Immune responses to inactivated influenza viruses

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Statement

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All other experiments described in this thesis represent my own work and were done by me.

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Abbreviations

-/- knockout
aa amino acid
APC antigen presenting cell
A/PC A/Port Chalmers/1/73
A/PR8 A/Puerto Ricko/8/34
aSFV avirulent SFV
ß2m ß2-microglobulin
BCR B cell receptor
BHK baby hamster kidney
CRBC chicken red blood cell
DC dendritic cell
DMEM Dulbecco’s modified Eagle’s medium
dsRNA double stranded RNA
EMEM Eagle’s minimum essential media with Earle’s salt
E:T effector to target ratio
FACS fluorescence-activated cell sorting
FCS fetal calf serum
FITC fluorescein isothiocyanate (fluorescent dye)
HA hemagglutinin
HAU hemagglutination unit
HIV human immunodeficiency virus
IFN interferons
IFN-I type I interferon
IFN-IR interferon-type Iα receptor
IFN-I&IIIR interferon type I and II double receptors knockout
IFN-II type II interferon
IFN-IIIR interferon-gamma receptor
IFN-γ interferon gamma
Ig immunoglobulin
IL-1 interleukin-1
i.n. intranasal
<table>
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<tr>
<th>Abbreviation</th>
<th>Full Form</th>
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<tbody>
<tr>
<td>INA</td>
<td>iodonaphyl-azide</td>
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<tr>
<td>IRF</td>
<td>Interferon regulatory factor</td>
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<tr>
<td>i.p.</td>
<td>intraperitoneal</td>
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<tr>
<td>i.v.</td>
<td>intravenous</td>
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<tr>
<td>kGy</td>
<td>kilogram</td>
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<tr>
<td>LCMV</td>
<td>Lymphocytic Choriomeningitis virus</td>
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<tr>
<td>MA</td>
<td>matrix protein</td>
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<tr>
<td>MDA5</td>
<td>melanoma differentiation-associated gene 5</td>
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<tr>
<td>MDCK</td>
<td>madin darby canine kidney</td>
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<tr>
<td>MHC</td>
<td>major histocompatibility complexes</td>
</tr>
<tr>
<td>MHC-I</td>
<td>MHC class I</td>
</tr>
<tr>
<td>MHC-II</td>
<td>MHC class II</td>
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<tr>
<td>MID_{50}</td>
<td>50% mouse infectious dose</td>
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<tr>
<td>MLD_{50}</td>
<td>50% mouse lethal dose</td>
</tr>
<tr>
<td>MyD88</td>
<td>myeloid differentiation primary response gene 88</td>
</tr>
<tr>
<td>NA</td>
<td>neuraminidase</td>
</tr>
<tr>
<td>NF-κB</td>
<td>nuclear factor kappaB</td>
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<tr>
<td>NK cell</td>
<td>natural killer cell</td>
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<tr>
<td>NLR</td>
<td>nod like receptor</td>
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<tr>
<td>NP</td>
<td>nucleoprotein</td>
</tr>
<tr>
<td>PB</td>
<td>polymerase basic protein</td>
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<tr>
<td>pDC</td>
<td>plasmacytoid dendritic cell</td>
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<tr>
<td>Perf</td>
<td>perforin</td>
</tr>
<tr>
<td>PFU</td>
<td>plaque forming unit</td>
</tr>
<tr>
<td>PI</td>
<td>post-immunization</td>
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<tr>
<td>PRR</td>
<td>pattern recognition receptor</td>
</tr>
<tr>
<td>RIG-I</td>
<td>retinoic acid inducible gene-I</td>
</tr>
<tr>
<td>SFV</td>
<td>Semliki Forest virus</td>
</tr>
<tr>
<td>S.D</td>
<td>standard deviation</td>
</tr>
<tr>
<td>TIV</td>
<td>trivalent influenza vaccine</td>
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### Abbreviations

(continued)

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>TLR</td>
<td>Toll-like receptor</td>
</tr>
<tr>
<td>TNF</td>
<td>Tumour necrosis factor</td>
</tr>
<tr>
<td>ssRNA</td>
<td>single stranded RNA</td>
</tr>
<tr>
<td>Tc cell</td>
<td>cytotoxic T cell</td>
</tr>
<tr>
<td>TCID50</td>
<td>50% tissue culture infectious dose</td>
</tr>
<tr>
<td>TCR</td>
<td>T cell receptor</td>
</tr>
<tr>
<td>Th cell</td>
<td>T helper cell</td>
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<tr>
<td>Th1</td>
<td>T helper type 1</td>
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<tr>
<td>Th2</td>
<td>T helper type 2</td>
</tr>
<tr>
<td>μMT</td>
<td>immunoglobulin μ-chain</td>
</tr>
<tr>
<td>WHO</td>
<td>World Health Organization</td>
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<tr>
<td>WTGR</td>
<td>129Sv/Ev</td>
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Abstract

In this thesis, I examined immunological responses induced by three different inactivated influenza A viruses: formalin-treated, ultraviolet- or gamma ray-irradiated virus. Both innate and adaptive immune responses were explored, with special emphasis on their importance in protective efficacy.

The mouse model of influenza clearly showed that a single dose of non-adjuvanted gamma-inactivated influenza virus administered intranasally confers robust cross-protection in mice against both seasonal and H5N1 avian influenza infections. A comparison of different inactivated virus preparations revealed that gamma-irradiation maintains the immunogenicity of influenza virus better than formalin or UV-inactivation methods. The superior protective efficacy of gamma-inactivated virus is demonstrated by: 1) heterosubtypic immunity that lasts for at least three months; 2) cross-protection against up to 50 x 50% mouse lethal dose (MLD50); 3) the lower requirement for the immunization dose; and 4) reduced pulmonary viral loads following heterosubtypic infections.

The induction of cross-protective immunity by gamma-inactivated influenza virus correlated with that of Tc cell responses, but neither of the responses were present in mice immunized with formalin- or UV-inactivated influenza virus preparations. Standard cytotoxicity assays revealed that upon intranasal influenza challenges mice pre-immunized with gamma-inactivated virus had earlier pulmonary Tc cell responses than naïve mice. The mechanism responsible for the observed cross protection was further investigated using various approaches: use of gene knockout mice defective in molecules of various adaptive immunity pathways, adoptive lymphocyte transfers, and passive serum transfers. Results from these studies point to Tc cells as a central player in the cross-protective immunity induced by gamma-inactivated influenza viruses.

Viral infections induce systemic partial lymphocyte activation, characterized by up-regulation of cell surface markers CD69 and CD86. However, unlike Semliki Forest virus, influenza viruses that are fully inactivated by any of the three different inactivation methods maintain their ability to induce partial lymphocyte activation. In Chapter 5, I describe experiments aimed at examining the cause of partial lymphocyte activation as a result of vaccination with inactivated influenza viruses with special emphasis on the role of Toll-like receptors in this process. Various gene knock-out mouse models revealed that in vivo partial lymphocyte activation induced by inactivated
influenza virus is highly dependent on type I interferon. The kinetics of the observed \textit{in vivo} partial lymphocyte activation correlated with the ability of the inactivated virus preparations to induce type I IFN responses. The biological significance of type I IFN dependent partial lymphocyte activation is at present not clear.

Collectively, data presented in this thesis shows superior immunogenicity of gamma-irradiated influenza virus over to conventional formalin- or UV-inactivated virus preparations. Gamma-ray irradiation may represent a simple and effective approach to prepare a universal influenza vaccine.
Chapter 1

Background Overview

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   1.2.2 Adaptive immunity

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1.4 Influenza vaccines
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1.1 Introduction

One of the most important milestones in the history of medical science is vaccination (Bland and Clements 1998; Bonanni 1999). A number of deadly infectious diseases have disappeared from some parts of the world or brought under control due to worldwide active vaccination programmes, such as the eradication of smallpox. In addition, vaccination is the most cost effective and efficient mean to combat infectious diseases. With the aid of our growing understanding of the human immune system and recent breakthroughs in molecular biology, vaccination targets have spread from infectious to non-infectious diseases such as cancer, autoimmunity and allergy, although development of these vaccines are still in very preliminary stages.

Emerging or re-emerging infection is defined as “infections that have newly appeared in a population or have existed previously but are rapidly increasing in incidence or geographic range” (Morse 1995). Pathogens such as H5N1 influenza virus, SARS, HIV, multi drug-resistant tuberculosis, drug resistant malaria, dengue, yellow fever, E.coli 0157:H7, plague and cholera are examples of emerging or re-emerging pathogens with significant burden of diseases worldwide.

Influenza, in particular, is a great concern. Epidemics of highly pathogenic avian influenza strain H5N1 in poultry and sporadic human infections have raised a great concern of the next influenza pandemic. In fact, despite availability of influenza vaccines since 1940s, influenza virus continues to circulate among humans and to cause seasonal infections and, sporadically, pandemics. Influenza virus mutates and readily escapes the strain-specific antibody responses directed against the surface glycoproteins hemagglutinin and neuraminidase. Their highly variable antigenicity raises a fear that virulent strains may emerge in a human population and cause the Spanish flu-like pandemic. Despite the massive effort of many researchers, a universal flu vaccine remains an illusive target. In fact, current vaccine companies still employ the conventional techniques of growing influenza virus in the allantoic fluid of embryonated hen’s eggs followed by chemical inactivation of purified virus. Unfortunately, this methodology is not well suited to produce vaccine for highly pathogenic strains, e.g. H5N1, due to the technical difficulties. Moreover, given that there is a 6 months lag time between identification of the virus and the availability of
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the first doses of vaccine (reviewed in (Gerdil 2003)), there is a clear need for an alternative approach.

The use of whole virus inactivated by gamma-irradiation may represent the alternative approach to current influenza vaccines. The possibility of using gamma-irradiated influenza virus as a cross-protective vaccine requires a clear understanding of the antiviral immune responses, the emergence of pandemic influenza, the limitation of currently available influenza vaccine and the requirement for a cross-protection, and the consequences of using gamma-irradiation as a mean of sterilisation.

1.2 Immune responses to viral infections

1.2.1 Innate immunity

The first line of defence against viral infections is often referred to as the innate immune response. Three main characteristics of innate immunity are; 1) lack of antigen-specificity; 2) rapid activation; 3) and lack of immunological memory. Innate immunity involves complex interactions and co-ordinating actions of the cells and molecules to eliminate or delay the growth of invading pathogens. The importance of innate immunity has been shown in a number of mice models where deficiencies in one or more components of innate immunity lead to fulminant viral disease (Levy 2001; White, Wang et al. 2001; Lobigs, Mullbacher et al. 2003).

Viral recognition by pattern recognition receptors

Host immune cells possess a number of receptors for sensing viral genomes, replication intermediates and glycoproteins. These receptors are called pattern-recognition receptors (PRRs) which include the Toll-like receptors (TLRs) (Diebold, Kaisho et al. 2004), the Nod-like receptors (NLRs) (Kanneganti, Body-Malapel et al. 2006), retinoic acid inducible gene-I (RIG-I) (Kato, Sato et al. 2005), melanoma differentiation-associated gene 5 (MDA5) (Siren, Imaizumi et al. 2006), dsRNA-dependent protein kinase (Williams 2001) and the DNA receptor DAI (Takaoka, Wang et al. 2007). These PRRs differ in their cellular localization, ligand specificity and
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downstream signalling pathways. It is becoming clear that the immune system does not rely on one detection pathway for innate viral recognition but possess a certain degree of redundancy to counteract viral immune evasion (if the virus evolves to circumvent one pathway or another). Thus, the immune system detects more than one viral component, stimulating two or more pathways that may act in a synergistic manner so as to augment the induction of the innate immune response.

TLRs are the most studied PRRs and comprise a family of at least 15 receptors, and the microbial ligands recognized by each of TLR have mostly been identified. With respect to respiratory viral infections, TLR-3, -7, -8 and -9 detect nucleic acid. TLR-3 is involved in recognition of double stranded RNA (dsRNA) intermediates of viral replication (Alexopoulou, Holt et al. 2001) while TLR-7 and -8 detects viruses that contain single stranded RNA (ssRNA) (Diebold, Kaisho et al. 2004; Heil, Hemmi et al. 2004; Lund, Alexopoulou et al. 2004). Unmethylated CpG sequences of viral DNA are recognized by TLR-9. TLR-3, -7, -8 and -9 are predominantly expressed in the late endosomal compartment. This cellular location serves two purposes: first, it allows detection of viruses that have entered the cell through endocytosis; second it allows avoidance of detecting host cell-derived genome. Other TLRs that are expressed on the cell surface, such as TLR-2 and -4, have been associated with viral glycoproteins (Kurt-Jones, Popova et al. 2000; Bieback, Lien et al. 2002).

While endosomal TLRs are present only in specialized cells, cytosolic sensors exist in almost all cells of both innate immune and non-immune cells. Two cytosolic RNA helicases, RIG-I and MDA5, mediate cytosolic recognition of actively replicating RNA viruses through detection of double stranded RNA (reviewed in (Yoneyama, Onomoto et al. 2008)). RIG-I was recently shown to be activated by single stranded viral genomic RNA bearing a 5' triphosphate end (Hornung, Ellegast et al. 2006; Pichlmair, Schulz et al. 2006), allowing the early detection of ssRNA viruses even before ssRNA viruses begin to generate replication intermediates.

Early inflammatory responses

The detection of virus through PRRs initiates a cascade of signal transduction pathways that ultimately results in activation of key transcription factors such as nuclear factor kappaB (NF-kB) (reviewed in (Liang, Zhou et al. 2004)) and interferon
regulatory factor (IRF) (reviewed in (Paun and Pitha 2007)). These transcription factors play an important role in transcriptional up-regulation of genes encoding pro-inflammatory cytokines and chemokines. The major pro-inflammatory cytokines of the innate immune response induced by viral infections include tumour necrosis factor (TNF), type I IFN (IFN-I), interleukin-1 (IL-1), IL-6, IL-12 and IL-18. Proinflammatory cytokines, together with chemokines, promote recruitment of innate immune cells such as neutrophils, monocytes and NK cells to a site of infection (Sprenger, Meyer et al. 1996). These cells, in turn, participate in the secretion of chemokines and cytokines to augment the inflammatory response (Banchereau and Steinman 1998; Eloranta and Alm 1999; Cooper, Fehniger et al. 2001). Some of these cytokines have direct antiviral roles or are involved in regulating innate and adaptive immunity (reviewed in (Ramshaw, Ramsay et al. 1997)).

Among numerous cytokines produced during the early inflammatory response, IFN-I are key players in innate immune responses due to its multi-immunoregulatory functions (reviewed in (Le Bon and Tough 2002)). Most cells can produce IFN-I subtypes, IFN-α and IFN-β, within a few hours of viral infections (Eloranta, Sandberg et al. 1997; Rodel, Groh et al. 1998; Celia, Jarrossay et al. 1999). IFN-I are antiviral cytokines that can inhibit one or more steps in the viral replication cycle, thereby limiting the extent of the viral spread (Isaacs and Lindenmann 1957; Sen and Ransohoff 1993). They are also involved in the recruitment of inflammatory cells to the site of infections and in activation of those cells to enhance their effector functions (Gallucci, Lolkema et al. 1999; Swann, Hayakawa et al. 2007). IFN-I can work in both auto- and paracrine fashion, leading to stimulation of neighbouring cells to secrete more IFN-I. This positive feedback loop by signalling through the IFN-α/β receptor results in high levels of IFN-I and heightened antiviral states (Levy, Marie et al. 2002).

**Innate immune responses lead to better adaptive immune responses**

An increasing body of evidence suggests that IFN-I not only provide a first line of defence, but also act as a link between the innate and adaptive immune response, facilitating the antigen specific responses (reviewed in (Kadowaki and Liu 2002; Tough 2004; Alsharifi, Mullbacher et al. 2008)). For example, IFN-I foster maturation of antigen presenting cells (Luft, Pang et al. 1998) and prolong the survival of T cells
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(Marrack, Kappler et al. 1999), thereby promoting generation of adaptive immune responses. In particular, the effect of IFN-I on APCs is important. IFN-I induces up-regulation of molecules involved in antigen presentation, such as MHC molecules, and co-stimulation molecules, such as CD40, CD80 and CD86 (Luft, Pang et al. 1998; Gallucci, Lolkema et al. 1999). Functionally matured APCs will efficiently activate CD4\(^+\) helper T cells through engagement of TCR and MHC-II. Activated CD4\(^+\) helper T cells will in turn play an important role in regulating adaptive immunity. For the above mentioned immunoregulatory role of IFN-I, adjuvants, such as TLR ligands, that can induce IFN-I responses have gained increasing attention in vaccinology.

1.2.2 Adaptive immunity

Unlike innate immunity, adaptive immune responses are highly antigen specific and holds immunological memory; two properties exclusive to lymphocytes. T and B lymphocytes are the constituents of adaptive immunity. Their specificity comes from their unique receptors on the cell surface specific to particular antigens. During lymphocyte development, a unique genetic mechanism generates a diverse repertoire of B cell receptors and T cell receptors from a small number of genes. Thus, although each lymphocyte expresses an antigenic receptor of single specificity, the lymphocyte receptor repertoire ensures that there is an antigenic specific lymphocyte for any given pathogen encountered during a lifetime. Upon binding antigen, the cells with an appropriate receptor specificity undergo clonal expansion; activation, proliferation and differentiation into effector cells to fight against infections. Some of the differentiated cells are maintained at higher frequency than their precursors and these memory cells are responsible for protection against secondary challenge.

**Cellular immunity**

Cellular adaptive immune responses are largely dependent on T lymphocytes. T lymphocytes are divided into two major subsets based on their cell surface markers, CD4\(^+\) and CD8\(^+\) T cells. CD8\(^+\) T cells function to eliminate the invading intracellular pathogens by directly killing the infected cells or secreting antimicrobial cytokines,
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whereas CD4+ T cells play an important role in orchestrating and directing immune responses. Thus CD4+ helper T cells and CD8+ cytolytic T cells have distinct but complementary functions.

**CD4+ helper T cells: activation via MHC-II**

Activation of naïve T cells involves complex processes. In the case of CD4+ helper T cells, the activation commences with an interaction between T cell receptor (TCR) and antigen-derived peptides bound to a major histocompatibility complex (MHC) class II molecules. Only so-called professional APCs, such as dendritic cells, macrophages and B cells, express MHC class II molecules. These professional APCs take up extracellular antigens by endocytosis and thus antigens enter the exogenous antigen presentation pathway. The engulfed proteins are enzymatically processed in the endosomal compartment and the generated short peptides (9-15 aa in length) bind to MHC class II proteins for export to the cell surface (Ramachandra, Song et al. 1999). The recognition of a cognate MHC class II-peptide complex by CD4+ helper T cells represents the first of at least two signals necessary for full activation. The second signal is provided by co-stimulatory molecules, B7 molecules on APCs and CD28 on lymphocytes (Green, Noel et al. 1994). Without the second signal, T cells become anergized and this is the fail-safe mechanism that prevents activation of self-reacting T cells (Schwartz 1990). The activation of naïve CD4+ helper T cells will lead to differentiation into at least two functionally distinct effector subpopulations, which are T helper 1 (Th1) and T helper 2 (Th2) cells. The ratio of Th1/Th2 population will be determined by the cytokine milieu, antigen presentation site, physical form and dose of antigen, and type of adjuvant (reviewed in (Del Prete 1998)).

**CD4+ helper T cell: effector functions**

The primary function of CD4+ T cells is to provide help for other immune effector cells, hence the name helper T cells. CD4+ T cells exert their helper function by either secretion of immunoregulatory soluble cytokines or direct interaction with other immune cells. Through these two mechanisms CD4+ T cells regulate B cell and CD8+ T cell responses.
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The classification of Th1 and Th2 subsets is based on the spectrum of cytokines that they secrete following activation. Th1 cells produce mainly IL-2, IFN-gamma, and TNF, whereas Th2 cells secrete IL-4, IL-5, IL-6, IL-10, and IL-13 (Fiorentino, Bond et al. 1989; Sornasse, Larenas et al. 1996). The balance of cytokines or "help" provided by Th1 and Th2 cells influences the type of immune response to be generated. For example, Th1 skewed responses are associated with cellular immune responses optimal for elimination of intracellular pathogens. In contrast, Th2 skewed responses are generally associated with development of humoral immune responses. Th2 responses are often seen in infections with extracellular pathogens, such as bacteria and parasites. Th2 cells are also implicated in allergy (Parronchi, Macchia et al. 1991).

In addition to secretion of distinct cytokines by Th1 and Th2 cells, both cell subsets share a common ability and that is to provide help for antigen-specific B cell expansion and antibody production. Abundant evidence suggests that T cell help for B cells is mediated through CD40-CD154 interaction (Noelle, Roy et al. 1992; Van den Eertwegh, Noelle et al. 1993; Brooks, Hamilton-Easton et al. 1999). Activated CD4⁺ helper T cells with correct TCR specificity can interact with B cells presenting antigens on MHC class II molecules. As a result of this first interaction, the B cells express CD40 on its surface and if the helper T cell expresses CD154 (CD40-ligand), further interactions can take place, leading to activation of B cells. This CD40-CD154 costimulation interaction is also used to indirectly stimulate CD8⁺ effector T cells (Bennett, Carbone et al. 1998; Ridge, Di Rosa et al. 1998; Schoenberger, Toes et al. 1998). CD154 on CD4⁺ helper T cells can engage with CD40 on DC resulting in activation or "licensing" of DC. The licensed DC in turn has an important role in sustaining or enhancing the effector function of CD8⁺ T cells. In addition to immunoregulatory functions, CD4⁺ helper T cells are also capable of cytolysis through perforin and/or FasL under a certain condition (Tite and Janeway 1984; Tite, Powell et al. 1985; Ju, Strack et al. 1988).

An important role of CD4⁺ T cells in maintaining CD8⁺ T cell effector functions during chronic viral infections has been clearly demonstrated in HIV patients, where loss of CD4⁺ T cells correlates with CD8⁺ T cell dysfunction and progression to AIDS progression (Klein, van Baalen et al. 1995; Klein, van der Burg et al. 1998; Kostense, Vandenberghe et al. 2002). This observation has been corroborated in several mouse models of chronic viral infections. For example, deficiency in CD4⁺ T cells significantly reduces LCMV-specific CD8⁺ Tc cell responses, leading to persistent...
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LCMV infections (Matloubian, Concepcion et al. 1994). Similarly, in the absence of CD4* help, immune system fails to control chronic murine gammaherpes virus infections (Ehtisham, Sunil-Chandra et al. 1993). The importance of other CD4+ T cell functions, i.e. secretion of Th1 cytokines and CD4+ T cell dependent antibody responses, in resistance to viral infections has been demonstrated for influenza (Eichelberger, Allan et al. 1991), varicella zoster virus (Maloy, Burkhart et al. 1999; Maloy, Burkhart et al. 2000) and vaccinia virus (Spriggs, Koller et al. 1992).

Several new subsets of helper T cells have been identified in recent years: Th17, T follicular helper (Tfh) and regulatory T (Treg) cells. Th17 cells, as name suggests, are a producer of IL-17 (Park, Li et al. 2005). An accumulating body of evidence strongly suggests that Th17 associated cytokines are involved in a number of autoimmune diseases such as multiple sclerosis (Matusevicius, Kivisakk et al. 1999), rheumatoid arthritis (Kirkham, Lassere et al. 2006), psoriasis (Krueger, Langley et al. 2007), inflammatory bowel disease (Duerr, Taylor et al. 2006). In addition, through production of IL-22 and IL-23, Th17 cells participate in anti-microbial immunity. IL-22, together with IL-17, induces antimicrobial peptides in keratinocytes (Aujla, Chan et al. 2008) and are essential for mucosal host defences (Huang, Na et al. 2004; Happel, Dubin et al. 2005; Aujla, Chan et al. 2008).

Tfh cells are a subset of CD4+ helper T cell cells that provide B cell help in the B cell follicles. High constitutive expression of B cell follicle homing receptor CXCR-5 on Tfh cells (Bryant, Ma et al. 2007) allows them to migrate into the B cell follicles, which are abundant in chemokine CXCL13 (Ansel, Ngo et al. 2000). Tfh cells are thought to exert their B cell help through secretion of IL-4, IL-10 and IL-21 that facilitate the survival, proliferation and differentiation of B cells (Schaeerli, Willimann et al. 2000; Kim, Rott et al. 2001; Bryant, Ma et al. 2007; Reinhardt, Liang et al. 2009). In addition to production of B cell-promoting cytokines, Tfh are characterized by high expression of inducible T-cell stimulator (ICOS) (Hutloff, Dittrich et al. 1999), programmed cell death 1 (PD1) (Haynes, Allen et al. 2007), and the transcriptional repressor B cell lymphoma 6 (BCL-6) (Chtanova, Tangye et al. 2004).

Treg are distinguished from other subsets of CD4 T cells by its immune suppressive role. Since its first discovery, a number of Treg associated immune suppressive functions has been described: induction of oral tolerance against dietary antigens (Chen, Kuchroo et al. 1994; Weiner 1997); induction of maternal tolerance to the fetus (Aluvihare, Kallikourdis et al. 2004), establishing and maintaining
immunologic self-tolerance (Sakaguchi, Sakaguchi et al. 1995; Wang, Song et al. 2007), suppression of pathogen-induced immunopathology (Asseman, Fowler et al. 2000; Hori, Carvalho et al. 2002); modulating effector class of the immune response (Alpan, Bachelder et al. 2004; Matzinger 2007). The mechanism/s behind the immunosuppressive function of Treg is still not fully understood. However, production of inhibitory cytokines such as IL-10 and transforming growth factor β (TGF-β) has been implicated in Treg immunosuppressive functions (Powrie, Carlino et al. 1996; Nakamura, Kitani et al. 2001).

**CD8⁺ T cells: activation via MHC-I**

Priming of CD8⁺ T cells occurs in response to cytosolic infections by intracellular pathogens. The antigens derived from intracellularly replicating pathogens enter endogenous antigen presentation pathway: the pathogen-derived antigens are processed within the cytosol by proteasome (Heemels and Ploegh 1995) and small peptides are loaded onto MHC I molecules in the endoplasmic reticulum to form MHC class I-peptide complexes (Klein and Sato 2000), which are then transported to the cell surface through the Golgi apparatus (Spiliotis, Manley et al. 2000). CD8⁺ T cells recognize these peptides bound to MHC class I molecules. Essentially all nucleated cells express MHC class I molecules and thus are able to process and present antigens to CD8⁺ T cells for activation (Steinmetz and Hood 1983). This also means that effector CD8⁺ T cells can detect to intracellular pathogens in most tissues. Recent studies suggest that DCs are able to cross present extracellular antigens on MHC class I molecules (Bevan 1976; Bevan 1976; Pletneva, Fan et al. 2009).

**CD8⁺ cytotoxic T cells: effector functions**

The name-giving effector function of Tc cell is their ability to induce cytolysis of infected cells through direct contact. Tc cells employ two distinct pathways for target cell killing: the granule exocytosis or the Fas-mediated pathways (Kagi, Vignaux et al. 1994; Lowin, Hahne et al. 1994; Clark, Walsh et al. 1995). Granule exocytosis is considered to be the primary mechanism utilized by Tc cells for killing virus-infected
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cells. The lytic granules of activated T cells carry the pore-forming molecule perforin and apoptosis inducing granzyme. The synergistic action and coordinated delivery of both perforin and granzymes mediates efficient target cell lysis (reviewed in (Trapani and Smyth 2002)). In contrast, the Fas mediated pathway involves ligation between cell death receptors Fas on the target cell and up-regulated Fas ligand on the effector CD8+ T cells (Fujimoto, Takizawa et al. 1998). This Fas-Fas ligand engagement induces a cascade of signalling events, which ultimately results in caspase-dependent apoptosis (Itoh, Yonehara et al. 1991).

CD8+ T cells are also cytokine producers. Effector CD8+ T cells secrete mainly TNF and IFN-gamma, antiviral cytokines that may directly interfere with intracellular pathogen replication (Paliard, de Waal Malefijt et al. 1988; Kuwano, Kawashima et al. 1993; Lohman and Welsh 1998). Effector CD8+ T cells also secrete chemokines that function, together with cytokines, to recruit and activate innate immune cells such as macrophages and neutrophils (Harty and Bevan 1999). Both cytolytic effector mechanisms and cytokine productions are tightly regulated through TCR-dependent signals.

The requirement of CD8+ T cells in viral clearance has been extensively studied by various approaches: acute in vivo CD8+ T cell depletion, use of mice deficient in genes encoding for molecules involved in cytolytic pathways, and adoptive CD8+ T cell transfer. These studies have clearly demonstrated that CD8+ T cells are one of the major mediators of viral clearance, although the required effector functions vary for each viral infection (Walsh, Matloubian et al. 1994; Kagi, Seiler et al. 1995; Doherty 1996).

T cell memory

The aim of vaccination is to generate immunological memory that can be rapidly recalled upon “first” infectious challenge. Memory immunity provides protection by generating more rapid and stronger adaptive immune responses. In contrast to antibody-based vaccines, effective T cell-based vaccines have not been developed to date, maybe due to a limited knowledge of how cellular memory is generated and maintained. Medically important infectious diseases of 21th century (e.g. HIV, malaria, tuberculosis) are often intracellular pathogens that require T cell immunity for pathogen clearance and, to a lesser extent, protection against infections. Thus, advancements in our
understanding of cellular memory are urgently required in order to help develop T cell based vaccine.

The quality of the cellular memory response is determined by three immunological factors: 1) frequency 2) functional phenotype; and 3) persistence of memory T cells. Memory T cells are derived from the effector T cell pool that was generated following initial antigenic stimulation (Opferman, Ober et al. 1999). These memory CD8⁺ T cells are known to exist in higher frequency, than their naïve precursors (Hou, Hyland et al. 1994), and the magnitude of antigen specific T cells reflects the frequency of effector response. The size of the memory T cell pool is thought to be dependent on the initial burst size, which in turn dependents on the amount of antigen at the time of initial activation (Oehen, Waldner et al. 1992; Hou, Hyland et al. 1994). More recent data suggests that other factors such as the cytokine milieu are also critical for activation of naïve T cells and their subsequent differentiation into memory T cells (Curtsinger, Schmidt et al. 1999; Schmidt and Mescher 1999; Curtsinger, Valenzuela et al. 2005; Mescher, Curtsinger et al. 2006).

Memory CD8⁺ T cells exist as a functionally heterogenous population. Memory T cells comprise of two major subpopulations: resting and effector cells. The resting memory T cells are characterized by the expression of CD62L (L-selectin)high and CCR7 and primarily circulate through the blood and secondary lymphoid organs (Dailey, Fathman et al. 1982; Arbones, Ord et al. 1994). These cells require restimulation to re-acquire effector functions but possess high proliferative capacity. Upon encounter with specific antigen in lymph nodes, resting memory cells rapidly generate large effector cell populations. By contrast, effector memory cells are CD62Llow and CCR7⁻ and reside in peripheral tissues, spleen and blood, monitoring these for their specific pathogen (Sallusto, Geginat et al. 2004). Effector memory cells can rapidly produce effector cytokines and mediate direct cytolytic activity ex vivo (Ehl, Kleenerman et al. 1997; Murali-Krishna, Lau et al. 1999; Opferman, Ober et al. 1999). Little is known about the contribution of the two subsets in providing protection against infectious diseases. In the case of LCMV (lymphocytic choriomeningitis virus), resting memory T cells are more effective than effector memory T cells at controlling the infections (Wherry, Teichgraber et al. 2003).

The requirement for antigen to sustain cellular memory has been a topic of debate. It was initially thought to require continuous or frequent re-exposure of antigens (Gray and Matzinger 1991; Kundig, Bachmann et al. 1996; Bachmann, Kundig et al. 2006).
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In contrast to the traditional view, more recent data consistently suggest that memory CD4⁺ and CD8⁺ T cells are in fact long living in vivo in the absence of re-exposure to antigens (Lau, Jamieson et al. 1994; Swain 1994; Flynn and Mullbacher 1997; Tanchot, Lemonnier et al. 1997; Murali-Krishna, Lau et al. 1999; Swain, Hu et al. 1999; Crotty, Felgner et al. 2003; Hammarlund, Lewis et al. 2003; Amara, Nigam et al. 2004). Thus, antigen does not appear to be absolutely required to maintain memory CD4⁺ or CD8⁺ T cells, however, whether these persisting memory T cells can afford protective immunity without re-stimulation i.e. vaccine booster immunization is another question.

