frozen down in 10%DMSO.FCS solution then stored in liquid nitrogen. Cryopreserved PBMCs were thawed by adding 5ml 37°C DNAseI solution drop by drop, then topped up with 40ml 37°C R10 (R0 with 10% FCS), centrifuged at 480 x g for 5 minutes, then re-suspended in 5ml H10 (R0 with 10% human AB serum) to rest in incubator (37°C, 5% CO_2) overnight before use.

Note:

- R0: RPMI 1640 Medium (Invitrogen, cat no: 21875091) supplemented with 25,000 units/ml Potassium Penicillin and 25,000 mcg/ml Streptomycin Sulfate (Lonza, cat no: 17-603)
- DNAseI solution: Deoxyribonuclease I from bovine pancreas (Sigma, cat no: DN25-1G) in R0 at 60ug/ml

2.6.4 *Ex vivo* IFN-γ ELISpot assay

Freshly isolated PBMCs were used in first round screening for *ex vivo* influenza A specific T cell response by detection of IFN-γ production. 250,000 cells per well and 1-2ug/ml peptides were used. Plates (Millipore, cat no: MAIPS4510) were coated with anti-human IFN-γ antibody at 10ug/ml (Mabtech, cat no. 3420-2A) every Monday and stored at 4°C (up to 7 days), then washed 4 times with sterile PBS (Sigma, cat no: D8537) and blocked with R10 for 1-2 hours at 37°C before adding cells and peptides. Cells and peptides were co-cultured for 18 to 24 hours then washed and developed according to Mabtech manufacture's instruction. Bio-Rad developing kit (cat no. 170-6432) was used following the manufacture's instruction.

2.6.5 Intracellular cytokine staining (ICS) of IFN-γ production by cultured influenza specific T cells

Cryopreserved PBMCs were thawed and rested overnight; cells were counted the next morning and washed once with H2 (R0 with 2% human serum). Between $0.3x10^6$ to $1x10^6$ cells were used for pulsing with pooled peptides (10ug/ml per peptide). Between 47 to 200 peptides were used in one pool (1 to 3 viral antigens per pool). Cells pulsed with peptides in a 100μl cell suspension for up to 2 hours then washed with 2-3ml of H2. Cells were re-suspended into 0.2 ml (for less than $0.5 x10^6$ cells) or 0.5ml (for more than $0.5 x10^6$ cells) of H10 with 25ng/ml IL-7 then cultured in 96 well round bottom or 48 well tissue culture plate respectively. 1000iu/ml IL-2 was added every 2-3 days and cells cultured for 13-14 days. At the end of the culture, cells were collected into 15ml Falcon tubes and topped up with R0 to 14ml and Cells were centrifuged at 480xg for 5minutes, then re-suspended into 1ml H10 and transferred to 5ml FACS tubes to rest for 30 hours. After having rested for 30 hours, 4 ml of R0 were added to each FACs tube, then centrifuge cells in 480xg and re-suspended in 200μl per test. Cultured T cells were pulsed with 2μg/ml pooled peptides in R10 for 1 hour at 37°C, 5% CO_2 with the addition of anti-CD28 and CD49d (1μg/ml, each, BD bioscience, cat no: 340975, 340976 respectively). After the initial one hour peptide stimulation, added 200μl of R10 supplemented with anti-CD28 and anti-CD49d (1ug/ml, each) and Brefeldin A (Sigma, cat no: B-7651 at final concentration of 10ug/ml), and then incubated for 5 hours further at 37°C, 5% CO_2. Cells were then incubated with 40μl of 20 mM EDTA (pH 7.2-4) (final concentration of 2mM) for 15 min at room temperature, and votexed, washed with 2ml of PBS. Washed cells were stained with 500μl of live/dead cell staining solution (Invitrogen,

cat no. L34955) for 30 minutes at 4°C. At the end of incubation, 2ml of PBS were added again to wash off the live/dead cell staining solution, and then stained for 20-30 min at 4°C with surface markers: anti-CD3 APC-H7 (BD bioscience, cat no: 641397), anti-CD4-PE (DAKO, cat no. R080501), anti-CD8-FITC (DAKO, cat no: F076501) and dumping panel antibodies consisting of anti-CD14-pacific blue (e-bioscience, cat no: 48-0149-42), anti-CD19-pacific blue (e-bioscience 48-0199-42 respectively) and anti-CD56-Pacific blue (Cambridge Bioscience, cat no: 304629). Cells were washed once again with 2ml FACS wash (1% FBS in PBS and 1% sodium azide), then permeabilized and fixed with 500µl of BD permeabilization buffer 2 (BD bioscience, cat no. 340457) for 15 min at room temperature. Fixed cells were washed with 2ml of FACS wash before staining with intracellular marker: IFN-γ-APC (BD bioscience, 341117) for 30 min at 4°C. Cells were washed one more time with 2ml FACS wash then re-suspended into 200µl FACS fix (PBS with 1% paraformaldehye), and kept at 4°C. Samples were analyzed on Cyan cytomation analyzer within 24 hours. The acquired data were analyzed with Flowjo software. Gating strategy is shown in Figure 2.4.

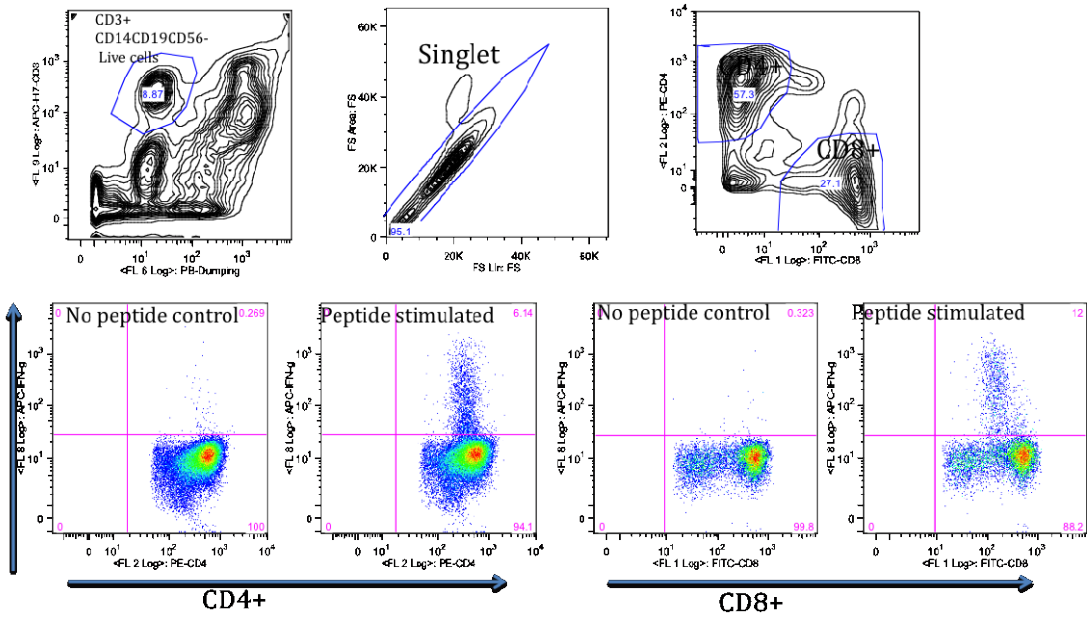


Figure 2.4: FACs analysis gating strategy for the characterization of CD4 and CD8 restrictions
On tope panel, from left to right, the CD3+ T live T cells were selected then those single cells were gated then either CD4+ or CD8+ T cells were selected for subsequent analysis. Bottom panel showed the IFN-γ producing CD4+ or CD8+ T cells were in the top right quarters.

2.7 Assay standardization

2.7.1 Assessing the impact of delayed PBMC isolation on the detection of influenza specific T cell responses measured by human IFN-γ ELISpot Assay

Forty milliliters of blood were taken from each of five independent healthy blood donors. PBMCs were separated at 0, 6, 12 and 15 hours after blood was taken. Until PBMC isolation, the blood was stored at room temperature. Influenza specific T cell responses ranged from 0 to 60 spot forming cells per 10^6 PBMCs and all of these responses remained stable over time (Figure 2.5)

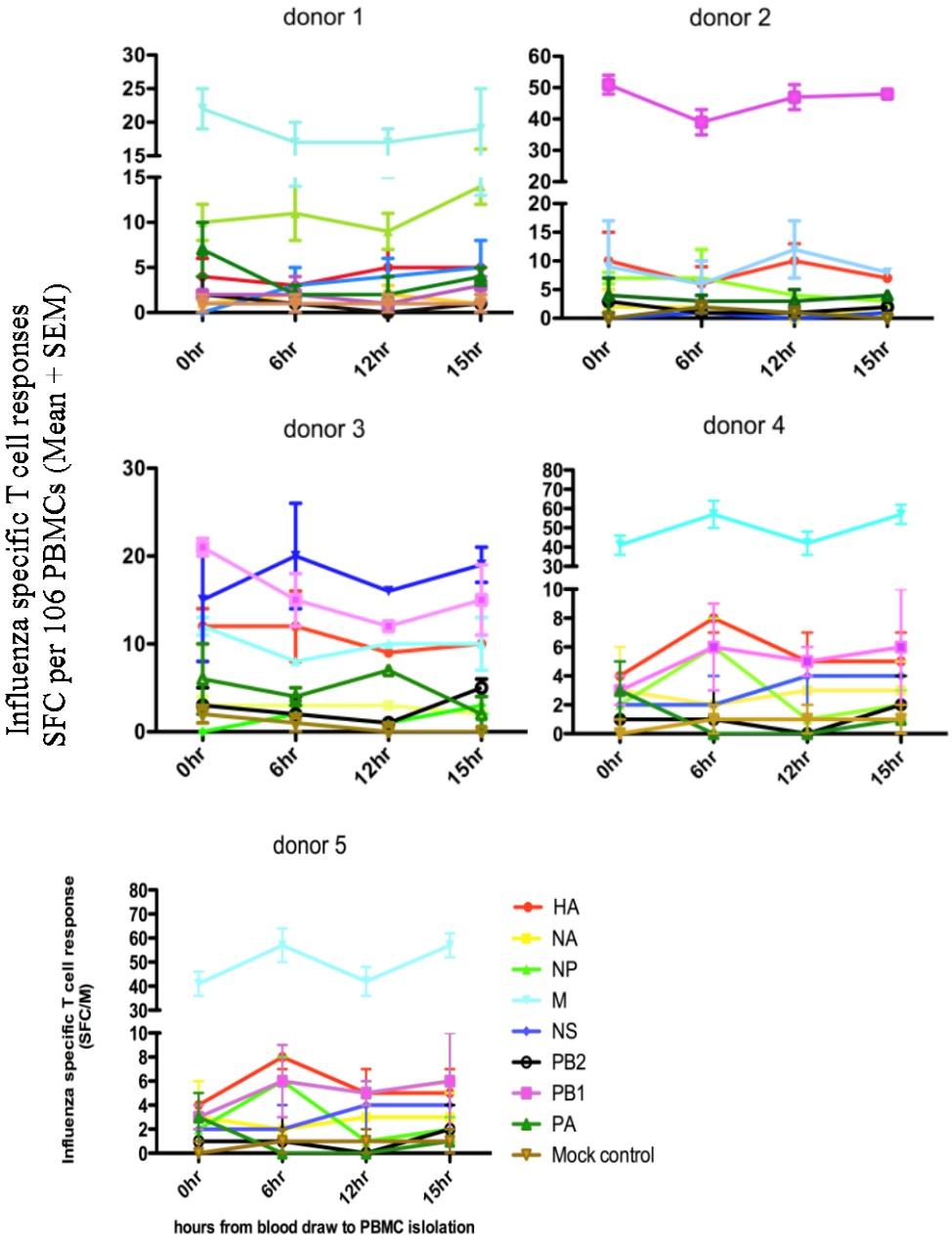


Figure 2.5: The delayed PBMC isolation did not significantly impact the *ex vivo* frequency of influenza A (H3N2) specific T cells against all influenza antigens

Fifty milliliters blood was taken from each of 5 healthy donors; PBMCs were isolated immediately and at 6, 12 and 15 hours. The blood was kept at room temperature before PBMCs isolation. T cell responses against each of eight influenza antigens were measured using *ex vivo*-human IFN-γ ELISpot assay. T cell responses presented here was background subtracted at mean+4 standard deviation of no peptides controls. Error bars shown on graph were standard error of mean of replicates. T cell responses remained relatively stable over 15 hours after venipuncture.

2.7.2 Assessing the impact of cryopreservation on the detection of influenza specific T cell responses measured by human IFN-γ ELISpot assay

Cryopreserved PBMCs from 7 Flu Watch participants were thawed and rested over night then used to repeat the ELISpot assay. A good liner relationship between T cell responses detected in fresh cells and frozen cells was observed (r2=0.68, P<0.0001) (Figure 2.6) and there was no difference in the magnitude of influenza A (H3N2) specific T cell responses measured using freshly isolated PBMCs and cryopreserved PBMCs (Figure 2.7)

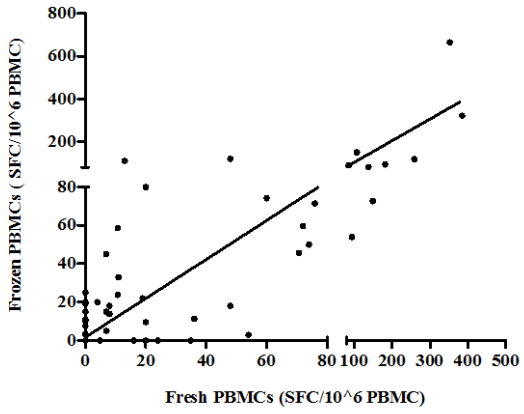


Figure 2.6: Cryopreservation did not impact the detection of influenza specific T cells using human IFN-γ ELISpot assay
Influenza specific T cell responses from 7 Flu Watch participants were assessed against 8 peptide pools (HA, NA, NP, M, NS, PB2, PB1, PA). There was a good liner relationship between the responses detected with freshly isolated PBMCs and cryopreserved PBMCs. Abbreviation used: SFC: spot forming cell. Liner regression analysis applied, r^2=0.68, P<0.0001. Statistical analysis was performed using Prism.

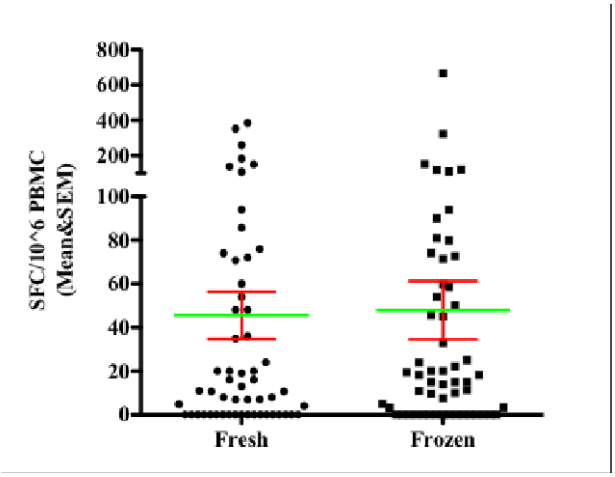


Figure 2.7: Cryopreservation did not impact the performance of PBMCs in human IFN-γ ELISpot assay

The magnitude of the influenza A (H3N2) specific T cell responses detected in human IFN-γ ELISpot using cryopreserved PBMCs were comparable to those using freshly isolated PBMCs. Samples were not normally distributed, Wilcoxon matched-pairs signed rank test were used to test the difference between this paired data set. P=0.8827ns.

Chapter 3: Characterization of *ex vivo* pre-existing influenza A (H3N2) specific T cells in a UK cohort

3.1 Introduction

Influenza causes annual epidemics that in temperate regions occur typically during their respective winter [218, 219]. In tropical countries, influenza cases occur throughout the year with one or two peaks that often coincide with the rainy season [218-221]. Consistent with ongoing exposure to 'seasonal' influenza, several studies have detected influenza A specific CD4+[212, 222-226] and CD8+ T cells [205, 206, 208, 212, 227, 228] in human peripheral blood. The immune correlates of protection conferred by T cell mediated immunity were investigated in human challenge studies. These studies produced encouraging results finding that pre-existing influenza A specific T cell immunity correlated with enhanced virus clearance against both homologous [212] and heterologous challenge [211]. The aim of this study is to build of previous challenge studies by investigating the role of T cell immunity in natural influenza A infection in the UK.

The Flu Watch study was set up in Autumn 2006. In total, 2,395 volunteers participated in this study over 4 years. Participants were randomly selected from participating GPs' register located 150 miles around Oxford. In this chapter, we characterize the pre-existing influenza A (H3N2) specific T cell responses from these volunteers, which included three pre-seasonal cohorts and one pre-pandemic cohort.

Aims:

- To investigate the magnitude and breadth of the pre-existing influenza A (H3N2) specific T cell responses
- To identify conserved immunodominant internal proteins as the target for the development of a potential T cell vaccine
- To define CD4+ and CD8+ phenotype of pre-existing influenza A (H3N2) specific T cells.
- To investigate the maintenance of pre- influenza A (H3N2) specific T cell responses over 5 to 8 months (from pre to post influenza season) and 1 year or 1.5 years.
- To identify novel influenza A (H3N2) specific T cell epitopes and characterize their CD4, CD8 and HLA restrictions.

3.2 Results

3.2.1 Defining a positive T cell response

Human IFN-γ ELISpot assay was used to measure the pre-existing influenza A specific T cell responses. An example of Flu Watch ELIspot result is shown in Figure 3.1. As the *ex vivo* pre-existing influenza A (H3N2) specific T cells existed in blood circulation at low frequency (reported in 3.2.3), and almost everybody has encountered influenza antigen by the age of 5 (reported in section 3.2.5), thus there was no influenza naïve population in which a biological cutoff could be defined. Therefore, to minimize false positives, we applied statistical method to define positive T cell responses. The background was defined as 4 standard deviations above the mean of no peptide controls. In addition, for a response to be classified as positive, the background subtracted value also needed to be more than twice of the noise detected in the whole study (a signal-to-noise ratio higher than 2:1); an approach recommended by Moodie et al. [229]. The noise was the 50th percentile of non-specific responses detected in no peptide controls from the whole study, which was 2 spot forming cells per million PBMCs in the Flu Watch study (Figure 3.2). Thus, the signal to noise ratio of 2:1 was 4 spot forming cells per million PBMCs. The background and positivity definitions described in this section were applied throughout this thesis.

3.2.2 Assessment of ELISpot intra-replicate variations

The detection of low frequency T cells is susceptible to technical variations. The intra-replicate variability can be assessed by the variance to median ratio (variance of triplicates / (median of triplicates+ 1)), which requires tests to have three or more replicates. The variance to median ratio was used to assess intra-replicate variation, the study of 717 triplicate ELISpot tests, including control and experimental wells, from nineteen different laboratories showed that the intra-replicate variation ranged between 0 to 95.4 with the middle 50% of the tests (between 25th to 75th percentiles) ranging from 0.31 to 2.47 and the 95% percentile at 10.13[229]. Therefore, it was recommended that tests with a median to variance ratio greater than 10 should be re-tested. The variance to median ratio (variance of triplicates / (median of triplicates+ 1)) was examined from 20,484 tests that were performed in triplicates in our study, which were all experimental wells and did not include controls. The variance to median ratio of ELISpot experimental wells was lower than previously reported with the middle 50% of variance to median ratio ranging from 0 to 1 and the 95th percentile at 3 (Table 3.). There were 42 (0.2%) of tests having a variance to median ratio of greater than 10, they were excluded from all analysis. Because of the high consistency between replicates, the single tests and duplicate tests were included in the analysis.

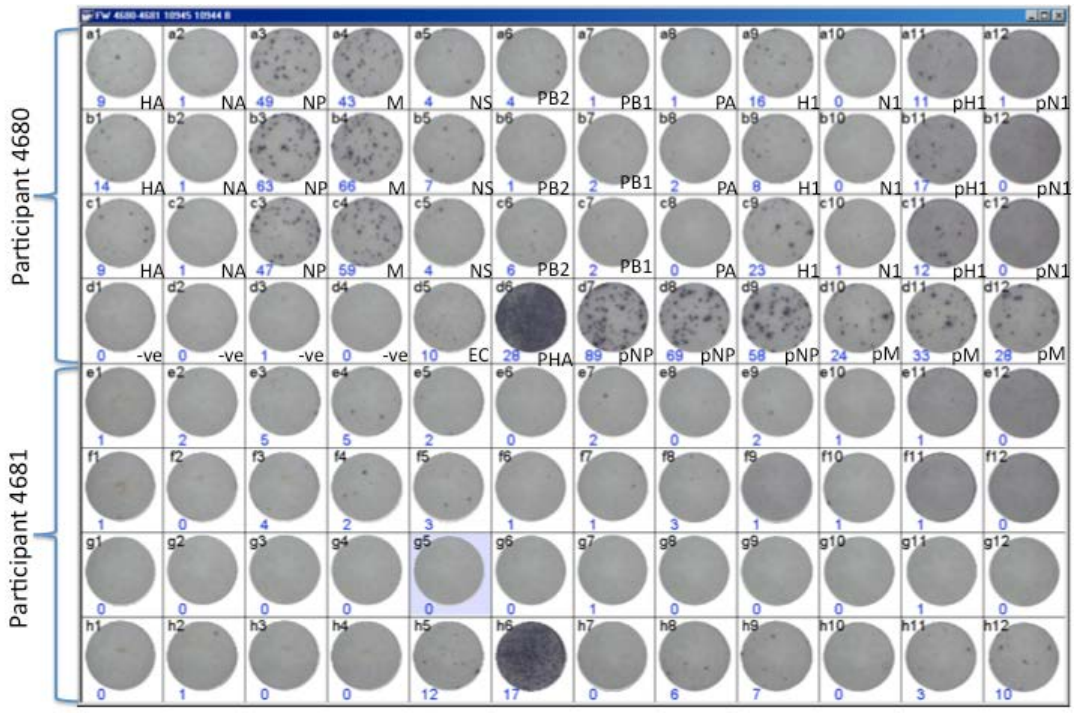


Figure 3.1: Example of Flu Watch ELISpot output
Two participants were tested on one plate. Tests were in triplicate. Controls were used are four no peptide controls, two peptide controls and one PHA control well. Abbreviations used: HA: hemagglutinin; NA: neuraminidase; NP: nucleoprotein; M: matrix protein; NS: non-structure protein; PB2: polymerase basic protein 2; PB1: polymerase basic protein 1; PA: polymersae acidic protein; -ve: no peptide mock control; EC: known EBV and CMV epitopes, served as positive peptide controls; PHA: Phytohaemagglutinin stimulated internal positive control. p: pandemic H1N1 2009 influenza virus.

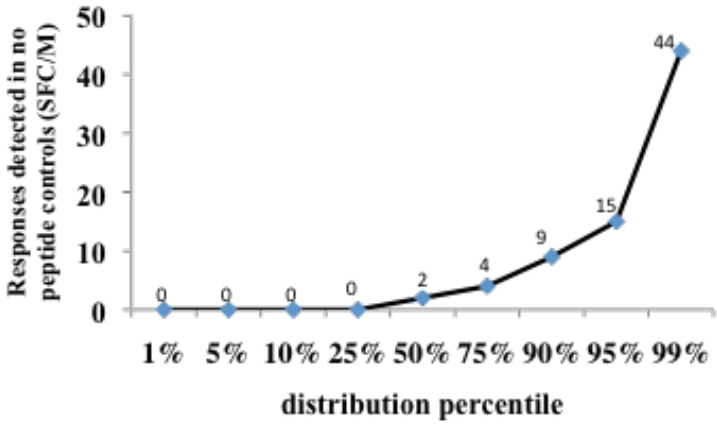


Figure 3.2: The non-specific responses detected in *ex vivo* human IFNγ ELISpot assay were extremely low
Means of non-specific responses (N=3695) detected in no peptide control well from the whole study, by the *ex vivo* human IFN-γ ELISpot assay, was extremely low. The median response from these means was at 2 SFC/M and 95% of non-specific responses were below 15 SFC/M, which was less than 4SFC/well. SFC/M: spot forming cells per 10^6 PBMCs.

Table 3.1: The distribution of variance to median ratios from triplicate experimental wells in the Flu Watch human IFN-γ ELISpot Assay

Variance to median ratio=variance/(median+1)					
Percentiles	Variance to median ratio	Smallest			
1%	0	0			
5%	0	0			
10%	0	0	number of Observations	20484	
25%	0	0			
50%	0		Mean	0.7883003	
		Largest	Standard deviation	4.594751	
75%	1	161			
90%	2	280	Variance	21.11174	
95%	3	363	Skewness	71.20888	
99%	5	408	Kurtosis	5649.801	

3.2.3 The breadth and magnitude of *ex vivo* pre-existing influenza A (H3N2) specific T cells

Pre seasonal influenza study:

In total, 1,538 participants were recruited into our pre-seasonal influenza T cell study cohort. Of this number, 396 participants were vaccinated before the baseline bleed was taken and were therefore excluded from the study of pre-existing T cell response. Subsequently, 1,142 participants were studied for their pre-existing T cell responses over the period of 3 years. The predominant circulating influenza strains had alternated between seasonal influenza H3N2 and H1N1 (Table 1.1). For the first season (Autumn 2006), influenza A (H3N2) specific T cell responses were measured by *ex vivo* human IFN-γ ELISpot assay against pooled peptides in a two dimensional matrix. Peptides in first dimension of the matrix were ordered by viral antigens that were subsequently used for the analysis of T cell responses against each of the influenza viral antigens. This peptide matrix also identified individual candidate reactive peptides/epitopes, which were deconvoluted and confirmed by ELISpot in 28 subjects for which there were sufficient PBMCs. Over the following two years (Autumn 2007 and Autumn 2008), *ex vivo* pre-existing influenza A (H3N2) specific T cell responses were measured against pooled peptides, each pool consisting of peptides spanning one of 8 viral antigens: haemagglutinin (HA), neuraminidase (NA), NP (nucleoprotein), M (matrix protein), NS (non-structural protein), and three viral polymerases: PB2 (polymerase basic protein 2), PB1 (polymerase basic protein 1), PA (polymerase acidic protein). Due to the limited PBMCs available for this study, measuring T cell responses against antigen pools would enable us to analyze responses in triplicate and allow the investigation of the intra-triplicate variation

(reported in Table 3.). Influenza A (H3N2) specific T cell responses were detectable in 72% of participants in pre-seasonal influenza studies (73%, 76% 68% from Autumn 2006, Autumn 2007, Autumn 2008 cohort respectively) (Figure 3.3).

Pre pandemic influenza study

Influenza A (H3N2) specific T cell responses from 818 participants were measured prior to H1N1 pandemic in 2009: before the first wave of pandemic H1N1 in Spring 2009 and before the second wave of pandemic H1N1 in Autumn 2009. Most participants (n=796) did not receive influenza vaccine before the baseline bleed and were included in the study of pre-existing T cell immunity. Consistent with pre-seasonal studies, pre-existing influenza A (H3N2) specific T cell responses were measured against pooled peptides with each pool containing peptides spanning each of the 8 influenza viral antigens. *Ex vivo* pre-existing influenza A (H3N2) specific T cell responses were detected in 74% of participants from pre-pandemic cohort, which was similar to the pre-seasonal cohort (Figure 3.3).

Observations from both pre seasonal and pre pandemic study

There was a broad range of *ex vivo* pre-existing influenza A (H3N2) specific T cell responses detected from Flu Watch participants ranging from approximately 1000 SFC per 10^6 PBMCs to no detectable T cell responses (Figure 3.4a). Approximately a quarter of participants had no influenza A (H3N2) specific T cell responses that were detectable by *ex vivo* human IFN-γ ELISpot assay, therefore T cell data were skewed towards zero (Figure 3.4b). The means of *ex vivo* pre-existing influenza A (H3N2) specific T cell responses directed at individual antigens were low, with the strongest T cell responses directed against NP (mean at 24 SFC per 10^6 PBMCs) (Figure 3.5).

The number of viral antigens recognized by individuals ranged from 1 to all 8 viral antigens.

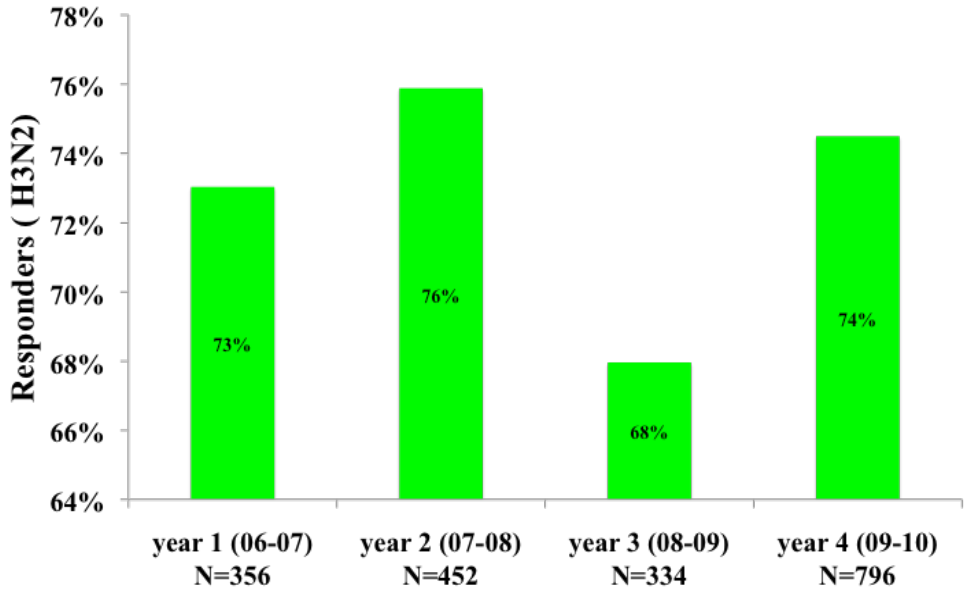


Figure 3.3: Most participants have influenza A (H3N2) specific T cell responses
The percentage of responders defined by *ex vivo* Human IFN-γ ELISpot assay was stratified by season. Each column represents percentage of responders from each season. The average responders from pre-seasonal influenza cohort (first three years) were 72%, which was similar to the pre-pandemic cohort (the 4th year) at 74%. Positivity was defined as background subtracted (mean+ 4 standard deviation of no peptide control wells) and the statistical cut-off was applied (described in 3.2.1).

