Immunological studies of cold-adapted influenza vaccine viruses in mice

A thesis submitted in fulfilment of the requirements for the degree of
Doctor of Philosophy

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Declaration

I certify that, except where due acknowledgement has been made, the work is that of the author alone; the work has not been submitted previously, in whole or in part, to qualify for any other academic award; the content of the thesis is the result of work which has been carried out since the official commencement date of the approved research program; any editorial work, paid or unpaid, carried out by a third party is acknowledged; and, ethics procedures and guidelines have been followed.

Lumin Xue

Signature: ______________________

Date: _______________________

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List of Abbreviations

aa	amino acid
Ab	antibody
Ag	antigen
AMs	alveolar macrophages
APCs	antigen-presenting cells
ASCs	antibody secreting cells
att	attenuated
β_2m	β-2 microglobulin
BAL	bronchoalveolar lavage
BALT	bronchus-associated lymphoid tissue
BCR	B cell receptor
BM	bone marrow
BSA	bovine serum albumin
bp	base pair
ca	cold-adapted
CDC	centre for disease control and prevention
CEK	chicken embryo kidney
CK	chicken kidney
CMI	cell-mediated immune response
CTL	cytotoxic T lymphocyte
DC	dendritic cell
DIP	defective interfering particle
DNA	deoxyribonucleic acid
ELISPOT	enzyme-linked immunospot
ER	endoplasmic reticulum
FDA	Food and Drug Administration

FITC	fluorescein isothiocyanate
FPLC	fast protein liquid chromatography
$\times g$	centrifugal force × gravity
GALT	gastrointestinal-associated lymphoid tissue
HA	haemagglutinin
HI	haemagglutination inhibition
HAU	haemagglutinin unit
HEF	haemagglutinin-esterase-fusion
H&E staining	Haematoxylin and Eosin staining
hr	hour
ICC	intracellular cytokine cytometry
IFN	interferon
Ig	immunoglobulin
IL	interleukin
i.m.	intramuscular
i.n.	intranasal
i.p	intraperitoneal
GMT	Geometric mean titre
Kb	kilobase
kDa	kilodalton
LAIV	live-attenuated influenza vaccine
LRT	lower respiratory tract
LN	lymph node
MALT	mucosal-associated lymphoid tissue
MDCK	Madin-Darby canine kidney
MHC	major histocompatibility complex
mIg	membrane Ig

mg	milligram
min	minutes
mL	millilitres
mM	millimolar
NA	neuraminidase
NALT	nasopharyngeal-associated lymphoid tissue
NEP	nuclear export protein
NIH	National Institutes of Health
NK cell	natural killer cell
nm	nanometres
NP	nucleoprotein
NS1	non-structural protein 1
NS2	non-structural protein 2
OD	optical density
ORF	open reading frame
PA	acidic polymerase protein
PBMCs	peripheral blood mononuclear cells
PB1	basic polymerase protein 1
PB2	basic polymerase protein 2
PCR	polymerase chain reaction
PD_{50}	50% protective dose
PE	phycoerythrin
PFU	plaque-forming unit
PMN	polymorphonuclear leukocytes
pIgR	poly Ig receptors
p.i.	post-infection
RBC	red blood cell

RDE	receptor destroying enzyme
RNA	ribonucleic acid
SEM	standard error of the mean
StDev	standard deviation
sIgA	secretory IgA
SPF	specific-pathogen-free
T_C	cytotoxic T cell
$TCID_{50}$	median tissue culture infectious dose
TCRs	T cell receptors
T_{CM}	central memory T cell
T_{EM}	effector memory T cell
TNF-α	alpha tumor necrosis factor
T_H	T helper
T_H1	Type I T_H cells
T_H2	Type II T_H cells
TIV	trivalent influenza vaccine
TLR	toll-like receptor
T_{reg}	regulatory T cell
ts	temperature-sensitive
μL	microlitre
URT	upper respiratory tract
VLP	virus-like particle
v/v	volume per volume
v/w	weight per volume
WHO	World Health Organization
wt	wild-type

List of Publications

Oral Presentation

Xue L, Lew AM and Tannock GA. Determinants of immunogenicity for cold-adapted LAIVS in mice. The 4th Orthomyxovirus Research Conference, September 21-24, 2007, Woods Hole, Massachusetts, USA

Poster Presentations

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Conference proceeding

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Summary

Cold-adapted (*ca*) live attenuated influenza vaccines (LAIVs) have been introduced as alternatives to existing inactivated influenza vaccines. The influenza A components of the FDA-approved *ca* LAIVs (*Flumist*®; Medimmune) have common internal genes derived from the donor strain A/Ann Arbor/6/60 *ca* and surface genes derived from current wild-type (*wt*) epidemic strains. The aim of this thesis was to investigate determinants of immunogenicity for reassortants of A/Ann Arbor/6/60 *ca*, using a range of immunological assays, including recently developed MHC tetramer techniques.

From the study, the extent of viral replication in the respiratory tract of mice, the primary site of inoculation, was a key factor in determining *ca* vaccine immunogenicity. Replication was shown to be influenced by both viral surface Ags and the host MHC. The H3 *ca* reassortants CR6, CR18, CR29 and CR6-35* exhibited greater replication efficiency (as determined by their PFU:HAU ratios) than the H1 *ca* reassortants CR35 and CR6-35. The H3 *ca* reassortant CR6 caused a 3.79% loss in body weight but no losses were observed for the H1 *ca* reassortant CR35 and the *ca* H2N2 donor strain A/Ann Arbor/6/60 *ca*. Higher HI responses were detected after 3 weeks in groups infected with the H3 *ca* reassortant CR6 (GMT 80) than with the H1 reassortant CR35 (GMT 10) and the H2 *ca* donor strain A/Ann Arbor/6/60 *ca* (GMT 13).

Recently developed techniques were used to evaluate specific T-cell response to *ca* LAIVs. Fluorescent-labelled tetramer is the key reagent for use in tetramer-based flow cytometry assays. The $NP_{366-374}$ peptide of influenza A viruses comprises an immunodominant epitope that is highly conserved between subtypes. Tetramers developed for A/PR/8/34 (H1N1) were able to detect NP-specific cytotoxic T lymphocytes (CTLs) induced by A/Ann Arbor /6/60 *ca* (H2N2). An attempt to prepare the A/Ann Arbor/6/60 *ca*-specific-NP-tetramer is described. H-$2D^b$ monomers were

successfully refolded with the peptide, but only 20% were able to form tetramers through biotin-streptavidin linkage, resulting in a poor capacity to stain. By contrast, an IFN-γ ICC assay developed in parallel demonstrated that peptide $NP_{366\text{-}374}$ was able to restimulate A/Ann Arbor/6/60 NP *ca*-specific CTLs and secrete IFN-γ when tested *in vitro*.

Specific-B and T cell responses induced in the lungs in response to infection by *ca* reassortants exhibited great variability that was determined by the growth characteristics of different viruses. Type I (CTL) responses were induced by low yielding *ca* reassortants, such as CR35 (H1N1). Viruses with enhanced growth characteristics, such as CR6 (H3N2), produced higher Type II (HA-specific Ab) responses. In addition, host factors, such as MHC type, were found to play an important role in responses to the same viruses. Susceptible mouse strains, such as C57BL/6, showed higher CTL but lower serum Ab responses than more resistant strains, such as BALB/c.

Throughout this PhD project, a fine balance between the humoral and CMI, local and systemic immune responses induced by *ca* LAIVs was demonstrated. The need to assess local immune responses, in addition to serum antibody levels, for the evaluation of vaccine efficacy was an important conclusion of the thesis. Further studies of regulation between the humoral and CMI responses to *ca* LAIVs and of cytokines secreted by T_C and T_H cells, such as IL-2, IL-4, IL-6, IL-12 and INF-γ, would allow the selection of better candidate vaccine strains.

Chapter 1: Literature Review

1.1 INFLUENZA VIRUSES

Influenza viruses can infect a wide range of animals from birds to humans. Subsequent respiratory disease in humans is common and often requires medical attention which imparts a huge economic burden to society. In a typical season, influenzal disease affects 5-40% of the population (Stamboulian et al 2000). Common symptoms of infection are fever, sore throat, muscle pain, severe headache, coughing, weakness and general discomfort. Pneumonia is a significant complication of influenza virus infections and a frequent cause of death in the elderly, the very young and patients with underlying cardiopulmonary conditions.

Because of their capacity to undergo changes, influenza viruses are able to emerge or re-merge as antigenically *new* viruses with the potential to spread rapidly through susceptible populations as the cause of epidemics or pandemics. The three most important influenza pandemics of the 20th century occurred in 1918/19, 1957 and 1968. The 1918/19 *Spanish* pandemic was responsible for more than 40 million deaths throughout the world and occurred in three waves. Twenty-five million people died during the first wave of 25 weeks. By comparison, the same number of deaths for the current HIV/AIDS epidemic was only reached after 25 years (Wright et al 2007). Although the 1957 *Asian* and the 1968 *Hong Kong* pandemics resulted in far fewer deaths (about 1 million for each), their impact was still substantial (Nicholson et al 2003; Luke and Subbarao 2006). Since 1997, a new pandemic threat has appeared from avian influenza viruses, which directly infect humans and have been responsible for limited human-to-human transmission (Lin et al 2000). Current plans to prepare for the next pandemic include the use of vaccination and antiviral drugs to minimize or mitigate its impact. Their use in prevention and prophylaxis will be discussed later in this chapter.

1.1.1 Nomenclature

Influenza viruses belong to the family *Orthomyxoviridae* and possess negative-sense, single-stranded, segmented RNA genomes. There are five different genera or types within the family: influenza A, B, and C viruses, thogotoviruses (isolated from ticks) and isaviruses (isolated from salmon). Reassortment between different genera does not appear possible, due to differences in the mechanisms of replication and the extent of evolutionary divergence between genes with similar functions. However, reassortment of individual genes can take place within the same genera (Wright et al 2007). Classical breakage and reunion does not appear to occur between influenza genomes from the same genera, despite suggestions of such a mechanism to explain the origin of the pandemic viruses of 1918-19 (Gibbs 2006).

Of the five genera, only type A influenza viruses can be further classified into subtypes, based on their two surface glycoproteins, the haemagglutinin (HA) and the neuraminidase (NA). There are now 16 different HA subtypes and 9 different NA subtypes of influenza A viruses (Wright et al 2007), which form the basis of the current system of nomenclature for describing individual influenza A viruses (Figure 1.1).

1.1.2 Virion and genome structure

The eight segments of influenza A and B viruses code for 11 known viral proteins (Figure 1.2B); influenza C viruses have seven segmented RNAs that code for 9 proteins. Each segment contains non-protein coding-regions at the 5'- and 3' terminals and a coding region within. Viral proteins have been categorised as structural and non-structural, according to their presence in complete virions. Structural proteins determine the host range, tissue tropism, and efficiency of transmission, antigenicity and, to a large extent, the pathogenicity of influenza viruses. Non-structural proteins are present only in infected cells and are not components of assembled viruses. However, they encode virulence factors that assist the virus to evade host defences (Yewdell and Garcia-Sastre 2002; Baigent and McCauley 2003).

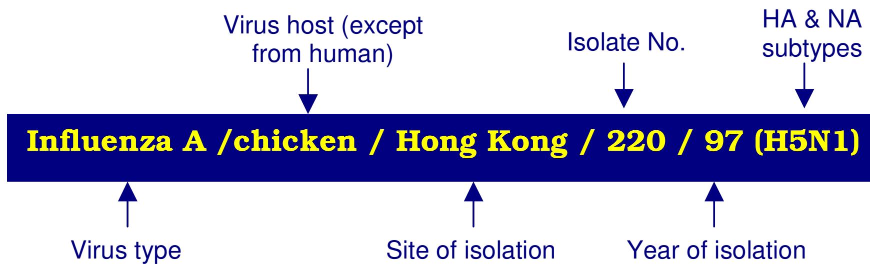


Figure 1.1 The nomenclature system for influenza A virus

A

B

(haemagglutintin)
HA
(neuraminidase)
NA
lipid bilayer
M2
(ion channel)
M1 (matrix protein)
NS2 or NEP (nuclear export protein)
RNPs

Infected cell (or non-structural) proteins
NS1
PB1-F2

PB2
5'
PA
3'
PB1
(-) sense ssRNA
3 polymerase subunits (PB1, PB2 and PA)
NP (nucleoprotein)

Figure 1.2

A: Electron micrograph image showing the position of the eight RNP segments (Noda et al 2006)

B: Schematic diagram of virus particle that shows the viral proteins (Digard et al 2007)

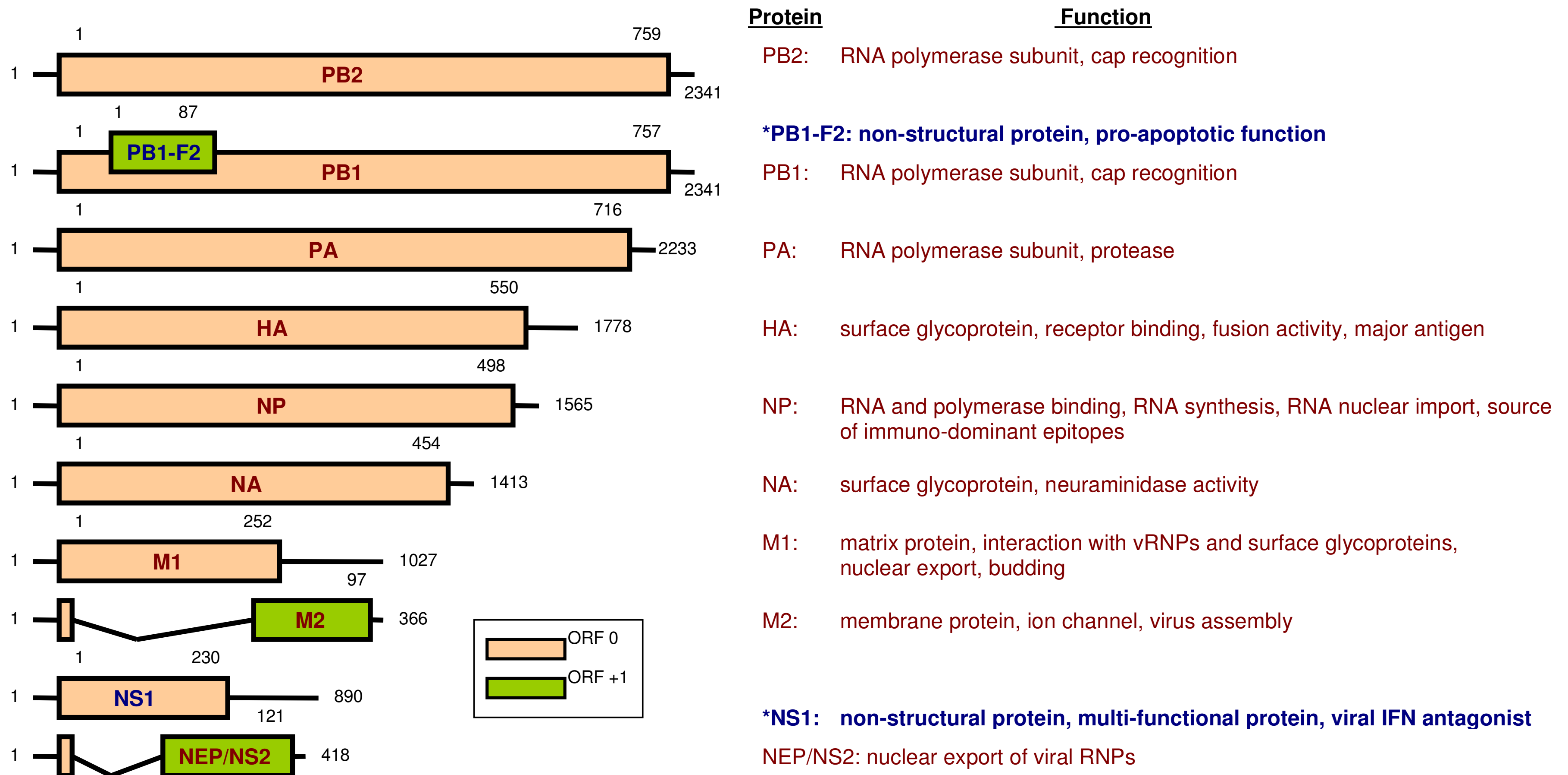


Figure 1.3 Genome structure of influenza A/ Puerto Rico/8/34 (A/PR8) virus and the functions of viral proteins (Wright et al 2007)

* Non-structure proteins showed in blue letters

1.1.2.1 Viral structural proteins

The virion outer layer and its proteins: HA, NA, HEF and M2

Influenza viruses possess an outer envelope consisting of a lipid bilayer membrane derived from the host cell during the budding process that results in release of progeny virus. There are several different proteins embedded within the viral membrane, which vary between different types of influenza viruses. For influenza A and B viruses, the HA and NA surface glycoproteins are present at an approximate ratio of 4:1. Only a single surface glycoprotein, haemagglutinin-esterase-fusion (HEF) protein, is present in influenza C viruses.

HA and NA differ in their three-dimensional structure and function. For influenza A viruses, the HA is a trimeric, rod-shape glycoprotein which is expressed in large quantities on the surface of infected cells (approximately 10^6 per cell) and in mature virions (approximately 10^3 per virion) (Bennink et al 1986). The major function of HA is to act as a receptor-binding protein. HA also plays an important role in viral host restriction and pathogenesis. By contrast, the NA glycoprotein consists of a homotetrameric head domain, a stalk, a trans-membrane region and a short tail (Figure 1.2B). NA is recognised as a receptor-destroying enzyme, which is responsible for the release of newly budded virus from infected cells. Both HA and NA are subtype-specific antigens.

Other tetrameric proteins present within the viral envelope are M2 for influenza A, NB and BM2 for influenza B and CM2 for influenza C viruses, all of which act as proton-selective transmembrane ion channels that are activated at acidic pH and are responsible for uncoating after virus entry into the cell (Pinto et al 1992).

The internal virion proteins: M1 and NEP/NS2

Beneath the viral envelope, is a layer of M1 matrix proteins - the most abundant structural protein. M1 proteins are present in all three influenza virus genera and are type-specific antigens. They form a bridge between membrane proteins and the inner core components. M1 proteins are non-

glycosylated and play a vital role in virus assembly. A recent study of virion structure using cryoelectrotomography demonstrated that some influenza A virions had substantial gaps in their matrix layer, while others appeared to lack a matrix layer entirely (Harris et al 2007). These findings indicate that M1 is not exclusively responsible for virion packaging and budding. In addition, M1 is a critical determinant of virion pleomorphology in determining whether the progeny are spherical or filamentous (Roberts et al 1998; Burleigh et al 2005). For influenza A viruses, the gene that codes for the M1 protein produces a spliced mRNA which encodes the M2 protein (Figure 1.3).

The NEP/NS2 (nuclear export protein / non-structural protein 2) is also present in influenza virions (Richardson and Akkina 1991). Both are associated with the M1 protein. NEP is responsible for exporting assembled viral core components from the nucleus to the cytoplasm of infected cells.

The virion core and its associated NP & polymerase proteins

The core of the influenza virion is the RNP, which comprises viral RNA complexed with nucleoprotein (NP) and polymerase proteins. Viral NPs and RNA are present in segmented form as double-helices, to which a copy of the segment-specific polymerase complex is attached (Lamb 1989). Recently, the internal arrangement of vRNP's has been visualised by electron microscopy (Figure 1.2A). However, an earlier study showed that both the supernatant of influenza-infected chicken embryo chicken cells and allantoic fluid containing non-virion NP account for 50% of total extracellular NP (Prokudina and Semenova 1991).

Viral NP possesses a highly compact monooligomer helical structure (Ortega et al 2000; Ye et al 2006). The oligomerization of NP is mediated by a tail loop structure, which is a potential target for antiviral compounds. The shape of NP resembles a crescent with a head and a body domain (Figure 1.2B). The viral RNA-binding groove is found between head and body domains, whereas the viral polymerase-binding site is located within the body domain. The RNA-NP interaction is

mediated by the positive charge carried by NP and the negative charge carried by RNA. Viral RNA is wrapped around the outside of the NP with one NP monomer consisting of approximately 24 RNA nucleotides (Ortega et al 2000). Uncoated viral RNAs have not been detected in infected cells and are always associated with NP. Besides being a type-specific antigen, NP is also a major source of immunodominant epitopes which are recognised by the host immune system.

Each individual RNP segment carries its own polymerase complex (Figure 1.2B). The viral polymerase is a heterotrimeric complex consisting of polymerase basic 1 (PB1), polymerase basic 2 (PB2) and polymerase acid (PA) subunits. The genomes encoding these three subunits are the largest RNA segments in the influenza virion (Figure 1.3). These subunits assemble in the nucleus to form a functional complex in which PB1 interacts with PA and PB2 at its C- and N- terminal regions with no direct contact between the PB2 and PA subunits. The influenza RNA-dependent RNA polymerase subunits are responsible for transcription and replication of the viral RNA genome within the nucleus of infected cells. Their roles in the virus life cycle, transmission and pathogenesis will be discussed in later sections.

1.1.2.2 Viral non-structural proteins

NS1

All three types influenza virus have been found to express a 230-amino acid (aa), dimeric non-structural protein, the so-called non-structural protein 1 (NS1) within the nucleus of infected cells. This protein has its RNA-binding-domain located within the N-terminal half and an effector-domain. The NS1 protein has been demonstrated to be a highly versatile viral protein because of its multiple roles in virus replication. The NS gene is the smallest of all viral RNA gene segments and not only encodes NS1 but also gives rise to a spliced mRNA that encodes NS2.

PB1-F2

PB1-F2 is the 11th viral protein and has been identified only in influenza A viruses. It consists of an 87-aa polypeptide that is only present in infected cells. The PB1-F2 is encoded by an alternate open reading frame (ORF) near the 5' terminus of the influenza A PB1 gene (Figure 1.3). PB1-F2 appears an accessory protein, since it is not present in all influenza A viruses. However, it has been shown to be present in 64 of the 75 influenza A isolates whose PB1 sequences are registered in GeneBank, most of which are human isolates, and has not been identified in influenza B or C viruses. Recent studies indicate that PB1-F2 accelerates cell apoptosis through its interaction with mitochondria (Yewdell and Garcia-Sastre 2002). Both NS1 and PB1-F2 appear to play influential, but not essential, roles in viral replication and are important contributing factors to viral pathogenesis. Their detailed functions in virus-host interaction will be discussed later.

1.1.3 Viral replication

Although significant advances have been made in recent years, mechanisms of influenza virus replication have not been completely determined. Replication is tightly regulated by both cellular and viral factors; and can be divided into four phases: (1) adsorption and entry; (2) fusion and uncoating; (3) viral RNA transcription and replication; (4) assembly/packaging and budding (Figure 1.4). One cycle of influenza virus replication takes about 4 to 6 hr to complete (Zambon 1999). Atypical events can also occur which result in the production of deficient viral progeny.

Phase 1: Adsorption and entry

The influenza virion enters the cell with the aid of its surface glycoprotein HA (receptor-binding molecule) and initiates infection. Attachment requires the precursor glycoprotein HA0, consisting of HA1 and HA2 subunits covalently linked by a disulphide bond that must be cleaved enzymatically. Cleavage makes the receptor-binding site at HA1 subunit accessible to the cell. Uncleaved HA is able to bind to the receptor, but not causing fuse. Infection is then initiated by the specific binding between HA1 and sialic acid cell receptors on epithelial cell surfaces.

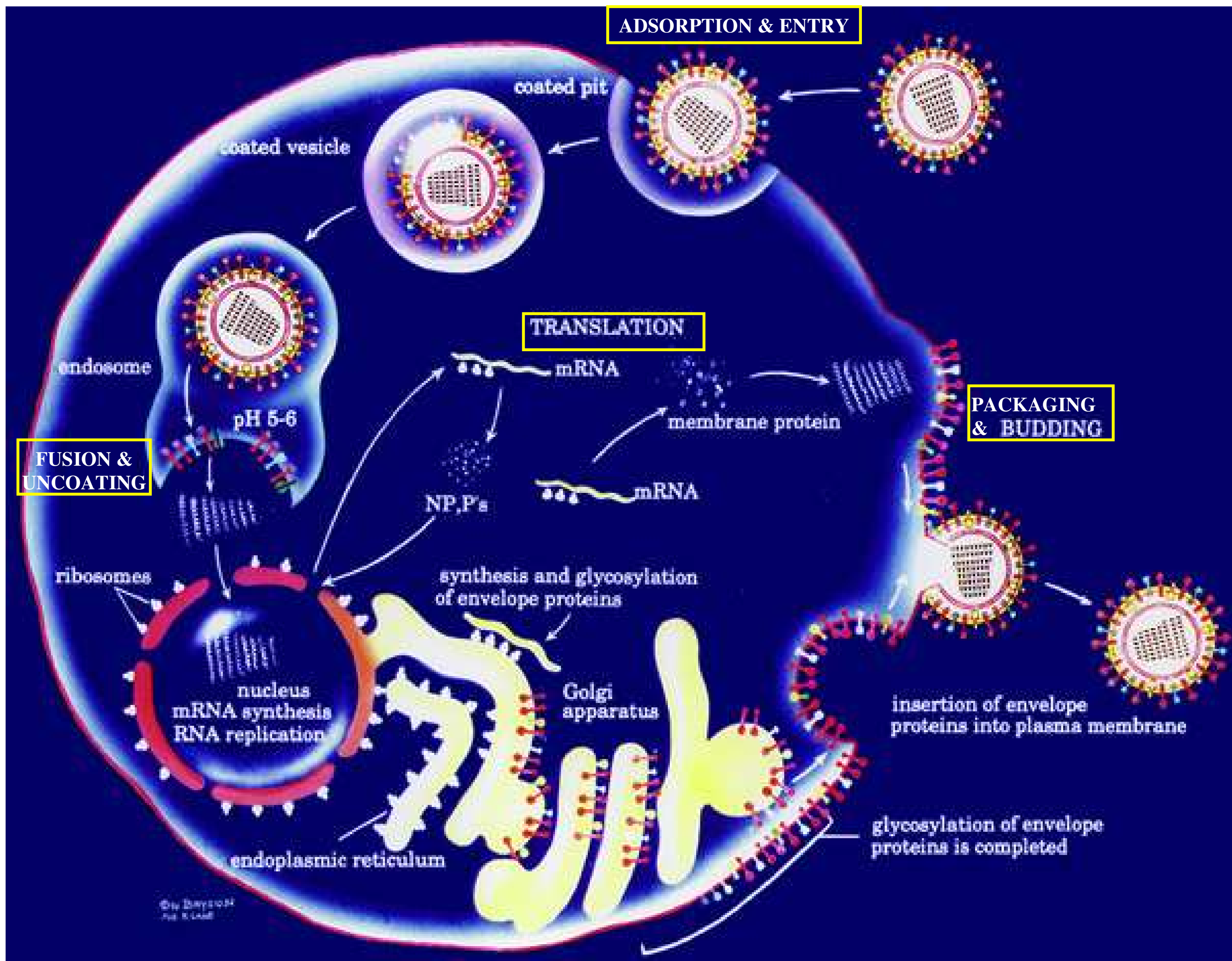


Figure 1.4

Influenza virus replication cycle (Wright et al 2007)

Multiple low-affinity interactions between HAs and their receptors induce endocytosis which facilitates virus entry into cell. Subsequently, the virus particle or virion is internalised within the endosome and transported to the cytoplasm.

Phase 2: Fusion and uncoating

The low-pH environment of the endosome induces the joining of viral and endosomal membranes. Under acidic conditions, the cleaved HA0 molecule undergoes a conformational change exposing the fusion peptide at the N terminus of the HA2 subunit. This allows the fusion peptide to interact with the endosomal membrane forming a fusion pore. At the same time, viral membrane ion-channel M2 proteins allow proton entry to the viral core from the endosome, resulting in the dissociation of vRNPs from M1 protein. The free vRNPs are then released from the interior of virion to the cytoplasm through the fusion pore.

Phase 3: Transcription and replication

Unlike other RNA viruses, the initiation of influenza viral (v)RNA synthesis entirely relies on the primers provided by cellular RNA polymerase II (Pol II). As a result, vRNA synthesis occurs only within the nucleus. This negative-sense vRNA serves as a template for both messenger (m)RNA transcription and complementary (c)RNA replication. The series of reactions, from vRNA → mRNA, vRNA → cRNA → progeny vRNA, are catalysed by a viral polymerase complex (Ps).

Once vRNPs enter the nucleus, the incoming vRNAs are transcribed into mRNAs by a primer-dependent mechanism. First, the PB1 subunit from the polymerase complex binds to the 5' end of each vRNA segment. This step results in attachment of the PB2 subunit to a cap structure on host pre-mRNAs attached to host Pol II. Then, PB1 acts as an endonuclease and cleaves host pre-mRNAs 10 to 13 nucleotides downstream of the cap structure, with the remainder acting as a primer for viral mRNA transcription. Newly synthesised mRNA is then exported to the cytoplasm where viral proteins are translated. Of the viral proteins, the NP and P travel back to nucleus, to

initiate the switch from vRNA synthesis of primer-dependent mRNA transcription to primer-independent cRNA replication. Then, newly synthesised negative-sense vRNAs, NP and P form vRNP complexes within the nucleus. Finally, the newly assembled vRNPs are exported to cytoplasm. The movement of vRNPs in and out the nucleus is complicated by processes that involve several viral proteins (NP, P, NEP, M1) and cellular factors, which are beyond the scope of this discussion.

Phase 4: Packaging and budding

Following export from the nucleus, viral mRNAs are translated into proteins at the endoplasmic reticulum (ER) in the cytoplasm. Viral internal proteins then travel back to the nucleus after post-translational processing. They then interact with segmented RNAs, resulting in vRNP assembly and subsequent export to the apical plasma membrane. Meanwhile, viral membrane proteins are processed through the ER, where they are folded, glycosylated, and assembled into trimers (HA) or tetramers (NA and M2). Finally, after passage through Golgi apparatus, they are inserted into the apical plasma membrane where the virus budding takes place prior to release (Figure 1.4). These actions are controlled by a signal network by mechanisms that are not fully understood.

However, recent studies on the viral genome have revealed the existence of packaging-signal sequences at each viral RNA segment. One study has demonstrated that the introduction of synonymous mutations within the highly conserved packaging-signal region in the PA gene resulted in more than 90% of progeny virions being assembled without the PA segment (Marsh et al 2007). Similar observations were made for the HA segment. These findings provide the basis for the current selective packaging hypothesis which suggests that influenza virus packaging is a segment-specific process mediated by specific RNA-RNA or protein-RNA interactions (Marsh et al 2007). Based on this hypothesis, influenza viruses are able to package the correct RNA segments into each virion to produce infectious particles.

Before infectious virions can be produced, budding involving the release of viral components in the vicinity of the apical plasma membrane must occur. The budding process starts after the cellular lipid bilayer becomes curved outwards following accumulation of the M1 protein. It ends with the viral envelope separating from the cell membrane (Figure 1.4). Until this point, virions remain attached to the cell surface or to themselves because of the binding of HA to the sialic acid receptor. The final release of influenza virions requires the enzymatic activity of NA (for Type A and B viruses) and HEF (for Type C viruses), which removes sialic acid receptors from the cell surface and mucus.

Defective interfering particles (DIPs)

The tendency to generate and propagate defective interfering particles (DIPs) is an innate feature of influenza, and many other viruses. DIPs are virus-like particles (VLPs) that are formed during both *in vivo* and *in vitro* replication. DIPs arise most readily under conditions of high multiplicity of infection (MOI), where many virus particles infect a single cell (Chambers and Webster 1987; Kirkwood and Bangham 1994).

Influenza A DIP genomes usually have a single, large deletion in their polymerase genes (PA, PB1 and PB2) but unchanged 3' and 5' termini (Chambers and Webster 1987; Mann et al 2006). The smaller sizes of DIP RNAs may provide a replicative advantage over the full-length RNAs due to their higher copying efficiency. When a certain ratio of DIP and infectious virus is reached, interference takes place, giving rise to progeny yields containing alternatively high and low amounts of infectious virus (Kirkwood and Bangham 1994). These changes can be measured from infectivity and haemagglutinin ratios during successive passaging of undiluted virus. However, this phenomenon does not occur if a diluted inoculum is used.

1.1.4 Viral genomic and antigenic evolution

Human influenza viruses have evolved continuously because of changes to the host-parasite relationship that are caused by viral genetic, cellular, environmental and social factors (Smirnov et al 2000; Fauci 2006). After infection, influenza viruses face pressure from the host for antigenic change due to innate and adaptive immune responses. Further changes may also arise from increases in the use of antivirals and vaccines.

Influenza A viruses existed for a long time in other species before becoming established in humans. A mathematical model, based on the origin and evolution of HA genes, suggests that influenza A, B and C viruses first appeared about 2, 4 and 8 thousand years ago, respectively (Suzuki and Nei 2002), but were not established in human populations until only a few centuries ago. Influenza B and C viruses have evolved at significantly slower rates than influenza A viruses. Influenza B viruses do not infect animals and produce less severe respiratory disease than influenza A viruses; they only infect humans and are the cause of localised infections. Influenza C viruses are an infrequent cause of localised cold-like upper respiratory infections in humans. Influenza A viruses, on the other hand, have the capacity to evolve rapidly and produce a range of disease syndromes throughout the entire respiratory tract.

1.1.4.1 Genomic evolution of human influenza A virus

Origin of human influenza A viruses

Based on phylogenetic analysis of host-restricted genes, such as NP, PB2, M and NS from avian and human viruses, all mammalian influenza A viruses are thought to have originated from an avian influenza virus pool (Webster et al 1992 & 2007). All known HA and NA subtypes are continuously maintained in avian species, especially ducks and shorebirds (Figure 1.5).

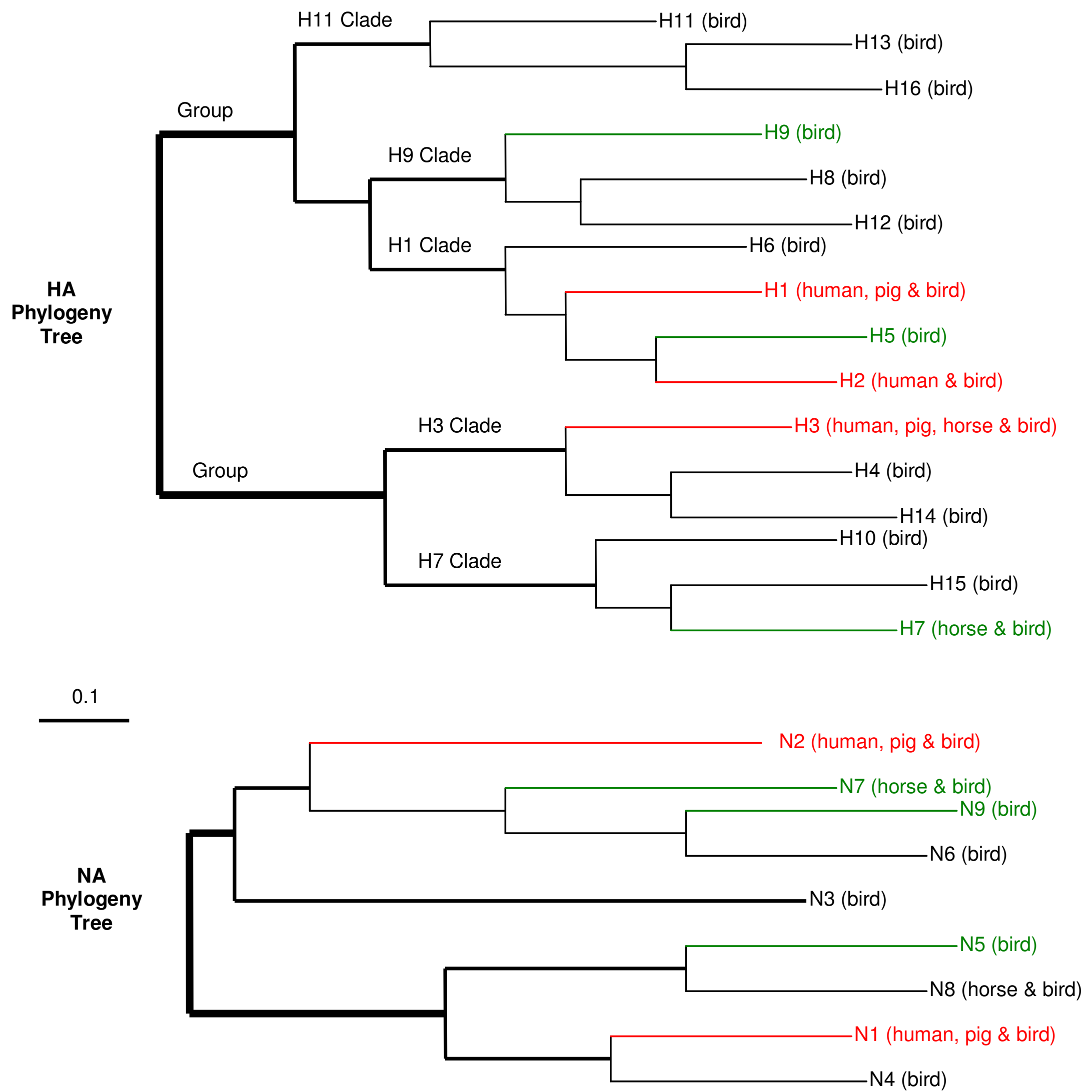


Figure 1.5 DNA maximum-likelihood trees of influenza A viruses (Fouchier et al 2005)

This dendogram was generated by comparing the nucleotide sequences of HA and NA sequences. The scale bars represent approximately 10% nucleotide changes between close relatives. The red arms indicate current circulating subtypes; green arms indicate potential pandemic subtypes.

Genomic evolution of human influenza A viruses

Only recent highly pathogenic avian influenza (HPAI) H5N1 viruses cause disease in wild aquatic birds. Many mutations in avian influenza viruses do not result in amino acid changes (Webster 2007). In contrast, aa substitutions have been detected frequently in all eight virus gene segments from mammalian or terrestrial bird isolates, including poultry (Scholtissek 1994, Widjaja et al 2004). These observations indicate that optimal adaptation of influenza A viruses in these species has not yet occurred (Lin et al 2000; Arora 2002; Choi et al 2004). Furthermore, the current *Influenza Genome Sequencing Project* has revealed diversity in the human influenza A genome. Multiple point mutations, deletions and segment exchanges have been detected in both surface and internal gene segments (Ghedin et al 2005). These mutational events have been reported previously in separate studies (Brown et al 1998; Barr et al 2003; Lindstrom et al 2004).

Among the eight gene segments, viral surface genes have evolved faster than internal genes due to the greater selective pressure they experience, resulting in a greater number of aa substitutions (Lindstrom et al 2004; Smith et al 2004). For example, 43% of all changes to the human H3 HA gene have resulted in aa changes, compared with only 10 and 4% for the PB2 and M1 genes (Webster et al 1992). However, the HA gene is subject to functional and structural constraints and aa substitutions occur mainly at HA antigenic sites (Gojobori et al 1994). These changes are linked to variations in viral antigenicity, which allow the virus to evade host immune responses.

1.1.4.2 Antigenic evolution in human influenza A viruses

Viral antigenicity is the primary criterion for vaccine strain selection and a main driving force in influenza A virus evolution (Ferguson et al 2003). As with the *phylogenetic tree* that has been used to measure genetic differences between strains, *antigenic mapping* had been developed to estimate the extent of immunogenic differences between strains (the so-called *antigenic distance*; Smith et al 1999). Mapping allows antigenic distances to be directly correlated with haemagglutination inhibition (HI) titres. One unit distance in the map corresponds to a twofold dilution of antiserum

in HI assays, two units to a fourfold dilution and three units to an eightfold dilution. When the antigenic distance between vaccine strains and epidemic strains is greater than two units, the influenza vaccine strains should be updated in order to achieve the maximum protection (Smith et al 2004). In a study where antigenic maps and phylogenetic trees of human influenza A H3N2 isolates from 1968 to 2003 were compared, it was demonstrated that antigenic changes occurred at irregular intervals, whereas genetic changes occurred continuously (Smith et al 2004). These findings confirm that not all of the nucleotide changes lead to aa substitutions and to consequent changes in antigenicity.

In summary, influenza A viruses are antigenically variable pathogens with great potential to evade host immune responses owing to their rapid mutation rates and broad host range. Human influenza A viruses have developed two strategies for varying their antigenicity in order to escape host immune defences, which are referred to as *antigenic drift* and *antigenic shift*.

Antigenic shift and influenza pandemics

Pandemic influenza occurs when a *new* influenza A virus variant emerges against which the entire human population has no pre-existing immunity. Three well-documented influenza pandemics have occurred in humans over the past century. The most significant of these occurred in 1918/19 (the so-called *Spanish* pandemic) which was caused by an H1N1 virus of avian origin that became adapted to humans through a series of point mutations (Taubenberger et al 2005). Based on recent phylogenetic analysis of the HA1 and M genes, the 1918 virus belongs to a clade, which is more closely related to avian strains than other human strains (Reid et al 1999 & 2002). In 1957, H2N2 influenza viruses entered human populations and were responsible for the *Asian* pandemic. The 1968 *Hong Kong* pandemic was caused by an H3N2 subtype; the H3 antigen had not been experienced by humans previously, but the N2 was derived from the *Asian* viruses.

Unlike the 1918/19 pandemic virus, both the 1957 and 1968 viruses arose through genetic reassortment between contemporary human and avian influenza A viruses. The 1957 H2N2 virus acquired PB1, HA and NA genes from an avian influenza A virus whereas, for the 1968 H3N2 virus, only the PB1 and HA genes were of avian origin (Wright and Webster 2001). Regardless of their different evolutionary paths, all three previous pandemic strains acquired an HA gene of an avian influenza virus that allowed them to infect humans unimpeded by any immunity from past infections.

Avian influenza H5N1 virus and the potential pandemic threat

Pandemic influenza A viruses of avian origin, especially H5N1 viruses, are of particular concern and have crossed the species barrier several times since 1997 (Hampson 2006; Thomas and Noppenberger 2007; Peiris et al 2007). Avian influenza H5N1 human infections caused more than 360 infections and 193 deaths worldwide by mid-2008 (WHO 2008). This situation could become dramatically worse if the infectivity of avian viruses for humans were increased by reassortment between the genes of human and avian viruses (Audsley and Tannock 2004). Such reassortment events, together with mutations at HA receptor binding site and/or in PB2 host-restriction gene, could lead to efficient replication in and transmission between human populations (Wright et al 2007). Fortunately, this has not yet taken place to date.

Co-infection of humans or an intermediate host with an avian strain and an existing human strain could produce new viruses of unknown pathogenicity, for which the entire population would be susceptible. The pig has been suggested as the host for co-infection (the *mixing vessel*) in the origin of new *shift* variants of influenza A viruses (Brown et al 1998; Xue et al 2007). However, during recent avian H5N1 outbreaks in humans, chickens appear to have played the key role in transmitting the viruses between wild birds (ducks) and humans (Gambaryan et al 2006).

Antigenic drift and seasonal influenza epidemics

In years subsequent to a pandemic, changes to the epidemic strains occur from immunological selection pressures caused, in part, by the high error rate of viral RNA replication. Circulating human influenza viruses do not appear to accumulate avian influenza virus genes during inter-pandemic periods (Shu et al 1996), but tend to change in adapting to humans. Mutations within the antigenic sites of HA and NA accumulate, resulting in changes to the antigenicity of both influenza A and B viruses (*antigenic drift*). Drifted strains may partially or completely evade neutralisation by antibodies induced by earlier infections. Variant viruses circulate in the general population, causing annual influenza epidemics of variable severity. Since 1977, two influenza A virus subtypes (H1N1 and H3N2) have co-circulated with influenza B viruses. Seasonal influenza viruses are responsible for 250,000-500,000 deaths throughout the world each year, mostly in older individuals (> 65 years), compared with pandemic influenza viruses which mainly targets young adults (aged 15 ~ 35 years) (WHO 2008).

1.2 HOST IMMUNE RESPONSES TO INFLUENZA A INFECTIONS

The course of a primary respiratory infection with Type A influenza virus is determined by the viral growth and spread and the development of immune responses (Doherty et al 1996 & 1997; Gerhard W et al 1997). Such immune responses (*innate* and *adaptive* immunity) are multifactorial and include the components for detecting infection and initiating defence reactions that lead to viral clearance. Innate and adaptive immunity are closely related, yet very different (Table 1.1). Innate immunity mediates antiviral effects at the earliest stages of primary infection and occurs before the optimum development of adaptive responses. Moreover, innate immunity provides regulatory signals for driving the appropriate cascades of adaptive subset responses that are required for viral clearance.

1.2.1 Host innate immune responses to influenza A virus infections

1.2.1.1 The respiratory tract as a physical barrier for the prevention of influenza infections

The respiratory tract can be considered as two parts of a single organ (1) the upper respiratory tract (URT) consisting of the nasal cavity and the pharynx; and (2) the lower respiratory tract (LRT) consisting of the trachea, bronchi, bronchioli, respiratory bronchioli and pulmonary alveoli (Tamura et al 2004). The surface of the entire tract is covered by a mucosal layer that contains epithelial and mucus-secreting goblet cells. This layer is primarily concerned with respiration (Iwasaki 2007).