There is good evidence that the presence of CD4⁺ T cell help during primary activation is required for the generation and maintenance of optimal CD8⁺ memory cells (Shedlock and Shen 2003; Sun and Bevan 2003; Sun, Williams et al. 2004). The exact role of CD4⁺ helper T cells is not fully understood but signals such as IL-2 family cytokines, a potent T-cell growth factor (IL-7 and IL-15) (Prlic, Lefrancois et al. 2002; Tan, Ernst et al. 2002; Oh, Perera et al. 2008) or CD40 ligand for the licensing of DC (Harty and Badovinac 2008) have been shown to be involved. Cytokine dependent homeostatic proliferation allows long term survival of memory T cells after clearance of infections (Sprent, Cho et al. 2008). APCs such as DCs are main producers of IL-15, which can be induced by CD4⁺ T cells licensing (Oh, Perera et al. 2008) or by other cytokines such as IFN-1 (Waldmann and Tagaya 1999).

**Humoral immunity**

Humoral immunity is mediated by antibodies (also called immunoglobulins), which are produced and released into blood or mucosal surfaces by effector B cells called plasma cells. Antibodies are Y-shaped glycoproteins with two ends of variable regions and one end of more conserved region called Fc region. The two variable regions are the antigen binding arms whereas the constant region binds to the Fc receptors of immune cells such as neutrophils and phagocytes. On the basis of constant regions, antibodies can be divided into 5 major subtypes: IgG, IgA, IgM, IgD, and IgE. Each of these isotypes has distinct biological roles and initiate appropriate antimicrobial pathways to clear the pathogens (reviewed in (Heinzel 2000)).
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**B cells: activation via MHC-II**

Naïve B cells can be activated in a T cell-dependent (reviewed in (Parker 1993)) or -independent manner (reviewed in (Vos, Lees et al. 2000)). T cell-dependent activation resembles the two-signal model of naïve T cell activation. The first signal is through the antigen receptor. Naïve B cells have surface-bound IgM receptors which bind to a specific antigen. The antigen-antibody complex is internalised, the antigen is degraded, and then peptides from the antigen are presented on the MHC class II molecules on the cell surface. The second signal is from the antigen specific CD4+ helper T cells. As described previously in “CD4+ helper T cells: activation via MHC-II” section, antigen presenting DCs interact with and stimulate CD4+ helper T cells. The activated CD4+ helper T cells, in turn, engage with B cells through TCR-MHC class II and CD40-CD154 interactions. These two interactions constitute the second signal and induce differentiation of naïve B cells into effector antibody secreting plasma cells. The functional phenotype of plasma B cells, antibody isotype and magnitude of antibody production, are determined by the CD4+ helper T cell derived cytokines, such as IL-2, IL-4, IL-5, IL-6, and IL-10 (Snapper, Peschel et al. 1988; Croft and Swain 1991; Croft and Swain 1991; Purkerson and Isakson 1992; Paul and Seder 1994).

Pathogens with multiple repetitive structures can activate B cells in the absence of MHC class II-restricted T-cell help and are called T-independent antigens (Vos, Lees et al. 2000). Antigens such as bacterial polysaccharides activate B cells by causing extensive cross-linking of B cell receptors. A distinctive feature of T-cell independent activation is that the generation of antigen-specific memory B cells is insufficient. Consequently, even repeated immunization induces poor memory antibody responses. This has been a problem for the vaccine development against polysaccharide encapsulated bacterial pathogens, such as *Haemophilus influenzae*, *Streptococcus pneumoniae*, and *Neisseria meningitidis* (Gold, Lepow et al. 1977; Borgono, McLean et al. 1978).

**B cell functions**

Antiviral activities of antibodies are mediated by a number of mechanisms. The most important function of antibody is the neutralization of invading pathogens.
Antibodies of a correct specificity will bind directly to a virus particle and inhibit virus attachment to host cells or interfere with the viral entry process. In addition, antibody can recruit other components of the immune system to destroy virus particles: complement-mediated virolysis and phagocytosis (reviewed in (Spear, Hart et al. 2001)). Virus infected cells are also a target for antibodies. Antibodies bound to the viral antigens expressed on the infected cells can inhibit the viral replication (Fujinami and Oldstone 1979; Levine, Hardwick et al. 1991), the release of viruses from infected cells (Gerhard 2001) and the cell-cell transmission of viruses (Pantaleo, Demarest et al. 1995). Furthermore, the Fc region of antibody mediates antibody-dependent cellular cytotoxicity (Fuson, Hubbard et al. 1983) or complement-dependent cytotoxicity (Tamerius and Hellstrom 1974). Two studies demonstrated an additional mechanism by which antibodies can directly neutralize intracellular viruses present inside the cells (Mazanec, Kaetzel et al. 1995; Bomsel, Heyman et al. 1998). Mucosal polymeric IgA and IgM can gain an entry into infected epithelial cell by binding to the polymeric immunoglobulin receptor and, upon entry, can mediate the intracellular neutralization of viruses.

Historically, antibodies are thought to be more important than T cells in memory immune responses. Antibodies not only prevent viral infections by neutralization but also eliminate virus-infected cells, whereas Tc cells generally act after infection has been established. In support of this, acute in vivo depletion of both CD4+ helper and CD8+ T cells has shown that antibodies alone can efficiently protect mice against secondary viral infections (Kim, Flano et al. 2002; Edghill-Smith, Golding et al. 2005; Morrison and Morrison 2005). However, this conclusion neglects the importance of CD4+ helper T cells for humoral immunity development. Nonetheless, other traditional approaches, such as passive transfer of immune sera, have consistently shown the protective property of antibodies for many different viruses in animal models (Ritchie, Oakes et al. 1993; Gupta, Mahanty et al. 2001) and in humans (Chanock, Crowe et al. 1993).

In addition to memory responses, humoral immunity provides non-specific innate immune responses. Recent studies have demonstrated the existence of pre-immune neutralizing antibodies (Haury, Sundblad et al. 1997; Baumgarth, Herman et al. 1999). These "natural" antibodies can directly neutralize viruses through formation of immune complexes and activation of complements.
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B cell memory

B cell memory is characterised by the high titre and affinity of the rapidly emerging antibody response. B cell memory responses are generated by two subsets of memory B cells, which are called long-lived plasma B cells or memory B cells (McHeyzer-Williams and Ahmed 1999). Both of these B cell subtypes can persist for a lifetime (Manz, Thiel et al. 1997; Slifka, Antia et al. 1998) and possess high-affinity B cell receptor. Long-lived plasma B cells continuously produce antibodies to maintain minimum levels of protective antibodies (Manz, Thiel et al. 1997; Slifka, Antia et al. 1998) and these antibodies represent the first line of B cell memory responses against secondary infections. The second line of B cell memory responses is mediated by memory B cells that rapidly proliferate and differentiate into antibody secreting cells upon stimulation. Activated memory B cells secrete large quantities of high affinity antibodies that can neutralize the residual pathogens. Thus, B cell memory response is a two-step process.

B cell memory responses are highly effective in preventing secondary infection. In fact, most of the currently available vaccines mediate their protective efficacy through the generation of B cell memory. For this reason, the correlates of protection for most vaccines are measured in terms of antibody titres or neutralizing activity of serum or mucosal antibodies specific for a pathogen after vaccination (Robbins, Schneerson et al. 1995).

Memory B cells and long-lived plasma cells can survive for years, or even a lifetime (Manz, Thiel et al. 1997; Bernasconi, Traggiai et al. 2002). The maintenance of long-lived plasma cells appear to be independent of antigenic stimulation (Manz, Lohning et al. 1998), whereas the antigen requirement for the survival of memory B cells remains controversial (Gray and Skarvall 1988; Maruyama, Lam et al. 2000). More recent studies have shown that antibody responses to viruses persist longer than those against non-replicating antigens, suggesting the importance of antigenic stimulation for the continued production of antibodies (Amanna, Carlson et al. 2007). What is clear is that repeated immunization is required for the maintenance of optimum antibody levels (Ochsenbein, Pinschewer et al. 2000).
1.3 The Emergence of Influenza pandemic

1.3.1 General remarks

Despite significant progress in modern medicine, approximately 10 million deaths worldwide annually are still caused by infectious diseases (WHO 2004). This number does not include millions of additional deaths due to the complications of infections. Infectious diseases have become the primary cause of death among people under the age of 50 years. These statistics clearly show how vulnerable we still are to microorganisms despite the intensive research and considerable effort that has been put into to eradicate infectious diseases. In addition to existing infectious diseases, such as tuberculosis, malaria and HIV, newly emerging pathogens, such as avian influenza, are great concern due to their potential to cause pandemics.

The majority of existing microorganisms have evolved to co-exist with their natural hosts. New pathological infectious diseases arise when a symbiotic relationship is breached and results in infectious microorganisms jumping from their natural hosts to a new host. In fact, it was estimated that 75 percent of emerging human pathogens, mainly viruses, originate from animals (Taylor, Latham et al. 2001). Crossing the species barrier occurs because the zoonotic virus maybe capable of causing human infection without any prior adaptations, as seen for many pathogens such as *Borrelia burgdorferi* (Pal and Fikrig 2003), *rabies virus* (Belotto, Leanes et al. 2005), and *Monkeypox virus* (Ligon 2004), or because the pathogen’s host tropism has changed. In either case the requirement for a human infection to occur is, obviously, the human contact with the infected animals, carcasses, animal parts, or animal excreta. Thus, the principal cause of a pathogen emergence or re-emergence is human factors associated with an increased risk of exposure to pathogens. However, most of the initial infections caused by human-animal contact result in a dead-end infection because of the absence of human-to-human transmissions. A recent example of dead-end infections is avian influenza H5N1 infection. Avian strain H5N1 has not gained the ability to efficiently transmit between humans and therefore, although the number of sporadic cases of H5N1 has exceeded 400 (WHO 2009) it has not resulted in a pandemic. Thus pandemic is a two-step process: introduction of new pathogen into a human population and subsequent spread and maintenance of new pathogen within the population. Both of
these steps are a complex multifactorial process involving both human and viral factors (Morse 1993; Wilson 1993).

Many of the emerging or re-emerging viral diseases are caused by RNA viruses. Viruses that use RNA as their genetic material or as a replicative intermediate represent the most abundant group of subcellular pathogens. They have a wide host range and are often able to transmit between different hosts (Murphy 1996). For these reasons, my introduction of emerging pathogens will be focussed on RNA viruses with emphasis on influenza virus.

1.3.2 Viral factors

RNA genome variability

A quasispecies is a model developed by Manfred Eigen, P. Schuster and colleagues to model the Darwinian evolution of self-replicating entities (Eigen and Schuster 1977). Later, this concept has been applied to RNA viruses to describe the extreme heterogenic nature of a viral population within its host. Viral genome analyses by modern technologies, such as sequencing, fingerprinting and cDNA cloning, have shown that RNA viruses do not form a homogenous population but exists as a group of related genotypes (Domingo, Sabo et al. 1978; Domingo and Holland 1997). The quasispecies model has numerous biological implications for the RNA viruses. Having a huge reservoir of variants will ensure that at least one mutant with beneficial phenotype will survive in the face of an environmental change. Three fundamental mechanisms exist to continuously generate new viral variants: 1) point mutation, 2) recombination, and, where the viral genome is segmented 3) genome segment reassortment.

Point mutation

The major contributing factor for RNA viral genomic variability is the high rate of point mutation that occurs during RNA replication. The mutation rate of a virus is the probability of a particular nucleotide position to change during a single viral replication. As a result of a high RNA polymerase error rate and the absence of error correction
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(Steinhauer, Domingo et al. 1992; Modrich and Lahue 1996), RNA viruses have high point mutation rates of approximately $10^{-4}$ to $10^{-5}$. In comparison, DNA viruses have mutation rates of $10^{-8}$ to $10^{-11}$. As an example, influenza virus NS gene has a mutation rate of approximately $1.5 \times 10^{-5}$ (Parvin, Moscona et al. 1986) ~ $2 \times 10^{-6}$ (Nobusawa and Sato 2006) mutations per base pair per replication cycle during the growth of a single plaque in MDCK cells. Thus, assuming this mutation rates apply to a whole viral genome (size of 13600 base pairs), there will be 0.2 mutation per genome per replication, giving one mutant for every 5 genomes generated. The high mutability of influenza virus is responsible for a phenomenon called antigenic drift. Point mutations leading to amino acid substitutions in the hemagglutinin, which is a major target for neutralizing antibodies, results in new drift variants and seasonal influenza outbreaks due to insufficient immunity. The continuous antigenic changes impose a difficulty in development of an effective vaccine against influenza virus.

Recombination

RNA viruses have a capacity to exchange genetic material with one another by two processes: recombination and reassortment. These two processes are the major factor responsible for the emergence of new viral strains. Recombination is an exchange of nucleotide sequences from one virus to another, generating a hybrid RNA genome containing genetic information from two separate sources. In the past, the term RNA recombination has been used synonymously with RNA segment reassortment for influenza virus. Unlike positive-stranded RNA viruses such as the Corononaviridae, which have high recombination rates, influenza viruses are thought to lack the ability to undergo genetic recombination. However, recent studies have shown that the recombination can contributes to the emergence of highly pathogenic avian influenza strains from low pathogenic precursor. The genetic comparison of highly pathogenic and low pathogenic H7N3 viruses isolated in 2002 from chickens in Chile showed a 30-nucleotide insert in the hemagglutinin cleavage site of the highly pathogenic strain (Suarez, Senne et al. 2004). This conversion of a non-virulence strain to virulence has occurred as the result of recombination between the hemagglutinin and the nucleoprotein genes of the same virus. A similar event has been reported in British Columbia, where the recombination event between the hemagglutinin and matrix genes...
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has led to outbreak of highly pathogenic H7N3 virus in poultry (Pasick, Handel et al. 2005).

Reassortment

Reassortment is another mechanism by which viruses exchange their genetic material. Gene reassortment occurs when viruses with a segmented genome, such as influenza virus or reovirus, simultaneously infect a host cells with two different virus strains. This is a crucial evolutionary mechanism for a number of viruses. Influenza virus is a great example of how genetic reassortment can contribute to emergence of new viruses. The previous two influenza pandemics are caused by reassortment of viral surface glycoprotein genes between previously circulating human viruses and avian influenza viruses. This major change in the genotype of influenza virus is called antigenic shift. Antigenic shift gives rise to pandemic strains with a new hemagglutinin or neuraminidase that is antigenically distinct from those of the previous circulating strains. Thus, reassortment has a great potential to create a highly pathogenic virus from previously unproblematic precursor viruses.

Biological implications

The biological implications of RNA genome heterogeneity are enormous. Even single amino acid substitutions can affect a number of biological phenotypes of a virus: host receptor specificity, resistance to monoclonal antibodies, growth property, immune evasion, antiviral drug resistance and virulence. Examples for each of these phenotypes are shown in Table 1.5.1. For emerging pathogens to cause a pandemic, most likely a combination of phenotypic changes are needed. For example, mutation in both hemagglutinin (Yamada, Suzuki et al. 2006) and basic polymerase protein 2 (Hatta, Hatta et al. 2007) are thought to be required for avian H5N1 strain to infect and transmit efficiently among humans. In contrast, in some cases a single amino acid mutation is sufficient for emerging pathogens to cause epidemics. Several recent epidemics of the mosquito-transmitted chikungunya virus were caused by a single amino acid change in the envelope protein that conferred the virus to be transmitted in a new vector (Tsetsarkin, Vanlandingham et al. 2007).
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1.3.3 Human factors

One of the principal causes of pathogen emergence or re-emergence is an increased exposure to these pathogens. A number of human factors have been identified that may bring the pathogens and humans in a close proximity. Table 1.5.2 shows a few examples of human factors that have contributed to pathogen emergence. An opening of previously closed ecosystems will lead to new contacts between unrelated species and spread the infectious diseases through previously thought to be unsusceptible hosts. Human intrusion into new ecosystems such as rainforests has led to exposure of humans to viruses like Ebola or Marburg that otherwise would not have occurred. With the rapid global transport, infected people can readily introduce emerging pathogens to new geographic areas. This certainly was the case for SARS. An initial infection occurred in China and the infected traveller from China was spreading the infection in a Hong Kong hotel. The virus coughed by the single man has transmitted to other hotel guests, whom carried back SARS-CoV to their home countries, further spreading the infection to almost twenty countries within a month (Mack 2005). Movement of pathogens from rural areas of the world into developed countries with a dense human population will provide the pathogen enough susceptible hosts for transmission to occur indefinitely, if the pathogens have adapted to a new host/environment. Seasonal human influenza virus and human immunodeficiency virus are two examples of zoonotic viruses that have permanently crossed the species barrier in the past century.

In the case of avian influenza virus, human infections have almost always been associated with large-scale poultry production. This has led to a hypothesis that modern intense poultry farming facilitates the emergence of highly pathogenic avian strains in humans (Zanella 2003; Webster 2005). In support of this, the influenza virus has been thought to co-exist in waterfowl as a harmless pathogen for millions of years (Kida, Yanagawa et al. 1980; Markwell and Shortridge 1982) and despite the first documented appearance of human influenza virus around 420 years ago (Potter 2001), human cases of highly pathogenic avian viruses has only recently recorded (Claas, Osterhaus et al. 1998; Subbarao, Klimov et al. 1998). In fact, there is a strong correlation between recent changes of worldwide poultry intensification with increasing scale and frequency of highly pathogenic avian influenza outbreaks around the world starting in the 1990s (Peiris, de Jong et al. 2007). Modern agriculture is characterized by a large numbers of genetically homogenous animals that are confined in small cages or enclosures.
Perhaps, the unnaturally high densities of potentially susceptible animals are providing a favourable niche for zoonotic pathogens to survive and to be amplified (Pearson, Salman et al. 2005).

The emergence of highly pathogenic avian influenza virus seems to have been the result of increasingly close and frequent contact with potentially infected domesticated animals, whereas the subsequent dissemination is due to other human factors such as exotic pet trade and livestock. Simultaneous outbreaks of H5N1 among 5000 chickens in eight different countries across Southeast Asia were recorded in 2004. It was later confirmed that these infected chickens were transported from China for human consumption (FAO 2004). In the U.S., newly emerged triple reassortant swine influenza virus H3N2, which originated in North Carolina pig farm in 1998, has spread to Texas, Minnesota, and Iowa within months of its first appearance (Zhou, Senne et al. 1999), and spread across the U.S. within a year (Webby, Swenson et al. 2000). In both of the above cases, the rapid dissemination of the newly emerged influenza virus was attributed to the long distance livestock transport (Wuethrich 2003).

Exotic pet trade have also been implicated in the spread of avian influenza virus. For example, H5N1 infected smuggled hawk eagles were seized at Brussels International Airport in 2004 (Van Borm, Thomas et al. 2005). A year later, on unrelated case, the British government announced that an imported parrot from South America was found to be infected with H5N1 (DEFRA 2005). Because South Africa is free of H5N1, the parrot is presumed to contract an infection while housed in a quarantine facility, which also had birds from Taiwan. That same month, smuggling from China into Taiwan of more than a thousand birds, some of whom were infected with H5N1, was caught (COA 2003). Thus, it was hypothesized that the infected birds from China spread H5N1 to Taiwanese birds, which in turn legally imported into the UK to further spread H5N1. Given that an estimated of 4 million birds are traded each year worldwide, wildlife trafficking present a huge risk in the spread of zoonotic disease such as highly pathogenic avian influenza virus.
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1.3.4 Influenza virus

Basic biology of influenza virus

Influenza virus is an enveloped, single-stranded, negative sense, segmented RNA virus of the family Orthomyxoviridae (Lamb and Krug 2001). They are classified into three types of influenza virus (A, B and C) on the basis of their internal proteins; nucleoprotein (NP) and matrix protein (M). The A type is the most virulent human influenza virus and it can be further subdivided based on the antigenicity of surface glycoproteins, hemagglutinin and neuraminidase. At least 16 hemagglutinin serotypes and 9 neuraminidase serotypes exist in their natural hosts (Webster, Bean et al. 1992). Aquatic birds and wild fowl are the natural reservoirs of influenza virus. Due to migratory property of aquatic wildfowl, “quarantine” or containment of influenza virus in a natural host is impossible and crossing the species barrier from birds into a mammalian host is likely to occur, as observed in at least 18 mammalian species.

Epidemiology of Influenza

The first documented influenza pandemic, known as “Spanish Flu”, was caused by the H1N1 subtype in 1918 which caused death of 20 to 50 million individual. The overall mortality rate was ~2%, however due to a high transmissibility almost a half of world population was infected and a significant death toll was recorded. Genome comparison between avian strains and human strains has indicated that the Spanish influenza H1N1 was an entirely avian strain that had emerged as a result of accumulated point mutation. The descendents, drifted variants, of Spanish flu H1N1 remained in a human population as a seasonal influenza virus until the mid-1950s when they were displaced by a new strain and it reappeared in 1977.

During the temporary disappearance of H1N1, the second influenza pandemic “Asian Flu” H2N2 emerged in China in 1957. Within 8 months, the virus spread to Hong Kong, Japan, the United States and the United Kingdom, killing at least 1.5 million people. This H2N2 strain was a reassortant virus possessing avian H2, N2 and PB1 gene and the other genes of the human virus. In 1968 the displacement of H2N2 by “Hong Kong Flu” H3N2 viruses occurred and this pandemic caused deaths of approximately 7000,000 individuals. Again, natural reassortment events appear to had
involved wild birds as the ultimate source of HA and PB1 (PAOH 2004). The less commonly known "Russian Flu" H1N1 caused an influenza outbreak in China during the winter season of 1977-1978. Genetic analysis revealed that this strain was re-emergence of the H1N1 viruses that have disappeared since 1950. A few sources suggest that it was an accidental leak from previously frozen laboratory strain.

Seasonal influenza is caused by descendants or drifted variants of “Hong Kong Flu” H3N2 and “Russian Flu” H1N1. Both H3N2 and H1N1 continue to co-circulate due to an antigenic drift that renders natural or vaccine induced immunity ineffective in preventing an infection. Despite the development of partial immunity in a human population, seasonal epidemics result in infection of 5-15% of the worldwide population and 500,000 deaths per year. The high-risk groups are young children, as they have no prior exposure to the influenza virus, and the elderly aged 65 years or older because of age-related decline in immunity. Immunocompromised people are also at risk.

Swine H1N1 outbreak

In mid February of 2009, a novel influenza A (H1N1) virus of swine origin emerged in Mexico (Fraser, Donnelly et al. 2009). Within a few weeks of its first identification, swine H1N1 rapidly spread to numerous countries and forced WHO to declare a pandemic in June 2009 (WHO 2009). Despite its zoonotic origin, swine H1N1 is highly adapted to human and maintains a high human-to-human transmission rate without requiring infected pigs as intermediates. This is in contrast to the human cases of avian H5N1 influenza that resulted from direct contact between humans and infected birds (Michaelis, Doerr et al. 2009). As of 15 November 2009, there have been more than 520,000 reported cases, including over 6700 deaths (WHO 2009). This gives the overall case-fatality rate of > 1 %, compared to < 0.1 % for seasonal influenza (Viboud, Boelle et al. 2004). However, these figures will likely to change considerably within next few months and, thus, it is difficult to conclusively assess whether swine H1N1 virus is more virulent than normal human influenza strains. Nevertheless, swine H1N1 is of great clinical concern. Furthermore, although current swine H1N1 strain is not as virulent as highly pathogenic H5N1 strains, there is a fear that it might mutate into a more pathogenic form.
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Future threat

From past influenza pandemics it is certain that pandemics occur when new influenza subtypes consisting of hemagglutinin and neuraminidase of zoonotic origin adapt to human hosts. For many years pigs were implicated as a mixing vessel because of their abundance, close proximity to farmers, and susceptibility to both avian and human influenza viruses, thus allowing co-infections by two virus strains to occur for reassortment (Ma, Lager et al. 2009). However, there is no direct evidence that the reassortment events culminating in the Asian Flu and Hong Kong Flu originated from pigs. The current epidemic of swine influenza H1N1 that started in February 2009 (Fraser, Donnelly et al. 2009) certainly supports the “vessel theory”. The swine H1N1 virus is a distinct strain of human seasonal H1N1 strain. It is reassortant swine influenza virus including genes from avian, human (H3N2) and swine strains (Dawood, Jain et al. 2009). Current swine flu outbreak reiterates the risk of the reassortant that can occur in intermediate hosts such as pigs and potentially provide highly pathogenic reassortant strain.

Direct infections of humans without passing through intermediate hosts have been observed for several avian influenza virus strains including highly pathogenic H5N1 (CDC 2004; Peiris, Yu et al. 2004; Tran, Nguyen et al. 2004) and moderately virulent H7 (Kurtz, Manvell et al. 1996; Fouchier, Schneeberger et al. 2004; Koopmans, Wilbrink et al. 2004), H9 (Peiris, Yuen et al. 1999; Uyeki, Chong et al. 2002) and H10 (PAOH 2004) subtypes. In almost all cases sustained human-to-human infections were absent. H5N1 strains in particular represent an eminent threat to humanity since they have proven to be highly pathogenic in humans. Since their appearance in 1997, WHO confirmed 445 human cases and 263 deaths due to H5N1 (WHO 2009), giving an overall mortality rate of ~60%. The extremely high mortality rate of H5N1 strain is unprecedented in the history of influenza. Thus, an important question to ask is whether they will adapt to efficiently transmit between humans. The 1918 H1N1 Spanish virus of avian origin did not reassort in an intermediate host and jumped directly to a human population (Taubenberger, Reid et al. 2005). The putative mutations that enabled the H1N1 avian virus to cross this species barrier were found to be 10 amino acid substitutions in the polymerase proteins. Genetic analysis has shown that the highly pathogenic H5N1 contains 5 of these 10 amino acid changes indicating that H5N1 is a
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halfway through a proven set of point mutations that result in human adaptation (Taubenberger, Reid et al. 2005).

Highly pathogenic avian strain H5N1 represents a continuous pandemic threat due to its high virulence, its ongoing mutations, and its widespread distribution in wild and domestic birds. Current scientific prediction is that future H5N1 pandemic is inevitable and what remains uncertain is the precise timing of the next influenza pandemic. Thus, it is imperative to have early warning system or disease surveillance so that effective control efforts to contain the disease outbreak can be placed should they arise. Given the nature of 6 months lag time in production of strain-specific influenza vaccine and unpredictability of future pandemic strain (Ferguson and Anderson 2002), the current influenza vaccine strategy will not be an effective preventive measure for combating next influenza pandemic. A further research into more effective and broad-spectrum influenza vaccines should be prioritised.

1.4 Influenza vaccines

1.4.1 General vaccinology

Brief historical background to vaccinology

In ancient history, people observed that those who got ill with what we now know to be infectious diseases and recovered never got the same illness again or at least had a certain period of protection against the same disease. Based on this observation, attempts were made in different parts of the world to ameliorate the severity of diseases by deliberately infecting the healthy individuals with infectious matters. The practice of variolation for prevention of smallpox has begun in China and India in 17th century, when people were inoculated with variola virus (causative agent of smallpox) (Fenner, Henderson et al. 1988). The method is thought to have spread slowly to various countries and was adapted to fight various infectious diseases such as syphilis, measles, yellow fever, bovine pleuropneumonia, ruminant anthrax and bovine plague (Fenner, Henderson et al. 1988).

Edward Jenner introduced the concept of vaccination in 1798. The world’s first vaccine, vaccinia virus, was highly successfully and resulted in eradication of smallpox. Although vaccination has proven to be highly effective, its application was limited to
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smallpox until the concept of artificial attenuation emerged in the 1800s (Pasteur 1880). Louis Pasteur developed the hypothesis that the virulence of infectious agents could be artificially attenuated and be used for immunizations. This hypothesis later led to the discovery of various attenuation techniques and allowed vaccination techniques to be applied for many infectious diseases.

The principal of using completely inactivated agents for immunization came from the work of Daniel Salmon and Theobald Smith (Salmon and Smith 1884-1886). In 1886, they demonstrated that heat-killed salmonella afforded protection in pigeons against challenge with live virulent salmonella. Inactivated vaccine soon became popular, due to the high reproducibility and lower risk of side effects. By the end of the nineteenth century, three inactivated whole bacterial vaccines for humans became available: salmonella, cholera and plague.

Formaldehyde is the most commonly used inactivant to prepare killed vaccines. In 1904 Glenny and Hopkins discovered that the formalin can be used to prepare inactivated diphtheria toxins or toxoids. This inactivation method provided stable and safe toxoids, and soon after the same procedure was applied to prepare tetanus toxoids (Ramon and Zoeller 1927). These two early acellular vaccines, diphtheria and tetanus toxoids, provided an important milestone towards development of subunit vaccines, as a means to bypass the toxicity of conventional whole inactivated bacterial/viral vaccines. Later, the advent of molecular biology has enabled the subunit vaccines to be developed.

The development of viral propagation methods based on eggs and tissue culture has accelerated the evolution of vaccinology in 1900s. Efficient virus production has allowed large-scale vaccine production. The use of mammalian cell cultures and eggs for the viral growth enabled many additional anti-virus vaccines to be developed for examples vaccines against measles, mumps, rubella, adenovirus, varicella, influenza, yellow fever, and poliomyelitis.

Future challenges

Almost all of the modern vaccines rely exclusively on antibody responses (Plotkin 2001). Even for live attenuated viral vaccines, such as those against mumps and polio, which can induce both cellular and humoral responses, the neutralizing
antibodies are thought to be responsible for the protective efficacy (Plotkin 2001; Pantaleo and Koup 2004). Generally antibody-based vaccines are highly efficacious, with a few exceptions, i.e. against acute infectious diseases. Most of the successful vaccines were empirically developed without a full understanding of how immune system protects against foreign pathogens (Hilleman 2000). Perhaps for the reason of the past successes, immunologists were not often involved in vaccine development. However, in the late 1980s, with the arrival of the HIV pandemic and the emerging drug resistance tuberculosis, it became clear that an understanding of what natural immune responses are required to clear infections and how these effector components can be artificially induced is vital for rational vaccine development. This is particularly true for those diseases that require T cell immunity such as influenza, respiratory syncytial virus, HIV, malaria and tuberculosis. For these intracellular pathogens, stimulation of both arms of adaptive immunity, antibodies and cell-mediated, will be needed for an optimum protection. Thus, current vaccinology is moving towards an era of T cell-based vaccines.

1.4.2 The History of Influenza Vaccine Development

The first influenza vaccine was inactivated whole virus vaccine. The finding that the magnitude of the antibody response in humans correlates with the amount of virus dose (Hirst, Rickard et al. 1942) has led to development of various virus concentration and purification methods such as adsorption on and elution from chicken erythrocytes (Francis and Salk 1942), freeze-thawing (Hirst, Rickard et al. 1942) and high speed centrifugation (Stanley 1945). These impure concentrated whole virus vaccines were associated with a high reactogenicity (Salk 1948), especially among young children (Quilligan, Francis et al. 1949). Unsurprisingly, the virus component itself was considered as a causative agent for side reactions (Salk 1948; Davenport, Rott et al. 1960) despite the fact that many of these early vaccines had a high content of egg-derived non-viral proteins. Later, it was found that the reactogenicity can be reduced by splitting (solubilization of the viral envelope) the virus using chemicals such as Tween-80 and ether (Davenport, Hennessy et al. 1964; Siegert and Braune 1964). Although, the mechanism behind the toxicity reduction was not elucidated, e.g. the splitting per se or
other processing steps involved, this finding evoked further investigation. Detergents such as sodium deoxycholate were used in favour over ether for split vaccine preparation because they are less hazardous than ether. Sodium deoxycholate treated virus vaccines were well tolerated in both animals (Webster and Laver 1966) and humans (Duxbury, Hampson et al. 1968). The reduced reactogenicity may be attributed to the action of deoxycholate induced dissociation of bacterial endotoxins (Tarmina, Milner et al. 1968; Badakhsh and Herzberg 1969), a common contaminant of egg-grown influenza vaccines (Rastogi, Hochstein et al. 1977), rather than the splitting per se. Nonetheless, currently used influenza vaccines are mostly split vaccine or subunit vaccines consisting of purified hemagglutinin.