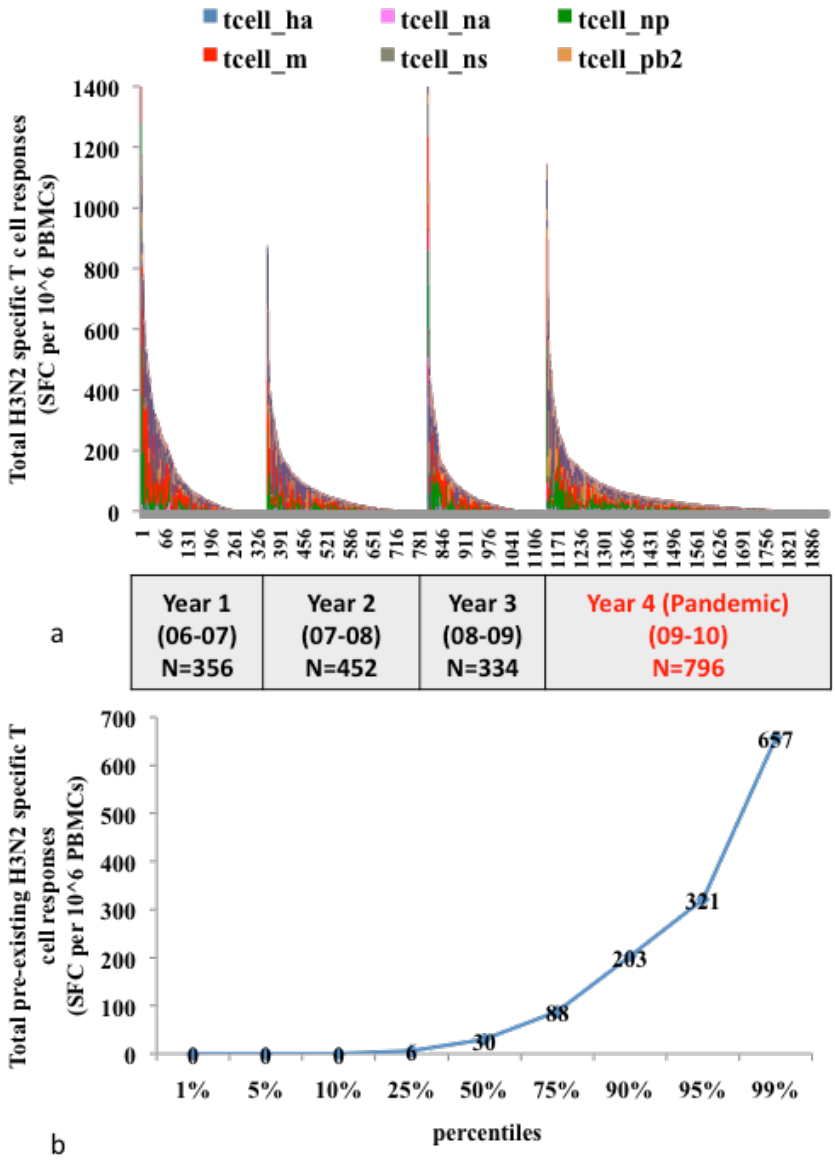
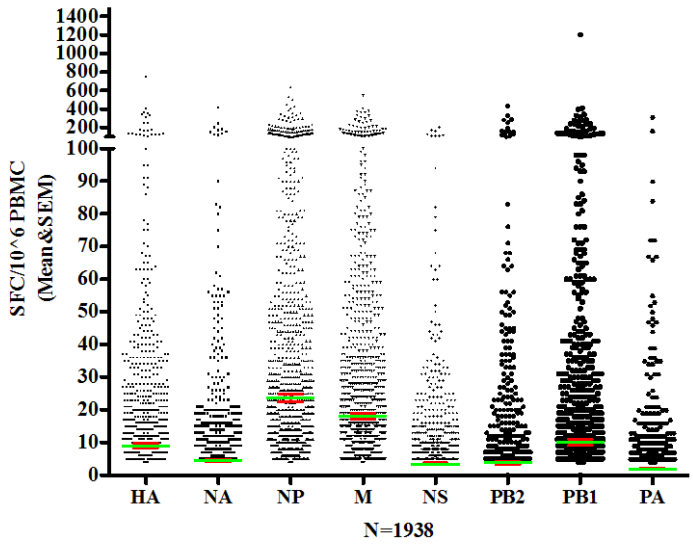


Figure 3.4: Considerable variations in *ex vivo* pre-existing influenza A (H3N2) specific T cell responses measured by human IFN-γ ELISpot assay

a) Pre-existing influenza A (H3N2) specific T cell responses directed against all eight antigens detected in Flu Watch participants. Each column represents responses from an individual participant; each colour represents responses directed at an individual antigen. These responses were grouped by season and ranked from high to low within each season. Considerable variations were detected in the magnitude of *ex vivo* pre-existing influenza A (H3N2) specific T cell responses from different individuals: from no detectable T cell response to approximately 1000 SFC per 10^6 PBMCs. **b)** The distribution of *ex vivo* pre-existing influenza A (H3N2) specific T cell responses displayed in percentiles. Numbers shown on the line are total *ex vivo* pre-existing H3N2 specific T cell responses (spot forming cells per 10^6 PBMCs) for a specific percentile. Background was subtracted (background=mean+4 standard deviation of no peptide controls).



Antigen	HA	NA	NP	M	NS	PB2	PB1	PA
Mean	9	4	24	18	3	4	10	2
Std. Error	0.72	0.39	1.18	0.94	0.28	0.47	0.96	0.24

Figure 3.5: *Ex vivo* pre-existing influenza A (H3N2) specific T cells were detected in the UK population at low frequency by human IFN-γ ELISpot assay

Ex vivo pre-existing influenza A (H3N2) specific T cells measured by human IFN-γ ELISpot assay were segregated by viral antigen specificity. Considerable variations were detected amongst the antigen specific T cell responses from different individuals with low mean T cell responses against all antigens. The strongest T cell responses were directed at NP with a mean response of 24 SFC per 10^6 PBMCs. Abbreviations used: HA: hemagglutinin; NA: neuraminidase; NP: nucleoprotein; M: matrix protein; NS: non-structure protein; PB2: polymerase basic protein 2; PB1: polymerase basic protein 1; PA: polymerases acidic protein. Positive responses were defined as background subtracted at mean+ 4 standard deviation of no peptide controls.

3.2.4 NP and M are immunodominant targets of influenza A specific T cells at the population level

In response to influenza A infection, many thousands of alternate antigenic peptides can be processed and presented to T cells; however only small fraction actually induce measurable antigen specific T cell responses. Generally, virus specific T cells recognize their target peptides in a hierarchy of dominance. Some peptides inducing strong virus specific T cell responses are known as immunodominant targets, other peptides inducing weak to barely detectable virus specific T cell responses are subdominant targets and still others that make little impact on the immune responses are known as silent targets. [230]. The term immunodominance is also used in another sense, to describe as those epitopes restricted by the same HLA alleles that are reproducibly recognized by T cells in different people expressing that allele [231]. T cell immunodominance has been described for influenza virus in mice with various haplotypes [232, 233] and in humans [234, 235]. It was demonstrated that in influenza infection, immunodominant CTLs provided more effective protection than subdominant CTLs upon lethal influenza virus challenge in peptide primed mice [236]. And for comparison, in HIV, immunodominant CTLs controlled virus replication therefore lower the virus set point: In the absence of the immunodominant HLA-B27 restricted Gag 263-272 CTLs response, HLA-B27 patients showed increased viral set points [237] and HIV virus escape at an immunodominant CTL epitope resulted in dramatic fitness cost of the virus [238] A vaccine that is less sensitive to the evolution of influenza virus could potentially offer broad protection across different strains. The identification of conserved and immunodominant targets

of influenza specific T cells would provide information for the design of a T cell inducing vaccine.

At the protein level, the Flu Watch study showed that more than 50% of the total magnitude of *ex vivo* pre-existing influenza A (H3N2) specific T cell responses detected were directed at the Matrix protein (M) and nucleoprotein (NP) (Figure 3.6) and more than 50% of volunteers in our study had *ex vivo* pre-existing influenza A (H3N2) specific T cell responses directed at these two viral proteins (Figure 3.7). This observation was consistent across all four seasons. Therefore NP and M were the most immunodominant of internal proteins with the capability of inducing the strongest T cell responses and were the most frequently recognized targets in a population with diverse TCR repertoires and HLA background.

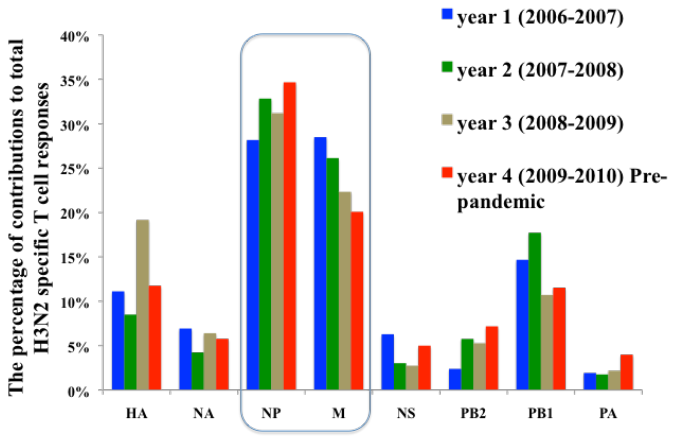


Figure 3.6: The percentage of contributions from H3N2 viral antigen specific T cell responses to the total magnitude of influenza A (H3N2) T cell responses in the Flu Watch cohort
The percentage of contributions from influenza A (H3N2) specific T cell responses against each of eight viral antigens was stratified by season. Each colour represents each season. The responses presented in this graph were measured by *ex vivo* human IFN-γ ELISpot assay. In terms of the magnitude of response, at population level, NP and M were the most immunodominant of internal proteins with over 50 % of *ex vivo* pre-existing T cell responses were directed at these two proteins. Background was subtracted (mean +4 Standard deviation of no peptide control wells)

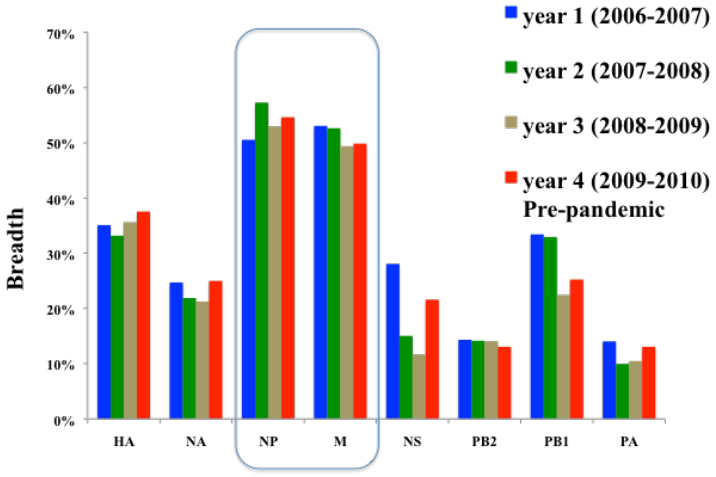


Figure 3.7: The breadth of *ex vivo* pre-existing influenza A (H3N2) specific T cell responses stratified by viral antigen specificity.
The percentage of responders defined by *ex vivo* human IFN-γ ELISpot assay were stratified by their detectable responses directed at each of eight influenza A (H3N2) antigens. Each colour represents each season. In terms of the breadth of H3N2 specific T cell responses, NP and M were the most immunodominant of the internal proteins with more than 50% of participants had T cell responses directed at NP and M. Abbreviations used: HA: hemagglutinin; NA: neuraminidase; NP: nucleoprotein; M: matrix protein; NS: non-structure protein; PB2: polymerase basic protein 2; PB1: polymerase basic protein 1; PA: polymerases acidic protein. Background was subtracted (mean ± 4 Standard deviation of no peptide control wells) and a statistical cut-off of 4 spot forming cells per million PBMCs was also use to define responders (described in 3.2.1).

3.2.5 CD4+ and CD8+ phenotyping of pre-existing influenza A specific T cells

As discussed in the introduction, influenza specific CD8+ T cells play a critical role in controlling influenza infection by eliminating virus-infected cells via exocytosis of cytolytic granules [239] [240] and Fas-Fas ligand medicated apoptosis [189, 241]. Influenza specific CD4+ T cells provide help in virus clearance by secreting cytokines and promoting the generation of high affinity antibodies to prevent from re-infection from the same strains [196, 197, 242-245] and also are required for the generation of functional influenza specific CD8+ recall T cells in subsequent infection [199]. In humans, the upregulation of CD107a expression was detected on *ex vivo* influenza NP and M specific CD4+ T cells upon antigen stimulation. This suggested that CD4+ T cells may act directly as cytotoxic T cells in human influenza infection [212]. Understanding the contribution from each subset of influenza A specific T cells in controlling natural influenza A infection and identifying the immunological targets that induce certain subset of T cell responses in the UK population would be informative for the design of a T cell based influenza vaccine.

In this section, the distribution of influenza A (H3N2) specific CD4+ and CD8+T cells was investigated. A subset of 174 participants was selected randomly, from participants who were not vaccinated before the baseline blood was taken. Their *ex vivo* pre-existing influenza A (H3N2) specific T cell responses were stimulated with pooled influenza A (H3N2) peptides and cultured in vitro for 14 days. The culture expanded CD4 mediated and CD8 mediated responses were elicited by intracellular cytokine staining (ICS) of IFN-γ production. The gating strategy for the FACs

analysis was shown in chapter 2, Figure 2.4. There was a correlation between *ex vivo* influenza A (H3N2) specific T cell responses measured by human IFN-γ ELISpot assays and those *in vitro* expanded responses measured by intracellular cytokine staining of IFN-γ production (the percentage of IFN-γ producing CD3+ H3N2 specific T cells) (Figure 3.8a, Spearman R=0.2794, P=0.0002***). Further investigation revealed that *ex vivo* influenza A (H3N2) specific T cell responses correlated with culture expanded CD8+ influenza A (H3N2) specific T cell responses (the percentage of culture expanded IFN-γ producing CD8+ CD3+ cells) (Figure 3.8c, Spearman R=0.3381, P<0.0001***), but not with culture expanded CD4+ influenza A (H3N2) specific T cell responses (the percentage of IFN-γ producing CD4+ CD3+ cells) (Figure 3.8d, Spearman R=0.1129, P=0.1381). This result suggested that influenza A specific CD8+ T cells could be proportionally cultured out *in vitro*, though not specific CD4 T cells. Overall, 93% of *ex vivo* non-responders had detectable cultured influenza A (H3N2) specific responses and 99% of *ex vivo* influenza A (H3N2) specific T cell responses were culture confirmed (Figure 3.8b). This indicated that almost everybody had low-frequency influenza A specific T cell responses, but only 72 to 74% of these responses can be detected by *ex vivo* human IFN-γ ELISpot assay, and these *ex vivo* responses detected in our ELISpot assay were true responses, could be consistently expanded *in vitro*. Within a 13-14 day culture period, 98% of study subjects had detectable influenza A (H3N2) specific T cell responses with 92% of culture responders having a mixture of the CD4+ and CD8+ mediated T cell responses. Four percent and 2% of responders had cultured CD4+ and CD8+ T cell responses only respectively.

Consistent with *ex vivo* influenza A (H3N2) specific T cell responses, at the protein level, NP and M were the most immunodominant of internal proteins. Sixteen and 15% of the total IFN-γ producing CD3+ culture expanded influenza A (H3N2) specific T cell responses were directed at NP and M respectively, and 87% and 91% of the participants had cultured responses directed at NP and M respectively (Figure 3.9). The breadth of culture expanded influenza A (H3N2) specific T cell responses directed at each antigen ranged from 74% to 91%. Within each antigen, relatively small proportion of responders had CD8+ response only (ranged from 1% to 12%)(Figure 3.9). Influenza nucleoprotein was the most potent viral protein in inducing CD8+ medicated T cell responses. In terms of population frequency, NP specific CD8+ T cells were detected in 64% of participants; this proportion was higher than those against other viral antigens. In terms of the magnitude, NP specific CD8+ T cells contributed 10% of the total IFN-γ+ CD3+ T cells against the whole viral proteome, a proportion that was higher than those directed at other viral antigens (Figure 3.9).

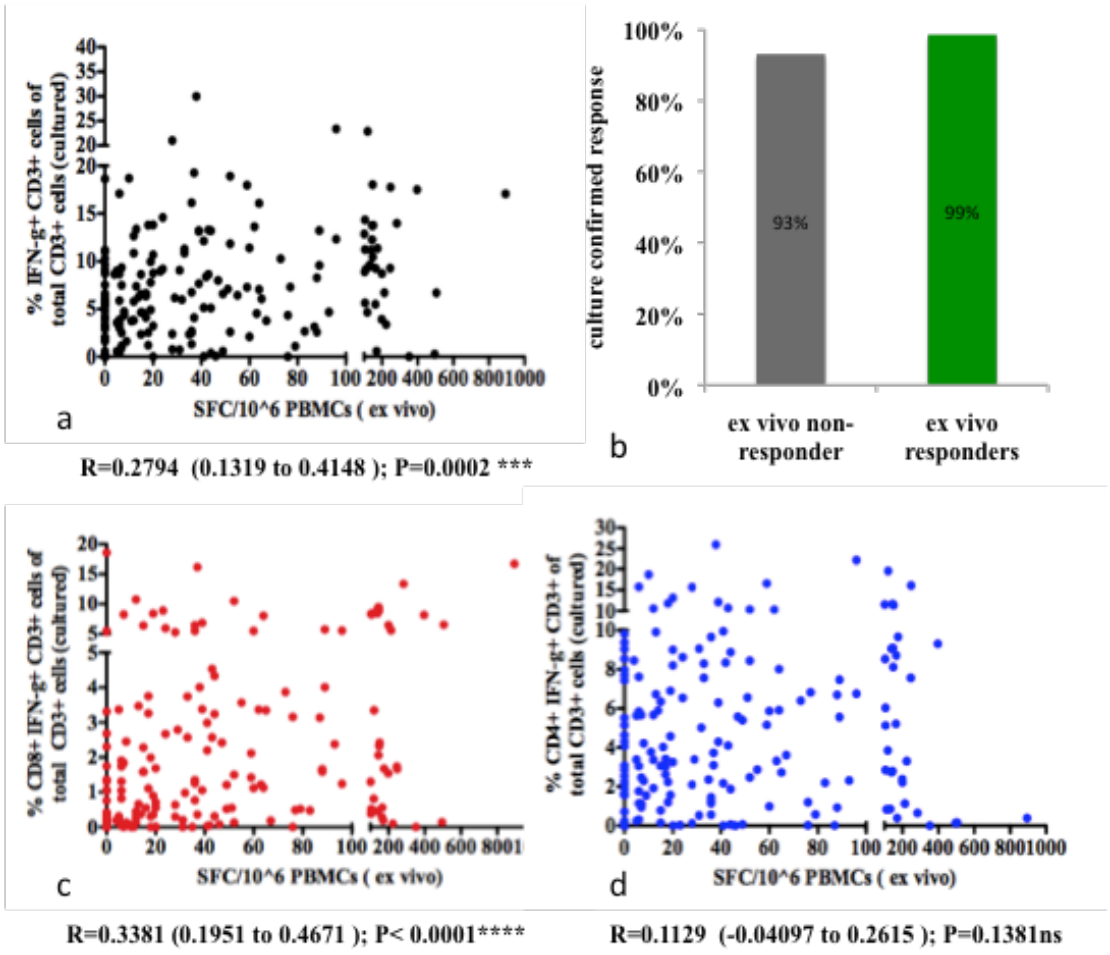
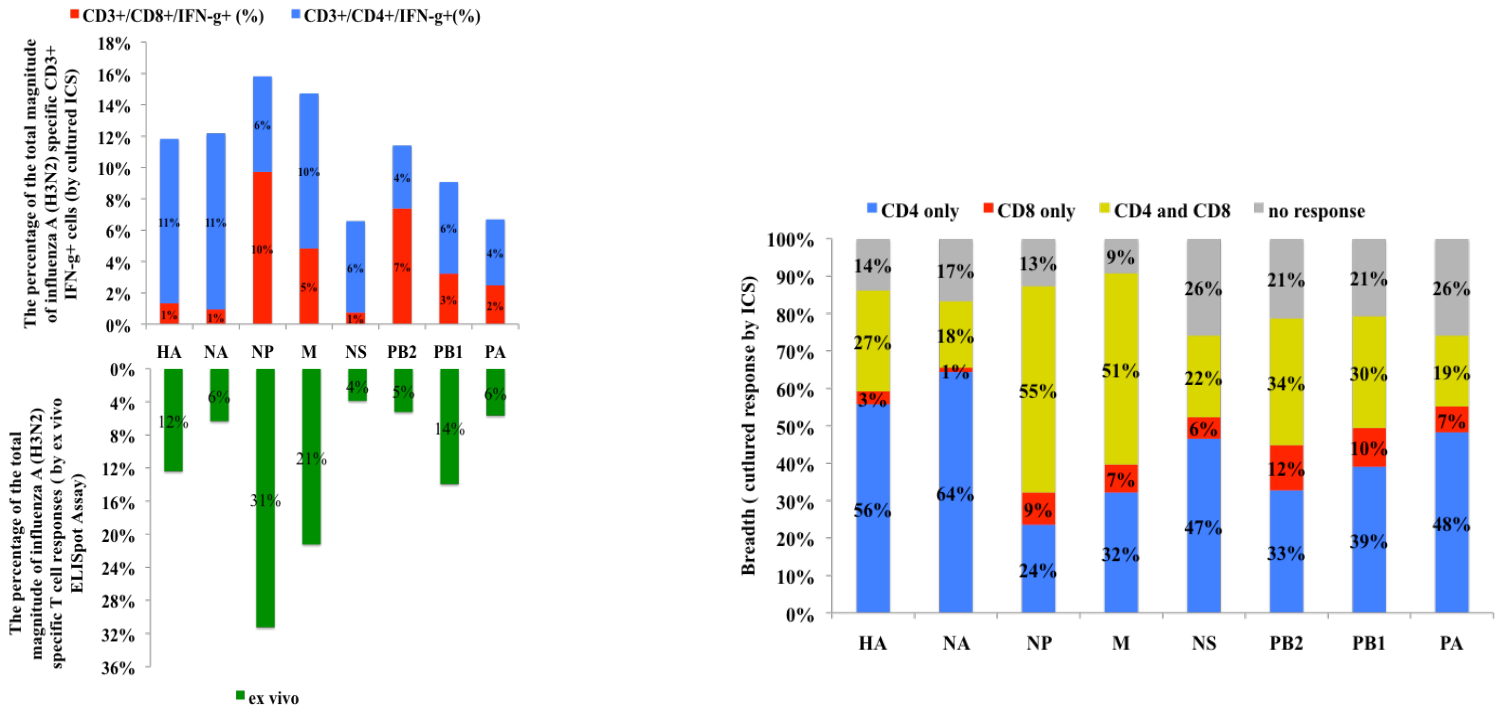


Figure 3.8: The relationship between culture explanded and *ex vivo* influenza A (H3N2) specific T cell responses.

Influenza A (H3N2) specific T cells from 174 randomly selected Flu Watch participants were stimulated with pool influenza A (H3N2) peptides then cultured for 13 to 14 days. Culture explanded CD4+ and CD8+ influenza A (H3N2) specific T cells were identified by intracelluar cytokine staining (ICS) of human IFN-γ production. *Ex vivo* H3N2 specific T cell responses were measured by human IFN-γ ELISpot assay (a) There was a correlation between *ex vivo* influenza A (H3N2) specific T cell responses and the percentage of IFN-γ producing CD3+ H3N2 specific T cells (R=0.2794, P=0.0002***). (b) Cultured influenza A (H3N2) specific T cell responses were detected from 93% of *ex vivo* non-responders. 99% of *ex vivo* responders were culture confirmed. (c) *Ex vivo* influenza A (H3N2) specific T cell responses correlated with the percentage of IFN-γ producing CD8+ CD3+ influenza A (H3N2) specific T cells (R=0.3381, P<0.0001). (d) No correlation was observed between *ex vivo* influenza A (H3N2) specific T cell responses and culture expanded IFN-γ producing CD4+ CD3+ influenza A (H3N2) specific T cell responses. Spearman ranked correlation analysis was performed using software Prism. Background was subtracted (higher than 0.04% and greater than 3 times of the no peptide control)



a) b)

Figure 3.9:The distribution of CD4+ and CD8+ influenza A (H3N2) specific T cell responses across the whole proteome of influenza A (H3N2) virus
Influenza A (H3N2) specific T cells from 174 randomly selected Flu Watch participants were stimulated with pooled influenza A (H3N2) peptides then cultured for 13 to 14 days. Culture explaned CD4+ and CD8+ influenza A (H3N2) specific T cells were identified by intracelluar cytokine staining (ICS) of human IFN-γ production. **a)** Proportional contributions to total magnitude of influenza A (H3N2) specific T cell responses from each subset of CD4+ and CD8+ responses are displayed on the top; and contributions from *ex vivo* responses are displayed at the bottom. Responses were grouped by viral antigens. **b**) Percentage of participants had either CD4+ or CD8+ or responses from both subsets of T cells directed at each antigen were shown. Abbreviations used: HA: hemagglutinin; NA: neuraminidase; NP: nucleoprotein; M: matrix protein; NS: non-structure protein; PB2: polymerase basic protein 2; PB1: polymerase basic protein 1; PA: polymerases acidic protein.

3.2.6 T cell epitope mapping and characterization of their CD4+, CD8+ phenotype and HLA restrictions

Influenza A specific T cell epitopes can be used to identify virus strain or subtype specific or cross-reactive T cell immunity against influenza, and could be useful for monitoring immune responses to circulating influenza viruses and also the evaluation of a new T cell based vaccine candidates.

Ex vivo influenza A (H3N2) specific T cell responses detected from a subset of 28 participants by two dimensional peptide matrices were mapped down to individual peptide responses by *ex vivo* human IFN-γ ELISpot assay using cryopreserved PBMCs. 51 peptides were identified (Table 3.2), including 8 peptides from viral matrix protein (M) and 12 peptides from viral nucleoprotein (NP). Twenty-one peptides have not been identified previously including 6 peptides from NP with 3 out of 6 newly identified NP peptides sharing identical amino acid sequence with pandemic H1N1 2009 virus. All except 2 participants had been HLA typed and the T cell epitope prediction tool from IEDB (immune epitope database and analysis resource) was used to predict epitopes and their HLA restrictions. T cell responses targeted at these mapped peptides were expanded via *in vitro* peptide stimulation, and then their CD4 and CD8 restrictions were characterized by the detection of human IFN-γ using intracellular cytokine staining (ICS). For those previously described peptides, the CD4 and CD8 phenotype determined by our ICS were consistent with those reported in the existing literature.

Consistent with immunodominance patterns detected at the protein level, at peptide level, influenza A (H3N2) specific T cell responses to internal protein of NP and M were the most immunodominant responses. This dominance was seen both in the magnitude of response and the frequency of recognition (Figure 3.10). The number of epitopes recognized by different individuals varied from 1 to 8 with over 50% of the participants recognized one or two epitopes. These individual peptide responses were further characterized into CD4+ and CD8+ T cell responses by cultured T cell ICS; more CD8+ T cell responses were mapped from M and NP than from other viral antigens (Figure 3.11). Amongst all peptides studied, 52% had both CD4+ and CD8+ T cell response and 44% had CD4+ T cell response only, 4% had CD8+ T cell response only.