Influenza viruses need to overcome host non-specific protective mechanisms present throughout the respiratory tract before they can infect respiratory epithelial cells. The respiratory mucin layer, ciliary action, and protease inhibitors are responsible for prevention of cell entry and virus uncoating (Wright et al 2007). In addition, local secretory macromolecules present in the fluid lining of the tract provide immediate inhibition of early viral growth and spread.

These mediators include: surfactant proteins A (SP-A) and D (SP-D), mannose-binding lectins, defensins, pentraxin-3 (PTX3), complement, entry blocker peptide and *natural* immunoglobulins, consisting of mainly IgM (Baumgarth et al 1999; Hartshorn et al 2003; van Eijk et al 2003; Gomi et al 2004; Hawgood et al 2004; Brown 2006; Jones et al 2007; Reading et al 2007). All of these mediators can inhibit infection by either neutralising or aggregating the viruses, or by binding to them and so enhancing the potential for viral phagocytosis.

1.2.1.2 The respiratory mucosal immune system

The respiratory tract mucosa is not only the primary site for influenza infections but also the site for the induction of protective immune responses. These responses are initiated at regional draining lymphoid structures, the mucosal-associated lymphoid tissue (MALT) located beneath the mucosal

layer. MALT from the URT is termed the nasopharyngeal-associated lymphoid tissues (NALT) in mice and Waldeyer's Ring (consisting of palatine and lingual tonsils, adenoid and paired tubal tonsils) in humans. In both mice and humans LRT, MALT consists of the inducible bronchus-associated lymphoid tissue (iBALT; Mayron-Quiroz et al 2004). Lymph from MALT drains to the secondary lymphoid organs, for instance, lymph from upper and lower respiratory MALT drains to cervical lymph nodes (LNs) and bronchial/ mediastinal LNs respectively.

NALT and iBALT play different roles in local responses against influenza infections. Earlier studies demonstrated that both NALT and cervical LNs are involved in the generation of local responses against influenza infections within the URT but are not essential for the induction of protection against viral challenge and viral clearance. However, the cervical LNs are essential in the induction of local immune tolerance against non-pathogenic antigens (Fokkens and Scheeren 2000; Wiley et al 2005). By contrast, iBALT functions as an inducible secondary lymphoid tissue, and generates protective immune responses throughout the entire course of an influenza infection (Nicod 1999; Mayron-Quiroz et al 2004). Detailed descriptions of their role in the induction of adaptive responses against influenza viruses were given in Section 1.2.2.

1.2.1.3 Mechanisms for signalling infections and initiating innate immune responses

Toll-like receptors (TLRs) are a family of trans-membrane proteins expressed by host cells that recognise pathogen-associated molecular patterns present early in infection, which trigger specific cellular responses (Seitz 2003). Eleven different TLRs have been described for humans, 12 for mice, with some (i.e.TLR2, 4, 6) being located on cell surfaces and others (TLR3, 7, 8, 9) on the ER (Boehme and Compton, 2004). Therefore, TLRs are capable of detecting pathogens that evade extracellular defences within the cell. Several complex signal transduction pathways can be activated after TLRs bind to their ligand. These pathways are mainly involved in the regulation of the host genes and are especially important in the production of inflammatory cytokines (Seitz 2003).

Table 1.1 Comparison of innate and adaptive immune responses to viral infections

	Innate	**Adaptive**
Reaction kinetics	Faster (non-specialised system; earliest times of primary infections)	Slower (specialised system: re-arranging gene segments to form antigen receptors; requirements for activation and expansion of a small pre-existing subset of antigen-specific cells)
Receptor diversity	Low (conserved receptors, broadly expressed, recognising non-protein viral products)	High (recognising viral protein products)
Pre-existing cell subset size	Large	Small
Cell types	Non-immune cells and innate immune cells: monocytes, monocytic cells (macrophages), DC, NK, PMN, neutrophil	T – and B – lymphocytes

DC: dendritic cells
NK: natural killer cells
PMN: polymorphonuclear leukocytes

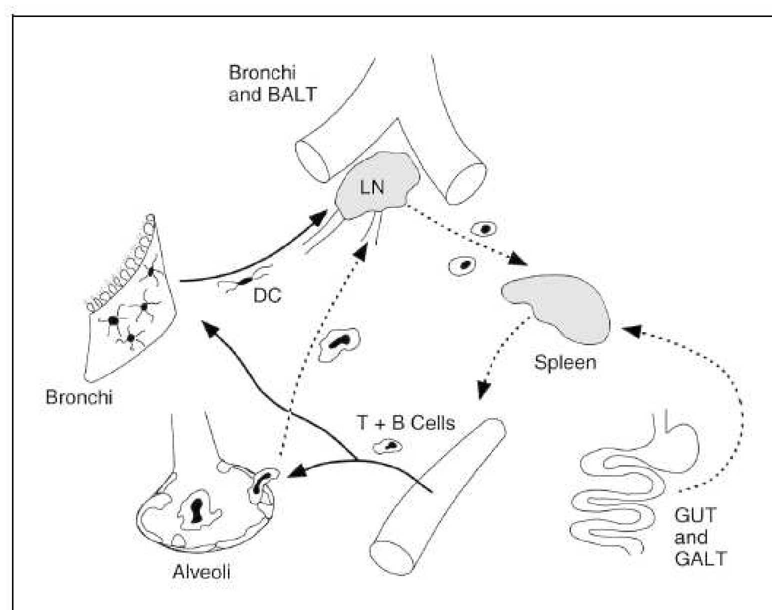


Figure 1.6 Circulation of dendritic cells (DC) from lung to lymphoid organs and recirculation of activated lymphocytes (Nicod 1999).

LN: lymph node
BALT: bronchus-associated lymphoid tissue
GALT: gastrointestinal-associated lymphoid tissue

TLR3 and -7 have been identified as major receptors for influenza viruses. They are both located within endosomal compartments where viral RNA is degraded. TLR3 is found constitutively expressed in human alveolar and bronchial epithelial cells, the primary targets for influenza virus infection. TLR3 is able to bind to viral replicative intermediate dsRNA and has a role in detecting events in influenza virus replication (Guillot et al 2005). By contrast, TLR7 is found in professional antigen-presenting cells (APCs), DCs and B cells and can identify influenza viral ssRNA prior to viral replication (Diebold et al 2004). Binding between TLR and viral RNA induces the interferon (IFN) pathway and other inflammatory cytokines that are involved in limiting viral replication and initiating innate immune responses (Lund et al 2004).

1.2.1.4 The effectors of innate immune response to influenza infection

Effector cells

There are two types of cells involved in innate responses, non-immune and innate immune cells. During influenza infections, non-immune cells, such as epithelial cells, are not only the main targets of virus infection but also the first line of host defence against infection.

- *Airway epithelial cells*

Following infection, epithelial cells undergo necrotic death (Gruta et al 2007). Cell necrosis triggers immune responses and the production of a number of chemokines, cytokines (i.e. IFNs, TNFs) and growth factors (i.e. GM-CSF). In response, epithelial cells up-regulate the expression of cell surface molecules, such as intercellular adhesion molecule-1 (ICAM-1) and the major histocompatibility complex (MHC). Through a complex signalling network, epithelial cells recruit and sustain innate immune cells at the site of infection. Moreover, they also have the capacity to directly present viral antigens to lymphocytes by MHC molecules (Nguyen et al 1998; Nicod 1999). Lastly, epithelial cells can facilitate the transport of specific secretory IgA (sIgA) within the airways following adaptive responses. The secretory process is initiated by poly Ig receptors (pIgR) which are expressed on the basolateral surfaces of the overlying epithelial cells (Tamura and

Kurata 2004). Beside non-immune cells, innate immune cells are involved in early responses to influenza infections. They are monocytes, alveolar macrophages (AMs), DCs, NK cells and neutrophils.

- *Alveolar macrophages (AMs)*

AMs comprise 85% of the cells in bronchoalveolar lavages (BAL) and are normally the only phagocytic cells present within the LRT (Nicod 1999). When a large amount of progeny virus is released from infected respiratory epithelial cells, AMs can also be infected. Within 24-48 hr after infection, infected AMs undergo apoptosis, which induces inflammatory cytokine and chemokine production in the LRT (Gruta et al 2007). The amount of cytokines produced by AMs has been shown to be proportional to the extent of virus shedding (Tamura and Kurata 2004). These cytokines are also responsible for fever and NK cell activation.

- *Dendritic cells (DCs)*

Over the past 35 years, DCs have been studied intensively because of their unique ability to prime naïve T cells (Banchereau and Steinman 1998; Reischl 2000). They also have a central role in initiating and modulating protective T-cell responses during respiratory infections (Dupuis and McDonald 1997; Andersson and Ahmed 2002). Respiratory DCs migrate continuously throughout the DC network (Figure 1.6). In the URT, DCs are present both within and beneath the epithelial layer but, in the LRT, they are also present in the lamina propria and alveolar lumen (Iwasaki 2007).

Many studies have indicated that DCs from different organs or parts of a single organ have different functions. For example, URT DCs have a rapid turnover time (< 72 hr), with 85% of the cells being renewed every 24-36 hr (Lambrecht et al 1996; Iwasaki 2007). During the early phase of pulmonary infections, URT DCs demonstrate an accelerated rate of migration towards the regional LNs. They occur in bronchial LNs as early as 6 hr (peak at 24-28hr) after encounter with

antigens in the airways mucosa (Hamilton-Easton and Eichelberger 1995). By contrast, DCs in the LRT have a much slower basal turnover rate and a weaker T-cell priming ability, possibly because of the inhibition of regulatory signals from AMs (Lambrecht et al 1996; Iwasaki 2007). At the commencement of a respiratory infection, such a lung antigen-tolerant microenvironment would help to prevent the development of immunopathology.

DCs reside in tissues as immature APCs but are highly efficient at facilitating antigen uptake. In the case of influenza infections, the maturation of airway DCs is initiated via TLR3, which can recognize viral dsRNA. During the process of maturation, the expression of co-stimulatory molecules and the MHC on the cell membranes of DCs increases but the capacity for antigen uptake is reduced (Reischl 2000). Following arrival in lymphoid tissues and under the influence of tumor necrosis factor (TNF-α) and GM-CSF, mature DCs are able to present antigen to and prime naïve T cells in an efficient manner (See Section 1.2.1.5 for more details on influenza viral antigen presentation).

However, the accelerated migration of respiratory DCs to the regional LNs occurs only during the early phase of respiratory infections. Studies with influenza infections have shown that activated respiratory DCs can continually accumulate at secondary LNs, such as cervical and bronchial/mediastinal LNs, and reach their peak by 18 hr post-infection. Then migration of DCs from infected airways and lungs to their draining LNs ceases by 48 hr, despite sustained virus replication in the lungs (which occurs up to 5-6 days). These observations indicate that such a transitional refractory state exhibited by respiratory DCs may lead to a diminished host adaptive response to a second stimulus with the same antigen (Legge and Braciale 2003).

- *Natural killer (NK) cells*

NK cells are large granular lymphocytes with increased lytic activity, and appear in the lungs 48 hr after influenza infections. They are IFN-γ producers and can lyse infected cells by a perforin-

dependent pore-forming mechanism (Brown 2006). However, their role in virus clearance is less important than T cells and neutralizing antibody (Ab), both of which appear later.

- *Neutrophils*

Neutrophils are also called PMN because of their multilobed nuclear appearance. Neutrophils are the most abundant immune cells in the body and constitute 60% of the circulating leukocytes (Fokkens and Scheeren 2000). However, they represent less than 2% of the cells in BAL (Nicod 1999). Activated neutrophils kill microorganisms or infected cells by a range of mechanisms, which include phagocytosis, the release of oxygen radicals and the production of cytotoxic peptides or proteins (Fokken and Scheeren 2000). In addition, toxic agents that are released from neutrophils can also cause tissue oxidative damage and inflammation.

There is only a brief transient increase in neutrophils in the lungs during mild influenza infections, and extravasation of neutrophils into the alveolar space is mediated by ICAM-1 receptors located on the alveolar epithelial cells. However, when cells in the alveoli are unable to restrain an infection, a massive influx of neutrophils with accompanying tissue damage can occur, which was frequently observed in post-mortem lung samples taken during both the 1918 pandemic and the more recent avian H5N1 outbreaks.

Chemokines and cytokines

Following influenza infection, epithelial cells and AMs die by necrosis/apoptosis which triggers the production of a range chemokines and cytokines. Levels of chemokines and cytokines in human nasal lavage fluids peak at day 2-3 post-infection (Table 1.2; Fritz et al 1999). These chemokines and cytokines play a major role in the control of infections. They are also involved in the induction of influenza-like symptoms (i.e. the sudden onset of malaise and fever, followed by upper and lower respiratory symptoms, myalgia and headache).

- *Chemokines*

Chemokines are small proteins which are released from a range of cells. At least 50 proteins have been identified within the human chemokine superfamily. They can be separated into two major groups, according to whether they have regulatory and inflammatory functions. Regulatory chemokines are constitutively produced at homing sites by specialised cells. By contrast, inflammatory chemokines of the CC and CXC subfamilies are found only where pathology is present and are responsible for modulating the recruitment of leukocytes to infected tissues (Kaufmann et al 2001). CC chemokines preferentially act on mononuclear cells, but most CXC chemokines favour neutrophils (Table 1.2; Fritz et al 1996; Lazarus et al 2003; Singh et al 2004). Recruitment and activation of leukocytes by chemokines also relies on the expression of specific chemokine receptors (CCR or CXCR) on the target cells (Kaufmann et al 2001; Debes et al 2004).

Induction of the chemokines (i.e. MCP-1, MIP-1α/β, IP-10 and RANTES), which attract mononuclear leukocytes, occurs within 2 days of an influenza infection. However, no significant production of neutrophil-attracting chemokines (IL-8 and GRO-α) occurs at the same time (Fritz et al 1999; Kaufmann et al 2001; Wareing et al 2004). These observations provide confirmation that infiltration of mainly monocytes and lymphocytes to infected tissues occurs during the early stages of influenza infections.

An earlier study with live and killed influenza viruses demonstrated that the secretion of inflammatory chemokines was related to the nature and extent of viral replication (Kaufmann et al 2001). For example, viral RNA replication was necessary for the maximal MIP-1α production. By contrast, the intact HA proteins, which are responsible for virus adsorption, endocytosis and fusion, were found to be sufficient for initiating the maximal MCP-1 production. Therefore, gradual chemokine release which depends on specific events in virus replication is necessary for an efficient antiviral response.

Table 1.2 Links between influenza infection and chemokine/cytokine-mediated immunopathogenesis (La Gruta et al 2007)

Chemokine /cytokine *	***Full name***	***Function***	***Produced by***
MIP-1β (CCL4)	macrophage inflammatory protein 1β	Monocyte, T cell chemoattractant ; activates neutrophils	Monocytes/macrophages, neutrophils, T cells, dendritic cells
MIG (CXCL9)	monokine induced by IFN-γ	Monocyte, T-cell chemoattractant	Respiratory epithelium, monocytes/macrophages
IP-10 (CXCL10)	interferon-inducible protein of 10kD	Monocyte, T-cell chemoattractant	Monocytes/macrophages, T cells, respiratory epithelium
RANTES (CCL5)	regulated up after activation, normal T cells expressed and secreted	Monocyte, T cell, DC chemoattractant. Activates T cells	T cells, respiratory epithelium
IL-8 (CXCL8)	interleukin 8	Neutrophil, T-cell chemoatrractant. Activates neutrophils	Respiratory epithelium, monocytes/macrophages, neutrophils
IFN-γ	interferon γ	Inhibits viral replication. Stimulates CTL-mediated killing. Increases MHC I expression. Activates macrophages and neutrophils. Promotes T-cell proliferation	T cells, NK cells
TNF-α	tumor necrosis factor α	Direct antiviral effects. Neutrophil chemoattractant. Stimulates macrophage phagocytosis and production of IL-1. Increases vascular permeability	T cells monocytes/macrophages, dendritic cells, neutrophils
IL-1	interleukin 1	Increases expression of adhesion factors on endothelium Increases vascular permeability Stimulates IL-6 production	Monocytes/macrophages, dendritic cells
IL-6	interleukin 6	Pro-inflammatory cytokine. Activates T cells.	Respiratory epithelium, T cells, dendritic cells, monocytes/macrophages

* Cytokines and chemokines listed here are induced by highly pathogenic influenza viruses in human and animal models. They are associated with extreme pathological damages (La Gruta et al 2007).

- *Cytokines*

Soon after intranasal influenza virus infections of humans or animals (i.e. mice and pigs), levels of proinflammatory cytokines, such as IFNs (especially type I IFN-α/β), TNF-α and interleukin-6 (IL-6), increase in nasal lavage fluids (Fritz et al 1999; Kaiser et al 2001; van Reeth et al 2002). Of these, IL-6 appears first and has proven to be one of the most reliable indicators of infections. IL-6 is a pro-inflammatory pleiotropic cytokine. It can induce terminal B cell differentiation and is also involved in activating type II immunity, leading to antibody production (Figure 1.8). TNF-α is a well-known mediator of local inflammatory reactions in the lungs (Table 1.2) and is responsible for gross lung lesions, weight loss and mortality (van Reeth et al 2002).

The IFN response is an important component of innate immunity because it is responsible for early anti-viral activity. IFNs were discovered in 1957 from studies on influenza virus-infected chicken cells (Isaacs and Lindenmann 1957). There are two types of IFN (I and II; Samuel 2001). Type II IFNs (i.e. IFN-γ) are secreted by NK cells and T cells (Table 1.2). Compared with type II IFN (IFN-γ), which is induced as a component of both innate and adaptive responses, type I IFNs (IFN-α/β) are only part of the innate response. In a study of human experimental influenza infections, high IFN-α/β titres were detected by day 1 after the commencement of virus shedding (Firtz et al 1999). Type I IFNs were secreted by infected host epithelial cells, macrophages, DCs and monocytes in the response to the presence of viral RNAs (Kadowaki and Liu 2002).

All type I IFNs have a common cell surface receptor. Binding between IFN-α/β and their receptors can result in the transcriptional stimulation of about 400 host genes (Garcia-Sastre 2001). Of them, those encoding the dsRNA-activated protein kinase (PKR), the 2'5'-oligoadenylate synthetase (2-5A synthetase) and the Mx proteins are known to interfere with viral replication and induce antiviral activity in nearby uninfected cells. In addition to their antiviral activities, both type I and II IFNs are involved in regulating cell growth, differentiation, apoptosis and modulating antigen-presentation by elevation of MHC expression on the surface of APCs (Samuel 2001). Besides IFN-

α/β, other inflammatory cytokines which are produced primarily by macrophages at the commencement of viral infections also play a role in the coordination of innate and adaptive immune responses (Fig 1.9; Murtaugh and Foss 2002). Their detailed functions are beyond the scope of this thesis and will not be discussed further.

1.2.1.5 Presentation of influenza viral peptide antigens to lymphocytes

Pulmonary antigen-presenting cells (APCs)

As mentioned above, APCs are part of the innate immune system and are also essential for the initiation of the adaptive responses to viral infections. APCs serve as a bridge linking innate and adaptive responses by (1) regulating T lymphocytes by the secretion of cytokines and chemokines, and (2) presenting peptides from viral antigens to T lymphocytes (Kadowai and Liu 2002). Non-immune cells which can be infected by influenza viruses have the capacity for antigen presentation and include both airway epithelial cells (Section 1.2.1.4) and M cells.

- *Membranous (M) cells of mucosal-associated lymphoid tissues (MALT)*

M cells are present in the epithelium between epithelial, columnar and mucous goblet cells, and above the MALT. After infection of human adenoid tissues with influenza A viruses, it has been shown by transmission electron microscopy that virions are endocytosed by M cells and then transported to surrounding macrophages, dendritic cells and lymphoid cells. This whole process occurs 30-90 min after infection (Fujimura et al 2003). Similar findings had also been demonstrated in studies in mice using a range of different mucosal pathogens, including Group A *streptococci*, reoviruses, poliovirus and HIV (Sminia and Kraal 1999; Park et al 2003). These findings suggest that M cells of the MALT are responsible for antigen presentation to the underlying lymphoid tissues during mucosal infections. They also suggest that the respiratory MALT, together with cervical and bronchial/mediastinal LNs, are the sites where antigen presentation takes place and where naïve T cells are primed.

APCs, AMs, DCs and B cells can present antigen to T lymphocytes more efficiently than non-immune cells, such as airways epithelial and M cells. These cells and especially DCs and B cells, the so-called professional APCs, can express MHCs and co-stimulators on cell surface which are required for initiating T cell proliferation (Hare et al 2003; Reinders et al 2003).

- *Lung dendritic cells (DCs)*

DCs are the most potent APCs and are present in most tissues. They capture antigens *in situ* and migrate to lymphoid organs where they activate naïve T cells (Cella et al 1996). After isolation from enzyme-digested murine lungs and BAL, mature DCs were shown to possess a lower buoyant density and to be loosely adherent, nonphagocytic and heterogeneous (Pollard and Lipscomb 1990). These DCs express costimulatory molecules including CD80, CD86, CD40L, CD2, CD54, and CD11a, which play important roles in lung DC-initiated T cell proliferation (Masten et al 1997). An earlier study showed that the numbers of MHC Class II-bearing lung DCs in mice were increased after treatment with INF-γ (Suda et al 1996).

Airway DCs are responsible for the uptake and processing of antigens and the consequent presentation of antigenic peptides to T lymphocytes. Beside their ability to capture virus directly, DCs also have the capacity to acquire antigens derived from necrotic or apoptotic cells, a process called *cross-priming* or *cross-presentation*. Cross-priming can generate virus-specific CD4 as well as CD8 cytotoxic T lymphocyte (CTL) responses (Abbas and Lichtman 2003), and is especially important for antigen presentation in the LRT. In contrast to URT DCs, those within the lung parenchyma do not readily engulf particulate antigens, including virus particles (Nicod 1999). By cross-priming, lung DCs can present antigenic peptides which are processed by lung interstitial macrophages or AMs to T cells (Gong et al 1994). Cross-priming is regulated by AMs.

When there is only a small number of mature DCs in lungs following infection, AMs tend to enhance the function of DCs, in part by releasing antigenic peptides; their ability to present antigen

to lung parenchyma DCs is only modestly increased following INF-γ stimulation. When more than 50% of lung DCs became mature, AMs start inhibiting DC function; probably by releasing soluble inhibitors (see Section 1.2.1.3). This change, from stimulation to inhibition, could prevent excessive immune responses and tissue damage in the lungs.

- *B cells*

B lymphocytes use their membrane Ig (mIg) as antigen receptors (so-called BCR) to bind and internalise soluble protein antigens and then to present processed peptides to T helper (T_H) cells. Because of their high affinity for antigens, B cells can effectively capture protein antigens that are present at very low concentrations in the extracellular fluid. The antigen-presenting function of B cells is essential for T_H-dependent Ab production, such as anti-HA and NA Abs, to influenza viruses (Abbas and Lichtman 2003).

Mechanisms of antigen presentation

- *The Major Histocompatibility Complex (MHC)*

MHCs were recognised initially as self-recognition proteins in graft rejection, but are now known to be integral parts of all immune response systems (Danchin et al 2004). The MHC region is a very large (2-4 Mb) multigene cluster within the cell genome and is among the most polymorphic genes of vertebrates (Parham and Ohta 1996). More than 881 different MHC class I alleles have been identified in humans (Marsh et al 2002). Due to the extreme polymorphism within the peptide binding region, individuals with different MHC molecules respond to different antigenic peptides derived from the same pathogen. MHC variation is found at both the population (allelism) and individual (many loci) levels. Some studies have suggested that populations with limited MHC diversity are more susceptible to viral infections (Evans et al 1996; Borghans et al 2004).

There are at least four sets of genes encoded within the MHC: (1) Class I MHC genes that are responsible for self-recognition. The classical Class I molecules are called H-2K, H-2D and H-2L in mice and HLA-A, HLA-B and HLA-C in humans. Class I MHC molecules present short

peptides (usually 8-10 aa) to $CD8^+$ T cells (Figure 1.7A), which can be induced to become CTLs that identify and destroy infected cells. (2) Immune response (*Ir*) genes encoding the Class II MHC molecules that determine the strength of an immune response. MHC Class II molecules present peptides derived from exogenous antigens to the MHC Class-II-restricted T_H cells. (3) *Tla* and *Qa* genes that encode cell surface proteins structurally similar to Class I MHC molecules (hence, often called non-classical Class I molecules) whose function is relatively unknown. (4) Genes that encode many components of the *complement system*, which is part of the innate immune system.

Class I molecules consist of two polypeptide chains, one of which is highly polymorphic (the α *chain*, containing a single peptide-binding cleft) and the other an invariant polypeptide, β-2 microglobulin (β_2m). Class I molecules are constitutively present on the surfaces of nearly all nucleated cells. By contrast, Class II molecules consist of two polymorphic chains and are found mostly on the surfaces of APCs (DCs, B lymphocytes and macrophages). However, the stable expression of MHC molecules on cell surfaces requires a bound antigenic peptide that stabilizes the MHC molecules (Figure 1.7). Many studies have also demonstrated that the cytokines secreted during innate and adaptive immune responses (i.e. IFNs and TNF) can enhance MHC expression by stimulating the transcription rate of Class I and II genes. The MHC molecule and antigenic peptide form the ligand for T cell receptors (TCRs). Binding of TCR to its ligand initiates adaptive immune responses.

- *T cell receptors (TCRs)*

The broad peptide-binding specificity of MHC molecules from a single host does not allow differentiation between foreign and *self* peptides (Barinaga 1992; Matsumura et al 1992; Parham 1992). The detection of foreign peptides is largely dependent on antigen receptors on the surface of T lymphocytes (TCRs). TCRs can only respond to antigens that have been processed and presented by APCs *via* MHC molecules (the phenomenon of *MHC-restriction*; Rudolph et al 2006; Figure

1.7B). Different T cell clones express different TCRs which recognize different antigenic peptides derived from the same pathogen (i.e. they are clonally distributed). Each TCR is a heterodimer that is comprised of a variable αβ complex (responsible for the antigen binding) and an invariant complex, CD3 (responsible for signaling) (Davis et al 1997).

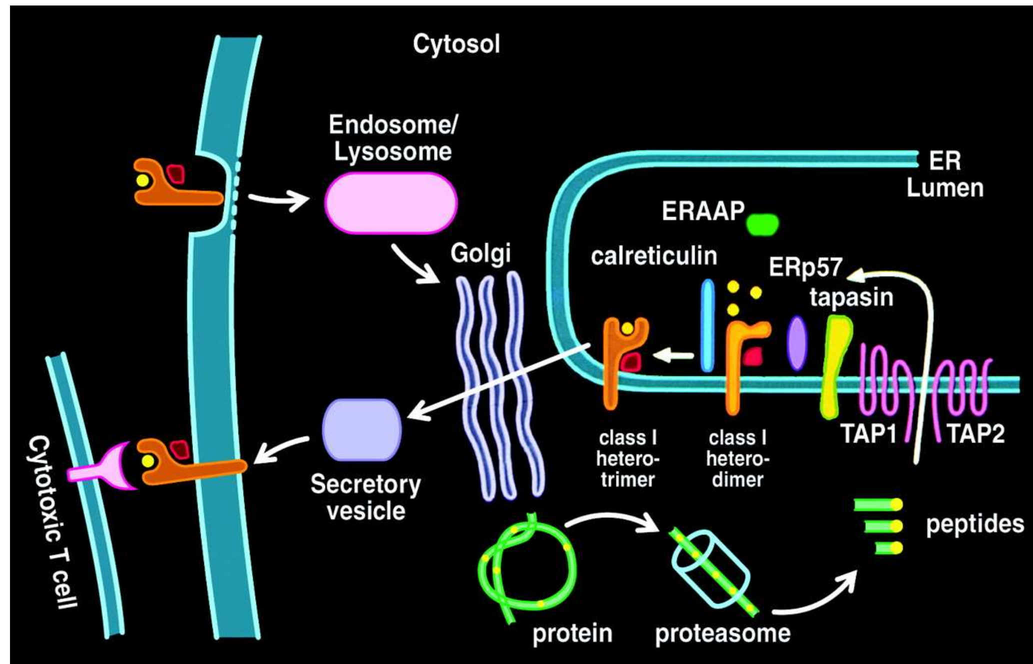
Unlike BCRs, which can recognize conformational determinants of protein antigens, TCRs only recognize linear peptide antigens. Accordingly during influenza infections, neutralizing Abs (i.e anti-HA and NA) are capable of distinguishing different subtypes whereas CTLs (NP or PA peptide-specific) are not (Ada 1994). However, an earlier study demonstrated that TCRs, especially those specific for NP, were mainly responsible for the CTL cross-subtype responses to influenza A viruses (Townsend and Skehel 1984). The reasons for this are: (1) viral internal proteins, such as NP, are relatively conserved proteins; (2) inherent properties of the αβ TCRs allow a degree of flexibility in peptide binding (Parham 1992; Mason 1998).

Apart from the cross-reactivity, TCRs have sufficient specificity to discriminate between many different antigenic peptides. *In vitro* studies had shown that TCRs can recognize multiple peptide ligands, but only those with the highest TCR affinity could fully activate T cells at low peptide concentrations (Demotz et al 1990). Because only limited amounts of antigenic peptides are present on the surfaces of host animals, cross-reactivity from T cells is restricted and the fine specificity of CTL recognition is only maintained to a limited extent (Burrows et al 2000).

- *Use of MHC tetramers in the measurement of T cell responses*

Altman et al (1996) first described the application of MHC tetramers in the measurement and analysis of Ag-specific T cell responses. CTLs, which were first described in 1968 (Brunner et al 1968), can now be directly phenotyped and enumerated without the need for a functional assay. Tetramer-based methodologies have further applications in studies of autoimmune disorders, transplantation, cancer and vaccination (Doherty 1998 & 2002; Klenerman et al 2002). They have

A



B

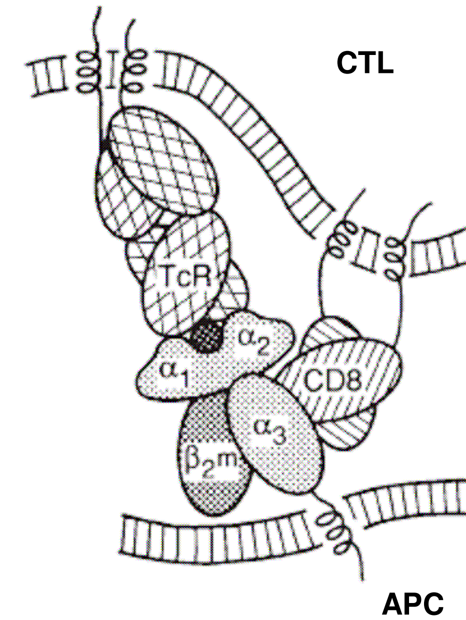


Figure 1.7 Diagram of the MHC Class I assembly and the peptide presentation (**A**, Petersen et al 2003). MHC Class I α chain and β_2m assemble with a peptide in a multimeric complex with calreticulin, ERp57, tapasin, and the TAP heterodimer in the ER. Once the peptide is bound, the complete MHC Class I molecule is released from ER and proceeds through the Golgi apparatus by vesicular transport. The MHC Class I complex reaches the cell surface where the peptide can be presented to CTL. After arrival at cell surface, certain virus proteins can undergo endocytosis. The cartoon shows interaction of TCR and CD8 co-receptor with the peptide-MHC I complex (**B**, Partham 1989).

been particularly useful for studies of virus-specific CTL responses to a range of viral infections, including influenza, HIV, viral hepatitis, Epstein-Barr virus, cytomegalovirus, human papillomavirus and herpes simplex virus infections (Ogg et al 1998; Kuroda et al 1998; Skinner et al 2000; Burrows et al 2000; Keiholz et al 2002).

The key reagent for the staining of Ag-specific T-cells is the MHC tetramer, which forms the TCR ligand. Fluorescent tetramers are comprised of four identical MHC molecules, each presenting an antigenic peptide (Figure 1.8a). Details of their preparation are given in Materials and Methods (Section 2.11). Briefly, complexes of soluble MHC α chain, β_2m and the peptide of interest are first assembled as monomers and then biotinylated at the terminus of α chain. The addition of fluorochrome-labelled streptavidin (containing four biotin-binding sites) leads to the formation of a final product, consisting of tetrameric peptide-MHC complexes or *tetramers* (Pittet et al 2001, Vollers and Stern 2007).

Before use, the newly generated tetramers need to be tested for the specificity and their optimal concentrations are determined. These pre-test procedures are critical for reducing non-specific background signals and for enhancing the specific fluorescent signals that can be measured by flow cytometry. In a two colour system, such as one using anti-CD8 and MHC I tetramers for cell staining (Figure 1.8b), cells which stain positively with both CD8 and MHC I tetramers correspond to an antigen-specific CTL population (Figure 1.8c). When additional cell surface molecules are stained, further analysis of T cell subsets can be achieved in a single assay (multi-colour flow cytometry; see Section 1.2.2.2).

The affinity of MHCs for their TCR ligands is greatly enhanced by the use of four MHC-peptide complexes (tetramers), compared with a single monomer complex for the detection of low-frequency T cell populations. The lower limit for detection by tetramers is approximately 0.01% of the total cells screened by flow cytometry (Dunbar et al 1998). Any smaller specific cell

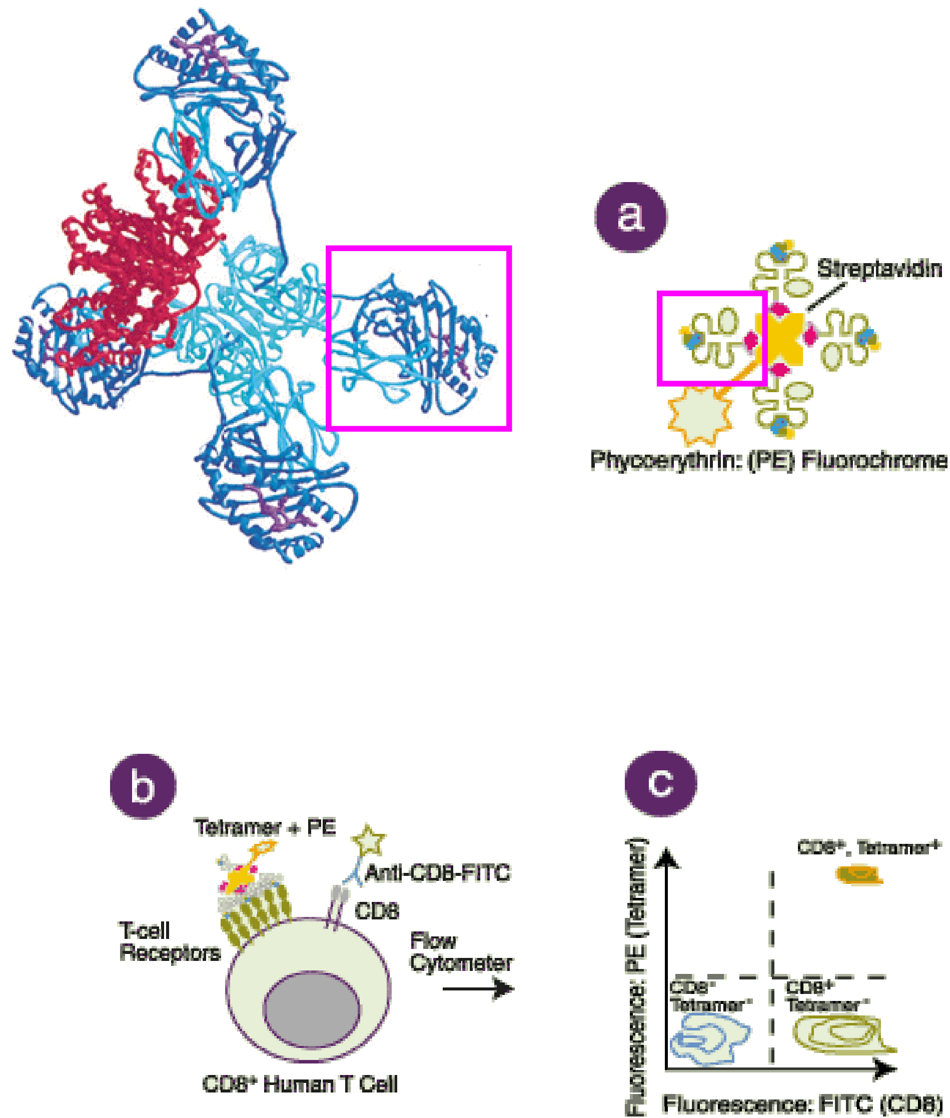


Figure 1.8 MHC tetramer staining for visualising antigen-specific T cells (McMicheal and O'Callaghan 1998; Beckman Coulter Website 2003).

(a) Schematic representation of a human MHC class I tetramer.The monomer is shown in the pink box;

(b) Complexes are bound to a distinct set of T cell receptors (TCRs) on a subset of human CD8^{+} T cells;

(c) The cellular response against one specific peptide or epitope can be detected by flow cytometry.

population cannot be distinguished from background non-specific staining (Pittet et al 2001; Keiholz et al 2002; Vollers and Stern 2007). Class I MHC tetramer staining is now commonly used to stain antigen-specific $CD8^+$ T cells directly in peripheral blood samples (Vollers and Sterm 2007). By contrast, Class II MHC tetramers are less commonly used because of low TCR-tetramer avidity and the low frequency of specific $CD4^+$ T cells (Klenerman et al 2002; Lucas et al 2004).

Tetramer-based assays are now amenable to standardization and can be used in the combination with well-established functional assays, such as the cytokine enzyme-linked immunospot (ELISPOT) and the intracellular cytokine cytometry (ICC) assays, (especially the latter) which are more time consuming and require *in vitro* stimulation with specific antigens (Murali-Krishna et al 1998; Riberdy et al 2001; Keilholz et al 2002).

- *Immunodominant epitopes of influenza A viruses*

When an individual is immunised or infected with a live virus (i.e. an influenza virus), the majority of the responding T cells recognise very few processed linear amino acid sequences from viral proteins (Thomas et al 1995; Abbas and Lichtman 2003). These linear sequences are termed the immunodominant epitopes.

Factors that contribute to immunodominance are: (1) IFN-γ production, (2) their MHC binding affinity, (3) the available TCR repertoire, (4) the epitope abundance and (5) the capacity of the target cells to process antigens, such as the cleavage patterns of whole proteins into small peptides that can bind to MHC (Boon et al 2002; Chen et al 2004; La Gruta et al 2006; Kedzierska et al 2004 & 2006; Jenkins et al 2006; Thomas et al 2007). The underlying mechanisms that govern these factors are largely unknown and are under intensive study (Sette and Sundaram 2006).

In contrast to natural infections, no obvious patterns of immunodominance have been observed following immunisation with inactivated virus by the parenteral route (Thomas et al 1995). In an

earlier study in mice, an even distribution among nine antigenic epitopes of HA1 was observed following injection with inactivated influenza viruses, whereas only two epitopes were identified following intranasal (i.n) immunisation with live attenuated influenza viruses (Thomas et al 1995).

Immunodominant epitopes are the main determinants of cellular immune responses. Most Class I MHC binding epitopes consist of 8-11 residues; Class II binding epitopes consist of 10-30 residues (Brown et al 1993). Pathways of assembly for MHC molecules ensure that Class II molecules preferentially display peptides that are derived from extracellular proteins and are taken up by vesicles in APCs. Class I molecules present peptides from cytosolic proteins. Consequently, $CD4^+$ T cells (i.e. T_H cells) mainly respond to epitopes derived from extracellular proteins, such as the influenza surface glycoproteins HA and NA. $CD8^+$ T cells (i.e. CTLs) respond mainly to epitopes from the cytosol, which include the influenza virus internal proteins NP, PA, PB, M and NS (Bennink et al 1987; Thomas et al 1995; Chen et al 2003).

However, there were several earlier reports that CTL-epitopes derived from influenza HA were not responsible for the cross-subtype responses (Bennink et al 1986; Spencer and Braciale 2000). More recently, a dominant T_H-epitope from the influenza A NP protein has been identified, in addition to the HA peptide (Table 1.3; Crowe et al 2006). However, downstream antibody responses to NP, alone, are not protective whereas antibodies to HA and NA are (Wright et al 2007).

Identification of influenza T cell epitopes, and especially conserved epitopes shared by different viral strains (*drift* viruses) or sub-types (*shift* viruses), is crucial for an understanding of host homosubtype and heterosubtype immunity to influenza infections (see also Section 1.2.2.3). However, until the recent application of MHC tetramer technology, screening for T cell epitopes was difficult for studies with humans, although possible with inbred mice (Crowe et al 2006; Wang et al 2007).

Most studies on T cell responses to influenza viruses have been conducted with A/PR8 (H1N1) and/or A/HK×31 (H3N2) in C57BL/6 mice. The A/HK×31 reassortant has the same six internal genes as A/PR8. Following primary infection with A/HK×31, a total of 16 Class I and 12 Class II epitopes have been identified (Crowe et al 2006). Within them, $NP_{366-374}$, $PA_{224-233}$ and $PB1_{703-711}$ are Class I immunodominant epitopes; whereas $HA_{211-225}$ and $NP_{311-325}$ are Class II immunodominat epitopes. A similar pattern of CTL immunodominance had been observed after secondary i.n infection with A/HK×31 following primary intraperitoneal infection with A/PR8 (Table 1.3; Doherty 2007). No epitopes were identified from NA protein of A/HK×31 in C57BL/6 (H-2^d) mice, but multiple epitopes derived from NA were found during a parallel screen in BALB/c (H-2^d) mice (Crowe et al 2006).

Identification of influenza T cell epitopes in humans is far more difficult than for inbred mice, due to the highly polymorphic nature of the HLA. At least 615 Class I isoforms are present among the human homologues HLA-A, -B, -C; and they can be grouped into 9 different *supertype*s with overlapping peptide binding specificities (Block et al 2003; Boon et al 2002). A peptide that binds to one allele within a supertype has a high probability of binding to other allelic members of the same supertype.

Using search tools involving bioinformatics, Wang et al (2007) predicted that 167 HLA-I peptides were derived from A/PR8, 13 of which were confirmed as CTL targets by INF-γ ELISPOT assays (Table 2.3). These 13 CTL-epitopes are highly conserved among human influenza A viruses and are also present in recent isolates of avian influenza viruses (Wang et al 2007). These findings confirmed the earlier view that critical epitopes should be included in vaccines that are capable of stimulating broad protective responses to *drift* and *shift* influenza viruses (Ben-Yedidia et al 1999; Thomas et al 2006).

Table 1.3 Influenza virus A/PR8/34 (H1N1) – derived immunodominant epitopes at C57BL/6 mouse lung and human peripheral blood mononuclear cells (PBMCs) after *in vivo* and *ex vivo* infection.

Host	MHC Class I restricted cytotoxic T lymphocyte (CTL) epitopes	MHC Class II restricted T helper (T_H) epitopes
Inbred mice C57BL/6	D^b – restricted $NP_{366\text{-}374}$, $PA_{224\text{-}233}$ and $PB1\text{-}F2_{62\text{-}70}$ K^b – restricted $PB1_{703\text{-}711}$, $M1_{128\text{-}135}$ and $NS2_{114\text{-}121}$	A^b – restricted $HA_{211\text{-}225}$ & $NP_{311\text{-}325}$
Humans	HLA-A1 – restricted $PB1_{591\text{-}599}$ and $NP_{44\text{-}52}$ HLA-A2 – restricted $PB1_{166\text{-}174}$ and $M1_{58\text{-}66}$ HLA-A26 – restricted $PB1_{41\text{-}49}$ HLA-B7 – restricted $PB1_{540\text{-}548}$ HLA-B8 – restricted $NP_{225\text{-}233}$ and $PA_{601\text{-}609}$ HLA-B27 – restricted $PB1_{349\text{-}357}$ and $NP_{383\text{-}391}$ HLA-B39 – restricted $M1_{173\text{-}181}$ HLA-B58 – restricted $NP_{199\text{-}207}$ HLA-B62 – restricted $PB1_{566\text{-}574}$ and $PB1_{347\text{-}355}$	Not been reported to date

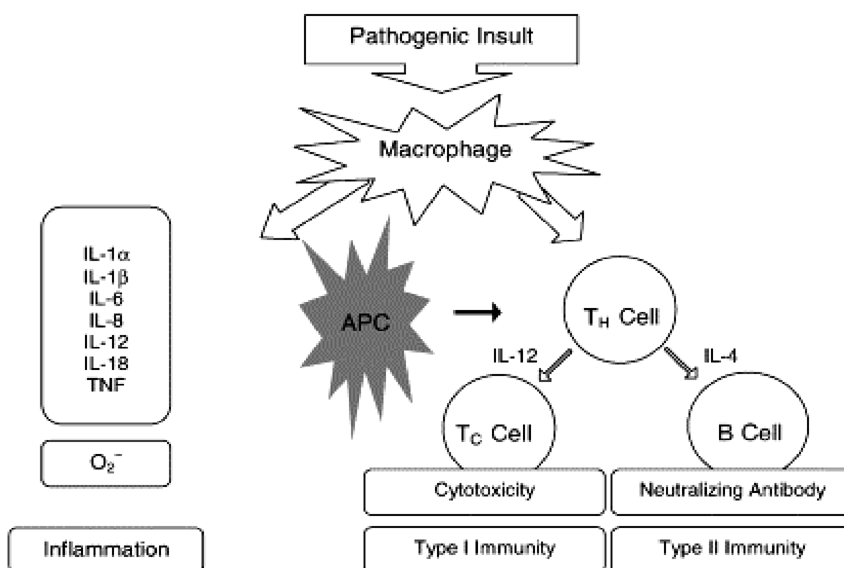


Figure 1.9 Host responses to proliferating pathogens (Murtaugh and Foss 2002). The response is characterised by an immediate non-specific innate response and a prolonged antigen-specific adapted response (type I or/and II immunity). During innate immune responses against infection, cytokines produced by macrophages or DCs play a central role in regulating the downstream adaptive immune responses.