### 1.4.3 Currently licensed influenza vaccines

The most commonly used influenza vaccines are endotoxin-free trivalent split or subunit influenza vaccines (TIV), which contain 15 μg of hemagglutinin from A/H1N1, A/H3N2, and influenza type B strain (Renfrey and Watts 1994). In the past, vaccine strains were made by selecting mutants with high growth characteristics that arise through tedious serial passage in embryonated eggs (Webby and Sandbulte 2008). Since 1971, with the emergence of genetic engineering, the seed viruses have been made by genetic reassortment between wild type virus and laboratory high-yield phenotype strain (Kilbourne 1969; Robertson, Nicolson et al. 1992). A reassortant virus that contains the surface glycoproteins of the wild type strain and has high growth properties is selected for a vaccine production. A seed virus is then propagated in embryonated chicken eggs, concentrated and purified by zonal centrifugation (Reimer, Baker et al. 1966). Purified virus is inactivated with β-propiolactone or formaldehyde and disrupted with detergent (Furminger 1998). Some vaccines are further purified to contain only viral surface glycoproteins.

Parenteral immunization with trivalent influenza vaccines induces predominantly serum antibodies to the major vaccine constituents, hemagglutinin and neuraminidase (Cox and Brokstad 1999), which are conserved only for a given strain. The efficacy of current influenza vaccine is therefore highly dependent on how well the vaccine strain matches the antigenicity of the newly emerging virus. Consequently, the
protection rates vary year-to-year but are expected to have 60-90% protection rate (Beyer, Palache et al. 2002). In order to ensure close antigenic match between the vaccine strain and the circulating strains, World Health Organization (WHO) have established the Global Influenza Network to monitor antigenic drift variants. Based on WHO recommendations, influenza vaccines are annually reformulated to take into an account of an ever-changing virus. However, despite these efforts a significant antigenic mismatch can still occur and results in reduced vaccine efficacy (Aymard, Valette et al. 1999; Klimov, Simonsen et al. 1999). This problem represents a major shortcoming of current strain-specific influenza vaccines.

In contrast to the strain-specific immunity induced by influenza vaccinations, natural influenza infection is known to generate heterosubtypic immunity in mice (Schulman and Kilbourne 1965; Floc'h and Werner 1978; Kreijtz, Bodewes et al. 2007) and humans (Slepushkin 1959; McMichael, Gotch et al. 1983; Sonoguchi, Naito et al. 1985). Recent mouse studies have shown that influenza vaccination against human influenza can prevent the development of cross-protective immunity against avian influenza H5N1 (Bodewes, Kreijtz et al. 2009). An implication of this report is that annual vaccination in immunologically naïve subjects against seasonal influenza may render them vulnerable to future pandemic strains of a different subtype. The mechanism behind the seasonal influenza vaccination-associated interference with the induction of heterosubtypic immunity was not fully investigated but the correlation with reduced influenza-specific T cell immunity was observed in vaccinated mice (Bodewes, Kreijtz et al. 2009). The lack of T cell immunogenicity is thought to account for declining cross-protective immunity against influenza, especially in the elderly (Provinciali, Di Stefano et al. 1994; Cox and Brokstad 1999; Webster 2000).

It is becoming clear that an optimal protection against influenza infections require balanced Th1/Th2 responses (Hovden, Cox et al. 2005; Huber, McKeon et al. 2006; Bungener, Geeraedts et al. 2008). However, immunizations with inactivated virus vaccine induce predominantly Th2-skewed antibody responses (Bungener, Geeraedts et al. 2008; Geeraedts, Bungener et al. 2008). Furthermore, adjuvant enhanced Th2-type antibody responses do not correlate with better protection, but intriguingly a higher morbidity was observed compared to control mice that received inactivated virus vaccine alone, supposedly as a result of Th2 immune response skewing (Bungener, Geeraedts et al. 2008). This raises a concern regarding the current use of
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hemagglutination inhibition titers as a correlate of protection for the evaluation of influenza vaccine efficacy.

1.4.4 Future Vaccine strategies – A universal vaccine

Although technologies have allowed development of new vaccine strategies, a major breakthrough has not been made for influenza vaccine. Thus we still rely on the traditional influenza vaccine approach. As described above, antibody mediated protection induced by the current influenza vaccine is highly strain-specific. Predicting the evolutionary change of the hemagglutinin is based on educated guess work and the future pandemic strain can be of any subtype of zoonotic origin. Thus, the ultimate goal of influenza vaccine is development of a highly cross-reactive vaccine that can provide protection not only against antigenic drift variants but also against the antigenic shifts in influenza A.

Vaccine candidates and their limitations

A common approach of new vaccine candidates is the exploitation of cellular immunity or antibody-based immunity directed against conserved epitopes of the viral proteins in influenza A viruses (Fiers, De Filette et al. 2004; Livingston, Higgins et al. 2006). Internal proteins such as nucleoprotein (Ulmer, Fu et al. 1998; Epstein, Kong et al. 2005) and matrix protein (Okuda, Ihata et al. 2001; Ozaki, Yauchi et al. 2005) are the principal targets of cell-mediated approach whereas for antibody-based vaccines the conserved regions of hemagglutinin (Chen, Matsuo et al. 1999; Mitchell, Green et al. 2003; Chen, Cheng et al. 2008) and the ectodomain of the matrix 2 proteins (Frace, Klimov et al. 1999; Neirynck, Deroo et al. 1999; De Filette, Min Jou et al. 2005; Zou, Liu et al. 2005) are the favoured targets. Several new vaccine approaches based on cross-reactive T cells or antibodies have been developed to achieve so called “universal influenza vaccine”. These include: 1) live attenuated virus vaccine; 2) replication defective influenza virus; 3) peptide or polypeptide based vaccine; 4) live vectors expressing recombinant HA, MA, or NP; and 5) DNA-based vaccines. The vaccination strategy is gradually shifting from whole virus vaccine to subunit, peptide and DNA
vaccine, in response to high reactogenicity associated with the whole virus vaccine. On the downside, newly developed vaccines are not as immunogenic as whole virus vaccine and often require adjuvants and/or multiple immunizations.

Since these vaccine candidates have not been tested side by side, it is difficult to conclusively judge which vaccine candidate is most superior. Their advantages and disadvantages are summarized in Table 1.5.3. New vaccine candidates have been extensively reviewed elsewhere (Epstein 2003; Cox, Brokstad et al. 2004; Cox 2005; Cinatl, Michaelis et al. 2007; Stambas, Guillonneau et al. 2008; Haynes 2009; He, Madhan et al. 2009; Tang, Zhang et al. 2009; Wilschut 2009) and thus will not be discussed here.

1.4.5 Gamma-irradiation for a universal flu vaccine

Superior immunogenicity of whole virus vaccine

Most of the current universal influenza vaccine candidates are longer-term options that are still in preclinical stages and more research will be needed to optimise each strategy and to test for safety profile before they can be commercially available. Meanwhile, utilizing conventional whole virus vaccines that can afford heterosubtypic immunity represents more immediate potential improvements. Inactivated whole virus vaccine has been abandoned since the advent of less reactogenic subunit or subvirion vaccines, despite the higher protective efficacy of whole inactivated virus vaccine in immunologically naïve animals and humans (Boyer, Cherry et al. 1977; McLaren, Verbonitz et al. 1977; Nicholson, Tyrrell et al. 1979; Beyer, Palache et al. 1998; Stephenson, Nicholson et al. 2003; Hovden, Cox et al. 2005; Geeraedts, Bungener et al. 2008). Superior immunogenicity of whole virus vaccines has been attributed to its ability to activate innate immune system through TLR-7 (Geeraedts, Goutagny et al. 2008), which leads to favourable Th1-skewed immune responses (Hovden, Cox et al. 2005; Bungener, Geeraedts et al. 2008; Geeraedts, Bungener et al. 2008). Furthermore, several recent mouse studies have changed our current thinking that the inactivated whole virus vaccine is a poor inducer of cross-protection. Induction of cross-protection against H5N1, when administered intranasally, with inactivated whole virus vaccines based on human influenza strains has been consistently demonstrated in mice (Takada, Kuboki et
al. 1999; Tumpey, Renshaw et al. 2001; Takada, Matsushita et al. 2003; Quan, Compans et al. 2008). Although the mechanism/s behind this cross-protection was not fully elucidated, the cross-protective potential of inactivated whole virus vaccine was clearly shown. However, these immunization regimes employed high vaccine dose and multiple priming with or without potentially harmful adjuvants, and thus will not be feasible for humans. Hence, further strategies are needed to reduce the vaccine dose and to enhance the immunogenicity in order to provide protective immunity after single priming.

**Gamma-irradiation is a superior inactivation method**

The above-mentioned vaccine improvements may be achieved by altering the method of inactivation. In the past, various inactivation methods were intensely investigated because it was a challenge at the time, and still is now, to fully inactivate the immunizing agent while preserving its immunogenicity (reviewed in (Ada 2001; Stauffer, El-Bacha et al. 2006)). The use of gamma-irradiation as an alternative method to chemical inactivation has been suggested several times in the literature based on the hypothesis derived in 1961 that, organisms can be rendered incapable of reproduction primarily by damage to genetic material rather than by structural destruction of proteins (Howard-Flanders 1961). This rationale was soon incorporated into vaccine development especially for those pathogens whose immunogenicity was destroyed by other means of inactivation. Gamma-irradiation was applied to prepare an experimental vaccine against a number of infectious diseases including parasitic (*Toxoplasma gondii* (Seah and Hucal 1975), *Dictyocaulus viviparous* (Jarrett, Jennings et al. 1960), *Ancylostoma caninum* (Miller 1965), *Schistosoma japonicum* (Hsu, Hsu et al. 1962; Radke and Sadun 1963), *Plasmodium berghei* (Wellde and Sadun 1967; Nussenzweig, Vanderberg et al. 1969), *Trypanosoma conglolense* (Duxbury, Anderson et al. 1972), *Toxoplasma gondii* (Seah and Hucal 1975)), bacterial (*E.coli*, *Pasteurella tularensis* (Gordon, Donaldson et al. 1964; Landay, Wright et al. 1968), *Mycobacterium tuberculosis* (Carpenter, Naylor-Foote et al. 1959), *Listeria monocytogenes* (Datta, Okamoto et al. 2006) viral diseases (bluetongue virus (Campbell 1985; Campbell, Barber et al. 1985), Venezuelan equine encephalitis virus (Reitman, Tribble et al. 1970; Reitman and Tonik 1971), rabies virus (Wiktor, Aaslestad et al. 1972), vaccinia virus,
mumps, influenza virus)). Among these studies that have directly compared gamma irradiation with other forms of inactivation method, the superior retention of immunogenicity by gamma-irradiation has been consistently reported (Wiktor, Aaslestad et al. 1972; Müllbacher, Ada et al. 1988; Hiramoto, Galisteo et al. 2002; Sanakkayala, Sokolovska et al. 2005).

Unsurprisingly, gamma-irradiation was also used for influenza virus vaccines. Polley et al was one of the first to successfully prepare gamma-inactivated influenza virus vaccine (Polley 1961). Intraperitoneal immunization in guinea pigs with gamma-inactivated A/PR8 (H1N1) induced high hemagglutination inhibition and serum neutralization titres and conferred homologous protection. Kulevich et al in 1974 shifted to intranasal route for immunization and showed that gamma-inactivated A2/HongKong/1/68 (H3N2) virus preparations provide homologous protection and induces antibody responses in serum and in lungs (Kulevich and Kosiakov 1974). An oral immunization route was also effective in eliciting homologous protection using gamma-inactivated H1N1 (Noack, Tischner et al. 1986). In 1988, Müllbacher et al first demonstrated the cross-protective potential of gamma-inactivated virus (Müllbacher, Ada et al. 1988). In their study, intravenously immunized mice with gamma-inactivated A/JAP (H2N2) virus survived a lethal heterosubtypic virus A/WSN (H1N1) and A/PC (H3N2) challenge. Intriguingly, unlike UV-irradiation, gamma-inactivation did not destroy the ability of the virus to induce Tc cell responses (Müllbacher, Ada et al. 1988). Pang et al in 1992 (Pang, Clancy et al. 1992) and Lidbury et al in 1997 (Lidbury, Grissell et al. 1997) confirmed the cross-protective property of gamma-inactivated virus using the adjuvant-like effect of co-delivered chicken erythrocytes. Such preparation was effective only when administered orally but not subcutaneously. Similarly, we have recently reported that a single intranasal administration of gamma-inactivated influenza virus protects mice against lethal H5N1 and other heterosubtypic infections (Alsharifi, Furuya et al. 2009). The above reports suggest that inactivation method as well as route of immunization are important factors for eliciting heterosubtypic immunity.

The question still remains whether the mechanism of observed cross-protection against influenza A viruses was humoral or cellular responses. Gamma-inactivated virus-induced cross-reactive antibodies or Tc responses have been observed but none of the previous studies have extensively investigated the mechanism behind the observed cross-protection. Full understanding of protective mechanisms will be crucial as such
knowledge may help to augment the efficacy of gamma-inactivated virus by manipulating the immune system with appropriate adjuvants.

It is important to distinguish gamma-ray from ultraviolet-ray, which is another form of radiation that has extensively been used to prepare experimental vaccines (Milzer, Oppenheimer et al. 1945; Rep. 1947). In contrary to gamma-radiation, UV has a number of undesirable properties. Firstly, a slight over-irradiation of UV can cause a significant antigenic reduction, for example, 2 times the minimum inactivation dose for rabies virus results in 75% loss of antigenicity (Milzer, Oppenheimer et al. 1945; Rep. 1947). Secondary, UV has a low penetrative strength implying that the antigens should be directly exposed to the UV source and the antigens should be suspended in a clear solution and/or in a thin layer. On the other hand, gamma-ray has superior penetration ability (Lowy, Vavrina et al. 2001), which permits the large volumes of viruses stored in an enclosed container to be irradiated. Thus, materials can easily be maintained in a frozen state with dry ice. Furthermore, gamma-radiation has a larger margin between the dose required to destroy the infectivity and that of detrimental impact on functional property of viral proteins (Jagger and Pollard 1956; De Flora, Badolati et al. 1969; Wiktor, Aaslestad et al. 1972; Goldblith 1975; Fridman, Ermolaev et al. 1979; Migunov, Aron et al. 1986). Finally, radiation inactivation does not require subsequent treatment to remove undesirable chemicals. These properties contribute to the attractiveness of gamma-inactivation method. Despite these obvious advantages, gamma-irradiation has not been applied for commercial human vaccines. This is probably due to the prior advent of chemically inactivated vaccines, which are highly effective against antigenically stable pathogens. Another possible reason is the public concerns about the use of irradiation. However, there has been an increasing public acceptance of irradiation in a past few years, especially in the food industry (Frenzen, DeBess et al. 2001; Spaulding, Wiegand et al. 2006).

**Gamma-irradiated pathogen generates Th-1 skewed immune responses**

The primary target of gamma ray-irradiation is the microbial genome (Lowy, Vavrina et al. 2001). It causes strand breaks in the genetic material, rendering the pathogen replication deficient without causing a detrimental impact on the structures and biological activities of proteins (Polley 1952; Jordan and Kempe 1956; Pollard
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1957; Kaplan 1960; Polley 1961; Smolk and Lombardo 2005). For this property, gamma ray-inactivation has permitted studies of protein chemistry of highly pathogenic pathogens such as Ebola, Marburg and Lassa virus, with relatively low risk of infection for experimenter (Lupton 1981; Elliott, McCormick et al. 1982). In an extreme example, a recombinant \textit{Brucella bortus} expressing \textit{E.coli} $\beta$-galactosidase exposed to a low dose of gamma-irradiation became replication deficient but its metabolic activity, namely expression of B-galactosidase, was maintained (Sanakkayala, Sokolovska et al. 2005). As a result of live bacteria-like properties, the gamma-inactivated preparation had a superior protective efficacy and was more effective than the heat-killed preparation in inducing antigen specific Th-1 type immune responses in mice (Sanakkayala, Sokolovska et al. 2005). A similar observation was made with gamma-irradiated \textit{Toxoplasma gondii} (Hiramoto, Galisteo et al. 2002). The gamma-irradiation destroyed the ability of parasites to reproduce but did not interfere with other cellular functions such as metabolic functions, protein and even nucleic acid synthesis. The irradiated parasites mimicked a natural infection, due to their preserved ability to infect target cells, and thereby induced strong antibody, Th-1 cytokine, and lymphoproliferative responses. T cell-dependent immunity has also been shown for gamma-irradiated \textit{Listeria monocytogenes} (Datta, Okamoto et al. 2006). Moreover, the phenomenon of retaining Tc cell immunogenicity after gamma-irradiation has been observed for viruses such as influenza virus (Müllbacher, Ada et al. 1988), alphavirus (Müllbacher, Marshall et al. 1979) and Bunyavirus (Müllbacher, Marshall et al. 1986). This suggests that the irradiated viruses could also invade target cells, although this was not formally demonstrated, and thereby allowing efficient uptake of viral antigens by APCs for MHC class I presentation pathway to elicit cellular immunity. A feature that is shared by the above-mentioned reports is that the gamma-irradiated pathogens retain T cell immunogenicity, an immune response previously thought to be generated only by live infection. Induction of T cell immunity by vaccine is a long-standing goal in vaccinology and such a vaccine is expected to be highly valuable against intracellular pathogens. It is highly promising to apply gamma irradiation for a number of different pathogens for a development of T cell based vaccine.
Maintained integrity of viral protein is a key factor

Another novel viral inactivation method worth mentioning is an approach based on hydrophobic photoactivatable compounds, i.e. 1,5-iodonaphthyl-azide (INA), which selectively targets the transmembrane domains of viral envelope proteins (Bercovici and Gitler 1978; Holowka, Gitler et al. 1981; Jorgensen, Karlish et al. 1982; Pak, Krumbiegel et al. 1994). Upon UV-irradiation, INA disrupts the viral-target cell membrane fusion activity and results in a complete loss of infectivity without changing the structural integrity of the virus (Raviv, Bercovici et al. 1984; Raviv, Viard et al. 2005; Warfield, Swenson et al. 2007). This approach has been applied to a variety of enveloped viruses including influenza virus (Raviv, Blumenthal et al. 2008), Ebola virus (Warfield, Swenson et al. 2007), Venezuelan equine encephalitis virus (Sharma, Raviv et al. 2007) and retroviruses (Raviv, Viard et al. 2005). In mouse models, induction of antibody and T cell responses similar to those after live virus infection has been reported using INA-inactivated Ebola virus (Warfield, Swenson et al. 2007) and influenza virus. A single immunization with a low dose INA-inactivated Zaire Ebola virus (equivalent to $5 \times 10^4$ PFU) conferred robust protection against lethal Zaire Ebola virus challenge in mice (Warfield, Swenson et al. 2007). In the case of influenza, both homologous and heterosubtypic protection was observed after a single intranasal immunization with 15 µg of INA inactivated influenza virus (Raviv, Blumenthal et al. 2008). This is a remarkable improvement in vaccine efficacy in comparison to the current influenza vaccine, which requires prime-boost strategy with multiple immunisations with adjuvant to provide cross protection in a mouse model (Ichinohe, Tamura et al. 2007). The authors proposed that the superior immunogenicity is attributed to the preserved integrity of viral structure.

The most commonly used inactivant for vaccine preparation is formalin (Cranage, Baskerville et al. 1992; Clemens, Safary et al. 1995; Shahzad and Kohler 2009), which induces extensive cross-linking of proteins and therefore potentially destroys immunological epitopes (Ferguson, Wood et al. 1993; Koch, Jensen et al. 1996). Such structural changes are perhaps the reason for the reduced immunogenicity of formalin-inactivated viruses relative to live virus (Lupton, Lambert et al. 1980; Nair and Sen 1992; Cranage, McBride et al. 1995; Rossio, Esser et al. 1998) and for the induction of Th-2 skewed immune responses (Bachmann, Bast et al. 1994) or, in some cases, disease exacerbation as seen in respiratory syncytial virus infection (Chin,
Magoffin et al. 1969; Kim, Canchola et al. 1969). Thus, preserving the conformational integrity of viral proteins may be the critical factor for enhancing the immunogenicity of the virus as demonstrated by gamma ray-irradiation and INA inactivation.

However, an approach based on preserving the integrity of virus structures can possibly be a double-edged sword. For example, prions, which consist primarily of proteins, are highly resistant irradiation (Gibbs, Gajdusek et al. 1978; Hoshi, Yoshino et al. 2000). In another example, gamma ray-irradiation does not significantly reduce the toxicity of endotoxin (Previte, Chang et al. 1967; Landay, Wright et al. 1968). This is probably because gamma-irradiation does not disrupt the protein structures; therefore irradiated endotoxin retains its toxicity. Thus for pathogens that may contain an intrinsic toxicity such as influenza (Ilyinskii, Gabai et al. 2007), whole virus vaccine with intact viral structures may have an increased reactogenicity in comparison to chemically inactivated preparations. Obviously, the safety profile of gamma- or INA-inactivated virus has not been tested in humans but the expected reactogenicity can be extrapolated from clinical trials on live attenuated virus vaccine. The safety of intranasal live attenuated vaccine has been extensively evaluated in a number of different settings: in children (Edwards, Dupont et al. 1994; Belshe, Mendelman et al. 1998), in adults (Edwards, Dupont et al. 1994; Nichol, Mendelman et al. 1999), in higher risk individuals i.e. individuals with HIV (King, Treanor et al. 2000; King, Fast et al. 2001) or asthma (Redding, Walker et al. 2002; Fleming, Crovari et al. 2006), in elderly with chronic diseases (Jackson, Holmes et al. 1999; Gorse, O'Connor et al. 2003) such as respiratory illness, cardiovascular disease, pulmonary disease, and diabetes. In the majority of these cases, the live attenuated vaccine was well tolerated except for those younger than 24 months, which had an increased rate of wheezing and a higher rate of hospitalisation for any cause (Belshe, Edwards et al. 2007). The statistically significant differences found in those older than 24 months were that vaccinees had more rhinorrhea (runny nose), sore throat and more low-grade fever than placebo recipients. These mild side effects might be acceptable considering the potential benefits of whole virus influenza vaccine during a pandemic.
## 1.5 Tables

### Table 1.5.1 Phenotypic changes in influenza virus associated with a single amino acid substitution

<table>
<thead>
<tr>
<th>Biological phenotypes</th>
<th>Description</th>
<th>Reference</th>
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<tbody>
<tr>
<td>Receptor specificity</td>
<td>A single amino acid substitution in hemagglutinin change binding preference for the human or avian receptor</td>
<td>(Glaser, Stevens et al. 2005)</td>
</tr>
<tr>
<td>Replication efficiency</td>
<td>Substitutions in basic polymerase 2 enhance their polymerase activity and more efficient replication of genome both <em>in vitro</em> and <em>in vivo</em></td>
<td>(Li, Ishaq et al. 2009)</td>
</tr>
<tr>
<td>Immune evasion, innate immunity</td>
<td>A single amino acid substitution in the non-structural protein NS1 affects its ability to inhibit induction of IFN-1 response</td>
<td>(Jiao, Tian et al. 2008)</td>
</tr>
<tr>
<td>Immune evasion, adaptive immunity</td>
<td>A glycine to argine substitution at position 135 within hemagglutinin prevents recognition by both HA-specific monoclonal antibodies and T cells</td>
<td>(Thomas, Skehel et al. 1987)</td>
</tr>
<tr>
<td>Attenuation/virulence</td>
<td>A single nucleotide deletion in the hemagglutinin renders the virus fusion incompetent.</td>
<td>(Haller, MacPhail et al. 2001)</td>
</tr>
<tr>
<td>Antiviral drug resistance</td>
<td>A single amino acid substitutions in the matrix 2 ion channel protein leading to amantadine resistance occur at residue 26, 27, 30, 31, 34, or 38.</td>
<td>(Lee, Song et al. 2008)</td>
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### Table 1.5.2 Human factors contributing to emergence of pathogens

<table>
<thead>
<tr>
<th>Human factor</th>
<th>Description</th>
<th>Examples of emerging or remerging infectious diseases</th>
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<tr>
<td>Human behaviour</td>
<td>Unsafe medical practice</td>
<td>SARS (Somogyi, Vesely et al. 2004), HIV (Zarocostas 2004).</td>
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<td></td>
<td>Sexual revolution</td>
<td>HIV (Lagarde, Auvert et al. 2001)</td>
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<td></td>
<td>Hunting for bush meat</td>
<td>HIV (Wolfe, Switzer et al. 2004), T-lymphotropic viruses (Wolfe, Heneine et al. 2005)</td>
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<td></td>
<td>Unplanned migration and refugee camps</td>
<td>Measles (Kamugisha, Cairns et al. 2003; Cranmer 2005), viral hepatitis (Chironna, Germinario et al. 2003)</td>
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<tr>
<td></td>
<td>Research study</td>
<td>Influenza virus (Check 2005), SARS (Senior 2003)</td>
</tr>
<tr>
<td></td>
<td>Pasturage practices</td>
<td>SARS (Xu, He et al. 2004), avian influenza virus (Ninomiya, Takada et al. 2002)</td>
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<tr>
<td>Technology and industry</td>
<td>Organ transplants</td>
<td>Cytomegalovirus (Michaels, Jenkins et al. 2001)</td>
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<tr>
<td></td>
<td>Development of agricultural activities sustain the virus main reservoir</td>
<td>Junin Junin virus (Maiztegui 1975), machupo virus (Vainrub and Salas 1994), guanaritovirus (Vainrub and Salas 1994), lassa virus (Richmond and Baglole 2003)</td>
</tr>
<tr>
<td></td>
<td>Encroachment on rain forest</td>
<td>Nipah virus (Enserink 2000), Hendra virus (Field, Young et al. 2001)</td>
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<tr>
<td>International travel and commerce</td>
<td>Introduction of emerging pathogens to new geographic areas by infected travellers, by contaminated food, or by transporting vehicle</td>
<td>Ebola virus (Peters and LeDuc 1999), West Nile virus (Russell 1987), Marburg virus (Slenczka 1999), monkeypox virus (Di Giulio and Eckburg 2004).</td>
</tr>
<tr>
<td>Ecological factors</td>
<td>Climatic events and global warming</td>
<td>Sin Nombre virus (Hjelle and Glass 2000), mosquito-borne diseases such as malaria, dengue and viral encephalitis (Patz, Epstein et al. 1996)</td>
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<td></td>
<td>Dam construction changing the surrounding ecology</td>
<td>Rift valley fever virus (Nabuth, Kane et al. 2001)</td>
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<table>
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<th>Vaccine strategy</th>
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<th>Advantages</th>
<th>Disadvantages</th>
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<tr>
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<tr>
<td>Split or subunit vaccine</td>
<td>Antibody</td>
<td>Low reactogenicity,</td>
<td>Strain specific</td>
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<td>High viral yield</td>
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<td>Currently used method</td>
<td>Minimum of two immunizations and two doses required</td>
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<tr>
<td>Cell culture vaccines</td>
<td>Antibody</td>
<td>Cells are easier to manage for mass production</td>
<td>Low efficacy in the elderly</td>
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<td></td>
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<td>Preservation of receptor specificity</td>
<td>Poor efficacy in immunologically naive individuals</td>
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<td>Abundant supply of substrate</td>
<td>Low production capability</td>
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<tr>
<td>Bovicovirus-expressed</td>
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<td>Adjuvanted virosome</td>
<td>Tc cell and</td>
<td>Large production capacity</td>
<td>Multiple and high doses required</td>
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<td></td>
<td>antibody</td>
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<td>Production capability uncertain</td>
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<td>Genetic (vectorised)</td>
<td>Tc cell and</td>
<td>Mimic natural infection</td>
<td>Safety issues, cases of Bell's palsy.</td>
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<td>Live attenuated vaccine</td>
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<td>Low reactogenicity</td>
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<td>Cross protection in children</td>
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<td>(6-59 months)</td>
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<td>Live virus vector</td>
<td>Tc cell and</td>
<td>Nasal administration</td>
<td>Low risk of reassortment with avian influenza strain and reversion to virulent form</td>
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<td>antibody</td>
<td>Large production capacity</td>
<td>Children under 5 are excluded</td>
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<td>Immunogenic</td>
<td>Moderate levels of cross protection in adults (18-46 years of age)</td>
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<td>Cross protection in animals</td>
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<td>Large production capacity</td>
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<td>DNA-based vaccine</td>
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<td>Absence of anti plasmid immune response</td>
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Chapter 2

Intranasal vaccination with gamma-inactivated influenza A virus induces cross-protection against seasonal and H5N1 avian influenza infections

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2.1 Abstract

Gamma-irradiation at a dose of 10 kGy rendered influenza A virus non-infectious but did not destroy its T cell immunogenicity. Similar to live virus infections, gamma-inactivated influenza virus induced the pro-inflammatory cytokines, IFN-gamma and TNF, and cross-reactive Tc cell responses in mice. Furthermore, a single dose of gamma-inactivated human influenza A virus provided robust cross-protection against both seasonal human and highly pathogenic avian H5N1 influenza infections. This cross-protective immunity was achieved when gamma-inactivated virus preparations were administered intranasally. These results warrant further investigation into the potential use of gamma-irradiation to prepare pandemic influenza vaccines.
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2.2 Introduction

Influenza A virus is a respiratory virus that causes high morbidity and mortality worldwide. The virus belongs to a family of the *Orthomyxoviridae* and carries a segmented, single-stranded RNA genome (Webster, Bean et al. 1992). Following the emergence of avian influenza strains in southern and eastern Asia, the need for more effective and cross-protective influenza vaccines is more urgent than ever. Thus the research for better influenza vaccines is gaining increasing attention.

Current influenza vaccines are either split virion vaccines or surface antigen vaccines from chemically inactivated viruses, containing the outer envelope proteins of influenza virus hemagglutinin and neuraminidase (Palese 2006). The efficacy of these vaccines is primarily due to induced specific antibody against viral surface antigens, mainly hemagglutinin (HA). This approach has the significant drawback of requiring reformulation almost every year to counter the continual antigenic drift and antigenic shift of the influenza virus. In contrast, the cytotoxic T cell response to influenza virus is predominantly directed against conserved internal viral proteins, which are less susceptible to antigenic variation and are antigenically similar among heterosubypic viral strains (Yewdell, Bennink et al. 1985; Bennink and Yewdell 1988). This property makes internal proteins ideal candidates for a universal influenza vaccine that could confer a protection against a broad range of existing and new influenza strains, including avian influenza. Therefore, vaccination strategies that elicit cytotoxic T cell responses could prove to be powerful weapons against seasonal and pandemic outbreaks of influenza.

Here, influenza A virus was tested for its ability to induce Tc cell responses after being fully inactivated by gamma irradiation. Gamma-inactivated influenza A virus was assessed as an experimental vaccine for human and avian influenza infections.
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2.3 Materials and Methods

Mice

Nine- to ten-week-old female BALB/c mice were routinely used in these studies. For H1 and H3 experiments, mice were obtained and housed in Biosecurity Level 2 containment facilities at the John Curtin School of Medical Research, the Australian National University, and ACT, Australia. For H5 studies, which were conducted at the Australian Animal Health Laboratory (AAHL; Geelong, Australia), mice were obtained from the Animal Resource Centre (Perth, Australia), and all work using live virus was carried out under Biosecurity Level 3 enhanced containment. All experimental procedures were approved by the institutional Animal Ethics Committees.

Viruses and cells

P815 mastocytoma, Madin-Darby canine kidney (MDCK) and baby hamster kidney (BHK) cells were grown and maintained in EMEM supplemented with 5% FCS, 2.2 g L⁻¹ NaHCO₃, 0.29 g L⁻¹ L-glutamine and PSN antibiotics (0.12 g L⁻¹ penicillin G, 0.2 g L⁻¹ streptomycin sulphate, 0.2 g L⁻¹ neomycin sulphate) at 37 °C in a humidified atmosphere with 5% CO₂.

The influenza A viruses, A/PR8 [A/Puerto Rico/8/34 (H1N1)], A/PC [A/Port Chalmers/1/73 (H3N2)] and A/Vietnam/1203/2004 [H5N1] were grown in 10-day-old embryonated chicken eggs. Virus stocks were prepared from allantoic fluid and stored in aliquots at -70 °C. A/Vietnam/1203/2004 was obtained from the WHO Collaborating Centre for Reference and Research on Influenza (Melbourne, Australia).

Virus titration

Virus content for A/PR8 and A/PC was determined by standard plaque assay on MDCK cells. Briefly, serial dilutions of virus stocks were pipetted on MDCK cells monolayers in 6-well tissue culture plates. After 1 h adsorption, monolayers were overlaid with Eagle’s minimum essential medium with Earle’s salts medium (EMEM) containing 1.8% Bacto-Agar and incubated for 2-3 days. Cell monolayers were stained
with 2.5% crystal violet solution and the plaques enumerated. Virus titres for these stocks were $2 \times 10^8$ PFU/ml and $1 \times 10^7$ PFU/ml, respectively.