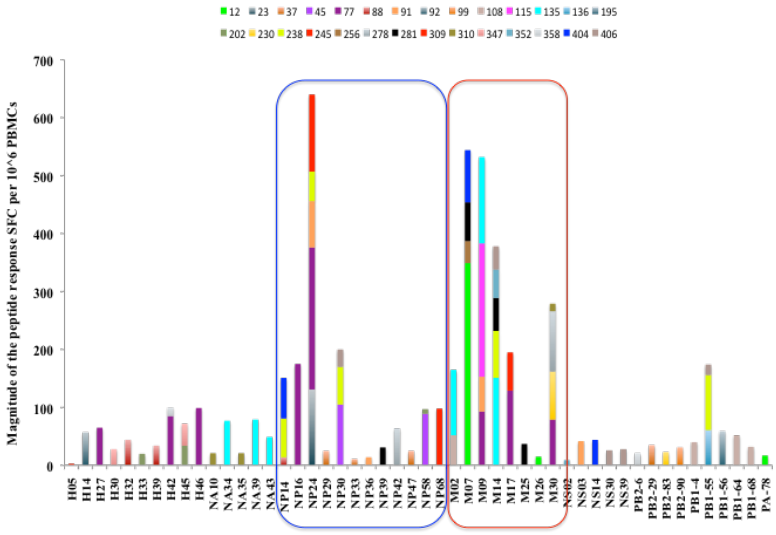
Table 3.2: List of epitopes identified in Flu Watch cohort

antigen	Peptide ID	Amino acid number	Sequence	Responders ID	CD4 /CD8 restriction	Previously described CD4/CD8 restriction	Reference	HLA class I restriction prediction
HA	H05	34-50	HAVPNGTIVKTITNDQI	FW245	CD4			
HA	H14	105-122	*ERSKAYSNCYPYDVPDYA	FW92	CD4	CD4	[246]	
HA	H27	205-220	#NDQISLYAQASGRITV	FW77	CD4&8			B*4501
HA	H30	225-242	SQQTVIPNIGSRPRVRDI	FW347	CD4			
HA	H32	241-258	DIPSRISIYWTIVKPGDI	FW88	CD4	CD4	[205]	
HA	H33	249-267	YWTIVKPGDILLINSTGNL	FW202	CD4	CD4		
HA	H39	295-310	SECITPNGSIPNDKPF	FW88	CD4	CD4	[205]	
HA	H42	317-333	TYGACPRYVKQNTLKLA	FW35 FW77	CD4 CD4 &8	CD4	[246]	B*0702
HA	H45	338-355	NVPEKQTRGIFGAIAGFI	FW202 FW347	CD4	CD4	[246] [205]	
HA	H46	346-363	GIFGAIAGFIENGWEGMV	FW245 FW77	CD4	CD4	[205, 246]	
M	M02	6-22	EVETYVLSIVPSGPLKA	FW108 FW135	CD4	unknown	[205]	
M	M07	40-57	EALMEWLKTRPILSPLTK	FW12 FW404 FW256 FW281	CD4 CD4&8	CD8	[205]	A*3001
M	M09	55-72	LTKGILGFVFTLTVPSER LTKGILGFVFTLTVPSER	FW77 FW115 FW91 FW135	CD4 CD8 CD4&8	CD4&8	[205] [247] [248]	A*0201 (a known A2 epitope)
M	M14	95-112	KAVKLYRKLKREITFHGA KAVKLYRKLKREITFHGA	FW406 FW352 FW115 FW238 FW77 FW135	CD4	CD4&8	[205]	
M	M17	116-132	ALSYSAGALASCMGLIY	FW309 FW77	CD8	CD8	[205]	A*2902
M	M25	173-189	IKHENRMVLASTTAKAM	FW281	CD4	CD4	[205]	
M	M26	180-199	VLASTTAKAMEQMAGSSEQA	FW12	CD4	CD4	[205]	
M	M30	210-227	RRMVQAMRAVGTHPSSST	FW278 FW230 FW310 FW77	CD4	CD4	[205]	
NA	NA10	66-82	VYLTNTTIEKEICPKLA	FW 310	CD4			
NA	NA34	245-262	SASGKADTKILFIEEGKI	FW135	CD4			
NA	NA35	252-268	KILFIEEGKIVHTSTL	FW 310	CD4			
NA	NA39	281-296	YPRYPGVRCVCRDNWK	FW135	CD8			B*0801
NA	NA43	308-322	KDYSIVSSYVCSGLV	FW135	CD4	unknown	[205]	

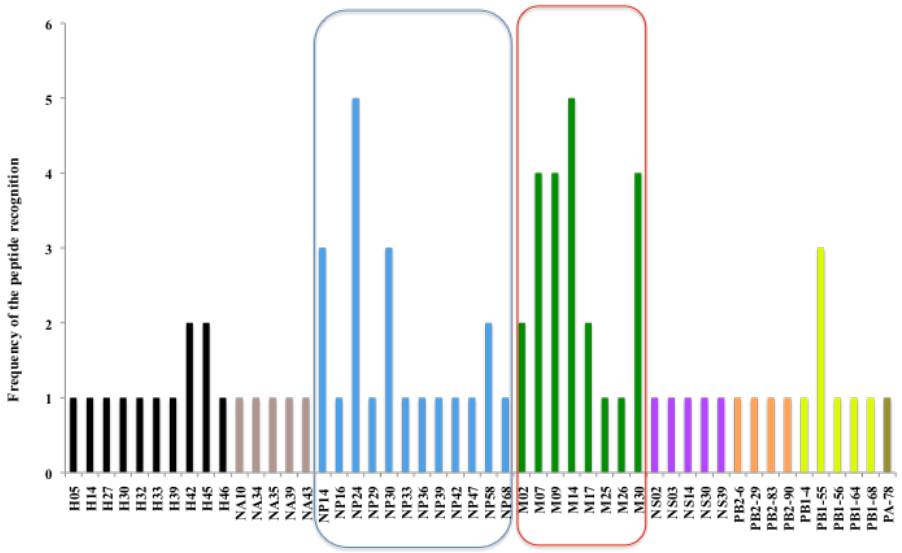
Table to be continued

	Peptide ID	Amino acid number	Sequence	Responders ID	CD4 /CD8 restriction	Previously described CD4/CD8 restriction	Reference	HLA class I restriction prediction
NP	NP14	95-111	PIYRRVDGKWMRELVLY	FW88 FW404 FW238	CD4 CD8&4			 A*0301
NP	NP16	110-129	LYDKEEIRRIWRQANNGEDA	FW77	CD4			
NP	NP24	170-186	STLPRRSGAAGAAVKG I	FW245 FW23 FW238 FW77 FW91	CD8			B*0702
NP	NP29	207-222	WRGENGRKTRSAYERM	FW37	CD8	CD4	[249]	unknown
NP	NP30	213-230	RKTRSAYERMCNILKGKF	FW406 FW238 FW45	CD4 CD8	 CD4	 [205]	 A*0301
NP	NP33	237-253	AMVDQVRESRNPGNAEI	FW37	CD4	unknown	[205]	
NP	NP36	258-273	FLARSALILRGSVAHK	FW91	CD4&8	CD4 and CD8	[226] [205] [250]	A*0201/06/ 11
NP	NP39	280-297	AYGPAVSSGYDFEKEGY	FW281	CD4			
NP	NP42	303-317	PFKLLQNSQI YSLIR	FW278	CD4			
NP	NP47	337-353	AFEDLRLLSFI RGTKVS	FW37	CD8			unknown
NP	NP58	411-428	TFSVQRNLPFEKSTIMAA	FW45	CD4&8	unknown	[205]	A*2402
NP	NP68	484-498	EGSYFFGDNAEEYDN	FW309	CD8	unknown	[249]	
NS1	NS03	14-30	FLWH IRKQVVDQELSDA	FW91	CD4			
NS2	NS14	97-112	EL SRNWFMLMPKQKVE	FW404	CD4			
NS3	NS2	5-23	VSSFQVDCFLWHI RKQVV	FW352	CD4&8			unknown
NS4	NS30	217-230	KQKRKMARTARSKV	FW406	CD4			
NS2	NS39	50-65	MRMGDLHLLQNRNGKW	FW406	CD4			
PA	PA-78	573-589	VKMKWGMEMRRCLLQSL	FW12	CD4			
PB1	PB1-4	21-38	TFPYTGDPPYSHGTGTGY	FW108	CD8	CD8	[205]	B*1501
PB1	PB1-18	123-140	TQGRQTYDWTLNRNQPAA	FW135	CD4	yes	not determined	
PB1	PB1-55	402-419	SLSPGMMMGMFNMLSTVL	FW136 FW195 FW406 FW238	CD4	CD4	[205]	
PB1	PB1-56	410-426	GMFNMLSTVLGVSILNL	FW195	CD4&8	CD4	[251] [205]	B*1501
PB1	PB1-64	470-486	CKLVGINMSKKKSYINK	FW108	CD4	unknown	[205]	
PB1	PB1-68	498-514	RYGFVANFSMELPSFGV	FW108	CD4&8	CD8	[205]	A*6802
PB2	PB2-29	205-221	YMLERELVRKTRFLPVA	FW37	CD8	CD8	[205]	unknown
PB2	PB2-6	32-49	KKYTSGRQEKNPSLRMKW	FW358	CD8			B*2705
PB2	PB2-83	598-615	TLFQQMRDVLGTFDTTQI	FW230	CD4	unknown	[205]	
PB2	PB2-90	654-670	PVFNYNKTTKRLT ILGK	FW99	CD4			

*Previously identified epitope regions are underlined.
#Predicted peptides with lowest IC50 were highlighted in red
Epitope prediction and their HLA restriction prediction were made based on the responders' HLA type by using online T cell epitope prediction tool of IEDB (immune epitope database and analysis resource)



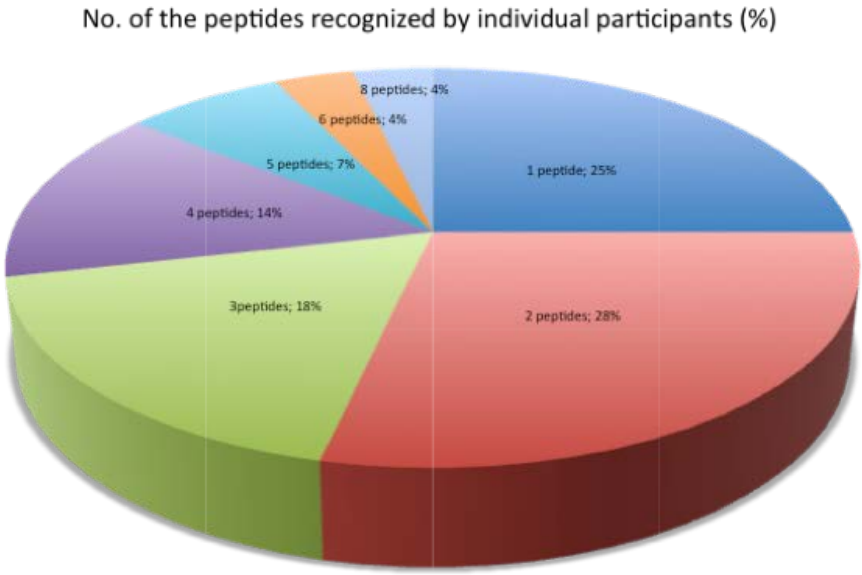
a



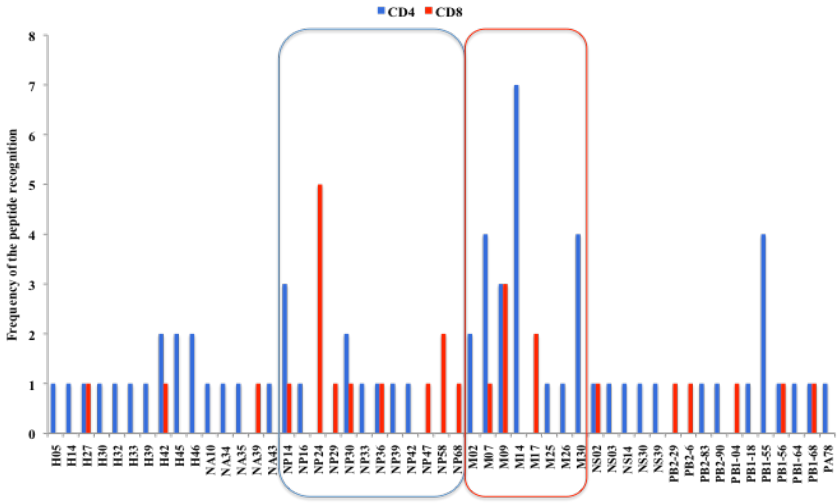
b

Figure 3.10: The distribution of mapped individual epitopes across full proteome of influenza A H3N2 virus

Twenty-seven participants had enough cryopreserved PBMCs for peptide mapping. H3N2 specific peptide responses were mapped from 27 participants by *ex vivo* human IFN-γ ELIspot assay using cryopreserved PBMCs. (a) The magnitude of individual peptide responses: each colour represents an individual participant. (b) the frequency of recognition: each colour represents an individual viral antigen. The influenza A specific T cell responses directed at NP and M were the most immunodominant proteins. More peptides were identified against NP than other antigens, but M peptides were recognized more frequently. Background was subtracted at mean+4 standard deviation of no peptide controls and statistical cutoff was applied to define responders. Abbreviations used: HA: hemagglutinin; NA: neuraminidase; NP: nucleoprotein; M: matrix protein; NS: non-structure protein; PB2: polymerase basic protein 2; PB1: polymerase basic protein 1; PA: polymerases acidic protein.



a



b

Figure 3.11: The distribution of peptides inducing CD4 and/or CD8 T cell responses across the whole proteome of influenza A (H3N2) virus

a. The number of peptides recognized by individual participants detected by *ex vivo* human IFN-γ ELISpot assay using cryopreserved PBMCs. Background substracted at mean+4 standard deviation of no peptide controls. Over 50% of the participants had a T cell response to 1 or 2 epitopes. (b) Peptide responses were cultured in vitro for 13 to 14 days, CD4+ and CD8+ mediated responses were elucidated by intracelluar cytokine staining of human IFN-γ production. Responses greater than 3 times of no peptide controls and higher than 0.04% were defined as true positive responses. The influenza A (H3N2) specific T cell responses directed at individual peptides from 8 antigens were a mixture of CD4 and CD8 responses. There were more CD8 responses directed at NP in comparison to responses directed at other antigens. Abbreviations used: SD: standard deviation; HA: hemagglutinin; NA: neuraminidase; NP: nucleoprotein; M: matrix protein; NS: non-structure protein; PB2: polymerase basic protein 2; PB1: polymerase basic protein 1; PA: polymerases acidic protein.

3.3 Discussion

Over a period of four years, influenza A (H3N2) specific T cell responses from 2395 participants were studied. These participants were randomly selected from participating GPs' registers located within a 150-mile radius of Oxford. 81 % of participants (N=1938) who had not received influenza vaccination before baseline blood samples bleed were entered the study (mean age at 47±0.4 (SEM)). *Ex vivo* influenza A (H3N2) specific T cell responses were quantified by human IFN-γ ELISpot assay and freshly isolated PBMCs from the blood of 12 to 15 hours old were used at 250,000 cells per well and their responses against pooled peptides were recorded. Assay standardization was performed before the start of the Flu Watch study and reported in section 2.7. The non-specific responses detected from no peptide controls in this study was very low with a median response at 2 spot forming cells per 10^6 PBMCs.

IFN-γ secretion is important in anti-viral protection against influenza infection [252] and ELISpot is one of the most sensitive and reproducible techniques in investigating the antigen specific T cell responses [253], with the ability to detect T cells as low as 1:100,000 and an inter-assay coefficient of variation of 15% to 25% [254-256]. The influenza A specific T cell responses detected by *ex vivo* IFN-γ ELISpot assay were previously described as a subset of circulating CD45RO memory T cells with the ability to provide immediate effector function without the need to differentiate or proliferate upon antigen encounter [257], IFN-γ secretion could be detected from these circulating memory T cells within 6 hours of *in vitro* peptide stimulation.

The influenza A (H3N2) specific T cell responses were detected in over 70% of Flu Watch participants. There was considerable variation in the magnitude and the breadth of influenza A (H3N2) specific T cell responses from different individuals. At the protein level, the breadth of these influenza A (H3N2) specific T cell responses ranged from 1 to all 8 antigens between individuals. The magnitude of *ex vivo* influenza A (H3N2) specific T cell responses detected by the human IFN-γ ELISpot assay were mostly below 1000 SFC per 10^6 PBMCs with low median response at 30 SFC per 10^6 PBMCs. The frequency of *ex vivo* influenza A (H3N2) specific T cells detected in the Flu Watch study were generally comparable with previous influenza studies [205, 212, 227, 258], and with the frequency of other acute respiratory viral infection such as respiratory syncytial virus[259], but significantly lower than the frequency of chronic viral infections such as HIV and CMV[260]. Although the frequency of these *ex vivo* influenza specific T cells was low, they were markedly above the background that had a median non-specific response of 2 SFC per 10^6 PBMCs from the whole study. Stringent statistical method was used to define the background; only those responses that were four standard deviations above the mean of no peptide controls were used in the analysis and all data reported in this thesis was background subtracted. In addition, a statistical cutoff of signal to noise ratio was also implemented when defining a positive responder.

Characterization of *ex vivo* CD4+ and CD8+ mediated antigen specific T cell responses by human IFN-γ ELISpot assay can be achieved by depleting CD4+ or CD8+ T cell subset prior to assay set up [261]. Cytokine flow cytometry allows simultaneous analysis of multiple parameters; it is a powerful immunophenotyping tool with the low limit of detection at around 1 in 50,000 (0.002%) [256]. It has been

used to identify responses mediated by *ex vivo* CD4+ and CD8+ T cells to different viral infections including CMV[262, 263], other Herpes viruses[263, 264], EBV[264, 265], HIV[265]. However, influenza specific T cells exist in periphery blood at low frequency. As described earlier in this chapter, half of Flu Watch participants had influenza A (H3N2) specific T cell responses below 30 spot forming cells per 10^6 PBMCs. This low frequency is below the lower limit of detection by conventional CD4/CD8 cell depleted human IFN-γ ELISpot assays and by intracellular cytokine flow Cytometry. *In vitro* expansion of antigen specific T cells can greatly enhance the frequency of antigen specific T cells enabling their reliable detection. Low frequency human tumor antigen specific T cells could be expanded 40 fold *in vitro*, measured by tetramer staining and more than 100 fold measured by ELISpot and intercellular cytokine staining [266, 267]. Also, the proliferative potential of T cells may be of importance for virus control. In a human influenza A virus intranasal challenge study with live attenuated influenza virus, the level of culture-expanded cytotoxic T cells correlated inversely with nasal viral shedding in the absence of specific antibody to the challenge strain [211]. Therefore, in this chapter, *ex vivo* influenza A (H3N2) specific T cells were antigen stimulated and cultured for 13 to 14 days, and influenza A (H3N2) specific CD4+ and CD8+ T cells were identified by intracellular cytokine staining of human IFN-γ secretion. *In vitro* expansion greatly enhanced the frequency of those influenza A (H3N2) specific T cells. Therefore, influenza A (H3N2) specific T cells were detected from 98% of participants by ICS using these *in vitro* expanded T cells, which was markedly higher than those detected by human IFN-γ ELISpot assay (around 70%). Influenza A (H3N2) specific T cell responses from 99% of *ex vivo* responders were culture confirmed, suggesting that the definition of background and the statistical cutoff for positivity in our human IFN-γ ELISpot assay (described

in section 3.2.1) was biologically valid. Ninety two percent of culture confirmed responders exhibited a mixture of CD4+ and CD8+ mediated T cell responses, while only 4% and 2% had CD4+ only and CD8+ only T cell responses respectively. Therefore it was not possible to study the function of each subset of T cells in controlling influenza infection.

Previous studies have demonstrated that CD45RB ^{low} memory CD4+ T cells specific for influenza hemagglutinin in TCR transgenic mouse were a heterogeneous population, consisting of a mixture of CD62L^{high} and CD62L^{low} memory T cells. The CD62L^{low} subset exhibited greater proliferation capacity ([^{3}H]thymidine assay) than CD62L^{high} upon peptide stimulation *in vitro*[268]. In humans, it was also demonstrated that CD45RA^{low} CMV specific memory CD4+ T cells in human periphery blood exhibited heterogeneity in activation threshold for IFN-γ and IL-2 secretion [269]. In our study, a correlation between the percentage of influenza A (H3N2) specific IFN-γ producing CD3+ culture expanded T cells and the *ex vivo* frequency of influenza A (H3N2) specific T cell responses was observed (Spearman R=0.2794, P=0.0002***). This suggested *ex vivo* influenza A (H3N2) specific T cells were those had proliferation capacity. There was a heterogeneous proliferation in CD4+ T cell compartment as demonstrated in this study, the correlation was observed between the *ex vivo* frequency and culture expanded CD8+ T cells, but not culture expanded CD4+ T cells (Figure 3.8), which may reflect the heterogeneity of CD4+ memory T cells as discussed above.

While T cells do not offer sterilizing immunity against influenza infection, they may offer partial protection by lowering the viral burden early after infection. There is a

growing interest in the development of T cell inducing influenza vaccines. Influenza A nucleoprotein (NP) and matrix protein 1 (M1) has been considered as potential candidates for MVA viral vector T cell based vaccine in humans [270, 271]. This is because NP and M1 are highly conserved; 90% and 92% of amino acid sequence, respectively, is shared across seasonal H1N1, H3N2 and pandemic H1N1 2009. Also, in humans, NP and M1 were previously described as the major targets of influenza specific cytotoxic T cells ([272, 273] and were also the dominant targets of cross-reactive T cells demonstrated in a human cohort of 90 participants from UK and Vietnam [205]. In this study, 1938 UK participants were randomly selected and studied. We have verified that at the protein level, in both *ex vivo* and *in vitro* expansion studies, the viral NP and M were the most immunodominant of internal proteins capable of inducing the strongest influenza A (H3N2) specific T cell responses. These two proteins were recognized by more than half of our study population. *In vitro* studies demonstrated that NP specific CD8+ T cells alone contributed to 10% of total T cell responses against all influenza antigens and this proportion was higher than CD8+ T cell responses against other influenza antigens; Also, over half of Flu Watch participants (64%) had CD8+ T cell responses directed at NP. This confirmed that NP was the most potent viral antigen at inducing CD8+ T cell responses. The immunodominance of nucleoprotein and matrix proteins were also observed at peptide level. Peptides mapped from nucleoprotein and matrix proteins induced the strongest *ex vivo* influenza A (H3N2) specific T cell responses and are the most recognized peptides with more CD8+ mediated peptide responses identified than from other viral proteins. Half (3 out of 6) of the novel NP peptides identified in this study shared identical amino acid sequence with pandemic H1N1 influenza and one of novel NP epitopes, NP 24 (B*0702 restricted), is one of the most frequently

recognized peptides and induced the strongest T cell responses that was exclusive mediated by CD8+ T cells. These results have verified that influenza nucleoprotein is the most immunogenic protein in inducing cytotoxic CD8+ T cell in the UK population and would be a suitable target for a T cell inducing vaccine for general UK public. The ability of these immunodominant T cells cross-react to other natural influenza virus stains were examined and reported in the next chapter. Further study is required to separate out T cell responses against Matrix 1 and Matrix 2 proteins in order to compare with previous findings of the T cell immunity against these two matrix proteins.

In summary, influenza A (H3N2) specific T cells were readily detectable from 70% of the UK population and largely (78%) at low frequency (below 100 spots forming cells per 10^6 PBMCs). Increased sensitivity in immunophenotyping was afforded by *in vitro* expansion of these low frequency influenza A (H3N2) specific T cells, and a mixture of CD4+ and CD8+ mediated influenza A (H3N2) specific T cell responses were detected from 92% of responders. Therefore it is not possible to address the importance of each subset of T cells in controlling influenza A infection. Nucleoprotein (NP) and Matrix (M) protein were the most immuodominant internal protein, and NP protein was the most potent protein in inducing CD8+ T cells, may be the ideal target of a protective T cell inducing vaccine for the UK population that comprises of individuals with different TCR repertoire and HLA background. The cross-reactivity of immunodominant T cell responses against nucleoprotein and matrix protein and the role of pre-existing influenza A (H3N2) specific T cell responses in controlling natural infection with seasonal influenza and pandemic influenza were examined and reported in the next chapter.

Chapter 4: Durability and cross- reactivity of *ex vivo* influenza A (H3N2) specific T cells

4.1 Introduction

Peripheral antigen specific memory T cells generally decrease after pathogen clearance [274]. Some infectious agents are capable of inducing long-term memory T cells. Vaccinia virus specific cytotoxic T cells, measured by ^{51}Cr release assay against autologous B cell lines expressing vaccinia antigen, could be detected in peripheral blood of vaccinees up to 50 years after immunization against smallpox in the absence of re-exposure to vaccinia virus [275]. Peripheral blood T cells that responded to measles infected EBV immortalized autologous B cells (by *ex vivo* human IFN-γ intracellular cytokine staining), within 24 hours of stimulation, were detected in healthy adults aged between 25 to 42 years with a childhood history of measles [276]. However, T cell memory to influenza appeared to be short lived with a half-live of approximately 2-3 years[277]. The cytotoxicity of human periphery influenza A specific CTLs measured by killing assay declined from 1978 and 1982 at population level, and this decline closely correlated with the decreased infection rate in the study community, which suggested the waning of memory influenza A specific T cells without infection[277]. Understanding the longevity of memory influenza A specific T cells without boosting through vaccination or natural infection is essential for the design of vaccination strategies that aim to boost the influenza A specific T cells.

In addition to durable T cells, another important feature that is particularly important for the development of a universal influenza vaccine is the ability of influenza specific T cells to cross react with the influenza virus that are serologically distinct subtypes. A previous study of a cohort of 90 volunteers from the UK and Vietnam assessed the cross-reactive anti-influenza specific T cells against the whole proteome

of novel influenza virus H5N1 using overlapping peptides and showed that influenza nucleoprotein and matrix protein were the major immunological targets of anti-influenza cross reactive T cells [205]. In the previous chapter, we verified that influenza nucleoprotein (NP) and matrix protein (M) were the most immunodominant proteins and would be the ideal vaccine immunogens. In this chapter, the cross-reactivity of *ex vivo* pre-existing influenza A (H3N2) specific T cells to pandemic H1N1 2009 was investigated, including the cross-reactivity of influenza A (H3N2) specific T cells against both immunodominant internal proteins of nucleoprotein (NP) and matrix protein (M). We investigated the potential of influenza NP and M as major immunological targets for T cell inducing vaccines against serologically distinct influenza strains in human.

Aims:

- To investigate the longevity of influenza specific T cells over 5 to 8 months and 1 to 1.5 years
- To investigate the half-life of influenza specific T cells in the absence of detectable influenza infection or influenza vaccination
- To investigate the cross-reactivity of influenza specific T cells against immunodominant internal proteins: nucleoprotein (NP) and matrix protein (M).

4.2 Results

4.2.1 The durability of *ex vivo* influenza A (H3N2) specific T cells in the absence of detectable influenza infection or influenza vaccination

In our Flu Watch study, a subset of 190 participants was studied for the maintenance of their influenza A (H3N2) specific T cell responses between pre- to post-season bleeds (approximately 5-8 months). These participants were selected based on the following criteria:

- Not vaccinated before the baseline and between the baseline and follow up bleed
- No seroconversion (HI sera titer of a 4-fold rise from pre to post season)

There was a correlation between *ex vivo* influenza A (H3N2) specific T cell responses detected at the baseline and at post influenza season (between 5 to 8 months after baseline bleed) (spearman r=0.39, P<0.0001***) (Figure 4.1). The correlation coefficient was slightly reduced but remained significant after the removal of 19 participants who had no detectable H3N2 specific T cell responses at both pre and post season (Spearman r= 0.26 and P<0.0006***), therefore the correlation was not driven by negative values. Although, there was no significant change in the magnitude of these T cell responses from pre to post season at a population level (non parametric paired T test, P= 0.105) (Figure 4.2a), some fluctuations in influenza A specific T cell responses were observed from individual participants from pre to post season (Figure 4.2b). Thirty five percent of participants had two-fold or more increase from pre to post season in H3N2 specific T cells. Conversely, responses from 27% of participants were reduced by half or more from pre to post season. However, these

fluctuations were mainly detected in low frequency responses. Ninety percent of fluctuations detected from responses below 100 spots forming cells per 106 PBMCs, which did not change the total magnitude at the population level (Figure 4.2c).

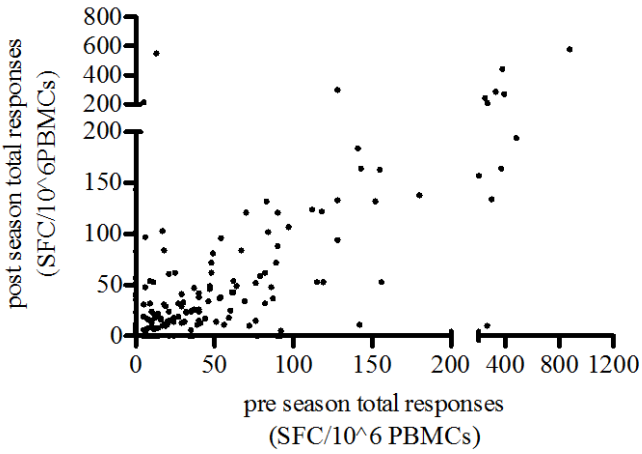


Figure 4.1: *Ex vivo* influenza A (H3N2) specific T cell responses detected at the baseline correlated with their corresponding post season responses (N=190)
H3N2 specific T cell responses were measured *by ex vivo* human IFN-γ ELISpot assay. Spearman non-parametric correlation analysis was performed using Prism. There was a correlation between the baseline T cell responses and their responses detected after influenza season (r= 0.38 and P<0.0001****). The correlation coefficient was slightly reduced but remained significant after the removal of 19 participants who had no detectable H3N2 specific T cell responses at both pre and post season (Spearman r= 0.26 and P<0.0006***). Abbreviation used: SFC/10^6PBMC: Spot forming cells per 10^6 PBMCs.

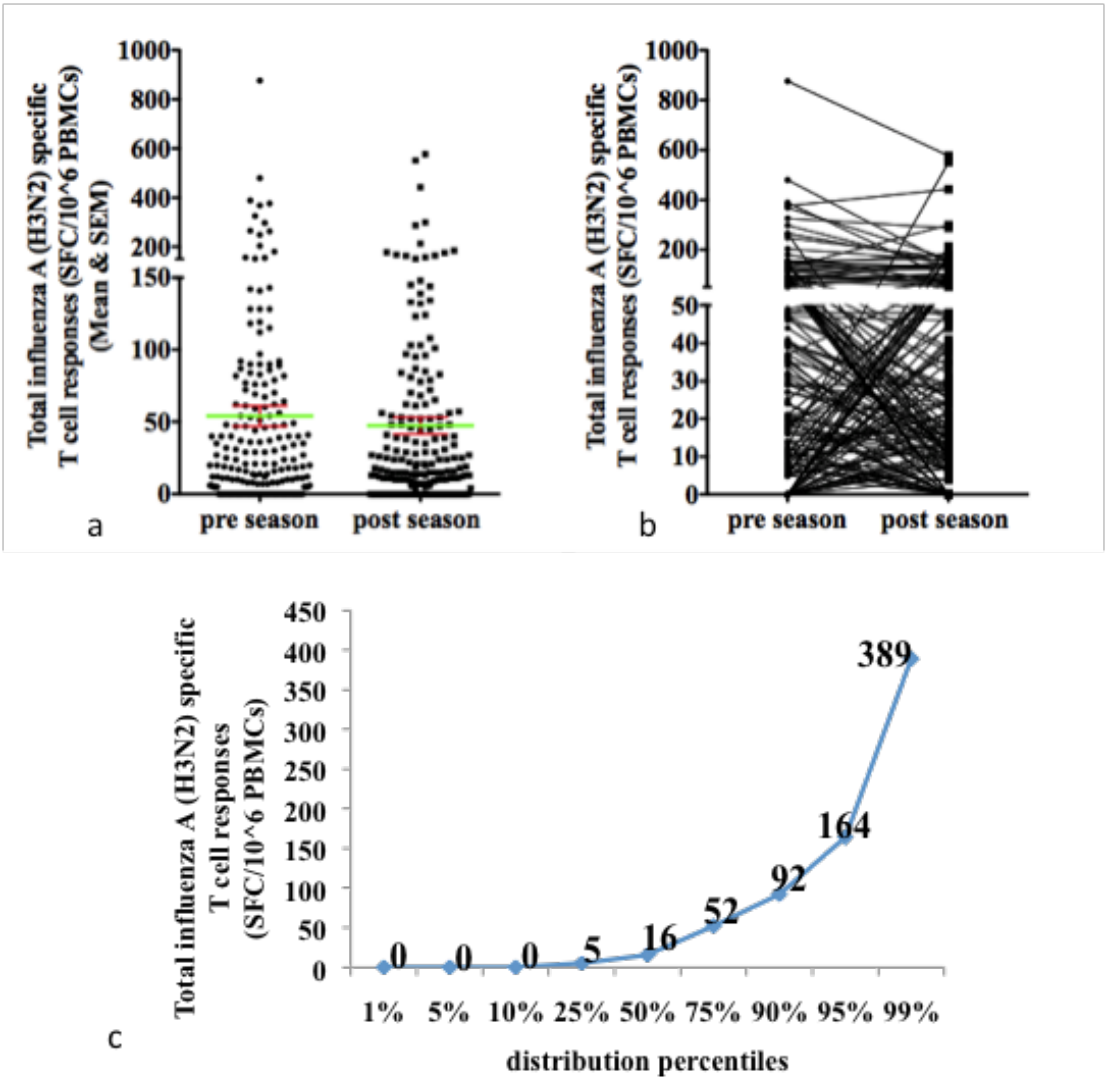


Figure 4.2: The durability of influenza A (H3N2) specific T cell responses at population level from pre season to post season (N=190)

a: Influenza A (H3N2) specific T cell responses measured by *ex vivo* human IFN-γ ELISpot assay remained unchanged at the population level with no difference between the mean of the magnitude of pre and post seasonal responses without influenza infection and vaccination. Non-parametric paired T test (Wilcoxon matched-pairs signed rank test) was performed using Prism, P= 0.105; **b:** There were some fluctuations in the total influenza A (H3N2) specific T cell responses at an individual level; **c:** The distribution of influenza A (H3N2) specific T cell responses from 117 participants who had a two-fold change from pre to post season (including two fold or more increase and reduction by half or less). 90% of fluctuations detected in influenza A (H3N2) specific responses were below 100 spot forming cells per 10^6 PBMCs. Numbers on the line indicated the H3N2 specific T cell responses for a specific percentile. Abbreviations used: SEM: standard error of mean; SFC/10^6 PBMCs: spot forming cells per 10^6 PBMCs. data presented here were background subtracted (background:mean + 4 Standard deviation of no peptide controls).