APC: antigen-presenting cell
T_H cell: helper T cell
T_C cell: cytotoxic T cell

1.2.2 Host adaptive immune responses to influenza A viruses infections

Following the presentation of antigenic peptides derived from influenza A viruses to T cells via APCs, specific adaptive immune responses (humoral and cell-mediated; local and systemic) can be initiated (Figure 1.9). Specific neutralizing antibodies and CTLs generated by adaptive responses contribute to viral clearance, recovery from the disease and complete or partial protection against re-infection.

Current understanding of immune responses (especially CMI responses) has largely come from the studies using inbred mice, which have also been used extensively for the studies of viral adaptation and virulence. However, such models do not always provide an indication of the situation in primed and outbred human populations (Eichelberger et al 2006). For instance, most mouse strains used in the laboratory lack biologically active Mx gene products (Staeheli et al 1988), which inhibit primary transcription of the viral PB2 polymerase and interfere with the viral replication (Section 1.2.1.4). Consequently, $Mx1^{-/-}$ mice are more susceptible than $Mx1^{+/+}$ mice or humans to influenza and show prolonged viral replication in the LRT (Doherty 2006; Salomon et al 2007). Overall, mouse studies are relevant to immunopathology in humans caused by influenza virus infections and the full recovery requires both innate and adaptive responses.

Although mice are not natural hosts for influenza infections, most mouse strains can be infected experimentally. Several earlier studies demonstrated that many egg-adapted human influenza viruses were capable of replicating asymptomatically in the respiratory tract of mice to high titre (Tannock et al 1984, 1995). Pathogenic lesions can be observed when the egg-grown viruses are multiply passaged in mice, implying that selection occurs within the viral population (Ward 1997; Sealy et al 2003). The great advantages of mouse models for influenza studies are their relatively low cost, their defined genetic status, and the large number of reagents available.

Many of the problems of extrapolation to human infections can now be overcome by the use of genetically modified (*humanised*) strains of mice (Thomas et al 2006).

1.2.2.1 Host humoral and cellular immune responses to natural influenza infection

Humoral immunity

Humoral immunity is mediated by antibodies (Abs) secreted by B cells. Innate and adaptive humoral immunities coexist in both humans and mice (Baumgarth et al 1999). Innate humoral immunity is mediated by B1 cells and their secretory products consist of *natural* Igs, mainly IgM (Kopf et al 2002). Adaptive humoral immunity is initiated by follicular B (B2) cells, and they secrete antigen-specific IgM, IgA, and IgG in response to infections (Bice et al 1993; Baumgarth et al 1999). Specific Abs and memory B cells are critical effectors against re-infection (Bachmann and Kopf 1999).

- *Antibody secreting cells (ASCs)*

Mature B-lymphocytes have membrane-associated IgM and IgD on their cell surfaces as BCRs for antigen recognition (see Section 1.2.1.5). After encounter with influenza viruses, mature B cells undergo activation and differentiate into influenza-specific ASCs. During the process of differentiation, two changes occur: (1) IgM is spliced to express a secreted form; (2) the heavy chain of immunoglobulin undergoes class switching to isotypes other than IgM and IgD. Compared with mature B cells, antigen-specific ASCs have reduced amounts of membrane-associated IgM or IgD but high rates of IgG secretion (Abbas and Lichtman 2003). Like most infections of immune competent individuals, influenza viruses initiate humoral immune responses consisting of an early increase of antigen-specific IgM followed by isotype switching and the production of anti-influenza IgG, IgA and IgE (Baumgarth et al 1999).

An earlier study demonstrated that ASCs appeared 3 days after the exposure of mice to an aerosolised preparation of human influenza A virus (WSN) grown in Madin-Darby bovine

kidney cells, which were mainly found in major airways and in consolidated lung parenchyma (Owens et al 1981). WSN-specific ASCs then appeared in the MLN and spleen after a delay of 4 days. Virus-specific IgA-secreting ASCs were the most abundant and comprised 40% of total ASCs found in the lungs. Accumulation occurred in the lamina propria of the major airways and close to the natural site of infection. By contrast, IgG-secreting ASCs were least frequently present in the airways and appeared mostly (85%) within consolidated lung lesions (Owens et al 1981). In the same study, IgM-secreting ASCs declined rapidly by 30 days post-infection (p.i.).

- *Anti-influenza antibodies*

Of the three antibody isotypes (IgM, IgA and IgG) induced in response to influenza infections, the short-lived IgM response has been shown to be insufficient to provide full protection (Bachman and Kopf 1999). By contrast, active sIgA (Section 1.2.1.4), the predominant isotype in human mucosal secretions, is able to provide full protection against re-infection (Czerkinsky et al 1987; Nicod 1999). The reasons for this are: (1) sIgA persists for up to 12 months after primary infections; (2) heterotypic immunity to drift viruses following natural infection is largely caused by local sIgA and not by serum Abs or CTLs (Liew et al 1984; Sealy et al 2003). Nevertheless, local IgG, together with the CTLs, are important in viral clearance from the lower lungs during primary infection. In all, both, IgA and IgG play important roles in the control of influenza infection in URT and LRT, respectively (Renegar et al 2003; Tamura and Kurata 2004).

Antibodies control influenza infections by impairing viral infectivity (*virus neutralization*) and viral release (*yield reduction*). Virus neutralization activity is mediated by HA-specific Abs, whereas yield reduction is induced by M2- and NA-specific Abs (Gerhard 2001; Mozdzanowska et al 2003). Neutralizing Abs are of central importance for the development for protective immunity against all cytolytic pathogens, including influenza viruses. For influenza A viruses there are five major antigenic sites, which surround the receptor-binding pocket on the surface

of the influenza A virus HA1 subunit. Antibodies to one or more of these regions are capable of neutralizing viral infectivity by blocking the entrance to the target cells (Thomas et al 1995). The neutralizing activity of influenza virus-specific Abs can be enhanced by serum components, Fc-receptor expressing cells (such as macrophages) and lectins from the innate immune system (Mozdzanowska et al 2006).

- *Influenza-specific T cell-dependent (TD) antibody responses*

Virus-specific antibody production by B cells takes place by both T cell-independent (TI) and -dependent (TD) pathways. For influenza viruses, the TD Ab response is more effective than the TI response and protection against infection is mainly mediated by TD neutralizing Abs (Mozdzanowska et al 2005). Within the influenza-specific TD Ab response, two types of interaction between $CD4^+$ T cells and B cells are responsible for the production of specific Abs. During the early-phase of influenza infections, a local neutralizing IgA response is generated, in the absence of B-T cell signalling, via the MHC II peptide/TCR or CD40/CD40L pathways. These non-specific or bystander $CD4^+$ T cells provide help only to viral-specific ASCs in the production of neutralizing IgA Abs against the viral surface HA glycoprotein (Sangster et al 2003). Unlike the IgA response, IgM and IgG responses require specific $CD4^+$ help through TCR or CD40L pathways and hence occur later than the IgA response. As a consequence of these two different types of B-T interaction, most IgAs are capable of neutralizing HAs. By contrast, IgG2a Abs contain substantial non-neutralizing components that recognise NA, NP and M2 (Kilbourne 2006).

Cellular immunity

The CMI response to influenza infections is mediated by antigen-specific $CD4^+$ and $CD8^+$ T cells and cells of the innate immune system [e.g. DCs, NK cells and macrophages (Section 1.2.1.3)]. Both effector $CD4^+$ and $CD8^+$ T cells produce a spectrum of cytokines that are involved in cell recruitment and activation. Overall, during viral infection of the respiratory

tract, $CD4^+$ T cells act mainly as helpers and $CD8^+$ T cells as killers (Doherty et al 1997). Specific $CD4^+$ and $CD8^+$ T cell responses can only be induced if an MHC molecule presents an appropriate antigenic peptide to T cells (*MHC-restriction*). Peptides of suitable length and capacity to bind to MHC receptors are the main determinants of adaptive cellular immune responses. By contrast, humoral antibody responses are determined by the 3-dimensional structure of the presenting antigen.

Other factors, such as the antigenic load or dose and the local presence of cytokines and accessory molecules, are also important in the development of viral immunity (Doherty et al 1997; Hozler et al 2003). For example, primary responses to replicating viruses are qualitatively different to those of purified non-replicating antigens prepared from the same virus (i.e. subunit influenza vaccines). Qualitative differences have also been observed between the responses to high-yielding virulent influenza strains and low-yielding attenuated strains (Mak et al 1982; Baumgarth and Kelso 1996; Wareing et al 2003 and 2005).

- *$CD4^+$ T helper (T_H) cell responses*

$CD4^+$ T_H cells are of central importance in the development of adaptive immunity against infectious pathogens (Figure 1.9), due to the assistance they provide to specific B cells and CTLs (Castellino and Germain 2006; Simpson 2008). Under the influence of cytokines IL-12 or IL-4, naïve CD4 cells can differentiate into Type I or Type II effector T_H cells (Doherty et al 1997; Murtaugh and Foss 2002). More recently, a third type of $CD4^+$ T_H cell, T_H17, has been described in response mainly to bacterial infections and was named after its ability to secrete IL-17 (Kaiko et al 2007).

Type I T_H cells (T_H1) secrete IFN-γ, IL-2 and TNF-α, which promote the clonal expansion of virus-specific CTLs and the production of IgG2a (Fazekas et al 1994). An *in vitro* study using human PBMCs demonstrated that IL-2 is the major cytokine responsible for the generation of

effective CTL responses (Boon et al 2005). Type II T_H cells (T_H2) produce IL-4, IL-5 and IL-13 to provide help for an effective B-cell response and, in particular, to drive IgG1 production (Brown et al 2004). Because T_H cells recognise many epitopes on influenza viral proteins (both surface and internal), they are able to provide assistance to B cells and CTLs during primary, secondary and memory responses to influenza viruses.

During primary infections in mice, low-level $CD4^+$ T cell responses are broadly heterogeneous. They commence in the MLN and CLN, followed by spleen, and then spread to other secondary lymphoid sites (i.e. NALT and BALT). By day 6-7 p.i., effector CD4+ T cells are recruited to the lungs and BAL (Roman et al 2002; Eichelberger et al 2006). Their response then declines rapidly in parallel with viral clearance. When effector $CD4^+$ T cells migrate to infected lungs, they undergo phenotypic changes that include: (1) loss of CD62L, down-regulation of the CCR7 *homing* marker; (2) stable expression of adhesion molecules such as CD44 and CD49d; and (3) discontinuance of IL-2 secretion (Baumgarth et al 1994; Brown et al 2004). Only fully divided and differentiated effector cells are available in the lungs and BAL for combating local infections.

Furthermore, in primary response to influenza infection, T_H cells are not the only source of help for the development of primary CTL responses, since DCs can also stimulate naïve $CD8^+$ cells (Diebold et al 2004). Consequently, CD4+ T cells are not essential for the development of an effective CMI during primary influenza infections. This was confirmed from earlier studies in which CD4-depleted mice were able to clear virus from lungs following primary infections, but with a delay of ~4 days compared with intact mice (Allan et al 1990; Eichelberger et al 1991).

However, T_H cells play a vital role in secondary CTL responses to influenza infections, especially in the establishment of immunodominance (Mintern et al 2002). Without the help from specific T_H cells induced during the primary response, memory CTLs and robust secondary

CTL responses cannot be generated (Riberdy et al 2000; Thomas et al 2006). Moreover, throughout the secondary influenza infections, specific T_H responses are relatively weak (2-3-fold less in the BAL) than CTL responses and immunodominance does not appear to be present (Wohlleben et al 2003; Eichelberger et al 2006; La Gruta et al 2007). These observations are inconsistent with the view that most viral infections favour cytotoxic Type I immune responses for the control of intracellular infections.

It has been recognised since the 1960s that T_H cells can help B cells in the production of Ab. With influenza infections, apart from natural IgM Abs, the specific Ab responses are in a T_H-dependent mode. Accordingly, the production of isotype-switched, high affinity anti-influenza neutralizing Abs requires the presence of T_H cells (Baumgarth and Kelso 1996). In addition, $CD4^+$ T_H cells are necessary for the generation of specific memory B cells (Gerhard 2001). T_H cells recognise both surface and internal proteins from influenza viruses. Specific T_H cells and ASCs recognise the viral epitopes derived from limited regions of the HA1 subunit. In addition, T_H cells that recognise M or NP can provide help to B cells for the production of HA-specific Abs (Marshall et al 1999). This feature of T_H cells has been useful in providing a mechanism for cross-protection against *drift* variants (Wright et al 2007).

Besides providing help for B and $CD8^+$ T cells, some $CD4^+$ T_H cells (less than 2% of the total $CD4^+$ T cell population) have been found to have direct cytotoxic effector function during herpesvirus, HIV and influenza infections (Eichelberger et al 1991). For influenza infections, $CD4^+$ CTLs appear early and before the production of specific-Abs. It was demonstrated in earlier studies that $CD4^+$ CTL cell lines isolated from the human PBMC were HA- and NA-specific (Sterkers et al 1985; Jameson et al 1998). They are strong IFN-γ producers, possess reduced proliferative capacity and do not provide help with the B cell differentiation. Several studies have demonstrated that a subset of $CD4^+$ T cells is able to kill infected cells by perforin-mediated cytolysis, as also occurs in $CD8^+$ CTLs and NK cells (Appay 2002; Brown et al 2006).

However, influenza-specific $CD4^+$ CTLs, alone, were shown to be insufficient to control relatively mild influenza infections (Topham and Doherty 1998; Thomas et al 2006).

- *$CD8^+$ cytotoxic T (T_C or CTL) cell responses*

Specific CTL responses are critical components of the defence against influenza infections; they allow the host to recognize and eliminate infected cells (Ennis et al 1978; McMichael et al 1986). More significantly, such responses are able to cross-react with cells infected with homologous and heterologous viruses (Webster and Askonas 1980; Lin and Askonas 1981). Through mainly CD95 (Fas) - or perforin-and granzyme-mediated cytotoxicity, effector CTLs are capable to kill target cells and subsequently terminate viral replication. In addition, certain cytokines (i.e. IFN-γ and TNF-α) produced by CTLs also play a role in the control of infections and in some cases can directly cause lung immunopathology (Carding et al 1993; Barry et al 2002; Xu et al 2004).

Previous studies have indicated that viral infectivity is essential for inducing specific CTL responses *in vivo* (Braciale and Yap 1977; Ada et al 1981). Sialic acid receptors with an alpha 2,6 linkage (SAα2, 6Gal) are specific for most human influenza viruses. Due to the differences in viral receptor distribution, viral replication in humans (except for H5N1 viruses) is usually restricted to the URT, whereas in mice replication largely occurs in the LRT. In addition, most healthy adults, unlike mice, have been exposed to influenza viruses many times and even partial pre-existing immunity probably reduces the chance of developing pneumonia following infection. In mice, peak CTL activity coincides with acute pneumonia and decreases during its resolution (Ennis et al 1978; Eichelberger et al 1991; Baumgarth et al 1994; Belz et al 1998).

There are further differences in the patterns of CTL responses following influenza infections of humans and mice. Bennink and Yewdell (1988) suggested that, because of the highly polymorphic nature of the human MHC gene, the peptides recognised by CTLs should vary

considerably in humans. This contrasts to the limited response (or *immunodominance*) observed following infection of inbred mice. By stimulating PBMCs with A/PR8 (H1N1), A/Japan/305/57 (H2N2) and A/Johannesburg/33/94 (H3N2) separately *in vitro*, human memory CTL responses to influenza A viruses were shown to be broadly directed to a range of epitopes derived from several different viral proteins (Jameson et al 1998). Ten CTL lines isolated from the PBMCs of three donors were shown to be specific for the epitopes of several viral proteins (including NP, M1, M2, NS1, PB1, PB2, and HA, NA), which may account for their subtype-specific and cross-reactive specificities (Jameson et al 1998).

An immunodominance hierarchy can be established during the influenza virus infections within several different inbred mice strains, including BALB/c (H-2D^d), C57BL/6 (H-2D^b), CBA/Ca (H-2D^K) (Tourdot et al 2001; Chen et al 2000 and 2002). However, specific CTL responses are directed at only a few epitopes and especially following secondary infections (Section 1.2.1.5). Individual epitopes differ in their capacity to induce T cell responses and, therefore, can be classified as *dominant, codominant, or subdominant*, according to their relative contributions to the total T cell response (Crowe et al 2003). However, in general, the immunodominance status is only a relative phenomenon and elimination of a dominant epitope can cause elevation of a subdominant epitope to the status of dominance (Belz et al 2000).

Following primary i.n. challenge of C57BL/6 mice with HK×31 (H3N2) virus, two CTL dominant epitopes ($NP_{366-374}$ and $PA_{224-233}$) and four subdominant epitopes ($PB1_{703-711}$, $NS2_{114-121}$, $M1_{128-135}$, $PB1\text{-}F2_{62-70}$) were identified (Belz et al 2000 and 2001; Table1.3). The $CD8^+D^bNP_{366}{}^+$, $CD8^+D^bPA_{224}{}^+$ and $CD8^+K^bPB1_{703}{}^+$ sets were detected by tetramer staining and comprised less than 5% of $CD8^+$ T cells in the spleen but more than 30% in the BAL. However, the secondary CTL response following i.n. infection with A/PR8 (H1N1) was shown to mostly take place in the $CD8^+D^bNP_{366}{}^+$ population, accounting for more than 70% of the total $CD8^+$ T cells in the BAL (Belz et al 1998).

Relationships between humoral and cellular immunity

The concept that Ab-mediated humoral immunity protects against extracellular pathogens and that CMI protects against intracellular pathogens is widely accepted (Abbas and Lichtman 2003). Many studies have demonstrated an inverse or sometimes antagonistic relationship between these two arms of the immune system (Casadevall 2003). In recent years, these relationships have been extended in mice by studies demonstrating the presence of T_H1/T_H2 or Type I/Type II systems (Figure 1.9). It would be highly desirable to understand the relative contributions of humoral and cellular immunity by the host and to examine the role of these relationships in the control of individual infections by vaccination.

Influenza infections of the respiratory tract in both mice and humans initiate mixed Type I (CTL) and Type II (neutralizing Ab) responses, which are consistent with the need to eliminate virus at both the intracellular and extracellular phases of the virus life cycle. For the optimal control of infections, both humoral and cellular immunity is required (Table1.4; Bachmann and Kopf 1999; Gerhard 2001). Some controversy exists as to whether CMI (which induces heterosubtypic responses) is less effective than the more strain-specific humoral immunity (Gerhard 2001). CMI responses, by themselves, in a mouse model have been shown to be less efficacious than humoral responses against the highly pathogenic mouse-adapted influenza strain A/PR8 (H1N1) (Subbarao et al 2006). However, without CMI responses, A/HK (H3N2)-primed mice did not survive lethal challenge with A/PR8 (Brown et al 2006). This indicates that CMI plays a critical role in eliciting a heterotypic, or memory-induced, protective immune response (Figure1.10).

While there is a general consensus that T_H cells promote specific-B cell responses (Jeurissen et al 2004), B cells also play a key role in promoting optimal and sustained T cell responses (Christensen et al 2003; Martin and Chan 2006). Using B cell-deficient mice infected i.n. with A/PR8, a significantly reduced $CD4^+$ cell response was observed (Kopf et al 2002). In further

study, DC-deficient mice were able to generate NP-specific CTL responses after receiving NP-expressing spleen B cells (Langlade-Demoyen et al 2003). This evidence is consistent with earlier observations that B cells can process and present endogenous peptides to $CD8^+$ T lymphocytes and, therefore, serve as APCs for the priming of specific-CTL responses (Billetta et al 1995).

Further evidence suggests that humoral responses can partially compensate for deficient CMI responses. An enhanced IgG antibody response was reported after naïve mice had been infected i.n. with influenza virus constructs (prepared by reverse genetics) with point mutations in their NP and PA genes (Webby et al 2003). One possible explanation is that mutations in the regions of immunodominant epitopes allowed these viruses to escape CTL activity (i.e. $CD8^+D^bNP_{366}^+$ or PA_{224}^+) and to grow to significantly higher titres, in comparison with *wt* virus. Therefore, the prolonged antigen-presenting process in the LNs and spleen may allow greater $CD4^+$ T cell and B cell stimulation. Interestingly, an increase in the size of the minor $CD8^+K_bPB1_{703}^+$ population appeared following infection with the NP/PA double mutant. Collectively, B and T cells can influence each other through several different pathways. Consequently, the relationship between protective humoral and cellular immunity is complicated, and interactions between the two can lead to variable outcomes.

1.2.2.2 Memory and cross immune responses to influenza infections

Immunological memory, the basis for vaccination, can be generated only by adaptive immune responses (Kaech et al 2002; Esser at al 2003). Naïve B and $CD8^+$ T_C cells can differentiate into effector cells during primary infection and in mice most die once the pathogens have been eliminated (Westermann et al 2001; Baz et al 2005). However, some survive and differentiate into long-lived memory cells with the assistance from $CD4^+$ T_H cells (Belz at al 2002; Swain 2005; Willams and Bevan 2006). At present, the mechanisms behind the generation and maintenance of antigen-specific memory lymphocytes are largely unknown.

Memory lymphocytes confer immediate protection in peripheral sites and mount recall responses to both homologous- and heterologous-antigens in secondary lymphoid tissues (Sallusto et al 2004). Cross-reactivity is particularly important in providing protection against pathogens that exhibit frequent antigenic variation, such as influenza viruses. Because of immunological memory, children who have previously experienced natural influenza infection or who received a live influenza virus vaccines exhibit a substantial reduction in both the amount and duration of virus shedding (Wright et al 2007).

Specific memory responses against influenza viruses

- *Memory B cells*

Specific memory B cells and the high levels of serum Abs are responsible for serological memory. Immunocompetent individuals who have recovered from respiratory infection with influenza A virus show long-lasting, high levels of sterilizing immunity to homologous viral challenge (15 months in mice; 20-25 years in humans) which is mainly mediated by anti-HA neutralizing Abs (Gerhard 2001; Zinkernagel 2002). These Abs are produced by influenza-specific ASCs that differentiate from the activated memory B cells after re-encounter with the same virus. These properties are used for the measurement of specific-resting memory B cells in ELISPOT assays using antigen restimulation (Welsh et al 2004). Nevertheless, because of high strain specificity, memory B cell responses are not efficient in providing protection from influenza infections caused by *drift* or *shift* strains (Figure 1.10).

- *Memory CTLs*

Memory $CD8^+$ T cells can mediate an accelerated and enhanced recall response to secondary virus infections. Acceleration is caused by increased numbers (~1000-fold) of antigen-specific T cells; enhanced responsiveness is caused by reduced co-stimulatory requirements and the higher activation state of the cells (Kambayashi et al 2003).

Table 1.4 All cell components of host adaptive immune responses were required for the optimal recovery from primary influenza virus infection in mouse (Gerhard 2001)

CMI		Humoral	Recovery from i.n. infection with A/PR8		
			50-200 $TCID_{50}$		10^7 $TCID_{50}$
CD8+ T	CD4+ T	B	Clearance (days)	Survival (%)	Survival (%)
+	**+**	**+**	**7-10**	**100**	**80**
-	+	+	10-14	100	10
-	+	-	>20	0	
-	-	+	>20	0	
+	+	-	10-14	35-85	
+	-	-	>14	20	
+	-	+	10-14	90	
-	-	-	>20	0	

CMI Cellular mediated immunity
$TCID_{50}$ 50% mouse infectious dose

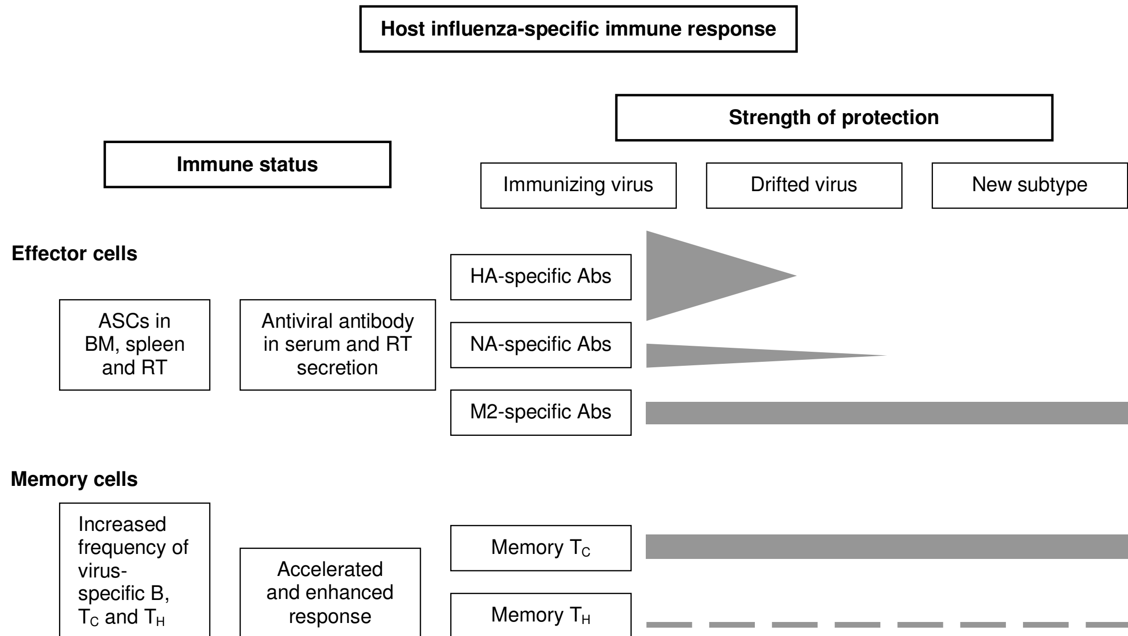


Figure 1.10 The role of cellular and humoral immune responses during secondary influenza infection (modified from Gerhard 2001)

ASC antibody-secreting cell
BM bone marrow
RT respiratory tract

Memory $CD8^+$ T cells (mostly $CD44^{high}$) have been broadly classified into (1) central memory cells (T_{CM} $CD62L^{high}$ $CCR7^+$) found in lymphoid organs that have no immediate lytic activity and (2) effector memory cells (T_{EM} $CD62L^{low}$ $CCR7^-$) that reside in nonlymphoid tissues with lytic functions (Sallusto et al 1999, Mackay 1999; Selin and Welsh 2004; Wherry and Ahmed 2004). Furthermore, optimal memory CTL-mediated protection against secondary influenza infection largely depends on the strength of T_{EM} responses which occur during the first 2-3 days of re-infection (Christensen et al 2000; Willams and Bevan 2006).

Compared with memory B cells that are able to mediate sterilizing immunity, the protection mediated by memory CTLs is infection-permissive which is required for the activation of resting CTLs (Gerhard 2001). Memory CTLs are more efficient than memory B cells in the induction of cross-reactive responses against influenza variants (Figure 1.10). Earlier studies demonstrated that memory CTL-mediated cross-reactivity can be maintained for at least 3 years following influenza infections (Doherty et al 1996; Flynn et al 1998).

Cross-immune responses to influenza virus infections

It has long been recognised that cross-immunity to other viruses within the influenza A sub-type occurs following recovery from any influenza A virus infection (Jordan et al 1958; Armerding and Liehl 1981; Tannock and Paul 1987). Cross-immunity between different *drift* strains within the same subtype is referred to as homosubtypic immunity; cross-immunity between different subtypes or *shift* strains is referred to as heterosubtypic immunity. Cross-immunity is mediated by pre-existing Abs and memory B and T cells that recognise conserved viral protein sequences shared by different variants (Epstein et al 1998). Although it cannot provide complete protection against influenza infections, it is vital in reducing morbidity and mortality (Doherty 2007).

There are quantitative and qualitative differences between homosubtypic and heterosubtypic responses against influenza A viruses. In cotton rats 25% of all cells recovered from the BAL as

part of the heterosubtypic response were specific $CD4^+$ T cells, compared with 70% for the homotypic response (Eichelberger et al 2006). A further study demonstrated that the homotypic Ab response requires a less diversified Ab repertoire than is necessary for the heterosubtypic Ab response (Nguyen et al 2007). These results indicate that a strong Ab response is induced by homotypic challenge and a strong CTL but weaker Ab response by heterosubtypic challenge. Furthermore, studies in mice infected i.n. with cold-adapted (*ca*) live-attenuated influenza vaccine (LAIV) virus demonstrate that heterotypic immunity, compared with homotypic immunity, is relatively short-lived, indicating the involvement of different effector pathways (Tannock and Paul 1987).

Influenza virus-specific $CD8^+$ T cells that recognise viral internal proteins (mainly NP and PA) are highly cross-reactive and are the primary contributors to heterosubtypic immunity in mice (Yewdell et al 1985; Bennink et al 1986 & 1987). A/PR8 H1N1-primed mice with relatively small numbers of influenza virus-specific memory $CD8^+$ T cells (<1% of total $CD8^+$ cells) were shown to clear A/HK×31 H3N2 infection 1-2 days earlier than naïve mice following i.n. challenge (Doherty et al 2006). In another study, pre-existing cross-reactive specific-memory CTLs, from A/HK×31 (H3N2)-primed mice were shown to have a higher survival rate (87.5%) than naïve mice (33%) 7 days p.i after challenge with a lethal dose of A/PR8 (H1N1) (Kreijtz et al 2007).

Several studies have demonstrated that the proportion of pulmonary specific CTLs determines the extent of cross-immunity (Riberdy et al 1999; Seo et al 2002). Therefore, heterosubtypic immunity can be enhanced by increasing specific memory in the CTL population. In support of this, mice primed i.n. with H1N1 and then boosted with H3N2 exhibited only transient weight loss and survived following i.n. challenge with highly pathogenic H5N1 and H7N7 viruses (Christensen et al 2000).

Generally, memory T cell-mediated heterosubtypic responses are weak and are easily overwhelmed by severe influenza infections and, to survive lethal influenza challenge, both memory B cells and pre-existing Abs are required. B cell and Ab-mediated heterosubtypic immunity can slow viral replication and thus facilitate the expansion of responding memory CTLs (Rangel-Moreno et al 2008). Cross-protection against secondary heterosubtypic infections of mice is related to levels of local sIgA Abs (Liew et al 1984). Strong Ab-mediated heterosubtypic immunity exists between influenza A H1 and H2 subtypes from the same clade (Figure 1.5). Similar observations have been found for influenza B viruses, with sIgA Abs providing strong cross-protection against strains from different lineages (Asahi-Ozaki et al 2004). Other Abs specific to viral NA, NP and M2 are also involved in the heterosubtypic responses to influenza variants (Kilbourne 2006; Straight et al 2008).

Significantly, several *ex vivo* studies have demonstrated the existence of heterosubtypic T cell and Ab responses against avian H5N1 influenza viruses in individuals who had been exposed to influenza H1N1 and H3N2 viruses through seasonal vaccination or infection (Goy et al 2007; Roti et al 2008; Kreijtz et al 2008). In one human study, it was reported that 58% of UK donors and 74% of Vietnamese donors exhibited detectable H5N1-specific $CD4^+$ and $CD8^+$ T-cell responses (Lee et al 2007). Currently, with the threat of possible influenza pandemic, a range of vaccines that are able to enhance heterosubtypic immunity and promote broad protection are under development (Ansaldi et al 2008; Quan et al 2008; Bright et al 2008; Zanvit et al 2008; Baras et al 2008).

1.2.2.3 Host local and systemic immune responses against influenza infection

The respiratory tract mucosa is both the primary site for influenza infection and for the induction of protective mucosal and systemic immune responses, involving the MALT and draining LNs (Section 1.2.1.2; Haanen et al 1999). For many years, local immunity has been considered in

isolation from humoral immunity but recent studies indicate that effective mucosal immunity depends on antigen-priming of both B and T cells (Woodland 2002).

Local and systemic humoral immune responses

For influenza, local specific humoral immunity involves the action of Abs (mainly sIgA and to a lesser extent IgG) and ASCs in the respiratory mucosa (Fokkens and Scheeren 2000; Renegar et al 2003). Following influenza virus infection, B cells were shown in mice to comprise up to 18% of cells isolated from mice lungs (Baumgarth and Kelso 1996); most long-term ASCs are present in the lung interstitium, giving rise to local Ab responses (Bice et al 1993). ASCs from draining or distant LNs or from the spleen play little or no role in the maintenance of local Ab memory responses to influenza infections. These cells produce most neutralizing Abs in the serum, and constitute the systemic humoral immunity.

Compared with sIgA Abs which acts primarily in the URT, serum Abs provide protection against influenza infections in the LRT. Recovery from infection in the LRT is related to the induction of a serum Ab response. Serum HI titres of 40 or greater are closely associated with protection against infections by influenza A viruses (Gross et al 1977). During primary i.n. infections of mice, the number of lymphocytes in the MLN was shown to increase from ~0.5- to 6-12 $\times 10^6$ within the first 3 days, and to reach a peak of 15-20 $\times 10^6$ by day 7 p.i (Gerhard 2001; Sealy et al 2003). These increases coincide with a significant increase in serum Ab titre. Such increases usually occur 1 week after primary infections (Gerhard 2001).

However, serum IgG Abs were found to have contributed little to mucosal immunity through passive transudation into the respiratory mucus (Wagner et al 1987). Compared with unanaesthetised mice, levels of IgG Abs in serum were found to be markedly higher in anaesthetised mice following i.n. challenge with influenza viruses; no differences in sIgA Ab levels were found (Jannkova et al 2002). Overall, during natural influenza infection, the stimulation of serum Ab is related to the extent of viral replication that occurs in the LRT.

Local T cell responses and mucosal vaccination

Influenza virus infections of mice result in both local and systemic T cell responses. Earlier studies showed that the ratio of CD4:CD8 T cells was 1:2 in airways, 1:1 in the lung parenchyma and 2:1 in the draining LNs following infection (Baumgarth and Kelso 1996). Following primary infection, the majority of specific memory $CD8^+$ T (T_{EM}) cells found in the in airways and the lung parenchyma (BALT) were able to provide prompt local cellular responses against secondary infection over long periods (Ely et al 2003) and to establish a second generation of memory T cells (Marsland et al 2004; Roberts and Woodland 2004). Importantly, memory CTLs in the MALT were associated with local protection against lethal heterosubtypic challenge (Nguyen et al 1999). However, local memory $CD8^+$ T cells (T_{EM}) have a half-life of about 40 days and their protective efficacy wanes over time in both humans and mice (Woodland 2002).

LAIVs or adjuvanted inactivated vaccines administered directly to the respiratory mucosa are able to promote local memory T cell pools in addition to stimulating systemic immunity (Tannock and Paul 1987; Asnuma 2001). Such presentation of antigens (Ags) to both the URT and LRT is referred as total respiratory tract immunization. In mice, this can be achieved by using i.n. inoculation administered under general anaesthesia and in humans by inhaling the Ags in the form of an aerosol (Janakova et al 2002). Total respiratory tract immunization has been found to be the only route that induces and maintains a significant memory CTL response in the MALT (Nguyen et al 1999; Haanen et al 1999).

1.2.2.4 Adaptive immune responses to influenza infections in aged populations

Aged individuals typically have an impaired capacity to clear infections and a lowered capacity to respond to vaccines, which coincides with declining innate and adaptive immunity (Kumar and Burns 2008; Nogusa et al 2008). Declining systemic and local Ab responses were observed following i.n. administration of cholera toxin-combined inactivated A/PR8 vaccines combined

with cholera toxin to 18-month old mice (Asanuma et al 2001). Another study demonstrated a marked decline in the frequency of memory B cells in 8-month- compared with 6-week-old mice- related to their reduced responsiveness to IL-7, which is required for sustaining memory responses (Miller and Allman 2003).

For primary T cell responses, it has been shown that numbers of peripheral naïve T cells decline throughout life and this decline is associated with severe functional defects. In 22-month-old mice, these include: (1) decreased T cell repertoire diversity leading to the development of immunodominance during the primary response, which impairs heterosubtypic immunity during secondary infections; (2) reduced reactivity to viral epitopes and (3) a 4-day delay in the maximum expansion of NP-specific CTLs (Po et al 2002; Yager et al 2008). Other impacts of age on the secondary T cell response to influenza infections are: (1) a significant loss of T_{EM} cells at peripheral sites over time; (2) an impaired rate of Ag-specific proliferation and (3) a decrease in the lytic capacity of effector cells (Boon et al 2002; Kang et al 2003; Ely et al 2007). These age-associated immunity changes need to be addressed when considering vaccination strategies against influenza infections in the elderly.

1.3 INFLUENZA VACCINES

Although specific immune responses occur in response to infection, influenza remains the major recurrent respiratory disease of humans because the viruses responsible have evolved several strategies to evade host defences. In addition to new pandemics, seasonal influenza epidemics cause significant morbidity and mortality. They account for, on average, 226,000 hospitalized cases and 36,000 excess deaths each winter in US (Subbarao and Murphy 2006) and 10,000 excess deaths in the UK (Barclay 2006), alone.

Measures for the control of influenza include: the development of global surveillance networks, fast diagnostic techniques, antivirals and vaccines (Wood 2002). Of these, vaccination has been proven to be the most cost effective and is most commonly used for high-risk populations (Table1.5) in developed countries. Currently, vaccination rates in all groups in US are <35%, compared with 60% in those aged >65 years (CDC 2007). Rates of vaccination have increased in recent years and the world market for seasonal vaccines is expected to increase to US$3.6 billion by 2009 as a consequence of recent WHO recommendations (Quigley 2006).

1.3.1 Inactivated trivalent influenza vaccines (TIVs)

Current vaccines are usually trivalent and contain representative influenza A H1N1, H3N2 and influenza B surface Ags, which are re-evaluated each year to ensure that there is antigenic match with current epidemic strains (Table 1.6). The majority of viruses used in the preparation of inactivated vaccines are still prepared by growth in the allantoic cavity of embryonated chicken eggs. Allantoic virus is purified, concentrated and inactivated. Reactogenicity associated with purified influenza virus is greatly reduced by treatment with a detergent or splitting agent (*split* vaccines). An additional step, involving the isolation and purification of surface glycoprotein Ags, is used by some vaccine manufacturers (*subunit* vaccines).

Purified whole virus vaccines are more immunogenic than split/subunit vaccines but are not widely used in interpandemic immunization programmes because of their reactogenicity. Influenza vaccines may still contain trace amounts of potentially reactogenic endotoxins, egg proteins, formaldehyde and preservative. Yields of egg-adapted viruses are usually high, but there are a number of problems associated with the use of eggs, which include (1) the limited flexibility they afford for expanded vaccine manufacture; (2) interruption of the embryonated-egg supply chain due to the presence of diseases in layer flocks; (3) the possibility of sterility problems arising during the processing of infected allantoic fluids; and (4) poor growth of some reassortant and non-reassortant vaccine strains in eggs.

Additionally, the growth of epidemic viruses in eggs often selects variants that differ in their glycosylation patterns from the original clinical isolates that are antigenically distinct. These problems do not occur with viruses isolated in the Madin-Darby canine kidney (MDCK) and African Green monkey kidney (Vero) continuous cell lines. The use of stable cell lines for vaccine virus growth could largely overcome these problems if yields were satisfactory and safety issues could be successfully addressed.

Many adjuvants are currently under examination to boost responses and reduce the amount of Ag required. These include immune-stimulating complexes (ISCOMS, liposome-like preparations or VLPs) and synthetic adjuvants (e.g. lipid A, muramyl peptide derivatives and cationic molecules). MF59 has been licensed in several European countries for use in pandemic influenza vaccines (Guy 2007; Aguilar and Rodríguez 2007). Other strategies under consideration to improve killed vaccines include the use conserved viral proteins (e.g. M2 or NP) (so-called *universal* vaccines), DNA vaccines and adjuvanted vaccines administrated by the i.n. route.

1.3.2 Cold-adapted live-attenuated influenza vaccines (*ca* LAIVs)

Live influenza vaccines are promising alternatives to inactivated vaccines (Subbarao et al 2006). They are administered i.n. and are, therefore, able to mimic natural infection. Restricted viral replication in the respiratory tract can induce both specific humoral and CMI, local and systemic and memory cross-immune responses (Section1.2.2; Wareing and Tannock 2001). LAIVs have been proposed for over 50 years as alternatives to intramuscular (i.m.) administered inactivated vaccines.

Table 1.5 Annual vaccine recommendations (CDC, October 23, 2007)

General group:

- all persons, including school-aged children, who want to reduce the risk of becoming ill with influenza or of transmitting influenza to others;

High risk groups:

- all persons aged 50 years and older;
- all children aged 6–59 months (i.e., 6 months–4 years);
- women who will be pregnant during the influenza season;
- children and adolescents (aged 6 months–18 years) receiving long-term aspirin therapy who therefore might be at risk for experiencing Reye syndrome after influenza virus infection;
- adults and children who have chronic pulmonary (including asthma), cardiovascular (except hypertension), renal, hepatic, hematological or metabolic disorders (including diabetes mellitus);
- adults and children who have immunosuppression (including immunosuppression caused by medications or by human immunodeficiency virus;
- adults and children who have any condition (e.g., cognitive dysfunction, spinal cord injuries, seizure disorders, or other neuromuscular disorders) that can compromise respiratory function or the handling of respiratory secretions or that can increase the risk for aspiration;
- residents of nursing homes and other chronic-care facilities;
- health-care personnel;
- healthy household contacts (including children) and caregivers of children aged <5 years and adults aged 50 years and older, with particular emphasis on vaccinating contacts of children aged <6 months; and
- healthy household contacts (including children) and caregivers of persons with medical conditions that put them at higher risk for severe complications from influenza.

Table 1.6 Recommended composition of seasonal human influenza virus vaccines used in northern hemisphere during recent four influenza seasons 2005-2009 (CDC Website 2008). It demonstrates the updating at least one component on a yearly base is required.

	Type A		**Type B**
	Subtype H1N1	**Subtype H3N2**	
2005-2006	A/New Caledonia/20/99 -like	A/California/7/2004 -like	B/Shanghai/361/2002 -like
2006-2007	A/New Caledonia/20/99 -like	A/Wisconsin/67/2005 -like	B/Malaysia/2506/2004 -like
2007-2008	A/Solomon Islands/3/2006 -like	A/Wisconsin/ 67/2005 -like	B/Malaysia/2506/2004 -like
2008-2009	A/Brisbane/59/2007 -like	A/Brisbane/10/2007 -like	B/Florida/4/2006 -like

Table 1.7 Phenotypes of the cold-adapted master donor virus used as attenuation markers for evaluating LAIVs (Maassab et al 1968; Wright at al 2007)

Phenotypes	**Definitions**
cold-adapted (*ca*)	Replicating efficiently (compatible to at 33ºC) in tissue culture at 25ºC, whereas no replication occurred for wild-type virus
temperature sensitive (*ts*)	Restricted replication at higher temperatures (37 ºC for Type B and 39ºC for Type A; temperatures permissive for the replication of wild-type virus)
attenuated (*att*)	Limited replication at upper respiratory tract and no replication in the lungs of infected ferrets (ferret attenuation assay); and is safe and attenuated in children and adults

Table 1.8 Mutations present in the *ca* A/Ann Arbor/6/60 master donor virus (Buonaguria et al 2006)

RNA segment	**Coding region**	**Nucleotide**[a] **[*wt* → ca]**	**Amino acid**[b] **[*wt* → *ca*]**	**Phenotype(s) assigned to master donor virus residue**
1	**PB2**	**A821G**	**N265S**[c]	*att, ts*
2	**PB1**	**A1195G**	**K391E**[c]	*att, ts*
		G1395T	E457D	
		A1766G	**E581G**[c]	*att, ts*
		G2005A	**A661T**[c]	*att, ts*
3	PA	A1861G	K613E	*att, ca*[d]
		TT2167/8CC	L715P	
5	**NP**	**A146G**	**D34G**[c]	*att, ts*
7	M1	None		
	M2	G969T	A86S	*att*
8	NS1	G483A	A153T	
	NS2/NEP	None		

a) Number indicates nucleotide position in the designated RNA segment;

b) Number indicates amino acid position in the designated protein;

c) Loci identified by reverse genetics as being major determinants of the ***att*** phenotype of master donor virus;

d) *ca* phenotypes have been assigned to the PA segment, but the amino acid responsible for this phenotype has not been identified.

Vaccines used today consist of reassortants are prepared from an attenuated donor strain (usually an antigenically distinct H2N2 strain) and a recent epidemic strain. Reassortants are selected for influenza A and B vaccines that contain the internal genes from donor strain and the surface Ag genes of the epidemic strain. Several strategies have been used to prepare attenuated donor strains. These include the use of: (1) *ca* mutants, (2) mutants containing a deletion in the NS1gene, and (3) replication-defective particles (Watanabe et al 2002; Müllbacher et al 2006; Wang et al 2008).