Titration of H5N1 infectivity was routinely undertaken by infection of replicate Vero cell monolayers with 10-fold serial dilutions of sample in EMEM containing 10% FCS and antibiotics. Plates were incubated at 37 °C for 5 days in 5% CO₂, and the number of replicate wells in each dilution series with cytopathic effect determined. Titration of stock virus in 13-week-old female BALB/c mice was performed by intranasal inoculation of groups of 5 mice with 35 µl of 10-fold serially diluted virus in PBS. Mice were monitored for development of signs consistent with influenza infection for 10 days and were euthanised if their clinical status met any of the following criteria: loss of 20% or more of the pre-challenge body weight, development of neurological signs or an inability to eat or drink. Both 50% tissue culture infectious dose (TCID₅₀) and 50% mouse infectious dose (MID₅₀) titres were calculated as described previously (Cottey, Rowe et al. 2001). For the stock H5N1 virus, the titre was $10^{9.0}$ TCID₅₀/ml or $10^{7.0}$ MID₅₀/ml.

**Virus inactivation**

Viruses were purified using chick erythrocytes as previously described (Laver 1969). Briefly, infectious allantoic fluids were incubated with chicken red blood cells (CRBCs) for 45 min at 4 °C allowing the virus to bind to CRBCs. Then, stocks were centrifuged at 1200 x g for 10 min and supernatants discarded. Pellets (CRBCs and attached viruses) were resuspended in normal saline and incubated for 1 h at 37 °C to release the CRBCs from the virus. Following incubation, samples were centrifuged (1200 x g for 10 min), the supernatants collected and virus titres determined by plaque assays on MDCK cells. The purified stocks with titres of $5 \times 10^8$ PFU/ml (A/PC) and $9 \times 10^8$ PFU/ml (A/PR8) were stored at -70 °C. For gamma-ray inactivation, influenza virus stocks (thawed and frozen stocks) were exposed to various doses of gamma-irradiation from a $^{60}$Co source (Australian Nuclear Science and Technology Organization – ANSTO) and tested for residual infectivity by plaque assay on MDCK cells and/or by standard hemagglutination assay, as previously described (Szretter, Balish et al. 2006), following incubation of irradiated viruses in embryonated chicken eggs.

**Protection experiment**
Mice were vaccinated with gamma-inactivated A/PC by different routes of immunization: intranasal, intravenous, intraperitoneal or subcutaneous vaccination. For intranasal and subcutaneous routes, avertin-anesthetized mice were vaccinated with 32 μL of normal saline containing $3.2 \times 10^6$ PFU equivalents. For intravenous and intraperitoneal routes, mice were vaccinated with 100 μL of normal saline containing $3.2 \times 10^6$ PFU equivalents of gamma-inactivated A/PC.

For H3 studies, 3 weeks following vaccination, mice were challenged intranasally with $7 \times 10^2$ PFU (MLD$_{50}$) of live virus A/PR8, and mice were weighed prior to infection and then daily for a period of 20 days. For analysis of lung histologies, 3 mice were euthanised on day 21 post-challenge.

For H5 studies, 4 weeks following vaccination with gamma-inactivated A/PR8, mice were challenged with 35 μl (3 x MID$_{50}$) of H5N1 virus. Two or three mice from each group were euthanised on day 3 and 6 post-challenge, respectively, and lungs were collected for determination of viral genetic load and infectivity. Tissues were diced using separate sterile disposable scalpels and stored in PBS, on ice, until transferred to -70 °C for longer term storage. The remaining 10 mice in each group were weighed daily (twice daily once 15% body weight loss was detected), examined twice daily for signs consistent with H5N1 infection, and euthanised according to the experimental endpoints described earlier for determination of MID$_{50}$.

**Lung-histology**

Lung tissue samples were fixed for a minimum of 24 h in 10% neutral buffered formaldehyde. 10 μm sections were stained with haemotoxilin-eosin by the John Curtin School of Medical Research histology unit and evaluated by light microscopy.

**$^{51}$Cr-release assay**

Influenza-specific Tc cells were generated by intravenously injecting 10-week-old BALB/c mice with various doses of either live A/PC or gamma-inactivated A/PC. For kinetics studies, splenocytes were harvested at different time points after immunization. For dose-response studies, splenocytes from infected or vaccinated mice were harvested at 6 days post immunization and tested for their killing activity against
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A/PC- or A/PR8-infected target cells using Cr\(^{51}\) release assays, as previously described (Mullbacher, Lobigs et al. 1999). RBC-depleted splenocytes (effectors) were mixed with labelled targets at different ratios and incubated for 8 h at 37 °C in a humidified atmosphere containing 5% CO\(_2\). The level of radioactivity in supernatants was measured and the specific lysis calculated using the formula: (experimental cpm – spontaneous cpm)/(maximal release cpm – spontaneous cpm) x 100.

For interpolation, a log regression equation was determined based on specific lysis values at E:T ratio of 120:1, 40:1, 13.3:1 and 4.4:1. E:T ratio of 40 was used to interpolate specific lysis values using an equation \(Y = a + bX\), where \(X\) is the E:T ratio and \(Y\) is the specific lysis value.

**TNF and IFN-gamma levels in blood**

Blood samples were collected from live A/PC or gamma-inactivated intravenously immunized BALB/c mice at day 1, 2 and 3 post immunizations (two mice per group). Serum TNF and IFN-gamma levels were tested using CBA mouse inflammatory cytokine kit (BD Biosciences).

**Homogenisation of tissues**

Lung and brain homogenates were generated in 1 ml of PBS using a Mini-BeadBeater-8 (Biospec Products, USA), and adjusted to 10% (w/v) in PBS prior to extraction of viral RNA or titration of infectivity.

**Real-time RT PCR**

Following the addition of 100 µl of lung homogenate into 600 µl of RLT buffer, RNA was extracted using an RNeasy Mini Kit (Qiagen) according to the manufacturer’s instructions. The total RNA concentration of each sample was determined by spectrophotometry and adjusted to 40 ng/µl with nuclease free water. Standardised amounts (200 ng) of template were subsequently reverse transcribed with random hexamer primers using the TaqMan Reverse Transcription Reagents kit (Applied Biosystems) in 20 µl reactions following the recommendations of the manufacturer. For quantitation of viral cDNA, universal influenza virus type A-specific primers and
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TaqMan probe, which amplified and detected a product from within the viral matrix gene, were used (Heine, Trinidad et al. 2007). Reactions were performed in triplicate and contained 12.5 μl of TaqMan 2X Universal PCR Master Mix, 900 nM of each primer, 250 nM of probe, 2 μl of cDNA template and 6.8 μl of water. Separate triplicate reactions to quantify 18S rRNA (TaqMan Ribosomal Control Reagents, Applied Biosystems) were also performed to exclude the presence of PCR inhibitors in all samples tested. Reactions were performed in 96-well plates using the 7500 Fast Real-Time PCR System (Applied Biosystems) and the following cycling parameters: 50 °C for 2 min; 95 °C for 10 min; 45 cycles of 95 °C for 15 sec and 60 °C for 1 min. For relative quantitation of viral genetic loads, a standard curve was generated using, as template, 10-fold serial dilutions of extracted stock virus RNA in 40 ng/μl of RNA prepared from uninfected mouse lung. To facilitate interpretation of data, 1 unit (1 U) of viral RNA was arbitrarily defined as the number of RNA molecules which, when reverse transcribed and subjected to real-time PCR, produced a C_T value of 38.

2.4 Results

2.4.1 Gamma irradiation with 5 kGy fully inactivates influenza A virus

Gamma-ray dose titration studies of frozen and room temperature-kept viral stocks were undertaken to define the conditions providing sterile virus preparations with optimal immunogenicity. Influenza virus stocks were exposed to a radiation dose of either 5, 10 or 20 kGy and hemagglutination assays were performed to detect any residual infectious viruses following amplification in embryonated eggs. A radiation dose of 5 kGy was sufficient to fully inactivate both frozen or room temperature-kept influenza viruses while maintaining T cell immunogenicity (Table 2.6.1). To grant a safety margin for sterility, a dose of 10 kGy was used to prepare gamma-inactivated influenza virus preparations throughout the study.

2.4.2 The induction and magnitude of cross-reactive Tc cell responses by gamma-irradiated A/PC is dose-dependent
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To characterize the cytolytic function of T cells in vaccinated mice, mice were intravenously immunized with either live or gamma-inactivated A/PC and the splenocytes were harvested at various times post immunization and tested for their killing activity on mock or A/PC-infected P815 target cells using a standard $^{51}$Cr-release cytotoxicity assay. Immunization with live A/PC or gamma-inactivated A/PC induced significant cytolytic activities, reaching their peaks at ~6 days (Figure 2.6.1 A & B). On day 6 post immunization with gamma-inactivated A/PC, the Tc cell response reached 44.3% of A/PC-infected target cell lysis and was similar to that developed in the live infection control.

I next examined the effect of the immunization dose of gamma-inactivated A/PC on the induction and magnitude of Tc cell responses. Mice were intravenously immunized with various doses of either live or gamma-inactivated A/PC and their splenocytes were tested for specific target cell killing 6 days post-immunization. Live A/PC elicited strong Tc cell responses over a wide range of immunization doses (Figure 2.6.1C). Immunization with a high dose ($10^8$ PFU equivalent) of gamma-irradiated A/PC also elicited strong Tc cell responses against both A/PC and A/PR8 infected targets (Figure 2.6.1D). However, immunization with low doses ($2 \times 10^7$ PFU equivalent or less) did not induce significant cross-reactive Tc cell responses. Thus, the magnitude of the Tc cell response by gamma-irradiated A/PC correlates closely with immunization dose.

2.4.3 Gamma-inactivated A/PC elicits IFN-gamma and TNF responses

I measured serum levels of IFN-gamma and TNF in mice at various time points following immunization with either live or gamma-inactivated A/PC (Figure 2.6.2A & B). Both live and gamma-inactivated A/PC induced elevated serum IFN-gamma levels, each reaching their peaks on day 1 post immunization. Serum IFN-gamma levels declined rapidly to near background levels on day 2 post immunization. As for TNF, a similar trend was observed in terms of the magnitude and kinetics. High serum TNF levels were seen in mice immunized with live or gamma-A/PC at day 1 post immunization and serum TNF levels dropped close to the background levels by day 2 post immunization. These results show that gamma-inactivated A/PC is capable of stimulating pro-inflammatory cytokine responses in vivo.
2.4.4 Intranasal immunization elicits superior heterosubtypic protection by gamma-inactivated influenza virus.

The cross-protective efficacy of gamma-inactivated influenza preparation was assessed for each of four different immunization routes: intranasal, intraperitoneal, intravenous and subcutaneous administration. 10-week-old BALB/c mice were immunized with gamma-inactivated A/PC (3.2 x 10^6 PFU equivalent) and challenged 3 weeks later with a lethal dose of heterosubtypic strain, A/PR8 (7 x 10^2 PFU). Mice immunized with intravenous, intraperitoneal or subcutaneous route developed significant weight loss and resulted in ~100% mortality rate (Figure 2.6.3A, C, D, E & F). In contrast, the intranasally immunized mice all survived with a mean maximum weight loss of ~5% (Figure 2.6.3B & F). These results show that intranasal administration is the most effective route to induce cross-protection by gamma-inactivated influenza virus preparation.

2.4.5 Minimal influenza infection-induced lung inflammation after gamma-inactivated A/PC vaccination

Three weeks following intranasal vaccination (3.2 x 10^6 PFU equivalent) with gamma-inactivated A/PC, mice were challenged with a heterosubtypic strain, A/PR8. Lungs of surviving mice were harvested 21 days post-challenge and lungs were processed for histology. Lungs of vaccinated mice exhibited localised inflammation with weak lymphocyte infiltration (Figure 2.6.4A & B), but overall similar to lungs of naïve mice (Figure 2.6.4C & D). In contrast, lung tissues from unvaccinated, A/PR8-challenged, mice contained extensive inflammatory infiltrates (Figure 2.6.4E & F). Thus, vaccination with gamma-inactivated A/PC prevents development of severe lung inflammation in mice during heterosubtypic infections.

2.4.6 Intranasal vaccination with gamma-inactivated A/PR8 protects against lethal H5N1 challenge
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To determine the extent to which the heterotypic immunity induced by gamma-inactivated preparations of human influenza A viruses extends to avian strains, I tested the protective efficacy of gamma-inactivated A/PR8 using a mouse model of H5N1 highly pathogenic avian influenza. BALB/c mice (15 mice/group) were vaccinated intranasally with a single dose of gamma-inactivated A/PR (3.2x10^6 PFU equivalents). Four weeks later, under Biosecurity Level 3 enhanced containment, mice were challenged intranasally with 3 x MID_{50} of A/Vietnam/1203/2004 (H5N1) and were monitored for development of clinical signs and loss of body weight (Figure 2.6.5). The experimental end points were based on: weight loss of 20% or more; development of any neurological sign; or inability to eat or drink were used for this protection experiment. The majority of unvaccinated mice rapidly lost weight, reaching an experimental end point by between days ~7 and 9 (Figure 2.6.5A & C). In contrast, all the vaccinated mice successfully survived the challenge and showed a mean maximum weight loss of 5.6% (Figure 2.6.5B & C). Thus, cross-protective immunity induced by gamma-inactivated influenza virus effectively protects mice against disease caused by avian H5N1 challenge.

I investigated the effect of vaccination on pulmonary viral load at days 3 and 6 after a H5N1 challenge. Two or three mice from vaccinated or mock treated group were euthanised on day 3 and 6 post-challenge, respectively, for determination of viral genetic load and infectivity in lung. Both viral infectivity and viral RNA were detected at day 3 and 6 in all unvaccinated mice (Table 2.6.2). In contrast, only 1 out of 2 vaccinated mice showed presence of H5N1 virus in lungs at day 3 and at day 6 viruses were undetected (Table 2.6.2). Thus, while vaccination with gamma-inactivated A/PR8 may not prevent H5N1 infections in mice, it facilitates faster viral clearance.

2.5 Discussion

A number of studies have emphasized the importance of infectivity and replicative capacity of the virus in inducing effective immune responses (Braciale and Yap 1978; Webster and Askonas 1980). This was demonstrated by a loss in immunostimulatory capacity with virus inactivation (Braciale and Yap 1978; Webster and Askonas 1980; Moran, Park et al. 1999; Blazevic, Trubey et al. 2000). Here I report
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that fully inactivated influenza virus, achieved by gamma-irradiation, is as effective as live virus in eliciting Tc cell responses. Most importantly, gamma-inactivated influenza virus preparations provided heterosubtypic immunity against both human and avian influenza virus strains.

It has previously been shown that exposure to 0.65 kGy of gamma-ray causes a total loss of influenza virus infectivity, but disrupting the hemagglutination activity requires an exposure to more than 200 kGy (Agency 1973). Thus, gamma-irradiation has a wide margin between the dose required to sterilize the virus and that of detrimental impact on antigenic properties. Consistent with the above, exposure of influenza virus to a dose of 5 kGy completely abolished its ability to replicate. Yet, even after exposure to irradiation doses of up to 20 kGy, influenza virus preparations maintained T cell immunogenicity (as demonstrated by a $^{51}$Cr release assay).

Immunization with gamma-irradiated influenza virus can elicit similar T cell responses to that induced by live virus, although gamma-irradiated virus requires > 100 fold higher immunization doses than infectious virus. A reduction in the immunizing dose of gamma-inactivated virus dramatically decreased the Tc cell response, whereas lowering the immunization dose of live virus had essentially no effect over the range investigated. For live viruses, a initial priming dose may not affect the magnitude of Tc cell responses because they can replicate to generate more antigens required for strong immune responses. An implication of this finding is that, if cross-protection is Tc cell dependent, low immunization doses may not elicit the full protective potential of gamma-inactivated virus.

Gamma-inactivated A/PC stimulated pro-inflammatory cytokines, IFN-gamma and TNF, responses. The levels of pro-inflammatory cytokines detected in mice immunized with the gamma-inactivated A/PC were similar to those detected in mice that received live A/PC. However, this comparison may not be appropriate since gamma-inactivated A/PC were inoculated at a higher dose than the live A/PC (1 x $10^8$ PFU equivalent for gamma-inactivated A/PC and 2 x $10^6$ PFU for live A/PC). The higher immunization dose was chosen for gamma-inactivated virus for the above-mentioned finding that gamma-inactivated viruses require higher doses than live virus to induce strong Tc cell responses. However, assuming that live virus replicates to some extent in mice the antigen load in vivo may be more similar than the above doses. In any case, pro-inflammatory cytokines IFN-gamma and TNF are indicators of Th-1 cell-mediated immune responses (Romagnani 1991). Thus, in contrast to the general notion
that inactivated viruses induce predominantly Th-2 responses (Moran, Park et al. 1999; De Swart, Kuiken et al. 2002), gamma-inactivated viruses are capable of inducing Th-1 responses. A complete cytokine profile, including Th2 cytokines, is needed to determine if Th1/Th2 polarization occurs as a result of vaccination with gamma-inactivated virus.

The most important finding in the present study is that gamma-inactivated human influenza virus confers protection against avian H5N1 challenge. However, this protection was not a sterilizing immunity since viral infectivity and viral RNA were detected in 1 vaccinated mouse at day 3, but not at day 6 post infection. An early viral clearance in vaccinated mice suggests that the observed cross-protection is mediated by an early influx of memory T cells into the lungs of challenged mice. The role of T cells in the observed cross-protection elicited by gamma-inactivated influenza virus is investigated and discussed in Chapter 4.

Given the high pathogenicity of the H5N1 strain, it was surprising that the cross-protective immunity induced by human influenza virus irradiated with gamma-ray is highly effective against H5N1 infections in mice. This was even more remarkable given that only a single dose, without any adjuvants, was used. If the observed cross-protective property of gamma-inactivated virus were reproduced in humans, this vaccine approach would be highly valuable in future influenza pandemics. Furthermore, a vaccine that is Tc cell-dependent can potentially accommodate the viral potential for antigenic changes and therefore expected to be effective in any influenza season, regardless of unpredictable emergence of antigenic variations.
2.6 Tables and Figures

Table 2.6.1 Dose response of gamma-irradiation and viral infectivity

<table>
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<tr>
<th>Irradiation condition(^a)</th>
<th>Gamma ray irradiation dose (kGy)</th>
<th>Hemagglutination activity*</th>
<th>Plaque formation*</th>
<th>\textit{Ex vivo} Tc cell responses(^b)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Room temperature</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>5</td>
<td>- (\dagger)</td>
<td>-</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>10.4</td>
<td>-</td>
<td>-</td>
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<td>+</td>
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<tr>
<td>20.5</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
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<tr>
<td>Frozen</td>
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<tr>
<td>4.9</td>
<td>-</td>
<td>-</td>
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<td>9.6</td>
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<td>+</td>
</tr>
<tr>
<td>19.2</td>
<td>-</td>
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<td>+</td>
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</tbody>
</table>

* Sterility determined by plaque assay on MDCK cells and hemagglutination assay following amplification of residual infectious virus in embryonated eggs.

\(^a\) Irradiations were carried on dry ice, and at ambient facility temperature (approx. 18 °C) at a dose rate of 4.40 kGy/hr, using ANSTO’s Gammacell 220 (containing \(^{60}\)Co sealed sources)

\(\dagger\) Undetectable

\(^b\) induction of Tc cell responses were assessed in a \(^{51}\)Cr release assay.
Table 2.6.2 H5N1 infectivity and viral genetic loads in lung

<table>
<thead>
<tr>
<th>Day post-challenge</th>
<th>Treatment</th>
<th>Mouse</th>
<th>Infectivity*</th>
<th>Genetic load*</th>
</tr>
</thead>
<tbody>
<tr>
<td>3</td>
<td>Mock</td>
<td>1</td>
<td>6.5</td>
<td>3.4 ± 0.03</td>
</tr>
<tr>
<td></td>
<td></td>
<td>2</td>
<td>5.8</td>
<td>2.0 ± 0.02</td>
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<tr>
<td></td>
<td>Vaccinated</td>
<td>1</td>
<td>-†</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td></td>
<td>2</td>
<td>7.4</td>
<td>3.6 ± 0.02</td>
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<tr>
<td>6</td>
<td>Mock</td>
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<td>6.4</td>
<td>4.2 ± 0.003</td>
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<td>3</td>
<td>7.4</td>
<td>4.4 ± 0.03</td>
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<td></td>
<td>Vaccinated</td>
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<td>-</td>
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<td>3</td>
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</tbody>
</table>

* Viral infectivity and relative viral genetic loads are expressed as log\textsubscript{10} TCID\textsubscript{50}/g and log\textsubscript{10} U / 20 ng of extracted RNA (geometric mean ± s.d. of triplicate reactions), respectively, where 1 unit (1 U) of viral RNA is arbitrarily defined as the number of RNA molecules which, when reverse transcribed and subjected to real-time PCR, produced a \( C_T \) value of 38.

† Undetectable (< 10\textsuperscript{3.2} TCID\textsubscript{50} g\textsuperscript{-1} (infectivity) or < 1 U per 20 ng of extracted RNA (genetic load)).
Chapter 2: Intranasal influenza vaccine

Figure 2.6.1 Kinetics and dose dependence of primary Tc cell responses induced by live A/PC and gamma-inactivated A/PC.

For kinetics study, BALB/c mice were intravenously immunized with either live A/PC (2 x 10⁶ PFU) or gamma-inactivated A/PC (1 x 10⁸ PFU equivalent) and splenocytes were harvested at various time points (A &B). For dose dependence study, groups of two BALB/c mice were immunized intravenously with various doses of either live A/PC or gamma-inactivated A/PC and splenocytes were harvested on day 6 post immunization. Splenocytes were used as effector cells against P815 target cells alone or infected with virus (A/PC or A/PR8). Each point/bar represents the mean percent ± S.D. Specific lysis values were interpolated from a linear regression curve at effector:target ratio of 60:1.
Figure 2.6.2 Comparison of serum inflammatory cytokine, IFN-gamma and TNF, levels in mice immunized intravenously with either live or gamma-inactivated A/PC.

Mice were infected with either live A/PC (2 x 10^6 PFU) or immunized with gamma-inactivated A/PC (1 x 10^8 PFU equivalent). Sera of individual mice were tested for indicated cytokines and means ± S.D of two mice per group are shown.
Chapter 2: Intranasal influenza vaccine

Figure 2.6.3 Intranasal vaccination with gamma-inactivated A/PC provides superior protection to heterosubtypic challenge.

Groups of ~10 BALB/c mice were either mock treated (A) or vaccinated with gamma-inactivated A/PC (3.2 x 10⁶ PFU equivalents) intranasally (B), intravenously (C), intraperitoneally (D) or subcutaneously (E). Mice were challenged intranasally after 3 weeks with a lethal dose (7 x 10² PFU) of A/PR. Survival (G) and weight loss of infected mice was monitored for 20 days (G). * P < 0.05 vs. control naïve group; Fisher’s exact test.
Figure 2.6.4 Vaccination with gamma-inactivated A/PC limits lung inflammation.

Three weeks following intranasal vaccination with gamma-inactivated A/PC, mice were challenged with A/PR8 (A & B). Lungs were harvested at day 21 post challenge and the lung histology was compared to that of naïve (C & D) and infected mice (E & F).
Figure 2.6.5 Intranasal vaccination with gamma-inactivated A/PR8 protects mice against H5N1 challenge.

Groups of 10 BALB/c mice were either mock treated (A) or vaccinated with gamma-inactivated A/PR8 (B) and at 4 weeks post immunization mice were intranasally challenged with 3 x MID50 of A/Vietnam/1203/2004[H5N1] intranasally. Survival (C) and weight loss of infected mice were monitored for 20 days. *: P < 0.05 vs. control naïve group; Fisher’s exact test.
2.7 References


Chapter 3

A comparison of the immunogenicity and vaccine efficacy of formalin-, gamma ray- and UV-inactivated influenza A viruses and split vaccine preparations in mice

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3.1 Abstract

Gamma-irradiation was utilized for candidate vaccine preparations (gamma-inactivated A/PC) to investigate its influenza strain cross-protective potential in comparison to formalin- and UV-inactivated virus. A single dose of gamma-ray inactivated A/PC (H3N2) conferred highly significant protection in mice against both homologous and heterosubtypic virus challenges. This contrast with only incomplete protection obtained with single doses of immunization using formalin-, UV-inactivated virus or the present commercially available trivalent influenza vaccine. A multiple immunization regime was required for formalin-inactivated virus preparation to protect mice against a homologous challenge, but even this regimen did not induce cross-protective immunity. The highly immunogenic gamma-inactivated A/PC, but not formalin- or UV-inactivated A/PC elicits T cell responses that are most likely responsible for the cross-protective, long-lasting immunity against highly lethal influenza A infections in mice.
3.2 Introduction

Infectious diseases, such as influenza, remain one of the leading causes of death in the human population and vaccination represents the most cost effective and efficient defence against virus induced morbidity and mortality. Live attenuated virus based vaccines have been the most successful for a number of infectious diseases including polio, yellow fever and smallpox. However, in regard to influenza, whole virus vaccines are associated with adverse reactions, especially in children, and consequently are little used (Salk 1948; Quilligan, Francis et al. 1949). To reduce reactogenicity, most influenza vaccines used today are split product vaccines or surface antigen vaccines from chemically inactivated viruses, containing only purified hemagglutinin and neuraminidase, the outer surface proteins of influenza virus. These vaccines, however, are less immunogenic than whole virus vaccines (Barry, Staton et al. 1974; Barry, Mayner et al. 1976; Ortbals and Liebhaber 1978), most likely because their antigenic structures have been altered by chemical treatment and they fail to induce T cell memory.

The protective efficacy of current subunit or split influenza vaccines rely exclusively on the induction of neutralizing antibodies against viral surface proteins, hemagglutinin and neuraminidase (Palese 2006). These neutralizing antibodies are generally not cross-reactive among subtypes (Ada and Jones 1986). Thus, the influenza vaccine is only effective against viruses whose antigenicity is the same or at least very similar to the vaccine strains. Accordingly, the composition of influenza vaccines needs to be updated each year to cover the prevailing circulating variants.

In general, preserving the integrity of the immunological epitopes is important for vaccine efficacy. A number of inactivation methods have been applied for influenza virus, including chemical treatment (Goldstein and Tauraso 1970; Redfield, Richman et al. 1981; Budowsky, Friedman et al. 1991; Budowsky, Smirnov Yu et al. 1993; Takada, Matsushita et al. 2003), UV-irradiation (Goldstein and Tauraso 1970; Kantorovich-Prokudina, Kaverin et al. 1978; Polianskaia 1979; Budowsky, Bresler et al. 1981; Zheleznova 1982), ionising radiation (Kulevich and Kosiakov 1974; Fridman, Ermolaev et al. 1979; Migunov, Aron et al. 1986; Noack, Tischner et al. 1986; Pang, Clancy et al. 1992; Lidbury, Grissell et al. 1997; Lowy, Vavrina et al. 2001), and heat treatment (De Flora and Badolati 1973; De Flora and Badolati 1973; Blazevic, Trubey et al. 2000).
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The currently used inactivation method for the influenza vaccines is chemical treatment with formalin or β-propiolactone (Knipe and Howley 2001). Chemical treatment induces extensive interaction and cross-linking among viral proteins (Jackson 1978; Martinson, True et al. 1979). In this process, the viral suspensions are kept unfrozen and physical as well as chemical agents degrade the antigenic virus proteins. In contrast, gamma-irradiation can be applied to frozen viral samples, preventing unwanted antigenic degradation. The primary target by which radiation brings about virus inactivation is via disruption of viral nucleic acids, in case of influenza, RNA, rather than of viral proteins (De Benedictis, Beato et al. 2007). Therefore, gamma-irradiation inactivates virus infectivity without having a detrimental impact on the antigenic structure and biological integrity of proteins (Lowy, Vavrina et al. 2001), presenting a potential advantage over traditional methods for inactivation of virus infectivity.

Müllbacher et al have previously suggested the use of gamma ray inactivated influenza A virus preparation as a vaccine candidate (Müllbacher, Ada et al. 1988; Müllbacher, Lobigs et al. 2006) and we have found that a single intranasal administration of gamma-irradiated A/PR8 induces cross-protective immunity against lethal heterosubtypic infections, including an avian H5N1 strain (Alsharifi, Furuya et al. 2009). Thus, gamma-irradiated influenza virus preparations represent a new approach for a cross-protective influenza vaccine. Here, I have compared the ability of gamma-ray, formalin- and UV- inactivated virus preparations to induce cross-protective immunity. I show that gamma-inactivated virus is substantially superior to other forms of inactivated virus preparations, including a currently used trivalent influenza vaccine.

3.3 Material and Methods

Mice

Nine- to ten-week-old female BALB/c mice were routinely used in these studies. Mice were obtained and housed in Biosecurity Level 2 containment facilities at the John Curtin School of Medical Research, the Australian National University, ACT, Australia. All experimental procedures were approved by the institutional Animal Ethics Committees.
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Viruses and cells

P815 mastocytoma, Madin-Darby canine kidney (MDCK) and baby hamster kidney (BHK) cells were grown and maintained in EMEM supplemented with 5% FCS, 2.2 gL⁻¹ NaHCO₃, 0.29 gL⁻¹ L-glutamine and PSN antibiotics (0.12 gL⁻¹ penicillin G, 0.2 gL⁻¹ streptomycin sulphate, 0.2 gL⁻¹ neomycin sulphate) at 37 °C in a humidified atmosphere with 5% CO₂.

The influenza type A viruses, A/PR/8 [A/Puerto Rico/8/34 (H1N1)] and A/PC [A/Port Chalmers/1/73 (H3N2)] were grown in 10-day-old embryonated chicken eggs. Each egg was injected with 0.1 ml normal saline containing 1 hemagglutinin unit (HAU) of virus, incubated for 48 h at 37 °C, and held at 4 °C for overnight. The amniotic/allantoic fluids were then harvested, pooled and stored at -80 °C. Titres were 10⁷ PFU/ml (A/PC) and 2 x 10⁸ PFU/ml (A/PR8) using plaque assays on MDCK cells. Viruses were purified using chicken red blood cells for vaccine preparation as previously described (Sheffield, Smith et al. 1954). Briefly, infectious allantoic fluid was incubated with red blood cells for 45 min at 4 °C allowing the hemagglutinin to bind red blood cells, and then centrifuged to remove the allantoic fluid supernatant. The pellets were suspended in normal saline, incubated for 1 h at 37 °C to release the red blood cells from the virus and then centrifuged to remove the red blood cells and collect the virus in the supernatant. Purified A/PC stock titre was 5 x 10⁸ PFU/ml.

Virus inactivation

For formalin inactivation, the viruses were incubated with 0.2% formalin at 4 °C for a week (Takada, Matsushita et al. 2003). The formalin was then removed by pressured dialysis using normal saline for 24 h at 4 °C. The dialysis method was adapted from Current Protocols in Immunology (Andrew, Titus et al. 2001). For UV inactivation, the viruses were placed in 60-mm petri dishes with a fluid depth of 10 mm. The virus was exposed to 4000 ergs per cm² for 45 min at 4 °C. For gamma ray inactivation, influenza viruses received a dose of 10 kGy from a ⁶⁰Co source (Australian Nuclear Science and Technology Organization – ANSTO). The virus stocks were kept
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frozen on dry ice during gamma irradiation. Loss of viral infectivity was confirmed by titration of inactivated virus preparations in eggs. The HAU titres of inactivated virus stock were determined to be 7.29 x 10^4 HAU/ml for gamma-inactivated A/PC, 2.43 x 10^4 HAU/ml for formalin- and UV-inactivated A/PC. For freeze-drying, one vial containing 0.5 ml of gamma-inactivated A/PC was placed in a Manifold Freeze-Dryer (FTS SYSTEMS, Dura-Dry™ MP).

Hemagglutination assay

Live and inactivated virus preparations were serially diluted in a 100 µl volume on 96-well U-bottom microtiter plate. 0.5% chicken red blood cell suspensions were added to all wells and plates were incubated for 30 min on ice. This method was adapted from Current Protocols in Microbiology (Szretter, Balish et al. 2006).

Protection experiment

BALB/c mice were immunized intranasally with inactivated virus preparations (3.2 x 10^6 PFU equivalent) or trivalent inactivated subvirion influenza vaccine (CSL fluvax vaccine; A/Solomon Islands/3/2006 H1N1, A/Brisbane/10/2007 H3N2, B/Florida/4/2006; 3 µg hemagglutinin). The formalin-inactivated A/PC vaccinated mice were re-immunized once or twice 2 and 3 weeks later. For lethal challenge, at 1-3 weeks post-immunization, mice were infected intranasally with 50% mouse lethal dose (MLD50). MLD50 was determined to be 7 x 10^2 PFU and 3.2 x 10^5 PFU for A/PR8 and A/PC, respectively, in preliminary experiments. For analysis of lung virus titers, 3 mice were euthanized on day 3 and 6 post-challenge. The remaining animals were monitored for body weight and mortality until day 20 post-challenge.