A subset of 88 participants was enrolled in the Flu Watch study for more than one influenza season. Thirteen participants who had no recorded influenza infection and vaccination were studied for the maintenance of their influenza A (H3N2) specific T cell responses over more than one season. Influenza A (H3N2) specific T cells had an overall trend of retaining their magnitude within the first 5-8 months then declining with time with a half-life of 1 to 1.5 years in participants who had total influenza specific T cell responses above 100 spot forming cells (SFC) per 106 PBMCs or had influenza specific T cells above 40 spot forming cells per 106 PBMCs against NP and M. But most of these participants had responses below 100 spot forming cells (SFC) per 106 PBMCs for total responses (69% of participants) and below 40 spot forming cells (SFC) per 106 PBMCs for NP specific and M specific T cell responses (77% of participants) and these influenza A (H3N2) specific T cell responses remained low over 1 to 1.5 years with some degree of fluctuation. Influenza A (H3N2) specific T cell responses directed at surface antigens HA and NA were very low, mostly below 20 SFC per 106 PBMCs and remained low over the study period (Figure 4.3).

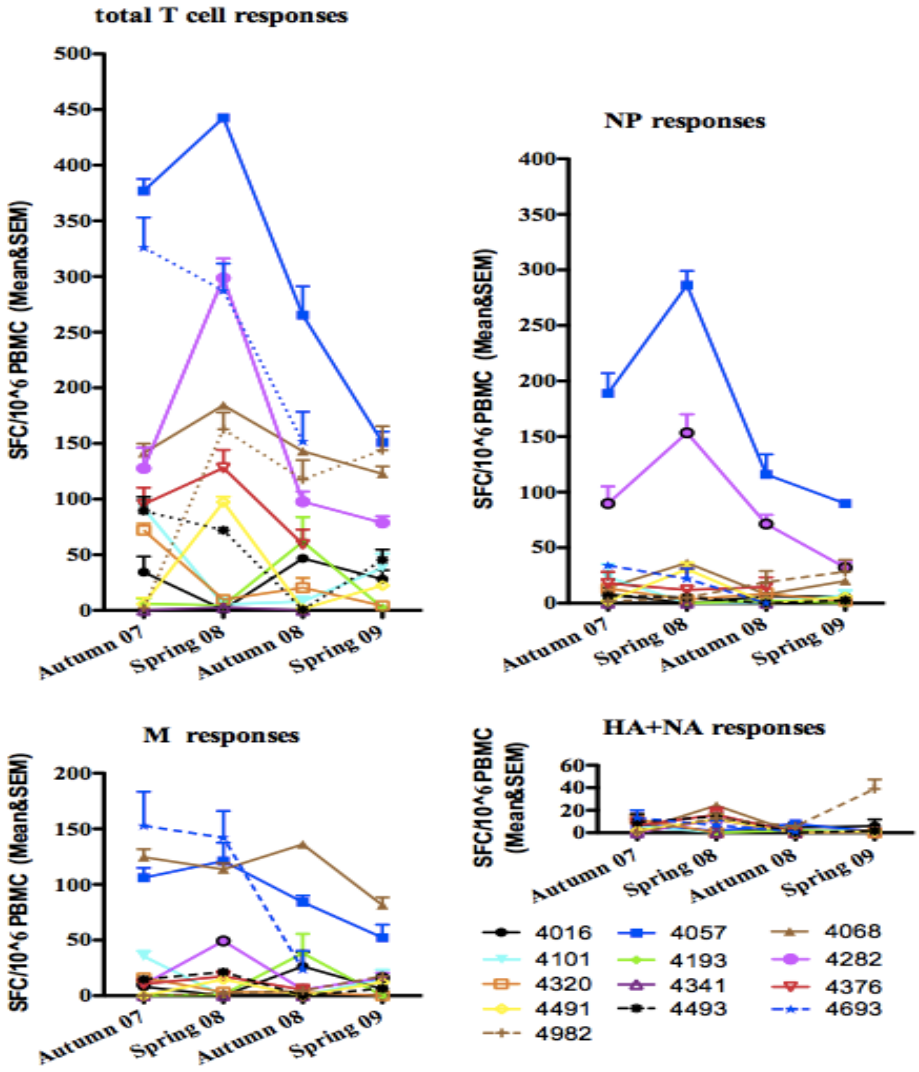


Figure 4.3: The durability of *ex vivo* influenza A (H3N2) specific T cells over a period of 1.5 years
Ex vivo influenza A (H3N2) specific T cell responses from 13 participants were measured over a period of 1.5 years by human IFN-γ ELISpot assay. These participants had no recorded influenza infection and vaccination within this period. Total responses were the responses against peptides spanning the whole viral proteome; HA+NA responses were overall responses directed at surface antigens HA and NA. Each color represents an individual participant. Abbreviations used: SEM: standard error of mean; SFC/10^6 PBMCs: spot forming cells per 10^6 PBMCs; NP: nucleoprotein; M: matrix protein; HA: hemagglutinin; NA: neuraminidase.

4.2.2 Assessing the level of subtype specific T cell immunity and the cross-reactivity of *ex vivo* T cells directed at immunodomiant internal proteins of NP and M

To investigate the level of *ex vivo* pre-existing T cell responses to different subtypes that were circulating in our Flu Watch cohort and the extent of cross-reactivity to pandemic H1N1, a subset of 552 samples collected before the first and second wave of 2009 H1N1 pandemic in spring 2009 and Autumn 2009 were also tested against the surface antigens (HA and NA) of seasonal H1N1 and pandemic H1N1 2009 and internal proteins (NP and M) of pandemic H1N1 2009. 411 out of 552 participants who had no detectable antibody against pandemic H1N1 were assessed for the T cell cross-reactivity against pandemic H1N1. T cell responses were measured by *ex vivo* human IFN-γ ELISpot assay. T cell responses against different antigens from each individual were tested on the same plate with blood that was taken from one given time point, and therefore was not influenced by the natural variation in blood taken at different time points (described in **Figure 4.3**).

Sequence alignment shows that these 3 strains share approximately 30-40% of amino acid identity in surface antigen HA and NA, because of the considerable variation in amino acid sequences of surface antigens (HA and NA) between different serotypes (Table 4.1), the T cell responses against these two surface antigens were considered to be subtype specific and could be used to assess the subtype specific T cell immunity. Considerable variation was detected in T cell responses against influenza surface antigens, ranging from approximately no detectable T cell response to 400 spot forming cells per 10^6 PBMCs to. T cell responses differed between different subtypes

(Figure 4.4). The ranked non-parametric T test which takes account of both high and low T cell responses showed that T cell responses directed at H3 and N2 were higher than T cell responses directed at seasonal H1 and N1 (Wilcoxon matched-pairs signed rank test, P<0.0001***).

Table 4.1: The amino acid sequence alignment of seasonal influenza H3N2, H1N1and pandemic H1N1 (pH1N1)

[1]Viral antigen	[2]HA	[2]NA	[3]NP	M1	M2	NS1	NS2	PB2	PB1	PA
				[3]M						
Length Amino acid	575	477	498	250	98	230	121	758	757	714
				350						
No. of amino acid differences	356	257	52	19	17	51	14	40	38	43
				37						
amino acid sequence homology	38%	46%	90%	92%	82%	78%	88%	95%	95%	94%
				89%						

[1]Amino acid sequences used in this alignment are from the isolate of H3N2 internal protein (A/New York 232/2004); H3N2 surface protein (AAZ74573: A/New York 388/2005); All antigens from H1N1 (ACA28844: A/Brisbane/59/2007) and Pandemic H1N1 (ACP41105: A/California/04/2009)
[2]The surface antigen HA and NA are the least conserved proteins sharing approximately 30-40% homology in their amino acid sequences.
[3]Most conserved internal proteins are polymerase proteins. There are 10% and 11% difference in amino acid sequence from NP and M respectively

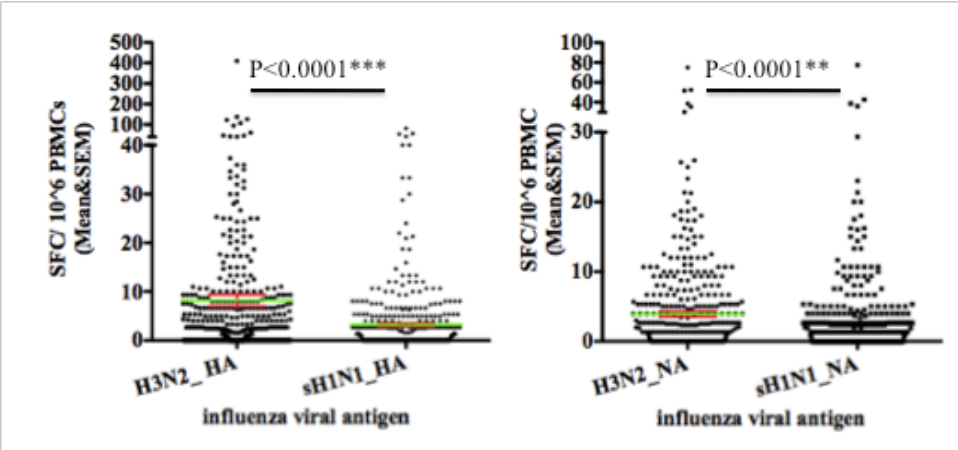


Figure 4.4: H3N2 specific T cell immunity was higher than H1N1 specific T cell immunity in the Flu Watch cohort

Ex vivo pre-existing influenza A specific T cell responses from 411 participants were measured by human IFN-γ ELIspot assay. T cell responses against surface antigens of HA and NA from different subtypes ranged from approximately 400 SFC per 10^6 PBMCs to no detectable response. The HA and NA specific T cells against H3N2 were higher than those against seasonal H1N1 (non parametric paired t test: Wilcoxon matched-pairs signed rank test, P<0.0001***). Mean T cell responses against surface antigens were very low, ranging between 8 to 2 SFC per 10^6 PBMCs Background was subtracted at mean+4 standard deviation of no peptide controls. Abbreviation used: HA or H: hemagglutinin; NA or N: neuraminidase; sH1N1: seasonal H1N1; SFC: spot forming cell.

The ability of *ex vivo* pre-existing NP specific and M specific T cells to cross-react with pandemic H1N1 virus 2009 was also investigated. Participants (N=411) had no detectable antibody against pandemic H1N1; therefore there was no evidence of pre-exposure to pandemic H1N1 2009 virus. Both NP specific and M specific T cell responses exhibited high level of cross reactivity (Figure 4.5). Large proportions of NP and M responders were able to recognize pandemic H1N1 2009 virus: 78% of H3N2 NP responders were able to recognize pandemic H1N1 virus, and 66% of H3N2 M responders cross reacted to pandemic H1N1 virus. The magnitude of cross-reactive T cell responses were investigated using a relative mean, which was calculated as dividing the magnitude of pH1N1 T cell responses by the magnitude of H3N2 T, cell responses. The relative mean for NP and M was at 0.7 and 0.62 respectively. The data from both the breadth and the magnitude of the T cells responses also suggested that NP was more efficient than M in generating cross-reactive T cells.

Further evidence of strong cross reactivity directed at nucleoprotein (NP) and matrix protein (M) was observed. Strong and significant correlations between the magnitude of H3N2 specific T cell responses and pandemic H1N1 specific T cell responses against nucleoprotein (NP) and matrix protein (M) were detected with Spearman r=0.7819 ($P<0.0001$*) and Spearman r= 0.6171 ($P<0.0001$***) respectively. This suggested that there were strong associations between having anti-H3N2 nucleoprotein and anti-pandemic H1N1 nucleoproteins T cell responses. The association in anti-matrix protein T cell responses was slightly weaker than the association observed in anti-nucleoprotein T cell responses (Figure 4.6).

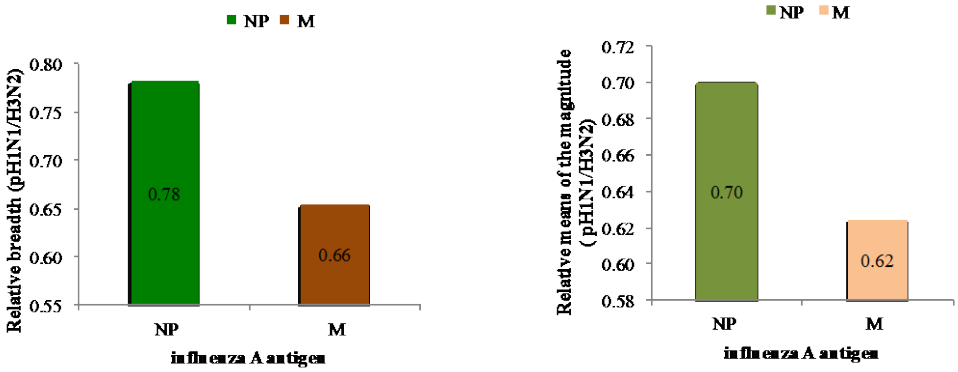
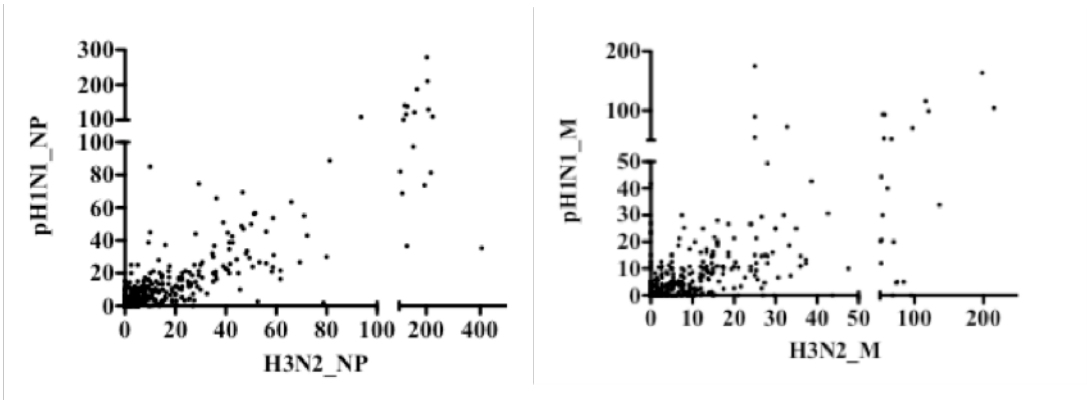


Figure 4.5: anti-NP and anti-M T cells were highly cross-reactive against pandemic H1N1 2009 virus

Left hand panel: Amongst H3N2 NP responders, 78% of them were able to recognized pandemic H1N1 2009 virus. Amongst H3N2 M responders, 66% of them were able to recognized pandemic H1N1 2009 virus. Right hand panel: the relative means of the magnitude of T cell responses. This is calculated amongst H3N2 NP and M responder as the magnitude of pH1N1/the magnitude of H3N2 for both NP and M specific T cell responses. The Abbreviation used: NP: nucleoprotein; M: matrix protein. ph1n1: pandemic H1N1 2009 virus.



Spearman r= 0.7819 (0.7399 to 0.8178),
P< 0.0001***

Spearman r= 0.6171 (0.5514 to 0.6752),
P< 0.0001***

Figure 4.6: Strong and significant correlations were observed between influenza specific (both NP specific and M specific) T cell responses against H3N2 and pandemic H1N1 (pH1N1)

All participants (N=411) had no detectable antibody against pandemic H1N1. T cell responses were measured by *ex vivo* human IFN-γ ELIspot assay. Non-parametric correlation analysis was performed (Spearman ranked correlation) using analytical software Prism. Background was subtracted at mean+4 standard deviation of no peptide controls. Abbreviation used: NP: nucleoprotein; M: Matrix protein.

4.3 Discussion

Previous studies have shown that circulating virus specific memory T cells determined by immediate cytokine secretion following direct *ex vivo* antigen stimulation declined gradually over time [278, 279] with more efficient maintenance in CD4+ T cell compartment than in CD8+ T cell compartment in humans [279, 280]. The half-life of influenza specific T cells was estimated at 2-3 years in a previous community study [277]. In the Flu Watch study, the durability of *ex vivo* influenza A (H3N2) specific T cells was assessed in the absence of detectable influenza infection, defined by a 4-fold sera antibody rise in hemagglutinin inhibition assay, and influenza vaccination (Figure 4.3). There was no significant reduction in *ex vivo* influenza A (H3N2) specific T cell responses from pre to post influenza season (between 5 to 8 months) at a cohort level (Figure 4.2a). High frequency *ex vivo* influenza A (H3N2) specific T cell responses, ≥100 spot forming cells per 106 PBMCs, had a overall trend of retaining their magnitude within the first 5-8 months then declining gradually with time with a half-live of around 1 to 1.5 years. The exact time of when these high frequency T cells was first generated prior to this study is not known, thus the half-life of circulating influenza specific T cells is at least to be 1 to 1.5 years. It is important to note that some fluctuations were detected and mainly in low responses that were below 100 spot forming cells per 106 PBMCs for total responses (Figure 4.2b, Figure 4.3), and below 40 spot forming cells per 106 PBMCs for responses against immunodominant influenza viral antigens of nucleoprotein and matrix protein (Figure 4.3). Approximately 60% of low responders exhibited a two-fold or more change in their magnitude of influenza A (H3N2) specific T cell responses as assessed in the paired comparison analysis of pre and post seasonal influenza A

(H3N2) specific T cell responses (Figure 4.2). These fluctuations could be the result of a mixture of factors. Various non–immunological factors that could introduce variations in PBMCs count that have been previously described, such as sleep deprivation [281] exercise, heat stress and hydration [282], which could result in approximately 12-15% of changes in lymphocyte count in humans. Also cross-reactivity between influenza A virus and unrelated virus such as hepatitis C virus have been described in humans [283]. These could boost the influenza A (H3N2) specific T cells, while other non cross-reactive antigens may proportionally reduce the frequency of influenza specific T cells [284-286]. It is very likely that low-level of T cell responses are more susceptible to minor technical variations. Caution should be exercised when interpreting findings supported by low frequency *ex vivo* antigen specific T cells, and further tertile analysis to compare high responses in the 1st tertile to low responses in the 3rd tertile would be advantageous compared to continuous variable analysis to minimise the effect of the fluctuation.

Seasonal H3N2 was first detected in the human population in 1968. Seasonal influenza H1N1 and H3N2 started to co-circulate from 1977. How these two viruses co-exist is not fully understood. It is suggested that evolution dynamics are different between these two influenza strains. Nucleotide sequence analysis of hundreds of human H3N2 and H1N1 isolates revealed more non-synonymous mutations were fixed in the HA globular site (HA1 gene) in H3N2 than H1N1 over the period of one to two decades[287, 288]. But some mutations in HA1 genes may be in receptor binding sites [289]. The difference in evolution dynamics could be a result of combination of factors including effective virus population size and host immunity selection pressure. In this chapter, the difference in T cell immunity against surface

antigens (hemagglutinin and neuraminidase) of H3N2 and H1N1 was investigated. T cell responses against surface antigens of H3N2 were higher than those against seasonal H1N1 (Wilcoxon matched-pairs signed rank test, P< 0.0001***). This suggested that the H3N2 was more immunogenic; this may reflect that H3N2 virus was more prevalent than H1N1 so that most of T cells in the UK community were more frequently exposed to the H3N2 subtype. The study of *ex vivo* pre-existing T cells against H3N2 has captured nearly all influenza specific T cell responses in the UK community.

The use of 'T cell' in this thesis refers to T cells expressing T cell receptors (TCRs) formed by an α chain and a β chain (αβ), which compose the majority of the T cells in the human immune system. A T cell response is initiated by T cell receptor recognition of an antigenic peptide-MHC complex. Theoretically, all peripheral T cells are thought to be cross-reactive, as they must be bound to at least one combination of self-peptide-MHC complex though at low affinity during positive selection in the thymus. The amino acid similarity could be a major component in T cell cross recognition[290]. Investigation of IFN-γ secreting CTL clones that recognized reference and mutant variants of HLA-A2 restricted HIV Gag epitope SLFNTVATL (SFL9) showed that the similarity in amino acid sequence correlated with the number of IFN-γ secreting cells measured by human IFN-ELISpot assay[290]. Thus, the high level of the T cell cross-reactivity against immunodominant internal proteins of NP and M observed in this study is likely to be due to the amino acid sequence homology, as these two antigens shared approximately 90% and 89% of amino acid homology, respectively, across different influenza strains (Table 4.1). This observation is also consistent with the previous

study, that T cells directed at nucleoprotein (NP) and matrix protein (M) were capable of mediating cross-reactive T cell responses against the serologically distinct influenza strains H5N1 [205]. The exact role of T cell immunity against these two highly cross-reactive immunodominant internal proteins of nucleoprotein (NP) and matrix protein (M) in natural influenza A infection were investigated and reported in the next chapter. Other mechanisms may also be involved in the T cell cross-reactivity. Experimentally, TCR cross recognition to peptides sharing structural and chemical features, known as molecular mimicry has been described. Molecular mimicry is the mechanism that sequence similarities between foreign and self-peptides are sufficient to result in cross-reactivity; which is a mechanism that may contribute to autoimmune disease. For example, a T cell clone from multiple sclerosis patients recognized peptides from both self-protein myelin basic protein (MPB) and Epstein-Barr virus (EBV) peptide and crystal structure revealed that these two peptides shared a marked degree of structural similarity on the surface presented for TCR recognition [291]. Further peptide stimulation assays showed that T cell clones could proliferate (^{3}[H] proliferation) [292, 293] and secrete cytokines of IL-2 and IL-3[294] in respond to peptides that do not necessarily share strong sequence homology. Alternative mechanisms to explain T cell cross-reactivity to peptides have little or no structural and chemical homology were identified and reviewed by *Yin and Mariuzza[295]* . The differential TCR docking was described by *Colf et al*. in crystal structure analysis of TCR binding to two unrelated peptides loaded on to two different mouse MHC molecules. The TCR global repositioning on the surface of peptide-MHC complex presented to TCR recognition allowed binding both shared and polymorphic residuals of the MHC molecule as well as the unrelated peptide[296]. Another mechanism, is known as induced fit, and is described using crystal structure

analysis of TCR binding to three distinct peptides loaded on to MHC H-2K. There were conformation changes in the flexible complementarity-determining region (CDR) loops permitted TCR to adapt structurally to different peptide-MHC complex. This adaptation was largely contributed by CDR3 loops with the largest shifts observed in CDR3 loops[297]. The mechanism of the Antigen-Dependent Tuning of Peptide-MHC Flexibility was also demonstrated. The human αβ TCR A6 recognized both HTLV-1 Tax peptide (LLFGYPVYV) and *Saccharomyces cerevisiae* peptide Tel1p (MLWGYLQYV) loaded on to HLA-A2 molecule. Crystallography demonstrated the substantial confirmation changes in the Tel1p-HLA-A2 peptide-MHC complex upon TCR binding, while Tax-HLA-A2 peptide-MHC complex was recognized without substantial adjustment[298]. The peptide-MHC conformational change upon TCR engagement could be the result of the flexibility in both peptide and MHC molecule that leads to different peptide dynamics in MHC binding grooves[298].

In summary, in the absence of natural influenza infection and vaccination, *ex vivo* influenza specific T cells remained largely at low frequency, total influenza (H3N2) specific T cell responses from 69% of participants were below 100 spot forming cells per 10^6 PBMCs and 77% of participants had T cell response below 40 spot forming cells per 10^6 PBMCs against immunodominant internal proteins of nucleoprotein (NP) and matrix protein (M). The influenza A (H3N2) specific T cells remained relatively stable over 5 to 8 months at population level. *Ex vivo* influenza A specific T cell responses that were above 100 spots forming cells per 10^6 PBMCs had a half-live of at least 1 to 1.5 years. Influenza specific T cells directed at immunodominant of internal protein of nucleoprotein (NP) and (M) exhibited high level of cross-

reactivity, and could be an ideal target for T cell inducing vaccine against serologically distinct influenza viruses.

Chapter 5: Assessing if sex and/or age may be confounders or modifiers

5.1 Introduction

Before the relationship between pre-existing T cells and influenza disease severity is assessed, it is essential to identify the potential factors that could confound and interact with this relationship so the appropriate statistical analysis can be implemented. Factors that satisfy the following criteria are to be considered as confounder: Factors associated with the outcome independent of the exposure (risk factor) under investigation and associated with the exposure under investigation in the source population. Factors also should not lie on the causal pathway between exposure and outcome. Confounders could inflate or undermine the association between exposure and outcome with the same magnitude and direction. If the effects differ in direction and/or magnitude then the factors may be modifiers. Gender and age of Flu Watch participants were available for the purpose of this thesis. In this chapter, investigations were carried out to assess if there were confounding and / or effect of modifications by age and/or sex. Statistical analysis was performed using STATA software.

The following steps were performed:

4.5.1 Examine if there were associations between *ex vivo* pre-existing influenza specific T cells and age, sex

4.5.1 Examine if there was association between disease outcomes of influenza infection and age, sex

4.5.1 Investigate if age and/ or sex confound or interact with the association between *ex vivo* pre-existing influenza specific T cell responses and influenza disease outcome, should the two criteria listed above are satisfied.

5.2 Results

5.2.1 Assessing if there were associations between *ex vivo* pre-existing influenza A (H3N2) specific T cell responses and age, sex

One of the most important biological changes to happen early in a human's life is puberty. Luteinizing hormone, a hormone that affects the function of the sex organs, is considered to be adequate in diagnosing puberty [299]. Serum luteinizing hormone of 0.2 international unit per litter indicated the start of puberty and the study of 307 (170 boys) children from randomly selected schools in the UK demonstrated that puberty could start to be detected at 9 year old in both sexes with over 99% of the children had started puberty by the age of 14 [300]. In this chapter, we divided the population in 5 years age group with first divisional point at the start of the puberty age of 9 years. Participants who were 70-years or over were grouped together to create a group of comparable size to the other adult groups (Figure 5.1).

There were some fluctuation in the total influenza A (H3N2) specific T cell responses between groups that were under the age of 50, but did not differ significantly (non-parametric one-way ANOVA: Kruskal-Wallis equality-of-populations rank test, chi-squared with ties =9.616 with 8 d.f., P= 0.2930). There was a significant decline in influenza A (H3N2) specific T cell responses in those who were 50-years old or over compared to those under this age (Two-sample Wilcoxon rank-sum (Mann-Whitney) test, p<0.00001***) (Figure 5.1). The decline in influenza A (H3N2) specific T cell responses in participants aged 50-years or over was contributed by T cell responses directed at both surface proteins (P=0.0006) and internal proteins (P<0.00001), which

included the reduction in T cell responses directed at immunodominant internal proteins of NP and M ($p<0.00001$ for both NP and M) (Figure 5.2).