Reassortants prepared from attenuated *ca* donor strains are the only LAIVs licensed for human use in the US and Russia. The US vaccine (FluMist®) was first licensed in 2003 by the Food and Drug Administration (FDA) for use in humans aged 5-49 years. Licensure has since been expanded to 2-49 years (CDC 2008; Luce et al 2008; Table 1.9). In addition, *ca* LAIVs for use as vaccines against equine and avian influenza viruses are currently under development (Song et al 2007).

1.3.2.1 Development and characteristics of ca donor strains

Although *ca* LAIVs were only recently licensed for use in US, the isolation of *ca* attenuated influenza A and B donor strains (used to prepare reassortant vaccine strains that comprise the FluMist® vaccine) was described more than 40 years ago (Maassab et al 1967 & 1968). Wild-type A/Ann Arbor/6/60 (A/AA/6/60) H2N2 virus is a naturally *ca* virus, capable of growing at both 25°C and 39°C and was found to be attenuated in ferrets (Herlocher et al 1993). However, further cold adaptation was required before it was considered for use as a donor strain. This was achieved by a stepwise lowering of the growth temperature, which allowed the selection of multiple lesions in the internal genes (Maassab and DeBorde 1985).

Under laboratory conditions, A/AA/6/60 *wt* virus was adapted to grow at 25°C by a step-wise reduction from 33°C (the permissive temperature) in chicken kidney (CK) cultures. Further

serial passage at 25°C was then undertaken until optimum yields of the donor strain (A/AA/6/60 *ca*) were obtained. Similar approach was also used to produce the cold-adapted donor strains, A/Leningrad/134/17/57 and A/Leningrad/134/47/57 (H2N2), in the former Soviet Union. Two donor strains were developed by passaging A/Leningrad/134/57 21 times at 33°C followed by 17 or 47 times in embryonated eggs at 25°C (Kendal et al 1982; Alexandrova et al 1984). These donor strains are referred to as Len/17 and Len/47.

Similar approaches have been used to prepare *ca* influenza B donor strains using the *wt* B/Ann Arbor/1/66 and B/USSR/60/69 for the preparation of reassortants for inclusion in trivalent LAIVs (Wareing and Tannock 2001). Recently, there have been attempts to develop *ca* donor strains other than A/AA *ca*. Human influenza A virus X-31, a high-yielding reassortant with genes derived from A/PR8 (H1N1) and A/Aichi/2/68 (H3N2), was successfully cold-adapted in both embryonated eggs and MDCK cells (Lee et al 2006; Liu et al 2008).

In general, *ca* viruses are accompanied by a reduction in virulence, attenuation (*att*), and reduced plaquing efficiency at body core temperatures (37°C in humans) and are referred to as temperature sensitive (*ts*) mutants. Previous studies have indicated that genetic lesions responsible for *ca/ts/att* phenotypes are all derived from the six internal genes (Cox et al 1988; Herlocher et al 1996). Importantly, the *ts* phenotype is responsible for the restricted replication of viruses in the URT and is associated with attenuation and vaccine safety. Recent site-directed mutagenesis analysis using reverse genetics has shown that the *ts* phenotype of A/AA/6/60 *ca* is associated with five major loci: $PB1^{1195}$, $PB1^{1766}$, $PB1^{2005}$, $PB2^{821}$, and NP^{146} (Table1.8; Jin et al 2003). These results indicate that the *ts* phenotype is a stable trait that requires mutations from three different gene segments (PB1, PB2 and NP) to act synergistically. The *ts* loci of A/AA/6/60 *ca* were found to be unusually stable during passage in CK cultures (Herlocher et al 1993). One possible explanation is that the lower temperature of 25°C slows down viral

replication and makes the viral polymerase less error-prone. These multiple genetic loci are the molecular markers for vaccine stability (Jin et al 2003; Chan et al 2008).

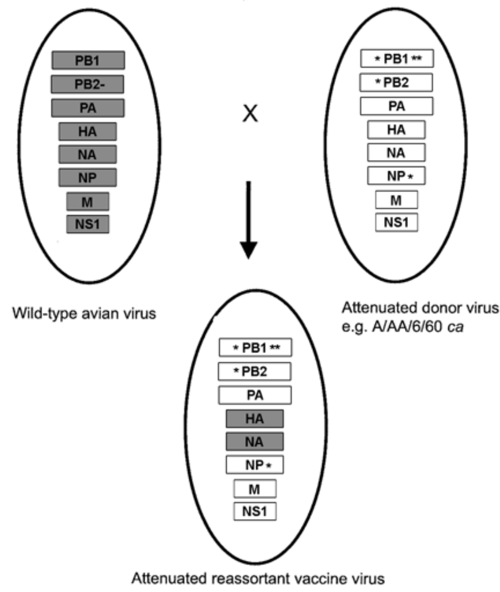
Similar findings were also observed with Russian *ca* donors Len/17 and Len/47, where a total of ten genetic lesions and eight amino acid substitutions from all six internal genes were shown to contribute to their *ca/ts/att* phenotypes (Kilmov et al 1992). Genetic stability studies found no aa changes occurred in these lesions after passage through ferrets (Marsh et al 2003).

1.3.2.2 Preparation of ca LAIVs

It has been was known for many years that certain characteristics of influenza A viruses can be transferred between different strains by genetic reassortment (Kilbourne 1975). Such an approach has been used for over 20 years for the generation of high-yielding influenza A viruses for use in inactivated vaccines that contain the internal genes of A/PR8 and the surface Ag genes of epidemic strains. The same approach is used for the development of attenuated vaccine reassortants that possess the 6 internal genes that specify *ca/ts/att* phenotype (from well-characterised *ca* donor strains) and the surface Ag genes of the epidemic strain. Currently, *ca* LAIV reassortants are prepared by (1) traditional co-infection, or (2) the application of reverse genetics (Figure 1.11).

Using classical genetic reassortment, new *ca* LAIVs are generated by co-infection of eggs or cell cultures with a *ca* donor virus and a contemporary virulent *wt* virus, followed by selection of reassortant progeny at 25°C in the presence of an antiserum to the H2N2 donor strain. To generate *ca* LAIV by reverse genetics, six plasmids encoding the internal genes of the attenuated donor virus are mixed with two plasmids encoding the HA and NA genes of a nominated epidemic virus. Accredited cells are transfected with the plasmids and the attenuated reassortant virus is isolated. Recently, the FDA has granted approval for the use of reverse genetics to prepare seasonal influenza vaccines, including FluMist® (Marsh and Tannock, 2005).

(1)



(2)

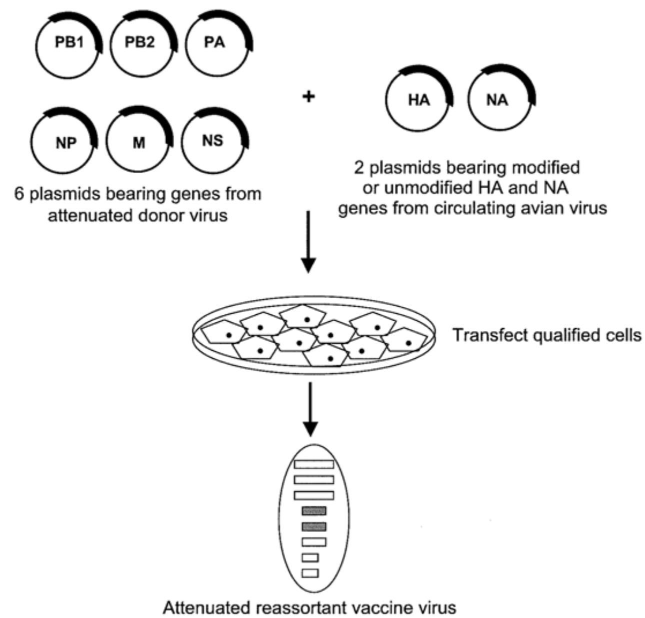


Figure 1.11 Two different systems for the generation of cold-attenuated LAIVs (Luke and Subbarao 2006)

(1) Generation of live, attenuated pandemic influenza vaccine viruses with the 6 internal genes from the attenuated donor virus bearing attenuating mutations (*) and the HA and NA genes from circulating avian virus by **classical reassortment**. The 6:2 reassortants generated by this method are selected in the presence of antiserum specific for HA and NA of the attenuated donor virus. **(2)** The 8-plasmid **reverse genetics system** to generate recombinant, live, attenuated pandemic influenza vaccines.

Table 1.9 The immunologic differences between US FDA-approved *ca* LAIV and TIV (CDC 2007)

Immunology		***ca* LAIV**	**TIV**
Vaccine-induced immune responses	Serum anti-HA Abs	Yes	Yes
	CMI (specially T cell)	Yes	Not detectable
	Mucosal IgA	Yes	No
	Cross-subtypes	Yes	No
	Cross-strains (within same subtype)	Yes	Yes but with less extent
	Long term specific-T and B memory cell	Yes	Yes but with much less extent
Evaluation	Correlate with	Anti-HA Abs, mucosal IgA	Anti-HA Abs
	Assays	Serologic assays, T / B cell assays (not available clinically yet)	Serologic assays
Protection rates	Young (15mo - 6yrs seronegative)	89% with one dose; 93% with two doses	27%
	Adults (18 - 64 yrs seropositive, against drifted strain)	86%	71%
	Elderly (≥ 65 yrs)	Not available	48%

LAIV live-attenuated influenza vaccine
TIV trivalent influenza vaccine

Table 1.10 Major differences between US FDA-approved seasonal influenza *ca* LAIV and TIV vaccines for using in humans (CDC 2008)

Factor	***ca* LAIV**	**TIV**
Route of administration	Intranasal spray (i.n)	Intramuscular injection (i.m)
Type of vaccine	Live virus ($10^{6.5\text{-}7.5}$ $TCID_{50}$ per strain)	Killed virus (15µg HA per strain)
No. of included virus strains	3 (2 influenza A, 1 influenza B)	3 (2 influenza A, 1 influenza B)
Vaccine virus strains updated	Annually	Annually
Frequency of administration	Annually	Annually
Approved age and risk groups	Healthy persons aged 2 – 49 yrs	Persons aged 6 months and older
Interval between 2 doses recommended for children aged < 9 years who are receiving influenza vaccine for the first time	6 –10 weeks	4 weeks

$TCID_{50}$ median tissue culture infectious dose

Reverse genetics involves the creation of infectious copy of the genome of negative-stranded RNA viruses from a full-length viral cDNA of their genome (so-called *infectious cloning*; Lee and Suarez 2008). Therefore, *ca* LAIVs could also be generated by direct attenuation through reverse genetics without the need to prepare reassortants. The introduction of five *ts* loci was found to be sufficient to confer the *ts* phenotype *in vitro* and the *att* phenotype in ferrets on A/PR8 *wt* and A/Guinea Fowl/HK/WF10/99 (H9N2) viruses (Jin et al 2004; Song et al 2007). Reverse genetics has also been used to allow the direct modification of influenza genome in order to remove the virulent motif from HA gene of the highly pathogenic avian influenza virus (Marsh and Tannock, 2005). It has also been used to generate recombinant LAIVs that carry foreign target genes. Such recombinant LAIVs can be used as gene delivery vectors for promoting specific mucosal and CMI responses. Currently, some recombinant LAIVs are under development as vectors for vaccines against pathogens such as malaria and *Chlamydia trachomatis* and range of cancers (Efferson et al 2003; González-Aseguionolaza et al 2003; Nimmerjahn et al 2003; He et al 2007).

1.3.2.3 The immunology of ca LAIVs

Restricted viral replication of *ca* LAIVs in the respiratory tract can induce both specific humoral and CMI, and local and systemic and memory cross-immune responses which are a feature of natural infections (Section1.2.2; Wareing and Tannock 2001). Consequently, *ca* LAIVs have several advantages over inactivated vaccines (Table 1.9). Of these, high vaccine efficacy in children is significant (Belshe et al 1998 & 2000; Jefferson et al 2008). Non-immune children play a central role in the transmission of influenza viruses (Bradshaw and Wright 2002) and here *ca* LAIVs could have a role in limiting the speed of transmission or even preventing an epidemic. However, in adults lower levels Ab responses are induced by i.n administered LAIVs compared with parenterally administered inactivated vaccines, possibly due to the presence of pre-existing neutralizing Abs (Edwards et al 1994). Despite this, *ca* LAIVs potentially provide better protection because they induce a broader range of immune responses (Belshe 2002).

These include vaccine-induced T cell and mucosal immunity but the role of other factors, such as the presence of particular vaccine genes and the age of vaccinees, on these responses is largely unknown (Greenberg 2006).

1.4 CONCLUSIONS AND RESEARCH AIMS

Vaccination remains the most cost-effective means for the control of influenza. However, vaccine efficacy is constrained by the capacity of influenza viruses to undergo antigenic variation. To increase protection rates *ca* LAIVs (FluMist®) are now used in the US in addition to traditional inactivated TIVs (Table 1.10). *Ca* LAIVs, unlike inactivated vaccines, are able to induce responses in all arms of the host adaptive immune response. These include humoral and cellular, local and systemic responses; which are required for optimal protection against influenza.

The immunogenicity of *ca* LAIVs depends on viral infectivity for the host, which determines the extent of immune stimulation. The immunogenicity of live vaccines is influenced by several viral and host factors. Viral factors include the nature of both internal and surface Ags. The six internal genes derived from *ca* donor strains are responsible for the attenuation phenotype of vaccine reassortants. Consequently, 6:2 *ca* reassortants are less immunogenic than their *wt* parental viruses. Earlier protection studies have shown that the surface HA glycoprotein is the major protective immunogen (Tannock et al 1984 & 1991; Marsh et al 2003; Wareing et al 2005).

However, CMI responses induced by internal Ags and their impact on the overall vaccine immunogenicity have not been examined in detail. With recent technological advances, such as the use of MHC tetramer techniques to measure T-cell responses, it is now possible to examine

mechanisms of CMI in the respiratory tract of mice and to provide further insights in the improvement of vaccine efficacy. The use of these techniques in examining responses to *ca* LAIVs in mouse models forms the basis of this thesis.

Specific research **aims** of this study are:

1) To examine the infectivity and pathogenicity of *ca* LAIVs;
2) To evaluate recent developed techniques (e.g. tetramer staining and I-PCR) in the measurement of specific T-cell response to *ca* LAIVs;
3) To investigate the role of the HA and NA viral surface Ags on CMI responses;
4) To compare the specific T- and B-cell responses to *ca* LAIVs in different mouse strains possessing different MHCs;
5) To investigate the B cell-mediated heterosubtypic immunities which induced by *ca* LAIVs.

Chapter 2: Materials and Methods

2.1 CHEMICALS

2.1.1 Inorganic chemicals

Chemical	Supplier
Aluminum potassium sulphate (alum)	BDH Chemicals, Poole, UK
Ammonium chloride (AnalaR)	BDH Chemicals, Poole, UK
Calcium chloride	BDH Chemicals, Poole, UK
Hydrochloric acid (Univar)	Ajax Chemicals, Auburn, NSW
Magnesium chloride (AnalaR)	BDH Chemicals, Poole, UK
Magnesium sulphate, heptahydrate (AnalaR)	BDH Chemicals, Poole, UK
Potassium chloride	Ajax Chemicals, Sydney, NSW
Potassium dihydrogen orthophosphate	BDH Chemicals, Poole, UK
Sodium azide	ICN Biomedicals Australia Pty Ltd, Seven Hills, NSW
Sodium bicarbonate	BDH Chemicals, Poole, UK
Sodium borate, anhydrous	BDH Chemicals, Poole, UK
Sodium carbonate	BDH Chemicals, Poole, UK
Sodium chloride (AnalaR)	Ajax Chemicals, Auburn, NSW
tri-Sodium citrate (AnalaR)	BDH Chemicals, Poole, UK
Sodium dihydrogen phosphate (AnalaR)	BDH Chemicals, Poole, UK
Sodium hydrogen carbonate (AnalaR)	BDH Chemicals, Poole, UK
di-Sodium hydrogen orthophosphate (AnalaR)	BDH Chemicals, Poole, UK
Sodium hydroxide (AnalaR)	BDH Chemicals, Poole, UK
Sodium iodide	BDH Chemicals, Poole, UK

2.1.2 Organic chemicals

Chemical	Supplier
Acetic acid, glacial (AnalaR)	BDH Chemicals, Poole, UK

Acrylamide (N,N'–methylenbisacrylamide)	Bio-Rad Laboratories, Hercules, CA, USA
Agarose (molecular biology grade)	Promega Corporation, Annandale, NSW
Ammonium persulphate (APS)	Sigma-Aldrich Inc., St. Louis, MO, USA
2-Amino-2-methyl-1-propanol (AMP)	Sigma-Aldrich Inc., St. Louis, MO, USA
L-Arginine hydrochloride	Sigma-Aldrich Inc., St. Louis, MO, USA
Brefeldin A (BFA)	Sigma-Aldrich Inc., St. Louis, MO, USA
5-Bromo-4-chloro-3-indolyl phosphate (BCIP)	Sigma-Aldrich Inc., St. Louis, MO, USA
Bromophenol Blue	Sigma-Aldrich Inc., St. Louis, MO, USA
Coomassie Brilliant Blue R250®	Sigma-Aldrich, Inc., St. Louis, MO, USA
Choral hydrate	BDH Chemicals, Poole, UK
Citric acid	BDH Chemicals, Poole, UK
D-glucose (AnalaR)	BDH Chemicals, Poole, UK
DE52 DEAE cellulose	Whatman Inc., Florham Park, NJ, USA
Diethylaminoethyl (DEAE) -Sepharose®	Sigma-Aldrich Inc., St. Louis, MO, USA
Diethyl polycarbonate (DEPC)	Sigma-Aldrich Inc., St. Louis, MO, USA
Dimethyl sulphoxide (DMSO) (AnalaR)	BDH Chemicals, Poole, UK
Dithiothreitol (DTT)	Progen Industries Ltd., QLD
DPX mountant	Sigma-Aldrich Inc. St. Louis, MO, USA
Ethanol (ethyl alcohol)	BDH Chemicals, Poole, UK
Ethidium bromide (EB)	Sigma-Aldrich Inc., St. Louis, MO, USA
Ethylenediamine tetra-acetic acid (EDTA), disodium salt (AnalaR)	BDH Chemicals, Poole, UK
Formalin (40% formaldehyde)	Sigma-Aldrich Inc., St. Louis, MO, USA
L-Glutathione oxidized	Sigma-Aldrich Inc., St. Louis, MO, USA
L-Glutathione reduced	Sigma-Aldrich Inc., St. Louis, MO, USA
Glycerol (AnalaR grade)	BDH Chemicals, Poole, UK
Glycine (AnalaR grade)	BDH Chemicals, Poole, UK
Guanidine hydrochloride (GuHCl)	Sigma-Aldrich Inc., St. Louis, MO, USA

N-(2-Hydroxyethyl) piperazine-*N'*-(2-ethanesulphonic acid) (HEPES)	Research Organics Inc., Cleveland, OH, USA
Isopropyl-β -D-thiogalactopyranoside (IPTG)	Progen Industries Ltd, QLD
Magnesium acetate	Sigma-Aldrich Inc., St. Louis, MO, USA
Methanol (AnalaR)	BDH Chemicals, Poole, UK
Paraffin	Sigma-Aldrich Inc., St. Louis, MO, USA
Pepstatin A	Sigma-Aldrich Inc., St. Louis, MO, USA
Phenol Red	Sigma-Aldrich Inc., St. Louis, MO, USA
Polyethylene-sorbitan monolaurate (Tween-20)	Sigma-Aldrich Inc., St. Louis, MO, USA
Phenylmethylsulphonylfluoride (PMSF)	Sigma-Aldrich Inc., St. Louis, MO, USA
Pyruvic acid	Sigma-Aldrich Inc., St. Louis, MO, USA
PVA glue	UHU Australia Pty Ltd, Smithfield, NSW
Saponin	Calbiochem Corporation, La Jolla, CA, USA
Sodium azide	Sigma-Aldrich Inc., St. Louis, MO, USA
Sodium deoxycholate (DOC)	Sigma-Aldrich Inc., St. Louis, MO, USA
Sodium dodecyl sulphate (SDS)	BDH Chemicals, Poole, UK
Sucrose (AnalaR)	BDH Chemicals, Poole, UK
Tetramethylethylenediamine (TEMED)	Sigma-Aldrich Inc., St. Louis, MO, USA
Tris (hydroxymethyl) aminomethane (Tris-HCl)	Research Organics Inc., Cleveland, OH, USA
Triton X-100	Sigma-Aldrich Inc., St. Louis, MO, USA
Triton X-405	Sigma-Aldrich Inc., St. Louis, MO, USA
Xylene	Sigma-Aldrich Inc., St. Louis, MO, USA
Xylene cyanol FF	Bio-Rad Laboratories Pty Ltd, Regents Park, NSW

2.1.3 Antibiotics and anaesthetics

Amphotericin B (fungizone)	Sigma-Aldrich Inc., St. Louis, MO, USA
Chloramphenicol	Sigma-Aldrich Inc., St. Louis, MO, USA
Ketamine (hydrochloride)	Troy Laboratries (Australia) Pty Ltd, Smithfield, NSW
Kanamycin sulphate	Roche Diagnostics GmbH, Mannheim, Germany
Penicillin G	CSL Ltd, Parkville, VIC
Ilium Xyalzine-20 (hydrochloride)	Troy Laboratories (Australia) Pty Ltd, Smithfield, NSW
Streptomycin sulphate	CSL Ltd, Parkville, VIC

2.1.4 Cell culture media

Eagle's Minimal Essential Medium (MEM) (modified)	ICN Biomedicals (Austtalia) Pty Ltd, Seven Hills, NSW
Leibovitz (L_{15}) medium	Trace Biosciences, Sydney, NSW
RPMI-1640 medium	Trace Biosciences, Castle Hill, NSW

2.1.5 Dyes

Neutral Red (cell culture tested)	Sigma-Aldrich Inc., St. Louis, MO, USA
Trypan Blue	ICN Biomedical (Australia) Pty Ltd, Seven Hills, NSW
Haematoxylin	Sigma-Aldrich Inc., St. Louis, MO, USA
Eosin Y	Sigma-Aldrich Inc., St. Louis, MO, USA

2.2 BIOLOGICAL REAGENTS

2.2.1 Virus stocks

The *ca* donor strain (A/AA/6/60 *ca*) and six *ca* reassortants, derived from A/AA/6/60 *ca*, were used in the present study. Table 2.1 summarises the origin and gene composition of these reassortants and the *wt* parental viruses that were used to prepare each reassortant (Tannock et al 1984 & 1995). CR6, CR18, CR29 and CR35 were obtained by co-infection of primary chicken embryo kidney (CEK) cultures with a *wt* virus; A/AA/6/60-*ca* was derived from the *wt* A/AA/6/60 (Maassab 1969) by adaptation to growth at 25ºC. A/AA/6/60 *ca*, CR6, CR18, CR29 and CR35 and the wt parental viruses A/AA/6/60 (H2N2), A/Qld/6/72 (H3N2), A/HK/123/77 (H1N1) were generously provided by Prof. H. F. Maassab, Department of Epidemiology, School of Public Health, University of Michigan, Ann Arbor, USA. CR6/35/1/19 and CR6/35/2/9 were prepared by co-infection of CEK cells with CR6 and CR35 (Tannock et al 1995).

The influenza viruses A/PR/8/34 (H1N1) and A/HK×31 (H3N2) were used as reagent controls for the tetramer staining technique. A/HK×31 was obtained from Dr. S. J. Turner, Dept. Microbiology and Immunology, University of Melbourne. The A/HK×31 and A/PR/8/34 viruses differed in their surface HAs and NAs, but shared the internal genes of A/PR/8/34 (Turner et al 2006). All viruses were passaged 1-2 times in 10 - or 11-day-old non-specific-pathogen-free (non-SPF) fertile chicken eggs for 2 or 3 days at 33-34°C. The allantoic virus was harvested and stored in 2mL aliquots at - 80°C.

2.2.2 Primary and continuous cell cultures

Primary CEK cells were prepared from 18-day-old chicken embryo kidneys using the method of Tannock et al (1985). The MDCK line was obtained from CSL Ltd, Parkville, VIC and stored in liquid nitrogen and was shown to be free of *Mycoplasma*. MDCK cultures prepared from resuscitated cells were passaged 25 times and then discarded in order to minimise the chances of further contamination.

Table 2.1 Gene composition of A/AA/6/60-*ca* and *ca* reassortants (CR) used in this study (Tannock et al 1984 & 1995).

ca Virus	Surface antigen	Parent viruses	Derivation of genes[a]							
			HA	NA	PA	PB1	PB2	NP	M	NS
A/Ann Arbor (AA)/6/60 *ca*	H2N2	A/AA/6/60 *wt*	A[b]	A	A	A	A	A	A	A
AA-CR6	H3N2	A/Queensland (Qld)/6/72 *wt* and A/AA/6/60 *ca*	W[c]	W	A	A	A	A	A	A
AA-CR18	H3N2	A/Scotland (Scot)/840/74 *wt* and A/AA/6/60 *ca*	W	W	A	A	A	A	A	W
AA-CR29	H3N2	A/Alaska/6/77 *wt* and A/AA/6/60 *ca*	W	W	A	A	A	A	A	A
AA-CR35	H1N1	A/HongKong (HK)/123/77 *wt* and A/AA/6/60 *ca*	W	W	A	A	A	A	A	A
AA-CR6/35/1/19	H1N2	CR6 and CR35	W	W	A	A	A	A	A	A
AA-CR6/35/2/9	H3N1	CR6 and CR35	W	W	A	A	A	A	A	A

[a] HA, haemagglutinin; M, matrix protein; NA, neuraminidase; NS, nonstructural protein; PA, PB1 and PB2 polymerase; NP, nucleoprotein.

[b] A indicates gene derived from A/AA/6/60-*ca*.

[c] W indicates gene derived from the wild-type parent.

2.2.3 Enzymes

BirA biotinlyation enzyme (recombinant)	Prepared and supplied by Dr. A. G. Brook, Department of Microbiology & Immunology, University of Melbourne, Parkville, VIC
Collagenase A, 0.18 U mg^{-1}	Roche Diagnostics GmbH, Mannheim, Germany
DNase I, 2000 U mg^{-1}	Roche Diagnostics GmbH, Mannheim, Germany
Lysozyme	Sigma-Aldrich Inc., St. Louis, MO, USA
Receptor destroying enzyme (RDE) - crude *V. Cholerae* filtrate, lyophilised	Denka Seiken Co., Ltd., Tokyo, Japan
Trypsin (crystalline)	Difco Laboratories, Detroit, MI, USA
Taq DNA Polymerase	Invitrogen Corp., Carlsbad, CA, USA

2.2.4 Antibodies

2.2.4.1 Primary antibodies

Monoclonal rat anti-mouse IFN-γ (IgG2a isotype)	Sigma-Aldrich Inc., St. Louis, MO, USA
Polyclonal goat anti-mouse IgA (α-chain specific)	Sigma-Aldrich Inc., St. Louis, MO, USA
Polyclonal goat anti-mouse IgG2a (heavy chain-specific)	Sigma-Aldrich Inc., St. Louis, MO, USA

2.2.4.2 Conjugated antibodies

Alkaline phosphatase (AP) conjugated:

Rabbit anti-goat IgG	Sigma-Aldrich Inc., St. Louis, MO, USA

Biotin conjugated:

Polyclonal rabbit anti-mouse interferon-gamma (IFN-γ)	PeproTech Inc., Rocky Hill, NJ, USA

Fluorescein isothiocyanate (FITC) conjugated:

Monoclonal mouse anti-mouse CD44 (IgG1 isotype)	Beckman Coulter Australia Pty Ltd, Gladesville, NSW
Monoclonal rat anti-mouse CD4 (Ig G2b), CD8 (IgG2a) and INF-γ (IgG1)	Beckman Coulter Australia Pty Ltd, Gladesville, NSW

R-phycoerythrin (PE) conjugated:

Monoclonal rat anti-mouse CD4 (IgG2b) and CD8 (IgG2a)	Beckman Coulter Australia Pty Ltd, Gladesville, NSW

2.2.4.3 Isotype controls

Rat IgG1, IgG2a FITC conjugated	Beckman Coulter Australia Pty Ltd, Gladesville, NSW
Rat IgG2a, IgG2b PE conjugated	Beckman Coulter Australia Pty Ltd, Gladesville, NSW

2.2.5 Recombinant cytokine

Recombinant mouse IFN-γ	PeproTech Inc., Rocky Hill, NJ, USA

2.2.6 Bovine products

Bovine serum albumin, Fraction V (BSA)	Trace Biosciences, Castle Hill, NSW
Gelatin, type B (cell culture tested)	Sigma-Aldrich Inc., St. Louis, MO, USA
Foetal bovine serum (FCS)	Thermo Trace Ltd, Melbourne, VIC

2.2.7 Products for biotinylation

Protein assay kit	Bio-Rad Laboratories Pty Ltd, Regents Park, NSW
Streptavidin beads	Sigma-Aldrich Inc., St. Louis, MO, USA

Extravidin® -R - phycoerythrin (PE)	Sigma-Aldrich Inc., St. Louis, MO, USA

2.2.8 Products for Immuno-PCR

QIAGEN Multiplex PCR Kit	QIAGEN Pty Ltd, Clifton Hill, VIC
QIAfilter™ Plasmid Midi Kit	QIAGEN Pty Ltd, Clifton Hill, VIC
QIAquick® PCR purification kit	QIAGEN Pty Ltd, Clifton Hill, VIC
Salmon testis DNA (for hybridization); 9.0 mg mL^{-1}	Sigma-Aldrich Inc., St. Louis, MO, USA
Streptavidin (1mg mL^{-1})	Amersham International Plc, Buckinghamshire, UK
Streptavidin-horseradish peroxidzae conjugated	Amersham International Plc, Buckinghamshire, UK

2.2.9 Oligonucleotides

Oligonucleotides were purchased as desalted preparations of approximately 30 nmol from GeneWorks Pty Ltd, Thebarton, SA. A summary of all oligonucleotides used in Immuno-PCR assays is presented in Table 2.2.

2.2.10 Viral peptide

A/A A/6/60 $NP_{366-374}$	Synthesised by Auspep Pty. Ltd., Parkville, VIC

2.2.11 Plasmids

D^b – Kan^R (used for the expression of MHC heavy chain of C57BL/6 mouse and bearing the kanamycin-resistance gene as a selectable marker)	supplied by Dr. S. J. Turner, Department of Microbiology & Immunology, University of Melbourne, Parkville, VIC
pBluescript® KS (used for the preparation of reporter DNA in Immuno-PCR assays)	Novagen, Merck Pty. Limited, Kilsyth, VIC

2.2.12 Bacterial strain

BL21 (DE3) pLysS (competent *E. coli*, used for the expression of MHC)	Novagen, Merck Pty. Limited, Kilsyth, VIC

2.2.13 MHC light chain

Human MHC β_2m	Department of Microbiology and Immunology, University of Melbourne, Parkville, VIC

2.2.14 Tetramers

$D^b/h\beta_2m/NP_{366\text{-}374}$ and $D^b/h\beta_2m/PA_{224\text{-}236}$	Department of Microbiology and Immunology, University of Melbourne, Parkville, VIC

The sequences of all the peptides used in the tetramers are listed in the Table 2.3.

2.2.15 Molecular weight markers

DNA marker, 1 kb Plus (100 bp – 10 kb)	Invitrogen Corp., Carlsbad, CA, USA
Protein marker, Kaleidoscope (prestained; 210 – 7.0 kDa)	Bio-rad Laboratories, Hercules, CA, USA

2.3 MICE AND EMBRYONATED EGGS

Non-SPF embryonated eggs	Research Poultry Farm, Research, VIC
SPF female C57BL/6 mice	Monash Animal Services, Clayton, VIC
SPF female BALB/c mice	Monash Animal Services, Clayton, VIC

Table 2.2 Oligonucleotides used in immuno-PCR assays

Oligo Name	DNA Sequence (5' → 3')
M13-20	Biotin-GT AAA ACG ACG GCC AGT
M13	GGA AAC AGC TAT GAC CAT G
IF	AGC GCG CGT AAT ACG ACT C
IR	ACC ATG ATT ACG CCA AGC G

Table 2.3 Peptides used in tetramer staining assays

Peptide	Virus Origin	Amino Acid Sequence (H → OH)
$NP_{366\text{-}374}$	A/AA/6/60 *ca*	Ala-Ser-Asn-Glu-Asn-Met-**Asp**-Thr-Met
$NP_{366\text{-}374}$	A/PR/8/34	Ala-Ser-Asn-Glu-Asn-Met-**Glu**-Thr-Met
$PA_{224\text{-}236}$	A/PR/8/34	Ser-Ser-Leu-Glu-Asn-Phe-Arg-Ala-Tyr-Val

Previous studies indicated that male mice allow more rapid rates of multiplication of influenza A than female mice and were used in immunologic study for *ca* reassortants (Tannock et al1984&1987; Wareing 2001&2005). However, female inbred mice have been used intensively in the studies of specific-CTL responses to influenza virus (Altman et al 1996; Doherty et al 1997; Belz et al 2000&2001). In a previous study (L. Xue, M. App. Sc. Thesis, RMIT University, 2003) no differences were found between serum Ab and lung B cell responses to *ca* viruses in male and female BALB/c mice.

2.4 BUFFERS AND SOLUTIONS

All buffers and solutions were prepared using Milli-Q® water, unless otherwise specified.

2.4.1 Egg inoculation reagents

Dulbecco's Phosphate Buffered Saline (PBS; pH 7.2)	0.14 M NaCl, 30 mM KCl, 82.1 mM $Na_2HPO_4.12H_2O$, 14.7 mM KH_2PO_4
Virus diluent	Chilled sterile PBS with 0.1% (v/v) penicillin (60 μg mL^{-1}) and 0.1% (v/v) streptomycin (100 μg mL^{-1})

2.4.2 HA and HAI reagents

Calcium saline (pH 5.6)	68.01 mM $CaCl_2.H_2O$, 1.54 M NaCl, 0.19 M H_3BO_3, 2.60 mM $Na_2B_4O_7.10H_2O$
1.5% (w/v) sodium citrate	5.44 mM $Na_3C_6H_5O_7.2H_2O$ in PBS
Receptor destroying enzyme (RDE) solution	One part RDE and one part calcium saline solution
Chicken red blood cells (RBC)	0.5% (v/v) chicken RBC suspended in PBS

2.4.3 CEK and MDCK plaque assay reagents

Citrate Saline	1.07 M NaCl, 27.8 mM KCl, 14.7 mM KH_2PO_4, 0.1 M D-glucose, 0.27 M $Na_3C_6H_5O_7.2H_2O$, 82.1 mM $Na_2HPO_4.12H_2O$

0.1% (w/v) Neutral Red	0.1 g Neutral Red in 100 mL H_2O
0.1% (w/v) crystalline trypsin	0.1 g crystalline trypsin in 100 mL H_2O
Trypsin/Versene (T/V)	0.5 mM EDTA, 0.1% (w/v) trypsin in PBS
Trypsinising Solution	1.25% (w/v) trypsin in citrate saline solution containing 60 μg mL^{-1} penicillin, 100 μg mL^{-1} streptomycin, and 1 μg mL^{-1} amphotericin B

2.4.4 Virus purification reagents

TNE Buffer (pH 7.4)	0.05 M Tris-HCl, 0.15 M NaCl, 1 mM EDTA
60% (w/v) sucrose in TNE Buffer	Autoclaved at 108°C for 30min
30% (w/v) sucrose in TNE Buffer	Autoclaved at 108°C for 30min

2.4.5 Reagents for lung cell suspensions

ATC Buffer (pH 7.2)	0.15 M NH_4Cl, 17 mM Tris-HCl
Hank's Balanced Salt Solution (HBSS)	0.137 M NaCl, 5.4 mM KCl, 0.25 mM Na_2HPO_4, 0.44 mM KH_2PO_4, 1.3 mM $CaCl_2$, 1.0 mM $MgSO_4$, 1.0 mM $MgCl_2$, 4.2 mM $NaHCO_3$, 0.1 M D-glucose and 0.01‰ (w/v) Phenol Red, pH 7.2
Lung Digestion Solution	100 mg collagenase A, and 5 mg DNase I in 50 mL HBSS with 60 μg mL^{-1} penicillin, 100 μg mL^{-1} streptomycin, and 1 μg mL^{-1} amphotericin B
0.4% (w/v) Trypan Blue	0.4 g Trypan Blue in 100 mL PBS

2.4.6 ELISPOT reagents

PBS-T	0.05% Tween-20 in PBS
BSA_5PBST	0.5% (w/v) BSA in PBS-T
$BSA_{10}PBS$	1.0% (w/v) BSA in PBS-T

$PBSN_3$	0.02% (w/v) NaN_3 in PBS
AMP Buffer (pH 10.25)	2.69 M AMP, 1.84 mM $MgCl_2.6H_2O$, 0.04 M NaN_3, 0.01% TritonX-405
Substrate Solution (BCIP)	1 mg mL^{-1} 5-bromo-4-chloro-3-indolyl phosphate in AMP buffer

2.4.7 Flow cytometry reagents

2.4.7.1 T lymphocyte phenotype reagents

Cell Wash and Staining Buffer	2% (v/v) FCS, 0.1% (w/v) NaN_3 in PBS
Cell Fixation Wash Buffer	0.02% (w/v) gelatine, 0.1% (w/v) NaN_3 in PBS
Cell Fixation Buffer	2% paraformaldehyde (w/v) in PBS

2.4.7.2 Intracellular cytokine cytometry (ICC) reagents

ICC Cell Wash Buffer	2% (v/v) FCS in PBS
ICC Cell Fixation Wash Buffer	0.02% (w/v) gelatine, 0.1% (w/v) NaN_3, 2.5 µg/mL BFA in PBS
ICC Cell Staining Buffer	2% (v/v) FCS, 0.1% (w/v) NaN_3, 5 µg/mL BFA in PBS
ICC Cell Permeablisation Buffer	2% (v/v) FCS, 0.1% (w/v) NaN_3, 0.5% (w/v) saponin in PBS
ICC Fixation Buffer	2% paraformaldehyde (w/v), 5 µg/mL BFA in PBS

2.4.8 Reagents for mouse MHC class I expression, refolding and tetramer formation

2.4.8.1 Inclusion body reagents

Resuspension Buffer (pH8.0)	50 mM Tris-HCl (pH8.0), 25% (w/v) sucrose, 1mM EDTA and 0.1% (w/v) NaN_3

Resuspension Solution I	0.2 mM PMSF, 0.001‰ (w/v) pepstatin A and 10 mM DTT in resuspension buffer
Resuspension Solution II	0.2 mM PMSF, 0.001‰ (w/v) pepstatin A and 10 mM DTT in H_2O
Lysis Buffer (pH8.0)	50 mM Tris-HCl (pH8.0), 1% (w/v) Triton-X 100, 1% (w/v) NaDOC, 100 mM NaCl and 0.1% (w/v) NaN_3
Lysis Solution	0.1% (w/v) lysozyme, 0.02% (w/v) DNase I, 5 mM $MgCl_2$ and 10 mM DTT in lysis buffer
Wash Buffer I (pH 8.0)	50 mM Tris-HCl (pH8.0), 0.5% (v/v) Triton-X 100, 100 mM NaCl, 1 mM NaEDTA, 1 mM DTT and 0.1% (w/v) NaN_3
Wash Buffer II (pH 8.0)	50 mM Tris-HCl (pH8.0), 1 mM NaEDTA, 1 mM DTT and 0.1% (w/v) NaN_3

2.4.8.2 SDS-PAGE reagents

Lower (separation) Gel Buffers (pH 8.8)	18% (v/v) acrylamide, 1.5 M Tris-HCl, 0.4% SDS, 0.05% APS and 0.1% (v/v) TEMED
Upper (stacking) Gel Buffers (pH 6.8)	1.25% (v/v) acrylamide, 0.5 M Tris-HCl, 0.4% SDS, 0.05% APS and 0.1% (v/v) TEMED
2 × Loading Buffer	100 mM Tris-HCl (pH 6.8), 4% SDS, 0.2% Bromophenol Blue, 20% glycerol with 200mM DTT added before use
5 × Electrophoresis Buffer (pH 8.3)	25 mM Tris, 250 mM glycine and 0.1% SDS
Coomassie Blue Gel Stain Buffer	0.1% R250 Coomassie Blue, 40% methanol and 10% acetic acid
Gel Destaining Buffer	40% methanol and 10% acetic acid

2.4.8.3 Monomeric MHC class I complex refolding reagents

Guanidine Buffer	6 M GuHCl in resuspending solution II, with 0.2 mM PMSF, 0.001‰ (w/v) pepstatin A with 10 mM DTT added immediately prior to use
Refolding Buffer (prepared fresh in an ice bath)	100 mM Tris, 2 mM EDTA (pH8.0), 400 mM L-arginine-HCl, 0.5 mM oxidised glutathione, 5mM reduced glutathione, with 0.2 mM PMSF, 0.001‰ (w/v) pepstatin A added immediately prior to use

2.4.8.4 Dialysis reagent

Dialysis Buffer	1 mM Tris-HCl (pH 8.0) in cold H_2O and stirred at 4°C for 12-24 hr before use

2.4.8.5 Biotinylation reagents

10 × BioMix Buffer A	5 M Tris-HCl in H_2O
10 × BioMix Buffer B	100 mM ATP and 100 mM magnesium acetate in H_2O

2.4.8.6 Tetramer forming reagent

Wash Buffer for streptavidin beads	10 mM Tris-HCl (pH 8.0), 150 mM NaCl, 1% Triton X-100 and 0.5 mM EDTA

2.4.9 Immuno-PCR reagents

Carbonate Coating Buffer (pH 9.6)	0.15 M Na_2CO_3, 0.35 M $NaHCO_3$ and 0.03 M NaN_3 in PBS
Blocking Buffer	0.1 mg mL^{-1} salmon sperm DNA, 1% (w/v) BSA and 0.02% (w/v) NaN_3 in PBS
Washing Buffer	0.05% Tween-20 (v/v) and 0.02% (w/v) NaN_3 in PBS

Antibody and antigen diluent	1% BSA (w/v), 5% FCS (v/v) and 0.02% (w/v) NaN_3 in PBS
Free Streptavidin Solution	1 mg mL^{-1} streptavidin and 1% (w/v) BSA in PBS
Reporter DNA Solution	1 ng L^{-1} DNA and 0.1% (w/v) BSA in PBS
DEPC H_2O	1% (v/v) diethyl pyrocarbonate (DEPC) in H_2O
Ethidium Bromide Solution	0.5 μg mL^{-1} ethidium bromide in H_2O
DNA Loading Buffer (6 ×)	0.25% (w/v) Bromophenol Blue, 0.25% (w/v) xylene cyanol FF and 30% (v/v) glycerol
TAE Buffer (1 ×)	40 mM Tris acetate and 10mM EDTA (pH 7.6)

2.4.10 Reagents for mouse lung histopathology

10% neutral buffered formalin (NBF)	10% (v/v) formalin in PBS
Mayer's Haematoxylin Solution	5% (w/v) alum, 5% (w/v) choral hydrate and 0.1% (w/v) haematoxylin in distilled water
Eosin Y Solution	1% (w/v) Eosin Y in distilled water

2.5 MEDIA

All media were prepared using Milli-Q® water, unless otherwise specified.

2.5.1 Cell culture media

2× L_{15} maintenance medium	2× L_{15} (with L-glutamine), 40 mM HEPES (pH 6.8), 0.056% (w/v) sodium bicarbonate, 60 μg mL^{-1} penicillin, 200 μg mL^{-1} streptomycin, 2 μg mL^{-1} amphotericin B

MEM maintenance medium	MEM (pH 7.2) with 20 mM Hepes, 0.14% (w/v) sodium bicarbonate, 60 μg mL^{-1} penicillin, 100 μg mL^{-1} streptomycin, and 1 μg mL^{-1} amphotericin B
MEM growth medium	MEM maintenance medium with 5% (v/v) heat-inactivated (56°C for 30 min) FCS for MDCK cultures; 10% for primary CEK cell cultures
T-cell medium (TCM)	RPMI medium (pH 7.2, with L-glutamine), 10% FCS, 2 mM sodium pyruvate, 6 mM HEPES (pH 6.8), 0.056% (w/v) sodium bicarbonate, 60 μg mL^{-1} penicillin, 100 μg mL^{-1} streptomycin and 1 μg mL^{-1} amphotericin B
CEK plaque assay overlay	Equal volumes of a 2× concentration of MEM maintenance medium and 1.8% agarose held at 46° C and mixed together
MDCK plaque assay overlay	Equal volumes of a 2× concentration of L_{15} maintenance Medium containing 100 μL 100 mL^{-1} of 0.1% crystalline trypsin and 1.8% agarose were mixed together at 46° C.
Neutral Red staining overlay	Agarose overlay for CEK or MDCK containing 1: 12,500 (w/v) final concentration of Neutral Red; held at 46° C.

2.5.2 Bacterial culture media

Luria-Bertani (LB) broth	1% (w/v) Bacto tryptone, 0.5% (w/v) yeast extract, 1% (w/v) NaCl with 34 μg mL^{-1} chloramphenicol and 50 μg mL^{-1} kanamycin.

Luria-Bertani (LB) agar	1% (w/v) Bacto tryptone, 0.5% (w/v) yeast extract, 1% (w/v) NaCl and 1.5% (w/v) agarose (bacteriology grade) with 34 μg mL^{-1} chloramphenicol and 50 μg mL^{-1} kanamycin.