Plaque assay

The lung tissue samples were collected 3 and 6 days after intranasal challenge. After removal, whole lungs were homogenized in normal saline. Homogenates were
centrifuged at 1500 rpm for 5 min. Supernatants were collected and were stored at -70 °C. Serial dilutions of the samples were inoculated on MDCK cells cultured on 6-well tissue culture plates. After 1 h adsorption, the cells were overlaid with EMEM medium containing 1.8% Bacto-Agar. After incubation for 2-3 days, cell monolayers were stained with 2.5% crystal violet solution and the plaques were enumerated.

$^{51}$Cr release assay

BALB/c mice were immunised intravenously (200 µl/mouse) or intranasally (32 µl/mouse) with live or inactivated (gamma-irradiated, formalin-, or UV-inactivated) A/PC. Seven days post immunization, RBCs depleted splenocytes or pulmonary lymphocytes were used as effector cells in $^{51}$Cr release assays, as described previously (Müllbacher, Hill et al. 1991). P815 target cells (1x10^6 cells) were infected with 1 PFU/cell of live A/PC or A/PR8 and incubated for 1 h in the presence of 100 µCi/ml of $^{51}$Cr. Targets were washed twice and incubated with effector cells at different ratios in a 8 h $^{51}$Cr release assay. The level of radioactivity in the supernatant was measured using Top Counter (company). Specific lysis is given as mean percent lysis of triplicate wells and values were calculated using the formula: (experimental release – spontaneous release)/(maximal release – spontaneous release) x 100.

For interpolation, a log regression equation was determined based on specific lysis values at E:T ratio of 120:1, 40:1, 13.3:1 and 4.4:1. E:T ratio of 40 was used to interpolate specific lysis values using an equation $Y = a + bX$, where X is the E:T ratio and Y is the specific lysis value.

3.4 Results

3.4.1 The effect of virus inactivation on hemagglutination activity

Hemagglutination activity after virus inactivation provides one indicator as to the denaturing effect of the sterilization treatment. Purified influenza stock was aliquotted into batches and treated with either formalin, UV- or gamma-irradiation. Following complete inactivation of infectivity as verified by the absence of virus
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growth in embryonated eggs, I compared hemagglutination activity of live and inactivated viruses (Table 3.6.1). Hemagglutination activity was reduced by 3-fold for gamma-inactivated viruses, whereas formalin- and UV-inactivation resulted in 9-fold reduced hemagglutination titres. These results provide evidence that, of these three virus sterilization methods, gamma-inactivation denatures viral protein structure least.

3.4.2 Gamma-irradiated, but not formalin- or UV-inactivated, virus preparations induce heterosubtypic immunity

I compared the protective efficacy of gamma-irradiated, formalin-, or UV-inactivated influenza virus preparations against homologous and heterosubtypic live virus challenges. 10-week-old BALB/c mice (~10 mice/group) were immunized intranasally with a single dose of inactivated A/PC (3.2 x 10^6 PFU equivalent). Three weeks later, mice were challenged in with 1 x MLD_{50} of homologous A/PC (3.2 x 10^5 PFU) or heterosubtypic A/PR8 (7 x 10^2 PFU). Mice were monitored for 20 days for mortality and weight loss. As shown in Figure 3.6.1 A, E, F & J, intranasal infection of naïve mice with A/PC or A/PR8 caused a rapid weight loss with 90-100% mortality (based on 25% weight loss as an end point). Mice immunized with either formalin-inactivated A/PC (Figure 3.6.1B & E) or UV-inactivated A/PC (Figure 3.6.1C & E) also developed significant weight loss and resulted in ~70% mortality when challenged with live homologous virus. When similarly vaccinated mice were challenged with the heterosubtypic strain A/PR8, the animals lost substantial body weight with 90~100% mortality (Figure 3.6.1G, H, & J). In both cases, homologous and heterosubtypic challenge, the induced protection was considered inadequate to be used as a vaccine (P-value > 0.05, Fisher's exact test). In contrast, mice immunized with a single dose of gamma-inactivated A/PC were not only protected against homologous virus challenge, but also against heterosubtypic challenge, with mice losing only 5% of their body weight on average (Figure 3.6.1D, E, I & J). Hence, intranasally administered gamma-inactivated influenza virus is the most effective vaccine preparation and route of administration to induce protective immunity against homologous and heterosubtypic influenza virus challenges.
3.4.3 Can multiple doses of formalin-inactivated influenza virus preparation enhance the protective effect?

Previous studies have shown that multiple intranasal administrations of formalin-inactivated preparations can provide protection against a lethal heterosubtypic challenge (Takada, Matsushita et al. 2003). Although gamma-inactivated A/PC was clearly more effective after only one dose, I determined whether the weak protective efficacy of formalin-inactivated A/PC could be improved by testing different immunization schedules. 10-week-old BALB/c mice were immunized either once, twice or three times with formalin-inactivated A/PC (2.3 x 10^3 HAU per single immunization, a dose equivalent to gamma-inactivated A/PC based on HAU). The vaccinated mice were challenged with a homologous strain, A/PC (3.2 x 10^4 PFU) at three weeks post immunization for mice that received single immunization or at one-week post immunization for mice that received double or triple immunizations. The group of mice that received a single immunization had no improved survival rate compared to that of unvaccinated mice (Figure 3.6.2A, B & E). In contrast, double immunization improved the survival rate to 60% (P < 0.05) although the majority of mice still showed a significant loss in body weight, indicating that they experienced severe illness (Figure 3.6.2C & E). The mice receiving triple immunization with formalin-inactivated A/PC showed complete protection with no mortality and little weight loss (Figure 3.6.2D & E). Triple immunization conferred partial protection from heterosubtypic challenge (P > 0.05) (Figure 3.6.2F, G & H). Thus, formalin-inactivated A/PC requires more doses and fails to elicit cross-protection by intranasal route suggesting that the induced immunity is not only quantitatively, but also qualitatively, substantially inferior to that induced by gamma-inactivated A/PC.

3.4.4 Trivalent flu vaccine is ineffective against drifted strains

For a direct comparison, the protective efficacy of a commercially available trivalent influenza vaccine was tested in my experimental approach. 10-week-old BALB/c mice were immunized once or twice with subvirion trivalent influenza vaccine (CSL fluvax vaccine; A/Solomon Islands/3/2006 H1N1, A/Brisbane/10/2007 H3N2,
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B/Florida/4/2006; 3 μg hemagglutinin) and were challenged at three weeks post immunization. As shown in Figure 3.6.3, single intranasal or double intramuscular immunization of mice conferred no statistically significant protection (P > 0.05) against both A/PC (3.2 x 10^5 PFU) (Figure 3.6.3A, B, C & D) and A/PR8 strain (7 x 10^3 PFU) (Figure 3.6.3E, F, G & H). This clearly shows that the current influenza vaccine does not confer appreciable cross-protection, even against strains within the same subtype.

3.4.5 Gamma-inactivated A/PC vaccine does not prevent infection but facilitates viral clearance

I evaluated the effect of vaccination on pulmonary viral load at days 3 and 6 after heterosubtypic challenge with A/PR8. Mice were vaccinated intranasally with gamma-, formalin-, or UV-inactivated A/PC. Three weeks later, mice were challenged with live A/PR8. Lungs were harvested and virus titres were determined by plaque assay using MDCK cells. High virus titres reaching 10^7 and 10^6 PFU/lung for days 3 and 6 post-infection, respectively, were detected in unvaccinated mice (Figure 3.6.4). Virus titres in the lungs of formalin- and UV-inactivated A/PC immunized mice were comparable to those detected in unvaccinated control mice. In contrast, the gamma-inactivated A/PC vaccinated group showed a > 100-fold reduction of A/PR8 lung virus titres both at days 3 and 6 post-challenge (P < 0.05 using Student’s T test) compared to that seen in unvaccinated control mice.

3.4.6 Gamma-irradiated, but not formalin- or UV-inactivated virus retains Tc cell immunogenicity

I compared the ability of inactivated A/PC virus (gamma-, formalin-, and UV) preparations to generate influenza virus-immune cytotoxic T (Tc) cell responses. BALB/c mice were intravenously immunized with live (10^7 PFU) or inactivated (10^8 PFU equivalent of gamma-, formalin-, or UV-) A/PC, and splenocytes harvested 7 days post immunization and tested for their killing activity against A/PC- and A/PR8-infected P815 target cells. The data show that effector splenocytes harvested from mice immunized with live or gamma-inactivated A/PC lysed A/PC- and A/PR8-infected
target cells, whereas splenocytes from formalin- or UV-inactivated A/PC immunized mice did not (Figure 3.6.5A). Similar results were obtained following intranasal vaccination (Figure 3.6.5B). Therefore, gamma-irradiated, but not formalin- or UV-inactivated, virus induces Tc cell immunity.

3.4.7 Intranasal immunization with gamma-inactivated A/PC confers protection against high dose heterosubtypic challenges.

Given the excellent protective capacity of gamma-inactivated A/PC to protect mice from heterosubtypic challenge, I then aimed at determining the limit of protection by challenging with increased influenza virus doses (Figure 3.6.6), up to 50 x MLD_{50}, which corresponded to the maximal dose experimentally administrable. Immunized mice receiving heterosubtypic challenge of 1 x MLD_{50} all survived and there was little or no weight loss (Figure 3.6.6C & F). Immunized mice given a challenge dose of 5 x MLD_{50} initially lost weight during the first 7 days post-challenge, but not significantly, and all fully recovered (Figure 3.6.6D & F). The mice receiving 50 x MLD_{50} lost on average 8% of body weight but here, too, all mice fully recovered (Figure 3.6.6E & F). Naïve mice receiving 1 x MLD_{50} or 5 x MLD_{50} progressively lost weight and 9/11 and 10/11 mice, respectively, failed to survive the challenge (Figure 3.6.6A, B & F). Thus, intranasal vaccination with gamma-inactivated A/PC confers potent protection against high doses, unlikely to be encountered naturally, of an heterosubtypic challenge.

3.4.8 Long-lived heterosubtypic protection conferred by gamma-inactivated preparations

A critical requirement for an effective influenza vaccine is the induction of persistent heterosubtypic immunity. To determine whether gamma-inactivated A/PC immunized mice maintain long-lasting heterosubtypic immunity, mice were challenged at 3 months post immunization. The vaccinated mice challenged with 1 x MLD_{50} A/PR8 lost in average only up to 10% body weight and fully recovered (Figure 3.6.7B & C). In contrast, the majority of challenged naïve mice lost substantial weight, reaching an end
point of 25% total body weight loss at around 7 days post challenge (Figure 3.6.7A & C).

3.4.9 Freeze-drying does not destroy the immunogenicity of gamma-inactivated-A/PC

A known shortcoming of the current liquid based influenza vaccine is the requirement of refrigerated storage that imposes a problem for vaccine distribution, particularly in developing countries. In an attempt to overcome the stringent storage requirement of the current influenza vaccine, I assessed freeze-drying gamma-inactivated influenza virus as a means to curtail refrigeration requirements. Freeze-dried gamma-inactivated A/PC was reconstituted with distilled water prior to intranasal vaccination. The majority of mice lost less than 10% total body weight and only 2/10 mice lost over 10% total body weight showing mild symptoms. All vaccinated mice survived the heterosubtypic challenge with A/PR8 (7 x 10^2 PFU) as opposed to 10% survival in naïve mice (Figure 3.6.8A, B & C). These data suggest that the dry freezing process does not markedly reduce the ability of gamma-inactivated A/PC to induce heterosubtypic immunity.

3.5 Discussion

Previously, we have reported on the use of gamma-irradiation as means to prepare inactivated influenza vaccine and that this vaccine was highly effective against not only human heterosubtypic strains (Müllbacher, Ada et al. 1988) but also against H5N1 (Alsharifi, Furuya et al. 2009). The current study evaluated in a comparative setting the protective efficacy of three types of inactivation regimens; gamma-radiation, and formalin-, or UV-inactivation, to assess whether the currently used chemical inactivation method, used since 1945, is the most suitable choice for influenza vaccine preparation.

I show that gamma-inactivated A/PC (3.2 x 10^6 PFU equivalent, 2.3 x 10^3 HAU) had superior immunogenicity compared to the other sterilization methods, and confers a
high level of protection against both homologous and heterosubtypic challenges. This superior protection was reflected in 100% survival and lower weight loss, which correlated with reduced lung viral load compared to naive and formalin- or UV-inactivated-virus vaccinated mice. In addition, single or double doses of a currently used trivalent influenza vaccine provided no protection against A/PC or A/PR8 challenges irrespective if the vaccine was given intranasally or intramuscular as at present given to humans.

High levels of protection similar to that afforded by gamma-inactivated A/PC can be induced with formalin-inactivated A/PC when three-fold higher doses (9.6 x 10^6 PFU equivalent, 2.3 x 10^3 HAU) and multiple immunization are administered. However, even under these conditions, protection is conferred only against homologous but not against heterosubtypic virus challenge. Therefore, an increase in dose and frequency of immunization only improves the strain-specific immunity of formalin-inactivated virus. It is important to note that for per virus particle inactivated, gamma-inactivated virus is more immunogenic than formalin-inactivated virus since formalin-inactivated virus preparation required three times more PFU for a comparable HAU dose and triple immunizations, as opposed to single priming for gamma-inactivated A/PC, to obtain strain specific immunity. These findings demonstrate that gamma-inactivation maintains superior antigenicity and immunogenicity relative to the other procedures. Thus gamma-inactivated virus could induce immunity that is not only quantitatively but also qualitatively superior to virus preparations inactivated by formalin treatment or UV-irradiation.

It is noteworthy that others have reported cross-protective efficacy of formalin-inactivated influenza vaccines (Takada, Kuboki et al. 1999; Tumpey, Renshaw et al. 2001; Takada, Matsushita et al. 2003) and conventional trivalent vaccine preparations (Ichinohe, Tamura et al. 2007). However, multiple, high dose, immunization regimens (with or without adjuvants) were used in those studies. In the event of a pandemic, a single dose regimen, as promised by gamma-inactivated virus, would be incomparably more desirable. Moreover, the fact that no adjuvants are required for gamma-inactivated influenza virus suggests that reactogenicity problems are less likely to be encountered. Alum is most commonly used adjuvant for human vaccines but it has been proven to be ineffective in enhancing the immunogenicity of influenza vaccine antigens (Skea and Barber 1993; Powers, Smith et al. 1995; Otto, Schmid et al. 1996). In addition, alum skews the immune response towards T helper (Th) type 2-supported humoral immune
Chapter 3: Superior immunogenicity of gamma-inactivated virus

responses (Singh and O'Hagan 1999; Aguilar and Rodriguez 2007), which may reduce the effectiveness of gamma-inactivated virus, as the latter is known to induce Th1-type cellular immune responses, including Tc cell responses that correlate with heterosubtypic protection. In any case, gamma-inactivated virus does not require adjuvants. Furthermore these studies of cross-protection achieved, using chemically inactivated virus preparations did not test the longevity of the observed cross-protective immunity. Challenge infections were routinely induced at only one or two weeks after the final immunization. The efficacy of gamma-inactivated virus is highlighted by the fact that after a single dose of intranasal priming, the immunized mice were able to resist heterosubtypic challenge doses for up to 50 times MLD50, and up to 3 months, underscoring the robust immunity induced.

In our previous study we highlighted the importance of the anatomical priming site for effective induction of heterosubtypic protection (Alsharifi, Furuya et al. 2009). I speculate that this cross-protection is mediated by mucosal Tc cell responses. The intranasal or mucosal route is known to be the only route by which cross-reactive Tc cells can be generated in the mucosa-associated lymphoid tissues from which Tc cells are recruited to the lungs during infections (Nguyen, Moldoveanu et al. 1999). An alternative hypothesis is induction of cross-reactive secretory IgA antibodies to internal viral proteins. Some secretory IgA antibodies are capable of intracellular neutralization of influenza virus during transcytosis into the infected epithelial cells (Mazanec, Kaetzel et al. 1992; Mazanec, Coudret et al. 1995; Mazanec, Kaetzel et al. 1995). My data suggests that cross-reactive Tc cells may be responsible for the cross-protection observed here as other forms of inactivated influenza viruses, are unable to prime for influenza-immune Tc cell responses (Müllbacher, Ada et al. 1988). Moreover, in line with previous reports (Agency 1973; Lowy, Vavrina et al. 2001), gamma-inactivation has less impact on hemagglutination activity than formalin- or UV-inactivation. Gamma-inactivated virus, retaining antigens similar to their native forms may partially account for its superior immunogenicity.

Numerous studies have suggested the essential role of mucosal immunity in protection against influenza virus. Thus an intranasal route of administration targets the lung mucosa associated lymphoid organ for inducing immunity in the respiratory tract. However, a previously marketed intranasally administered influenza vaccine, although eliciting protective immunity, was associated with an increase in the number of Bell’s palsy cases – facial paralysis (Mutsch, Zhou et al. 2004) and consequently resulted in
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market withdrawal of this vaccine preparation. This adverse event has been attributed to the mucosal adjuvant used; *Escherichia coli* heat-labile toxin. Such safety concern would not be an issue with gamma-inactivated influenza vaccine as it does require the inclusion of potentially harmful adjuvants in its vaccine formulation.

Apart from the strong protective efficacy observed, several additional factors contribute to the attractiveness of gamma-inactivation for influenza vaccines. Firstly, freeze-dried gamma-inactivated A/PC maintained its cross-protective property. Since dry powder formulations have been shown to have improved stability compared to liquid formulations under various storage conditions (Ada and Jones 1986), this could enormously facilitate distribution of the vaccine in an event of a pandemic. Secondly, the intranasal route of delivery, which requires little training or medical qualified personnel, would provide additional advantages for developing countries. Thirdly, gamma-inactivated influenza vaccine would be comparatively easy and inexpensive to manufacture when compared to other vaccine production processes. Most importantly with regards to manufacturing considerations and availability, the robust heterosubtypic protection induced by gamma-inactivated influenza virus may render annual reformulation of influenza vaccines obsolete.

In conclusion, I have characterized a vaccine candidate that provides long lasting, cross-protective immunity in mice. This study demonstrated that sterilization by gamma-ray is more effective than the currently used vaccine preparation methods, chemical inactivation, in preserving immunogenicity of the virus and in induction of cross-protective immunity.
### 3.6 Tables and Figures

**Table 3.6.1 Hemagglutination activity of inactivated influenza virus A/PC preparations.**

<table>
<thead>
<tr>
<th>Strain</th>
<th>Method of inactivation</th>
<th>HAU/ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>A/PC</td>
<td>Original live purified stock</td>
<td>$2.2 \times 10^5$</td>
</tr>
<tr>
<td></td>
<td>Gamma-inactivation</td>
<td>$7.3 \times 10^4$</td>
</tr>
<tr>
<td></td>
<td>Formalin-inactivation</td>
<td>$2.4 \times 10^4$</td>
</tr>
<tr>
<td></td>
<td>UV-inactivation</td>
<td>$2.4 \times 10^4$</td>
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</table>
Chapter 3: Superior immunogenicity of gamma-inactivated virus

Figure 3.6.1 Gamma-inactivated influenza virus A/PC protects mice against both homologous and heterosubtypic challenge.

Groups of 9-10 BALB/c mice were mock treated (A, F) or immunized either with formalin- (B, G), UV- (C, H) or gamma-inactivated A/PC (D, I) (3.2 x 10^6 PFU equivalent) and at week 3 after the immunization, naïve and immunized mice (9~10 mice per group) were intranasally infected with A/PC (MLD_{50}; 3.2 x 10^5 PFU) or A/PR8 (MLD_{50}; 7 x 10^5 PFU). Survival of infected mice was monitored daily for 20 days (E, J). *: P < 0.05 vs. control naïve group; Fisher’s exact test.
Figure 3.6.2 Formalin-inactivated influenza virus A/PC requires multiple immunizations for homologous protection.

Groups of 9-10 BALB/c mice were mock treated (A, F) or immunized either once (B), twice (C) or three times (D, G) with formalin-inactivated A/PC (9.6 x 10^6 PFU equivalent or HAU dose equivalent to that of gamma-inactivated A/PC; 2.3 x 10^3 HAU). Mock treated or single dose immunized mice were challenged with A/PC (MLD_{50}; 3.2 x 10^3 PFU) at three weeks post immunization. Double or triple dose immunized mice were intranasally infected with A/PC (MLD_{50}; 3.2 x 10^3 PFU) or A/PR8 (MLD_{50}; 7 x 10^2 PFU) one week after the final immunization. Survival of infected mice were monitored daily for 20 days (E, H). *: P < 0.05 vs. control naïve group; Fisher’s exact test.
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Figure 3.6.3 Trivalent influenza vaccine failed to provide protection against drifted strains.

Mice were immunized intranasally (i.n.) or intramuscularly (i.m.) with trivalent influenza vaccine (CSL fluvax vaccine; A/Solomon Islands/3/2006 H1N1, A/Brisbane/10/2007 H3N2, B/Florida/4/2006; 3 μg hemagglutinin) and at 3 week post immunization, naïve (A, E), i.m. immunized (B, F) and i.n. immunized mice (C, G) were i.n. challenged with either A/PC (3.2 x 10^3 PFU/mouse) or A/PR8 (7 x 10^2 PFU). Survival of infected mice was monitored daily for 20 days (D, H).
Figure 3.6.4 None of the inactivated virus preparations prevents infection, but immunization with gamma-inactivated A/PC leads to early viral clearance.

BALB/c mice were intranasally immunized either with gamma-, formalin- or UV-inactivated A/PC (3.2 x 10^6 PFU equivalent) and at week 3-post immunization, naïve and immunized mice were intranasally challenged with A/PR8 virus (MLD_{so}). On day 3 and 6 post infection, three mice per group were sacrificed and the viral titres in lungs were determined by the plaque assay as described in Materials and Methods. (*) : P < 0.05 vs. control nil group, Student’s T test)
Figure 3.6.5 Tc cell responses induced by live and inactivated A/PC.

Mice were immunized intravenously (A) or intranasally (B) with live, gamma-irradiated, formalin-, UV-inactivated A/PC or mock treated. Splenocytes or lung-derived lymphocytes were harvested 7 days post immunization and were used as effector cells against A/PC and A/PR8 infected P815 target cells. Mean values ± SD of two mice per group are shown. Specific lysis values were interpolated from regression curves at effector:target ratio of 50:1.
Figure 3.6.6 Intranasal immunization with gamma-inactivated A/PC provides protection against high-dose A/PR8 lethal challenge.

Groups of 9-10 BALB/c mice were mock treated (A, B) or immunized intranasally with gamma-inactivated A/PC (C, D, E) (3.2 x 10^6 PFU/ml equivalent) and at 3 weeks post immunization mice were intranasally challenged with either 1 x MLD_{50} A/PR8 (A, C), 5 x MLD_{50} A/PR8 (B, D), or 50 x MLD_{50} A/PR8 (E). Survival and weight loss of infected mice was monitored for 20 days (G). *: P < 0.05 vs. control naive group; Fisher’s exact test.
Figure 3.6.7 The cross-protective immunity following immunization with gamma-inactivated influenza virus is long lasting.

Group of 9-10 BALB/c mice were either mock treated (A) or immunized intranasally with gamma-inactivated A/PC (B) (3.2 x 10⁶ PFU equivalent) and at 3 months post immunization mice were intranasally challenged with MLD₃₀ A/PR8 (7 x 10² PFU). Survival and weight loss of infected mice was monitored for 20 days (C). *: P < 0.05 vs. control naïve group; Fisher's exact test.
Figure 3.6.8 Heterosubtypic protective property of gamma-inactivated A/PC is maintained after a dry freezing process.

Gamma-inactivated A/PC stock was freeze-dried and resuspended in distilled water immediately prior to intranasal administration (3.2 x 10^6 PFU equivalent). Groups of 9-10 BALB/c mice were either mock treated (A) or immunized with freeze-dried gamma-inactivated A/PC (B) and challenged with heterosubtypic strain A/PR8 (7 x 10^2 PFU) at week 3-post immunization. Survival and weight loss of mice was monitored daily for 20 days (C). *: P < 0.05 vs. control naïve group; Fisher’s exact test.
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3.7 References


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Chapter 4

Gamma-ray inactivated influenza A viruses induce cross-protective immunity in mice that is primarily mediated by cytotoxic T cells

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Chapter 4: T cell mediated cross-protective influenza vaccines

4.1 Abstract

Previously, I have demonstrated that a single dose of non-adjuvanted intranasal gamma-inactivated influenza A virus can provide robust protection in mice against both homologous and heterosubtypic influenza A virus challenges, including an H5N1 avian strain. I investigated the mechanism behind the observed cross-protection to define which arms of the adaptive immune response are involved in mediating this protection. Studies with gene knock-out mice showed that the induction of cross-protective immunity was dependent on the cytolytic effector molecule perforin and both CD4\(^+\) and CD8\(^+\) T cells. Adoptive transfer of memory T cells from gamma-inactivated A/PC immunized mice, but not memory B cells, protected naive recipients against lethal heterosubtypic influenza challenge. Furthermore, protection was not transferable by serum, and cross-reactivity between H1N1 and H3N2 was seen by gamma-inactivated A/PC induced Tc cells but not by antibodies in the serum of immunized mice. However, a decrease in protection of \(\mu\)MT\(^{-/+}\) mice suggests that B cell factors other than antibody are contributing to T cell mediated cross-protection. Thus, heterosubtypic protection induced in mice immunized with gamma-inactivated influenza virus is mainly provided by cellular immunity.
4.2 Introduction

Natural infections with influenza A viruses induce immune responses that provide protection against not only homologous but also heterosubtypic influenza A viruses (Webster and Askonas 1980; Epstein, Lo et al. 1997; Nguyen, Moldoveanu et al. 1999; Heinen, de Boer-Luijtze et al. 2001; Seo and Webster 2001; Kreijtz, Bodewes et al. 2007). The mechanism for this cross-protection has been extensively studied in mice immunized with live, replicating influenza virus, and has been attributed to cross reactive cytotoxic T (Tc) cells (Webster and Askonas 1980; Ulmer, Fu et al. 1998; Cerwenka, Morgan et al. 1999; Nguyen, Moldoveanu et al. 1999; Sambhara, Kurichh et al. 2001; Seo and Webster 2001; Kreijtz, Bodewes et al. 2007). In addition, influenza-immune Tc cells are directed predominantly against the internal viral proteins, which are commonly shared among influenza A viruses (Townsend, McMichael et al. 1984; Yewdell, Bennink et al. 1985; Taylor, Davey et al. 1987; Wraith, Vessey et al. 1987; Jameson, Cruz et al. 1998; Jameson, Cruz et al. 1999). Despite these understandings, the immunological basis of the heterosubtypic immunity against influenza A virus infection and the contribution of Tc cell response remain an important area of research. Importantly, in order to investigate the underlying mechanism for heterosubtypic immunity, many researchers have used low doses of live viruses to prime animals prior to heterosubtypic challenge (Liang, Mozdzanowska et al. 1994; Benton, Misplon et al. 2001; Heinen, de Boer-Luijtze et al. 2001; Nguyen, van Ginkel et al. 2001; Tumpey, Renshaw et al. 2001; Quan, Compans et al. 2008). It has been reported that in vivo depletion of CD4+ or CD8+ T cells (Liu and Mullbacher 1989; Liang, Mozdzanowska et al. 1994; Epstein, Lo et al. 1997; Benton, Misplon et al. 2001; Quan, Compans et al. 2008), or both T cell subsets (Epstein, Lo et al. 1997), had only a minor effect on heterosubtypic protection. Benton et al have shown that immunization with sublethal doses of live viruses protected Ig− /−, CD1− /−, and γδ− /− mice from lethal heterosubtypic challenges (Benton, Misplon et al. 2001). Acute depletion of CD4+ or CD8+ T cell subsets in these knock-out animals, but not their wild-type counterpart, abrogated heterosubtypic protection (Benton, Misplon et al. 2001). In addition, heterosubtypic immunity has been reported in β2-microglobulin-deficient (Tc cell response deficient) (Epstein, Lo et al. 1997; Nguyen, van Ginkel et al. 2001; Tumpey, Renshaw et al. 2001) and IFN-gamma deficient mice (Nguyen, van Ginkel et al. 2000; Sambhara, Kurichh et
al. 2001). Therefore, it appears that heterosubtypic immunity induced by live influenza virus is a multifaceted phenomenon. However, considering the negligible heterosubtypic immunity induced by current inactivated influenza vaccines, the role of both antibody and cell-mediated immunity require further investigation.

Inactivated virus-based vaccines provide strain-specific protection (Cate and Mold 1975; Armerding, Rossiter et al. 1982; Ada and Jones 1986). This is largely due to their ability to induce only humoral immunity, but not Tc cell responses (Braciale and Yap 1978). The major targets of anti-influenza antibodies are the viral surface antigens, hemagglutinin and neuraminidase, which are highly susceptible to antigenic variations due to antigenic shift and drift (reviewed in (Couch 2003)). This renders antibody responses ineffective in providing protection against antigenically drifted strains that emerge frequently to cause seasonal influenza outbreaks. However, recent studies have suggested that inactivated influenza viruses, when administered intranasally, may elicit B cell dependent cross-protective immunity (Tamura, Ito et al. 1992; Tumpey, Renshaw et al. 2001; Takada, Matsushita et al. 2003; Ichinohe, Tamura et al. 2007; Quan, Compans et al. 2008). In addition, several groups have reported antibodies specific for conserved regions of transmembrane matrix protein 2 to be cross-protective against different subtypes (Slepushkin, Katz et al. 1995; Frace, Klimov et al. 1999; Neirynck, Deroo et al. 1999; Tompkins, Zhao et al. 2007). These reports suggest that in mice immunized with inactivated influenza virus, B cells and antibodies participate in cross-protective immunity as opposed to the contribution of T cells in mice primed with live virus.

Müllbacher et al have reported previously that gamma-ray inactivated influenza viruses can confer cross-protective immunity in mice (Mullbacher, Ada et al. 1988) and hypothesized that such inactivated influenza preparations may potentially be effective as universal influenza vaccines (Mullbacher, Lobigs et al. 2006). More recently we reported that a single dose of intranasally administered, gamma-irradiated influenza virus affords mice with protection against different subtypes of influenza A virus, including the highly pathogenic avian influenza strain H5N1 (Alsharifi, Furuya et al. 2009). While a role of Tc cells in heterosubtypic immunity remains contentious the present study aimed to define in more detail the immune mechanisms responsible for the observed cross-protection in our gamma-ray inactivated influenza virus protection model.
4.3 Materials and Methods

Mice

BALB/c, C57BL/6, 129Sv/Ev, β2-microglobulin knockout (β2m⁻/⁻) (Koller, Marrack et al. 1990), Ig μ-chain knockout (μMT⁻/⁻) (Kitamura, Roes et al. 1991), perforin knockout (Prf⁻/⁻) (Kagi, Ledermann et al. 1994), interferon-γ receptor knockout (IFN-IIR⁻/⁻) (Huang, Hendriks et al. 1993) and MHC II knockout (H₂AB⁻/⁻) (Madsen, Labrecque et al. 1999) mice were bred under specific pathogen-free conditions and supplied by the Animal Services Division at the John Curtin School of Medical Research, Canberra. 10~14-week-old females were used. All experimental procedures were approved by the Institutional Animal Ethics Committee.

Cells and viruses

P815 mastocytoma (Ralph, Moore et al. 1976), Madin-Darby canine kidney (MDCK) (Gaush, Hard et al. 1966), PU5-1.8 lymphoid tumour (Ralph and Nakoinz 1974), EL4 T lymphoma (Gorer and Kaliss 1959), JAWS II monocyte (MacKay and Moore 1997), RAW 264.7 macrophage (Raschke, Baird et al. 1978) and fetal-skin-derived dendritic cell (FSDC) (Lutz, Granucci et al. 1994), RMA T lymphoma (Karre, Ljunggren et al. 1986) and RMA-S peptide transporter (TAP-) deficient RMA subclone (Ljunggren and Karre 1985; Karre, Ljunggren et al. 1986) were grown and maintained in EMEM supplemented with 5% FCS, 2.2 gL⁻¹ NaHCO₃, 0.29 gL⁻¹ L-glutamine and PSN antibiotics (0.12 gL⁻¹ penicillin G, 0.2 gL⁻¹ streptomycin sulphate, 0.2 gL⁻¹ neomycin sulphate) at 37 °C in a humidified atmosphere with 5% CO₂.