Age group	minimum age	maximum age	Number of participants
0	5	8	25
1	9	14	108
2	15	19	102
3	20	24	59
4	25	29	71
5	30	34	97
6	35	39	141
7	40	44	176
8	45	49	178
9	50	54	182
10	55	59	226
11	60	64	294
12	65	69	114
13	70	93	165

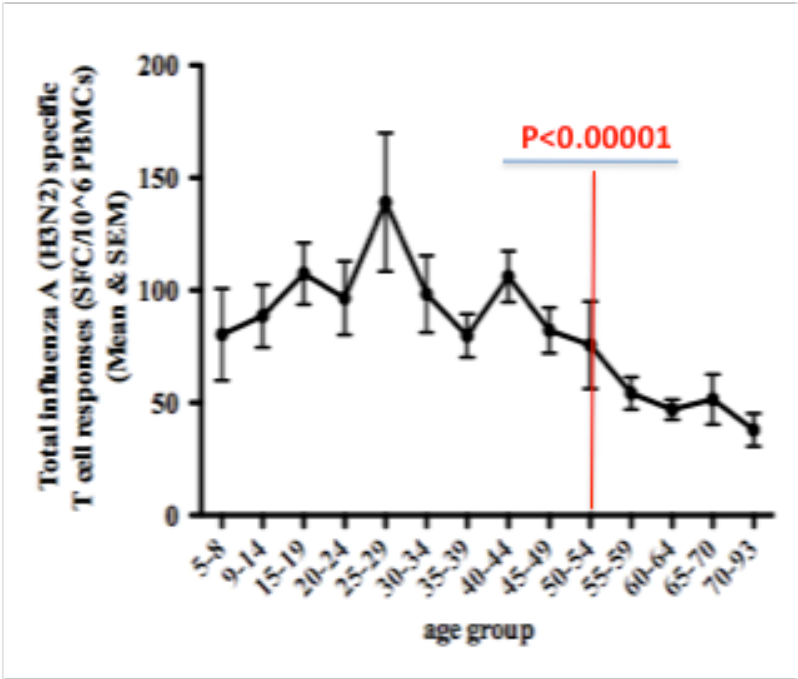
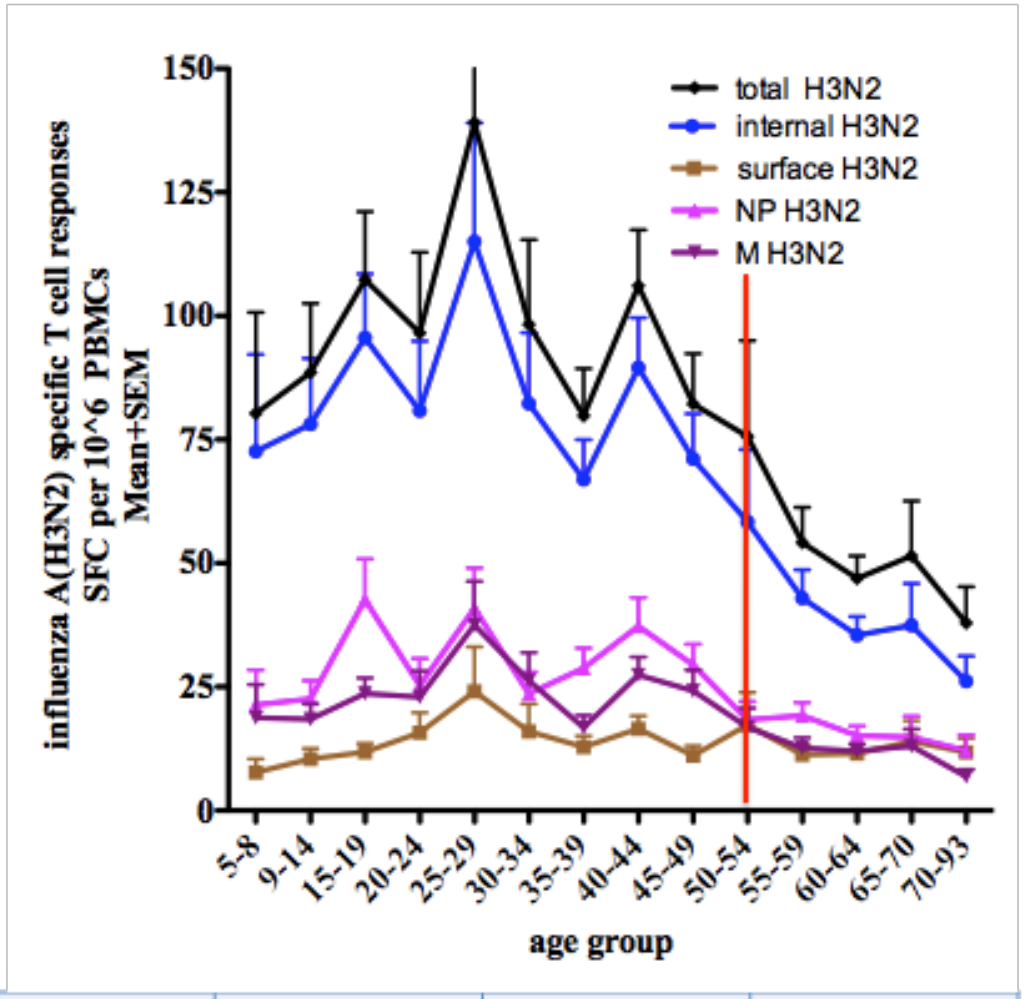


Figure 5.1: ***Ex vivo*** **pre-existing influenza A (H3N2) specific T cell responses declined after the age of 50**
Age related influenza A (H3N2) specific T cell responses were investigated. Left hand table: Participants were divided into 13 groups with the first divisional point at the start of the puberty, age of 9, then grouped with 5 years of age gap until the age of 70. All the 70-year or older participants were grouped together to create a group size that is comparable to the rest of adult groups. Right hand graph: There was no significant difference in influenza A (H3N2) specific T cell responses between the groups that were under the age of 50 (Kruskal-Wallis equality-of-populations rank test, chi-squared with ties =9.616 with 8 d.f., P= 0.2930). These T cell responses decreased in those aged 50 or over compared to those under 50 years old (Two-sample Wilcoxon rank-sum (Mann-Whitney) test, p<0.00001***). *Ex vivo* influenza A (H3N2) specific T cell responses were measured with human IFN-γ ELISpot assay, and data presented here were background subtracted at mean±4xstandard deviation of no peptide control wells. No participants (N=1938) had received an influenza vaccine at before the blood was taken.



Age group	minimum age	maximum age	Number of participants
Under 50 years	5	49	957
50 years or over	50	93	981

Figure 5.2: The age related decline in *ex vivo* influenza A (H3N2) specific T cell responses in participants aged 50-year or over was not antigen specific

Influenza A (H3N2) specific T cell responses directed at all antigens in participants aged 50 years or over was decreased. Two-sample Wilcoxon rank-sum (Mann-Whitney) test revealed, comparing T cell responses in participants age 50-year or over to those under the age of 50, a significant decrease in T cell responses directed at internal proteins ($P<0.00001$), surface proteins ($P=0.0006$) and also immunodominant internal proteins of nucleoprotein (NP) ($P<0.00001$) and matrix protein (M) ($P<0.00001$). Participants (N=1938) had not received influenza vaccination and data presented here was background subtracted at mean+4 x standard deviation of no peptide controls. *ex vivo* influenza A (H3N2) specific T cell responses were measured by human IFN-γ ELISpot Assay. SEM: standard error of mean. SFC: spots forming cells.

The association between *ex vivo* pre-existing influenza A (H3N2) specific T cell responses and gender was assessed. The group sizes of male and female were similar (Figure 5.3). Overall, *ex vivo* pre-existing influenza A (H3N2) specific T cell responses were significantly lower in female participants than those in male participants (Two-sample Wilcoxon rank-sum (Mann-Whitney, P=0.0003). There was no difference in either baseline influenza A specific antibodies (Figure 5.4) or the influenza infection rate between the male and females (Table 5.1), suggesting that the higher frequency of circulating influenza A (H3N2) specific T cells in male was not a result of higher incidence of influenza A infection.

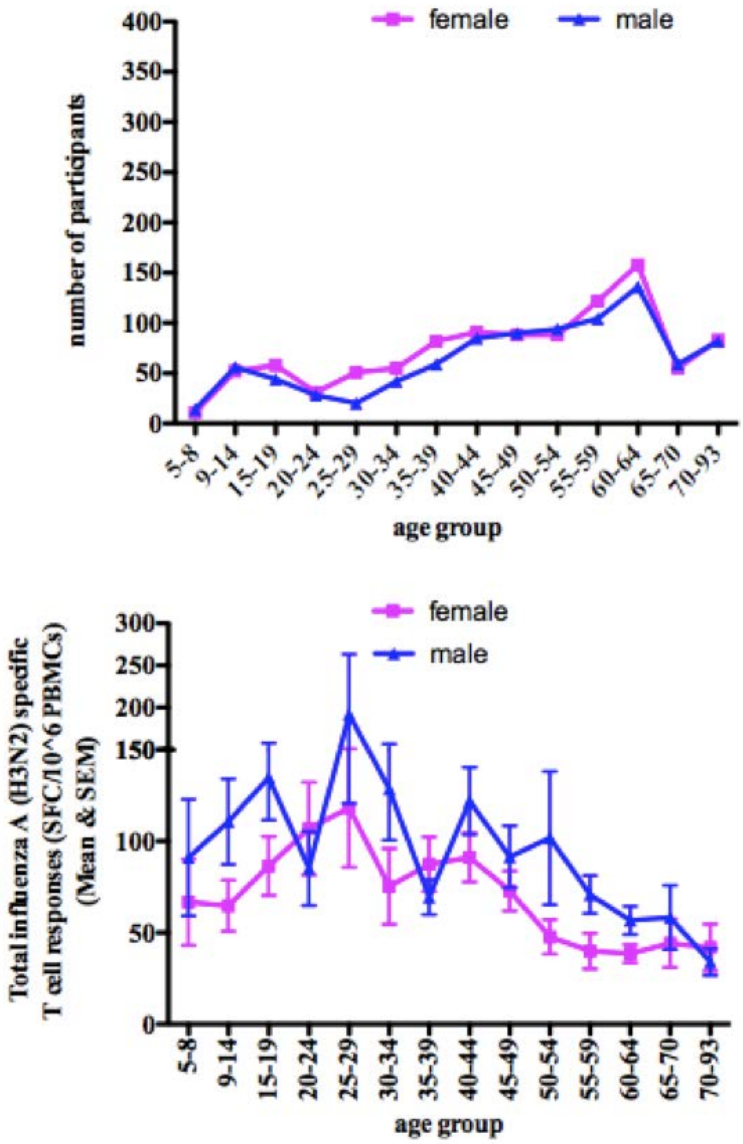


Figure 5.3: *Ex vivo* pre-existing influenza A (H3N2) specific T cell responses were lower in female participants compared to male participants

Top graph: numbers of female and male participants in each age group were similar. Bottom graph: Overall, *ex vivo* pre-existing influenza A (H3N2) specific T cell responses were significantly lower in female participants than those in male participants (Two-sample Wilcoxon rank-sum (Mann-Whitney, P=0.0003). Participants (N=1938) had not received influenza vaccination. *Ex vivo* pre-existing influenza A (H3N2) specific T cell responses were measured by human IFN-γ ELISpot assay and were background subtracted at mean+ 4 standard deviation of no peptide controls. SEM: standard error of mean. SFC: spots forming cells.

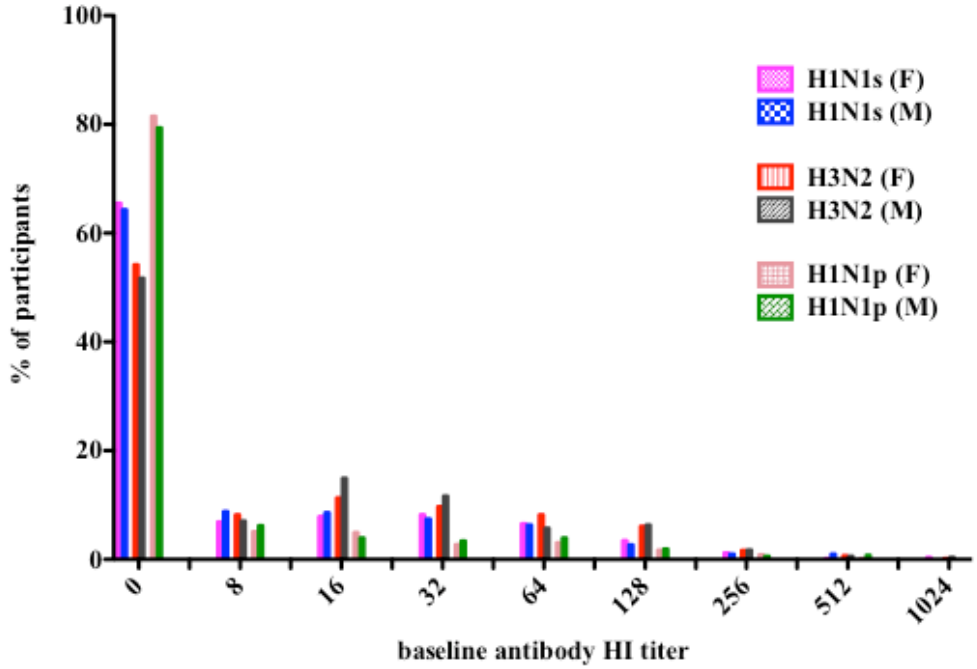


Figure 5.4: There was no difference in the baseline influenza A specific antibodies between female and male participants

Influenza A specific antibody against different subtypes was measured using Haemagglutination Inhibition (HI) Assay. Participants (N=1938) had not received influenza vaccination before the baseline blood was taken (before the influenza season). H1N1s: H1N1 seasonal influenza. H1N1p: pandemic H1N1 2009. F: female. M: male.

Table 5.1: The influenza infection rates did not differ between females and males

	sex		
	female	**male**	**Total**
influenza infection (seroconversion)			
no	695	627	1,322
	84.86%	87.08%	85.90%
yes	124	93	217
	15.14%	12.92%	14.10%
Total	819	720	1,539
	100%	100%	100%
Pearson chi2(1) = 1.5644 Pr = 0.211			

Seroconversion: a 4-fold rise in influenza specific antibodies from pre to post influenza season measured by Haemagglutination Inhibition (HI) Assay. All participants (N=1539) had paired sera from pre and post influenza season and had not received influenza vaccination before the baseline and between the baseline and follow up bleed. Chi square test was performing using STATA analytical software.

5.2.2 Assessing if the age and gender influenced the disease severity if infected with influenza

Participants who met the following criteria were subsequently included in the analysis to investigate the role of *ex vivo* pre-existing influenza A (H3N2) specific T cell responses in the disease severity of natural influenza infection:

1 Had not received influenza vaccination before the baseline bleed or between the baseline and follow up bleed

2 Had paired sera (serum samples from before and after influenza season), therefore influenza infection could be identified by seroconversion (a 4-fold rise in influenza specific antibody titer from pre to post influenza season measured by the haemagglutination inhibition assay).

3 Had no detectable influenza serotype specific antibody that was against the serotype they were infected with.

Over the period of 4 years, 195 participants from the Flu Watch study had met the above criteria, 43 of whom were infected with pandemic H1N1 2009 and 152 were infected with seasonal influenza (H3N2 and /or H1N1).

Only 43 participants were included in the pandemic study, which is too few to investigate the effect of age and sex. The effect of sex and age were investigated for seasonal influenza infection. As demonstrated in the previous section, age and sex were associated with the magnitude of *ex vivo* pre-existing influenza A (H3N2) specific T cell responses, and this suggested that age stratums of dividing participants into under 50-years old and 50-years or older would be appropriate. The sample size from each of these stratums is shown in Table 5.2. Logistic regression analysis was carried out to assess whether age and sex associated with the influenza disease severity if infected with seasonal influenza. The logistic regression analysis showed

there was little evidence that age and sex associated with the disease severity (Table 5.3). In addition, the number of participants in some of the stratums was small (below 10), which is not suitable for statistical modeling, suggesting this study is not powered to study the confounding and/or the effect of modifications. Therefore, stratification by age and sex will not be considered for subsequent analysis

Table 5.2: Clinical outcomes of seasonal influenza infection in the absence of serotype specific antibodies stratified by age and sex

	Female		Male	
	Under 50-year	50-year or older	Under 50-year	50-year or older
Clinical symptom scores				
0	24	18	14	8
1	5	6	4	4
2	13	8	10	5
3	15	5	7	6
*PCR+				
no	51	30	28	20
yes	6	7	7	3

*PCR+: Influenza virus was detected in nasal swab by PCR detection
All participants (N=152) were infected with either or both seasonal influenza H1N1 and H3N2 and had no detectable influenza A specific antibody against the subtype they were infected with. Influenza infections were defined by seroconversion (a 4-fold rise in influenza specific antibody titers from pre to post influenza season measured by Hemagglutinin Inhibition Assay).

Table 5.3: There was little evidence that age and sex associated with the disease severity if infected with seasonal influenza virus

Ordered logistic regression			[1] Number of obs	=	152	
			[2] LR chi2(2)	=	0.96	
			[3] Prob > chi2	=	0.6188	
Log likelihood = -196.64551			Pseudo R2	=	0.0024	
[4] symptom scores	Odds Ratio	[7] Std. Err.	z	[8]P>z	[95% Conf.	Interval]
[5] 50.age50	0.7992467	0.2420104	-0.74	0.459	0.441508	1.446848
[6] 1.sex	1.209791	0.3673014	0.63	0.53	0.6672355	2.193519

Logistic regression			Number of obs	=	152	
			LR chi2(2)	=	0.5	
			Prob > chi2	=	0.7787	
Log likelihood = -64.347523			Pseudo R2	=	0.0039	
[9] Nasal PCR+	Odds Ratio	Std. Err.	z	P>z	[95% Conf.	Interval]
50.age50	1.214961	0.5568181	0.42	0.671	0.4948298	2.983109
1.sex	1.297737	0.5952091	0.57	0.57	0.5281797	3.188538

[1]All participants (N=152) were infected with seasonal influenza of H3N2 and/or H1N1 serotypes and no detectable antibody against the influenza serotypes they were infected with at baseline. Influenza infection was defined by seroconversion (a 4-fold rise in influenza specific antibodies from pre to post influenza season measured by Hemagglutinin Inhibition Assay.
[2]LR chi2(2): Likelihood ratio chi-square test with 2 degree of freedom
[3]Prob > chi2: probability of obtaining LR chi-square, if there is in fact no effect of the independent variables, P<0.05 indicates there is statistically significant influence from predictors (in this case, age and sex are the predictors);
[4]Symptom scores: 0= no symptom, 1= any respiratory illness, 2= influenza like illness with no raised temperature but feeling feverish. 3= influenza like illness with confirmed fever.
[5]age50: age group categorized into under 50-year and 50-year or over which was labeled as 50.
[6]Sex: female=0, male=1.
[7]Std. Err.: the standard errors associated with odd ratio.
[8]P>z: 2-tailed p-value, a value of less than 0.05 could indicate a predictor has influence on the outcome.
[9]nasal PCR+: nasal swabs contain influenza virus, detected by polymerase chain reaction.
Statistical analysis was performed using analytical software STATA.

5.3 Discussion

The frequency of influenza A (H3N2) specific T cells in blood circulation was measured by *ex vivo* human IFN-γ ELISpot assay before the start of influenza season. No participants had received influenza vaccination before the blood was taken. The magnitude of these *ex vivo* pre-existing influenza A (H3N2) specific T cell responses did not differ in participants aged between 5 to 49 years old, but decreased in participants aged 50-years or older when compared to those from participants aged under the age of 50. The reduction in *ex vivo* influenza A (H3N2) specific T cell responses was seen in both T cells directed at variable influenza surface antigens and conserved internal proteins including immunodominant and non-immunodominant internal proteins. This finding is consistent with a previous study in a small cohort of 85 unvaccinated participants that *ex vivo* influenza A (H3N2) specific T cell responses measured by human IFN-γ ELISpot assay decreased with age and the decrease was seen in T cell responses against all influenza proteins [258]. This suggests that aging had significant influence on the T cell immunity against influenza. The term of immune senescence is collectively used to describe the complex changes involved in the aging of immune system. (Reviewed in reference [301-303]). Immune senescence at the level of cell population stems from primary changes in T cell production that stems from thymic involution. Thymuses from 204 humans who had suffered sudden death, aged between 1 month to 107 years, were studied using stereological and morphometrical methods. The silver impregnation technique showed that thymus atrophy started from the first year to the end of life. The volumes of the thymic epithelium (cortex and medulla) space in which thymopoiesis occurs continuously decreased with age [304]. Hematoxylin and eosin staining revealed,

between ages 1 and 50 years, the thymic epithelial space decreased to approximately 10% of its original volume[305]. A study of naïve T cell output from thymus in mice showed there was over 90% of reduction of FITC labeled thymocytes exported from thymus in mice age 6 months compared to mice aged 3.5 to 5.5 weeks old [306]. Homeostatic proliferation refers to the process of spontaneous T cell proliferation in a lymphopenic host. In response to reduced T cell output from thymus, in pathogen free condition, naïve T cells underwent homeostatic proliferation to maintain naïve T cell population in periphery. Adoptive transfer of naïve CD8+ T cells to mice that had no mature B and T cells (RAG 1-/- mice) and irradiated mice showed that naïve CD8+ T cells underwent increased homeostatic cycling, from approximately once every month to four or more divisions per month similar to the level that was seen in the T cell memory pool. And naïve T cells acquired a memory like phenotype during homeostatic driven proliferation by expressing high levels of CD44, Ly-6C, IL-2Rβ, and LFA-1, and producing IFN-γ within 8 h of anti-CD3ε stimulation[307, 308]. While homeostatic proliferation of naïve T cells seem to have crucial influence on the T cell aging in laboratory mice kept under pathogen free condition, clonal expansion of antigen specific T cells provide another level of influence in pathogen-exposure mammals. The diminished functional repertoire of the immune system at older age may be the result of expansion of certain T cell clones at the expense of others. For example, T cell responses to human cytomegalovirus (HCMV) from 33 HCMV seropositive and 10 seronegative participants of diversed HLA background were studied by flow cytometry analysis. All participants had no HCMV viremia detectable by real-time PCR. T cell responses to 13,687 overlapping peptides (15mer overlapping by 10 amino acid) spanning 213 predicted HCMV proteins were measured by flow cytometry. The human cytomegalovirus virus (HCMV) induced

broad CD8+ T cell responses, Of 213 HCMV proteins tested, 151 proteins elicited CD4+ and/or CD8+ T cell responses in seropositive participants and on average, about 10% of circulating memory T cells in blood were HCMV specific T cells. On the contrary, in the vast majority tests (100% of CD4 and 99% of CD8), seronegative participants had no detectable HCMV specific T cells. This demonstrated the skewing of the T cell receptor repertoire through repeated antigen stimulation by chronic infection [309]. The combination of the thymic involution with marked reduction in naïve T cell production and age related changes in memory T cell pool described above may provide explanation of the age related decrease in *ex vivo* influenza A (H3N2) specific T cell responses in the Flu Watch study.

The impact of repeated antigen stimulation on the antigen specific memory T cell pool could explain the age-related reduction in *ex vivo* influenza A (H3N2) T cell responses. Repeated antigen stimulation may result in T cell exhaustion and lost of essential functional activity. As described in HIV study, PD-1 (the inhibitory receptor programmed death 1) a marker of exhaustion was associated with HIV specific T cell exhaustion. The blockage of the PD-1/PD-L1 pathway could significantly increase the expansion of HIV specific CD8+ T cells [310]. For reference, a study of mice infected acutely and chronically with lymphocytic choriomeningitis virus (LCMV) showed that, comparing to acute infection, repeated antigen stimulation by chronic infection induced upregulation of PD-1 (the inhibitory receptor programmed death 1) mRNA expression and the loss of IFN-γ production in virus specific CD8+ T cells [311]. The loss of capacity for replication as a result of telomere erosion and/or unrepaired DNA damage was also known to result from repeated antigen stimulation, this is known as replicative senescence[312]. As for influenza specific T cells, study

of the *ex vivo* frequency of influenza M1-specific T cells using M1 tetramer revealed that the frequency of M1-specific T cells in the elderly (aged 67-88 years) was similar to those in the young (aged 21 to 35 years), but the frequency of IFN-γ secreting M1-specific T cells in the elderly was 48% of less than those in the young and with reduced replicative capacity when stimulated with influenza virus *in vitro*. [313].

The effect of sex on the *ex vivo* frequency of influenza A (H3N2) specific T cells measured by human IFN-γ ELISpot assay was also assessed. *ex vivo* pre-existing influenza A (H3N2) specific T cell responses detected in male participants were higher than those detected in female participants. There are many factors that could influence the size of the antigen specific memory T cell pool such as risk of antigen encounter, precursor frequency of influenza specific T cells, antigen dose, burst size of clonal expansion and the effect of sex hormones on the function of antigen specific T cells. Our data suggested that the risk of contracting influenza infection was unlikely to be the cause of the difference in *ex vivo* pre-existing influenza A (H3N2) specific T cells as there was no difference in baseline influenza specific antibody titers between males and females, and the influenza incidence rate in males was similar to females. The difference in influenza specific T cells between male to female was unlikely due to amount of T cells in blood circulation as previous studies have suggested that, although there was no difference in the CD4+ to CD8+ T cell ratio between two sexes, the number of circulating T cells in peripheral blood was higher in females than males. This was described in a study of leukocyte counts in 15 healthy women aged 26 to 37 years and 20 healthy men aged 27 to 45 years old. It showed that the total number of lymphocyte in males was similar to females but the number of CD3+ T lymphocytes in blood circulation was lower in males than females

with no difference in the ratio of T helper cells (CD8-CD3+) to cytotoxic T cells (CD8+CD3+) [314]. A study of 10 male to female (aged 20 to 41years) and 10 female to male treated Caucasian transsexuals (aged 18 to 40 years) confirmed that sex hormones had no impact on the total number of lymphocytes and the CD3+CD4 to CD3+CD8+ T cell ratio. Four months after cross sex hormone administrations, there was no change in the total number of lymphocytes and the CD4/CD8 T cell ratio [315]. In a larger study of 468 healthy blood donors (263 males, mean age 41.2±14.9 for male and 39.6±15.9 for female), it was reported that the number of CD8+ T cells per cubic millimeter was similar in males and females, but the number of CD4+ T cells was significantly less in males than those in females, and further analysis of family members suggested that both genetic and environmental factors can influence the CD4/CD8 ratio [316]. While there was no evidence that the sex hormone influenced the frequency of circulating T cells, the study in a mouse model reported that the sex hormone influenced the function of antigen specific T cells. In mice intracranially infected with lymphocytic choriomeningitis virus (LCMV), *in vitro* ^{51}Cr release assay showed that the addition of estrogen at female physiological concentration could enhance the cytotoxicity of LCMV specific CTL from CD8 deficient (β_2m-/-) male mice[317]. At day 8 after infection, the percentage, also the absolute number of LCMV tetramer positive CD4+ T cells in cervical lymph node and brain were significantly less in β_2m-/- males than in β_2m-/-females but no sex difference in immune cells was detected beyond day 8. And after orchidectomy, mice with unaffected estrogen levels and approximately three-fold reduction in testosterone generated increased LCMV tetramer-positive cells in cervical lymph node to a level that was 2-3 fold higher than those mice that underwent sham surgery [318]. Cervical lymph nodes are the draining lymph nodes for the central nervous system (CNS), it

was possible the T cell priming was less efficient in males than in females. The increased frequency of influenza specific T cells in males than those found in females observed in our Flu Watch study may be the results of the functional differences in the ability of T cell eliminating influenza virus infection, which may lead to a difference in infection resolution and the burst size of the clonal expansion, which in turn could affect the influenza specific memory T cell pool. Thus, further studies that investigate the effect of sex hormones on the human T cell proliferation, cytotoxicity and survival of clonal expansion after the resolution of influenza infection would contribute to our understanding of the sex related difference in the T cell immunity observed in this study. It was not possible to rule out the effects of other environmental factors such as personal hygiene that could introduce the variation in the initial antigen dose and difference in susceptibility to other infectious agents, which could skew the memory T cell pools.

In the Flu Watch study, there was little evidence that age or sex associated with the influenza disease severity and nasal viral shedding, although an association may exist if the sample size is larger. However, that was beyond the power of detection in this study, therefore stratification by age and sex were not considered in the subsequent analysis.

In summary, age related decrease in the *ex vivo* frequency of influenza specific T cells was observed with a significant reduction seen in those 50-years or older compared to those who were under the age of 50. The age related decrease was seen in T cell responses against all influenza antigens, may suggest that there is an overall reduction in the frequency of influenza specific memory T cells in blood circulation. There were sex related differences in *ex vivo* pre-existing influenza specific T cells, overall,

the *ex vivo* frequency of influenza A specific T cells in males was higher than females. Further study of the functional difference in these influenza specific T cells would provide valuable information in understanding the sex related difference in the influenza T cell immunity. However, there was little evidence that the sex and age associated with the disease outcomes in this study, and the further stratification on age and sex yield stratums with samples size less than 10 which would not be suitable for multi-level statistical modeling, suggesting this study was not powered to study any confounding and/or effect of modification by age and sex. Therefore no further stratifications on age and sex were considered when investigating the relationship between pre-existing influenza A specific T cells and the disease outcome of natural influenza A infection in the next chapter.

Chapter 6: The role of T cell immunity in natural influenza A infection

6.1 Introduction

Studies of the burden of natural influenza A infection in the UK has heavily relied on data generated through sentinel surveillance schemes through primary care such as Royal College of General Practitioners (RCGP) Research and Surveillance Centre, which is a network of approximately 100 participating GPs well distributed throughout England & Wales, and the Scottish national surveillance system (SISRS) which includes aggregated data from almost all GP practices in Scotland. In addition, the HPA QSurveillance clinical surveillance system collects data from 3,400 general practices spread throughout the UK. Identification of influenza cases through these surveillance networks are based on self-reporting of clinical symptoms. Clinical signs and symptoms of influenza infection are similar to other viral infections such as rhinovirus or coronavirus infection [319] and not all influenza infections present symptoms[94]. Therefore, it has not been possible to accurately estimate the influenza burden in the UK. The identification of true natural influenza infection with or without symptoms will be valuable in assessing the disease severity of seasonal influenza and of the 2009 H1N1 pandemic influenza. Also, the accurate identification of natural influenza infection would enable the study of the immune correlate of protection conferred by T cell immunity against symptoms.

The role of T cell immunity in controlling influenza infection have previously been investigated mostly in challenge studies as discussed in chapter I section 1.10.2. This is the first comprehensive study to address the question whether T cell immunity confers protection against natural influenza infection in humans. In the absence of serotype specific antibody, the role of *ex vivo* pre-existing influenza A (H3N2)

specific T cells in controlling same and different serotypes of seasonal influenza and pandemic influenza infection were investigated in this chapter.

6.2 Results

6.2.1 The influenza burden in the UK and the specificity of clinical signs and symptoms

To investigate the immune correlate of protection conferred by *ex vivo* pre-existing influenza specific T cells, accurate identification of influenza infection with and without symptoms was required. The burden of influenza infection in the UK was investigated in this study. In total, 1,539 out of 2,395 randomly selected participants were analyzed. These participants had not received influenza vaccination before the baseline bleed or between the baseline and follow up bleed. These participants also had paired sera from pre and post influenza season. The Hemagglutinin Inhibition (HI) assay is an important diagnostic approach for influenza infection when it is not possible to carry out virus isolation and is the test of choice for WHO global influenza surveillance. The Flu Watch followed WHO guidelines and influenza infection was defined as a 4-fold HI titer rise from pre to post influenza season; this is also known as seroconversion. The HI assay was performed by the respiratory unit at the Health Protection Agency, UK; The clinical symptoms were closed monitored and recorded by the Department of Primary Care and Population sciences at the University College of London.

In the Flu Watch cohort, approximately 14% of the 1,539 participants (217 participants) seroconverted. A small proportion of infected participants were infected with more than one subtypes of influenza virus (Table 6.1). The vast majority (over 80%) of influenza-infected individuals had no detectable baseline serotype specific antibody against the influenza subtype with which they were infected. The influenza

serotype specific antibody was also measured by haemagglutinin inhibition assay. Eighty three percent (H3N2), 87% (seasonal H1N1) and 88% (pandemic H1N1) of those infected had no pre-existing serotype specific antibody (Figure 6.1).

Table 6.1: Flu Watch participants infected with more than one subtypes

*Subtypes of influenza infection	Season 1	Season 2	Season 3
Seasonal H1N1 and H3N2	*2 (4%)	6 (13%)	4 (6%)
Seasonal H3N2 and pandemic H1N1			1 (1.4%)

* Influenza infection was defined by seroconversion—a 4-fold rise in influenza serotype specific antibody from pre to post influenza season measured by haemagglutinin inhibition assay.