2.6 EQUIPMENT

Analytical balance, Sartorius BP 210S	Sartorius AG, Goettingen, Germany
Centrifuges:	
Eppendorf centrifuge, 5415C	Eppendorf, Geratebau, Germany
Refrigerated bench top centrifuge, Sigma 3K10	Quantum Scientific Pty Ltd Milton, QLD
Ultracentrifuge, Optima L-80 XP	Beckman Instruments Inc., Palo Alto, CA, USA
Camera, Olympus DP70	Olympus Australia Pty Ltd., Mount Waverley, VIC
Columns:	
Econo-column®	Bio-Rad Laboratories Pty Ltd, Regents Park, NSW
Superdex™ PD-10 desalting column	Amersham Biosciences Co. Piscataway, NJ, USA
Electrophoresis gel tanks	Bio-Rad Laboratories, Hercules, CA, USA
Electrophoresis power supply	Bio-Rad Laboratories, Hercules, CA, USA
Filter units:	
Acrocap 0.2μm	Gelman Sciences, Ann Arbor, MI, USA
Acrodisc syringe filter 0.2μm	Gelman Sciences, Ann Arbor, MI, USA
Centricon® centrifugal filter unit with Ultracel YM-100 membrane	Millipore Australia Pty Ltd., North Ryde, NSW
FALCON® Cell strainer (100 μm Nylon)	Becton Dickinson Labware Franklin Lakes, NJ, USA
Tissue sieve (100 μm Metal)	RMIT Workshop, Melbourne, VIC

Flow cytometers:

FACScan (locate at WEHI)	Becton Dickinson, Australia Pty Ltd. North Ryde, NSW
EPICS Altra (located at RMIT)	Beckman Coulter, Australia Pty Ltd. Gladesville, NSW
FPLC™ system, Pharmacia LCC-500	Amersham Biosciences Co. Piscataway, NJ, USA
Haemocytometer, Improved Neubauer (Brightline)	Selby Scientific Instruments, Notting Hill, VIC
Homogenisers:	
Polytron	Glen Mills Inc., Clifton, NJ, USA
Ultraturrax T25 basic	IKA-Werke, Staufen, Germany
Incubators:	
CO_2 Incubator, Sanyo MCO-17A1	Quantum Scientific Pty Ltd, Milton, QLD
CO_2 Incubator, Flow 210	Flow Laboratories, Selby-Biolab, Clayton, VIC
Electronic egg incubator, Multiplo E1	Multiplo Pty Ltd, Sydney, NSW
Microscopes:	
Light microscope, BX45	Olympus Australia Pty Ltd, Mount Waverley, VIC
Inverted microscope, IX51	Olympus Australia Pty Ltd, Mount Waverley, VIC
PCR machine, GeneAmp PCR System 2400	Perkin-Elmer Corp., Foster City, CA, USA
pH meter, ATI Orion Model 420A	Orion Pacific, Frankston, VIC
Plates:	
HI & HA plates, 96-well V-bottomed	Nalge Nunc International, Rochester, NY, USA
Nunc-Immuno™ plates, 96-well flat-bottomed (MaxiSorp™ surface)	Nalge Nunc International, Rochester, NY, USA
Cell culture plates 6, 24 & 96-well	Greiner Labortechnik, Frickenhausen, Germany

Item	Supplier
Vinyl flat-bottomed Microtiter® plates, polyvinyl chloride (PVC)	Thermo Labsystems, Franklin, MA, USA
Rotary microtome, Leica RM2265	Leica Microsystems Pty Ltd., Gladesville, NSW
Software:	
FACS analysis, EXPO32™	Applied Cytometry Systems, Dinnington, Sheffield, UK
FACS analysis, FlowGel	Becton Dickinson, Australia Pty Ltd North Ryde, NSW
Statistic, MINITAB® 14	Minitabs Inc., State College, PA, USA
Photo process, DP Controller and Manager	Olympus Australia Pty Ltd., Mount Waverley, VIC
Cell culture flasks 25, 75 & 175cm^2	SARSTEDT Australia Pty Ltd., Technology Park, SA
Tissue processor, Shandon Pathcentre®	Thermo Electron Corporation, Waltham, MA, USA
Tubes:	
BD Falcon™ round-bottomed test tube	Becton Dickinson, Australia Pty Ltd., North Ryde, NSW
Polyallomer centrifuge tubes	Beckman Instruments, Inc., Palo Alto, CA, USA
Ultra-Clear™ centrifuge tubes	Beckman Instruments, Inc., Palo Alto, CA, USA
Ultracentrifuge swinging bucket rotor, SW41	Beckman Instruments Inc., Palo Alto, CA, USA

2.7 CELL CULTURES

2.7.1 MDCK cell line

Cultures of the MDCK line were prepared in MEM growth medium in 75 cm^2 cell culture flasks and incubated at 37°C in a 5% CO_2 incubator. Confluent monolayers were split at ratios of 1:5 - 1:10, depending on when they were needed.

2.7.2 Primary CEK cultures

Chicken embryo kidney (CEK) cultures were prepared from 18-day-old chicken embryos by the method of Tannock et al (1985). Kidneys were removed and washed in citrate saline solution before being finely minced, transferred to trypsinising solution and held overnight at 4°C. The trypsinised kidneys were then gently shaken to break up clumps, passeded through a pair of conical gauze filters, one inside the other, and pelleted at 700 *g* for 5 min. The cell pellet was washed with chilled PBS and centrifuged to determine the packed cell volume. A 1:200 dilution of CEK cells was prepared in MEM Growth Medium and passeded again through a pair of gauze filters. Finally, 3 mL cell suspension were dispensed into each well of a 6-well plate and incubated for 48 hr at 37°C in 5% CO_2.

2.8 VIRUS GROWTH

Viruses were passaged in 10 or 11 day-old embryonated chicken eggs in a 34°C egg incubator for 2 or 3 days.

2.9 VIRAL TITRATIONS

2.9.1 HA assays

HA assays were performed in 96-well V-bottomed microtitre plates using 0.5% chicken RBC. To detect haemagglutination, endpoints were read by tilting the plates at 45°C. The HA titre was defined as the reciprocal of the highest virus dilution at which complete haemagglutination was observed.

2.9.2 Plaque assays

Plaque assays were performed using monolayers of MDCK or CEK cells in 6-well culture plates, as described by Tannock et al (1984 & 1985). Tenfold dilutions were prepared and 200 µL aliquots of each were inoculated to washed monolayers. After adsorption for 30 min, 3 mL of

overlay were added to each well. MDCK or CEK cells cultures were incubated for 4 days at 34 and 39°C, or 10 days at 25°C in the presence of 5% CO_2. Two millilitres of a staining overlay were then added to each well. Any plaques present were counted after further overnight incubation.

2.10 VIRUS PURIFICATION

Allantoic virus was clarified at 3,000 × *g* for 5 min. The supernatant was then centrifuged at 100,000 × *g* for 60 min at 4°C using an SW41 rotor. The virus pellet was resuspended in a 0.5-1.0 mL of TNE buffer. Concentrated virus was then centrifuged onto a 60% sucrose cushion through a 30% sucrose interface at 100,000 × *g* for 90 min, using an SW41 rotor (isopycnic ultracentrifugation). Virus appeared as a sharp opalescent band at the interface between the 30% and 60% sucrose solutions.

This band was recovered by inserting a syringe needle through the side of the tube and, using a syringe, carefully aspirating the opalescent layer (1-2 mL), together with a small amount of the adjacent sucrose fractions. The virus band was diluted 1:5 in TNE buffer and centrifuged at 100,000 × *g* for 60 min at 4°C using an SW41 rotor. The pellet was resuspended in a small volume of TNE buffer and stored at -80°C after HA assay. The virus was further purified by velocity ultracentrifugation in order to separate free virus from membrane-attached particles of similar density.

2.11 MOUSE MHC CLASS I EXPRESSION, REFOLDING AND TETRAMER FORMATION

MHC tetramers consist of complexes of four MHC molecules associated with a specific peptide and bound to a fluorochrome (Figure 1.8). They have been used extensively in studies of pathogenicity and vaccine-induced antigen-specific T-cell immunity in humans and in animal

model systems (Section 1.2.1.5). The tetramer $D^b/h\beta_2m/NP_{366\text{-}374}$ specific for influenza A/AA/6/60 *ca* was prepared by a modification of the method described in NIH tetramer core facility protocols (2003).

2.11.1 Heat shock transformation

The H-2D^b heavy chain expression plasmid was transformed into competent cells BL21 (DE3) by heat-shock method. Competent cells contain the chloramphenicol resistance gene as a selectable marker. In a chilled sterile Eppendorf tube, 1 µL of plasmid DNA was mixed with 10 µL of competent cells. The transformation mixture was placed on ice for 30 min and then at 42 ºC for 30 sec. After this heat-shock step, the mixture was held on ice for a further 2 min to complete transformation. After adding 150 µL of LB medium, the mixture was incubated at 37 ºC for 1 hr with constant shaking. Transformed cells were then pelleted by centrifugation at 5,000 × *g* for 30 sec and resuspended in 100 µL LB medium. Finally, the cells were plated onto LB agar plates containing 34 µg mL^{-1} chloramphenicol and 50 µg mL^{-1} kanamycin. Plates were left for incubation overnight at 37 ºC.

2.11.2 Induction of mouse MHC (H-2D^b) heavy chain expression in *E. coli*

A single colony was picked from the plate and transferred to a 10 mL LB medium with appropriate antibiotics and the suspension incubated overnight at 37 ºC. Competent BL21 (DE3) cells contained an IPTG-inducible T7 RNA polymerase that served to repress basal expression of target genes under the control of the T7 promoter. On the following day, 10 mL cultures were used to inoculate a 2 L LB culture. The inoculated culture was grown by shaking at 37 ºC until an optical density (OD) between 0.6 and 0.9 was observed at 600 nm. At this point, the culture was induced with 2 mL of 1 mM IPTG to allow H-2D^b heavy chain expression. After induction at 37 ºC for a further 4 hr, cells were harvested and pelleted at 1,000 × *g* for 20 min at 4 ºC. The pellet was then resuspended in 10 mL of resuspension buffer.

2.11.3 Preparation of inclusion bodies from *E. coli*

After the IPTG induction, recombinant protein accumulated in *E. coli* cells as inclusion bodies from which the desired protein H-2D^b heavy chain could be prepared. After induction, cells were lysed by adding 2.5 mL lysis solution per mL of cell suspension and incubated at room temperature for 20 min with constant rocking. For further cell degradation, the lysate was mixed with 0.5 M of EDTA and homogenised for 30 sec. The inclusion bodies were then pelleted by centrifugation at 4 ºC 5,000 × *g* for 20 min. After washing twice with 100 mL of Wash Buffer I and once with 100 mL Wash Buffer II, inclusion bodies resuspended in 10 mL Resuspending Solution II and held at – 70 ºC until next step. The concentration of inclusion bodies was estimated by SDS-PAGE in comparison with dilutions of known quantities of bovine serum albumin (BSA).

2.11.4 Estimation of the concentration of inclusion bodies by SDS-PAGE

Proteins were separated by discontinuous SDS-PAGE. Polyacrylamide gels consisted of 18% separating gel and a 1.25% stacking gel. Gels were cast as slabs in a Mini-PROTEAN 3 system (Bio-Rad, USA). Ten microlitres of 2 × Loading Buffer were added to 10 µL of each sample and heated to 100 ºC for 5 min before loading. The proteins were then electrophoresed at 200 V for 60 min or until the Loading Buffer reached the end of the gel. The SDS-PAGE gels were stained with Coomassie Blue for 30 min and then destained for 1 hr at room temperature with constant rocking.

2.11.5 Formation of mouse MHC class I monomer (D^b/$h\beta_2m$/NP$_{366\text{-}374}$)

Recombinant H-2D^b heavy chains were prepared from the inclusion body suspension. Briefly, the thawed suspension of inclusion bodies (6 - 12 mg) was transferred to SS34 rotor tubes and pelleted by centrifugation at 5,000 × *g* for 15 min at 4 ºC. To release mouse H-2D^b heavy chains from inclusion bodies, 10 mL of guanidine buffer were added to the pellet and the resulting suspension incubated at room temperature for 30 min with constant rocking. The supernatant was then collected after centrifugation 5,000 × *g* for 30 min at 4 ºC and 8 mg of $h\beta_2m$ were added. At the

same time, peptide was prepared by dissolving 6 mg $NP_{366-374}$ in 100 µL DMSO before adding 200 mL of fresh Refolding Buffer.

For monomer refolding, the MHC heavy- and light chain mixture was injected to the refolding buffer through a freshly rinsed pump in a 4 ºC room. The refolding buffer was stirred constantly during the injection process. After injection, the mixture was held for 2 -3 days at 4 ºC to allow the monomer ($D^b/h\beta_2m/NP_{366-374}$) to form. After refolding, the monomer mixture was transferred to dialysis tubing which was placed in 5 L freshly prepared cold Dialysis Buffer. Dialysis was carried out with constant stirring at 4 ºC overnight. The dialysed monomer was then further purified through FPLC.

2.11.6 Purification of MHC class I monomer $D^b/h\beta_2m/NP_{366-374}$ by fast protein liquid chromatography (FPLC)

2.11.6.1 Anion exchange purification

DEAE sepharose was used to pack the anion exchange column. Briefly, 50 mL of DE52 resin was mixed with 200 mL MilliQ water at 4 ºC. Excess water was removed after the resin had settled. It was then rinsed twice with 200 mL 10 mM Tris-HCl. The chromatography column was packed to three quarters of its volume with washed resin, which was then equilibrated by washing with 50 mL 10 mM Tri-HCl 1 M NaCl for about 20 min. It was then washed with 100 -200 mL 10mM Tis-HCl at pH 8.0 to remove residual Na^+.

The column was then loaded with 200 mL of filtered and dialysed MHC I monomer solution in a 4 ºC cold room. Protein was eluted by washing the column using the Liquid Chromotography Controller LCC-500, first with 10 mM Tris-HCl and then with10 mM Tris-HCl 1 M NaCl. The fractions within the peak were collected and examined by SDS-PAGE. Selected fractions were pooled together and then concentrated to a final volume of 0.5 mL using a Millipore Centricon tube.

2.11.6.2 Gel filtration

Further purification of protein was achieved by gel filtration. Briefly, a column was equilibrated with 100 -150 mL 10 mM Tris-HCl for 1hr prior to loading. The sample was loaded onto the column and protein was eluted by washing with the same buffer, using the Liquid Chromotography Controller LCC-500. Fractions within the peak were then collected and examined by SDS-PAGE and concentrated to a final volume of 0.8 mL.

2.11.7 Biotinylation of D^b/*hβ2m*/$NP_{366\text{-}374}$ monomer

D^b/*hβ2m*/$NP_{366\text{-}374}$ monomers were biotinylated before the tetramer refolding process. To achieve this, 100 µL 10 × Biomix A buffer, 100 µL 10 × Biomix B buffer, 100 µL Biotin and 15 µL BirA enzyme were added to 800 µL purified MHC monomer. The mixture was then held at 28 °C overnight. On the following day, any free biotin was removed by loading on a PD-10 column and eluting with 2.5 mL of 10 mM Tris-HCl. All eluates were checked for protein using Bio-Rad protein assay. Samples containing protein were pooled together and then concentrated to 0.8 mL by using a Millipore Centricon 10's tube and centrifugation at 3,500 rpm at 4 ºC for 30 seconds.

To measure the proportion of monomers that were biotinylated, 50 µL of streptavidin beads were washed three times with 1 mL of Wash Buffer by centrifugation at 5,000 × g for 1 - 2 sec and then resuspended in 500 µL Wash Buffer. Fifteen microlitres of biotin-labelled protein were then added and the mixture held at 4 ºC overnight. On the following day, the bead-protein mixture was washed three times with 1 mL of Wash Buffer to remove non-biotinylated monomers. The final biotinylated: non-biotinylated monomer ratio was determined by SDS-PAGE gel**.**

2.11.8 Generation of tetramer D^b/*hβ2m*/$NP_{366\text{-}374}$

For the generation of tetramers, suspensions of Extravidin®-R-PE conjugated beads were added to biotinylated monomers in the final ratio of 1:4. At hourly intervals, 10% of the total volume of conjugated beads was added to the monomer preparation in an ice bath. The tetramers were then

stored at 4 ºC after the addition of NaN_3 (final concentration 0.02%) and titrated by flow cytometry prior to use, using the lymphocytes from influenza virus A/Ann Arbor/6/60 *ca*-infected C56BL/6 mice.

2.12 MOUSE EXPERIMENTS

2.12.1 Anaesthetic

Mice were anaesthetised by intraperitoneal (i.p) injection with Ketamine/Ilium Xylazine-20 (150 mg / 50 mg kg^{-1} body weight).

2.12.2 Immunization procedures

Groups of 6 mice were inoculated i.n under anaesthesia with 50 µL allantoic virus, diluted in PBS to contain 1.45×10^5 PFU. Control mice were inoculated with normal allantoic fluid prepared in PBS at the same dilution used to prepare the virus. Mice were inoculated twice with the same dose at an interval of 3 weeks. Seven or nine days after inoculation, they were anaesthetised before being heart bled, and each lung pair was then removed for a series of assays. Sera for HAI tests were prepared from blood collected by heart bleed.

2.12.3 Preparation of single cell suspensions from mice lung and spleen

Single cell suspensions from lung were prepared by a modification of the method of Harling-McNabb et al (1999). Pairs of lungs from individual mice were removed and placed in PBS, and washed with 3 mL of HBSS containing 60 µg mL^{-1} penicillin and 100 µg mL^{-1} streptomycin. Lung tissues were then finely minced with curved scissors and digested with 3 mL Lung Digestion Solution at 37°C for 30 min. To break up the clumps, the digested lung tissues were forced through metal sieves using a 5 mL- syringe into a 10 mL- centrifuge tube containing 5 mL of ATC Buffer.

Spleen tissues were forced through sieves directly into ATC Buffer without the digestion step. Mixtures were then allowed to stand for 2-5 min at room temperature to deplete erythrocytes. Cells in the supernatant were pelleted by centrifugation at 4 °C 400 g for 5 min and briefly mixed with 5 mL of TCM using a vortex mixer. Cell suspensions were then filtered through 100µm Nylon Cell Strainers to remove dead cells and debris. The resulting single-cell suspensions were centrifuged at 400 *g* for 5 min and the cell pellets resuspended in 0.5 mL of TCM prior to counting.

2.12.4 Inactivation of non-specific inhibitors in sera

Thermostable inhibitors in mouse sera were inactivated by the addition of one part serum to four parts RDE solution and incubating at 37°C overnight. The reaction was stopped by the addition of an equal volume of 1.5% sodium citrate and further incubation at 56°C for 30 min in order to inactivate thermolabile inhibitors.

2.13 DETERMINATION OF VIRUS MORBIDITY AND LUNG VIRUS TITRES

For the determination of viral morbidity, mice were weighed immediately before the infection. On day 3 p.i, they were sacrificed and their body and lung were weighted individually. Morbidity was indicated from changes of the body weight pre- and post infection, and changes in the ratio of body: lung (w/w) between infected and uninfected mice. To compare the capacity of different influenza strains to replicate in mice, individual lung extracts were prepared by a modification of the method of Tannock et al (1984). Briefly, the right lobe of each lung was removed and homogenised in 2 mL of sterile HBSS. Extracts were stored at –80 ºC until titrated for infectious virus by plaque assays in MDCK cells (see Section 2.9.2 for plaque assay).

2.14 HISTOLOGICAL STUDIES ON LUNG TISSUES

Histopathological changes in the mouse lung were determined by Haematoxylin and Eosin (H&E) staining. Mice were sacrificed by an intraperitoneal injection with Ketamine/Ilium Xylazine-20

(375 mg /125 mg kg^{-1} body weight) at days 3 and 21 after primary infection. The large left lobe of each lung was then removed and fixed by inflation with 4 mL of 10% neutral buffered formalin for the H&E staining. The fixed lung tissues were then processed in Tissue Processor by the standard processing schedules. At the end of this procedure, the tissues were completely infiltrated with melted paraffin and were then placed into embedding moulds. The blocks were allowed to cool until ready for sectioning with a microtome.

Three sections from 250 µm levels of bronchial tree were prepared and stained with H&E. Briefly, glass slides with 5- to 8-µm-thick paraffin sections attached were progressively dipped through xylene, 100% ethanol, 70% ethanol and tap water for dewaxing and hydration. The slides were first placed in haematoxylin solution and in Scott's blueing agent. Finally, they were counter-stained with Eosin Y solution. The slides were then dehydrated with alcohol and cleared in the resin solvent, xylene. Cover slips were mounted on the dry slides with DPX resin and slides were left to dry at room temperature.

2.15 IMMUNE RESPONSE ANALYSIS

2.15.1 Humoral immune responses analysis

2.15.1.1 Serological analysis –HAI assays

HAI assays were performed in 96-well V-microtitre plates using 0.5% chicken RBCs, as previously described by (CDC 1982). Haemagglutinin end-points were read by tilting the plates at an angle of 45°. The HI titre was defined as the reciprocal of the highest serum dilution causing complete inhibition of haemagglutination. Geometric mean titres (GMTs) for each vaccine group were calculated. Sera from mice, inoculated with normal allantoic fluid were prepared in each experiment for use as controls.

2.15.1.2 Virus-specific ASCs analysis - ELISPOT assay

Influenza virus-specific ASCs were enumerated by an ELISPOT assay based on that described by Sedgwick & Holt (1983). Briefly, 96-well flat-bottomed polyvinyl chloride microtitre plates were coated with 50 μL PBS containing 200 HAU of purified virus per well for *in vivo* stimulation of murine B cells; control titration plates were coated with normal allantoic fluid. The plates were then incubated overnight in a humidified atmosphere at room temperature. Plates were washed three times with PBS-T and blocked with 100 μL of $BSA_{10}PBS$ per well. After 1 hr of incubation in a humidified atmosphere at room temperature, the plates were washed three times with PBS-T and then 50 μL of TCM were added to each well. Aliquots 50 μL of ATC-treated lung or spleen cell suspensions were added to triplicate wells, and six serial two-fold dilutions performed. The plates were then placed in a vibration-free incubator at 37°C in 5% CO_2 for 6 - 8 hr.

After incubation, the cells were lysed and removed by rinsing the plates twice with Milli-Q water and twice with PBS-T. Goat anti-mouse IgA and IgG2a isotype-specific antibodies, diluted 1:400 in BSA_5PBS-T, was added (50 μL per well) to a triplicate titration series and the plates further incubated for 2 hr in a humidified atmosphere at room temperature. Plates were then washed three times with PBS-T and 50 μL per well of alkaline phosphatase-conjugated rabbit anti-goat IgG antibody, diluted 1:1000 in BSA_5PBS-T, were added and the plates incubated for a further 2 hr in a humidified atmosphere at room temperature. Plates were washed with PBS-T three times and once with Milli-Q water. The assay was then developed by adding to each well 50 μL of BCIP substrate solution and held at room temperature for 1 hr. The plates were washed three times with Milli-Q water and spots counted using an inverted microscope. Each spot represented a single influenza virus-specific ASC and results were expressed as the mean number of ASC ± standard error of the mean (SEM) or standard deviation (StDev) per 10^6 cells. ASCs in lungs or spleens from normal allantoic fluid inoculated mice when assayed on normal allantoic fluid-coated plates were used to determine background levels.

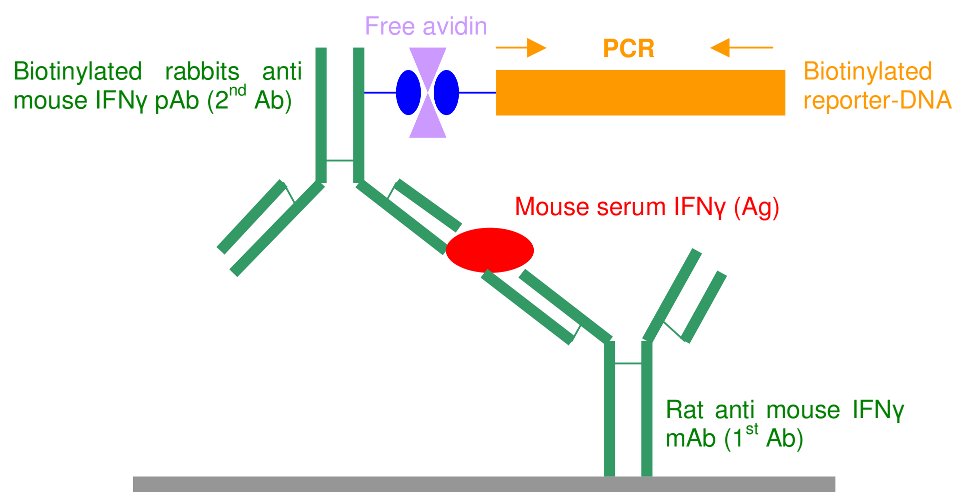


Figure 2.1 Schematic representation of immuno-PCR reaction for IFN-γ

mAb: monoclonal antibody

pAb: polyclonal antibody

2.15.2 Cell mediated immune (CMI) responses analysis

2.15.2.1 Serum IFN-γ analysis – Immuno-PCR assay

Immuno-PCR assays are highly sensitive and have been used for the detection of variety of antigens, including cytokines (Anne Mckie 2002). A DNA label had been used as report marker that can be detected by PCR in this assay. The principle of the assay is shown in Figure 2.1.

Preparation of biotinylated reporter DNA

Biotinylated report DNA (227 bp) was prepared from plasmid Bluescript by PCR amplification with the biotinylated M13-20 primer and the non-biotinlated M13 reverse primer. The PCR reaction mixture was prepared according the protocol given in the QIAGEN Multiplex PCR Kit. The reaction mixture was contained 10 mM Tris-HCl buffer (pH 8.3), 50 mM KCl, 3.0 mM $MgCl_2$, 0.2 mM dNTP, 0.2 μM of each primer, 2 units of Taq DNA polymerase, and 5 pg of pBluescript. The reaction was allowed to take place in the following steps: (1) an initial denaturation at 95°C for 5 min, (2) 30 cycles of denaturation at 94°C for 30 s, (3) annealing at 58°C for 1 min, (4) extension at 72°C for 30 s, and finally (5) extension at 72°C for 5 min. The PCR product was purified using QIAquick® PCR purification kit.

Immuno-PCR assay for the detection of IFN-γ

For coating 96-well ELISA plates (Nunc), 1 μg primary anti-IFNγ monoclonal antibodies in 50 μL of carbonate Coating Buffer were added to each test well and the plate incubated at 37°C for 1 hr. The plates were washed three times with the Wash Buffer and then 200 μL Blocking Solution was added to each well. The plate was then held at room temperature for 1 hr. After washing three times, 50 μL of each diluted serum samples were added to individual wells and the plate was incubated at 37°C for 1 hr. After washing, 0.025 μg of biotinylated-rabbit anti-mouse IFNγ antibody was added to each well and plate incubated at 37 °C for a further hr. Free avidin was added to each well and the plate incubated at 37 °C 30 min to allow it to bind to biotinylated secondary antibody. After washing, 0.1 pg of reporter-DNA was added to each well and the plate incubated for 30 min at 37 °C to allow biotinylated reporter-DNA to bind to the complex.

Unbound reporter-DNA was removed by washing and the bound reporter-DNA was used as template DNA for PCR amplification. To obtain template DNA, the plate was heated to denature the reporter DNA. PCR was carried out using IF and IR primers, which are nested to M13-20 and M13 reverse primers. Briefly, 50 μL DEPC H_2O were added to each well and the plate was sealed with Parafilm and heated at 95°C for 5 min. Then 5 μL of each supernatant was used as template DNA in the following PCR reactions. Reactions were performed using a Gene Amp PCR system 2400 (Applied Biosystems, USA). Reactions were heated to 95°C for 5 min, followed by 40 cycles of denaturation at 95°C for 30 s, annealing at 58°C for 1 min and extension at 72°C for 1 min. A final extension at 72°C for 5 min completed the reaction and samples were stored at 4°C until required.

2.15.2.2 Effector T cell analysis

Phenotype: *in vivo* peptide-specific $CD8^+$ T cell and $CD44^{high}$ T cell

The PE labelled D^bNP_{366} and D^bPA_{224} used in these experiments were supplied by Dr. Stephen Turner from the Department of Immunology, University of Melbourne. In addition, D^bNP_{366} prepared from influenza A/AA *ca* and H-2D^b in Dr. Andrew Brook's Lab, Department of Immunology, and University of Melbourne was tested through these experiments. CD44 had been chosen as an effector and potential memory T cell marker for the T lymphocytes phenotype. Lymphocytes isolated from the lungs and spleens of virus-infected mice at the third day post primary and secondary intranasal infection were prepared by the method same as described in section 2.12.3. The cells were diluted to a final concentration of 1×10^6 cells 50 μL^{-1} in FACS stain buffer and then transferred to 96-wells U-bottomed tissue culture plate.

For tetramer staining of peptide-specific $CD8^+$ T cells, cells were stained with the tetrameric complexes D^bNP_{366} or D^bPA_{224} at room temperature avoiding exposure to the light. After 60 min incubation time, lymphocytes were washed twice with stain buffer for removing extra tetrameric complexes and stained with anti-CD8-FITC for 30 min at room temperature. Moreover, for

identification of $CD44^{high}$ T cell population, cells were stained with anti-CD44-FITC and either anti-CD8 or CD4-PE for 30 min at room temperature. Cells were fixed for the preservation of their morphology straight after staining. Briefly, lymphocytes were washed once with stain buffer and twice with fix wash buffer containing gelatine. Then cells were diluted to a final concentration of 1×10^6 cells 200 μL^{-1} in the fixation buffer with 2% paroformaldehye. On the following day, fixed cells were washed twice with fix wash buffer before they were transferred to the FALCON® FACS tube respectively to each sample well. Cells were analysed by flow cytometer within one week.

Functional study: *in vitro* intracellular IFN-γ staining with stimulation

This assay used the single cell preparations from lung and spleen that were RBCs lysed and cultured for 5 hr in 96-well round-bottom plates at a concentration of $1 \sim 4 \times 10^6$ cells per well in 200 µl of T-cell medium containing 1 µg ml^{-1} peptide $NP_{366\text{-}374}$ and 10 µg ml^{-1} BFA. The reason for using BFA here was to prevent excretion of protein thus leading to intracellular accumulation and more intense staining of cytokines. The lymphocytes were then washed and stained with anti-mouse CD8-PE at room temperature for 30 min. The cells were fixed in ICC fix buffer for 15 min, then 10 min in ICC cell permeablisation buffer before staining with a FITC conjugated mAb to mouse IFN- γ. The percentage of $CD8^+$ IFN- γ^+ T cells was detected through flow cytometer.

2.16 STATISTICS

Normal distribution of data was determined by the Kolmogorov-Smirnov test ($P \geq 0.05$, Appendix); Statistical significance was determined by the Student's t-test ($P < 0.05$, Appendix). Where data did not assume normal distribution, each datum was converted to a natural logarithm. The converted data were again checked for normal distribution before being applied to a statistical model.

Chapter 3:

The relative infectivity of reassortants of the cold-adapted influenza A donor strain A/Ann Arbor/6/60 in C57BL/6 mice

3.1 INTRODUCTION

Earlier studies have indicated that the immunogenicity of *ca* reassortants was directly related to the extent of their replication in the respiratory tract of mice (Tannock et al 1995). In seronegative humans, *ca* LAIVs have also been found to be highly immunogenic (Treanor et al 1990). Both findings were not unexpected because viral replication can enhance both MHC Class I and II antigen presentation pathways (Section 1.2.1.5). However, immunogenicity is also influenced by both the presence of pre-existing Ab and by the intrinsic properties of individual reassortants. Detailed studies in mice have shown that: (1) the immunogenicity of both US and Russia *ca* donor strains was 100-1000× less than their *wt* parental viruses, (2) *ca* donor strains or reassortants, when administered by the i.n route in two low doses, are as immunogenic as a single dose of the corresponding parental virulent virus that was used to prepare each reassortant, and (3) the immunogenicity of each reassortant is determined by both the genes for cold-adaptation and HA glycoproteins (Table 3.1).

In the current study, all *ca* reassortants had been used in previous studies conducted by the Tannock group and their *ts* and *ca* phenotypes were confirmed before inclusion in the present experiments. Of the reassortants, CR6 was the first developed for use as a live human vaccine in 1975. It was evaluated *in vitro* and *in vivo* had all the desired characteristics of an LAIV. It grew to high titres at 25 and 33°C, was attenuated for ferrets and showed no evidence of reversion to virulence (Maassab and DeBorde 1985). Another early reassortant, CR18, possessed the internal NS gene from the *wt* parental virus and was also included in this study (Table 2.1). Earlier experiments on the growth of these viruses in the respiratory tract of outbred mice showed that the presence of *wt* NS gene in CR18 did not provide any growth advantage (Tannock et al 1984). Unlike ferrets, all *ca* reassortants tested grew to higher titres in the lungs of mice compared with turbinates (Table 3.1). These findings are explicable in terms of the different distribution of α-2,3 glycoprotein receptors in mice and ferrets (Wright et al 2007).

Table 3.1 Summary of properties of *ca* reassortants with similar internal genes *in vitro* and *in vivo* (Maassab and DeBorde 1985; Tannock et al 1995)

6:2 *ca* reassortant	***wt* parent**	**Dosage required to induce maximum Ab in seropositive adults**	**PD_{50} with two doses (in outbred male mice)**	**HAI Ab levels in outbred male mice (GMT)[a]**		**Clinical signs (Ferrets)**	**Histopathology (Ferrets)**	
				One dose[b]	**Two dose[c]**		**Turbinates**	**Lung**
CR6 (H3N2)	A/QLD/6/72	n.a.	$10^{1.17}$ $TCID_{50}$	71	243	Absent	1+ out of 4+	0
CR18 (H3N2)	A/Scot/840/74	n.a.	$10^{2.30}$ $TCID_{50}$	71	202	n.a.	n.a.	n.a.
CR29 (H3N2)	A/Alaska/6/77	$10^{6.5}$- $10^{7.0}$ $TCID_{50}$	$10^{2.50}$ $TCID_{50}$	40	211	Coryza 1 day	2+	0
CR35 (H1N1)	A/HK/123/77	n.a.	$10^{3.00}$ $TCID_{50}$	40	64	Absent	1+	0
A/AA/6/60 ca (H2N2)	Master Donor	$10^{5.3}$ -$10^{5.7}$ $TCID_{50}$	$10^{1.13}$ $TCID_{50}$	64	113	n.a.	n.a.	n.a.

[a] Titres were determined against the homologous virus. Each figure represents the GMT for the results from six mice.

[b] Each dose consisted of 10^5 $TCID_{50}$ of virus. Six weeks after single dose, mice were bled and sera were collected.

[c] There was a 3-week-interval period between the two inoculations; mice were bled 3 weeks after the last dose.

n.a. not available

$TCID_{50}$ 50% tissue culture infectious dose

PD_{50} 50% protective dose; the amount of virus administered i.n as two identical doses 3 weeks apart which completely inhibits multiplication of a standard challenge, consisting of $10^{4.5}$ $TCID_{50}$ of a wt parental virus with similar surface Ags, in the lungs of 50% of mice.

GMT geometric mean titre

In outbred mice, H3N2 reassortants (CR6, CR18 and CR29) were shown to grow to higher titres in lungs by day 3 p.i and are to be more immunogenic (Tannock et al 1984). By contrast, CR35 (H1N1) is a slower-growing virus that could not be detected by day 3 and is a weaker immunogen. A recent study with A/HK/2188-49/06 H3N2 and H1N viruses in MDCK cultures similarly indicated that the H3N2 virus formed large plaques and grew to high titres, compared with the H1N1 virus. These differences are associated with differences in levels of phosphorylation by extracellular-regulated kinase (ERK; Marjuki et al 2007). ERK belongs to the MAPK signalling pathway which, when induced late in the replication cycle, plays a crucial role in efficient virus replication.

In the present study, the relative infectivity of *ca* reassortants with common internal genes was compared in genetically defined inbred mice, in order to provide information on the role of viral surface Ags in the absence of a variable MHC background. Viral infectivity was measured from changes in lung pathology, virus yields in lungs and body weight loss. In addition, inherent differences in the replication characteristics of *ca* reassortants and Ab responses were measured.

3.2 RESULTS

3.2.1 Viral replication characteristics

All viruses were first passaged in embryonated eggs and virus stocks prepared at each passage were tested for their infectious titre (expressed as PFU in MDCK monolayers at 34ºC) and HAU content. The replication efficiency of egg-grown viruses was calculated from the PFU: HAU ratio. The average PFU: HAU ratio for each virus used is shown in Figure 3.1. Results indicate that all the H3N2 *ca* viruses have higher ratios than H1N1 *ca* viruses. Substitution of the H3 HA gene of CR6 for the H1 HA gene of CR35 to produce the new reassortant CR6-35 H1N2 resulted in a reduction of the PFU: HAU ratio of almost 50%, compared with CR6. By contrast, the ratio for

the CR6-35* (H3N1) reassortant (possessing the H3 HA gene of CR6 and the N1 gene of CR35) was increased by a factor of almost 3, compared with CR35. However, this phenomenon was not observed with *wt* A/PR8 (H1N1) and it's reassortant A/HK×31 (H3N2), both of which share the same internal genes. The PFU: HAU ratios of A/PR8 and A/HK×31 are 71.16×10^3 and 22.80×10^3 respectively.

Besides egg-adapted viruses, two recent human epidemic strains and non-egg-adapted isolates, A/Fujian/411/02 (H3N2) and A/New Caledonia/20/99 (H1N1) were examined in MDCK cells. A/Fujian was found to be highly virulent for embryonated eggs and caused embryo death prior to the achievement of a detectable HA titre. However, A/Fujian could be grown in MDCK cultures where they produced low HA titres (2-32 HAU in contrast to 640-1280 HAU for A/PR8) but comparatively high PFU titres. After eight passages in MDCK cells, a PFU: HAU ratio of 31.25×10^3 was achieved. By contrast, the HAU titre of A/New Caledonia/20/99 was 512 after the first passage in MDCK culture cells but it's PFU: HAU ratio was only 6.15.

In addition to variations observed in their replication efficiency, these viruses exhibited different plaque characteristics (Figure 3.2). CR29 forms small plaques of uniform size; other *ca* viruses generate plaques of both medium and large size. The plaques of *wt* viruses are generally large, whereas those of A/HK×31 are mainly small. Plaques of human non-egg-adapted isolates A/Fujian and A/New Caledonia were large and irregular. Their plaquing ability *in vitro* and virulence *in vivo* were confirmed with full-length of PB1-F2, in contrast to the truncated form that is present in A/AA *ca* (Zell et al 2006).

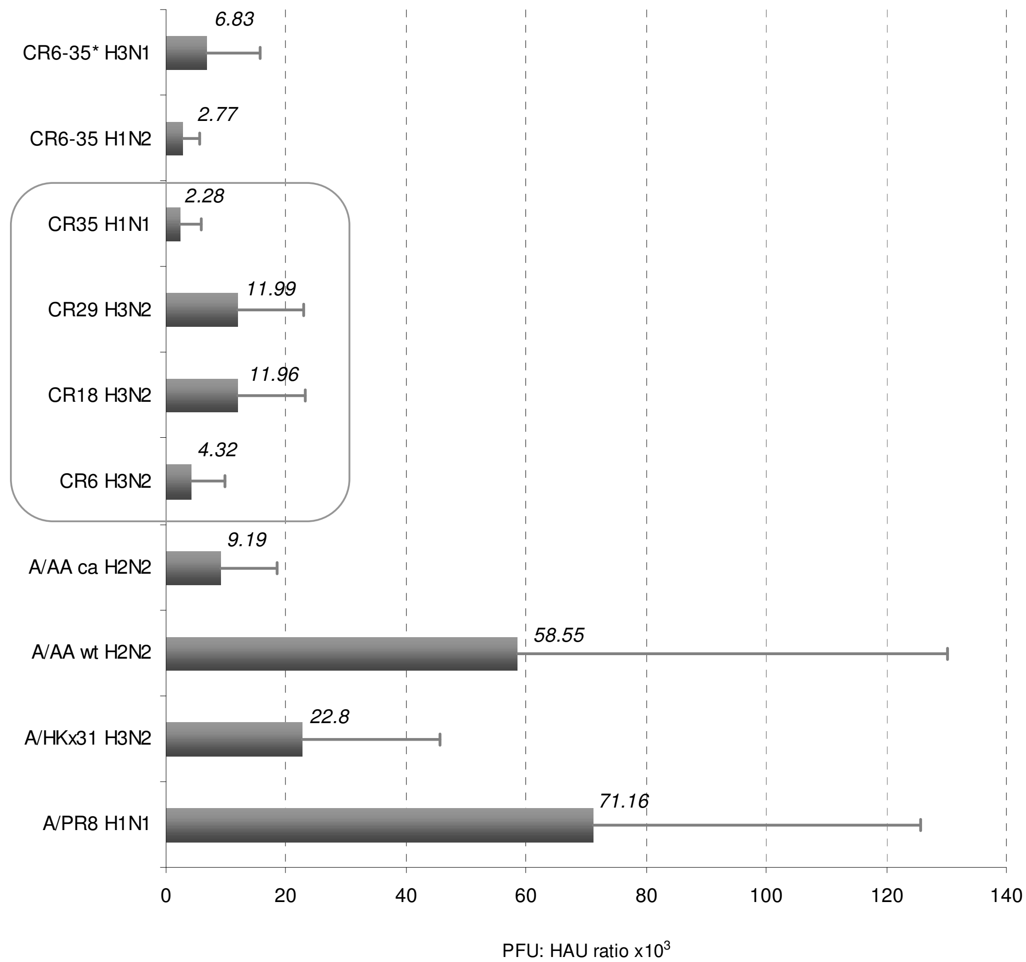


Figure 3.1 Viral replication efficiency of *ca* and *wt parental* in embryonated eggs, as measured by the average PFU: HAU ratio. The *ca* reassortants used in previous studies are showed inside the box.

PFU plaque forming unit
HAU haemagglutination unit

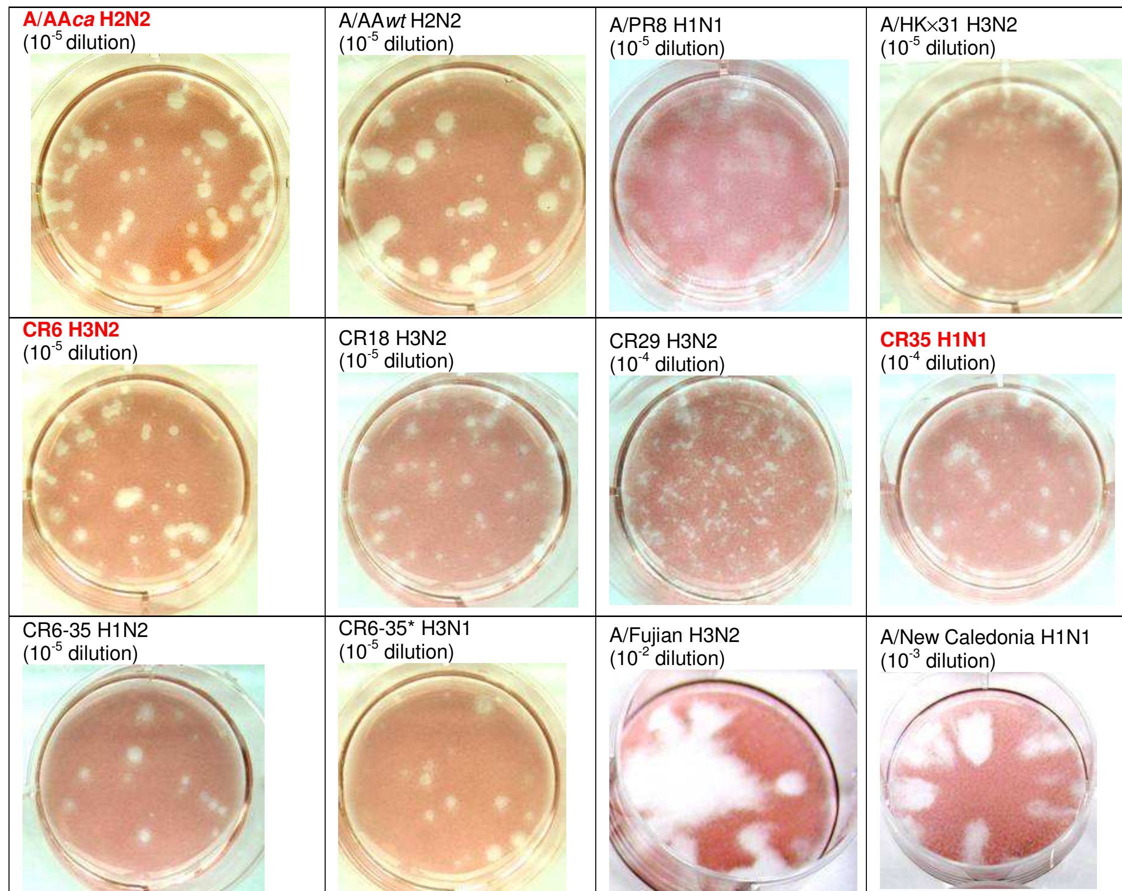


Figure 3.2 Differences in plaque shape and size between different influenza viruses.