The influenza type A viruses, A/PR/8 [A/Puerto Rico/8/34 (H1N1)] and A/PC [A/Port Chalmers/1/73 (H3N2)] were grown in 10-day-old embryonated chicken eggs. Each egg was injected with 0.1 ml normal saline containing 1 hemagglutinin unit (HAU) of virus, incubated for 48 h at 37 °C, then held at 4 °C for overnight. The amniotic/allantoic fluids were harvested, pooled and stored at -80 °C. Titres were 10⁷ PFU/ml (A/PC) and 2 x 10⁸ PFU/ml (A/PR8) using plaque assays on MDCK cells. Viruses were purified using chicken red blood cells (CRBCs) for vaccine preparation as previously described (Sheffield, Smith et al. 1954). Briefly, infectious allantoic fluid
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was incubated with CRBCs for 45 min at 4 °C allowing the viral hemagglutinin to bind CRBCs, and then centrifuged to remove the allantoic fluid supernatant. The pellets were re-suspended in normal saline, incubated for 1 h at 37 °C to release the CRBCs from the virus and then centrifuged to remove the CRBCs and collect the virus in the supernatant. Purified A/PC stock titre was 5 x 10⁸ PFU/ml.

Virus inactivation and vaccination

For formalin inactivation, the viruses were incubated with 0.2 % formalin at 4 °C for a week (Takada, Matsushita et al. 2003). Formalin was then removed by pressure dialysis using normal saline for 24 h at 4 °C. The dialysis method was adapted from Current Protocols in Immunology (Andrew, Titus et al. 2001). For UV inactivation, the viruses were placed in 60-mm petri dishes with a fluid depth of 10 mm. The virus was exposed to 4000 ergs per cm² for 45 min at 4 °C. For gamma-ray inactivation, influenza viruses received a dose of 10 kGy from a ⁶⁰Co source (Australian Nuclear Science and Technology Organization – ANSTO). The virus stocks were kept frozen on dry ice during gamma-irradiation. Loss of viral infectivity was confirmed by titration of inactivated virus preparations in eggs. The HAU titres of inactivated virus stock were determined to be 7.3 x 10⁴ HAU/ml for gamma-inactivated A/PC, 2.4 x 10⁴ HAU/ml for formalin- and UV-inactivated A/PC.

Mice were immunized intranasally with inactivated virus preparations (3.2 x 10⁶ PFU equivalent) or live viruses (7 x 10¹ PFU). For lethal challenge, at 3 weeks post-immunization, mice were infected intranasally with A/PR8 (7 x 10² PFU). Mice were weighed daily and monitored for mortality until day 20 post-challenge.

Adoptive immune lymphocyte transfer experiment

10-week-old donor BALB/c mice were immunized intravenously with gamma-irradiated A/PC (1 x 10⁸ PFU equivalent). Splenocytes were collected at week 3 post immunization. Single-cell suspensions were prepared and red blood cells were lysed. The splenic lymphocytes were separated into B and T cell populations by passing the cells through nylon wool columns. 2 ml of 5 x 10⁷ cells/ml were loaded onto columns
and incubated for 2 h at 37 °C. The columns were washed with warm (37 °C) Hanks-balanced salt solution + 5% FCS and non-adherent T cells in the first effluent were collected. Nylon wool-bound B cells were then collected by washing the columns with cold (4 °C) Hanks-balanced salt solution. Purity of T (82.8%, + 7.94% B cell) and B (84.2%, + 8.3% T cell) cell populations was confirmed by flow cytometric analysis. Small samples of purified splenocytes were washed in PBS with 2% FCS. Fc receptors were blocked by incubation with mouse CD16/CD32 (Fcγ III/II receptor) Ab (BD Pharmingen) for 20 min at 4 °C. Cells were washed and further incubated with a mixture of fluorescent-conjugated anti-CD3, anti-CD8, anti-CD19 (BD Pharmingen) Abs. Dead cells were labelled with 7-aminoactinomycin D (Sigma-Aldrich). Stained cells were quantitated using a FACS Calibur (Becton Dickinson). Purified T or B cells (1.1 x 10^7 cells in a volume of 0.2 ml) were intravenously injected into recipient mice, which were then challenged with A/PR8 (7 x 10^1 PFU) intranasally at 3 h after the adoptive cell transfer. Mice were monitored for body weight loss and mortality until day 20 post-challenge.

**Passive serum transfer experiment**

Sera from intranasally immunized mice with gamma-irradiated A/PC were collected at 3 weeks post-immunization. The pooled immune sera were heated for 30 min at 56 °C to inactivate complement. Recipient mice received 200 μL of immune sera intravenously. After two hours, the recipient mice were challenged with A/PR8 (7 x 10^2 PFU). Mice were monitored for body weight and mortality until day 20 post-challenge.

**Plaque reduction assay**

Immune sera were collected 3 weeks post-immunization from mice vaccinated with live, gamma-irradiated, formalin or UV-inactivated A/PC. After heat inactivation of serum samples at 56 °C for 30 min, 190 μL of serially diluted (x10, x30, x90, x270) serum was mixed with 10 μL virus (A/PC or A/PR8 strain) suspension containing roughly 100 PFU. After 60 min incubation at 37 °C the residual virus infectivity was measured by plaque assay on MDCK cells.
**51Cr release assay**

BALB/c mice were injected intranasally with gamma-irradiated (3.2 x 10^6 PFU equivalent) A/PC. The vaccinated mice and unvaccinated control mice were challenged with live A/PR8 (7 x 10^1 PFU) at 3 weeks post immunization. Lungs were harvested at various time points post-challenge and red blood cell-depleted cell suspensions were prepared for use as effector cells. Another group of intravenously primed mice received an intravenous secondary immunization at 3 months post primary immunization and splenocytes were harvested at 7 days post-immunization and used as effector cells. Target cells were prepared by incubating P815, RAW, FSDC, PU5, EL4, or JAW II cells with either A/PC, A/PR8 or K^d^ restricted nucleoprotein derived peptide TYQRTRALV (NPP), followed by a 1 h incubation in medium containing 100–200 μCi of 51Cr. After washing, target cells were mixed with effector cells at different ratios in a 8 h chromium release assay. The level of radioactivity in the supernatant was measured in a gamma counter. Specific lysis is given as mean percent lysis of triplicate wells and values were calculated using the formula: (experimental cpm – spontaneous cpm)/(maximal release cpm – spontaneous cpm) x 100.

For interpolation, a log regression equation was determined based on specific lysis values at E:T ratio of 120:1, 40:1, 13.3:1 and 4.4:1. E:T ratio of 40 was used to interpolate specific lysis values using an equation Y = a + bX, where X is the E:T ratio and Y is the specific lysis value.

### 4.4 Results

#### 4.4.1 Role of immune sera and B and T lymphocytes in heterosubtypic immunity induced by gamma-irradiated influenza virus

To determine the role of antibodies in cross-protective immunity, mice were intranasally immunized with either live A/PR8 (7 x 10^1 PFU) or gamma-irradiated A/PC (3.2 x 10^6 PFU equivalent), and 3 weeks later blood was collected. Groups of
naïve mice injected intravenously with 200 μl of either gamma-irradiated A/PC immune serum, hyper-immune serum (from mice that received two doses of live A/PR8 at three weeks intervals) or pre-immune serum and challenged with a lethal dose of A/PR8 virus (7 x 10^2 PFU) 2 h post serum transfer. Naïve mice that received gamma-irradiated A/PC immune serum developed clinical signs and weight loss similarly to those that received pre-immune serum (Figure 4.6.1A, C & D). These mice rapidly lost weight to reach the end-point of 25% weight loss and accordingly were not protected from heterosubtypic challenge. In contrast, mice that received the hyper-immune serum were fully protected with virtually no weight loss when challenged with homologous A/PR8 (7 x 10^1 PFU) (Figure 4.6.1B & D). These data indicate that gamma-irradiated A/PC induced antibodies are not cross-protective.

Secondly, I used B cell-deficient μMT^/-^ mice to assess the role of B cells in cross-protective immunity. 10-week-old μMT^/-^ mice were immunized intranasally with gamma-irradiated A/PC (3.2 x 10^6 PFU equivalent) and challenged with the heterosubtypic strain A/PR8 (7 x 10^2 PFU) three weeks post-immunization. The vaccinated μMT^/-^ mice displayed a survival rate similar to that of naïve mice (Figure 4.6.2A, B & C), implying that an absence of B cells does impair the development of cross-protective immunity. Furthermore, intranasal vaccination with gamma-irradiated A/PC failed to protect MHC-II^/-^ mice against heterosubtypic challenge with A/PR8 (Figure 4.6.3A, B & C). This provides evidence that B and CD4^+^ T cells participate in the induction of cross-protective immunity by gamma-irradiated influenza virus.

I next used β2M^/-^ mice, which are deficient in CD8^+^ Tc cell responses (Zijlstra, Bix et al. 1990), to evaluate the contribution of CD8^+^ T (Tc) cells in the cross-protective immunity induced by intranasal immunisation with gamma-irradiated A/PC. A heterosubtypic challenge with A/PR8 (7 x 10^2 PFU) caused a mortality rate of 60%, with the surviving mice losing over 10% of their body weight prior to their recovery (Figure 4.6.4B & C). Controls, unvaccinated mice infected with the same virus strain suffered 100% mortality (Figure 4.6.4A & C). This demonstrates a critical role for CD8^+^ T cells in the cross-protective immunity induced by gamma-irradiated influenza virus.

Although these results show a role for B, CD4^+^ T and CD8^+^ T cells in the cross-protective immunity against influenza, defective primary immune responses in the knock-out mice may obscure the cross-protective potential of both humoral and cellular
memory responses. As an alternative approach to assess the nature of the effector cells, I used an adoptive transfer model, with splenocytes from 3 week earlier intravenously gamma-irradiated A/PC (1 x 10^8 PFU equivalent) immunized mice as donor cells. Splenocytes were nylon wool-enriched T cells (82.8% T cells, 7.9% B cell) or B cells (84.2% B cells, 8.3% T cells) and intravenously transferred into naïve mice. Three h post-transfer mice were challenged with 0.1 x MLD_{50} A/PR8 (7 x 10 PFU). T cell recipients were partially protected against A/PR8 challenge (Figure 4.6.5A, B & D). In contrast, no protection was observed in B cell recipient mice, which developed disease symptoms similar to that of controls (unvaccinated with no lymphocyte transfer) following A/PR8 challenge (Figure 4.6.5A, C & D). These adoptive transfer studies further support a critical role for T cells, but not B cells, in cross-protective immunity against A/PR8 challenges.

CD8^+ T cells exert antiviral effects by either directly killing virus-infected cells or secreting cytokines such as IFN-gamma and TNF (Doherty, Allan et al. 1992). To delineate which effector function of T cells provides heterosubtypic immunity, I used prf^{−/−} mice, which lack perforin-mediated lytic function, and IFN-IIR^{−/−} mice, whose immune cells are unresponsive to IFN-gamma. Vaccination with gamma-irradiated A/PC failed to confer significant cross-protection to prf^{−/−} mice (Figure 4.6.6A, B & C). This strongly suggests that cross-protection induced by gamma-irradiated A/PC requires perforin-mediated lytic function, which is associated with CD8^+ T and NK cells. In contrast, IFN-IIR^{−/−} mice immunised with gamma-irradiated A/PC were fully protected against a lethal challenge with A/PR8 (Figure 4.6.7A, B & C). Thus, IFN-gamma function is dispensable for the induction of the cross-protective immunity.

4.4.2 Absence of cross-neutralizing activity in the serum of gamma-irradiated A/PC immunized mice

I have shown elsewhere that immunisation with gamma-irradiated, but not formalin- or UV-inactivated, influenza viruses induce cross-protective immunity (see Chapter 3). Here I tested the cross-neutralizing activity of the immune sera induced by variously inactivated influenza formulations against homologous and heterosubtypic strains of influenza A viruses. Mice were immunized with either infectious, or gamma-irradiated, formalin- or UV-inactivated A/PC, and sera were collected 3 weeks post-
immunisation. Immune sera collected from all vaccinated animals contained high levels of neutralizing activity against the homologous strain A/PC (H3N2) (Figure 4.6.8A). The same immune sera, when tested against a heterosubtypic strain A/PR8 (H1N1), showed levels of neutralizing activity similar to that of naïve sera (Figure 4.6.8B). These data demonstrate that immunization with any of the inactivated influenza virus preparations, including gamma-irradiated influenza viruses, induces highly strain-specific neutralizing antibodies with limited, if any, cross-neutralising activity.

4.4.3 Gamma-vaccination generates rapid pulmonary recall Tc cell responses

The absence of serum cross-neutralising activity between H3N2 and H1N1 influenza viruses, lack of cross-protective immunity in defined Tc cell deficient knock-out mice, and the results of adoptive transfer experiments indicate that cellular rather than humoral immunity plays a pivotal role in protecting mice against heterosubtypic influenza virus challenges. In addition, we have shown previously that gamma-irradiated influenza virus preparations induce cross-reactive Tc cell responses (Mullbacher, Ada et al. 1988; Alsharifi, Furuya et al. 2009). Thus, I tested the effect of vaccination on the kinetics of Tc cell responses in the lung following intranasal infection with A/PR8. I used a low infectious dose of 70 PFU/mouse of A/PR8 to enable this investigation. Lungs from unvaccinated or gamma-A/PC vaccinated mice were harvested at different times after heterosubtypic challenge with A/PR8, and lung homogenates were tested for their cytolytic activity using a ⁵¹Cr-release assay. I did not detect any killing activity in the unvaccinated group prior to day 7 after A/PR8 challenge (Figure 4.6.9A). In contrast, lung homogenates from the gamma-A/PC vaccinated group showed an early cytotoxic activity at days 3 and 5 after A/PR8 challenge (Figure 4.6.9B). In addition, this cytotoxic activity was cross-reactive, since killing activity was detected against A/PR8-infected, A/PC-infected and NPP-labelled targets. Therefore, this data illustrate that intranasal vaccination with gamma-irradiated influenza virus induced early cross-reactive Tc cell responses in the lung following heterosubtypic influenza virus challenge.
4.4.4 Induction of memory Tc cells by gamma-irradiated A/PC

I have shown elsewhere that immunization with gamma-irradiated A/PC could provide cross-protection lasting at least 3 months (see Chapter 3). Here I evaluated the longevity of memory T cell immunity that may account for this long lasting protection. Mice were primed with gamma-irradiated A/PC (1 x 10^8 PFU equivalent) and boosted 3 months later with either live A/PC (2 x 10^6 PFU) or A/PR8 (1 x 10^7 PFU). Splenic Tc cell responses were analysed 7 days after the secondary immunization (Figure 4.6.10). My data clearly show that initial vaccination with gamma-irradiated A/PC resulted in an enhanced Tc cell responses following boosting with a live heterosubtypic strain A/PR8. In addition, while the overall killing activity of these splenocytes was high against all tested targets when compared to primary Tc cell responses, the killing activity against challenge A/PR8 virus infected targets was prominent. In contrast, secondary immunisation with the live homologous strain A/PC did not increase the potency of the Tc cell responses most likely due to the presence of neutralizing antibodies to the challenge virus.

4.4.5 Gamma-irradiated, but not formalin or UV-inactivated, virus preparations can sensitise selective cells for lysis by Tc cells

As the replication deficient gamma-irradiated A/PC is able to induce Tc cell immunity, it must be able to deliver antigens to the MHC-I antigen presentations pathway. To test this I investigated whether gamma-irradiated A/PC could sensitise target cells, derived from a range of different origins, for Tc cell lysis; P815 mastocytoma cells, RAW 264.7 macrophages, FSDC immature dendritic cells, PU5-1.8 macrophages, EL4 T thymoma, JAWS II immature dendritic cells/monocytes. Target cells were incubated with a multiplicity of infection of 1 for live A/PC and 10 for gamma-irradiated A/PC for 1 h. These cells were used as target cells for ^51Cr release by primary gamma-irradiated A/PC immune Tc cells (Figure 4.6.11A & B). Live A/PC was able to sensitise all cell lines tested. In contrast, gamma-irradiated A/PC sensitised only EL4 T cell lymphomas for lysis by influenza-immune Tc cells.

I then tested if this ability to sensitise EL-4 targets was applicable also to other cell lines of T cell origin. Thus, I compared the ability to sensitise EL4 and RMA T cell...
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lymphomas by live A/PC and inactivated A/PC (gamma-, formalin-, and UV-) (Figure 4.6.11C & D). Both live and gamma-irradiated A/PC sensitised T cell lymphomas to give significant lysis above mock treated control targets (P < 0.05, student’s T test). In contrast, formalin-, UV-inactivated A/PC or low pH (4) treated gamma-irradiated A/PC failed to sensitise EL4 and RMA cell lines for Tc cell lysis. The transporter associated with antigen processing (TAP)-deficient T lymphoma RMA-S cells (Ljunggren and Karre 1985; Karre, Ljunggren et al. 1986) were refractory to sensitisation by any of the virus preparations tested (Figure 4.6.11E).

4.5 Discussion

We have reported previously that gamma-ray inactivated influenza A virus, especially when administered intranasally, confers robust protection against lethal homologous and heterosubtypic virus challenges, including the virulent avian H5N1 strain (Alsharifi, Furuya et al. 2009). I now show that cross-reactive cellular immunity mediated predominantly by Tc cells, and not humoral immunity, is the essential element responsible for the cross-protective immunity induced by gamma-irradiated influenza virus. This conclusion is based on several independent lines of evidence: 1) β2M<sup>−</sup> mice did not generate cross-protective immune responses; 2) transfer of immune sera from gamma-inactivated influenza immune donor mice did not confer cross-protective immunity to naïve recipients; 3) transfer of enriched T cells, but not of B cells, from gamma-inactivated influenza immune donor mice conferred cross-reactive immunity to naïve recipients; and 4) cross-protective immunity induced by gamma-irradiated influenza preparations was dependent on the essential Tc cell cytolytic effector molecule perforin. The latter observation, together with the induction of cross-protective immunity induced in IFN-IIβ<sup>−</sup> mice strongly suggests that the cytolytic component of Tc cells provide protection through killing of virus-infected cells via their granule-exocytosis pathway of cytotoxicity, and not by CD8<sup>+</sup> T cell IFN-gamma-mediated virus control.

Consistent with the above, immunization with gamma-irradiated influenza virus can elicit cross-reactive Tc cell responses similar to that induced by live virus (Mullbacher, Ada et al. 1988). The data presented in this chapter also show that these
cross-reactive Tc cells persist for at least 3 months and are responsible for the induction of more potent secondary Tc response upon in vivo re-immunization. This correlates with the observation that mice immunised with gamma-irradiated influenza virus are protected against heterosubtypic challenge for at least 3 months (see Chapter 3). However, re-immunization with the homologous virus did not enhance the secondary Tc response. This is best explained by invoking that the primary immunization elicited a highly strain specific antibody responses which neutralized the secondary challenge with the homologous virus, thus limiting memory Tc cell activation. Moreover, I have shown that the cross-protection induced did not provide sterilizing immunity (see Chapter 3). Instead, my data show an accelerated virus clearance in the lungs of vaccinated mice following heterosubtypic infection (see Chapter 3). This accelerated virus clearance correlates with rapid pulmonary recall Tc cell responses that appeared at least 3 days earlier than in unvaccinated control mice.

With Tc cells identified as the dominant factor in providing heterosubtypic protection, I nevertheless found evidence of a contribution by B cells to this protective response, since μMT+ mice also showed increased susceptibility to heterosubtypic challenge. The absence of cross-neutralizing antibody responses in serum as well as a lack of protective effect of transferred serum or adoptively transferred B cells suggests that the contribution of B cells is independent of their principle soluble product, antibody. The susceptibility of μMT− and MHC-II− mice to influenza infection may be related to their contribution to the adaptive immune response other than antibody, namely antigen presentation and/or cytokine milieu modification. Indeed, several authors have reported the ability of naïve B cells to restore immunity in μMT− mice against secondary infections in an antibody-independent manner (Elkins, Bosio et al. 1999; Mozdzanowska, Maiese et al. 2000). In addition, a role of B cells and B cell-derived soluble factors, in promoting effector Tc cell function in a mouse influenza model, has been reported (Liu and Mullbacher 1989; Kos and Mullbacher 1992; Kopf, Brombacher et al. 2002). The requirement for MHC-II dependent activation of CD4+ T cells in the activation and maintenance of CD8+ T cell responses is less clear-cut and in the response to influenza virus in mice conflicting results have been reported (Liu and Mullbacher 1989; Riberdy, Christensen et al. 2000). Therefore, while our data clearly show a negligible role for cross-neutralising antibodies, an overall positive contribution of B cells to cross-protective immunity cannot be ignored. In fact, previous studies have demonstrated the induction of cross-protection in mice vaccinated intranasally with
inactivated influenza viruses and observed that cross-protection correlated with antibody responses in lungs (Tumpey, Renshaw et al. 2001; Takada, Matsushita et al. 2003; Quan, Compans et al. 2008). It is known that internal viral proteins, e.g. matrix proteins, contain B-cell epitopes that are conserved among influenza A viruses (Frace, Klimov et al. 1999; Neirynck, Deroo et al. 1999) and that mucosal IgA have broader specificity than serum IgG (Mazanec, Coudret et al. 1995). Reports from others with a live, attenuated, intranasal influenza vaccine in patients showed that mucosal cross-neutralizing antibodies may contribute to heterosubtypic immunity (Belshe, Gruber et al. 2000; Belshe, Gruber et al. 2000). The observed partial cross-protection, although not statistically significant, in $\beta_2M^{++}$ and prf$^{-/}$ mice supports this notion. Moreover, passive immunity obtained by adoptive T cell transfer was only partial successful in protecting against a sublethal dose of virulent A/PR8. Thus, both antibodies as well as Tc cells may ultimately contribute to optimal cross-protection. Mucosal antibody response studies would be considered in further work with gamma-irradiated influenza virus.

Exposure of T cell lymphomas (EL4 and RMA cell), but not other cell lines such as macrophages and dendritic cells, to gamma-inactivated A/PC resulted in specific target cell lysis. The mechanism behind this selective sensitisation by gamma-inactivated A/PC is not known. It is possible that macrophages and dendritic cells used in my experiments may differ from their in vivo counterparts that are involved in presenting antigens from gamma-inactivated virus. In any case, MHC-I processing pathways are a requirement for target sensitisation by gamma-inactivated A/PC since RMA-S cells, deficient in TAP peptide transporter (Ljunggren and Karre 1985; Karre, Ljunggren et al. 1986), did not get sensitised. Furthermore, low pH treatment of gamma-inactivated A/PC resulted in a loss of target sensitisation. Thus, any treatments that disrupt viral protein structures, such as formalin- or UV-inactivation, renders virus unable to sensitise targets. The above observations indicate that virus retains ability to infect cells after gamma-ray irradiation and therefore allow their antigens to enter MHC-I presentation pathway, leading to induction of Tc cell responses.

In conclusion, I have provided evidence to illustrate the roles of cross-protective Tc cells in the heterosubtypic immunity induced by gamma-irradiated influenza A virus. Formulating fully inactivated influenza vaccines capable of inducing cross-reactive T cell responses with high protective efficacy against antigenic variants is a global aim to replace the current antibody based vaccines with their limited efficacies.
Figure 4.6.1 A passive serum transfer fails to transfer the heterosubtypic immunity, induced by gamma-irradiated A/PC, to naïve mice.

Serum samples were pooled from donor mice immunized with either a single dose of gamma-irradiated A/PC (3.2 x 10^6 PFU equivalent) or two doses of live A/PR8 (7 x 10^2 PFU) (hyper immune). Recipient mice (9–10 mice per group) were given intravenously 0.2 ml of immune sera or pre-immune sera as a control. At 2 h post serum transfer, mice were challenged intranasally with A/PR8 (7 x 10^2 PFU). Mice were monitored daily for weight loss (A, B & C) and mortality (D). Endpoint: 25% weight loss. *: P < 0.05 vs. control pre immune sera group; Fisher’s exact test.
Figure 4.6.2 Absence of heterosubtypic protection in B cell-deficient mice.

μMT<sup>−/−</sup> mice were immunized intranasally with gamma-irradiated A/PC (3.2 × 10<sup>6</sup> PFU equivalent). 3 weeks post-immunization, naïve (A) and immunized (B) mice (9–10 mice per group) were challenged with heterosubtypic strain A/PR8 (7 × 10<sup>2</sup> PFU). Mice were monitored daily for weight loss (A & B) and mortality (C) for 20 days.
Figure 4.6.3 Absence of heterosubtypic protection in MHC II deficient mice.

MHC II⁺ mice were immunized intranasally with gamma-irradiated A/PC (3.2 x 10⁶ PFU equivalent). 3 weeks post-immunization, naïve (A) and immunized (B) mice (9–10 mice per group) were challenged with heterosubtypic strain A/PR8 (7 x 10³ PFU). Mice were monitored daily for weight loss (A & B) and mortality (C) for 20 days.
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Figure 4.6.4 Absence of heterosubtypic protection in β2M deficient mice.

β2M−/− mice were immunized intranasally with gamma-irradiated A/PC (3.2 x 10^6 PFU equivalent) and at week 3 post-immunization, naive and immunized mice (9~10 mice per group) were challenged with heterosubtypic strain, A/PR8 (7 x 10^2 PFU). Mice were monitored daily for weight loss (A & B) and mortality (C) for 20 days.
Figure 4.6.5 Adoptively transferred T cells, but not B cells, protect mice against heterosubtypic challenge.

Splenic lymphocytes were collected 3 weeks post-immunization from 20 mice immunized intravenously with gamma-irradiated A/PC (1 x 10^8 PFU equivalent). Enriched T and B cells were obtained by nylon wool separation and were transferred to naïve mice intravenously (10 mice per group). 3 hours after the adoptive cell transfer, mice were challenged with A/PR8 (7 x 10^1 PFU). Mice were monitored daily for weight loss (A, B & C) and mortality (D) for 20 days. *: P < 0.05 vs. control nil group; Fisher’s exact test.
Figure 4.6.6 Absence of heterosubtypic protection in perforin deficient mice.

Prf<sup>c</sup> mice were immunized intranasally with gamma-irradiated A/PC (3.2 x 10<sup>6</sup> PFU equivalent). 3 weeks post-immunization, naïve and immunized mice (9~10 mice per group) were challenged with the heterosubtypic strain A/PR8 (7 x 10<sup>2</sup> PFU). Mice were monitored daily for weight loss (A & B) and mortality (C) for 20 days.
Figure 4.6.7 Heterosubtypic protection in type II IFN receptor knock-out mice.

IFN-IIR\textsuperscript{-} mice were immunized intranasally with gamma-irradiated A/PC (3.2 x 10\textsuperscript{6} PFU equivalent). 3 weeks post-immunization, naïve and immunized mice (6~7 mice per group) were challenged with the heterosubtypic strain A/PR8 (7 x 10\textsuperscript{2} PFU). Mice were monitored daily for weight loss (A & B) and mortality (C) for 20 days. *: P < 0.05 vs. control nil group; Fisher’s exact test.
Figure 4.6.8 No cross-neutralizing activity found in serum of immunized mice.

Viral neutralizing activities against A/PC (H3N2) (A) or A/PR8 (H1N1) (B) were determined by plaque reduction assays for sera collected 3 weeks after the immunization with live, gamma-irradiated, formalin- or UV-inactivated A/PC. After heat inactivation of serum samples at 56 °C for 30 min, 190 µl of serially diluted (x10, x30, x90, x270) sera were mixed with 10 µl virus suspension containing roughly 1 x 10² PFU. After 60 min incubation the virus/serum mixtures were added to six well plates for plaque assay. Each bar represents the mean percent ± S.D (n=3). *: P < 0.05 vs. pre immune sera, student’s T test.
Figure 4.6.9 Gamma-inactivated virus vaccination generates rapid pulmonary recall Tc cell responses.

BALB/c mice were either mock treated (A) or vaccinated intranasally with gamma-irradiated A/PC (B). Three weeks later, vaccinated and mock groups were challenged intranasally with A/PR8. Lungs were harvested at different time points and lung homogenates were used as effector cells in a \(^{51}\text{Cr}\) release assay. Target cells were, mock, A/PC- or A/PR8-infected or K\(^d\)-restricted nucleoprotein derived peptide (NPP)-pulsed P815 cells. Specific lysis values were interpolated from a regression curve at an effector:target ratio of 40:1. Each bar represents the mean percent lysis ± S.D. N.T.: not tested.
Secondary ex vivo Tc cell response

Figure 4.6.10 Long lasting memory Tc cell responses.

Groups of two BALB/c mice were intravenously immunized once or twice with either live A/PC (2 x 10⁶ PFU), A/PR8 (1 x 10⁷ PFU) or gamma-irradiated A/PC (1 x 10⁸ PFU equivalent), as indicated. The secondary immunization was given 3 weeks after priming. Splenocytes were harvested 7 days after the second immunization and used as effector cells against mock, A/PC- or A/PR8 infected P815 target cells or labelled with K⁺ restricted nucleoprotein derived peptide (NPP), as shown in the legend. Each bar represents the mean percent ± S.D. Specific lysis values were interpolated from regression curve at effector:target ratio of 40:1.
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Figure 4.6.11 Target cell sensitisation.

Target cells were incubated with a multiplicity of infection of 1 for live A/PC or 10 for gamma-inactivated A/PC. BALB/c or C57BL/6 mice were intravenously immunized with gamma-inactivated A/PC (10⁸ PFU equivalent). Splenocytes were harvested on day 7 post-immunization and were used as effector cells against targets: live A/PC, gamma-inactivated A/PC or mock treated target cells, P815, RAW, FSCD, PU5, EL4, and JAWS II cells (A & B); live-A/PC, inactivated A/PC (acid treated gamma-, gamma-, formalin- or UV-) or mock treated EL4 (C), RMA (D) or RMA-S cells (E). Each bar represents the mean percent ± S.D. Specific lysis values were interpolated from regression curve at effector:target ratio of 40:1.
4.7 References


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Chapter 5

Pathways of partial lymphocyte activation by inactivated influenza viruses

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5.1 Abstract

Partial activation of lymphocytes, characterized by a significant increase in the cell surface expression of CD69 and CD86, but not CD25, is induced by a wide spectrum of live viruses including Semliki Forest virus but not when the virus is inactivated. This partially systemic activation of lymphocytes is mediated by type-I interferon, is rapid and results in a period of type-I interferon unresponsiveness including heightened disease susceptibility. In contrast, here I report that inactivated influenza A viruses, are able to elicit partial lymphocyte activation in vivo. Using a variety of inactivation methods (UV, ionising radiation and formalin treatment) and commercially available influenza vaccines I show that influenza virus trigger partial lymphocyte activation via both viral genome- and protein-dependent pathways. The existence of multiple pathways, in vivo, to trigger induction of partial lymphocyte activation during influenza infections, suggests an important function during the early immune response to a primary infection, especially as it later leads to higher vulnerability to a second infection of the host.
Chapter 5: Partial lymphocyte activation

5.2 Introduction

Recently, Alsharifi et al have discovered that live viral infections cause type-I interferon (IFN-I) dependent, generalized and systemic partial activation of lymphocytes (Alsharifi, Lobigs et al. 2005; Alsharifi, Regner et al. 2006). This partial activation is characterized by elevated expression of the early activation marker CD69 and the co-stimulatory molecule CD86, but not the IL-2Rα chain, CD25 (Alsharifi, Lobigs et al. 2005; Alsharifi, Regner et al. 2006). The vast majority of lymphocytes undergo partial lymphocyte activation within 24 h of viral infections and cell surface marker expression returns to a base state at around day 5 post infection. A few recent reports have hinted at some insights into a possible biological significance of this phenomenon. In vitro studies have revealed that IFN-I induced up-regulation of cell surface markers CD25, CD69 and CD86 reduced the activation threshold for naïve B cells, allowing for augmented B cell responses (Braun, Caramalho et al. 2002). In another study, an implication of CD69 in immunoregulatory function has been reported (Shiow, Rosen et al. 2006). The early activation marker CD69 was found to play a role in temporarily retaining lymphocytes in secondary lymphoid organs, presumably promoting antigen specific activation of lymphocytes via antigen presenting cells.