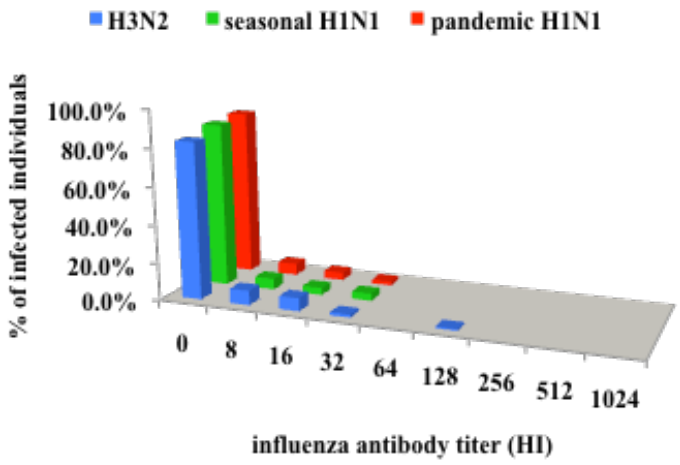
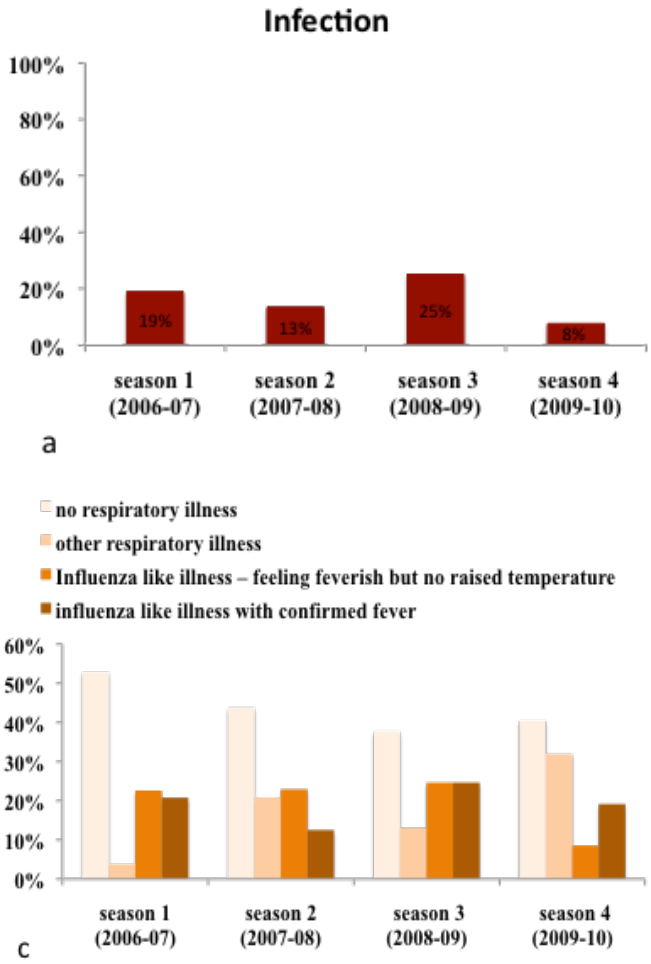


Figure 6.1: Over 80% of participants infected were seronegative
Influenza antibody titer was measured at baseline by haemagglutinin inhibition assay. Starting dilution was at 1/8 and subsequent 2 fold dilutions were made.

Over the period of the Flu Watch study, annual influenza infection rate ranged from 8% to 25% with the pandemic H1N1 2009 giving the lowest infection rate at 8% (Figure 6.2a). The predominant circulating strains alternated between seasonal H3N2 and H1N1 during the first 3 years (Table 1.1). There were no seasonal H3N2 and H1N1 antibody titers available for year 4 at the time of writing this thesis and therefore all participants were tested for infections with pandemic H1N1 2009 virus only. However, only 2.5% of 3059 influenza virus isolates detected by the Respiratory Virus Unit of the Health Protection Agency was non-pandemic H1N1 2009 viruses from mid-May 2009 to mid-May 2010[320]. Therefore, it was very likely that vast majority of infections in season 4 were caused by pandemic H1N1 2009 virus. A considerable proportion of influenza infection did not present any illness. The asymptomatic infection rate amongst influenza infected individuals ranged from 38% to 53% in each influenza season (Figure 6.2).

The specificity of clinical symptoms presented by influenza-infected individuals was assessed. The influenza like illness with confirmed fever was equivalent to the influenza like illness (ILI) defined by Center of Disease Control, which is sudden onset of fever >38°C and cough and/or sore throat in the absence of a known cause other than influenza. The proportion of individuals presenting various symptoms with or without influenza A infection was presented in Table 6.2. A considerable proportion of individuals (over 42%) with no detectable influenza infection reported various symptoms. Only small proportion of influenza like illness recorded over the period of influenza in circulation was contributed by seroconverted influenza-infected individuals. The attributable risk of influenza like illness with confirmed fever by natural influenza infection was less than 14% and the influenza like illness with

confirmed fever (same as CDC definition) was the only combination that was associated with natural influenza A infection (Table 6.2, Pearson Chi-square=43.8022, P<0.001). Therefore, this determination will be used in the subsequent analysis to investigate the role of *ex vivo* pre-existing influenza A specific T cells in protection against clinical outcomes.



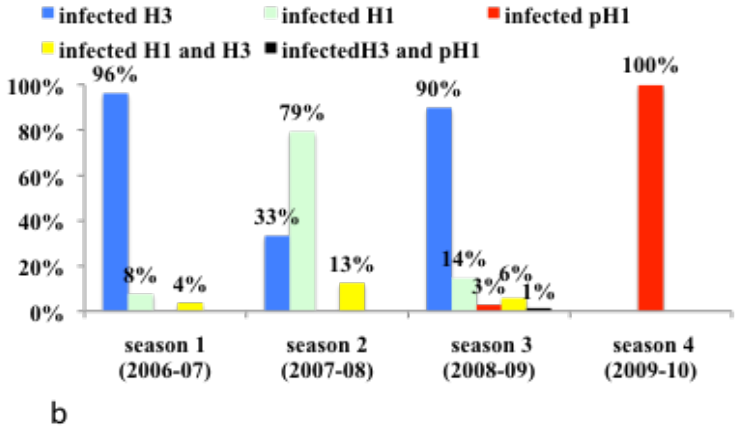


Figure 6.2: Influenza infection rates in seasonal and pandemic influenza and the disease severity

In total, there were 1539 participants who had paired sera and did not receive influenza vaccination either before the baseline or between the baseline and the follow up bleed. Paired sera from these participants were analyzed. Pre and post seasonal antibody titers were measured by Hemagglutinin Inhibition (HI) Assay. A 4-fold rise in HI titer from pre to post influenza season was defined as infection. **a.** Influenza infection rate by season. **b.** Proportion of influenza infections caused by different subtypes each year (N=217) **c.** Clinical symptoms from those who were infected with influenza viruses. N=281 (season 1), N=358 (season 2), N=276 (season3), N=624 (season4). Abbreviations used: H1: seasonal H1N1; H3: seasonal H3N2; pH1: pandemic H1N1 2009

Table 6.2: Influenza like illness with confirmed fever was associated with natural influenza infection

	Infected (seroconversion)		Attributable risk	Significance?
	No	Yes		
	764	94		
no respiratory illness	**57.79%**	**43.32%**	**-14.47%**	
	253	36		
other respiratory illness	**19.14%**	**16.59%**	**-2.55%**	
Influenza like illness – feeling feverish but no raised temperature	220	44		**Pearson chi2(1) = 1.7331**
	16.64%	**20.28%**	**3.64%**	**Pr = 0.188**
influenza like illness with confirmed fever	85	43		**Pearson chi2(1) = 43.8022**
	6.43%	**19.82%**	**13.39%**	**Pr < 0.001**
	1,322	217		
Total	**100%**	**100%**		

Pr: probability

6.2.2 Investigating the role of *ex vivo* pre-existing influenza A (H3N2) specific T cells in protection against symptoms and nasal viral shedding in natural seasonal and pandemic influenza infection

Ex vivo pre-existing influenza specific T cells can be readily detected in human peripheral blood as demonstrated in previous chapter. This is the first study to investigate whether these circulating influenza specific T cells correlated with protection against natural influenza infection. If so, stimulation of T cell immune responses will have potential to complement antibody inducing vaccines to improve the protection against natural influenza infection with or without antigenic drift and shift in humans.

The majority (over 80%) of influenza A infected individuals had no detectable serotype specific antibody against the subtype with which they were infected. These seronegative influenza infected participants were then analyzed for the role of their T cell immunity in protection against symptoms. Participants were divided into tertiles according to their *ex vivo* pre-existing influenza A (H3N2) specific T cell responses using analytical software STATA. The unequal group size was the result of various degrees of ties within each tertile (ties are the numbers of data points that have equal value, it is the cluster of data). The immune correlates of protection against symptoms and nasal viral shedding of influenza virus (influenza virus was detected in nasal swab by PCR), conferred by T cells, was assessed by comparing symptomatic cases between top and bottom tertiles. Dividing the participants into tertiles for analysis was to minimize the effect of natural fluctuation in low frequency influenza specific T cells as discussed in chapter 4.

6.2.2.1 Investigating the role of *ex vivo* pre-existing influenza A (H3N2) specific T cell immunity in protection against symptoms and nasal viral shedding if infected with pandemic influenza

The role of *ex vivo* pre-existing influenza A (H3N2) specific T cells in natural pandemic influenza infection was investigated. In total, there were 43 participants infected with pandemic H1N1 2009 influenza virus had no detectable pre-existing antibodies against this pandemic strain, they were also not vaccinated before the baseline bleed or between the baseline and follow up bleed. The *ex vivo* frequency of cross-reactive T cells against pandemic H1N1 2009 nucleoprotein and matrix protein were determined in 22 out of 43 pandemic H1N1 infected participants. T cell responses against the whole proteome of H3N2, and T cell responses directed at nucleoprotein and matrix protein of pandemic H1N1 2009 were investigated (N=43). Consistently, total H3N2 specific T cells (Table 6.3), NP specific T cells (Table 6.4) and M specific T cells (Table 6.6) (Table 6.7) showed a trend towards protection against influenza like illness with confirmed fever and nasal viral shedding (nasal swab PCR+ for influenza); this was evident in the decreased rate of influenza like illness with confirmed fever and nasal swab PCR+ in the 3rd tertile (responders with higher frequency of influenza specific T cells) compared to the 1st tertile (responders with lower frequency of influenza specific T cells). The immune correlate of protection against nasal viral shedding conferred by NP specific T cells (Table 6.4) (Table 6.5) was statistically significant (one-sided Fisher's exact test P=0.043 for anti-H3N2 NP and P=0.044 for cross-reactive anti-pandemic NP), and those infected individuals who shedding virus were all in the 1st tertile (with low influenza specific T cells).

Table 6.3: Immune correlate of protection conferred by total *ex vivo* pre-existing H3N2 specific T cells against pandemic H1N1 2009

Tertile	Number of participants	Minimum T cell responses (SFC/M)	Maximum T cell responses (SFC/M)
1	18	0	10
2	11	10.667	33.681
3	14	35	893.666

a.

Tertile	Influenza like illness with confirmed fever		Total
	0	1	
1	12 (67%)	6 (33%)	18
2	10 (91%)	1 (9%)	11
3	12 (86%)	2 (14%)	14
Total	34 (79%	9 (21%)	43
Compare 1st and 3rd tertile			
1-sided Fisher's exact=0.207			

b.

Tertile	PCR+ for influenza		Total
	0	1	
1	15 (83%)	3 (17%)	18
2	9 (82%)	2 (18%)	11
3	13 (93%)	1 (7%)	14
Total	37 (86%)	6 (14%)	43
Compare 1st and 3rd tertile			
1-sided Fisher's exact=0.403			

c.

a. The number of participants and the range of T cell responses within each tertile
b. The number of participants presented influenza like illness with confirmed fever within each tertile.
c. The number of participants who had nasal swabs that contained influenza virus, detected by polymerase chain reaction (PCR).
0=No, 1=Yes.
All 43 participants were infected with pandemic H1N1 2009, and had no detectable antibody specific for pandemic H1N1 2009. Infection was defined by sera-conversion of a 4-fold rise in HI titer against H1N1 2009 from pre to post influenza season. Participants were divided into tertiles based on their total pre-existing *ex vivo* H3N2 specific T cell responses that were measured by human IFN-γ ELISpot Assay. The unequal group size was the result of ties within each tertile. Analytical software STATA was used to create tertiles and carry out Fisher's exact tests. All data was background subtracted at mean+ 4 standard deviations of no peptide controls then applied the statistical cutoff of 4 SFC/M. SFC/M: spot forming cells per 10^6 PBMCs. HI: Hemagglutinin Inhibition. PCR+: influenza virus was detected in nasal swab by polymerase Chain Reaction (PCR). Ties: data points had equal value.
Though it was no statistically significant, there was a trend that those with higher frequency of H3N2 specific T cells were protected from influenza like illness with confirmed fever and nasal swab PCR+ for influenza; the rate of influenza like illness with confirmed fever and nasal swab PCR+ was lower in the 3rd tertile than in the 1st tertile.

Table 6.4: Immune correlate of protection conferred by *ex vivo* pre-existing H3N2 NP specific T cells against pandemic H1N1 2009

Tertile	Number of participants	Minimum T cell responses (SFC/M)	Maximum T cell responses (SFC/M)
1	23	0	0
2	6	5	6.667
3	14	7.5	50

a.

Tertile	Influenza like illness with confirmed fever		Total
	0	1	
1	17 (74%)	6 (26%)	23
2	5 (83%)	1 (17%)	6
3	12 (86%)	2 (14%)	14
Total	34 (79%)	9 (21%)	43
Compare 1st and 3rd tertile			
1-sided Fisher's exact=0.34			

b.

Tertile	PCR+ for influenza		Total
	0	1	
1	17 (74%)	6 (26%)	23
2	6 (100%)	0 (0%)	6
3	14 (100%)	0 (0%)	14
Total	37 (86%)	6 (14%)	43
Compare 1st and 3rd tertile			
1-sided Fisher's exact=0.043			

c.

a. The number of participants and the range of T cell responses within each tertile
b. The number of participants presented influenza like illness with confirmed fever within each tertile.
c. The number of participants who had nasal swabs that contained influenza virus, detected by polymerase chain reaction (PCR).
0=No, 1=Yes.
All 43 participants were infected with pandemic H1N1 2009, and had no detectable antibody specific for pandemic H1N1 2009. Infection was defined by seroconversion of a 4-fold rise in HI titer against H1N1 2009 from pre to post influenza season. Participants were divided into tertiles based on pre-existing *ex vivo* T cell responses against H3N2 nucleoprotein (NP) that were measured by human IFN-γ ELISpot Assay. The unequal group size was the result of ties within each tertile. Analytical software STATA was used to create tertiles and carry out Fisher's exact tests. All data was background subtracted at mean+4 standard deviations of no peptide controls then applied the statistical cutoff of 4 SFC/M. SFC/M: spot forming cells per 10^6 PBMCs. HI: Hemagglutinin Inhibition. PCR+: influenza virus was detected in nasal swab by polymerase Chain Reaction (PCR). Ties: data points had equal value.
Though it was no statistically significant, there was a trend that those with higher frequency of H3N2 NP specific T cells were protected from influenza like illness with confirmed fever; the rate of influenza like illness with confirmed fever was lower in the 3rd tertile than in the 1st tertile. Pre-existing H3N2 NP specific T cells protected against nasal swab PCR+ for influenza.

Table 6.5: Immune correlate of protection conferred by *ex vivo* pre-existing cross-reactive anti-NP T cells against pandemic H1N1 2009

Tertile	Number of participants	Minimum T cell responses (SFC/M)	Maximum T cell responses (SFC/M)
1	13	0	0
2	2	5	6
3	7	7.5	85

a.

Tertile	Influenza like illness with confirmed fever		Total
	0	1	
1	12 (92%)	1 (8%)	13
2	1 (50%)	1 (50%)	2
3	6 (86%)	1 (14%)	7
Total	19 (86%)	3 (14%)	22
Compare 1st and 3rd tertile			
1-sided Fisher's exact=0.589			

b.

Tertile	PCR+ for influenza		Total
	0	1	
1	7 (54%)	6 (46%)	13
2	2 (100%)	0 (0%)	2
3	7 (100%)	0 (0%)	7
Total	16 (73%)	6 (27%)	22
Compare 1st and 3rd tertile			
1-sided Fisher's exact=0.044			

c.

a. The number of participants and the range of T cell responses within each tertile
b. The number of participants presented influenza like illness with confirmed fever within each tertile.
c. The number of participants who had nasal swabs that contained influenza virus, detected by polymerase chain reaction (PCR).
0=No, 1=Yes.
All 22 participants were infected with pandemic H1N1 2009, and had no detectable antibody specific for pandemic H1N1 2009. Infection was defined by seroconversion of a 4-fold rise in HI titer against H1N1 2009 from pre to post influenza season. Participants were divided into tertiles based on pre-existing *ex vivo* anti-pandemic nucleoprotein (NP) T cell responses mediated by cross-reactive anti-H3N2 nucleoprotein (NP) T cells that were measured by human IFN-γ ELISpot Assay. The unequal group size was the result of ties within each tertile. Analytical software STATA was used to create tertiles and carry out Fisher's exact tests. All data was background subtracted at mean± 4 standard deviations of no peptide controls then applied the statistical cutoff of 4 SFC/M. SFC/M: spot forming cells per 10^6 PBMCs. HI: Hemagglutinin Inhibition. PCR+: influenza virus was detected in nasal swab by polymerase Chain Reaction (PCR). Ties: data points had equal value.
Ex vivo pre-existing NP specific cross-reactive T cells against pandemic H1N1 2009 protected against nasal swab PCR+ for influenza.

Table 6.6: Immune correlate of protection conferred by *ex vivo* pre-existing H3N2 M specific T cells against pandemic H1N1 2009

Tertile	Number of participants	Minimum T cell responses (SFC/M)	Maximum T cell responses (SFC/M)
1	23	0	0
2	7	4.667	10
3	13	10.667	75

a.

Tertile	Influenza like illness with confirmed fever		Total
	0	1	
1	18 (78%)	5 (22%)	23
2	5 (71%)	2 (29%)	7
3	11 (85%)	2 (15%)	13
Total	34 (79%)	9 (21%)	43
Compare 1st and 3rd tertile			
1-sided Fisher's exact=0.501			

b.

Tertile	PCR+ for influenza		Total
	0	1	
1	19 (83%)	4 (17%)	23
2	7 (100%)	0 (0%)	7
3	11 (85%)	2 (15%)	13
Total	37 (86%)	6 (14%)	43
Compare 1st and 3rd tertile			
1-sided Fisher's exact=0.631			

c.

a. The number of participants and the range of T cell responses within each tertile
b. The number of participants presented influenza like illness with confirmed fever within each tertile.
c. The number of participants who had nasal swabs that contained influenza virus, detected by polymerase chain reaction (PCR).
0=No, 1=Yes.
All 43 participants were infected with pandemic H1N1 2009, and had no detectable antibody specific for pandemic H1N1 2009. Infection was defined by seroconversion of a 4-fold rise in HI titer against H1N1 2009 from pre to post influenza season. Participants were divided into tertiles based on pre-existing *ex vivo* T cell responses against H3N2 matrix protein (M) that were measured by human IFN-γ ELISpot Assay. The unequal group size was the result of ties within each tertile. Analytical software STATA was used to create tertiles and carry out Fisher's exact tests. All data was background subtracted at mean+ 4 standard deviations of no peptide controls then applied the statistical cutoff of 4 SFC/M. SFC/M: spot forming cells per 10^6 PBMCs. HI: Hemagglutinin Inhibition. PCR+: influenza virus was detected in nasal swab by polymerase Chain Reaction (PCR). Ties: data points had equal value.
Though it was no statistically significant, there was a trend that those with higher frequency of H3N2 M specific T cells were protected from influenza like illness with confirmed fever and nasal swab PCR+ for influenza; the rate of influenza like illness with confirmed fever and nasal swab PCR+ was lower in the 3rd tertile than in the 1st tertile.

Table 6.7: Immune correlate of protection conferred by *ex vivo* pre-existing cross reactive anti-M T cells against pandemic H1N1 2009

Tertile	Number of participants	Minimum T cell responses (SFC/M)	Maximum T cell responses (SFC/M)
1	11	0	0
2	4	5	5.121
3	7	5.667	175

a.

Tertile	Influenza like illness with confirmed fever		Total
	0	1	
1	9 (82%)	2 (18%)	11
2	3 (75%)	1 (25%)	4
3	7 (100%)	0 (0%)	7
Total	19 (86%)	3 (14%)	22
Compare 1st and 3rd tertile			
1-sided Fisher's exact=0.359			

b.

Tertile	PCR+ for influenza		Total
	0	1	
1	8 (73%)	3 (27%)	11
2	2 (50%)	2 (50%)	4
3	6 (86%)	1 (14%)	7
Total	16 (73%)	6 (27%)	22
Compare 1st and 3rd tertile			
1-sided Fisher's exact=0.485			

c.

a. The number of participants and the range of T cell responses within each tertile
b. The number of participants presented influenza like illness with confirmed fever within each tertile.
c. The number of participants who had nasal swabs that contained influenza virus, detected by polymerase chain reaction (PCR).
0=No, 1=Yes.
All 22 participants were infected with pandemic H1N1 2009, and had no detectable antibody specific for pandemic H1N1 2009. Infection was defined by seroconversion of a 4-fold rise in HI titer against H1N1 2009 from pre to post influenza season. Participants were divided into tertiles based on pre-existing *ex vivo* anti-pandemic nucleoprotein (NP) T cell responses mediated by cross-reactive anti-H3N2 nucleoprotein (NP) T cells that were measured by human IFN-γ ELISpot Assay. The unequal group size was the result of ties within each tertile. Analytical software STATA was used to create tertiles and carry out Fisher's exact tests. All data was background subtracted at mean+4 standard deviations of no peptide controls then applied the statistical cutoff of 4 SFC/M. SFC/M: spot forming cells per 10^6 PBMCs. HI: Hemagglutinin Inhibition. PCR+: influenza virus was detected in nasal swab by polymerase Chain Reaction (PCR). Ties: data points had equal value.
Though it was no statistically significant, there was a trend that those with higher frequency of H3N2 specific T cells were protected from influenza like illness with confirmed fever and nasal swab PCR+ for influenza; the rate of influenza like illness with confirmed fever and nasal swab PCR+ was lower in the 3rd tertile than in the 1st tertile.

6.2.2.2 Assessing the role of *ex vivo* pre-existing influenza A (H3N2) specific T cell immunity in protection against influenza like illness and nasal viral shedding if infected with seasonal influenza

The role of *ex vivo* pre-existing influenza A (H3N2) specific T cells in natural seasonal influenza infection was also investigated. *Ex vivo* pre-existing influenza A (H3N2) specific T cells against whole proteome of H3N2 (Table 6.8) and immunodominant internal proteins of nucleoprotein (Table 6.9) and matrix protein (Table 6.10) were investigated. There were no detectable immune correlates of protection against influenza like illness or nasal viral shedding from either total H3N2 specific T cells or T cells directed at H3N2 nucleoprotein and matrix protein.

Table 6.8: Total *ex vivo* pre-existing H3N2 specific T cells did not protect against either influenza like illness or nasal viral shedding if infected with natural seasonal influenza H3N2 and/or H1N1

Tertile	Number of participants	Minimum T cell responses (SFC/M)	Maximum T cell responses (SFC/M)
1	57	0	0
2	45	4.191	42
3	50	43.71	1445.335

a.

Tertile	Influenza like illness with confirmed fever		Total
	0	1	
1	46 (81%)	11 (19%)	57
2	36 (80%)	9 (20%)	45
3	37 (74%)	13 (26%)	50
Total	119 (78%)	33 (22%)	152
Compare 1st and 3rd tertile			
Pearson chi2(1) = 0.6876, Pr = 0.407			

b.

Tertile	PCR+ for influenza		Total
	0	1	
1	51 (89%)	6 (11%)	57
2	36 (80%)	9 (20%)	45
3	42 (84%)	8 (16%)	50
Total	129 (85%)	23 (15%)	152
Compare 1st and 3rd tertile			
Pearson chi2(1) = 0.7017, Pr = 0.402			

c.

a. The number of participants and the range of T cell responses within each tertile
b. The number of participants presented influenza like illness with confirmed fever within each tertile.
c. The number of participants who had nasal swabs that contained influenza virus, detected by polymerase chain reaction (PCR).
0=No, 1=Yes.
All 152 participants were infected with pandemic either/or seasonal H3N2 and H1N1, and had no detectable antibody against the serotype they were infected with. Infection was defined by seroconversion of a 4-fold rise in HI titer from pre to post influenza season. Participants were divided into tertiles based on total *ex vivo* pre-existing H3N2 specific T cell responses measured by human IFN-γ ELISpot Assay. The unequal group size was the result of ties within each tertile. Analytical software STATA was used to create tertiles and carry out Pearson's Chi-squared tests. All data was background subtracted at mean+4 standard deviations of no peptide controls then applied the statistical cutoff of 4 SFC/M. SFC/M: spot forming cells per 10^6 PBMCs. HI: Hemagglutinin Inhibition. PCR+: influenza virus was detected in nasal swab by polymerase Chain Reaction (PCR). Ties: data points had equal value.

Table 6.9: *Ex vivo* pre-existing H3N2 NP specific T cells did not protect either influenza like illness or nasal viral shedding if infected with natural seasonal influenza H3N2 and/or H1N1

Tertile	Number of participants	Minimum T cell responses (SFC/M)	Maximum T cell responses (SFC/M)
1	84	0	0
2	18	4.015	8
3	50	8.762	350.667

a.

Tertile	Influenza like illness with confirmed fever		Total
	0	1	
1	67 (80%)	17 (20%)	84
2	15 (83%)	3 (17%)	18
3	37 (74%)	13 (26%)	50
Total	119 (78%)	33 (22%)	152
Compare 1st and 3rd tertile			
Pearson chi2(1) = 0.5989, Pr = 0.439			

b.

Tertile	PCR+ for influenza		Total
	0	1	
1	72 (86%)	12 (14%0	84
2	14 (78%)	4 (22%)	18
3	43 (86%)	7 (14%)	50
Total	129 (85%)	23 (15%)	152
Compare 1st and 3rd tertile			
Pearson chi2(1) = 0.0021, Pr = 0.963			

c.

a. The number of participants and the range of T cell responses within each tertile
b. The number of participants presented influenza like illness with confirmed fever within each tertile.
c. The number of participants who had nasal swabs that contained influenza virus, detected by polymerase chain reaction (PCR).
0=No, 1=Yes.
All 152 participants were infected with pandemic either/or seasonal H3N2 and H1N1, and had no detectable antibody against the serotype they were infected with. Infection was defined by seroconversion of a 4-fold rise in HI titer from pre to post influenza season. Participants were divided into tertiles based on their *ex vivo* pre-existing H3N2 nucleoprotein (NP) specific T cell responses measured by human IFN-γ ELISpot Assay. The unequal group size was the result of ties within each tertile. Analytical software STATA was used to create tertiles and carry out Pearson's Chi-squared tests. All data was background subtracted at mean+4 standard deviations of no peptide controls then applied the statistical cutoff of 4 SFC/M. SFC/M: spot forming cells per 10^6 PBMCs. HI: Hemagglutinin Inhibition. PCR+: influenza virus was detected in nasal swab by polymerase Chain Reaction (PCR). Ties: data points had equal value.

Table 6.10: *Ex vivo* pre-existing H3N2 M specific T cells did not protect either influenza like illness or nasal viral shedding if infected with natural seasonal influenza H3N2 and/or H1N1

Tertile	Number of participants	Minimum T cell responses (SFC/M)	Maximum T cell responses (SFC/M)
1	88	0	0
2	14	4.908	12.624
3	50	13.333	376

a.

Tertile	Influenza like illness with confirmed fever		Total
	0	1	
1	67 (80%)	17 (20%)	84
2	15 (83%)	1 (6%)	18
3	37 (74%)	15 (30%)	50
Total	119 (78%)	33 (22%)	152
Compare 1st and 3rd tertile			
Pearson chi2(1) = 1.3227, Pr = 0.250			

b.

Tertile	PCR+ for influenza		Total
	0	1	
1	73 (83%)	15 (17%)	88
2	11 (79%)	3 (21%)	14
3	45 (90%)	5 (10%)	50
Total	129 (85%)	23 (15%)	152
Compare 1st and 3rd tertile			
Pearson chi2(1) = 1.2771, Pr = 0.258			

c.

a. The number of participants and the range of T cell responses within each tertile
b. The number of participants presented influenza like illness with confirmed fever within each tertile.
c. The number of participants had nasal swab contained influenza virus that was detected by polymerase chain reaction (PCR).
0=No, 1=Yes.
All 152 participants were infected with pandemic either/or seasonal H3N2 and H1N1, and had no detectable antibody against the serotype they were infected with. Infection was defined by seroconversion of a 4-fold rise in HI titer from pre to post influenza season. Participants were divided into tertiles based on their *ex vivo* pre-existing H3N2 nucleoprotein (NP) specific T cell responses measured by human IFN-γ ELISpot Assay. The unequal group size was the result of ties within each tertile. Analytical software STATA was used to create tertiles and carry out Pearson's Chi-squared tests. All data was background subtracted at mean+4 standard deviations of no peptide controls then applied the statistical cutoff of 4 SFC/M. SFC/M: spot forming cells per 10^6 PBMCs. HI: Hemagglutinin Inhibition. PCR+: influenza virus was detected in nasal swab by polymerase Chain Reaction (PCR). Ties: data points had equal value.