Plaques were observed in MDCK cultures after 4 days at 34 ºC p.i. Non-egg-adapted A/Fujian and A/New Caledonia viruses used in this study are human isolates with limited passages (less than 10) in MDCK cells. All the other viruses are egg-adapted. A/AA *ca*, CR6 and CR35 were used for the following histological studies.

3.2.2 The relative infectivity of *ca* reassortants and a donor strain in C57/BL6 mice

3.2.2.1 Early pathogenic changes in mice lungs after a single i.n inoculation

In the following study inbred female C57/BL6 mice were inoculated with *ca* viruses and their corresponding parental *wt* viruses with H2N2, H3N2 and H1N1 surface antigens. Early virus-host interactions were assessed by determining body weight loss, lung virus titres and histopathology within the lungs. Groups of three 8-week-old female C57/BL6 mice were administered 50 µL containing 10^5 $TCID_{50}$ of each virus by the i.n route under general anaesthetic. All mice were sacrificed on the day 3 p.i and the lungs harvested. Body weight losses were determined from measurements taken immediately before and 3 days after infection (Figure 3.3). No infectious virus could be detected on day 3-p.i in the lungs of mice infected with A/AA *ca*, CR6 (QLD) and CR35 (HK) (Figure 3.3B), in agreement with a previous study using outbred mice (Tannock et al 1982 & 1984).

By contrast, mean titres of 6.37 and 72.62 × 10^3 pfu per lung were observed with the *wt* parental viruses A/AA and A/QLD infected mice, respectively. However, with A/HK *wt* virus, no infectious viruses could be detected on day 3 (Figure 3.3B). All groups infected with *wt* viruses experienced more than 5% weight loss within 3 days (Figure 3.3D). For *ca*-infected mice, only those infected with CR6 exhibited a 3.8% decline and there was no body weight loss observed after A/AA *ca* and CR35 infection.

The lung: body weight ratio was used as an index of lung consolidation in response to infection (Figure 3.3C). Mice infected with A/AA *ca* and CR6 at day 3 p.i exhibited slight consolidation, with lung: body weight ratios of 0.94 and 0.93×10^{-2}, respectively, compared with uninfected controls where the ratio was 0.82×10^{-2}. However, mild consolidation was observed in the lungs of CR35-infected mice and the ratio was 1.08×10^{-2}. By contrast, *wt*-infected mice exhibited mild lung consolidation and their lung: body weight ratios were all greater than 1×10^{-2}.

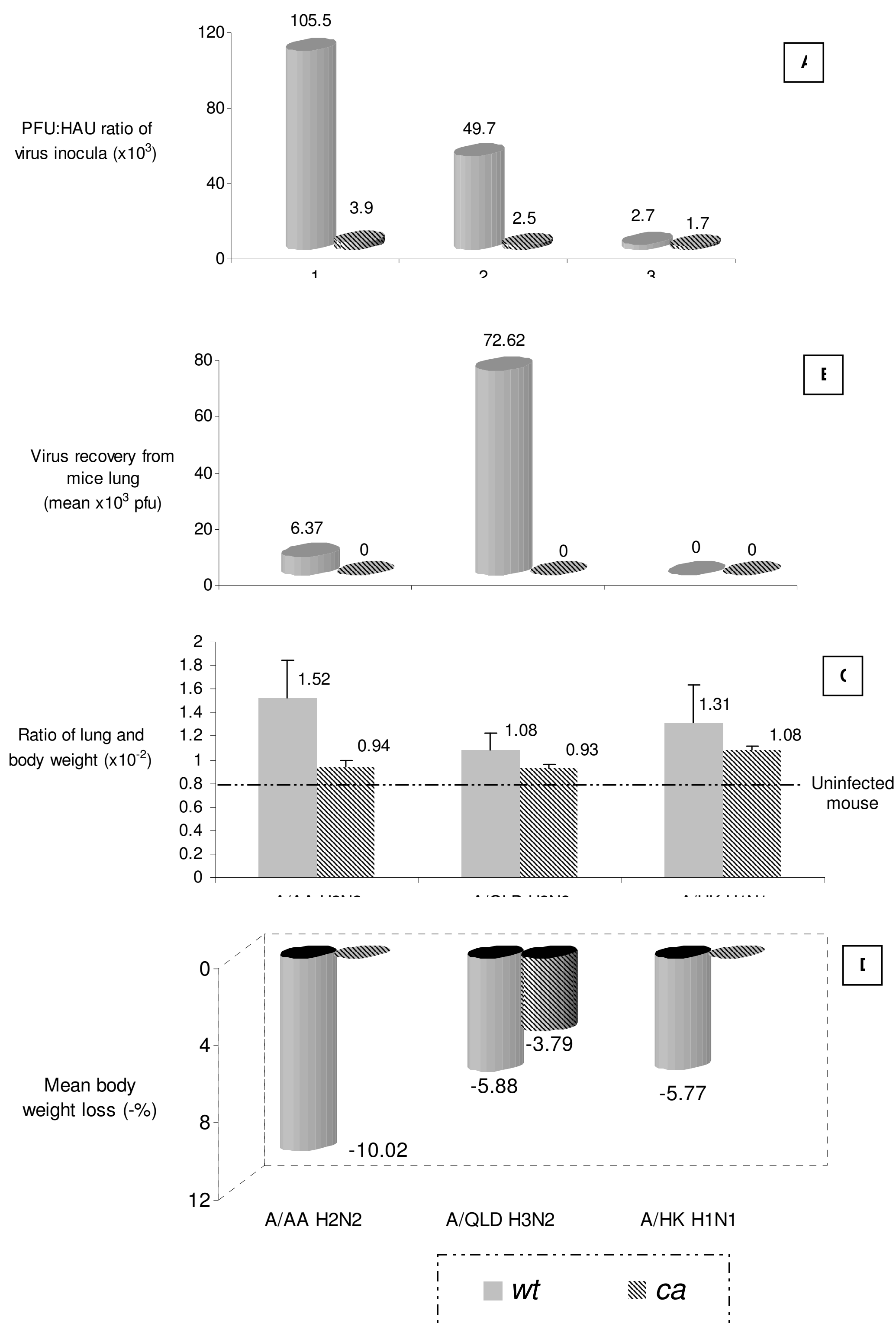


Figure 3.3 Early virus-host interactions indicated by lung virus yield (**B**), levels of lung consolidation (**C**) and weight loss (**D**). Graph **A** showed that the PFU: HAU ratio of each virus used to infect mice. Each virus group consisted of three 8-week-old female C57BL/6 mice. Mice were administered 10^5 $TCID_{50}$ viruses i.n under general anaesthetic and were sacrificed at day 3 p.i.

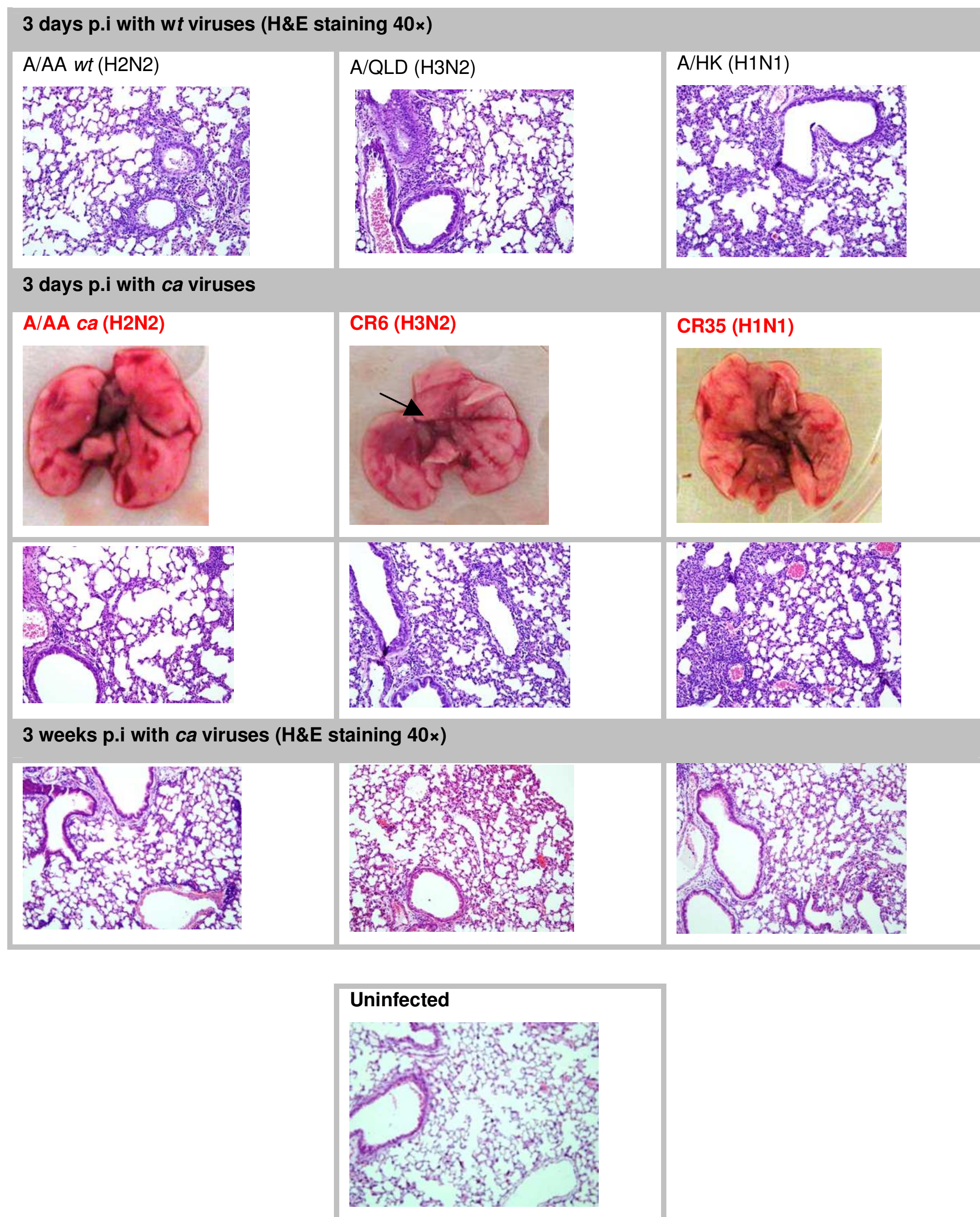


Figure 3.4 Lung pathological changes after infection with *wt* and *ca* influenza viruses with similar surface antigens.

The lungs of *ca*-infected mice appeared normal, except for small patches of consolidation in the left lung (see arrow) and cardiac lobe of the right lung. From H&E staining, lungs from both *wt*- and *ca*- infected mice exhibited similar levels of mild lymphocytic peribronchitis with mild alveolitis at 3days, compared with uninfected mice. However, *ca*-infected mice regained weight and the inflammation in lung had declined markedly by 3-week-p.i. By contrast, *wt*-infected mice had to be euthanized within the first week p.i due to continuing body weight losses.

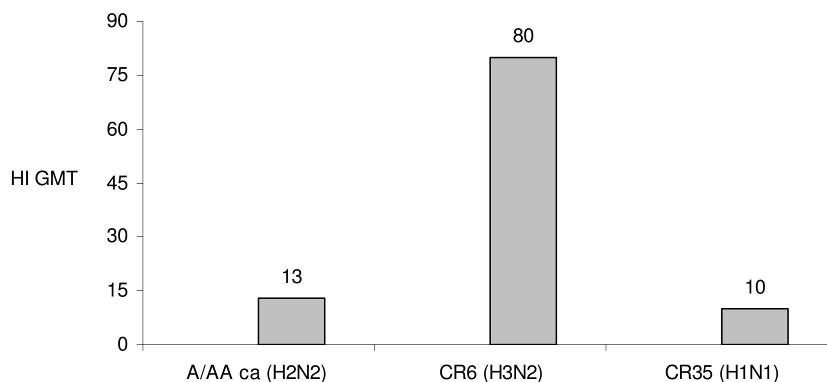


Figure 3.5 Serum Ab responses to *ca* influenza viruses, which share the same internal genes but possess different surface antigens, 3-week-p.i. Groups of four 8-week-old female C57BL/6 mice were given 10^5 $TCID_{50}$ of each virus, and were held for further 3 weeks. Individual mice were then bled and their serum HI titres tested for the calculation of group GMTs.

Table 3.2 Summary of responses at 3-days to infection with *ca* influenza A viruses with the same internal genes but different surface antigen genes.

	A/AA *ca* (H2N2)	**CR6 (H3N2)**	**CR35 (H1N1)**
Virus recovery from lungs ($\times10^3$ pfu)	0	0	0
Mean Lung: Body weight ratios (w/w $\times10^{-2}$)	0.94	0.93	1.08
Average body weight loss (- %)	0	3.79	0

3.2.2 Histopathological changes in the lungs of infected mice

Female C57BL/6 mice were administered 10^5 $TCID_{50}$ of a particular virus by the i.n. route and were sacrificed at either 3 days or 3 weeks p.i. Their lungs were harvested and then processed for H&E staining. The lungs of mice infected with all *ca* viruses appeared normal except for small patches or foci of consolidation that were mainly seen in the left and cardiac lobes of the right lung (Figure 3.4 middle panel). H&E staining also showed that, at 3 days p.i, the lungs of both *wt*- an *ca*-infected mice had mild lymphocytic peribronchitis and mild alveolitis (Figure 3.4 upper panel), compared with uninfected controls. However, inflammation caused by *ca* viruses was much reduced after 3 weeks (Figure 3.4 lower panel). Comparison was not possible at 3 weeks with *wt*-infected mice, which, (under the direction of the RMIT Animal Care and Ethics Committee) were euthanized within the first week because of increasing weight loss.

3.2.3 Serum Ab responses

Three weeks p.i, Ab responses to a single dose of each *ca* virus were determined and are expressed as GMTs for groups of four C57BL/6 mice (Figure 3.5). All four mice from the CR6-infected group had individual titres of 80, and from the CR35-infected group of 10. One mouse HI titre from the A/AA *ca*-infected group was had a titre of 20 and the other three mice were 10. Therefore, the GMT for H3N2 reassortant CR6-infected mice is 80 and for the A/AA *ca* (H2N2) - and CR35 (H1N1)-infected mice 13 and 10, respectively.

3.3 DISCUSSION

Viral growth and spread are major determinants of the course of an influenza infection. Viral load is important in the development of immunity and determines the extent of immune stimulation (Doherty et al 1997; Hozler et al 2003; Ahmed et al 2007). In the present study, early virus-host interactions were investigated at day-3 after i.n inoculation of C57BL/6 mice with different *ca*

reassortants or their parental *wt* viruses. Infectivities were determined for (1) *ca* viruses, in comparison with parental w*t* viruses with the same surface Ags but different internal genes; and (2) *ca* viruses, with common internal genes but different surface Ags.

3.3.2 Early systemic reactions to influenza infection are related to internal viral genes

An earlier study indicated that the *ca/ts/att* phenotypes of *ca* reassortants were responsible for reduced infectivity and immunogenicity (Tannock et al 1995). The current study extended these findings and showed that *ca* viruses possessing surface antigens from three different subtypes that exhibited markedly reduced viral replication efficiency both *in vitro* and *in vivo* (Figure 3.3 A&B). At day-3-p.i, *ca*-infected mice exhibited no or little body weight loss, while losses for *wt*-infected mice were 5-10% (Figure 3.3 D). Therefore, at day-3, fewer systemic reactions were induced by *ca* viruses than *wt* parental viruses with the same surface Ags. The attenuation of *ca* viruses for mice is considered to be mainly due to mutations in their internal genes, especially PB1 and PB2, which are responsible for the induction of cytokines and chemokines (La Gruta et al 2007; Section 1.2.1.4).

In contrast to the marked differences in body weight between mice inoculated with *ca*- and *wt* viruses, no substantial differences lung pathology were noted at day 3-p.i (Figure 3.4), indicating that the extent of primary local responses induced by either *ca* or *wt* viruses was similar during the early stages of infection. These findings were not unexpected, since SPF mice were used that were immunologically naïve to influenza A viruses; specific adaptive responses involving the recruitment of lymphocytes to the lungs do not usually occur before 1 week (Eichelberger et al 2006; Section 1.2.2.1). Consequently, by day 3, lung consolidation in *ca*-infected mice was only marginally less than in mice infected with *wt*-viruses with the same surface antigens (Figure 3.3C). However, by 3-week-p.i, inflammation in *ca*-infected lungs was much less than at day 3 p.i and similar to that of control uninfected mice (Figure 3.4).

The results demonstrated, for the first time, that, in the early stages of infection, *ca* viruses are able to induce similar local responses in the lungs to their *wt* parental viruses in absence of systemic symptoms (i.e. body weight loss). This stimulation of CMI responses following i.n administration has been proposed as a major advantage of i.n-administered *ca* LAIVs over parenterally administered inactivated vaccines (Tannock 1991).

3.3.2 Surface HA glycoprotein is a major determinant of the infectivity for *ca* LAIV

The relative infectivity of *ca* viruses with surface from three different subtypes (H3, H2 and H1) was investigated in an attempt to elucidate a relationship between their replication efficiency and immunogenicity. The PFU: HAU ratios for H3 reassortants are >1,000 times greater than those of H1 reassortants (Figure 3.1). Importantly, a reassortant possessing the H3 gene (CR6-35*) had a PFU: HAU ratio that was >1,500 times higher than CR35 with the same N1 gene (Figure 3.1). A possible explanation is that viral H3 glycoproteins are stronger inducers of cellular ERK than H1, and can accumulate more efficiently on the cell surface (Marjuki et al 2007). The accumulation of HA, that is expressed late in the replication cycle is necessary for efficient packaging and budding of progeny virions (Marjuki et al 2006).

In previous protection experiments, mice were immunized i.n with *ca* LAIVs twice before being challenged by a standard dose of the *wt* parental virus (Tannock et al 1984 & 1987). Only 10 infectious units of a H3N2 *ca* reassortant, compared with 1,000 units of a H1N1 *ca* reassortant was required to protect mice against homologous challenge. Therefore, the H3N2 *ca* reassortant tested was a superior immunogen than the H1N1 *ca* reassortant by a factor of 100. Both viral replication efficiency and/or the surface protein HA appear to play a significant role in determining the immunogenicity of *ca* LAIVs.

However, not all the H3 viruses replicate more efficiently than H1 viruses. The parental A/PR8 (H1N1) exhibited superior replication efficiency (>50,000 times) than the reassortant A/HK×31

with H3N2 surface antigens (Figure 3.1). Other studies suggest that viral internal proteins, especially PB1 and PB2, also play a role in the cellular ERK pathway that is related to HA accumulation on the cell surface (Marjuki et al 2006 & 2007), indicating a role for the genes of both surface- and non-surface-Ags in replication.

3.3.3 Primary Ab responses induced by *ca* influenza viruses are related to viral pathogenicity

In the study, serum Ab levels were measured 3 weeks after a single i.n inoculation with *ca* viruses. As expected, the H3 subtype *ca* virus induced a stronger Ab response than the H2 subtype *ca* virus (Figure 3.5) as the H3 replicated more efficiently, as indicated by its PFU: HAU ratio. The H3 *ca* virus (CR6) was the only *ca* virus tested in present study that caused weight loss (Table 3.2), which is consistent with an earlier study indicated that the H3 *ca* reassortant is able to induce a stronger Ab response than the H1 reassortant 6 weeks after the second vaccination (Table 3.1; Tannock et al 1995). By contrast, A/AA *ca* (H2N2) also had high *in vitro* replication efficiency but did not induce strong Ab responses or cause body weight loss. The reasons for these differences is unknown but could be related to mutations that specify the *ca* phenotype. Overall, humoral responses to *ca* viruses are more closely related to systemic reactions than viral replication rates measured *in vitro*.

3.4 SUMMARY

Early virus-host interactions were studied at day-3 after i.n inoculation of C57BL/6 mice with (1) *ca* viruses and their parental *wt* viruses with the same surface Ags but different internal genes, or (2) *ca* viruses with common internal genes but different surface Ags. Parameters studied were body weight changes, viral growth in the lungs and lung histopathology for consolidation.

Results from lung histopathology studies demonstrated no differences at day-3 p.i between the mice infected with *ca* viruses and their parental *wt* viruses. However, as expected, when measured by body weight loss, all *ca* reassortants and the *ca* donor strain were found to be less pathogenic than their *wt* parental viruses at day-3 p.i. The H3 *ca* reassortants CR6, CR18, CR29 and CR6-35* exhibited greater replication efficiency (determined by their PFU:HAU ratios) than H1 *ca* reassortants CR35 and CR6-35. The H3 *ca* reassortant CR6 caused a 3.79% loss in body weight; no losses were observed for H1 *ca* reassortant CR35 and the *ca* H2N2 donor strain A/AA *ca*.

Primary serum responses to *ca* reassortants and the donor strain were compared at 3-weeks p.i. Levels of HI antibody were found to be related to viral pathogenicity in mice. Higher HI responses were induced in groups infected with the H3 *ca* reassortant CR6 (GMT 80) than the H1 reassortant CR35 (GMT 10) and the H2 *ca* donor strain A/AA *ca* (GMT 13).

Chapter 4:

Evaluation of tetramer staining and immuno-PCR techniques for the measurement of specific CMI responses to *ca* LAIVs in C57BL/6 mice

4.1 INTRODUCTION

The measurement of immune responses is essential for determining the efficacy of human influenza vaccines. Traditional serum Ab tests, such as the HI test, are simple, specific and sensitive and are used for measuring responses to individual HA antigens in inactivated TIVs. Others, such as the neutralization test, are more complex but provide a greater sensitivity than HI assay and are used to measure responses to inactivated H5N1 vaccines (Cassetti et al 2006; Eichelberger et al 2008).

For LAIVs administered directly to the respiratory tract, measurement of serum Ab, alone, does not provide a complete indication of immunity induced within the respiratory tract (Romanova et al 1994; Treanor et al 2000; Belshe 2002). Optimal protection to respiratory challenge is determined by both specific humoral and CMI responses, both of which can be induced by LAIVs (Reiss and Schulman 1980; Section 1.2.2.1). Previous human studies have indicated that vaccine-induced IFN-γ and local IgA, rather than serum Ab responses, are associated with *ca* LAIV efficacy (Tomoda et al 1995). Moreover, protection can be induced by *ca* LAIVs in the absence of detectable serum Ab (Belshe et al 2000).

In addition to measuring serum Ab, results from clinical trials in which reductions in infection (vaccine efficacy) and illness (vaccine effectiveness) provide a more comprehensive evaluation of *ca* LAIVs. Clinical studies have shown that vaccine efficacy depends largely on vaccine-induced pre-existing neutralizing Ab levels that can lead to sterilizing immunity, whereas vaccine effectiveness is more likely to be related to memory CMI responses (Sasaki et al 2007; Wright 2007). When an antigenic mismatch occurs between the epidemic strain and the vaccine strain, live vaccines are significantly more effective than killed vaccines (Belshe et al 2000; Halloran et al 2007). Therefore, the measurement of vaccine-induced CMI responses is required to evaluate the performance of *ca* LAIVs (Eichelberger et al 2008).

Several tests on human PBMCs, including ELISPOT assays and tetramer flow cytometry, have been developed over 30 years to measure B and T cell responses (Keiholz et al 2002). However, it is not possible to directly measure CMI or IgA responses to infection or vaccination in the lower respiratory tract of humans. The extent to which vaccine-induced T cell and mucosal immunity and other factors (e.g. properties of the vaccine virus and the age of the vaccinee) influence immunity in humans is largely unknown (Arvin and Greenberg 2006).

Mouse models are convenient due to the availability of immunological reagents and are used in many studies of both live and killed influenza vaccines that may provide preliminary evidence of the likely situation in humans (van der Lann et al 2008). As discussed in Section 1.2.1.5, fluorescent labelled MHC tetramers have been widely used for the staining of Ag-specific T-cells, especially CTLs (Figure 1.8). This chapter describes attempts to refold a mouse MHC tetramer carrying the $NP_{366\text{-}374}$ peptide from the *ca* donor strain A/AA *ca* in order to evaluate responses in infected C57BL/6 mice.

In addition, the immuno-PCR (I-PCR) was used to measure IFN-γ levels in cell culture supernatants after co-culture of mouse lung lymphocytes with the same viruses used for vaccination. The I-PCR assay has been used to detect TNF-α and IL-18 and is a modification of an ELISA, in which the enzymes used for detection are replaced with a biotinylated reporter DNA (Section 2.15.1.2; Figure 2.1; Sanna et al 1995). The reporter DNA is bound to the biotinylated Ag-Ab complex through a streptavidin linkage. The I-PCR assay is highly sensitive and has a detection limit of 2.5 pg/l, which is 1.6×10^4 lower than that of the ELISA (Furuya et al 2000).

4.2 RESULTS

4.2.1 The formation and titration of a tetramer ($D^b/h\beta_2m/NP_{366-374}$) specific for A/AA

Tetramers were prepared from four monomers (Figure 1.8), each of which contained a MHC Class I heavy chain (D^b) specific for C57BL/6 mice, a human light chain (β_2m) and a 9-aa peptide [NP366$_{-374}$ (ASNENMDTM)] derived from *ca* donor strain A/AA *ca*. The fluorescent-labelled tetramers are the ligands for specific TCRs on the CTLs, allowing them to be characterised by flow cytometry (Section 1.2.1.5).

4.2.1.1 Expression vector for recombinant H-2D^b protein

The expression vector D^b-Kan^R was used to transform *E. coli* BL21 (DE3) by the heat shock method, and expression of heavy chain H-2D^b was induced by adding IPTG (Section 2.11.2). After induction, recombinant protein was expressed largely in the form of inclusion bodies from which H-2D^b was isolated and purified. By comparing different dilutions of BSA on the SDS-PAGE gel, it was estimated that the yield of inclusion bodies after induction was 4~8 mg/mL. Recombinant H-2D^b protein contains a peptide recognition sequence for the BirA, a biotinylation enzyme (O'Callaghan et al 1999).

4.2.1.2 Refolding and biotinylation of monomeric $D^b/h\beta_2m/NP_{366-374}$

Recombinant eukaryotic proteins expressed from *E. coli* need to be refolded in order to retain their original configuration. The monomer refolding process was carried out in the presence of the peptide (2.11.5) and was the critical step for the preparation of tetramers that bind to TCRs. After refolding, monomers were purified by FPLC to remove contaminants derived from *E. coli* (Figure 4.1A). Electrophoresis in 18% SDS-PAGE gels of unpurified preparations and fractions collected within the peak contained both monomers and contaminant proteins derived from *E. coli* (Figure 4.1B).

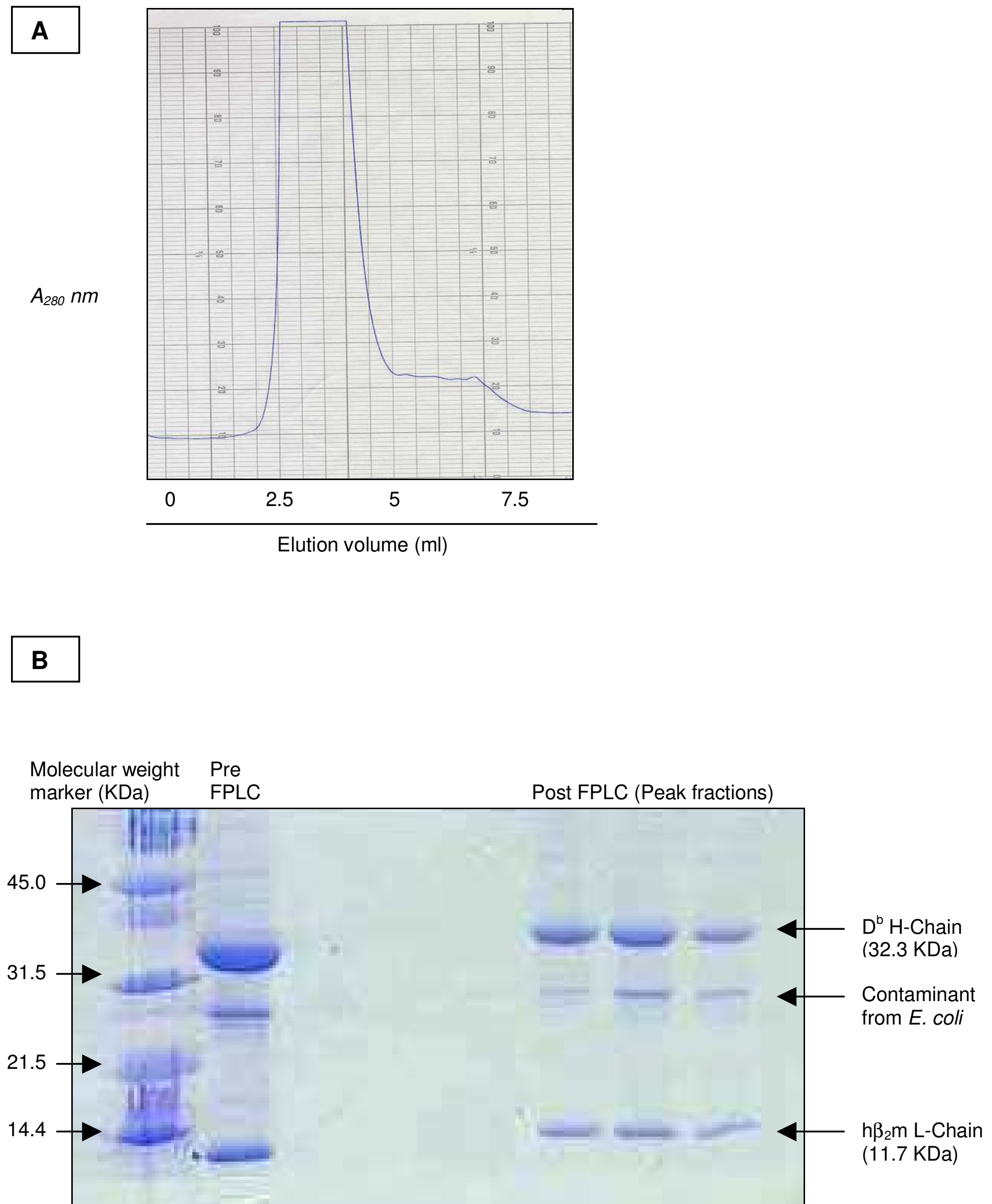


Figure 4.1 Purification of D^b/hβ_2m/$NP_{366-374}$ monomers specific for influenza A/Ann Arbor/6/60

A Gel filtration chromatogram after FPLC purification showing a peak of monomer eluted with 10mM Tris-HCl after absorbance at 280nm (A_{280} nm).

B 18% SDS-PAGE reducing gel analysis of monomer purified by gel filtration.

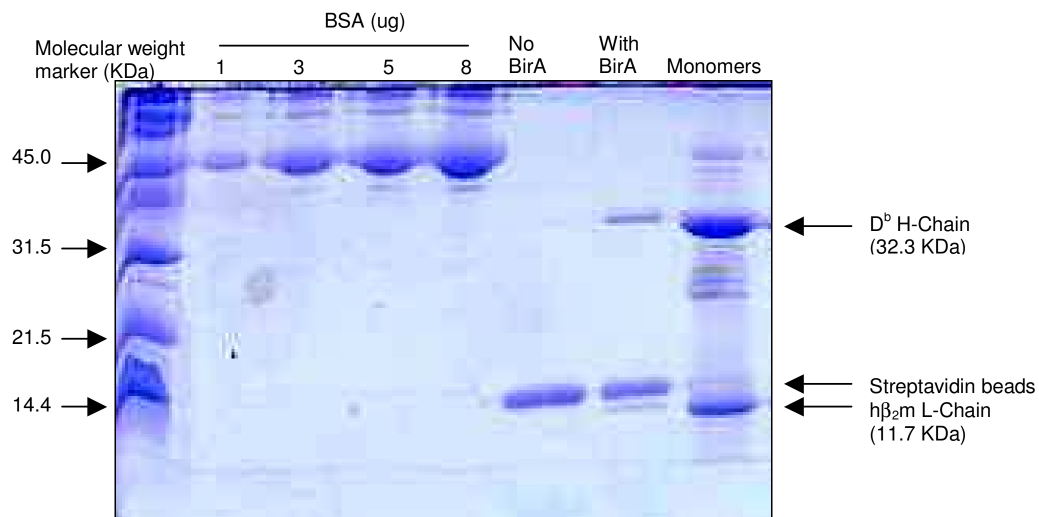


Figure 4.2 **T**etramer formation of D^b/hβ_2m/$NP_{366\text{-}374}$ specific for influenza A/Ann Arbor/6/60

18% SDS-PAGE was used to demonstrate pre- and post-biotinylation and tetramer formation. In the absence of biotinylation by BirA, monomers could not bind to SA-bead and were eluted. By contrast, biotinylated monomers that bound to SA-beads were saved from purification steps. By comparing the quantity of tetramer to monomer on SDS-PAGE gel, the final extent of biotinylation was estimated to be 20%.

4.2.1.3 Formation of $D^b/h\beta_2m/NP_{366\text{-}374}$ tetramers

Refolded and purified monomers were then biotinylated by using the enzyme BirA. Only after biotinylation could the monomers bind to PE-labelled streptavidin beads, each with four sites. Tetramers were formed by mixing the biotinylated monomers with streptavidin-PE at a 4:1 molar ratio. By comparing different dilutions of BSA on the SDS-PAGE gel, it was estimated that about 20% of $D^b/h\beta_2m/NP_{366\text{-}374}$ monomers formed tetramers with streptavidin-PE (Figure 4.2).

4.2.1.4 Staining of CTLs with $D^b/h\beta_2m/NP_{366\text{-}374}$ tetramers

The newly formed tetramers were tested using lymphocytes isolated from C57BL/6 mouse lungs following i.n infection with A/AA *wt*. A/AA *wt* was used instead of A/AA *ca* to infect mice in the tetramer titration study because: (1) an earlier study demonstrated that peptide $NP_{366\text{-}374}$ sequences for A/AA *ca* and A/AA *wt* were identical (Cox et al 1988). Therefore, $D^b/h\beta_2m/NP_{366\text{-}374}$ tetramers are able to recognise $NP_{366\text{-}374}$ - specific CTLs from either virus when used to infect C57BL/6 mice; (2) the local CTL immune response and subsequent $NP_{366\text{-}374}$ specific-CTL population induced by A/AA *wt* in lungs was expected to be significantly greater than that induced by A/AA *ca* (Figure 3.3), which would increase the sensitivity of the assay.

Four mice were infected with 10^5 pfu of A/AA *wt* and two uninfected mice were used as controls. All mice were held for further 10 days before being sacrificed and their lungs harvested. Lymphocytes from the lungs of infected and uninfected control mice were then isolated and pooled separately. Two parallel assays were carried out on those lymphocytes using (1) *in vivo* tetramer staining and (2) *in vitro* intracellular IFN-γ staining after stimulation with peptide $NP_{366\text{-}374}$. Because the positive control for the tetramer $D^b/h\beta_2m/NP_{366\text{-}374}$ specific for A/AA *ca* was not available, the intracellular cytokine cytometry (ICC) assay for IFN-γ was included as a peptide control.

IFN-γ ICC is a functional assay since peptide-specific CTLs can be restimulated to secrete IFN-γ by co-culture with the relevant peptide. By staining intracellular IFN-γ following a peptide pulse, peptide-specific CTLs can be identified by flow cytometry. The IFN-γ ICC assay has been used widely for the screening of CTL-epitopes (Crowe et al 2006; Wang et al 2007). Previous studies have demonstrated that there is a good correlation between the tetramer and IFN-γ ICC assays (Doherty 1998; Murali-Krishna et al 1998).

For tetramer titrations, 1 μL of anti-CD8-APC and 3 or 10 μL of tetramer-PE were added to 50 μL staining buffer containing 10^6 cells from the lungs of infected or uninfected mice. The results indicate that NP-specific CTLs were not stained by either 3 or 10 μL of tetramer-PE, even though the percentage of $CD8^+$ cells increased more than two-fold from 4.21 to 9.72 following infection (Figure 4.3 A&B). After staining with 10 μL tetramer, 0.3% of total lung lymphocytes from infected mice were NP^+ $CD8^+$ (Figure 4.3A), compared with 0.11% for uninfected mice (Figure 4.2B). Similar results were observed after staining with 3 μL of tetramer where NP^+ $CD8^+$ cells accounted for 0.1% of total lung lymphocytes from both infected and uninfected mice.

During the IFN-γ ICC assay, 50% of the lung lymphocytes isolated from infected or uninfected mice were cultured with peptide $NP_{366-374}$, which was added to cell cultures (1 μg to 10^6 cells per mL) in the presence of BFA. The cultures were then incubated for 4 hr at 37 ºC. The remaining lymphocytes were incubated under the same conditions in the absence of peptide. After incubation, each sample of cells was washed and divided into two tubes. Cells from one tube were stained with anti-CD8-APC and anti-IFN-γ-PE, both at a 1: 50 dilution. Cells from the other tube were used as an isotype control for measuring the non-specific intracellular staining background. The percentage of cells showing background staining in the lung lymphocytes of infected and uninfected mice was low, irrespective of whether a peptide pulse was applied (0.08-0.13%).

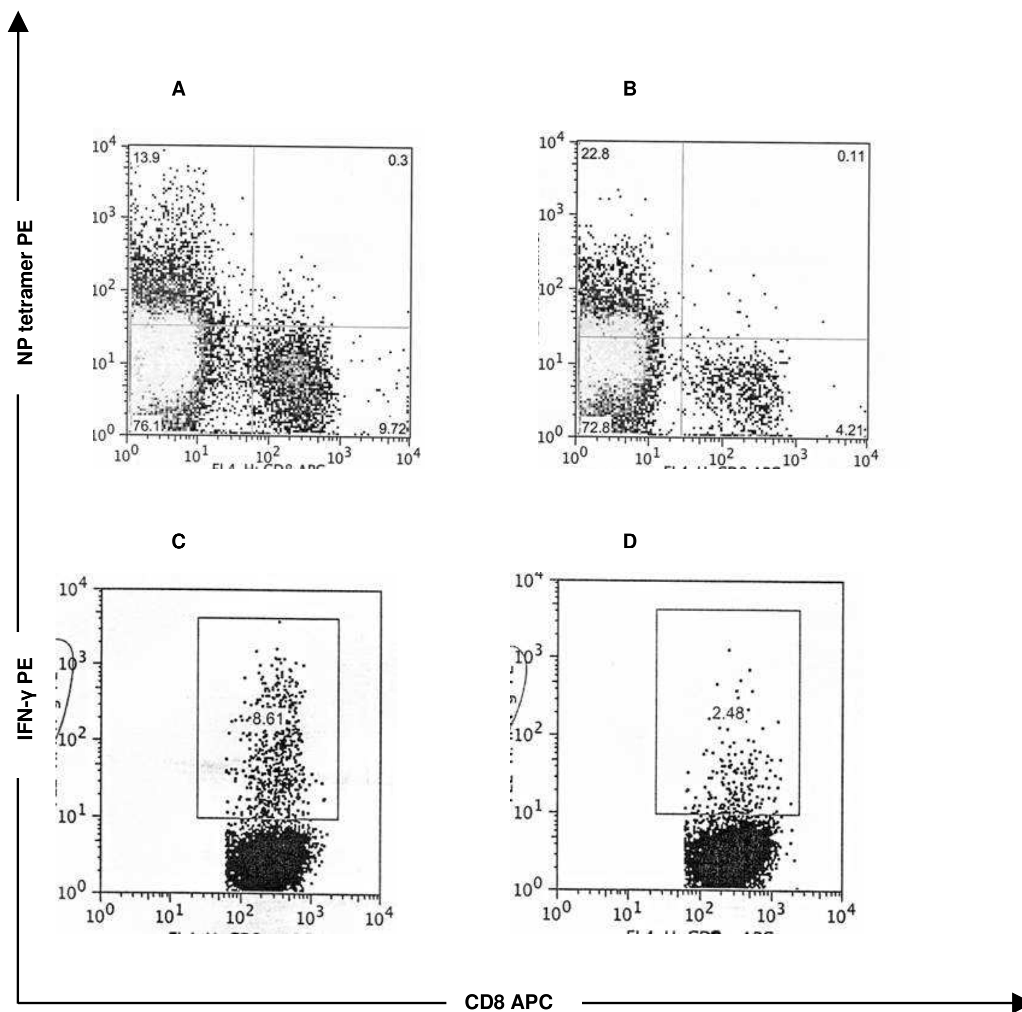


Figure 4.3 Flow cytometry profiles of A/Ann Arbor/6/60 $NP_{366-374}$-specific $CD8^+$ T cells from lungs stained directly with NP-tetramer (Panels **A** and **B**; gated on lung lymphocytes) or with INFγ intracellular following the application of an NP-peptide pulse *in vitro* (panels **C** and **D**; gated on lung $CD8^+$ T cells)

To demonstrate the specificity of the NP-tetramer, lung lymphocytes from (**A**) infected or (**B**) uninfected mice were compared. Results indicate that the NP-tetramer was unable to identify NP-specific CTLs, although $CD8^+$ cells of total lung lymphocytes comprised 9.72% for infected compared with 4.21% for uninfected mice.

To demonstrate the specificity of the NP-peptide in IFN-γ intracellular staining, lung lymphocytes from infected mice were cultured with (**C**) or without (**D**) NP-peptide and IFN-γ^+. $CD8^+$ T cells as a percentage of total lung $CD8^+$ cells were compared. Results indicate that $NP_{366-374}$-peptide was able to restimulate NP-specific CTLs and that peptide-specific $IFN\gamma^+$ $CD8^+$ T cells increased by a factor of 3.5 (2.48 to 8.61%) following the peptide pulse.

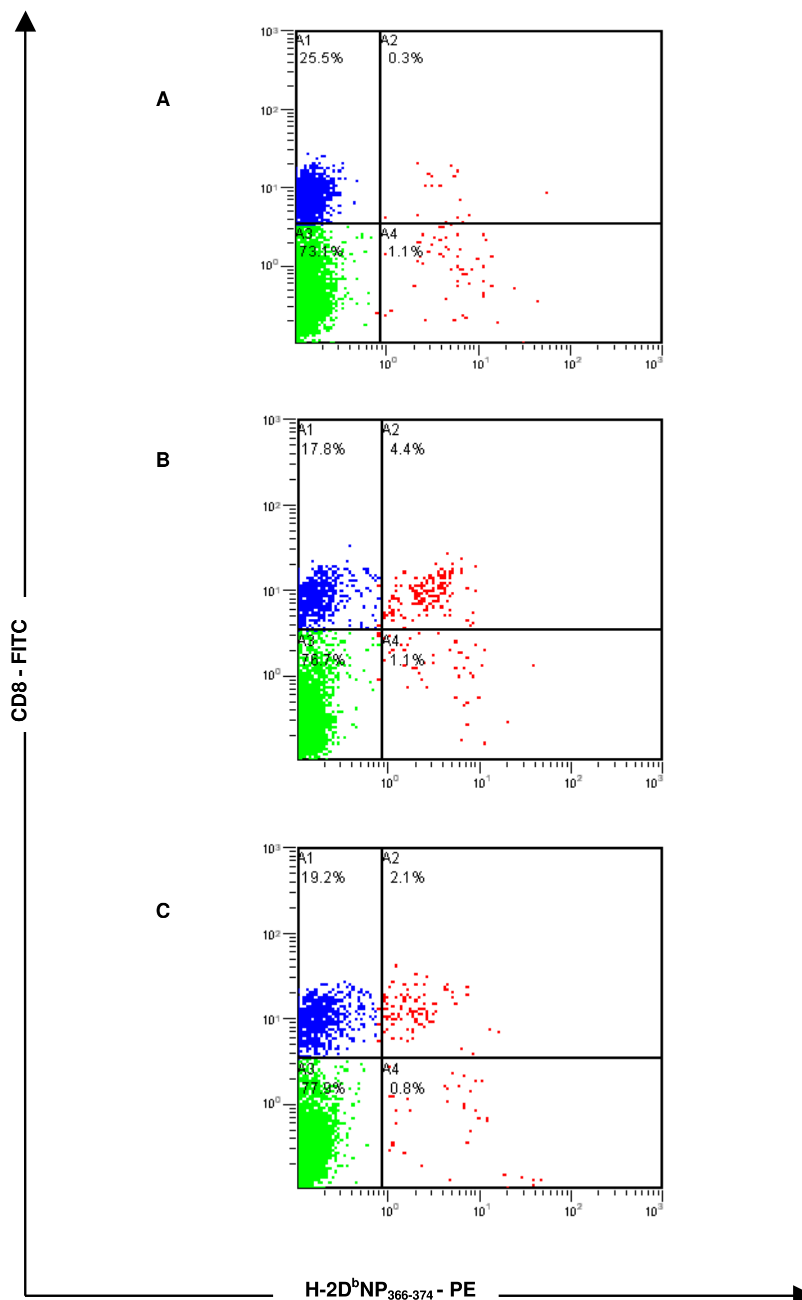


Figure 4.4 Mouse lung lymphocytes flow cytometry results demonstrating that MHC class I (H-2D^b) tetramer containing peptide derived from A/PR/8/34 nucleoprotein (NP) 366-374 is also able to stain lung NP$_{366-374}$-specific-CTLs 7-days-post secondary i.n vaccination with A/AA/6/60 ca

A Mice in uninfected control group inoculated with normal allantoic fluid i.n in two doses, 3 weeks apart;

B Mice in tetramer control group were first inoculated with reassortant A/HK×31 (H3N2) and, 3 weeks later, cross-pulsed with the parent virus A/PR/8/34 (H1N1);

C Mice from A/AA/6/60 ca infected group were vaccinated twice i.n at an interval of 3 weeks.