While the biological role of partial lymphocyte activation during a primary infection is not yet understood, its down side, namely the refractory period of an interferon response to a consequently secondary infection, has attracted more attentions due to its clinical implications (Alsharifi, Regner et al. 2006; Alsharifi, Mullbacher et al. 2008). This is particularly evident in severe cases of influenza infection, which often lead to secondary bacterial infections (Louria, Blumenfeld et al. 1959; Morens, Taubenberger et al. 2008; Hussell, Wissinger et al. 2009). Clinical observations indicate that such secondary infections occur most frequently between 5 ~ 10 days following the primary infection and suggests a transient immunosuppression may be responsible. This is consistent with observation in a mouse model of viral disease, showing that a primary viral infection results in an enhanced susceptibility to secondary, unrelated viral episode (Alsharifi, Regner et al. 2006; Zuniga, Liou et al. 2008). It has been shown that an unrelated secondary viral infection fails to trigger partial lymphocyte activation for a period of 5-9 days post-primary infection and that this is a result of IFN-I exhaustion (Alsharifi, Regner et al. 2006). An investigation into how viruses elicit partial lymphocyte activation is therefore of high clinical importance.
Induction of partial lymphocyte activation in vivo by live replicating virus is well characterized (Alsharifi, Regner et al. 2006). Alsharifi et al have previously reported that the magnitudes of IFN-I response and partial lymphocyte activation correlate with virus dose and strain virulence and that inactivated avirulent Semliki Forest virus failed to induce such phenomenon (Alsharifi, Lobigs et al. 2005; Alsharifi, Regner et al. 2006). In this study, I used inactivated influenza virus to investigate whether viral replication is an absolute requirement for partial lymphocyte activation to occur. I demonstrate that, unlike aSFV, fully inactivated influenza virus, regardless of inactivation method used, is capable of provoking partial lymphocyte activation in vivo. And this may occur via a number of different pathways. I hypothesize as to the biological relevance of this striking phenomenon.

5.3 Materials and Methods

Mice

C57BL/6, 129Sv/Ev (WTGR), interferon-type Iα receptor knockout (IFN-IR') (Muller, Steinhoff et al. 1994), interferon-gamma receptor knockout (IFN-IIR') (Huang, Hendriks et al. 1993), interferon type I and II double receptors knockout (IFN-I&IIR'), β2-microglobulin (β2m') (Koller, Marrack et al. 1990), MHC II knockout (H2AB') (Madsen, Labrecque et al. 1999), myeloid differentiation primary response gene 88 knockout (MyD88') (Adachi, Kawai et al. 1998), Toll like receptor 2 (TLR-2') (Takeuchi, Hoshino et al. 1999) and Toll like receptor 4 (TLR-4') (Hoshino, Takeuchi et al. 1999) mice were bred under specific pathogen-free conditions and supplied by the Animal Services Division at the John Curtin School of Medical Research, Canberra. 10–14-week-old females were used in these experiments. All animal procedures were carried out under the guidelines of the animal ethics committee. Mice deficient in MyD88, TLR-2, and TLR-4 were used with permission of Prof. S. Akira.

Viruses and cells

Madin-Darby canine kidney (MDCK) and baby hamster kidney (BHK) cells were grown and maintained in EMEM supplemented with 5% FCS, 2.2 gL⁻¹ NaHCO₃.
and PSN antibiotics (0.12 gL⁻¹ penicillin G, 0.2 gL⁻¹ streptomycin sulphate, 0.2 gL⁻¹ neomycin sulphate) at 37 °C in a humidified atmosphere with 5% CO₂.

The Influenza type A virus, A/PR/8 [A/Puerto Rico/8/34 (H1N1)] was grown in 10-day-old embryonated chicken eggs. Each egg was injected with 0.1 ml normal saline containing 1 hemagglutination unit (HAU) virus, incubated for 48 h at 37 °C, and held at 4 °C overnight. The amniotic/allantoic fluids were then harvested, pooled and stored at -80 °C. Viruses were purified using chicken red blood cells (CRBCs) as previously described (Sheffield, Smith et al. 1954). Briefly, infectious allantoic fluid is incubated with CRBCs for 45 min at 4 °C allowing the viral-hemagglutinin to bind CRBCs, and then centrifuged to remove the allantoic fluid supernatant. The pellets were resuspended in normal saline, incubated for 1 h at 37 °C to release the virus from the CRBCs and then centrifuged to remove the CRBCs and collect the virus containing supernatant. Purified A/PR8 stock had a titre of 1 x 10⁸ PFU/ml determined by plaque assays on MDCK cells.

The avirulent alphavirus, Semliki Forest virus, (aSFV) was grown by infecting semi-confluent Baby Hamster Kidney (BHK) cell monolayers at a multiplicity of infection of 0.5 PFU per cell. Infected cells were incubated for 24 h, culture supernatants were harvested and centrifuged at 1200 x g for 4 min and stored in single-use aliquots at -70 °C. Viral titres were determined by plaque assay on Vero cells to be 10⁷ PFU/ml.

**Virus inactivation**

For formalin inactivation, the viruses were incubated with 0.2% formalin at 4 °C for a week (Takada, Matsushita et al. 2003). The formalin was then removed by pressure dialysis using normal saline for 24 h at 4 °C. The dialysis method was adapted from Current Protocols in Immunology (Andrew, Titus et al. 2001). For UV inactivation, viruses were placed in 60-mm petri dishes with a fluid depth of 10 mm. The virus was exposed to 4000 ergs per cm² for 45 min at 4 °C. For gamma-ray inactivation, influenza viruses and aSFV received a dose of 10 kGy and 40 kGy respectively from a ⁶⁰Co source (Australian Nuclear Science and Technology Organization – ANSTO). The virus stocks were kept frozen on dry ice during gamma irradiation. Loss of viral infectivity was confirmed by titration of inactivated virus.
preparations in eggs or by plaque assay for A/PR8 and aSFV, respectively. The HAU
titres of inactivated virus stock were determined to be $7.29 \times 10^4$ HAU/ml for gamma-
inactivated A/PR8, $2.43 \times 10^4$ HAU/ml for formalin-inactivated A/PR8 and $8.1 \times 10^3$
HAU/ml for UV-inactivated A/PR8.

CSL fluvax split vaccine 2008 is egg derived, β-propiolactone inactivated and
contains 90 µg/ml of the hemagglutinin antigens of influenza A/Solomon
Islands/3/2006 H1N1, A/Brisbane/10/2007 H3N2 and B/Florida/4/2006 (CSL Limited,
Australia).

Solvay influvac subunit vaccine 2009 is egg derived, formaldehyde inactivated
and contains 90 µg/ml of the hemagglutinin antigens of influenza A/Brisbane/59/2007
H1N1, A/Brisbane/10/2007 H3N2 and B/Brisbane/60/2008 (Solvay Biologicals LLC,
Spain)

Hemagglutination assay

Live, inactivated virus and trivalent influenza vaccine preparations were serially
diluted in a 100 µl volume on 96-well U-bottom microtiter plate. 0.5% CRBC
suspensions were added to all wells and plates were incubated for 30 min on ice. This
method was adapted from Current Protocols in Microbiology (Szretter, Balish et al.
2006).

ELISA assays for serum IFN-α, -β levels

Serum samples were collected from influenza or aSFV immunized C57BL/6
mice at various time points post-immunization. IFN-α and -β levels in these serum
samples were determined by ELISA. Briefly, Nunc-Immuno 96 Microwell plates were
coated with monoclonal rat anti-mouse IFN-α (HyCult Biotechnology). Plates were
then sequentially incubated with serum samples or a recombinant mouse IFN-α
standard (HyCult Biotechnology) for 2 h at room temperature followed by a polyclonal
rabbit anti-mouse IFN-α (PBL InterferonSource) for 2 h at rt and followed by horseradish
peroxidase conjugated goat anti rabbit IgG (Sigma) for 2 h at rt and followed by
peroxidase substrate (TMB substrate reagent set, Biosciences) for 30 min.
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Optical density was measured at 450 nm. Serum IFN-α concentrations were estimated using a standard curve and expressed as Units/ml.

For IFN-β ELISA, monoclonal rat anti mouse was used IFN-β (PBL InterferonSource) as capturing antibody and polyclonal rabbit anti-mouse IFN-β (PBL InterferonSource) as the detecting antibody. A recombinant mouse IFN-β (PBL InterferonSource) was used for establishment of a standard curve.

Flow cytometric analysis

For in vivo studies, mice were immunized intravenously with either live or inactivated virus preparations (2 x 10⁷ PFU equivalent) or trivalent inactivated influenza vaccine (CSL fluvax or Solvay influvac: 18 μg hemagglutinin). Spleens were harvested 1, 2 or 3 days post immunization and red cell depleted. For in vitro studies, red cell depleted splenocytes from naïve mice were cultured at 10⁶ cells/well with or without various stimuli for 12-14 h. Splenocytes were washed in PBS with 2% FCS. Fc receptors were blocked by incubation with mouse CD16/CD32 (Fcy III/II receptor) Ab (BD Pharmingen) for 20 min at 4 °C. Cells were washed and further incubated with a mixture of fluorescent-conjugated anti-CD3 (BD Pharmingen), anti-CD8 (BD Pharmingen), anti-CD19 (BD Pharmingen), anti-CD11c (BD Pharmingen), anti-CD25 (BD Pharmingen), anti-CD40 (abcam), anti-CD69 (BD Pharmingen), anti-CD80 (abcam) or anti-CD86 (BD Pharmingen) Abs. Dead cells were labelled with 7-aminoactinomycin D (Sigma-Aldrich). Stained cells were quantitated using a FACSCalibur (Becton Dickinson).

Thymidine proliferation assay

Red blood cell-depleted splenocytes were prepared in EMEM medium supplemented with 10% fetal calf serum (FCS). Splenocytes were cultured (10⁶ cells/0.2 ml) in the wells of flat-bottomed microtiter plates in triplicates in the presence of various doses of gamma-, formalin-, UV-inactivated or live influenza A/PR8. Positive and negative control was 1 μg Concanavalin A or medium alone, respectively. Cultures were incubated at 37 °C in a humidified atmosphere of 5% CO₂ in air for up to 42 h. To measure proliferation, 0.5 μCi [³H]thymidine was added to each well for a 16 h
labelling period. Cells were harvested onto glass fibre filters using an automatic cell harvester and thymidine incorporation was determined by liquid scintillation counting.

Statistical analysis

Student's $t$ test (two-tailed distribution, two-sample equal variance) was used. A $P$ value of $< 0.05$ was considered statistically significant.

5.4 Results

5.4.1 General gating strategies for flow cytometry analysis

For analysis of CD25, CD40, CD80, CD86 expressions on CD3$^+$, CD8$^+$, CD11c$^+$ and CD19$^+$ cells, lymphocytes and monocytes were gated on the forward scatter and side scatter profiles (Figure 5.6.1A). The non-viable cells were excluded from the analysis using 7AAD (7-amino-actinomycin D) staining (Figure 5.6.1B). 7-AAD penetrates cell membranes of non-viable cells and intercalates into exposed double-stranded nucleic acids and therefore 7AAD positive cells were considered non-viable. The viable lymphocytes/monocytes (7AAD negative cells) were then gated for CD3$^+$, CD8$^+$, CD11c$^+$ or CD19$^+$ cells on a histogram (Figure 5.6.1C). These gated populations were analysed for cell surface marker expressions using various fluorochrome-conjugated antibodies (Figure 5.6.1D). The above gating strategies were applied consistently in all flow cytometry experiments described in this chapter.

5.4.2 Live and inactivated influenza viruses induce partial lymphocyte activation

Alsharifi et al have previously reported that for the alphavirus SFV, viral replication and subsequent IFN-I production is essential for the induction of partial activation of lymphocytes (Alsharifi, Lobigs et al. 2005). To test whether this is a general phenomenon for immune stimulating activity by viruses, I inactivated influenza
A virus by three different means (gamma-irradiation, formalin treatment or UV-inactivation), and compared their immune stimulatory ability in vivo with that of live and gamma-irradiated aSFV. The expression of cell surface molecules on mouse splenocytes were analyzed at day 1, 2 and 3-post immunization (2 x 10^7 PFU or PFU equivalent/mouse) (Figure 5.6.2 & 5.6.3). Consistent with the previous report (Alsharifi, Lobigs et al. 2005), gamma-inactivated aSFV failed to induce partial lymphocyte activation (Figure 5.6.4). This contrasts, with inactivated influenza viruses, which induced up-regulation of CD69 and CD86 expression on CD3^+ T, CD8^+ T, CD19^+ B and also on CD11c^+ dendritic, cells as shown in Figure 5.6.2. CD25 and CD40 expressions were slightly elevated on CD11c^+ dendritic and CD19^+ B cells. No elevation of CD80 expression was observed on any of the leukocyte subpopulations.

5.4.3 Effector mechanism of inactivated influenza viruses responsible for partial lymphocyte activation.

I addressed the question as to why influenza virus but not aSFV retains its ability to stimulate partial lymphocyte activation after inactivation. The similar kinetics of partial lymphocyte activation induced by live and inactivated viruses suggested that IFN-I may likewise be responsible. To test this, I investigated lymphocyte activation in mice deficient in type I and/or type II IFN receptor(s) (IFN-IR^−, IFN-IIR^−, and IFN-I&IIR^−). Indeed, elevated expression of activation markers was only observed in IFN-IIR^− and in their wild-type 129 counterparts but not in IFN-IR^− or IFN-I&IIR^− mice following immunization with live (2 x 10^7 PFU/mouse) or gamma-inactivated A/PR8 (2 x 10^7 PFU equivalent/mouse) (Figure 5.6.5A, B, C, E).

I also compared serum levels of IFN-α and IFN-β following immunization with live or inactivated influenza viruses (gamma-, formalin-, UV-A/PR8) or live and gamma-aSFV; (2x10^7 PFU equivalent/mouse) and tested at various times post immunization (Figure 5.6.6A, B, C & D). Both live A/PR8 and aSFV induced elevated IFN-α levels in sera, reaching peak values at 6 and 12 h, respectively, post immunization. Serum IFN-α levels returned to background levels 2 days post immunization (Figure 5.6.6A & C). Elevated IFN-α levels induced by inactivated influenza virus preparations peaked at 3 h post immunization, and gradually declined to background levels by day 1 for formalin- and UV-inactivated A/PR8 and day 2 for
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gamma-inactivated A/PR8 respectively (Figure 5.6.6A). No elevation of serum IFN-α was detected in mice immunized with gamma-inactivated aSFV (Figure 5.6.6C). Interestingly, the peak IFN-α levels in mice immunized with gamma- and formalin-inactivated A/PR8 were substantially higher than those seen with live A/PR8 suggesting a capacity of live replicating virus to interfere with IFN-α responses (Figure 5.6.6A). Among all inactivated influenza virus preparations, gamma-inactivated A/PR8 induced the highest serum IFN-α levels in this study (P < 0.05 vs. formalin- or UV-inactivated A/PR8), except at the 3 h time point. Live A/PR8 failed to increase IFN-β, serum levels at any time point sampled after infection (Figure 5.6.6B). IFN-β secretion induced by live aSFV had similar kinetics to that of IFN-α, reaching the peak serum level at 6 h post infection (Figure 5.6.6C & D). Of all inactivated viruses tested, elevated serum IFN-β levels were detected only in mice immunized with gamma- or formalin-inactivated A/PR8 (Figure 5.6.6B & D). Thus, differential induction of type I IFN-α and -β can be seen with both live and inactivated influenza virus preparations and the secretion of IFN-I correlates with partial lymphocyte activation.

Influenza virus is a known lymphocyte mitogen (Anders, Scalzo et al. 1985). The mitogenicity of inactivated virus preparations was assessed to determine if potential differential mitogenicity between aSFV and influenza virus could provide a possible explanation for the observation that influenza virus retains its immunostimulating potential following full inactivation where as aSFV does not. Influenza virus is thought to mediate its mitogenic effect by the binding of the viral hemagglutinin to MHC II molecules (Anders, Scalzo et al. 1986; Poumbourios, Anders et al. 1987). Hence, I tested whether partial lymphocyte activation is absent in H2AB knock out mice, which are deficient in α and β chains of MHC II (Madsen, Labrecque et al. 1999). Surprisingly, partial lymphocyte activation induced by inactivated influenza virus was seen even in the absence of MHC II molecules (Figure 5.6.5D). However, the possibility that the mitogenic trigger is mediated through other cell surface molecules cannot be ruled out. I therefore performed proliferation assays to assess mitogenic activity present in the inactivated viral preparations. Splenocytes from naïve mice were co-cultured in vitro with different virus preparations (1x10⁵ PFU equivalent/culture). Lymphocyte proliferation was induced by live influenza virus but not by live aSFV from both wild type and MHC II⁺⁺ splenocytes (Figure 5.6.7A & B). In addition, virus inactivation did not significantly affect the mitogenicity of influenza virus. Most
importantly, all influenza virus preparations induced strong proliferative responses in splenocytes from MHC II−/− mice, albeit to a lesser extent than wild-type splenocytes, indicating that the mitogenic effect is only partially mediated via MHC II. All influenza virus preparations exhibited mitogenic effects in a dose dependent manner with all inactivated preparations giving similar levels of stimulation but marginally less than live virus.

The difference in the mitogenicity between influenza virus and aSFV suggests that influenza virus hemagglutinin may activate innate immune cells and trigger IFN-I synthesis. To test this we intravenously immunized mice with either of two different formulations of commercially available influenza vaccines: subvirion vaccine containing all the viral proteins (CSL fluvax vaccine; A/Solomon Islands/3/2006 H1N1, A/Brisbane/10/2007 H3N2, B/Florida/4/2006; 18 μg hemagglutinin) or subunit vaccine containing purified hemagglutinin (A/Brisbane/59/2007 H1N1, A/Brisbane/10/2007 H3N2 and B/Brisbane/60/2008; 18 μg hemagglutinin). Splenocytes were harvested at 24 h post immunization and analysed for expression of activation markers. The magnitude of CD69 and CD86 up-regulation induced by immunization with subvirion was comparable to that induced by live A/PR8 (Figure 5.6.8). In contrast, subunit vaccine did not induce upregulation of either CD69 or CD86 expression (Figure 5.6.9).

Various gene knock out mice, MHC I−/−, MHC II−/−, IFN-IR−/− and MyD88−/− were used to delineate further the mechanism behind induction of partial lymphocyte activation by subvirion vaccine. Subvirion vaccine elicited up-regulation of cell surface markers in MHC I−/−, MHC II−/− and wild-type 129 but not in IFN-IR−/− and MyD88−/− mice (Figure 5.6.10A, B, C, D & E), albeit to a lower magnitude compared to that induced by live or gamma-inactivated A/PR8, suggesting that the in vivo up-regulation of activation markers induced by trivalent influenza vaccine is mediated by IFN-I via a MyD88 (myeloid differentiator factor 88) dependent pathway. However, the subvirion vaccine preparation was significantly dependent on both MHC-I and II expression for efficient up-regulation of CD69 and CD86 whereas live or gamma-inactivated virus was not (Figure 5.6.10A & B).

Recent studies have shown a role of TLRs in the innate recognition of different viruses (Kurt-Jones, Popova et al. 2000; Alexopoulou, Holt et al. 2001; Lund, Sato et al. 2003; Melchjorsen, Jensen et al. 2005). Viral protein dependent induction of IFN-I has been shown to occur through TLR-2 (Barbalat, Lau et al. 2009). Furthermore, the hemagglutinin of measles virus can stimulate production of pro-inflammatory cytokines
and cell surface expression of CD150 via TLR-2 signalling (Bieback, Lien et al. 2002). Thus, I investigated the possible involvement of TLR-2 in the induction of partial lymphocyte activation. Mice deficient in TLR-2 were capable of undergoing partial lymphocyte activation upon immunization with live or inactivated influenza virus (2 x 10^7 PFU or PFU equivalent) (Figure 5.6.11A). Similar results were obtained with TLR4^{-} mice (Figure 5.6.11B). TLR-4 is a known receptor for bacterial endotoxins (Poltorak, He et al. 1998; Hoshino, Takeuchi et al. 1999). Given that the bacterial endotoxin is a common contaminant of egg grown viruses (Rastogi, Hochstein et al. 1977), it was important to eliminate the possibility of endotoxin mediated partial lymphocyte activation as the reason for my observed phenomenon. I further tested the requirement of TLR systems by using MyD88^{-} mice (Figure 5.6.11C). MyD88 is an adaptor molecules utilized by the majority of TLR mediated signalling with the exception of TLR-3 (O'Neill, Fitzgerald et al. 2003). A requirement of TLR-3 was not tested here because it is unlikely that fully inactivated negative sense, single stranded RNA virus could generate double stranded RNA, the ligand for TLR-3. Both live and gamma-inactivated influenza virus were able to induce partial lymphocyte activation in MyD88^{-} mice at a dose of 2 x 10^7 PFU equivalent or 1.5 x 10^4 HAU. In contrast, formalin-inactivated virus preparation required a three-fold higher virus dose (6 x 10^7 PFU) to induce a similar magnitude of partial lymphocyte activation. This however translates into the same dose of HAU (1.5 x 10^4 HAU) when compared to live and gamma-inactivated virus preparations. Thus, the magnitude of partial lymphocyte activation correlates closely with HAU rather than with PFU equivalence. Collectively, these data show that inactivated influenza virus triggered in vivo partial lymphocyte activation is independent of known TLR mediated activation.

The partial dependency of formalin-inactivated virus on MyD88 to induce activation in vivo prompted an investigation of the dose response effect in vitro. I stimulated splenocytes harvested from naïve mice with different doses of virus preparations and the expression of CD25 and CD69 on CD3^{+} T cells and CD19^{+} B cells were determined after 12-16 hr incubation (Figure 5.6.12A). Consistent with the in vivo data, even at the highest dose of 10^5 PFU/well, gamma-inactivated aSFV did not stimulate expression of either CD25 or CD69. Surprisingly, live aSFV failed to elicit partial lymphocyte activation in vitro in contrast to in vivo. Interestingly, unlike in vivo, influenza virus mediated CD25 up-regulation on CD3^{+} T cells and CD19^{+} B cells. This up-regulation of CD25 suggests that different induction mechanisms for partial
lymphocyte activation exist in vivo and in vitro. Indeed, unlike in vivo, the CD25 and CD69 expression in vitro in response to influenza virus stimuli was highly dependent on MyD88 (Figure 5.6.12B). Similar results were obtained with formalin-inactivated A/PR8, UV-irradiated A/PR8, and subvirion vaccine but not with subunit vaccine (Figure 5.6.12C & D). All of the influenza virus preparations, except for subunit vaccine, induced MyD88 dependent up-regulation of CD25 and CD86 on CD3+ T cell and CD19+ B cells in vitro. Interestingly, although with reduced magnitude, even splenocytes from IFN-IR−− mice expressed partial lymphocyte activation. Thus, IFN-I is not an absolute requirement for partial lymphocyte activation in vitro.

5.5 Discussion

We originally reported the novel finding that infection of mice with live viruses from a range of virus families (pox-, flavi-, alpha- orthomyxo- and adenoviridae) results in rapid, systemic, but partial, lymphocyte activation, which is mediated by IFN-I (Alsharifi, Lobigs et al. 2005; Alsharifi, Regner et al. 2006). Here I analysed this phenomenon in more detail, and surprisingly found that inactivated influenza viruses were as effective as live virus in stimulating IFN-I mediated partial lymphocyte activation in vivo, which strongly contrasts with our previous studies (mainly with SFV) that suggested that viral replication was necessary for the induction of this phenomenon.

The difference between aSFV and influenza virus in their ability to induce partial lymphocyte activation after gamma-irradiation sterilization was surprising, given that both are enveloped ssRNA viruses. This may be due to a difference in their intrinsic mitogenicity. Past studies have focused on the viral genome/replication intermediates in mediating induction of IFN-I response (Diebold, Kaisho et al. 2004; Lund, Alexopoulou et al. 2004; Barchet, Krug et al. 2005; Guillot, Le Goffic et al. 2005; Pichlmair, Schulz et al. 2006; Le Goffic, Pothlichet et al. 2007; Lee, Lund et al. 2007; Pothlichet, Chignard et al. 2008) but more recent studies have found a genome-independent pathway of IFN-I induction, in which viral glycoproteins are recognized as the primary stimuli triggering IFN-I production. For example, Miller and Anders (Miller and Anders 2003) showed that fixed influenza virus-infected cells were a sufficient stimulus to induce IFN-I production in vitro. The authors argued that fixed influenza virus-infected
cells may present arrays of viral glycoproteins at their cell surface that may interact with as yet unidentified receptors of IFN-I producing cells. This suggests that viral entry into the IFN-I producing cells may not be an absolute requirement for IFN-I production. Consistent with these observations are in vitro studies that showed up-regulation of costimulatory molecules and MHC-II on B cells in response to purified hemagglutinin (Poumbourios, Anders et al. 1987), which occurred via a MyD88-dependent pathway (Marshall-Clarke, Tasker et al. 2006). This suggests that, under certain circumstances, viral glycoproteins are able to directly activate immune responses. My data support this notion. My results show that inactivated influenza virus, but not inactivated aSFV, induces splenocyte proliferation, an IFN-I response, and consequent partial lymphocyte activation. This indicates that the viral glycoproteins of influenza virus can act as a ligand for an as yet unknown receptor to trigger an innate immune response. In support of this interpretation is the fact that trivalent influenza subvirion vaccine containing all the viral proteins was also able to induce partial lymphocyte activation in vivo. Recent studies have shown that the lower immunogenicity of subunit and subvirion vaccines, compared to whole inactivated virus vaccine, is in part due to a substantially reduced amount of viral RNA and thus absence of viral genome-mediated TLR signalling (Geeraedts, Goutagny et al. 2008). Thus, induction of partial lymphocyte activation by subvirion, but not subunit, vaccines in the present study demonstrates that in the case of influenza virus recognition of the viral genome may not be essential for the induction of partial lymphocyte activation.

This raises the question why subvirion, but not subunit, vaccine maintains the capacity to induce partial lymphocyte activation, despite both containing hemagglutinin. Three possible explanations can be envisaged: 1) the subvirion vaccine contains more viral RNA, sufficient to stimulate IFN-I responses. However, this is unlikely as Geeraedts et al. showed that both subunit and subvirion vaccines contain very little RNA (500 to 5000-fold less than whole virus vaccines) (Geeraedts, Goutagny et al. 2008); 2) the additional viral antigen/s present in subvirion but not in subunit vaccine, i.e. viral internal proteins, are the inducer of partial lymphocyte activation; 3) the viral surface proteins such as the hemagglutinins require a particular spatial arrangement, i.e. multivalency, or structural integrity that is disrupted during the vaccine processing step (i.e. splitting and purifying). Indeed, the HAU/virion of the subunit vaccine is significantly lower compared to that of intact live virus (Table 5.6.1). Also, numerous reports have identified TLR recognition of various viral surface proteins (Kurt-Jones,
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Popova et al. 2000; Rassa, Meyers et al. 2002; Boehme, Guerrero et al. 2006; Georgel, Jiang et al. 2007) and viral structural proteins (Triantafilou and Triantafilou 2004; Jiang, Georgel et al. 2005; Barbalat, Lau et al. 2009). For example, measles virus hemagglutinin is a ligand for TLR-2 (Bieback, Lien et al. 2002), and Barbalat et al (Barbalat, Lau et al. 2009) proposed that a variety of viral fusion proteins would be a suitable target for the innate immune system because of their structural homology among unrelated viruses (Steven and Spear 2006) and the absolute requirement of the fusion apparatus for viral infections. In this context, it is conceivable that the conserved structural regions of the influenza hemagglutinin are a PPR ligand.

In support of the hypothesis that the influenza hemagglutinin is involved in induction of partial lymphocyte activation, I have shown that in the absence of MHC molecules, the mitogenicity receptors (Scalzo and Anders 1985; Scalzo and Anders 1985; Marshall-Clarke, Tasker et al. 2006), the subvirion induced partial lymphocyte activation is significantly reduced. This data, together with the previously reported finding that trivalent influenza vaccines have a little viral RNA, strongly suggests that influenza hemagglutinins are involved in triggering partial lymphocyte activation by these preparations. Live or inactivated whole virus, on the other hand, induced partial lymphocyte activation in both MHC-I and -II deficient mice. Most likely, in the case of whole virus preparations that contain viral RNAs, genome-dependent innate immune recognition system, e.g. via TLR or RLR (RIG-I like receptor), are compensating for the loss of viral protein-mediated signalling pathway/s.

An important observation was that partial lymphocyte activation was dependent on MyD88 in vitro but not in vivo. This raises concerns about the physiological relevance of in vitro data, such as the reduced activation threshold obtained in B cells (Braun, Caramalho et al. 2002). The reason for the discrepancy between in vivo and in vitro results may be a presence of multiple, partially redundant, innate immune recognition mechanisms in vivo, which may compensate for a loss of MyD88-dependent pathways. For example, it has previously been shown that production of serum IFN-α is not substantial lower in MyD88 deficient mice in response to a systemic viral infection (Kumagai, Takeuchi et al. 2007). Almost all cells of immune and non-immune origin can produce IFN-I via RLH-dependent mechanism (Diebold, Montoya et al. 2003; Kato, Takeuchi et al. 2006; Kumagai, Takeuchi et al. 2007). Thus, serum IFN-α levels are largely unaffected by an absence of MyD88 signalling. On the other hand, in vitro IFN-α production by splenocytes has been shown to be very low, when
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cocultured with inactivated influenza viruses (Asselin-Paturel, Boonstra et al. 2001). The deficiency of MyD88 may further reduce IFN-I levels to below the threshold required for partial lymphocyte activation. Furthermore, it has been shown that the influenza hemagglutinin mediated B cell activation in vitro is dependent on MyD88 (Marshall-Claire, Tasker et al. 2006). Thus, it is not surprising that the magnitude of in vitro partial lymphocyte activation was greatly reduced in the absence of MyD88. In line with this interpretation, the capacity of subvirion to induce partial lymphocyte activation in MyD88"mice was reduced due to the lack of viral RNA in the subvirion formulation that may have otherwise allowed the compensatory effects by RLH signalling.

In contrasts to the absolute in vivo dependency of partial lymphocyte activation on the IFN-IR, cells from IFN-IR"mice were capable of up-regulating cell surface markers in response to influenza virus stimuli in vitro, albeit to a lesser extent. A possible explanation is that IFN-I may be acting intracellularly in interferon producing cells. While such an intracrine mechanism has indeed been described for IFN-II (Sanceau, Sondermeyer et al. 1987; Will, Hemmann et al. 1996), intra-cyttoplasmic or nucleic injection of IFN-I does not induce an antiviral state in mouse or human cells (Higashi and Sokawa 1982; Huez, Silhol et al. 1983; Riviere and de Maeyer-Guignard 1990), although the expression of cell surface markers was not assessed in these studies. The absence of autocrine or paracrine signalling due to the lack of IFN-IR explains the reduced magnitude of partial lymphocyte activation among IFN-IR"cells in vitro and total absence of partial lymphocyte activation in vivo. It is likely that partially activated IFN-IR" splenocytes in vitro are those cells that came into direct contact with influenza virus. In support of the notion of contact mediated activation, the number of IFN-IR" cells with CD25 or CD69 is similar (i.e. CD25"CD3" / CD3"cells: 13%, CD69"CD3" / CD3"cells: 11%) whereas wild-type splenocytes have twice as many CD69" cells (CD69"CD3" / CD3" cell: 59%) compared to CD25" cells (CD25"CD3" / CD3" cells: 29%). We have recently shown that co-culture of naïve splenocytes with recombinant IFN-β does not up-regulate CD25 expression on T cell (Wijesundara et al, manuscript under review). Thus, influenza virus may stimulate CD25 and CD69 expressions on lymphocytes via direct contact, in a non-antigen specific manner, and the subsequent secretion of IFN-I mediates further up-regulation of CD69 in an autocrine fashion. This could provide an explanation for the observed CD25 up-regulation observed in vitro but not in vivo.
Overall, the data are consistent with the notion that the mitogenic activity of influenza virus does not directly elicit partial lymphocyte activation but acts as the stimulus to initiate IFN-I responses, which are responsible for partial lymphocyte activation downstream.

The observation that trivalent influenza vaccine triggers partial lymphocyte activation may be of clinical relevance, because partial lymphocyte activation correlates with negative consequences for the host, manifest in a greater susceptibility to a secondary infection with an unrelated virus (Alsharifi, Regner et al. 2006; Zuniga, Liou et al. 2008). Thus, the possibility that trivalent influenza vaccine could render vaccinees transiently immunosuppressed needs to be considered and investigated.