6.2.3 Power of detection

The original power calculation for this study was based on the detection of the difference in the proportion of symptomatic infections between those who had *ex vivo* pre-existing influenza-specific T cells and those who did not have *ex vivo* pre-existing influenza-specific T cells. However, it was not possible to define positive T cell responders due to the low frequency of *ex vivo* influenza specific T cells and the lack of an influenza naïve population. This is because of the low frequency of *ex vivo* pre-existing influenza specific T cells in blood circulation were susceptible to natural fluctuation in peripheral blood cells and minor technical variations, which could lead to false negative results. It was difficult to define a biological cutoff for T cell positivity, as there was no influenza naïve population that could be used to define a threshold that eliminates the false positive results. Although we have demonstrated that our statistical cut-off was also biologically valid as influenza-specific T cells in 99% of *ex vivo* responders defined by our statistical cutoff could be expanded in culture. 93% of *ex vivo* non-responders could also be expanded in cultured assays (Figure 3.8 b). These results demonstrated that almost all individuals by the age of 5 have established influenza specific T cells either through natural infection or vaccination. Therefore, an alternative analysis was performed by grouping individuals into tertiles based on the magnitudes of their *ex vivo* influenza A specific T cells then compared the disease outcome of T cell responders in the first (low) and third (high) tertile. This approach minimizes the influence by the natural fluctuation in periphery blood lymphocytes and enabled us to examine the relationship between *ex vivo* pre-existing influenza specific T cells and influenza disease outcome. As reported in the section above, *ex vivo* pre-existing H3N2 nucleoprotein specific T cells correlated with no nasal viral shedding in people infected with a serologically distinct influenza

virus (Table 6.4c and Table 6.5c). Having high T cell responses appeared to confer 100% protection against nasal viral shedding with 26% of low T cell responders shedding virus and none of high T cell responders shedding virus.

It is unfortunate for this study that, amongst those individuals who did not receive influenza vaccination, the infection rate in the pandemic was lower than expected. Only 8% compared to the previous seasonal outbreaks, which were between 13 to 25%. Thus, there was not enough power in the current study to drawn conclusion on a protective effect by NP specific T cells (Table 6.11). Approximately 2.2 times more participants would have been required to achieve a desired power of 80% (Table 6.12) assuming an infection rate of 8%. In total, 859 volunteers participated in the pandemic H1N1 2009 study, therefore a sample size of 1890 would be required to achieve a desirable power of 80% if the infection rate remains as low as for pandemic H1N1 2009 virus. To study the role of T cell immunity in seasonal influenza, further investigation is needed to identify the rates of true seronegative participants, which would be highly informative for future power calculations.

Table 6.11: The Flu Watch study was underpowered to detect the protection against nasal viral shedding conferred by *ex vivo* pre-existing cross-reactive NP specific T cells

	Low T cells	High T cells	Ratio	Total
[1]**Sample size**	23	14	1.64	37
% shedding virus	26	0		
[2]**Confidence**	95%			
[3]**Power**	33%			

[1]Participants infected with pandemic H1N1 2009 virus in the Flu Watch
[2]Confidence: 95% confidence a protective effect detected is true.
[3]Power 33%: There was 33% confidence to detect a protective effect.
The infection rate, the influenza specific T cell responses and the proportion of individuals shedding virus use in this power calculation is from the Flu Watch study
Power calculation was performed using online tool of OpenEpi at http://www.openepi.com/OE2.3/Menu/OpenEpiMenu.htm

Table 6.12: Infected individuals that would be need to power a study in detecting heterotypic immunity against nasal viral shedding

	Low T cells	High T cells	Ratio	Total
[1]**Sample size**	51	31	1.64	[4]82
% shedding virus	26	0		
[2]**Confidence**	95%			
[3]**Power**	80%			

[1]Participants infected with pandemic H1N1 2009 virus in the Flu Watch
[2]Confidence: 95% confidence a protective effect detected is true.
[3]Power 33%: There was 33% confidence to detect a protective effect.
[4]The total participants required to achieve 80% of power is approximately 2.2 times higher of what Flu Watch had (reported in Figure 6.11)
The infection rate, the influenza specific T cell responses and the proportion of individuals shedding virus use in this power calculation is from the Flu Watch study of pandemic H1N1 2009 virus.
Power calculation was performed using online tool of OpenEpi at http://www.openepi.com/OE2.3/Menu/OpenEpiMenu.htm

6.3 Discussion

This is the first comprehensive study to investigate the role of T cell immunity against disease outcomes in natural influenza infection. Over a period of four years, a cohort of 2,395 family based volunteers was established through random selection. Subjects' clinical symptoms were recorded during the period that influenza was known to circulate. Influenza like illness with confirmed fever in this study refers to the CDC influenza like illness (ILI) definition which is sudden onset of fever >38°C and cough and/or sore throat in the absence of a known cause other than influenza. Various symptoms were recorded in our study but the CDC influenza like illness was the only symptom set that was associated with natural influenza infection. This is consistent with a previous study of 3744 (2470 influenza-infected by seroconversion) individuals enrolled in phase 2 and 3 clinical trial that was designed to study the antiviral agent Zanamivir. The combination of cough and fever (body temperature ≥ 37.8°C or ≥ 37.2°C for 65years or older) was the best predictor of influenza infection with a positive predictive value of 79% (P<. 001) [94]. The overall infection rate for seasonal influenza (first three years) was approximately 19%, but pandemic H1N1 2009 produced an infection rate of 8% that was lower than seasonal influenza. This infection rate of 8% was also slightly lower than the infection rates estimated based on the data collected from those who presented influenza like illness in primary care and through national surveillance in the UK (at 11.2%)[321], and outpatients in HongKong (at 10.7%) [322]. The attributable risk of CDC influenza like illness due to natural influenza infection was low (below 14%) and there was a trend that *ex vivo* pre-existing influenza A (H3N2) specific T cells protected against CDC influenza like illness if infected with pandemic influenza, but not seasonal influenza. The immune

correlate of protection against nasal viral shedding (nasal swab PCR+ for influenza) conferred by T cells was also observed if infected with pandemic H1N1 2009 virus, but not seasonal influenza; *ex vivo* pre-existing T cells directed at H3N2 nucleoprotein (NP) associated with preventing the occurrence of nasal viral shedding if infected with serologically distinct pandemic H1N1 2009 subtype (Fisher's exact test, P=0.043). This heterotypic protection was confirmed by the detection of the association between *ex vivo* pre-existing cross-reactive NP specific T cells and the prevention of the occurrence of nasal viral shedding cases (Fisher's exact test, P=0.044) if infected with pandemic H1N1 2009.

Heterotypic immunity in influenza has been demonstrated in experimental challenge studies as discussed in chapter 1, section 1.10.2. The mechanism of T cell heterotypic immunity was studied extensively in mice immunized with live replicating influenza virus. The important role of T cells in heterotypic immunity was demonstrated in mice. Antibody depletion of CD4+ and/or CD8+ T cells just before and following heterosubtypic challenge abrogated the reduction in lung virus clearance[323, 324] and virus clearance in the nose[324]. Moreover, depletion of CD8+ T cells was more effective in the reduction of heterotypic immunity than depletion of CD4+ T cells [324]. Cytotoxic T cell (CTL) deficient ($\beta_2m^{-/-}$) mice primed with γ-irradiated influenza A viruses showed greater weight lost than CTL competent mice upon challenge with a heterotypic strain suggesting that cytotoxic T cells play a predominant role in cross protection against heterologous challenge [325]. It was also suggested heterotypic immunity was multifaceted event and not mediated by T cells alone. *In vivo* depletion of CD4+ and CD8+ T cells suggested that in the absence of CD4+ and CD8+ T cells, γδ T cells conferred protection against lethal heterosubtypic challenge [323]: in CD4+ and CD8+ T cell depleted mice, compared with normal

mice, γδ TCR knockout mice ($\gamma\delta^{-/-}$) abrogated heterosubtypic protection. In addition, B cell deficient mice (IgH-$6^{-/-}$ [326] and disruption of the immunoglobulin μ heavy chain [327]) did not generate effective heterotypic immunity suggesting an important role for B cells in heterotypic immunity.

In our study, T cells directed at influenza nucleoprotein (NP) contained the largest proportion of CD8+ T cells compared to T cells directed at other proteins as reported in chapter III in this thesis. Approximately 10% of total T cell responses were from viral nucleoprotein (NP) specific CD8+ T cells, which was twice as much as the CD8+ T cell responses induced by viral matrix protein (Figure 3.9). This may provide an explanation as why the protective effect was observed from T cells directed at nucleoprotein (NP), but not matrix protein (M) despite of the fact that both nucleoprotein and matrix proteins were immunogenic in inducing *ex vivo* influenza specific T cells.

Although T cell heterotypic immunity was observed in pandemic influenza infection in this study, no protective effect observed from *ex vivo* pre-existing influenza specific T cells against seasonal influenza. It is possible that pre-existing antibodies against seasonal influenza that were not detected by hemagglutinin inhibition assay may interfere with the investigation of the role of T cell immunity in natural seasonal influenza infection. Seasonal H3N2 and H1N1 have been circulating in humans since 1970's. Giving the long history of these two seasonal influenza subtypes in human population, one may speculate that large proportion of human population would have encountered these two subtypes of influenza virus before and possess antibody immunity against these two subtypes. As demonstrated in Chapter 3 (3.2.3) 98% of participants had detectable influenza specific T cells that can be expanded following *in vitro* stimulation. This suggested that almost all UK participants had encountered

antigen from seasonal influenza by the age of 5. However, over 80% of individuals infected with influenza had no detectable influenza specific antibodies measured by hemagglutinin inhibition (HI) assay and these HI sera negative participants were selected for the investigation of the role of T cell immunity in natural influenza infection. It was possible these seronegative individuals may have influenza specific antibodies induced by previous exposure(s) that were not detected in hemagglutinin inhibition (HI) assay. The accurate detection of influenza antibodies in sera by the hemagglutinin inhibition (HI) assay was influenced by nonspecific serum inhibitors, differences in the ability of viruses to agglutinate erythrocytes and the frequency of antigenic drift [328]. Although the hemagglutinin inhibition assay was performed by the Health Protection Agency to a highest quality, it was not possible to overcome the problem with the variability in the ability of virus to agglutinate erythrocytes and antigenic drift amongst influenza viruses circulating in human population. Genome sequence analysis of 1250 human influenza virus isolates from individual patients with minimal *in vitro* passage revealed a high level of mixed infection in human populations and within individual hosts. Virus diversity included different hemagglutinin (HA) lineage of the same influenza virus subtype and those hemagglutinins that were likely to differ in antigenicity [329]. In contrast, the hemagglutinin inhibition assay should provide relative accurate detection of pandemic H1N1 2009 specific antibodies due to the homogeneity amongst these virus isolates. Phylogenetic analysis of nucleotide sequence of all pandemic H1N1 human virus isolates from Mexico and the United States up to May 2009 revealed 99.9% of sequence identify within each gene segments [10]. Alternative antibody detection methods such as detection of NP specific antibody by ELISA [330] may be more

appropriate in assessing antibody immunity in humans with mixed influenza infection for the purpose of identifying true seronegative individuals.

This is the first time that heterotypic immunity conferred by T cells was observed in natural influenza infection in human and demonstrated that nucleoprotein may be a suitable vaccine target for a T cell inducing human vaccine. It should be possible to amplify pre-existing NP specific T cell responses several fold with a suitable vaccine, putting the T cell responses of all recipients in or above the top tertile presented in this thesis (Table 6.4) (Table 6.5). T cell heterotypic immunity conferred by influenza nucleoprotein specific T cells has been demonstrated in mice[331]: Intramuscular injection of plasmid cDNA encoding H1N1 nucleoprotein driven by Rous sarcoma virus or cytomegalovirus promoter induced both nucleoprotein specific antibody and CTLs. These CTLs were capable of lysing MHC-class I matched H1N1 and H3N2 NP epitope-pulsed or virally infected target cells in vitro, with antigen specific stimulation or without antigen specific stimulation but activated with concanvalin A and IL-2. Those NP-DNA vaccinated mice showed 50% or more survival rate than control mice when challenged with lethal H3N2. Those NP-DNA vaccinated mice had either no virus or 3 times less virus titer in the lung on day 7 compared to control mice when challenged with sublethal H3N2 and much less weight loss was observed in NP-DNA vaccinated mice. This study further demonstrated that the NP-specific antibodies did not offer heterotypic protection, as passive transfer of NP –specific antibodies induced by NP-DNA vaccine to naïve mice did not offer protection against H3N2 challenge, nor did the high titer of NP-specific antibodies induced by intramuscular injection of purified NP protein. Therefore the heterotypic immunity observed in this model was likely mediated by

NP specific CTLs. However, research in developing a vaccine in inducing nucleoprotein (NP) specific T cells that could confer protection in human is still yet to be achieved.

It is important to note that the heterotypic immunity observed in this study can only be seen as a trend due to the lack of statistical power (Table 6.11), consequent as the low frequency of natural infection by the pandemic H1N1 2009 virus. Heterotypic immunity conferred by nucleoprotein specific T cells against natural influenza infection could be the primary hypothesis for a future study that would required just under 2000 participants (Table 6.12) in natural pandemic influenza infection assuming 8% of seroconversion rate. The sample size could potentially need to be much greater in the studies of seasonal influenza, because of smaller proportion of true seronegative participants.

In summary, in unvaccinated persons, the mean of the natural seasonal influenza infection rate for the first three years of the Flu Watch study was approximately 19%, but the infection rate in pandemic H1N1 2009 was only 8%. As the result of this unexpected low infection rate and the mild disease outcomes seen in the pandemic H1N1 2009, this study did not have sufficient statistical power in detecting a protective effect. Various symptoms were measured in this study, but the influenza like illness defined by Center of Disease Control (CDC) was the only symptom associated with natural influenza infection. The observation of heterotypic protection against nasal viral shedding and influenza like illness conferred by *ex vivo* pre-existing influenza specific T cells can only be seen as a trend, and further study is required to achieve statistical power.

Chapter 7: General discussion

The Flu Watch study was a co-ordinated effort by the Department of Primary Care and Population sciences of University College of London (UCL), the Health Protection Agency (HPA) and the Medical Research Council, Human Immunology Unit at the University of Oxford (HIU, MRC). Flu Watch participants were recruited by UCL and their clinical outcome data used in this thesis were also contributed by UCL. The HPA conducted the serological study; their serology data was used to identify the true influenza infection cases in this thesis. The T cell study was conducted at the HIU, MRC.

7.1 The importance of the accurate identification of influenza infection

Mathematical models have been used to understand the transmission dynamics of influenza, being first used to describe an influenza epidemic in 1927 by Kermack and McKendrick. This mathematical model is known as the Susceptible-Infectious-Recovered (SIR) model [332]. The Basic Reproduction Number (R_0) is defined as the numbers of new infections generated by one infected individual, in an entirely susceptible population, during the time they are infectious. The R_0 can be obtained by fitting the SIR model to the incidence data collected during the initial exponential growth phase. The R_0 was used to assess the severity of the epidemic and the initial rate of increase. Subsequent modifications were made based on SIR model to assess the effect of environmental factors on influenza pandemics, such as demographic models to include inflow and outflow of individuals (e.g. the effect of air travel on influenza pandemics[333, 334]); the effectiveness of biomedical intervention such as vaccination, anti-viral drug and prophylactic treatment with antiviral, and therapeutic treatment [335-337] and the effectiveness of behavioral interventions such as closing schools, quarantining infected individuals or imposing travel restrictions [14, 338-341]. As reported in section 6.2.1 in this thesis, approximately half of influenza infections were asymptomatic and influenza like illness was not specific to influenza infection. Therefore the attributable risk of influenza like illness was only 14%. The estimated R_0 based on the incidence rates collected through sentinel surveillance and observational studies with self-reporting symptoms largely depends on the incidence rates of other infections that also present influenza like illness and the rates of asymptomatic influenza infections. In total, 3482 volunteers participated in the Flu

Watch serological study, of which, 2685 also participated in the T cell immunity study. In addition, several thousand volunteers were scheduled for serological study beyond the scope of this study. The identification of true infection would provide accurate incident rates for the derivation of an accurate $R_{0,}$ which would be valuable in informing planning of control and prevention of influenza infection. While the clinical and serological data from Flu Watch study should contribute to the planning in control and prevention of natural influenza infection, they were also vital for the study of the T cell immune correlates of protection against disease outcome if infected with natural influenza.

7.2 Highly cross-reactive and immunogenic influenza A nucleoprotein (NP) was the most attractive target for a T cell based influenza vaccine

The level of *ex vivo* pre-existing influenza A specific T cell responses from a cohort of 1539 randomly selected volunteers who were aged between 5 to 88 years was investigated in chapter 3. Investigation using the *ex vivo* human IFN-γ ELISpot assay reported a range of influenza specific T cells from these volunteers. The frequency of *ex vivo* influenza A (H3N2) specific T cells ranged from individuals having approximately 1000 spot forming cells per 10^6 PBMCs to those who had no detectable influenza specific T cells. *Ex vivo* influenza A (H3N2) specific T cells were detectable from over 70% of the volunteers, which was similar to previous smaller UK cohort studies of influenza A specific T cell immunity [205]. The median *ex vivo* influenza specific T cell response was at 30 spot forming cells per 10^6 PBMCs, which was comparable to T cell responses reported from previous influenza studies [205, 212, 227, 258], and from other acute respiratory viral infection such as respiratory syncytial virus [259], but significantly lower than those from chronic viral infections such as HIV and CMV [260].

Data from this cohort study suggested that influenza nucleoprotein could be the most attractive target for a T cell based vaccine for the following four reasons:

1. In terms of the breadth and magnitude, the most immunodominant T cell responses were directed at nucleoprotein (NP). Over 50% of Flu Watch volunteers had *ex vivo* T cells recognized NP and, out of all influenza

antigens, approximately 30% of total detectable *ex vivo* influenza (H3N2) specific T cells were directed at NP.

2. Influenza nucleoprotein was the most potent viral antigen at eliciting influenza specific CD8+ T cells. CD8+ T cells were proportionally expanded *in vitro* as reported in chapter 3, and the enhanced frequency allowed their detection by flow cytometry analysis of intracellular cytokine staining of human IFN-γ. *In vitro* expanded H3N2 nucleoprotein specific CD8+ T cells contributed to 10% of total culture expanded influenza specific T cells and this proportion was larger than proportions of *in vitro* expanded H3N2 specific CD8+ T cells directed at other H3N2 influenza antigens. As discussed in chapter 6, cytotoxic CD8+ T cells were important in eliminating influenza virus infected cells, which led to effective resolution of influenza infection. This has been demonstrated in mice, that the delayed virus clearance in the lung was observed in cytotoxic T cell deficient mice (β_2m-/-) at day 8 post challenge compared to cytotoxic T cell competent mice[195].
3. Nucleoprotein specific T cells were highly cross-reactive to pandemic H1N1 2009 as reported in chapter 4. Although it is likely that multiple mechanisms (as discussed in chapter 4) are involved in generating cross-reactive T cell responses in humans due to the heterogeneity in human T cell populations and as the result of life long exposures to many antigens, the cross reactivity seen in T cells directed at conserved internal proteins may largely be attributed to sequence homology.
4. The CD8+ enriched T cell responses directed at H3N2 NP correlated with preventing the occurrence of nasal viral shedding if infected with pandemic H1N1 2009 virus. This observation is consistent with results from a previous

human challenge study, in which, the frequency of *in vitro* expanded pre-existing cytotoxic T cells correlated inversely with nasal viral shedding in all ages of experimentally challenged volunteers[211].

The likely value of a vaccine that induces NP specific T cells has been demonstrated in mice. NP specific cytotoxic T cells induced by intramuscular injection of a NP-DNA vaccine promoted lung virus clearance and reduced weight loss when challenged with a sublethal heterotypic influenza strain; and conferred 50% more protection against mortality when challenged with a lethal heterotypic influenza strain [331]. The depletion of NP specific CD8+ T cells in DNA-NP vaccinated mice abrogated the protection from mortality and weight loss; but the deletion of NP specific CD4+ T cells abrogated the protection from mortality but not weight loss [342], suggesting that both subsets of NP specific T cells induced by DNA-NP vaccination play a role in heterotypic protection with greater contribution from CD8+ T cells than CD4+ T cells. The first human T cell inducing influenza vaccine in phase I clinical study was reported in 2011[271]. A modified Vaccinia Virus Ankara (MVA) vector encoding nucleoprotein and matrix protein 1 was determined to be immunogenic and safe to be used in healthy adults aged 18-50 years; adverse effects were comparable to other licensed influenza vaccines. The following phase 2a trial demonstrated that this T cell inducing influenza vaccine reduced both disease severity and cases of nasal viral shedding upon experimental challenge [270].

7.3 The likely value of this study for the development of a T cell based vaccine

As discussed in chapter 6, although heterotypic immunity in influenza infection has been reported in animal and human challenge studies, evidence of the importance of such heterotypic immunity in natural influenza A infection has yet to be generated, especially in a natural population with varying age, genetic background and underlying medical conditions. This study provides the first evidence that heterotypic T cell immunity can reduce the occurrence of nasal viral shedding after natural infection with a serologically distinct pandemic influenza virus, as reported in chapter 6. The evidence generated from this study is consistent with results from previous studies, suggesting that a T cell inducing influenza vaccine may be valuable in generating heterotypic protection.

However, it is unfortunate, that despite of the great effort involved in the Flu Watch, there was not enough statistical power to conclude that the T cell heterotypic immunity in natural influenza A infection (Table 6.11). The lack of statistical power is primarily due to the mild disease outcome that produced fewer symptomatic infection and the lower than expected infection rate. Even so, this study provides essential information as listed below for powering future studies:

1. *Ex vivo* influenza specific T cells in blood circulation existed at low frequency as reported in chapter 3 with median response of 30 spot forming cells per 10^6 PBMCs. The accurate quantification of such low frequency T cells was susceptible to the natural fluctuation in the frequency of PBMCs as reported in chapter 4. Thus, it is technically challenging to identify true responders from non-responders. Also, most of the UK participants had influenza specific T

cells that can be detected by *in vitro* assays as reported in chapter 3, suggesting the lack of influenza naïve individuals. Therefore it was not possible to measure the protective effect of T cell immunity by comparing the rates of symptomatic infections in those who had pre-existing T cells with those who did not have pre-existing T cells. An alternative approach should be taken by dividing individuals into tertile or quartile groups according to their influenza specific T cell responses as described in chapter 6, and comparing the disease severity in top tertile/quartile to those in lowest tertile/quartile to minimize the noise introduced by the natural fluctuation in the frequency of PBMCs. For the purpose of future power calculation, the ratio between top tertile T cell responders to lowest tertile T cell responders was approximately 1 to 1.6 in this study.

2. Influenza-like illness was not a robust measurement of disease severity of natural influenza infection; and was not suitable for study of natural influenza infection, as reported in chapter 6. Definitive measurement of virus burden in influenza-infected individual such as nasal viral shedding should be used. Future studies should be powered using the rate of virus shedding infected cases at approximately 26% and the virus shedders were all in T cell responders of the lowest tertile.
3. The natural influenza infection rate in those who do not receive influenza vaccination is likely to be less than 25%, which was the maximum infection rate observed in influenza season during this study. Approximately 80% of these unvaccinated individuals would have no detectable influenza serotype specific antibodies.

In addition, the robust experimental procedures employed in this study could be applied to future studies of this kind. And data from this study could be combined with future studies to achieve the desired statistical power.

Lastly, the information gained from the establishment of the research framework necessary for the study, which included epidemiologists, general practitioners, research nurses, virologist and immunologist, will be invaluable for setting up of future efforts in the event of pandemic. Similarly, the increased awareness and public interest that was achieved by this study will be critical for successful recruitment for future studies.

7.4 Challenges of this study

It is particularly challenging to study low frequency T cells. Quantification of low frequency T cells requires assays with high precision and low background noise. As demonstrated in chapter 3 and chapter 2 (assay standardization section), high precision amongst replicates was achieved and standard operating procedures (SOPs) were developed to ensure that 12-15 hours old blood specimens performed equally well, including low backgrounds, to freshly drawn blood. The SOPs are attached as Appendix in this thesis.

It was not possible to apply an existing definition of T cell positivity cutoff to this study; this is due to the lack of an influenza naïve population and the low frequency of influenza specific T cells. An alternative approach of applying statistical cutoff then validating with *in vitro* assays was used to define positivity in this study as described chapter 3.

Finally, it has been a great challenge to run a study of this size. I was responsible for assay design and standardization, staff training, consumable management, laboratory set up and equipment maintenance. Staff training was required every 6 months with 2-3 trainees each time as 15 out of 19 staff who worked on this project were temporary researchers from an exchange training programme, agency and fellow colleagues of other labs within the institute. To ensure adequate standards were achieved and maintained during the 4-year study, technicians were first required to demonstrate competency at training sessions. This kind of organization was essential for a study of this scale and will be needed for a future study of this kind.

7.5 Future directions

To draw conclusions on the protective role of T cell immunity in natural influenza A infection in humans, more studies are needed. It would be preferable that these studies be conduced in a way that is compatible with this study in terms of the parameters measured and participant recruitment; this would enable this study to be combined with future studies to achieve the desired statistical power.

Alternatively, a challenge study that closely resembles the natural influenza infection could also be used to investigate T cell heterotypic immunity in influenza infection. Though ethically, it would not be possible to study with a new pandemic virus in a challenge study and further studies are also required to assess the effect of influenza heterotypic T cell immunity in the young, elderly and people with concerning underlying medical conditions. Influenza virus used in any influenza challenge study should only go through minimal *in vitro* passage to minimize mutations that may impact the virulence and subsequently alter the immune response.

Although influenza nucleoprotein (NP) is highly conserved, mutations in nucleoprotein that were associated with the escape of recognition by cytotoxic CD8+ T cells have been reported [343-346]. However, it has also been reported that mutations to conserved influenza matrix and nucleoprotein sequence could lead to impaired influenza virus replication, which limited the emergence of these mutant influenza viruses [347]. The vast majority of influenza epitopes identified were from H3N2 and H1N1 viruses, and a relatively large proportion of influenza epitopes were derived from prototype strains used for model studies [348]. Further comprehensive T cell epitope mapping from a natural population will be of great value in identifying

highly conserved epitopes that limit influenza virus escape from cytotoxic T cells by fitness costs. This information could be used to evaluate T cell inducing influenza vaccines.

7.6 Conclusions

This is the first comprehensive study to investigate the role of T cell immunity in natural influenza A infection in a randomly selected UK cohort. There was minimal selection bias in this study due to the random selection of participants and therefore the scientific findings generated from our study population, the Flu Watch cohort, could be applied to the entire UK population. Also, this is the first time that the role of T cell immunity in natural influenza infection was analyzed with the support of clinical illness record, influenza virus shedding and sera influenza antibody status, so the influenza specific T cell immunity could be related to symptomatic and asymptomatic influenza infections.

It is essential to consider the following aspects in the development of a cross-protective T cell based human influenza vaccine:

1) It is safe to be used in a natural population, which consists of people with different ages and health status.
2) It can protect a natural population from serologically distinct influenza strains
3) It is immunogenic in a natural population with different ages and genetic diversity

The data from this study showed encouraging trends to support the view that the T cell immunity fulfills the above considerations and future research with the aim to develop a T cell based human influenza vaccine would be valuable. There was no evidence observed from this study that influenza specific T cells are

immunopathogenic; There was some evidence that pre-existing influenza specific T cells directed at the most immunogenic influenza nucleoprotein prevent the occurrence of virus shedding in people infected with a pandemic, serologically distinct influenza virus. The influenza specific T cells could contribute to the better control of influenza virus replication and may reduce subsequent transmission.

This study is likely to contribute to the design of future studies of T cell immunity in natural influenza A infection, and data from this study could be combined with future studies to test the hypothesis that heterotypic immunity is important in natural influenza infection.

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Appendixes:

Appendix 1: Preparation of peripheral blood mononuclear cells (PBMCs) from whole blood

TITLE: PBMC and Plasma isolation and cryopreservation using density gradient centrifugation.	**NUMBER:** 021006
EFFECTIVE DATE:021006	**REPLACES:** N/A

ORIGINATED BY: LiLiWang _____ _____

(Researcher) (Signature) (Date)

REVIEWED BY: Andrew McMichael _____ _____

(Principal Investigator) (Signature) (Date)

Weatherall Institute of Molecular Medicine
MRC human Immunology unit
John Radcliff Hospital
OX3 9DU

PREPARATION OF PERIPHERAL BLOOD MONONUCLEAR CELLS (PBMC) FROM WHOLE BLOOD

1. PURPOSE

1.1 This Standard Operating Procedure (SOP) details the method to be followed when preparing whole blood samples from Flu Watch Cohort

2. INTRODUCTION

2.1 Peripheral blood mononuclear cells (PBMC) are isolated from Flu Watch patients for cryostorage, use in Enzyme Linked ImmunoSPOT (ELISPOT) assay and Flow Cytometry (FACS) analysis.

3. MATERIALS AND EQUIPMENT

3.1 Sterile medium R0
500ml RPMI 1640
5ml penicillin-streptomycin

3.2 Sterile medium R2
500ml RPMI 1640
5ml penicillin-streptomycin

10ml of FBS from Sigma- heat inactivated and sterile filtered. Lot number: 085K3395

3.3 Sterile medium R10
500ml RPMI 1640
5ml penicillin-streptomycin
50ml of FBS from Sigma- heat inactivated and sterile filtered. Lot number: 085K3395

3.4 Sterile medium R20
500ml RPMI 1640
5ml L-glutamine
5ml penicillin-streptomycin
100ml of FBS from Sigma- heat inactivated and sterile filtered. Lot number: 085K3395

3.5 50ml Sterile cell separation tubes containing a polyethylene, porous barrier, called a frit, and containing no density gradient medium: i.e. "Leucosep" tubes (Vendor Greiner Bio-One, cat no: 227290);

3.6 Sterile Ficoll density gradient medium: Lymphoprep (Vendor Axis-shield PoC AS, cat no: 1114547), protected from light.

3.7 Fetal Bovine Serum (FBS), heat –inactivated, sterile, filtered store aliquots at -20°C (vendor Sigma)

3.8 Dimethyl Sulfoxide (DMSO) sterile, high quality grade, protected from light

3.9 15ml, 50ml Polypropylene sterile conical tubes

3.10 5ml, 10ml, 25ml Polyethylene sterile transfer pipettes

3.11 2ml sterile Cryovials suitable for storage in Liquid Nitrogen (LN2)

3.12 Ice Bucket and Crushed ice

3.13 Tabletop centrifuge, low –speed (minimum capability of 250 to 800xg), with swinging bucket rotor

Note: This SOP specifies all centrifugation speeds as relative centrifugal force (RCF) in "g". Centrifuge/rotor manuals usually include RCF to revolutions per minutes (rpm) conversion charts to allow users to convert between RCF and rpm. If the laboratory does not have an RCF/rpm chart for the rotor, obtain one from the centrifuge/rotor manufacturer. It is recommended that the required g to rpm conversions be posted on the centrifuge for easy reference.