Results from flow cytometry indicate that peptide $NP_{366\text{-}374}$ was able to restimulate a specific-CTL population *in vitro*; 8.61% of $CD8^+$ T cells isolated from infected mouse lungs were NP-specific after the peptide pulse, compared with 2.48% in its absence (Figure 4.3 C&D). In addition, the proportion of $CD8^+$ T cells within the total lung lymphocytes was 22% for infected and 4% for uninfected mice. By contrast, no differences were detected in uninfected mice where 2.51% were positive following the pulse, compared with 2.34% where it was not applied.

4.2.1.5 MHC class I tetramer H-2D^b containing A/PR/8/34 nucleoprotein ($NP_{366\text{-}374}$) peptide stains A/AA/6/60-specific CTLs

Tetramers carrying $NP_{366\text{-}374}$ of A/PR/8/34 were used in the following comparative study of lung CTL responses to different *ca* LAIVs. In a preliminary validation study, three groups of three C57BL/6 (H-2D^b) mice were inoculated i.n twice at an interval of 3 weeks with A/AA *ca*, A/HK×31 plus A/PR/8/34, or normal allantoic fluid. The local lung CTL responses were investigated a week later after the secondary infection.

In the uninfected control group, mice were inoculated i.n. with normal allantoic fluid. Mice from tetramer control group were first inoculated with 1.45×10^5 PFU A/HK×31 (H3N2) and then cross-primed with 1.45×10^5 PFU A/PR/8/34 (H1N1) 3 weeks later. A/HK×31 is a reassortant virus with all six internal genes derived from A/PR/8/34. This cross-priming strategy often used to maximize pre-existing specific-CTL populations specific for influenza A internal Ags by avoiding anti-HA or NA Abs-mediated suppression by different surface antigens (Doherty et al 1998). Finally, mice from the infected test group were vaccinated i.n twice with 1.45×10^5 PFU A/AA/6/60 *ca*.

The results indicate that the tetramer carrying $NP_{366\text{-}374}$ of A/PR/8/34 is able to stain the CTLs primed by A/AA/6/60 *ca*. From uninfected group, only 0.3% of total lung lymphocytes were

NP-specific-CTLs. In contrast, a significant expansion of these cells was detected in infected mouse groups. $NP_{366\text{-}374}$-specific-CTLs comprised 4.4% of lung lymphocytes in A/HK×31 plus A/PR/8/34-infected mice and 2.1% in A/AA *ca*-infected mice (Figure 4.4).

4.2.2 Evaluation of the Immuno-PCR assay for the measurement of IFN-γ in cell culture supernatants

The immuno-PCR (I-PCR) technique was used to measure INF-γ levels in the lung lymphocytes of vaccinated mice after *in vitro* restimulation mice with the same virus.

4.2.2.2 Reporter DNA preparation and optimization of I-PCR conditions

For I-PCR assays, biotinylated DNA was used as a reporter marker that could be detected by PCR (Section 2.15.1.2). This reporter DNA was developed from a Bluescript plasmid by PCR amplification with the biotinylated M13-20 primer and the non-biotinylated M13 reverse primer (Figure 4.5A). The optimal I-PCR reporter concentration that did not cause non-specific binding and amplification was determined using blocking buffer (1% BSA in PBS) in place of recombinant mouse IFN-γ. Different dilutions of reporter DNA were tested and results are shown in Figure 4.5B. Bands were observed at concentrations of 10^6, 10^4 and 10^3 ng/L DNA, indicating that excess reporter DNA was present and non-specific binding led to false-positive PCR results. However, the band was not seen at 1 ng/L, the concentration used subsequently for I-PCR assays.

4.2.2.2 Application of I-PCR in the detection of IFN-γ in supernatants from mouse lung lymphocytes cultures

The ca donor virus A/AA *ca* and reassortants CR6, CR18, CR29, and CR35 that had been prepared from A/AA *ca* were inoculated to groups of 6 C57BL/6 mice. Each mouse was inoculated twice by the i.n. route with 1.45×10^5 PFU of individual viruses at an interval of 3 weeks (Section 2.12). A week after the last inoculation, all mice were euthanased and single cell

suspensions of individual lungs were prepared and incubated at 96-well cell culture plate coated with a purified preparation of the same virus used for inoculation (Section 2.15.2.1). Mice from an uninfected control group were inoculated with normal allantoic fluid, and lung lymphocyte preparations were incubated in a plate coated with normal allantoic fluid.

Following incubation, cell culture supernatants were collected and pooled within the same virus group and I-PCR assays were performed to detect IFN-γ in cell culture supernatants. From the intensity of the PCR products on 2% agarose gels, IFN-γ levels in mice vaccinated with A/AA ca, CR18, CR29 and CR35 were found to be similar (Figure 4.6). However, no increase in IFN-γ could be detected in CR6-infected mice, where the band intensity was similar to that for uninfected control mice (Figure 4.6).

4.3 DISCUSSION

The measurement of specific CMI responses in the respiratory tract remains a challenge in determining the efficacy of *ca* LAIVs for humans. In most studies, tests on blood and nasal wash samples are used to provide surrogate measurements of responses in humans. In the present study in a mouse model the need to include specific measures of CMI, in addition to humoral immunity for monitoring responses to LAIVs was clearly indicated from assays involving the use of tetramer staining and I-PCR.

4.3.1 Application of the tetramer containing peptide from A/PR/8/34 $NP_{366-374}$ for the identification of influenza A/AA/6/60 *ca*-specific CTLs by flow cytometry

As discussed previously in Section 1.2.1.5, fluorescent-labelled MHC tetramers can directly measure Ag-specific T cell responses by binding to TCRs. Viral peptide $NP_{366-374}$ includes one of the immunodominant CTL epitopes in the C57BL/6 mouse for influenza A viruses (Table 1.3).

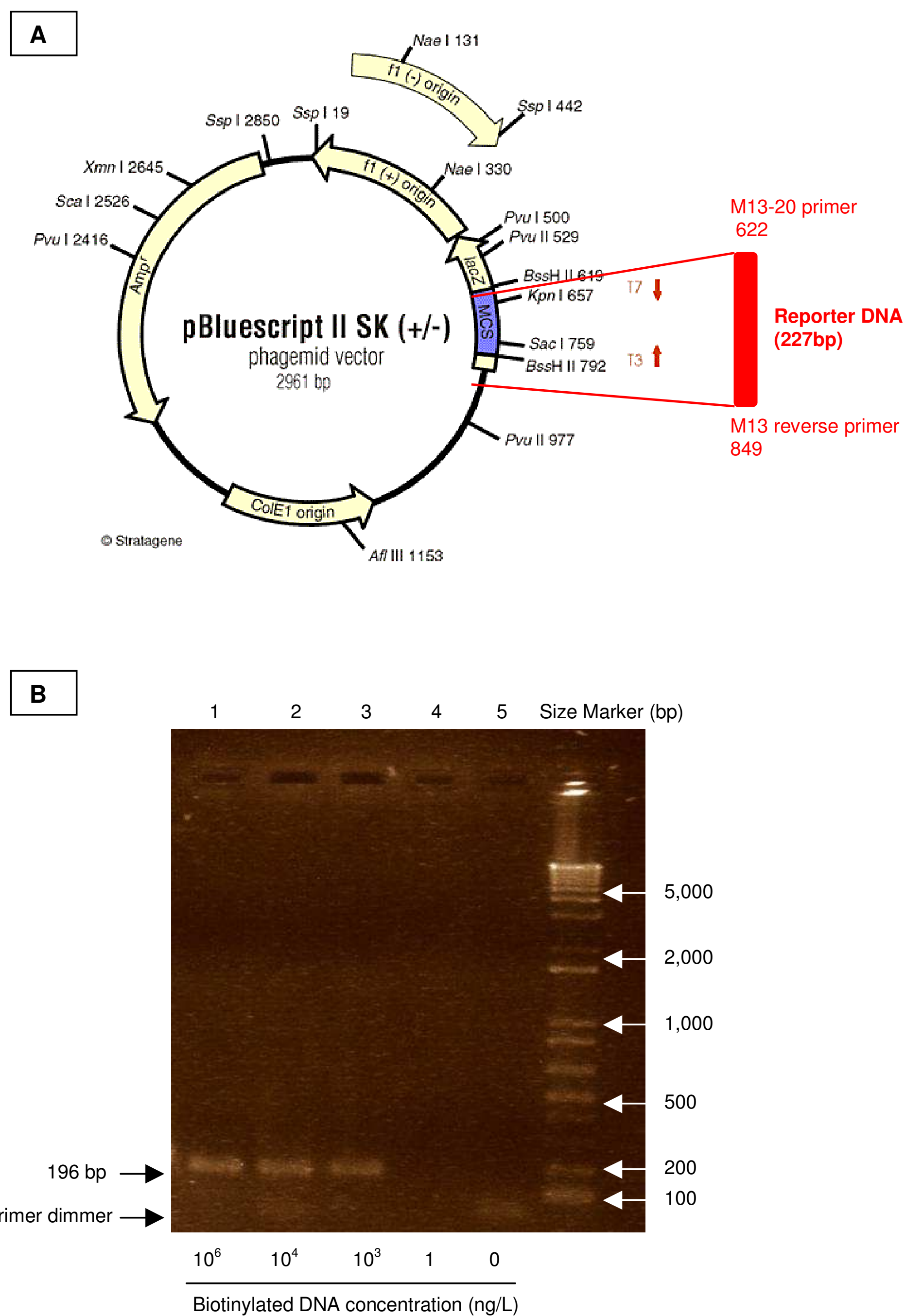


Figure 4.5 Preparation and titration of biotinylated reporter DNA. Reporter DNA (227 bp) was prepared from the Bluescript plasmid by PCR amplification with the biotinylated M13-20 primer and the non-biotinylated M13 reverse primer (**A**). The optimal concentration of reporter DNA used in I-PCR was determined using 1% BSA in PBS instead of recombinant mouse IFNγ (**B**). Final PCR products (196 bp) were obtained with IF and IR primers nested to M13-20 and M13 primers. PCR products were electrophoresed in 2% agarose gels.

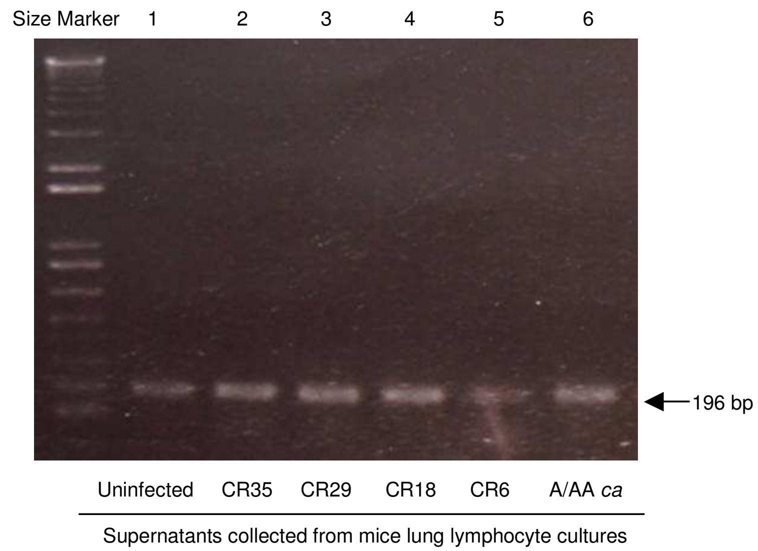


Figure 4.6 Measurement of IFN-γ levels by comparing the intensity of the PCR product bands in I-PCR assays. Following i.n vaccination, mouse lung lymphocyte suspensions were prepared and co-cultured with purified viruses. After incubation, cell culture supernatants were collected and I-PCR was performed.

In a previous study, in which mice were challenged with A/HK×31 (H3N2) after priming with A/PR/8/34 (H1N1), $NP_{366\text{-}374}$-specific CTLs accounted for 57% of total BAL cells by the tetramer technique (Belz et al 1998). Therefore, the peptide $NP_{366\text{-}374}$ from influenza A/AA/6/60 was selected to form MHC I tetramers in this study.

For reasons unknown, newly formed $NP_{366\text{-}374}$ tetramers failed to detect NP-specific CTLs from lungs, even though the peptide itself was found effective in stimulating the secretion of IFN-γ in the same cells under *in vitro* conditions. Overall, 8.61% of total lung $CD8^+$ T cells or 1.89% of total lung lymphocytes were identified as $CD8^+$ IFN-γ^+ T cells - the functional CTLs (Figure 4.3C). Similar results were reported in an earlier study, using a cross-priming strategy, where 7.1% of BAL cells were shown to be the functional CTLs (Belz et al 2001).

Examination of full-length protein sequence alignments from a selection of 130 sequences available in GeneBank demonstrated that 80% of NP was conserved (Powell et al 2007). Comparison of the peptide $NP_{366\text{-}374}$ from A/PR/8/34 and A/AA/6/60 indicated that the only difference found was at the 7th aa where Asp (E) was replaced by Glu (D) (Table 2.3). As the 7th aa is outside the two anchor points (aas 2 and 9), this change should not have affected binding between the peptide and MHC I peptide-binding cleft (Parham 1992). It was, therefore, not unexpected that the tetramer containing the peptide from A/PR/8/34 $NP_{366\text{-}374}$ would be able to identify influenza A/AA/6/60 *ca* NP-specific CTLs (Figure 4.4). After two i.n vaccinations with A/AA/6/60 *ca* and staining with A/PR/8/34 $NP_{366\text{-}374}$ tetramers, 2.1% of total lung lymphocytes were identified as NP-specific CTLs (Figure 4.4C).

A good correlation between tetramer staining and IFN-γ ICC assays was demonstrated in this study. Ten days after primary infection with A/AA/6/60 *wt*, 1.89% of total lung lymphocytes were functional NP-specific CTLs that were detected by IFN-γ ICC. Compared with the tetramer assay, 2.1% of these cells were identified as NP-specific CTLs 7 days after secondary

i.n vaccination with A/AA/6/60 *ca*. Consequently, in the following chapter, IFN-γ ICC assays were used for measuring the NP-specific CTLs in BALB/c (H-2^d) mice for which the tetramer was unavailable.

4.3.2 Detection of IFN-γ in C57BL/6 mice by I-PCR after i.n. administration of a second dose of *ca* LAIVs

I-PCR is a modified ELISA and a DNA-enhanced immunoassay with broad applications for the detection of antigens. The coupling of biotinylated Abs and biotinylated DNA to a linker protein, such as streptavidin, has been widely used (Sano et al 1992). The major advantage of I-PCR is the significant increase in assay sensitivity which is achieved using PCR-amplified DNA as a signal reporter instead of the traditional enzyme and substrate of an ELISA. Currently, the precise Ag quantitation could be achieved by the test such as real-time PCR (Adler et al 2008).

IFN-γ is produced locally in lungs by T and NK cells in response to the i.n vaccination of mice with LAIVs and has critical roles in both the innate and adaptive immune responses to influenza infections (Table 1.2). However, secretion occurs over a very short time in tissues (Morris 1988), which makes the detection of IFN-γ *in vivo* very difficult. In the present study, an I-PCR assay for the detection of IFN-γ *in vitro* was established and IFN-γ induced by different *ca* LAIVs was compared. Lung lymphocytes were prepared from *ca* LAIV-primed mice and then pulsed with the same viruses used for priming under *in vitro* conditions. However, the priming viruses used were purified and inactivated and, therefore, IFN-γ secreting T cells were more likely to be the HA or NA- than NP-specific.

Increased IFN-γ levels were detected in lung lymphocytes prepared after 7 days from mice reinfected with A/AA *ca*, CR18, CR29 and CR35. In contrast, no increases could be detected in mice reinfected with CR6 (Figure 4.6). Interestingly, from Chapter 3, serum HI titres induced by CR6 (GMT 80) were much higher than those induced by CR35 (10) 3 weeks after the first dose

(Figure 3.5). Moreover, CR6 caused on average 3.79% weight loss at day 3 p.i. compared with CR35 where no loss could be detected (Table 3.2). The results suggest that CR6 produces a maximum response after one dose, unlike A/AA/6/60 *ca* and the other reassortants that require two doses.

Unlike CR35 which possesses a PFU: HAU ratio of 1.7×10^3, CR6 grows to high titres with a PFU: HAU ratio of 2.5×10^3 and could, therefore, be expected to elicit stronger immune responses than CR35 (Figure 3.3A). The replication efficiency of individual *ca* viruses (see Chapter 3) was directly proportional to the primary immune responses induced in mice. However, the same pattern was not observed with secondary responses, probably due to the presence of pre-existing immunity. This was evidenced by using I-PCR to measure IFN-γ post secondary vaccination with *ca* LAIVs. A higher level of IFN-γ level detected in cultured CR35-infected mouse lung lymphocytes than in CR6-infected mice.

These results indicate that Ab levels generated from primary responses play a role in the secondary response to the same Ag, which may have important implications for the use of *ca* LAIVs in humans. However, unlike mice that are naïve to influenza virus infections, most humans have pre-existing immunity from natural infections. Therefore, during the evaluation or selection of candidate *ca* LAIV, both pre-existing immunity and the viral replication efficiency need to be considered.

The I-PCR results presented here had limitations because the test is not amenable to quantitation. Therefore, in Chapter 5, the tetramer staining and IFN-γ ICC assays were used in conjunction with ASC ELISPOT and serum HI tests to compare immune responses to different *ca* LAIVs in different strains of mice. Such tests may provide further reliable insights to the correlation between the humoral and CMI responses to *ca* LAIVs.

4.4 SUMMARY

Reliable sensitive assays are required to compare CMI responses to different *ca* LAIVs in mice and in this chapter the use of tetramer staining, ICC and I-PCR were evaluated. For tetramer-based flow cytometry assays, the fluorescent-labelled tetramer is the key reagent for estimation of *in vivo* epitope-specific CTL responses. The NP$_{366-374}$ peptide of influenza A viruses comprises the immunodominant epitope that is highly conserved between subtypes. For A/PR/8/34 (H1N1) and A/AA/6/60 (H2N2), there is only one aa difference within peptide NP$_{366-374}$. Therefore, the peptide NP$_{366-374}$ was used to prepare tetramers for detecting NP-specific CTLs against all influenza A viruses used in this study.

H-2D^b monomers were successfully refolded with the NP$_{366-374}$ peptide, but only 20% were found to form tetramers through biotin-streptavidin linkage. Newly formed NP-tetramers were unable to identify NP-specific CTLs isolated from mouse lungs 10 days after infection with A/AA/6/60 *wt*. In contrast, an IFN-γ ICC assay conducted in parallel demonstrated that peptide NP$_{366-374}$ was sufficient to restimulate NP-specific CTLs to secrete IFN-γ *in vitro*. Tetramers carrying the A/PR/8/34 peptide NP$_{366-374}$ also stained NP-specific CTLs prepared from the lungs of A/AA/6/60 *ca* mice after two doses.

Like the IFN-γ ICC assay, I-PCR is a functional assay which requires *in vitro* Ag restimulation. However, unlike IFN-γ ICC in which cells were pulsed by peptide NP$_{366-374}$, inactivated whole viruses were used as the stimulus in I-PCR. Consequently, IFN-γ detected by I-PCR was more likely to be secreted by HA- and NA-specific lymphocytes. In the present study using the I-PCR assay, increased IFN-γ levels were detected in lung lymphocytes prepared after 7 days from mice reinfected with A/AA *ca*, CR18, CR29 and CR35. By contrast, no increases could be detected in mice reinfected with CR6 which induced a higher primary humoral response. In the following chapter, tetramer and IFN-γ ICC assays were used to compare CMI responses elicited by a range of *ca* LAIVs.

Chapter 5:

Analysis of the cellular and humoral responses in mice following intranasal administration of *ca* LAIVs

5.1 INTRODUCTION

Ca LAIV viruses replicate in the respiratory tract of mice, mostly the LRT, where they induce and maintain significant levels of memory ASCs and CTLs and broad-protection across influenza A subtypes (Section 1.3.3.3). The immunogenicity of *ca* LAIVs is related to the infectivity of individual *ca* viruses for mice (Wareing and Tannock 2001; Wright et al 2007; Chapter 3), and is determined by both viral and host factors. In the following chapter, CMI and humoral responses induced by range of *ca* reassortants prepared from the A/AA *ca* donor strain were compared in different strains of mice, using methodologies developed in earlier chapters.

5.2 RESULTS

5.2.1 Relative immune responses to reassortants of A/AA *ca* in C57BL/6 mice

5.2.1.1 Local and systemic humoral responses to viral surface Ags of ca LAIVs

This experiment was designed to investigate humoral responses, both local and systemic, against the surface Ags of *ca* LAIVs at different time points. Groups of six 6-8 week-old female C57BL/6 mice were inoculated twice by the i.n. route, at an interval of 3 weeks, with inocula containing 1.45×10^5 PFU of the *ca* donor strain A/AA *ca*, the H3N2 reassortants CR6, CR18 and CR29 and an H1N1 reassortant CR35. Three mice from each group were sacrificed at day 7 p.i. and the remaining 3 on day 9. Mouse lungs were harvested and single cell suspensions prepared for use in the ELISPOT assay to measure local virus-specific IgA- or IgG2a-Ab secreting B cells. Individual, mouse sera were prepared in parallel and HI tests were performed to measure HA-specific-Ab levels (Section 2.15.1). Higher local ASC responses (both IgA and IgG2a) were observed on day 9 than day 7 p.i. for A/AA *ca*-, CR6-, CR18- and CR29-infected mice (Figure 5.1A&B). Although, the IgG2a ASC response to CR35 was higher on day 9 than

on day 7, the IgA ASC response was lower (Figure 5.1A&B). Furthermore, from day 7 to day 9, an increase in anti-HA Abs occurred in groups infected with A/AA *ca*, CR18 and CR29 but a decrease occurred in groups infected with CR6 and CR35 (Figure 5.1C). Overall, of the five *ca* LAIVs tested at day 7, CR35 induced the highest local B cell responses.

Further statistical analysis using combined data from days 7 and 9 revealed no significant differences in humoral responses induced by A/AA *ca*, CR6, CR18 and CR29 (two sample *t*-test; $P > 0.05$; Table A.1&A.2; Figure 5.2). However, the response to CR29 was significantly different (Table A.2; Figure 5.2). CR29 induced a significantly weaker IgA ASC response than that induced by CR18 and CR35, and a weaker IgG2a ASC response than CR35. HI titres induced by mice infected with CR29 were significantly lower than in mice infected with A/AA *ca* (Figure 5.2).

In the following experiment, local B cell and serum Ab responses in the tetramer-positive control group (Section 4.2.1.5) and the CR35-vaccinated group were compared. Each group consisted of seven C57BL/6 mice. Mice in the tetramer control group were first primed i.n with 1.45×10^4 PFU of A/HK×31(H3N2) and 3 weeks later were challenged i.n with 1.45×10^4 PFU of A/PR8 (H1N1). Mice from the CR35 group received two identical i.n inocula containing of 1.45×10^4 PFU 3 weeks apart. All mice were killed 1 week after the last inoculation, and ELISPOT and HI assays were performed. In the ELISPOT assays, lung lymphocytes from the tetramer control group were tested against either A/HK×31 or A/PR8. Sera from this group were tested against A/HK×31.

ELISPOT assays indicated that a higher IgA ASC response was induced in response to CR35 (189 per 10^6 lung lymphocytes compared with 53 per 10^6 for the tetramer control group when tested against A/HK×31 or 75 per 10^6 against A/PR8). However, no differences were observed between the IgG2a ASC responses of the two groups (Figure 5.3B). By contrast, much higher

serum Ab levels were induced by the tetramer control, compared with the CR35 group (GMT 320 versus 24.37; Figure 5.3C).

5.2.1.2 Local and systemic CMI responses to viral internal Ags of ca LAIVs

In order to investigate CMI responses to *ca* LAIVs, groups of four 6-8 week-old female C57BL/6 mice were inoculated by the i.n. route. Mice were infected twice at an interval of 3 weeks, with inocula containing 1.45×10^5 PFU of *ca* LAIVs (A/AA *ca*, CR6, CR18, CR29 and CR35) or A/AA *wt*. Mice from an uninfected (negative) control group were inoculated with normal allantoic fluid; mice from the tetramer (positive) control group were first inoculated with 1.45×10^4 PFU of A/HK×31 and, 3 weeks later, were challenged i.n with 1.45×10^4 PFU of A/PR8. On day 7 p.i., all mice were euthanased and lungs, spleens and sera were collected. Single cell-suspensions were prepared from individual organs. Cell samples from each group were pooled for T cell phenotype analysis. Individual mouse sera were tested for HI Ab and group GMTs calculated.

Two-colour flow cytometry was performed at same time on each group in order to compare cell populations with markers specific for $NP_{366-374}^{+}$ CD8 $^{+}$, $CD8^{+}$ CD44 $^{+}$ and $CD4^{+}$ $CD44^{+}$, within the lungs and spleens. Since viral peptide $NP_{366-374}$ contains one of the immunodominant epitopes for C57BL/6 mice, especially during secondary infections (Section 1.2.2.1), $NP_{366-374}$-specific CTL populations in the lungs have critical roles in the clearance of influenza viruses following challenge (Section 1.2.2.3). Expression of the activation or memory marker, CD44, was expected to increase in T cells following exposure to Ags (Unger et al 2003). The expression of CD44 is required to mediate lymphocyte extravasation and migration to virus-induced inflammatory sites (Flynn et al 1998).

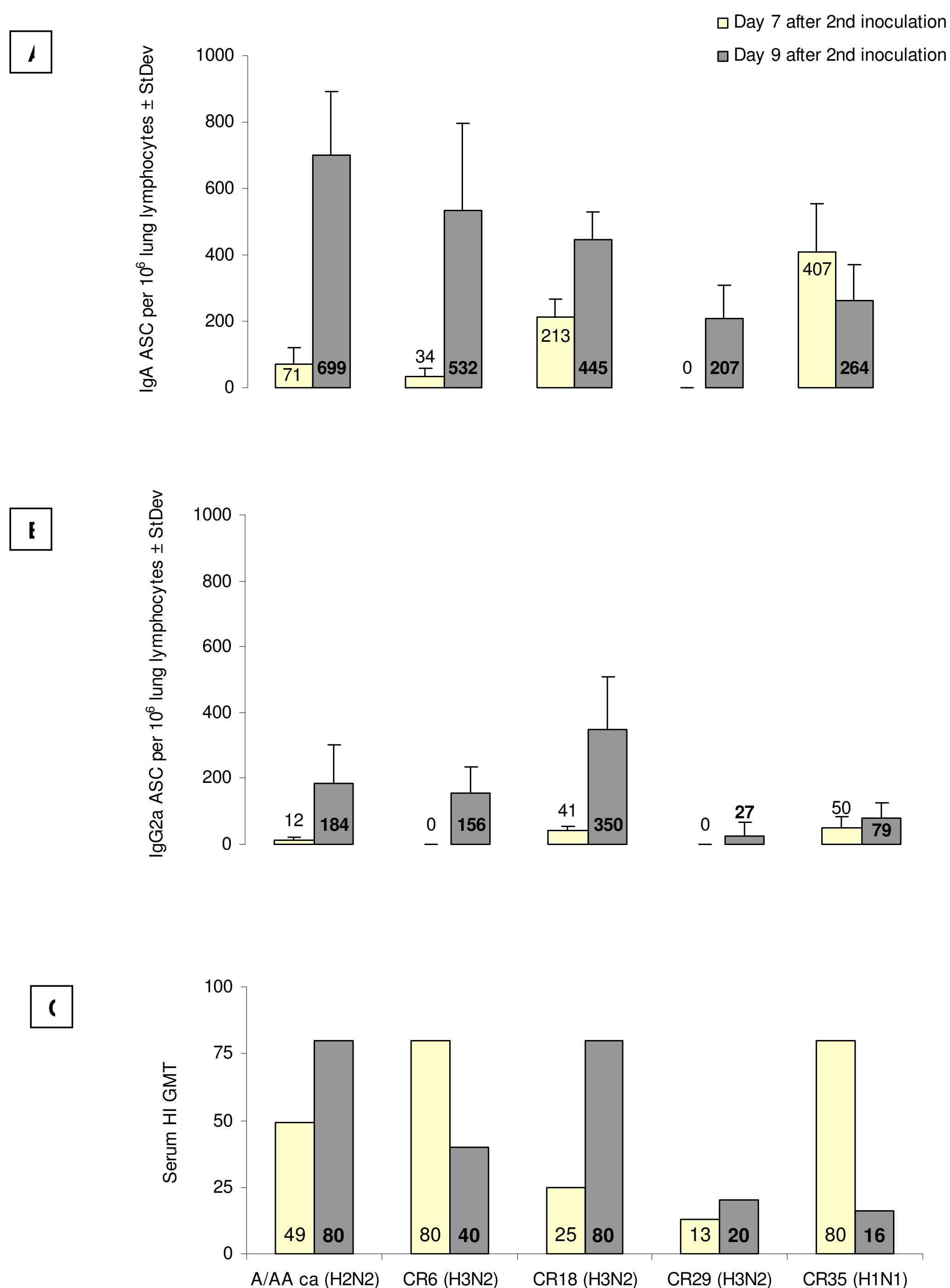


Figure 5.1 Local and systemic humoral responses to *ca* LAIVs in C57BL/6 mice on days 7 and 9 after the second inoculation. Six-eight week-old female C57BL/6 mice were vaccinated twice i.n with 1.45 $\times 10^5$ PFU of *ca* LAIVs at an interval of 3 weeks, to groups of 6. On days 7 or 9 after the second inoculation, three mouse lungs and three serum samples from each group were prepared. ELISPOT and HI assays were carried out for the measurement of lung specific IgA (**A**) or IgG2a (**B**) ASCs and serum Abs(**C**).

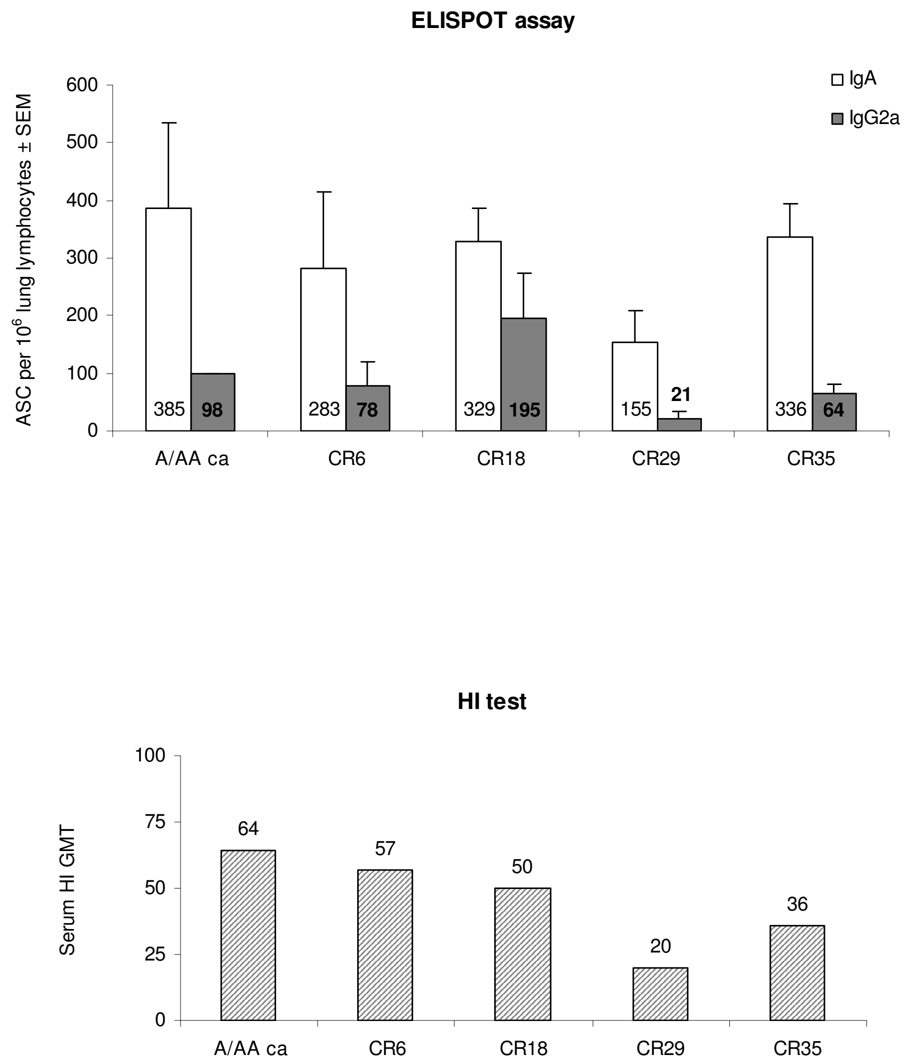
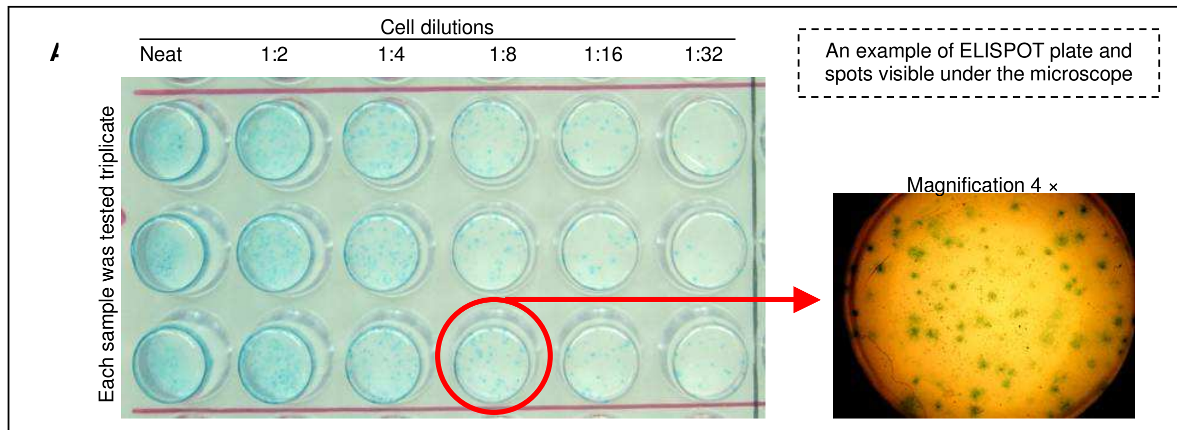
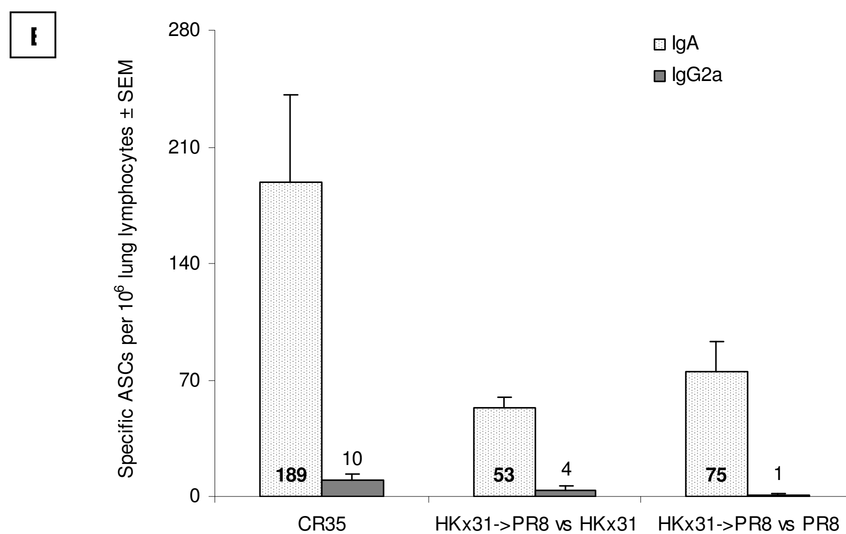


Figure 5.2 Overview of local and systemic humoral responses to *ca* LAIVs in C57BL/6 mice. The graph was generated from combined data for days 7 and 9.





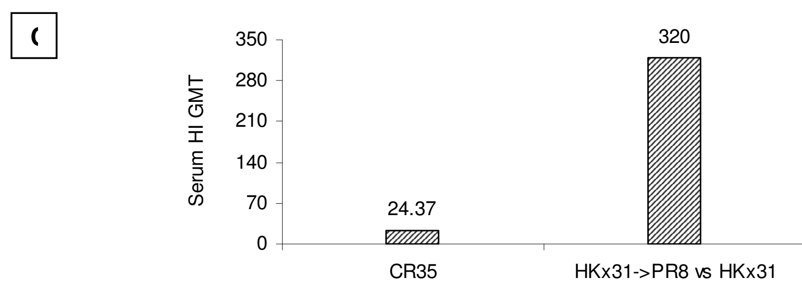


Figure 5.3 Comparative studies of local and systemic humoral responses to CR35 and HK×31. For CR35, seven 6-8 week-old female C57BL/6 mice were inoculated i.n twice with an identical dose at an interval of 3 weeks. Another group of 7 mice were first infected with HK×31and then with A/PR8 after 3 weeks. This cross-priming system was used as the tetramer control in T cell flow cytometry analysis.

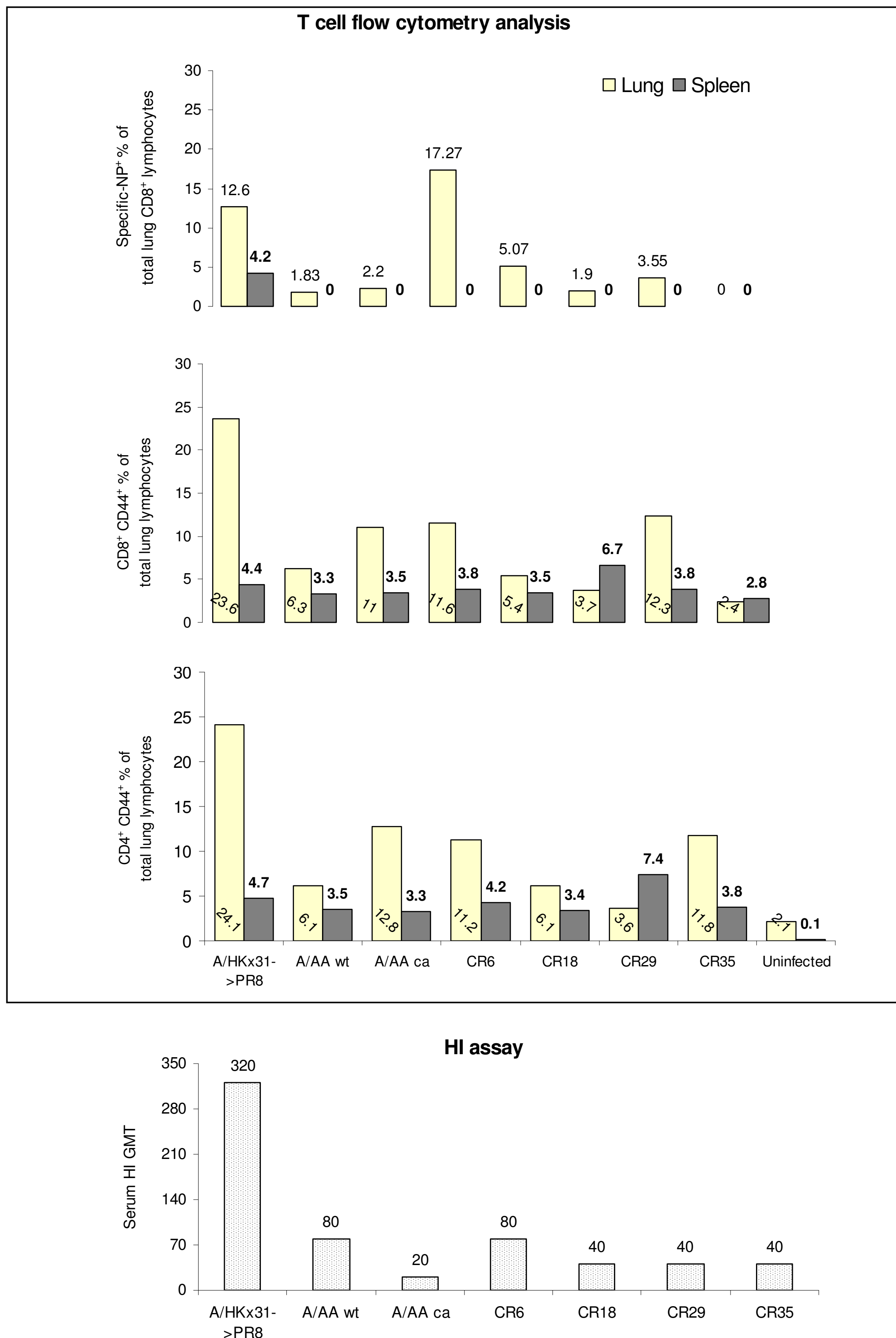


Figure 5.4 Phenotype of virus-specific T cells in mouse lungs (Experiment I). Four 6-8 week-old female C57BL/6 mice from each group were inoculated twice at an interval of 3 weeks. Lungs and spleens were harvested and pooled 1 week after the 2nd dose. T cells from lungs and spleens were tested for their phenotype and individual sera were prepared for use in HI tests (see also Figure A.1 & 2 for flow cytometry data of lung T cell tetramer NP staining and phenotype).

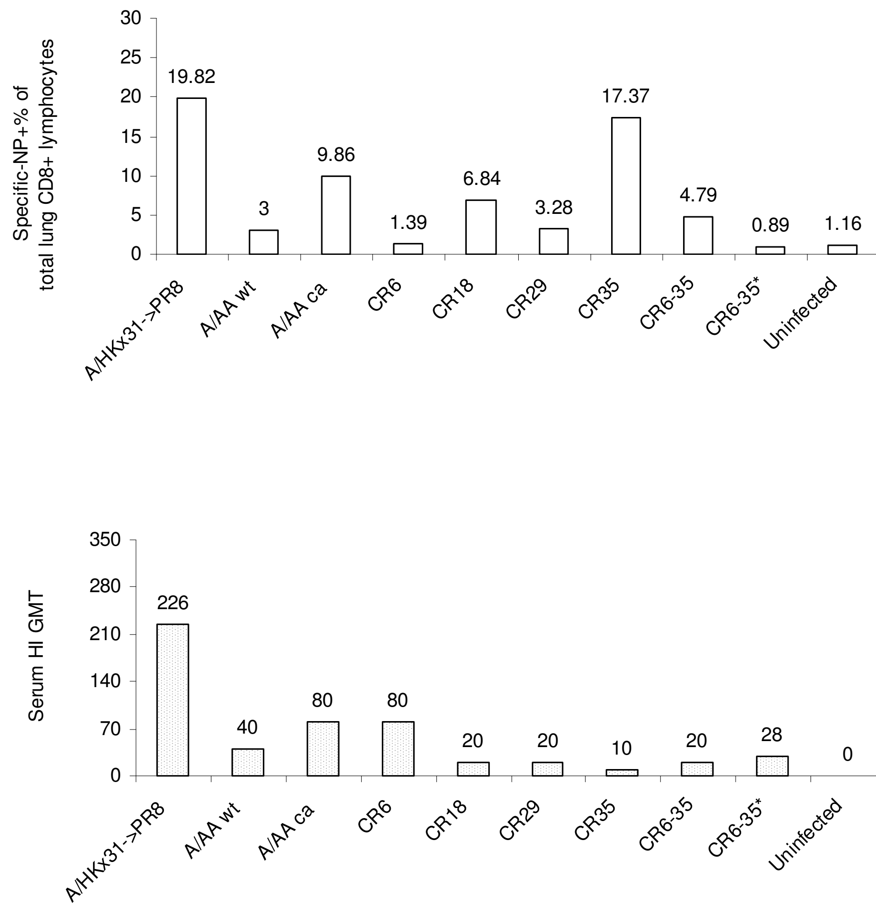


Figure 5.5 Phenotype of virus-specific T cells in mouse lungs (Experiment II)

Table 5.1 Lung NP-specific CTL and serum anti-HA responses in Experiments I and II

Virus	NP-specific CTL response (% of total lung lymphocytes)		Serum HI GMT		PFU:HAU ratio ($x10^3$ pfu/HAU)[a]	
	Experiment I	Experiment II	Experiment I	Experiment II	Experiment I	Experiment II
A/AA *ca*	**0.50**	**2.09**	20	80	29.00	6.25
CR6	**3.80**	**0.34**	80	80	N.D.[c]	3.40
CR18	0.70	0.76	40	20	N.D.	36.93
CR29	0.00	0.37	40	20	14.20	12.78
CR35	**0.90**	**2.92**	40	10	0.79	1.67
A/HK×31->PR8	5.4	4.47	320 (40)[b]	226 (80)	19.71 (70.31)	27.34 (136.72)
A/AA *wt*	0.30	0.40	80	40	105.47	70.31
Uninfected	0.00	0.30	0	0	N.A.[d]	N.A.