From an evolutionary perspective this increased susceptibility to a secondary infection points to a strong selective advantage to activate partial systemic lymphocyte activation after a primary virus infection. We have hypothesized that partial lymphocyte activation may reduce the activation threshold of B and T cells (Alsharifi, Mullbacher et al. 2008) but recently found evidence to the contrary for T cells (Wijesundara et al, manuscript submitted). Alternatively, partial lymphocyte activation may be essential to generate a large pool of phenotypically ready lymphocytes to participate in “passive” antigen receptor acquisition, a striking phenomenon reported recently (Quah, Barlow et al. 2008; Chaudhri, Quah et al. 2009). Such a scenario would solve the dilemma highlighted by the ‘protection hypothesis’ of Langmann and Cohn – every sized species have evolved immune system that are equally efficient in generating immunological responses despite their significant lymphocyte repertoire differences (Cohn and Langman 1990). The findings that CD 69 expression mediates LN retention (Shiow, Rosen et al. 2006) does support this hypothesis, as it does ensure close proximity between antigen-specific, fully activated and antigen non-specific, partial activated lymphocytes.

In conclusion, infectivity and replicative capacity are not essential for influenza virus to induce partial lymphocyte activation, which contrasts with other viruses such as the alphavirus, aSFV. Thus, partial lymphocyte activation appears to be a universal immune response to viruses but the immune system may employ a range of strategies to achieve it. Together with the corresponding increased susceptibility for secondary infection, this strongly points to an important as yet unidentified biological role for partial, rapid, systemic lymphocyte activation after a primary viral infection.
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5.6 Tables and Figures

Table 5.6.1 Virion particle and HAU contents of different viral preparations

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<td>Red blood cell solution</td>
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<td>$4 \times 10^5$</td>
<td>$4 \times 10^3$</td>
</tr>
<tr>
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<td>$4 \times 10^5$</td>
<td>$1.3 \times 10^5$</td>
</tr>
<tr>
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<td>$4 \times 10^5$</td>
<td>$1.3 \times 10^5$</td>
</tr>
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<td>90</td>
<td>$5 \times 10^4$</td>
<td>$1 \times 10^{12}$</td>
<td>$5 \times 10^4$</td>
</tr>
<tr>
<td>TIV-subvirion</td>
<td>90</td>
<td>$1.5 \times 10^5$</td>
<td>$1 \times 10^{12}$</td>
<td>$1.5 \times 10^7$</td>
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*Virions/ml and HAU/virion were calculated using known PFU/ml values and based on the following: molecular weight of HA is 80000 (Wrigley 1979); 500 HA per virion (Compans, Klenk et al. 1970; Inglis, Carroll et al. 1976); and 40 defective virions per PFU (Hutchinson, Curran et al. 2008)).
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Figure 5.6.1 Gating strategies used for cell surface marker expression analysis by flow cytometry.

The gated populations R0 are selected based on forward scatter and side scatter (A). 7-AAD negative cells were then gated for further analysis (B). Cell surface marker positive cells were gated on a histogram (C). These gated cell populations were then stained and analysed for various cell surface marker expressions. An example of CD86 expression analysis on CD3+ cells is shown.
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Figure 5.6.2 Inactivated influenza virus preparations induce partial lymphocyte activation.

Two mice per group were immunized intravenously with either live (2 x 10^7 PFU) or inactivated viruses (2 x 10^7 PFU equivalent); live A/PR8 (pink), gamma-inactivated A/PR8 (blue), formalin-inactivated A/PR8 (green) or UV-inactivated A/PR8 (yellow) and mock treated (black). Splenocytes from >10-week-old C57BL/6 mice were harvested 24 h post immunization. Cells were stained and analysed by FACS. Histograms show activation marker expression levels in gated, CD3^+^, CD8^+^, CD11c^+^ or CD19^+^ cell populations. N.T.: not tested.
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Figure 5.6.3 Kinetics of partial lymphocyte activation on splenocytes from immunized mice.

C57BL/6 mice were immunized intravenously with either live (2 x 10^7 PFU) or inactivated viruses (2 x 10^7 PFU equivalent); live A/PR8, gamma-inactivated A/PR8, formalin-inactivated A/PR8 or UV-inactivated A/PR8 and mock treated (dotted line). Splenocytes from >10-week-old mice were harvested at 1, 2 and 3 days post immunization and were analysed for indicated cell surface antigen expressions on CD3^+, CD8^+, CD11c^+ or CD19^+ cell populations. The percentage of cell surface antigen expressing cells is calculated by flow cytometry. Data represent the mean ± SD of two mice per group. N.T.: not tested.
Figure 5.6.4 Gamma-inactivated aSFV does not induce partial lymphocyte activation.

Two mice per group were immunized intravenously with either live aSFV ($10^7$ PFU) or gamma-inactivated aSFV ($10^7$ PFU equivalent). Splenocytes from >10-week-old C57BL/6 mice were harvested 24 h post immunization. Cells were stained and analysed by FACS. Histograms show activation marker expression levels on splenocytes from mock (black), live aSFV infected (pink) and gamma-aSFV immunized mice (blue) in gated, CD3+, CD8+, CD11c+ or CD19+ cell populations.
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A: IFN-IR^- mice

CD25 cell counts

N.T.

CD40 cell counts

N.T.

CD69 cell counts

N.T.

CD86 cell counts

CD25 → CD40 → CD69 → CD86
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B: IFN-IIR-/- mice

N.T.
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C: IFN-I&IIR^{-} mice

CD3^+ cell counts

CD86

CD25

CD40

CD69

CD86

N.T.
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D: H₂AB⁻/⁻ mice

CD³⁺ cell counts

CD⁸⁺ cell counts

CD³⁺α/β cell counts

CD⁸⁺ cell counts

CD25 → CD40 → CD69 → CD86

N.T.
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E: 129 (WTGR) mice

Figure 5.6.5 Generalized partial lymphocyte activation depends on IFN-IR but not on IFN-IIR or MHC-II.

Splenocytes from IFN receptor deficient mice ((A) IFN-IR−/−, (B) IFN-IIR−/−, (C) IFN-I&II−/− mice), (D) MHC-II deficient mice (H2AB−/−), and (E) wild type 129 mice were analysed for CD25, CD40, CD69 and CD86 expression on CD3+, CD8+, CD11c+ and CD19+ cells following in vivo immunization. Histograms are comparing fluorescence profiles from mock (black), live A/PR8 infected (pink), gamma-A/PR8 immunized (blue) and formalin-A/PR8 immunized mice (green). Day 1 post immunization data are shown above.
Figure 5.6.6 Comparison of IFN-α (A&C) and IFN-β (B&D) serum levels in mice immunized intravenously with either live and or inactivated viruses.

Mice were infected with either $10^7$ PFU aSFV or A/PR8 or immunized with inactivated aSFV or A/PR8 (dose equivalent to $2 \times 10^7$ PFU). Serum of individual mice was tested for IFN-α and IFN-β concentrations and means were expressed as Units/ml. One unit of mouse interferon alpha/beta is the amount of interferon alpha/beta which protects 50% of the indicator cell population from viral destruction. Each column represents the mean and 1 standard deviation of two mice per group.
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Figure 5.6.7 Proliferative response of splenocytes to live or inactivated virus preparations.

A) Splenocytes of C57BL/6 mice and MHC-II deficient mice (H2AB-) mice were incubated in culture medium at $1 \times 10^6$ cells/ml with different virus preparations ($1 \times 10^5$ PFU/culture), as indicated on the graph, for 42 h followed by further 16 h incubation with 0.5 µCi $[^3H]$thymidine. Each column represents the mean and 1 standard deviation of triplicate cultures. *: P < 0.05 vs. corresponding media control. **: P < 0.05 vs. MHC II+.

B) Splenocytes of C57BL/6 were cultured with various HAU/ml concentrations of live or inactivated A/PR8 (H1N1) influenza virus preparations. Each column represents the mean and 1 standard deviation of triplicate cultures. *: P < 0.05 vs. A/PR8.
Figure 5.6.8 Subvirion vaccine, containing all the viral proteins, induces partial lymphocyte activation.

Two mice per group were immunized intravenously with either live ($2 \times 10^7$ PFU) or trivalent influenza vaccine (CSL fluvax vaccine; A/Solomon Islands/3/2006 H1N1, A/Brisbane/10/2007 H3N2, B/Florida/4/2006; 18 μg haemagglutinin); live A/PR8 (pink), trivalent influenza vaccine (blue), and mock treated (black). Splenocytes from >10-week-old C57BL/6 mice were harvested 24 h post immunization. Cells were stained and analysed by FACS. Histograms show activation marker expression levels in gated, CD3⁺, CD8⁺, CD11c⁺ or CD19⁺ cell populations.
Figure 5.6.9 Influvac subunit vaccine, containing purified hemagglutinin and neuraminidase, failed to induce partial lymphocyte activation.

Two mice per group were immunized intravenously with either live (2 x 10^7 PFU) or trivalent influenza vaccine (Solvay Biologicals Influvac subunit vaccine 09/10; influenza A/Brisbane/59/2007 H1N1, A/Brisbane/10/2007 H3N2 and B/Brisbane/60/2008: 18 μg hemagglutinin); live A/PR8 (pink), trivalent influenza vaccine (blue), and mock treated (black). Splenocytes from >10-week-old C57BL/6 mice were harvested 24 h post immunization. Cells were stained and analysed by FACS. Histograms show activation marker expression levels in gated, CD3\(^+\), CD8\(^+\), CD11c\(^+\) or CD19\(^+\) cell populations.
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A: B2M<sup>−/−</sup> (MHC I<sup>−/−</sup>) mice  B: H2AB<sup>−/−</sup> (MHC II<sup>−/−</sup>) mice

C: IFN-IR<sup>−/−</sup> mice  D: MyD88<sup>−/−</sup> mice  E: 129 (WTGR) mice

Figure 5.6.10 IFN-I, MHC molecules and MyD88 all play a role in subvirion vaccine induced partial lymphocyte activation.

Splenocytes from (A) MHC-I deficient mice (B2M<sup>−/−</sup>), (B) MHC-II deficient mice (H2AB<sup>−/−</sup>), (C) IFN-I receptor deficient mice (IFN-IR<sup>−/−</sup>), (D) MyD88 deficient mice (MyD88<sup>−/−</sup>) and (E) wild type 129 mice were analysed for CD25, CD40, CD69 and CD86 expression on CD3<sup>+</sup>, CD8<sup>+</sup>, CD11c<sup>+</sup> and CD19<sup>+</sup> cells following in vivo immunization. Histograms are comparing fluorescence profiles from mock (black), live A/PR8 infected (pink), gamma-A/PR8 immunized (blue), and CSL fluvax vaccine immunized mice (green). Day 1 post immunization data are shown.
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A: TLR-2\(^{-/-}\)

- CD8\(^+\) cell counts
- N.T.
- CD3\(^+\) cell counts
- N.T.
- CD11c\(^+\) cell counts
- N.T.
- CD19\(^+\) cell counts

- CD25
- CD40
- CD69
- CD86
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B: TLR-4\(^{-/-}\)

- CD3\(^+\) cell counts
- CD8\(^+\) cell counts
- CD11c\(^+\) cell counts
- CD19\(^+\) cell counts

CD25  CD40  CD69  CD86
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C: MyD88−/− mice

Naive

Live-A/PR8: 2 x 10⁷ PFU,
1.5 x 10⁴ HAU

Gamma-A/PR8: 2 x 10⁷ PFU,
1.5 x 10⁴ HAU

Formalin-A/PR8: 2 x 10⁷PFU,
5 x 10³ HAU

Formalin-A/PR8: 6 x 10⁷ PFU,
1.5 x 10⁴ HAU

Figure 5.6.11 Inactivated influenza virus induced partial lymphocyte activation is partially dependent on MyD88.

Splenocytes from (A) Toll-like receptor-2 deficient mice (TLR-2−/−), (B) Toll-like receptor 4 (TLR-4−/−) and (C) Myeloid differentiator factor 88 deficient mice (MyD88−/−) were analysed for CD25, CD40, CD69 and CD86 expression on CD3+, CD8+, CD11c+ and CD19+ cells following in vivo immunization. Histograms are comparing fluorescence profiles from mock (black), live A/PR8 infected (pink), gamma-A/PR8 immunized (blue) and formalin-A/PR8 immunized mice (green). Day 1 post immunization data are shown above.
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A: C57BL/6
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B: MyD88⁻/⁻

[Graphs showing CD25⁺, CD3⁺ cells, CD19⁺ cells, and CD69⁺ cells with PFU/well on the x-axis and percentage on the y-axis for different treatments: Live A/PR8, Gamma-A/PR8, Live aSFV, Gamma-aSFV.]
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C: C57BL/6

![Graphs showing partial lymphocyte activation](image-url)
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D: MyD88

- CD25, CD3^+ cells / CD3^+ cells (%)

- CD25, CD19^+ cells / CD19^+ cells (%)

- CD69, CD3^+ cells / CD3^+ cells (%)

- CD69, CD19^+ cells / CD19^+ cells (%)

HAU/well

- Purified A/PR8
- Gamma-A/PR8
- Formalin-A/PR8
- UV-A/PR8
- CSL subvirion
Figure 5.6.12 Up-regulation of cell surface markers CD25 and CD69 by whole and split influenza virus is MyD88 dependent.

C57BL/6 (A & C), MyD88<sup>−/−</sup> (B & D) and IFN-IR<sup>−/−</sup> (E) plenocytes were cultured with various concentrations of different virus preparations or commercially available trivalent influenza vaccines and expression of cell surface markers was assessed at 12-15 h. The percentage of cell surface antigen expressing cells is calculated by FACS. Each column represents the mean and 1 standard deviation of triplicate wells. Dotted line represents background levels.
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Chapter 6

Concluding Remarks
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In this thesis I investigated the influence of three different viral inactivation methods, formalin treatment, UV-, and gamma-ray irradiation, on the ability of the influenza virus to induce innate and adaptive immune responses. The emphasis was on a comparison of the protective efficacy of these three different inactivated influenza virus preparations, in a side-by-side experimental setting, to investigate which inactivation method confers superior immunity against a heterosubtypic influenza virus challenge in a mouse model.

Factors such as the nature of the antigen, the route of immunisation, the vaccine dose, and the use of adjuvants can greatly influence the potency of vaccines. The comparison of different viral preparations in eliciting immune responses has clearly indicated that the inactivation method has a profound impact on the immunogenicity of the virus. Of the three inactivation methods investigated, only gamma-irradiation, which had the least detrimental impact on influenza hemagglutination activity (see Chapter 3), preserved the cross-protective potential of the influenza virus (see Chapters 2, 3 and 4) (Alsharifi, Furuya et al. 2009). Most importantly, the immunity induced by gamma-inactivated human influenza type A virus, H1N1 (A/Puerto Rico/8/34) cross-protected mice against challenge with not only different human seasonal strains, but also the highly pathogenic avian influenza virus, H5N1 (A/Vietnam/1203/2004) (see Chapter 2) (Alsharifi, Furuya et al. 2009). Further, gamma-irradiation maintained the T cell immunogenicity of the influenza virus (see Chapter 3), a phenomenon that was absent in formalin or UV-inactivated influenza virus preparations (see Chapters 2 and 3).

Gamma-inactivated virus induced immune responses that were qualitatively and quantitatively superior to those induced by the other two inactivation methods. For example, a single immunisation with gamma-inactivated virus conferred robust homologous protection, whereas formalin-inactivated virus required higher doses and multiple immunisations to achieve a similar effect. In addition, mice immunised with gamma-inactivated virus had higher levels of serum type I IFN (see Chapters 5). Thus, gamma-irradiation better preserves the viral immunogenicity and consequently, results in a superior vaccine in comparison to other methods of viral inactivation.

Inferior immunogenicity of the commonly used vaccine formula (subunit and split influenza vaccines) to whole virion vaccines is well documented (Gross, Ennis et al. 1977; Ortbals and Liebhaber 1978; Profeta, Bigatello et al. 1980; Stephenson, Nicholson et al. 2003). In my model system live virus, inactivated whole virus and split vaccine (CSL fluvax 2008 formulation) maintained an ability to induce type I IFN
dependent partial lymphocyte activation, whereas subunit vaccine (Solvay Biologicals influvac 2009 formulation) did not (see Chapter 5). The latter's lower immunogenicity is probably because of the additional step of protein purification, which may have disturbed the spatial arrangement of the hemagglutinin, a suspected inducer of partial lymphocyte activation. This notion is supported by the comparison of the split and the subunit vaccine in terms of hemagglutination activity, which revealed that the subunit vaccine contained one third less HAU/HA μg than the split vaccine, indicating that the integrity of influenza hemagglutinin has indeed been severely affected by an additional purifying step during vaccine production. However, due to the differences in the vaccine strains (same influenza B strain but different type A strains), the absence of partial lymphocyte activation with the subunit vaccine cannot definitively be attributed to the additional steps of vaccine production. Nonetheless, that above data suggests that virus-processing treatments, in addition to the mode of viral inactivation, can affect the viral immunogenicity. The physiological significance of \textit{in vivo} partial lymphocyte activation for vaccine efficacy is not known. However, based on the assumption that the naturally occurring immune response is most superior, unnecessary virus antigen processing or destructive inactivation methods should be avoided to maintain the natural adjuvant and antigenic structures.

The cross-protection conferred by gamma-inactivated influenza virus is multifactorial. Given the complexity of the immune system, it is not surprising that multiple components of immune system, MHC class-I, -II, perforin, B cells and T cells, are all necessary for the development of cross-protective immunity. However, the data presented in Chapter 4 provide evidence that the gamma-inactivated influenza virus induced cross-protective immunity is mainly mediated by cross-reactive Tc cells. Generally, inactivation is thought to render pathogens unable to stimulate T cell immunity (Ertl, Gerike et al. 1977; Braciale and Yap 1978; Bachmann, Kundig et al. 1993). Thus, the above finding leads to a question: How does a virus that is fully inactivated by gamma-irradiation elicit Tc cell responses? At least two speculations can be made. (1) In contrast to inactivation by chemical treatment, gamma-irradiation leaves the functional domains of the viral surface proteins intact allowing efficient uptake and uncoating of viral particles. This may provide sufficient viral antigens (all viral products with exception of NS1 protein that is expressed only in infected cells but not detected in the virion (Lamb and Choppin 1983; Richardson and Akkina 1991)) into the cytoplasm of antigen presenting cells for introduction into the MHC-I antigen presentation
pathway. In support of this, gamma-irradiation, but not formalin or UV-inactivation, retained the ability of the influenza virus to sensitise target cells for Tc cell lysis, indicating that a gamma-inactivated virus is able to efficiently deliver their antigens into MHC-I presentation pathway. (2) Defective ribosomal products (prematurely terminated and misfolded gene products) are considered a dominant source of viral antigen for MHC class I antigen presentation (Yewdell, Anton et al. 1999). Partial genome transcription and abortive translation of fragmented influenza virus genomic RNA may occur and allow the priming of virus-specific Tc cell immunity. In support of this, preserved protein and nucleic synthesis has been reported for parasitic protozoa (Hiramoto, Galisteo et al. 2002) and bacteria (Sanakkayala, Sokolovska et al. 2005). However, the latter is a less probable scenario because gamma-inactivation does destroy the ability of flavivirus, whose major Tc cell determinants are derived from non-structural proteins, to induce Tc cell response. Thus, protein synthesis does not appear to occur for gamma-irradiated viruses. A plausible explanation for the retained protein synthesis for microorganisms with more complex genomic structures is that only a few lethal DNA strand breaks are required to inhibit the microbial replication, which leaves large proportions of the genomes intact. This may allow adequate function of the genes needed for some protein synthesis.

In mice, T cell-mediated heterosubtypic immunity to influenza type A virus is a well established phenomenon (Grebe, Yewdell et al. 2008). The facts that the majority of Tc cell against influenza A virus are cross-reactive among subtypes (Zweerink, Courtneidge et al. 1977; Askonas, Taylor et al. 1988), derived from conserved internal proteins (Yewdell, Bennink et al. 1985; Gotch, McMichael et al. 1987), have given rise to the notion that the T cell-mediated cross-protection observed in mice may also exist in humans. However, due to the limited amount of human studies, there has been no conclusive answer for the existence of heterosubtypic immunity in humans or for the importance of T cells in the cross-protection (Grebe, Yewdell et al. 2008). McMichael et al. were the first and last to demonstrate in humans that in the absence of influenza virus strain specific antibodies, the presence of Tc cell memory correlates with a significant reduction in the level of nasal virus shedding following an experimental challenge (McMichael, Gotch et al. 1983). This unique study has shown that T cell immunity is not sterilising immunity but rather facilitates viral clearance. In our mouse model, mice immunized with gamma-inactivated virus can indeed be infected with heterosubtypic strains, but recover faster in comparison to unvaccinated mice (see
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Chapters 2 and 3). Other supporting data for a cross-protective role of T cells in humans are circumstantial evidence. Epidemiological data from 1957 suggests that individuals with prior exposure to H1N1 strains were more resistant to the newly emerged pandemic H2N2 strain (Slepushkin 1959; Epstein 2006). The observed partial protection cannot be attributed to antibodies against the viral surface antigens since both the hemagglutinin and neuraminidase had shifted. Cross-protective immunity has also been shown to exist during seasonal influenza (Sonoguchi, Naito et al. 1985). Although the above correlative evidence does not guarantee high efficacy of Tc cell-inducing vaccines, it encourages further experiments to be pursued in other models of human influenza infection, such as ferrets.

Two scenarios can be envisaged in which T cell-based gamma-inactivated virus vaccine is useful in the event of a pandemic. Firstly, the incubation period and the course of infection with avian influenza viruses are longer than those for human influenza viruses (Beigel, Farrar et al. 2005). Thus, the acceleration of the cross-reactive Tc cell response by even half to one day by prior immunisation with gamma-inactivated virus vaccine could be beneficial when antibodies are ineffective. Secondly, high levels of Tc cells are expected to reduce the amount and the period of viral shedding (McMichael, Gotch et al. 1983), thereby limiting the human-to-human transmission rate. Thus, having cross-reactive T cell immunity in a community may reduce both the rate and the extent of pandemic spread.

Inevitably, the maintenance of memory T cells will be crucial for T cell dependent vaccines. CD8 T cell-mediated immunity has been shown to rapidly decline with time compared to long-lasting B cell mediated immunity (Liao, Li et al. 1998; Maruyama, Lam et al. 2000; Tuaillon, Tabaa et al. 2006). In humans, an estimated half-life of two to three years has been reported for Tc cell activity (McMichael, Gotch et al. 1983). Thus, gamma-inactivated virus vaccine could be incorporated into the seasonal influenza vaccination programme to allow for a regular boosting in order to maintain memory CD8 T cells at the protective levels. Therefore, in the event of pandemic, gamma-inactivated virus vaccine should provide partial protection to allow enough time for the antigen-matched vaccine to be prepared. This is particularly important because with current egg-based vaccine production, manufacturing antigen-matched vaccines is expected to require six to eight months (Gerdil 2003) and even longer for highly pathogenic avian influenza strains due to their toxicity in eggs (Takada, Kuboki et al. 1999). Further, pre-pandemic immunisation with a broadly cross-reactive vaccine
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would avoid the huge financial burden of the pandemic vaccine stockpiling strategy that is currently implemented (Straetemans, Buchholz et al. 2007). Given that predicting the future pandemic strain is not possible, stockpiling strain-specific vaccines based on H5N1 strains would seem highly ineffective and wasteful compared to the proposed strategies using gamma-irradiation.

It is important to note that gamma-inactivated virus vaccine is not intended to be a sole measure of defence against future pandemics, but rather should be promoted to supplement or expand current intervention strategies. This, of course, is because the gamma-inactivated influenza vaccine would not confer sterilising immunity against heterosubtypic strains, but provide protection from what might otherwise be a lethal outcome. Thus, gamma-inactivated virus vaccine should be used with the acceptance that disease of a certain degree associated with heterosubtypic influenza infection cannot be avoided during pandemics.

An ideal vaccine would confer protection to all segments of the population. However, current influenza vaccines have suboptimal protective efficacy of 30 to 40 per cent among the elderly (Govaert, Thijs et al. 1994), in contrast to 65 to 80 per cent efficacy in young adults (Muszkat, Friedman et al. 2000). Consequently, ~90 percent of influenza related mortality occurs among the elderly (Thompson, Shay et al. 2003). The mechanism behind the poor vaccine efficacy in the elderly is not fully understood, but waning immunity due to increasing age is thought to be the primary reason (Gavazzi and Krause 2002). Among elderly individuals, the current influenza vaccine has a limited boosting effect on the antibody responses (Brandriss, Betts et al. 1981; Gross, Quinann et al. 1989). However, a recent study shows that even with hemagglutination titers that are generally considered to be protective, the elderly are still susceptible to influenza infections (Gravenstein, Drinka et al. 1994). The inconsistent association between high antibody levels and disease protection in the elderly has led to a suggestion that a measure of T cell immunity is a better vaccine protection correlate among this group (McElhaney, Xie et al. 2006; McElhaney, Ewen et al. 2009). Failure of antibody-mediated protection by current vaccines also implies that T cells may be important in the elderly against not only heterosubtypic strains, but also against homologous strains. Further, original antigenic sin, where memory B cells mount antibody responses against previous viral strains at the expense of simulating naïve B cells for new strains (Kim, Skountzou et al. 2009), has been consistently demonstrated for antibody responses against influenza vaccines (Virelizier, Allison et al. 1974;
Webster, Kasel et al. 1976; McElhaney, Meneilly et al. 1993). Thus, in the light of these findings, Tc cell inducing influenza vaccines are highly valuable for vaccination targeting elderly people.

Current influenza vaccines are highly effective in boosting pre-existing immune responses in young adults, whereas a larger amount of antigen and multiple doses are required to obtain protective antibody levels in immunologically naïve individuals (Treanor, Campbell et al. 2006; Leroux-Roels, Borkowski et al. 2007). Similarly, in our mouse model, formalin-inactivated virus preparations required a threefold higher dose with double to triple immunisation to achieve statistically significant homologous protection (see Chapter 3). In fact, this requirement represents one of the major challenges of facing influenza pandemics due to the limited production capacity of the antigen-matched vaccine (Nicholson, Colegate et al. 2001; Treanor, Wilkinson et al. 2001; Bresson, Perronne et al. 2006; Treanor, Campbell et al. 2006). In this context, dose sparing is considered an important aspect for pandemic vaccine development. Thus, gamma-inactivated virus vaccine that can confer long-lasting immunity after single low-dose immunisation is highly relevant, especially in developing countries where populations may not have easy access to vaccination services. The dose of gamma-inactivated virus can be indirectly compared with trivalent influenza vaccine. One study has reported that intranasal triple priming with two-thirds µg of adjuvanted seasonal influenza vaccine conferred cross-protection against H5N1 in mice (Ichinohe, Tamura et al. 2007). Two µg of influenza type A hemagglutinin is equivalent to $3 \times 10^{10}$ viral particles (based on the following: molecular weight of HA is 80000 (Wrigley 1979); 500 HA per virion (Compans, Klenk et al. 1970; Inglis, Carroll et al. 1976); and 40 defective virions per PFU (Hutchinson, Curran et al. 2008)). The dose of gamma-inactivated virus preparation that provided cross-protection against H5N1 was $3.2 \times 10^6$ PFU equivalent, which is $1 \times 10^8$ viral particles. Thus, in mice, the antigen content of gamma-inactivated virus preparation is at least 1/300 of the current trivalent influenza vaccine even without an adjuvant. However, due to the differences in the virus strain used for the challenge and vaccination, the vaccine doses from two different experiments cannot be directly compared. Nevertheless, if this antigen-sparing effect of gamma-inactivated virus in mice were mirrored in humans, the gamma-irradiation method would certainly ameliorate the potential shortage of vaccines during a pandemic.
Adjuvantation represents an important antigen sparing strategy (Stephenson, Gust et al. 2006). Currently, Alum and MF59 are the only adjuvants that are approved for human influenza vaccines. Of these, only MF59 has been included in human influenza vaccines, mostly in Europe, due to its ability to elicit balanced IgG1:IgG2a responses and CD8+ T cell responses (Podda and Del Giudice 2003; Radosevic, Rodriguez et al. 2008). Similarly, adjuvanting T cell-based gamma-inactivated virus vaccine with Th-1 skewing MF59 adjuvant could possibly allow a dose-reduction without the concomitant loss of immunogenicity. In contrast, alum is not expected to enhance the efficacy of T cell-based vaccines due to its potential to inhibit the development of CD8 Tc cells (Simon, Edelman et al. 2006). Other mucosal adjuvants, such as cholera toxin or Escherichia coli enterotoxin, are highly effective but their high reactogenicity limits their use in humans (Lewis, Huo et al. 2009).

Another aspect of a successful vaccination strategy is the need to distribute and administer the effective vaccine as rapidly as possible. Current influenza vaccines, inactivated and live attenuated formulations, are in aqueous and frozen forms, respectively, with stringent storage requirements. In contrast, the smallpox vaccine, the most successful vaccine, is in dry powdered form. Dry powder formulation allows rapid distribution of the vaccine even in the developing countries and facilitates an active vaccination programme. Interestingly, the freeze-drying (lyophilization) process maintains the immunogenicity of gamma-inactivated influenza virus (see Chapter 3). Currently, further investigations are underway to determine the storage life of lyophilised gamma-inactivated virus preparations. If lyophilised formulation is proven to be room temperature stable, it could relieve enormous financial pressures in developing countries by reducing the cost associated with the transport and storage of vaccines.

Intranasal immunisation was clearly superior to intravenous, intraperitoneal, or intramuscular immunisation for the induction of cross-protective immunity (see Chapter 2). Although most vaccines traditionally have been administered by an intramuscular route, the mucosal administration of vaccines offers several important advantages. These include easier administration, reduced vaccine dose, and the potential for frequent boosting. In addition, local immunisation induces mucosal immunity at the sites where majority of pathogens initially establish infections. This is particularly relevant for the vaccine strategy against avian influenza strains. A distinctive feature of highly pathogenic avian influenza virus such as H5N1 is that the infection spreads to a lower
respiratory track (Tran, Nguyen et al. 2004; Uiprasertkul, Puthavathana et al. 2005). Thus, one could anticipate that in humans, gamma-inactivated virus will need to be delivered to the lower respiratory track for the optimum protection against influenza strains with broad tissue tropism. In support of this, only total respiratory tract immunization (anaesthetised), but not upper respiratory tract immunization (unanaesthetized), with live influenza virus generated heterosubtypic protection against pulmonary challenge and induced Tc cells in the mucosa-associated lymphoid tissue from which Tc cells are recruited to the lungs (Nguyen, Moldoveanu et al. 1999). For all the protection experiments described in this thesis, mice were anaesthetised for intranasal vaccination with large inoculum of 32 µL. This allowed the vaccines to penetrate deep into the lower lungs (Yetter, Lehrer et al. 1980) and ensured longer antigen residence time in lungs for an optimum immunisation (Foster, Walters et al. 2001). However, this method of vaccination will not be feasible for humans. In humans, delivery of vaccines to the lower respiratory tract is difficult, requiring sophisticated technology, and is associated with potential risk of reactogenicity due to lung deposition of foreign materials (Vajdy and OiHagan 2001). The nasal spray devices used for the current live attenuated influenza vaccine may not be sufficient for the delivery of gamma-inactivated virus preparation. The majority of antigens administered intranasally are deposited in the anterior regions of the nose (Soane, Frier et al. 1999; Suman, Laube et al. 1999), leaving large proportions of the respiratory tract unexposed to the antigens. This property does not affect the efficacy of the live attenuated influenza vaccine due to its target site of immunisation being the upper respiratory tract and, therefore, delivery to the lower lungs is not required. The difficulty in controlling the vaccine dose and the site of antigen deposition within the respiratory tract are the limitations of the current nasal delivery technologies. Intranasal vaccine delivery technology is an intense field of research and gamma-inactivated virus is likely to benefit from this rapidly developing technology for human administration.

In conclusion, the advantage of gamma-inactivated whole virus vaccine is threefold. Firstly, cross-protection against a range of virus subtypes would be useful during pandemic and seasonal influenza seasons. Secondly, use of inactivated whole virus vaccine will avoid the time and resources required to purify antigens for subunit vaccines or to create genetically attenuated live vaccine strains. Thirdly, the rapidity of gamma-irradiation, in comparison to chemical treatments that require longer incubation time and additional steps to remove the chemicals, will accelerate the vaccine
production during influenza pandemic. Together with the above-mentioned advantages, the data presented in this thesis strongly suggests gamma-irradiation to be considered as an alternative vaccine preparation strategy.
References


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