3.14 2-8°C refrigerator

3.15 -70-80°C freezer (for plasma storage and short term PBMC storage)

3.16 -20°C freezer

3.17 37°C water bath

3.18 Liquid Nitrogen storage tank (≤-140°C)

3.19 Cell counter: automated cell counter (Pentra 60)

3.20 Freezing apparatus:
Stratacooler (Vendor: Stratagene)
Note: Chilled to 2-8°C before being used

3.21 Micropipettors and sterile tips to fit

3.22 1 Litre Beaker

3.23 500ml 1% Virkon

3.24 Pipette man

3.25 Class II Biological laminar flow safety cabine

3.26 Tube rack 12 x 50ml tubes

4 PROCEDURE

4.1 All manipulations should be performed in a certified category II MSC at all times and all waste will be disposed of by soaking in 1% VIRKON for 15 minutes then discarded into the sink

4.2 Samples should be processed within 15hrs or immediately upon receipt. Receipt of samples should be recorded with time and date on sample receipt form Flu Watch blood collection form 021006.

4.3 Preparation of Ficoll Cell Separation Tubes:

4.3.1 Pipette 15ml Ficoll into 50ml unfilled LeucoSep tubes containing the frit alone

4.3.2 Centrifuge for 1 minutes at 800-1000xg at 18-26°C so that the Ficoll settles below the frit

4.3.3 Tubes can be prepared and stored at 4-26°C until the expiration date of the Ficoll batch

Note: Large bubbles between the frit and Ficoll indicate that insufficient Ficoll was added to the tube

4.4 Density Gradient Centrifugation:

4.4.1 Ensure the Ficoll is below the frit. If there is Ficoll above the frit, centrifuge for 1 minute at 800-1000xg at 18-26°C so that the Ficoll settles below the frit.

4.4.2 Allow the Ficoll-loaded Leucosep tubes to reach room temperature (18-26°C) before use

4.4.3 Mix blood vaccutainer tubes by gently inverting the tube several times

4.4.4 Remove whole blood from vaccutainer tubes by pouring directly onto the frit of ficoll-loaded Leucosep tubes:

If blood volume is between 15-30ml, use 50ml Ficoll-Loaded leucosep tube
If blood volume is between 8-15ml, dilute blood with R0 (1:1) and mix well then use 50ml Ficoll-Loaded leucosep tube
If the blood volume is between 4-8ml, use 14ml Ficoll-Loaded leucosep tube
If the blood volume is between 1-4ml, dilute blood with R0 (1:1) and mix well then use 14ml Ficoll-Loaded leucosep tube

4.4.5 Record the total volume of whole blood in the Flu Watch blood processing record

4.4.6 Centrifuge for 15minutes at 800 x g for 20minutes with the brake off at 18-26°C, make a note when the centrifugation started on the FluWatch blood processing record

Note: For processing clotted blood specimens see troubleshooting procedures in Appendix A.

4.5 PBMC isolation:

4.5.1 Gently remove samples from the centrifuge so as not to disturb the layers

Centrifugation results in the tube contents dividing into six distinct layers, from the top to the bottom of the tube, these are:

Plasma
PBMC layer
Ficoll
Frit
Ficoll
Packed red blood cells (RBC) and granulocytes

Check for a distinct PBMC layer. If you do not see a distinct PBMC layer, go to Appendix B.
Check for haemolysis. Grossly Haemolysed samples should be discarded (4+Hemolysis-i.e. dark red mahogany color in plasma) and record the haemolysed blood condition on the PBMC Processing Worksheet.

4.5.2 Using a 3ml Pasteur pipette to remove most of the plasma to leave approximately 1.0cm fluid above the PBMC layer. Be careful not to disturb the PBMC layer beneath the plasma then using a Pasteur pipette, carefully transfer the PBMC layer to a 50ml conical centrifuge tube labeled with the Flu Watch Laboratory ID, using one fresh tube for every Ficoll tube.

4.5.3 Re-cap the Ficoll tube and discard it as biohazardous waste.

4.5.4 Dilute the PBMC fraction to approx. 45ml by adding R0

Note: Discard the unwanted plasma and lymphoprep into a waste bottle containing 1% virkon

4.6 Wash and resuspension of PBMC

4.6.1 Pellet cells by centrifugation for 10 minutes at 700 x g at 18-26°C with brake on and watch the centrifuge reach 700 x g to check it is balanced. Check for a cell pellet after centrifugation. If there is no visible cell pellet, confirm that the centrifuge is working and settings on the centrifuge are correct and repeat centrifugation.

.

4.6.1 Gently pour the supernatant into the discard bucket containing 1% virkon and flick the bottom of the centrifuge tube to fully resuspend the cells in the residual volume of 200-500ul PRMI

4.6.2 Resuspend cells in 5ml R2 with Dnase (dilute 1:1000 in R2, so 5ul in 5ml R2), Swirl a couple of times every 5-10 second for 30-50 second. Then add 40ml R2 and centrifuge for 10 minutes at 210 x g,18-26°C with brake on

After centrifugation, check for a cell pellet. If there is no visible cell pellet, confirm that the centrifuge is working and that the settings on the centrifuge are correct and repeat centrifugation.

4.6.3 Gently pour the supernatant into the discard bucket containing 1% virkon and flick the bottom of the tube to fully resuspend cells in the residual volume of R2.

4.6.4 Resuspend cells in 10ml R2 and mix to homogeneity and perform cell count using an automated cell counter (Pentra 60).

Using the PBMC processing worksheet to record the viability, viable cell concentration (expressed as $x10^6$ per ml), the total viable cell number (expressed as $x10^6$ per ml) for each sample and the viable cell yield

4.7 Prepare cells for ELISpot

4.7.1 Take enough cells (refer to cell counting excel worksheet) into 15 ml falcon tube pre-labeled with sample ID and spin at 480x g for 5 minutes at 18-26°C

4.7.2 Discard supernatant, and then flick the 15 ml falcon tube to resuspend cells in the residual of R10 (200-500ul).

4.7.3 Resuspend cells to 5x10^6 per ml in R20 and add 50ul (250,000 cells) to each well

4.8 Preparation of cryopreservation solution (CPS)

4.8.1 To prepare the CPS, mix 9 parts of FBS to 1 part DMSO in a 15-50ml screw-cap centrifuge tube to a final DMSO concentration of 10%.

4.8.2 Place the freshly prepared CPS on ice and allow the CPS to cool for at least 15 minutes so it is ice cold before use. This is an exothermic reaction and the liberated heat will result in cell damage if the CPS is not cooled before use.

4.8.3 Label cryopreservation tubes with Flu Watch PBMC labels.

4.8.4 Place cryovials in a rack and force the rack into the ice so that the base of cryovials is covered with ice, ensuring that the lids are free from the ice.

4.8.5 Allow cryovials to cool for at least 10 minutes so that they are ice cold before use

4.8.6 Wipe clean outside of the StrataCooler with 70% alcohol and place in the biological safety cabinet.

Note: CPS must be freshly prepared for each batch of stored cells

4.9 PBMC cryopreservation

4.9.1 Pellet cells by centrifugation for 10 minutes at 250xg,and 18-26°C with bake on

4.9.2 After centrifugation, check for a cell pellet and gently pour the supernatant into the discard bucket then flick the bottom of the centrifuge tube to resuspend cells in the residual volume of R10 (around 200-500ul)

4.9.3 Resuspend cells in ice-cold CPS to 5-10x10^6 per ml by add ice-cold CPS slowly to the cell suspension, drop by drop, mixing the suspension by swirling the tube between drops.

4.9.4 Aliquot 1ml of the suspension to each cryovial labeled with Flu Watch PBMC label and transfer cryovials to pre-chilled StrataCooler then place StrataCooler in a -70/-80°C freezer for overnight

4.9.5 Transfer the cells the following day to LN2 for long term storage

6. REVISION HISTORY
7. References:
This SOP is adapted from CHAVI-A0001 Version: 3.0 and Oxford PANVAC Phase II Colorectal Cancer Trail CPL01.01

Appendix A:

Trouble-shooting: Processing anticoagulated blood that has clotted

A.1 If the blood in a vacutainer tube is completely clotted, then discard the tube then make a note on the FW PBMC Record.

A.2 If only some blood clots in a vacutainer tube, remove the clots carefully with pipette then process the blood, then make a note on the FW PBMC Record.

Appendix B

Trouble-shooting: Recovery of PBMC in the absence of a defined PBMC band after density gradient centrifugation

B.1 Background:

B1.1. If something has gone wrong during the density gradient centrifugation of the blood, the Ficoll and plasma layer will mix and there will be no visible PBMC layer. Do not panic, PBMC can be retrieved with additional steps.

B.2 Identify the problem:

B.2.1 Remove the tubes from centrifuge and transfer to a rack.
B.2.2 Try to identify why the sample became mixed. Possible causes are listed below:
B.2.2.1 The tube were dropped
B.2.2.2 The brake was left on.
B.2.2.3 The centrifugation speed was too high. Check the settings is at 800, the centrifuge used in Flu Watch study can display both rpm and g force speed, so make sure you are using display for g force. The correct bucket needs to be applied, please refer to the user's guidebook provided by the centrifuge manufacture, which is kept in the box under the centrifuge.
B.2.2.4 The centrifuge stopped due to a discontinuity in the electricity supply
B.2.2.5 The frit dislodged. (This is often due to a centrifugation speed that was too high, but occasionally there is a defective tube in the batch.)
B.2.2.6 The centrifuge was misbalanced.

B.3 Of the above causes, the first five causes are easily fixed. If the cause is due to a misbalance centrifuge, determine why the centrifuge was misbalanced. Check the following:
B.3.1 Check that the tubes were balanced
B.3.2 Check that the centrifuge buckets were balanced
B.3.3 Check that the centrifuge arms and buckets were properly greased and oiled

NOTE: If ever in doubt about a centrifuge, use another one.

B.4 Assuming the problem is fixed, re-centrifuge the samples as follows:

B.4.1 Use a pipette to slowly remove the contents of the mixed sample from the Leucosep tube. (Typically, the frit will have dislodged.)
B.4.2 Transfer up to 30ml of the mixed sample to the a Ficoll-loaded Leucosep tube (tube is warmed up to room temperature)
B.4.3 Repeat this for all mixed samples.
B.4.4 Centrifuge for 30-40 minutes at 400xg with the brake off at 18-26°C
B.4.5 A PBMC layer should now be visible. (often you will have lost cells, so the layer could be thin).
B.4.6 The top layer, which is plasma potentially contaminated with Ficoll, may be collected at this stage and processed as in Sections "PBMC and Plasma Isolation" and "Plasma Storage) of the main protocol. However, make a note on the FW PBMC Record in the comment section that the plasma is potentially contaminated with Ficoll.
B.4.7 Carefully transfer the PBMC layer to a 50ml centrifuge tube labelled with lab ID. Use one new tube for every Ficoll tube.
B.4.8 Re-cap the Ficoll tube and discard it as Biohazardous waste.
B.4.9 Return to "Wash and resuspension of PBMC" step of the main protocol.

NOTE: In the "comments and Deviations" section of the FW PBMC Record, record the details of deviation from the SOP (i.e. that steps from "Appendix B" were taken to recover PBMC due to the absence of a defined PBMC band after density gradient centrifugation.) In addition, note how long the re-centrifugation took, in order to provide an estimate of how long cells were in Ficoll. Ficoll is toxic to the cells, so these recovery steps should be carried out efficiently.

Appendix II: *Ex vivo* ELISpot assay for the Flu Watch study

TITLE: *Ex vivo* ELISpot using Peripheral Blood Mononuclear Cells from the Flu Watch study	**NUMBER: 021006**
EFFECTIVE DATE:	**REPLACES:** N/A

ORIGINATED BY: LiLi Wang _____ _____

(Researcher) (Signature) (Date)

REVIEWED BY: Andrew McMichael _____ _____

(Principal Investigator) (Signature) (Date)

Weatherall Institute of Molecular Medicine
MRC Human Immunology
John Radcliff Hospital
Oxford, OX3 9DU

***Ex vivo* ELISPOT for Flu Watch study.**

1. PURPOSE

1.1 This Standard Operating Procedure (SOP) details the method to be followed when carrying out *ex vivo* ELISPOT on peripheral blood mononuclear cells from Flu Watch study.

2. INTRODUCTION

2.1 Peripheral blood mononuclear cells (PBMC) from the blood of Flu Watch participants are tested for interferon gamma release in response to peptides using Enzyme Linked ImmunoSPOT (ELISPOT).

3. MATERIALS AND EQUIPMENT

3.1 15ml Polyethylene conical tube

3.2 Sterile medium R0

500ml RPMI 1640 with L-glutamine
5ml penicillin-streptomycin

3.3 Sterile medium R20

500ml RPMI 1640
5ml L-glutamine
5ml penicillin-streptomycin
100ml Foetal Calf Serum (FCS)

3.4 10ml Polyethylene sterile transfer pipettes

3.5 1 Litre Waste bottle

3.6 1 Litre Beaker

3.7 500ml 1% Virkon

3.8 Tube rack 12 x 50ml tubes

3.9 96 well round bottom plate

3.10 20µl pipette

3.11 200µl pipette

3.12 200µl pipette tips

3.13 ELISPOT plate

3.14 Sterile PBS

3.15 AP colour development solution

3.16 Petri dish

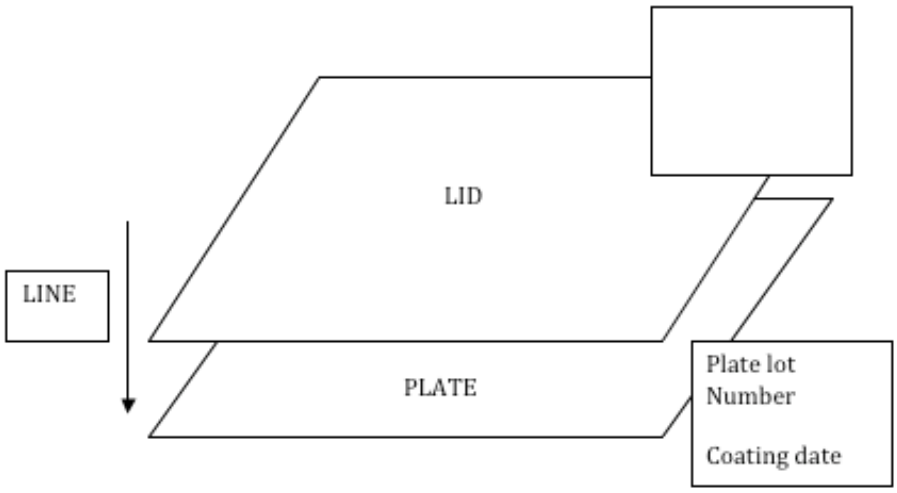
4. PROCEDURE

4.1 All manipulations should be performed in a certified category II MSC at all times and all waste will be disposed of by soaking in 1% VIRKON for 15 minutes then discarded into waste for autoclaving.

4.2 In a sterile 15ml or 50ml conical tube dilute coating antibody (1-D1K green top, 1mg/ml)) to 10µg/ml (1:100) in sterile PBS. Mix by pipetting up and down x 5 with the Gilson pipette or invert 5 times

Use a sterile pipette to transfer the prepared coating solution to a sterile Petri dish, then coat wells with 50ul of diluted coating antibody

4.3 Using a black cryo-marker pen label the ELISPOT plate in the top right hand corner: the date that the plate is coated and the plate lot number Draw a connecting line down the left side of the lid and plate.



4.4 Place the lid on the plates and incubate at 4-8°C in a fridge overnight (5-24hr). Take care to ensure the membrane of all wells is covered with antibody solution by tapping each side of the plate gently.

4.4.1 Plates may be stored with coating antibody solution in the refrigerator for up to 7 days. For storage time greater than overnight, place plates in a ziplock bag in the refrigerator

4.4.2 Plates may also be incubated at room temperature (18-22°C) for 4 hours or 37°C for 2 hours

4.3 Under aseptic conditions in a safety cabinet, discard antibody by flicking plate into a 1 litre beaker and wash with 200µl/well of sterile SIGMA PBS using a multichannel pipette and 200µl sterile tips, invert plate over a beaker to decant the wash solution, (repeat 3 times). Take care not to touch the membrane and rotate the plate 180 degrees after each wash.

4.4 Block the plates: Using a sterile pipette to transfer RPMI medium containing 10%FCS (R10) to a sterile Petri dish, add 200ul R10 to each coated and washed well. Place the lid on plates and place plates in a 37°C 5%CO2 incubate for at least 2 hours.
Note: plates may be incubated for 1 hour

4.5 Take prepared Flu Watch peptides plates from -80° C freezers to tissue culture hood, and remove the inner plastic seal immediately. Place the lid back on and thaw in an incubator or at room temperature (place blue towel under the plate to absorb excess water).

4.6 Remove the blocked plates from the incubator to the hood. Label the first plate with the first subject's Flu Watch Laboratory ID (numerical

number start from 1, e.g.: Flu Watch 0001) on top left hand corner of the plate and the lid, then invert the plate over a virkon trough to decant the blocking solution.

4.7 Ensure the plate is correctly oriented with the top of the plate (row A) closest to the back of the hood

4.8 Gently swirl the tubes containing the first subject's PBMC to mix.

4.9 Pour cells into a dispensing tray

4.10 Using an multi-channel pipette (with only 5 tips on it), transfer 50ul of 5x10^6 per ml cell suspension to each coated wells (250,000 cells/well. Take care not to let wells become dry, if an unexpected delay occurs after removing medium from plate, add 100µl R10 to the plate until ready.

4.11 Cap the cell tube and discard into tissue culture waste. Discard tips into 1 litre beaker containing 1% Virkon.

4.12 Using a multi-channel pipette (with only 5 tips on it), transfer 50µl of the peptides from the Flu Watch peptide plate to the ELISpot plate. Take care to add peptides to the correct wells, change tips between each pipetting.

4.13 Record the time on ELISPOT plate lid bottom left corner

4.14 Incubate plate at 37°C 5% CO_2 for 18-24hrs. Minimise subsequent disturbance to the plate during incubation. Take care to ensure plate is level and no air bubbles are present. To remove air bubbles use a 200µl pipette set to 100µl and draw up the air from the bubble.

4.15 At the end of incubation, remove the cells by flicking into a 14 cm sterile Petri dish for recycling the cells. Empty every five plates, and place lid back on straightaway, and then pass on to another person to wash immediately. Using sterile pipette, transfer the PBMC from the five ELISpot plates into 15ml Flacon tubes, one tube per samples, do not contaminate samples with each other.

4.16 Using the immuno-washer on the bench, wash the plates 6 times with 0.01% Tween 20.PBS. Take care not to touch the membrane and rotate the plate 180 degrees after each wash. Dab the plate dry on a paper towel between each wash. Leave the last wash on until ready to apply the Biotinylated antibody (7-B6-1-Biotin yellow top). May process every 5 plates together.

4.17 Dilute biotinylated antibody (7-B6-1-biotin yellow top, 1mg/ml) to 1µg/ml (1:1000) in PBS 0.5%BSA. Make a volume equal to 3.3ml per plate. Keep the 0.5% BSA.PBS in the fridge, only open in the hood.

4.18 Add 50µl/well diluted biotinylated antibody 7-B6-1-biotin using a multichannel pipette and 200µl tips. Incubate for 2-4hrs at room temperature. Record the start time on ELISpot processing sheet for the specific sample.

4.19 Remove the biotinylated antibody (7-B6-1-biotin) by flicking into a sink with running water and wash plate 5 with PBS using an immunowasher. Take care not to touch the membrane and rotate the plate after each wash. Dab the plate dry on a paper towel between each wash. Leave the last wash on until ready to apply the Diluted Streptavidin-ALP

4.20 Dilute Streptavidin-ALP (white top, 1mg/ml)) to 1µg/ml (1:1000) in PBS. Make a volume equal to 3.3ml per plate.

4.21 Add 50µl/well diluted Streptavidin-ALP using a multichannel pipette and 200µl tips. Incubate for 45minutes to 1hour at room temperature. Record the start time ELISpot processing sheet for the specific sample.

4.22 Step 5.19 to 5.21 may be done with every five plates as one group as Streptavidin-ALP can not be left for more than an hour in the ELISpot plate, it will give background

4.23 Remove the Streptavidin-ALP antibody solution by flicking into a sink with running water and wash plate 6 times with PBS using an immunowasher. Take care not to touch the membrane and rotate the plate 180 degrees after each wash. Dab the plate dry on a paper towel between each wash, leave the last wash on until ready to apply the BioRAD substrate solution

4.24 Make up substrate solution BIORAD AP colour development solution: 10 ml = 9.6ml milliQ water, 400µl 25x buffer, 100µl reagent A, 100µl reagent B. make up 7ml for each plate In a 15ml or 50ml conical tube using a 200µl pipette and 10ml transfer pipette.

4.25 Add 100µl per well substrate solution and leave to develop until distinct spots emerge. Leave for 10-15minutes.

4.26 Remove the colour development substrate solution by flicking into a sink with running water. Wash plate by rinsing under the running tap.

4.27 Remove ELISPOT plate underdrain. Place plate inverted on a paper towel and leave to dry in a cupboard overnight.

4.28 Seal lid to plate with tape and store in a dark cupboard.

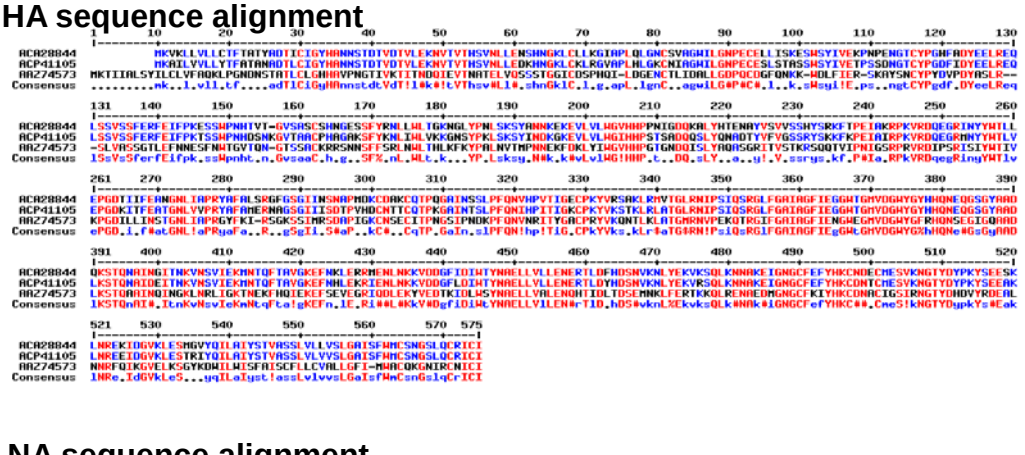
4.29 Refer to ELISPOT Plate Reader SOP.

5. REVISION HISTORY

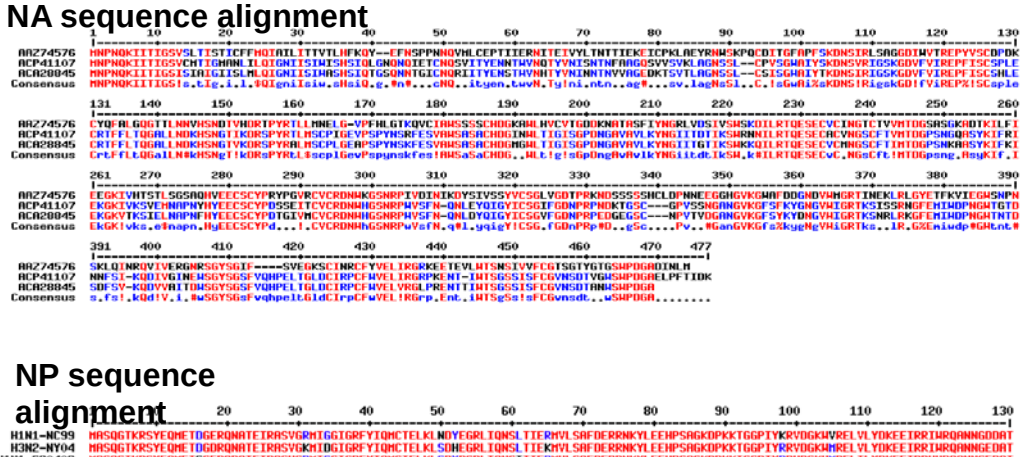
Appendix III The amino acid sequence alignment of seasonal H1N1, H3N2 and pandemic H1N1

The sequence alignment output is shown below, red indicating shared sequence, and blue and black indicating sequence differed amongst different influenza virus strains

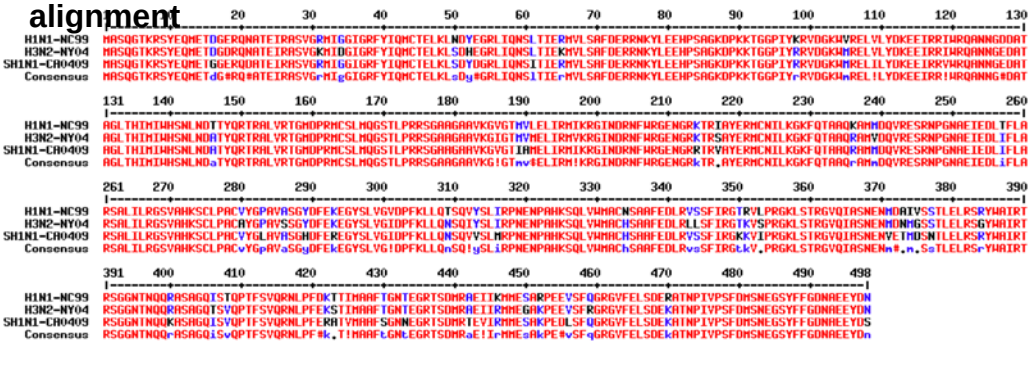
HA sequence alignment



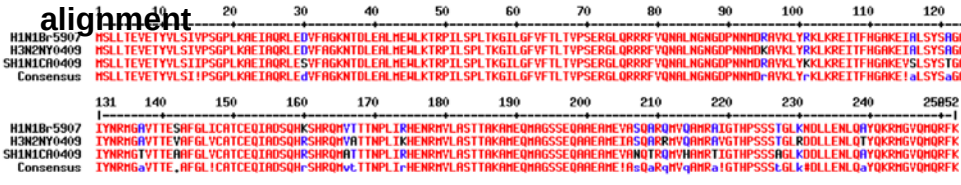
NA sequence alignment



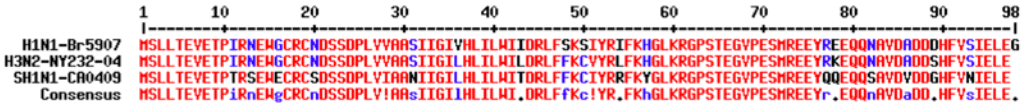
NP sequence alignment



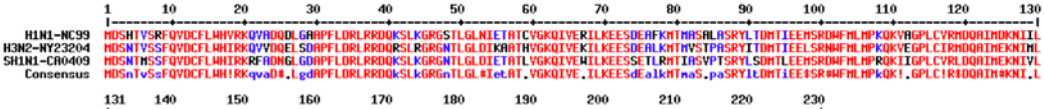
M1 sequence alignment



M2 sequence alignment



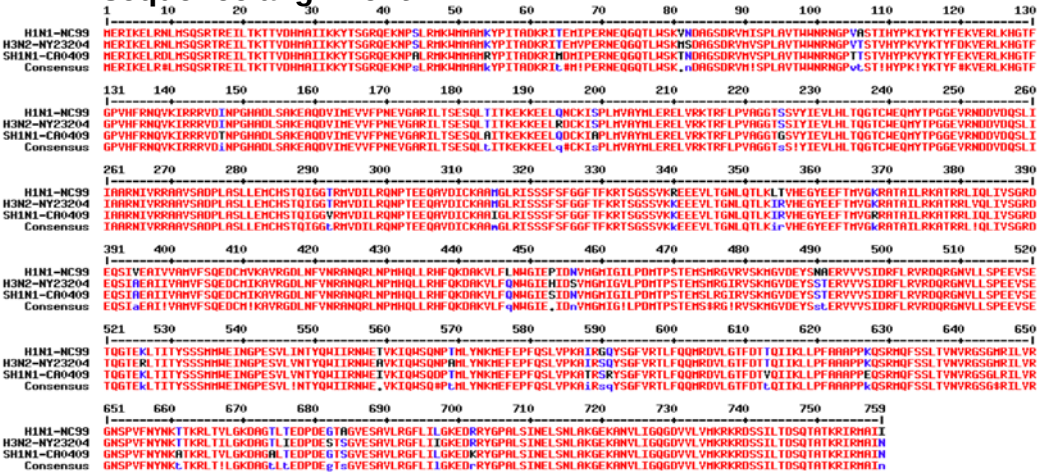
NS1 sequence alignment



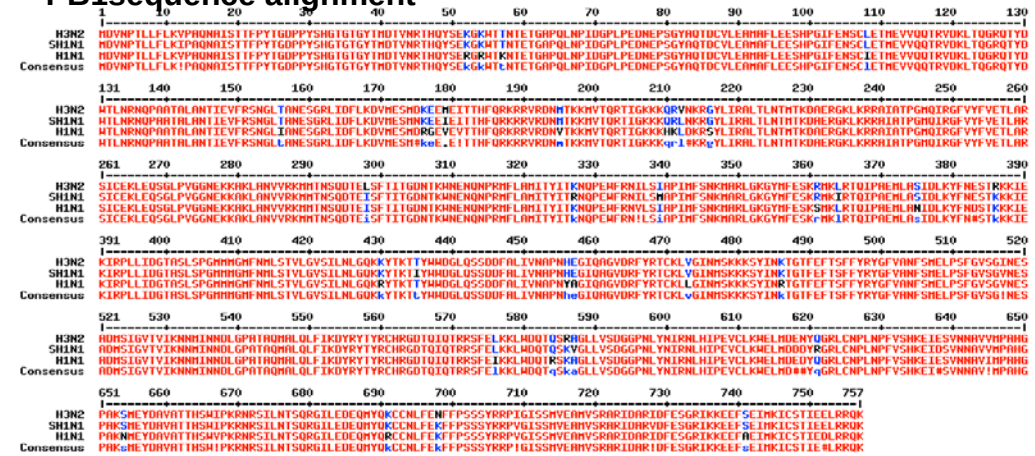
NS2 sequence alignment



PB2 sequence alignment



PB1sequence alignment



PA sequence alignment