[a] PFU: HAU ratios of virus stocks used for preparation of mouse inocula. A/HK×31 and PR8 were diluted to 1.45×10^4 PFU per 50 uL and the remaining viruses were diluted to 1.45×10^5 PFU per 50 uL prior to inoculation;

[b] Bracketed values are titres against PR8.

[c] N.D.: not done;

[d] N.A.: not applicable.

Table 5.2 Lung NP- and PA-specific CTL and serum anti-HA responses to *ca* reassortants with varies surface Ags

Virus inoculum	Surface Ags	$NP_{366-374}$-specific CTL response (% of total lung $CD8^+$ T cells)	$PA_{224-236}$-specific CTL response (% of total lung $CD8^+$ T cells)	Serum HI GMT
CR6	H3N2	1.55	2.10	67.27
CR6-35	H1N2	1.03	2.06	25.14
CR35	H1N1	2.22	1.06	29.72
CR6-35*	H3N1	0.62	0.67	47.57

Table 5.3 B cell cross-reactions to different HA subtypes in C57BL/6 and BALB/c mice

Experiment setup[a]	Specific-IgA ASCs $/10^6$ lung lymphocytes		Specific-IgG2a ASCs $/10^6$ lung lymphocytes		Serum HI GMT	
	C57BL/6	BALB/c	C57BL/6	BALB/c	C57BL/6	BALB/c
H1-H1	86	142	44	70	60.63	113.14
H1-H3	76	192	49	71	0	0
H3-H3	25	46	21	32	40	64.98
H3-H1	25	49	18	28	0	0

[a] Six 6-8 week-old female C57BL/6 and BALB/c mice were inoculated twice at an interval of 3 weeks and lung and serum samples were prepared after 7 days. Virus inocula were CR35 (an H1 virus) or CR6-35* (an H3 virus). ELISPOT plates were coated with either homologous- or heterologous viruses.

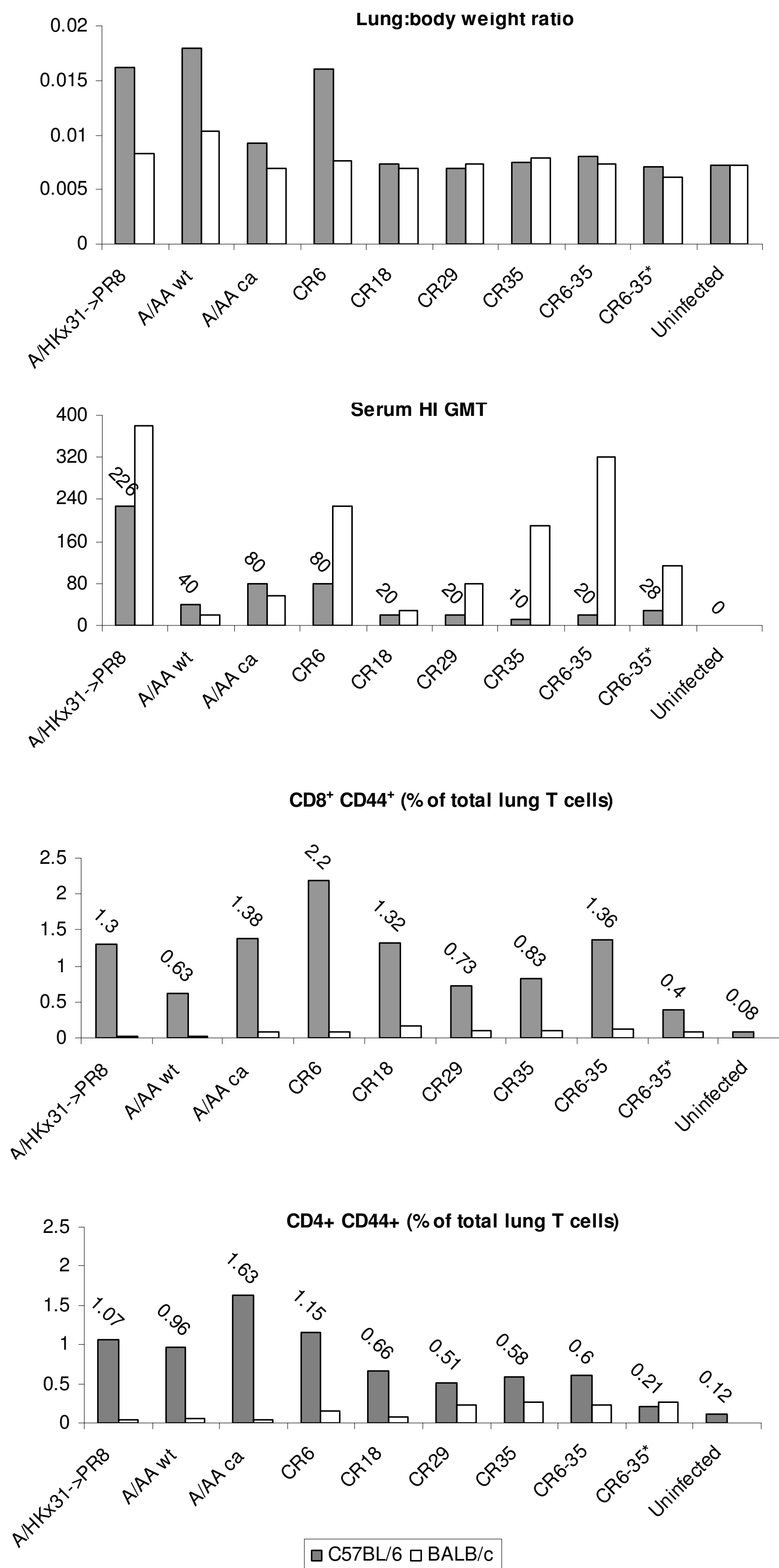


Figure 5.6 Comparison of immune responses in C57BL/6 and BALB/c mice. Two of 6-8 week-old female C57BL/6 or BALB/c mice from each group were inoculated twice at an interval of 3 weeks. Lungs were harvested and pooled 1 week after the 2nd dose for T cells phenotype analysis. Individual sera were prepared for HI tests.

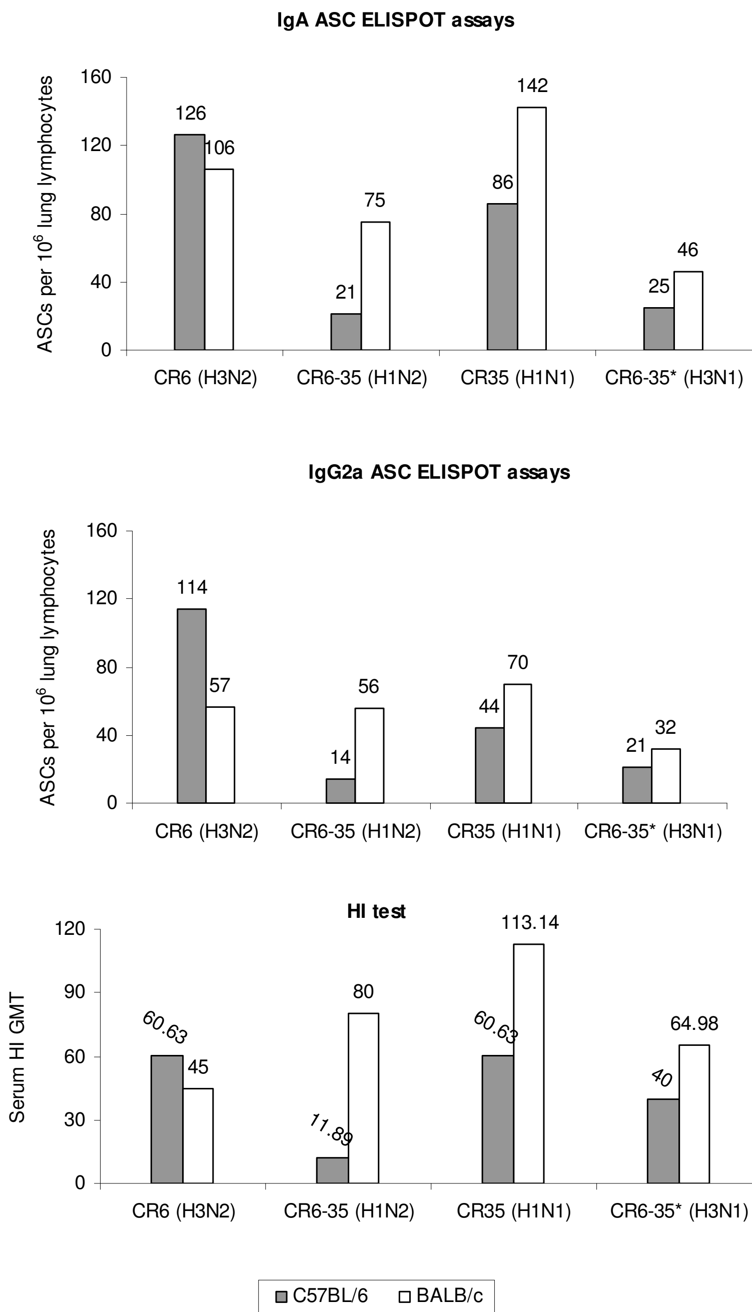


Figure 5.7 Comparative studies of local specific-ASC responses in C57BL/6 and BALB/c mice to selected *ca* reassortants. Six 6-8 week-old female C57BL/6 or BALB/c mice from each group were inoculated twice at an interval of 3 weeks. Lungs were harvested 1 week after the 2nd dose and cell suspensions were prepared individually for ASCs ELISPOT assays. Individual sera were prepared at the same time for HI tests.

The results for phenotyping of virus-specific T cells indicate that, overall, a much stronger T cell response was induced in the lungs than in the spleens following inoculation by the i.n route (Figure 5.4). No NP-specific CTL could be detected in the spleens of mice from all, except the tetramer control group. Furthermore, after secondary infection, the CD4:CD8 T cell ratios in the lung parenchyma of all infected mice remained at 1:1 (Figure A.3). Of the *ca* LAIVs, CR6 induced the strongest CTL response (Figure A.1). An increase of CD44 expression on T cells was observed in all infected groups but was absent in the uninfected control group (Figure 5.4 & A.3). Except for the CR29 group, the increase of CD44 on T cells occurred more in the lungs than in the spleens (Figure 5.4).

In addition to the lung T cell responses, serum HA-specific Ab levels for each group were determined by HI assays. The highest HI GMT (320) was detected in the tetramer control group and the lowest (20) in the A/AA *ca*-infected group. Other infected groups showed smaller increases (80 for the A/AA *wt*- and CR6-infected groups; 40 for the CR18-, CR29- and CR35-infected groups; Figure 5.4).

An additional T cell phenotype analysis was undertaken after 12 months with other preparations of the same viruses and two additional *ca* reassortants derived by co-infection with CR6 and CR35 (Tannock et al 1995) - CR6-35 (H1N2) and CR6-35* (H3N1). The viruses used in the second experiment were prepared following an additional passage and differences were noted in the PFU:HAU ratios of the same viruses used in the first experiment (Table 5.1). In the second experiment, the NP-specific CTL responses to A/AA *ca*-, CR6- and CR35-infected mice differed from those observed in the first experiment (Figure 5.5 & A.2). Both A/AA *ca* and CR35 induced larger NP-specific CTL populations (2.09 and 2.92% of total lung lymphocytes) than had been observed previously (0 and 0.9%). By contrast, a weaker CTL response was induced by CR6 (0.34 compared with 3.8% in the first experiment). However, levels of NP-specific CTL responses induced by the other viruses were similar in both two experiments (Table 5.1).

To further investigate the role of surface Ags in the local CTL responses to *ca* reassortants, mice were inoculated with two pairs of reciprocal *ca* reassortants CR6 (H3N2), CR6-35 (H1N2), CR35 (H1N1) and CR6-35* (H3N1) (i.e the only difference between the two viruses within each pair was the surface Ag HA gene; the remaining 7 genes were the same). Eight 6-8-week old female C57BL/6 mice in each group were administered two identical doses containing of 1.45×10^5 PFU of each virus, at an interval of 3 weeks by the i.n. route. At day 7 after the second dose, single cell suspensions and sera were prepared for each mouse and flow cytometry and HI assays were performed on all samples.

In the flow cytometry assay, lymphocytes samples from individual lungs were detected for $PA_{224-236}$- and $NP_{366-374}$-specific CTLs. As mentioned earlier (Table 1.3), peptide $PA_{224-236}$ of influenza A viruses, together with $NP_{366-374,}$ are immunodominant epitopes for C57BL/6 mice. The results indicate that there were no significant differences in the CTL responses induced by CR6 and CR6-35, when H3N2 or H1N2 viruses were compared (two sample *t*-test *P*-values > 0.05; Table 5.2). By contrast, higher responses (two sample *t*-test $P < 0.05$) was detected in the CR35 (H1N1)- than the CR6-35* (H3N1)-infected group (Table 5.2).

5.2.2 Relative immunogenicity of *ca* reassortants in C57BL/6 and BALB/c mice

MHC Class I or II molecules on the surface of APCs are responsible for the initiation of adaptive immune responses (Section 1.2.1.5). Different mouse strains carry different types of MHC molecule i.e H-2^d for BALB/c mice; H-2^b for C57BL/6 mice. Furthermore, different types of MHC molecules recognise different epitopes derived from the same Ag. Earlier studies demonstrated that no epitopes could be detected for the influenza NA protein of A/HK×31 in C57BL/6 mice, but multiple epitopes could be identified for the same protein in BALB/c mice (Crowe et al 2006). Therefore, immune responses to the same Ag in different hosts could be expected to differ. In the following experiments, viral infectivity (measured by changes to

lung:body weight ratios following infection) and immune responses (determined by serum HI tests and lung T cell phenotyping) to range of influenza viruses were compared in C57BL/6 and BALB/c mice.

Results for lung:body weights, indicated that C57BL/6 mice were more susceptible to A/HK×31, A/PR8, A/AA *wt*, A/AA *ca* and CR6 than BALB/c mice, but no differences were observed for the other *ca* viruses (Figure 5.6). In general, the levels of serum HI Ab to *ca* reassortants were higher in BALB/c than C57BL/6 mice. However, lower $CD8^+$ $CD44^+$ and $CD4^+$ $CD44^+$ T cell numbers were found in the lungs of BALB/c, compared with C57BL/6 mice, 7 days after secondary inoculation (Figure 5.6).

The ASC ELISPOT assay was used to detect local B cell responses to CR6 (H3N2), CR35 (H1N1), CR6-35 (H1N2) and CR6-35* (H3N1) in both C57BL/6 and BALB/c mice. Six 6-8 week-old female mice from each group were inoculated twice at an interval of 3 weeks and individual lung and serum samples were then prepared after 7 days. The results indicate a good correlation between local B cell and serum Ab responses (Figure 5.7). CR6 induced higher local B cell and serum Ab responses in C57BL/6 compared with BALB/c mice. However, for the other viruses (CR35, CR6-35 and CR6-35*), higher responses were obtained in BALB/c mice.

5.2.3 Heterosubtypic specific immunity induced by *ca* LAIVs in mice

Cross-immunity mediated by memory T and B cells to different influenza A subtype viruses has long been recognised (see Section 1.2.2.2). In the present study, B-cell based cross-immune responses between the CR35 (H1N1) and CR6-35* (H3N1) infected mice were demonstrated (Table 5.3). Six 6-8 week-old female C57BL/6 or BALB/c mice from each group were inoculated twice at an interval of 3 weeks and lung and serum samples of each were then prepared. ELISPOT plates were coated with either homologous or heterologous viruses. For

CR35-infected mice, lung lymphocytes were co-cultured *in vitro* with either CR35 (an H1 virus) or CR6-35* (an H3 virus). Lung lymphocytes, isolated from CR6-35* (H3N1)-infected mice were tested against both H3 virus and H1 viruses. The results indicate extensive local cross-reactivity in B cell responses to different HA subtypes in both C57BL/6 and BALB/c mice (Table 5.3). By contrast, no cross-HI reactivity could be detected.

5.3 DISCUSSION

Influenza A reassortant viruses in *ca* LAIVs approved by the FDA have common internal genes (NP, M, P, and NS) derived from the donor strain A/AA /6/60 *ca* and surface Ag genes (HA and NA) derived from current epidemic strains. Previous studies in outbred mice demonstrated that such reassortants are less immunogenic than *wt* parental strains with the same surface Ags (Tannock et al 1984). In mouse challenge/protection studies, viral surface HA Ags were identified as major determinants of immunogenicity for all reassortants (Tannock et al 1995). However, the immunological basis for these observations has not been investigated. In the present study, the interaction between the two arms of the adaptive immune system in the induction of responses to *ca* reassortants was studied in two inbred mouse strains (C57BL/6 and BALB/c). Specific immune responses were assessed by quantitation of lung virus-specific CTLs and ASCs by tetramer staining and ELISPOT assays. Results obtained were compared in parallel with serum antibody responses.

5.3.1 Specific humoral and CMI responses to *ca* reassortants in C57BL/6 mice

5.3.1.1 Local and systemic humoral responses induced by CR6, CR18, CR29 and CR35

Earlier challenge/protection studies in outbred mice demonstrated that the *ca* reassortant CR6 is a superior immunogen to CR35 since its 50% protective dose (PD_{50}) after administration of two identical vaccinating doses was $10^{1.17}$ $TCID_{50}$, compared with 10^{3} $TCID_{50}$ for CR35 (Tannock et

al 1995; Table 3.1). These results indicated that only 10 infectious units of this H3N2 reassortant were sufficient to induce a protective response in outbred mice whereas, for the H1N1 reassortant, 1,000 units were required. In the same study, a direct relationship was observed between serum HA-specific Ab levels induced by *ca* reassortants and their PD_{50}s CR6 induced highest HI responses (GMT 243) and CR35 the lowest (64).

These results were partially supported by results from ELISPOT studies in inbred C57BL/6 mice in the current study. GMTs for sera prepared days 7 and 9 after secondary inoculation (Figure 5.2) indicate that HA-specific Ab levels in the CR6-infected-group (GMT 64) were higher than for the CR29- and CR35-infected groups (20 and 36). However, the lung ELISPOT data indicates that CR35 elicited stronger IgA- and IgG2a-ASC responses at day 7 than CR6 (i.e. 2 days earlier than for the other reassortants; Figure 5.1). This observation was unexpected, given that CR35 grows more slowly than the other *ca* reassortants (Figure 3.3).

Unexpected lung IgA ASC and serum Ab responses to CR35 and A/HK×31 A/PR8 were also observed in comparative studies (Figure 5.3). Results showed that CR35 induced a strong IgA ASC response (189 per 10^6 lung lymphocytes) but a weak serum Ab response (HI GMT 24.37). By contrast, a strong serum Ab response occurred in the tetramer control group (320 when tested against A/HK×31), but a relatively weak IgA ASC response in the lungs (53 or 75 per 10^6 lung lymphocytes when tested against A/HK×31 and A/PR8, respectively). The reason for the weak local ASC responses observed in tetramer control group can be explained by the fact that only primary B cell responses were being measured. In the cross-priming protocol, maximum T cell responses could be induced since each inoculum had distinct surface Ags (e.g. A/HK×31 H3N2 and A/PR8 H1N1). Nevertheless, after secondary inoculation, CR35 induced strong ASC responses but a small increase in serum HI Ab (Figure 5.1 & 5.3). A possible explanation for this phenomenon could be that an enhanced mucosal ASC response after second inoculation was possible because of low levels of pre-existing immunity from the primary inoculation. Further

evidence came from an earlier experiment using a single dose (Chapter 3; Figure 3.5), in which CR35 induced a weak serum Ab response (HI GMT of 10 at week 3 p.i.). Low specific-Ab levels would probably result in a reduced capacity to neutralise virus from the secondary inoculation. Similar results have been observed with the killed subunit influenza vaccines. From clinical trials, vaccinees with initially low antibody levels responded better than those possessing higher levels, leading to the conclusion that there was little additional benefit from the second dose of vaccine (Feery et al 1976).

Influenza-specific IgA and IgG play important roles in the control of influenza infections in the URT and LRT, respectively (Chapter1; Section 1.2.2.1). In addition, the induction of IgG2a is closely related to the secretion of Type I cytokines (e.g IFN-γ, IL-2 and TNF-α) by T_H1 cells. From the current study, the highest IgG2a ASC response was found in CR18-infected mice (Figure 5.2). This was not unexpected, considering that CR18 is the only *ca* reassortant studied that possessing a *wt* NS gene whose product is an IFN inhibitor (Garcia-Sastre 2001). Overall, lung ELISPOT results demonstrated that *ca* LAIVs induced higher superior IgA ASC responses, in comparison with IgG2a at days 7 or 9 p.i. (Figure 5.1). Similar observations had been reported in studies using different *ca* donor strains (Wareing and Tannock 2003). High local mucosal IgA responses are required for the control of influenza, especially in the URT and provide full protection against re-infection (Chapter1; Section 1.2.2.1).

In general, serum Ab responses to the same *ca* reassortant in C57BL/6 mice were lower than in outbred mice by a factor of 7.5. The reasons for this could be related to mouse genotype and/or the size of virus inocula used or to the inherent variability that occurs between experiments performed at different times. However, a direct correlation between the local and systemic humoral or protective responses was observed for all *ca* reassortants, except for CR35.

5.3.1.2 Local and systemic CMI responses induced by ca reassortants

Previous studies with different *ca* donor strains demonstrated that T_H1/T_H2 cytokine responses and an increase in $CD4^+$ or $CD8^+$ T cells occurred primarily in the lungs, the primary site of infection. (Wareing et al 2001 & 2005). In the present studies, T cell immune responses to a range of *ca* reassortants derived from A/AA/6/60 *ca* following i.n. administration were investigated, for the first time, in C57BL/6 mice by tetramer staining and T cell phenotyping techniques. T cell responses in spleens were studied in parallel.

Results from tetramer staining demonstrated that the *ca* reassortants CR6, CR18, CR29 and CR35 induced a detectable NP-specific CTL response in mouse lungs on day 7 after secondary infection (Figure 5.4). However, no CTL responses could be detected in the spleen after infection with any of the reassortants. Similar observations were made in the T cell phenotyping study. Here $CD8^+$ $CD44^+$ and $CD4^+$ $CD44^+$ T cell populations were higher in the lungs than in the spleens following i.n. inoculation with CR6, CR18 and CR35. However, for CR29-infected mice, these cell populations were higher in the spleens than in the lungs.

$CD8^+$ $CD44^+$ and $CD4^+$ $CD44^+$ T cell populations in the spleens of CR29-infected mice were higher than for mice infected with the other *ca* reassortants studied, accounting for 6.7 and 7.4% of total spleen lymphocytes (Figure 5.4). Interestingly, CR29 induced the lowest NP-specific CTL response in lungs (1.9% of total lung $CD8^+$ T cells) compared with 17.27, 5.07 and 3.55% for CR6, CR18 and CR35, respectively. Furthermore, the lung ASC and serum HI Ab responses to CR29 were lower in comparison with the other three *ca* reassortants (Figure 5.2). The exact reason for the lack of stimulation in lung by CR29 remains unknown, but it is likely to be associated with its growth characteristics. An indication of this, reported in Chapter 3, was the capacity of CR29 to produce minute plaques in MDCK cells compared with other viruses (Figure 3.2).

Further variability was observed in lung NP-specific CTL responses to CR6, CR35 and A/AA *ca* during a later experiment (Figure 5.4&5.5). CR6 induced a high CTL response (3.8% of total lung lymphocytes were NP-specific CTLs) in the earlier experiment, but a lower response (0.34%) in the later experiment (Table 5.1). For CR35 and A/AA *ca*, 0.9 and 0.5% of the same cells were induced in the first experiment and 2.92 and 2.09% in the second. Differences in the inocula used for the same virus in both experiments could be a cause of such variability. Influenza virus stocks contain variable proportions of infectious particles, non-infectious particles and DIPs (Chapter Section 1.1.3). Non-infectious particles and DIPs could be expected to induce lower CTL responses. Differences in the PFU:HAU ratio were noted in the same virus preparations used in the two experiments (Table 5.1). The variability presented in current studies indicates that factors, such as properties of the viruses used, should be taken into consideration when comparing results between experiments.

5.3.1.3 Interaction between local and systemic, humoral and CMI responses to ca reassortants

During the course of an influenza infection, the serum Ab response is proportional to the extent of viral replication in the LRT (Chapter 1; Section 1.2.2.3). Therefore, lower serum Ab responses could be expected from *ca* reassortants because of their restricted capacity to replicate in the LRT (Figure 5.3). In the present study, anaesthetised C57BL/6 mice were infected by the i.n. route with *ca* and *wt* viruses and the serum Ab and lung ASC responses were compared.

No direct correlation between serum Ab and local ASC responses was observed. Infection with the H1N1 reassortant CR35 resulted in a low serum Ab response (HI GMT 36) but a high IgA ASC response in the lungs ($366/10^6$ lung lymphocytes; Figure 5.2). For the H3 *ca* reassortant CR6, the serum Ab response was higher (57) but the IgA ASC response ($283/10^6$) was lower. High serum Ab levels were observed in the A/HK×31 A/PR8-infected group, (GMT 320

when tested against A/HK×31), but IgA and IgG2a ASC responses were much lower (53 and $1/10^6$, respectively; Figure 5.3).

Virus stocks of CR35 that were used in two experiments had PFU:HAU ratios lower than those of the other viruses. Stocks of A/HK×31 had the highest ratio (approximately 10-fold greater than for CR35; Figure 3.1). These results suggest that the stimulation of local ASC and serum Ab responses is influenced by the kinetics of viral replication in the lungs. High-yielding viruses, such as CR6 or A/HK×31, generally induce higher serum Ab responses than low-yielding viruses, such as CR35, although the latter was capable of inducing good local ASC responses.

All four *ca* reassortants elicited detectable NP-specific CTL responses in the lungs. However, NP-specific CTLs could not be detected in the spleens of infected C57BL/6 mice (Figure 5.4), which confirms the previous observation that most specific-T_{EM} cells found in the airways and lung parenchyma (BALT) are a direct consequence of primary infection (Ely et al 2003).

An inverse relationship appeared to exist between local NP-specific CTL and serum HA-specific Ab responses. For the CR35-infected group, low serum Ab and high lung NP-CTL responses were induced by day 7 after secondary infection (Figure 5.5; Table 5.3). For the CR6-infected group, higher serum Ab but lower local NP-CTL responses were detected. Both CR6 (H3N2) and CR35 (H1N1) were derived from same *ca* donor strain A/AA/6/60 *ca* and the differences in their growth characteristics appears to result from differences in their surface Ags.

A further study with two additional reassortants (CR6-35 H1N2 and CR6-35* H3N1) that were derived by co-infection with CR6 and CR35 confirmed the role of surface Ags in the induction of CTL responses. When the HA gene of CR35 was replaced by H3, the PFU:HAU ratio of the resulting CR6-35* reassortant increased from 2.28 to 6.83×10^3 (Chapter 3; Figure 3.1) and an

increase in serum HI GMT from 30 to 48, and a decrease in the lung specific-CTL response (Table 5.2). However, increases in the specific-CTL responses also occurred in the presence of N2 (i.e. CR6 H3N2 and CR6-35 H1N2). Taken together, the efficiency of viral replication at the site of infection appears to be a key factor in determining the relative contributions of local and systemic, humoral and systemic responses in the control influenza.

5.3.2 Immune responses to *ca* reassortants in C57BL/6 and BALB/c mice

MHCs vary considerably and are present on the APCs of different hosts. As a consequence, the immune responses to the same *ca* reassortants in different strains of inbred mice could be expected to differ. In an earlier study, virus-specific IgG responses were investigated in C57BL/6, BALB/c and CBA/CaH mice following i.n. infection with CR6 (Hocart et al 1989). After two i.n. doses, an IgG2a dominant response was detected in the serum, lungs and salivary secretions of BALB/c mice. For C57BL/6 mice, virus-specific IgG1 was dominant in the serum but no dominant subclass could be detected in lung and salivary secretions. In the present study mucosal IgA responses in different strains of mouse are described for the first time.

A range of influenza A viruses, including six *ca* reassortants, was administered twice by the i.n. route to both C57BL/6 and BALB/c mice and lung specific-ASC (IgA and IgG2a), T cell and serum Ab responses were compared. In addition the extent of lung consolidation caused by different viruses was compared in parallel to determine endpoints. Results indicate that C57BL/6 mice were more susceptible than BALB/c mice to CR6-induced lung consolidation (Figure 5.6; top panel). No consolidation was observed for the remaining five *ca viruses* in either strain of mice.

An inverse relationship between humoral and CMI responses was observed. In C57BL/6 mice, all *ca* reassortants elicited higher serum Ab but lower T cell responses than in BALB/c mice

(Figure 5.6). These results suggest that (1) BALB/c mice are more likely to develop Type II (B cell) responses and (2) C57BL/6 mice are more likely to develop Type I (CTL) responses against *ca* reassortants. These observations were confirmed in ELISPOT studies, in which higher lung IgA- or IgG2a responses were detected in the BALB/c than C57BL/6 mice in response to all viruses, except CR6. From earlier measurements of lung:body weight ratios, C57BL/6 mice were shown to be more susceptible to CR6 than BALB/c mice (Figure 5.6), resulting in higher local ASC responses (Figure 5.7).

5.3.3 Cross-reactive B- and T- cell responses induced by *ca* reassortants

Tannock and Paul (1987) demonstrated that significant cross-immune responses to all H3N2 and H1N1 *wt* viruses were present 6-8 weeks after the administration of mice with *ca* LAIVs. Other more recent studies have indicated that memory CTLs in the MALT are associated with local protection against lethal heterosubtypic challenge (Nguyen et al 1999). Local T_{EM} have a half-life of about 40 days (Woodland 2002). Viral internal Ags, especially the highly conserved NP and PA protein antigens, have a significant role in the development of heterosubtypic immune responses by influenza viruses (Yewdell et al 1985; Bennink et al 1986 & 1987)

In the present studies, the tetramer developed for A/PR8 (H1N1) was also able to detect an NP-specific CTL population induced by A/AA *ca* (H2N2) and other reassortants of A/AA *ca* (Figures A.1 & A2). This finding is consistent with a recent study, in which CR29-primed mice survived a lethal challenge of A/PR8 (Powell et al 2007). In the present study lung ASC ELISPOT data revealed that local B cell can also promote such responses against H1 and H3 viruses (Table 5.3). However, B cell heterosubtypic immunity is also likely to be mediated by common epitopes present in the Ags of different of NA and HA subtypes. Overall, i.n. immunization with *ca* LAIVs i.n. could provide short-term protection against both *shift* and *drift* variants of influenza A viruses.

5.4 SUMMARY

Results from this chapter indicate that humoral and CMI immune responses were induced by *ca* LAIVs and that these responses were both local and systemic. These responses were variable and were influenced by both viral and host factors. Type I (CTL) responses were induced by low-yielding *ca* reassortants with reduced growth characteristics, such as CR35 (H1N1). Viruses with enhanced growth characteristics, such as CR6 (H3N2), produced higher Type II (HA-specific Ab) responses. In addition host factors, such as the MHC type, were found to play important roles in responses to the same viruses. Susceptible mouse strains, such as C57BL/6 showed higher CTL but lower serum Ab responses than more resistant strains, such as BALB/c. The efficiency of viral replication at the site of inoculation appears to be a key factor in determining the relative contributions of local or systemic and humoral or cellular immune responses to *ca* LAIVs

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Appendix: Statistics and Flow Cytometry Analysis

Table A.1. Normality test statistical analysis distribution of C57BL/6 mice lung specific IgA or IgG2a ASC responses to various *ca* LAIVs after two identical i.n inoculation doses

Group		N	Mean	StDev	SE Mean	*P*-Value
A/AA *ca*	**IgA**	**6**	**385**	**366**	**149**	**>0.15**
	IgG2a	6	98	121	NA	0.04
CR6	**IgA**	**6**	**283**	**320**	**131**	**>0.15**
	IgG2a	6	78	99	41	>0.15
CR18	**IgA**	**6**	**329**	**142**	**58**	**>0.15**
	IgG2a	6	195	196	80	>0.15
CR29	**IgA**	**6**	**103**	**130**	**53**	**>0.15**
	IgG2a	6	14	28	12	>0.15
CR35	**IgA**	**6**	**336**	**139**	**57**	**>0.15**
	IgG2a	6	64	41	17	>0.15

Table A.2. Two-sample *t*-test statistical analysis of differences between two groups of C57BL/6 mice for lung specific IgA, IgG2a ASC and serum Ab responses to ca LAIVs after two identical i.n doses

P-value of two-sample t-test for **IgA** data:

	CR6	CR18	CR29	CR35
A/AA ca	0.622	0.74	0.13	0.77
CR6		0.76	0.25	0.73
CR18			**0.018**	0.94
CR29				**0.015**

P-value of two-sample t-test for **IgG2a** data:

	CR6	CR18	CR29	CR35
A/AA ca	0.76	0.33	0.16	0.54
CR6		0.23	0.17	0.76
CR18			0.075	0.17
CR29				**0.037**

P-value of two-sample t-test for **HI titre** data:

	CR6	CR18	CR29	CR35
A/AA ca	0.91	0.82	**0.027**	0.56
CR6		0.91	**0.050**	0.65
CR18			0.071	0.73
CR29				0.12

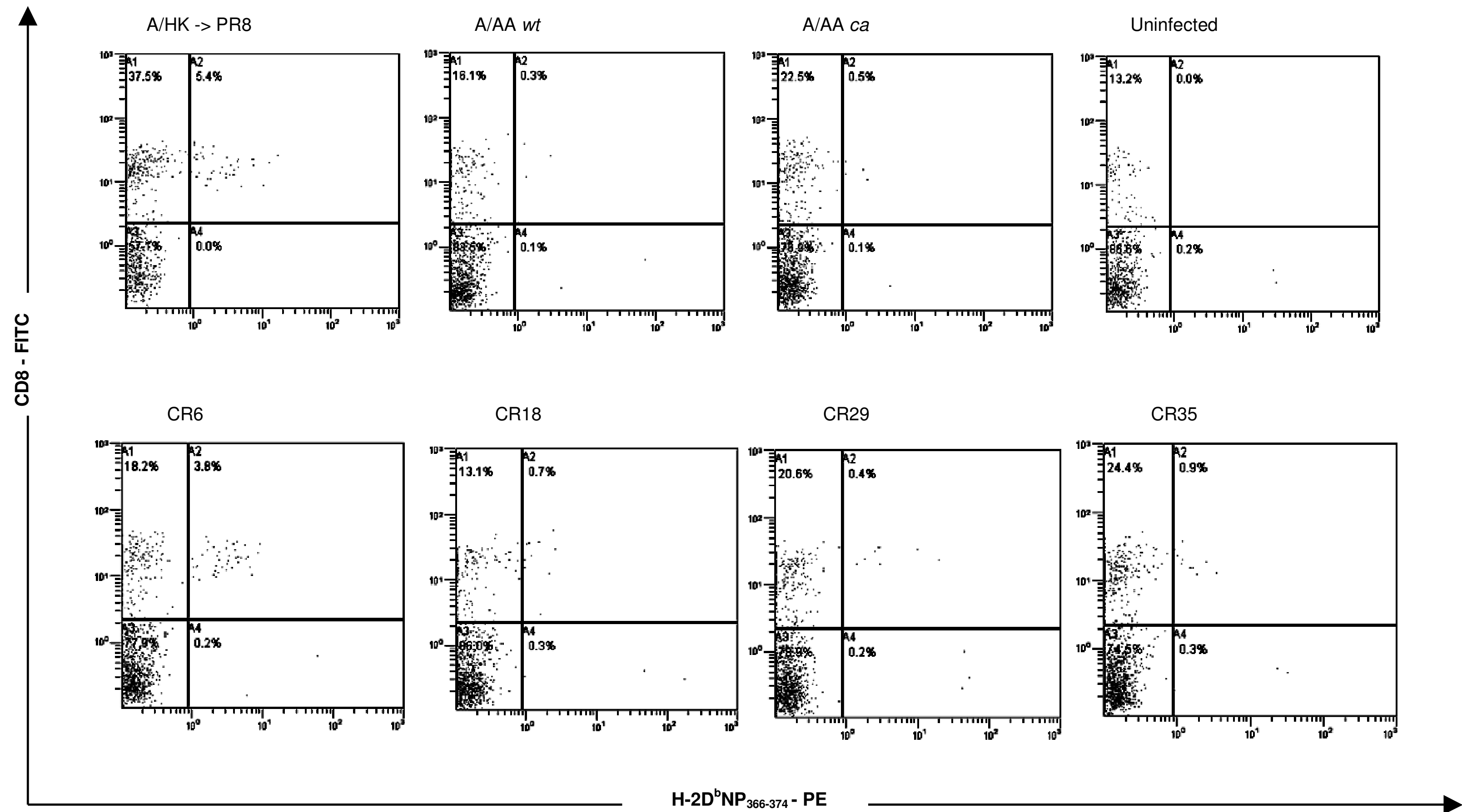

Figure A.1 Flow cytometry analysis for lung influenza $NP_{366\text{-}374}$-specific T cell responses to different influenza viruses (gated on lymphocytes, Experiment I). Four 6-8 week-old female C57BL/6 mice from each group were inoculated twice at an interval of 3 weeks. One week after the 2nd dose, lung single cell suspensions were prepared and pooled for each group. Tetramer staining and flow cytometry assays were performed to measure NP-specific CTLs.

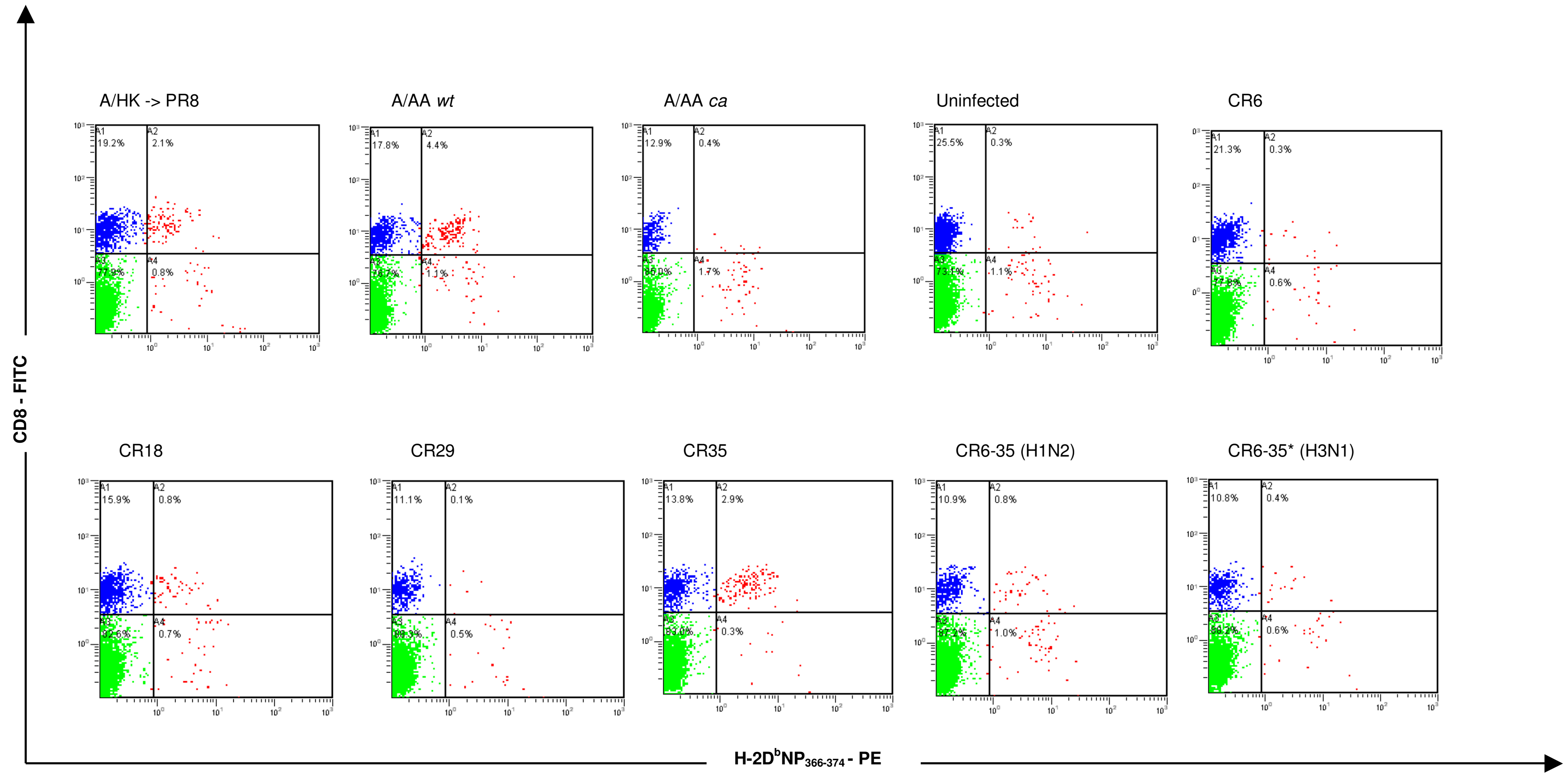

Figure A.2 Lung influenza $NP_{366\text{-}374}$-specific T cell responses to different influenza viruses (gated on lymphocytes; Experiment II)

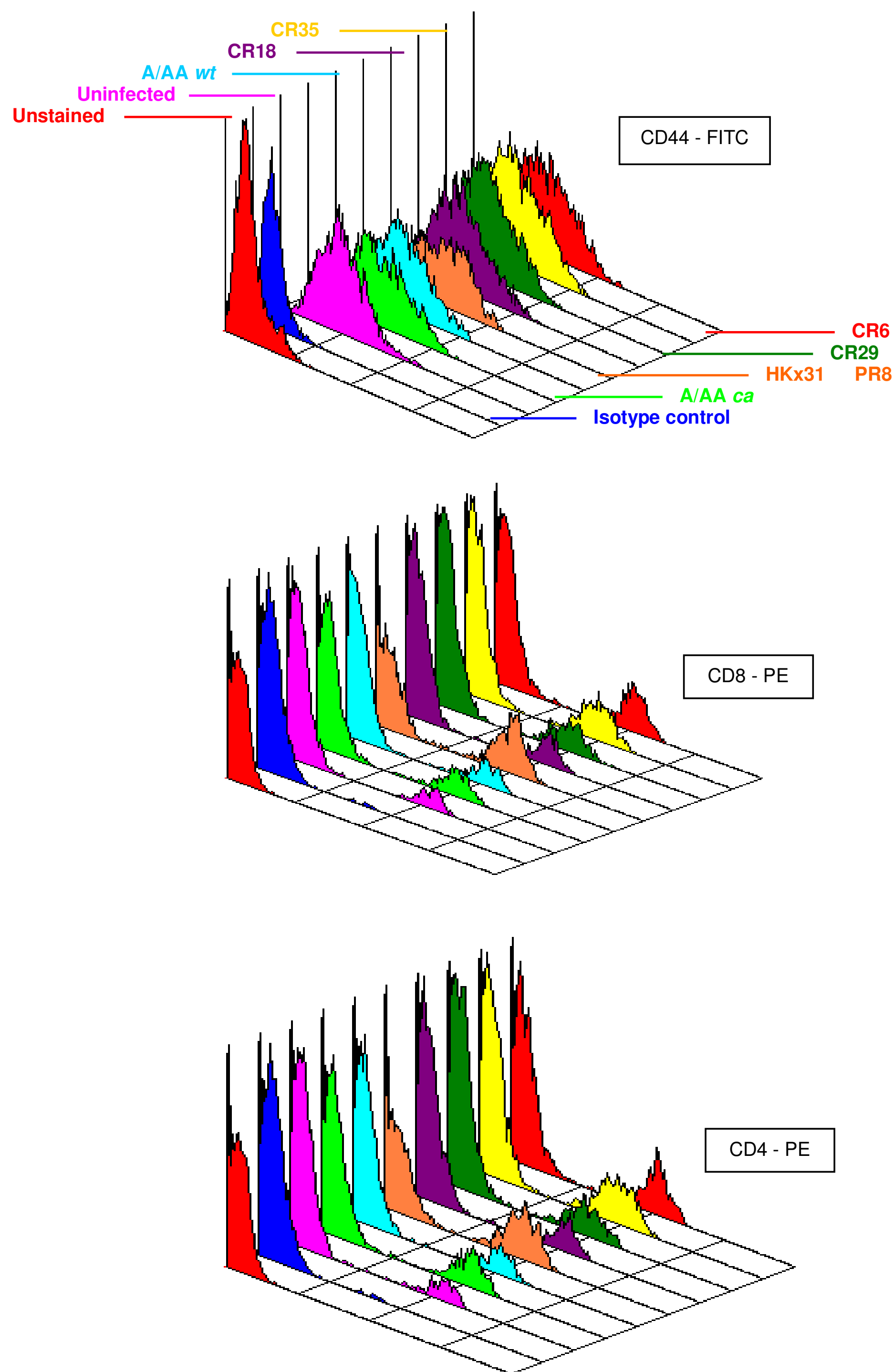

Figure A.3 Overlay plots of mice lung lymphocytes showing different cell populations that stained with CD44-FITC, CD8-PE or CD4-PE at day 7 after secondary inoculation with different viruses. Cell samples were collected from the same mouse study as Figure A.1.