Recombinant Influenza viruses as a vaccine vector for HIV

By

Mohammed Jasim Mohammed Shallal

Submitted in fulfillment of the requirements for the degree of

Doctor of Philosophy

Deakin University
March, 2015
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Date: 2nd of March 2015
Preface
The work presented in this thesis represents of my original laboratory work performed at CSIRO (AAHL) under an agreement with Deakin University.

The pseudo-challenge experiments in Chapter 5 were performed in the laboratory of Dr. Charani Ranasinghe, Head of the Mucosal Immunology group, at the John Curtin School of Medical Research, Australian National University.

Publications


Conference presentations
- AIDS Vaccine Conference, October 2013 (Barcelona, Spain). "Prime boost HIV vaccination with recombinant influenza virus vectors stimulates specific and mucosal CD8+ T cell immune response in BALB/c mice".

- Australian Society of Immunology (ASI), Annual Conference, December 2012 (Melbourne, Australia). "Highly expressed α4β7+CD8+ T cells induced following intranasal prime boost vaccination by recombinant influenza-HIV vaccine in a mouse model".

- Immunology Group of Victoria (IGV) Meeting, September 2012 (Melbourne, Australia). "Recombinant influenza viruses as a vaccine vector for HIV".
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To my family and friends

Mother and Father, I would like to thank you for the patience and love you have provided me throughout my life and I promise you that I will not forget the sacrifices you have made to support my studies.

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<th>Description</th>
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<tr>
<td>AAHL</td>
<td>Australian Animal Health Laboratory</td>
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<tr>
<td>AIDS</td>
<td>Acquired Immunodeficiency Syndrome</td>
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<tr>
<td>ADH</td>
<td>Alcohol dehydrogenase</td>
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<tr>
<td>ALVAC</td>
<td>Recombinant Canary Pox Vector vaccine expressing HIV Gag</td>
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<tr>
<td>APC</td>
<td>Antigen Presenting Cell</td>
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<tr>
<td>CD8α/β</td>
<td>CD8⁺ T cell Alpha/Beta</td>
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<tr>
<td>CSIRO</td>
<td>Commonwealth Scientific Industrial Research Organization</td>
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<tr>
<td>CTL</td>
<td>Cytotoxic T lymphocyte cell</td>
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<tr>
<td>DMEM</td>
<td>Dulbecco’s Modified Eagle Medium</td>
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<tr>
<td>DNA</td>
<td>Deoxyribonucleic Acid</td>
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<tr>
<td>Env</td>
<td>HIV envelope glycoprotein</td>
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<tr>
<td>FCS</td>
<td>Fetal Calf Serum</td>
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<tr>
<td>Gag</td>
<td>HIV group-specific antigen</td>
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<tr>
<td>Gag197</td>
<td>AMQMLKETI peptide spanning residues 197-205 of Gag</td>
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<td>HBSS</td>
<td>Hank’s buffered salt solution</td>
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<tr>
<td>HA</td>
<td>Haemagglutinin</td>
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<td>HEVs</td>
<td>High Endothelial Venules</td>
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<td>HIV</td>
<td>Human Immunodeficiency Virus</td>
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<tr>
<td>HLA</td>
<td>Human Leukocyte Antigen</td>
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<tr>
<td>HNE</td>
<td>Human Neutrophil Eastase inhibitor</td>
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<tr>
<td>ICS</td>
<td>Intracellular Cytokine Staining</td>
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<tr>
<td>IFN-γ</td>
<td>Interferon gamma</td>
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<td>IL-2</td>
<td>Interleukin 2</td>
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<tr>
<td>i.n</td>
<td>Intranasal</td>
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<td>i.v</td>
<td>Intravaginal</td>
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<td>i.n-i.n</td>
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<td>LPAM</td>
<td>Lymphocyte Peyer’s Patches Adhesion Molecule</td>
</tr>
<tr>
<td>MAdCAM</td>
<td>Mucosal Adhesion Molecules</td>
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<tr>
<td>MBL</td>
<td>Mannose Binding Lectin</td>
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<tr>
<td>MDCK</td>
<td>Madin–Darby Canine Kidney</td>
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<tr>
<td>MDCs</td>
<td>Myeloid Dendritic cells</td>
</tr>
<tr>
<td>MHC-1</td>
<td>Major Histocompatibility Complex class I</td>
</tr>
<tr>
<td>N or NA</td>
<td>Neuraminidase</td>
</tr>
<tr>
<td>NP</td>
<td>Viral Nucleoprotein</td>
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<tr>
<td>NP147</td>
<td>TYQRTALV peptide spanning residues 147–155 of NP</td>
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PBS  Phosphate Buffer Saline
PCR  Polymerase Chain Reaction
PDCs  Plasmacytoid Dendritic Cells
pfu  Plaque Forming Unit
PR8  A/PR8/34 (H1N1)
PR8NA-Gag197  Recombinant PR8 influenza virus expressing Gag197 peptide
PR8NA-Tat17  Recombinant PR8 influenza virus expressing Tat17 peptide
rIL-2  Recombinant interleukin 2
RALDH  Retinal Aldehyde dehydrogenase
RNA  Ribonucleic Acid
RT-PCR  Reverse Transcriptase-Polymerase Chain Reaction
RV144  Thailand clinical trial of HIV vaccine 2009
SD  Standard Deviation of the mean
SHIV  Simian-Human Immunodeficiency Virus
STD  Sexually Transmitted Disease (s)
SEM  Standard Error of the Mean
Tat  HIV Transcriptional transactivator
Tat17-25  QPKTACTNC peptide spanning residues 17–25 of Tat
TB  Tuberculosis
TGF-β  Transforming Growth Factors-β
Th1  T helper 1
Th2  T helper 2
TNF-α  Tumor Necrosis Factor alpha
UNAIDS  United Nations Program on HIV/AIDS
VCAM-1  Vascular Adhesion Molecules
VLA  Very Late Antigens
VRC  The Vaccine Research Center
WHO  World Health Organization
X31  A/HK/X31 (H3N2)
X31NA-Gag197  Recombinant X31 influenza virus expressing Gag197 peptide
X31NA-Tat17  Recombinant X31 influenza virus expressing Tat17 peptide
Abstract

Human immunodeficiency virus/Acquired immunodeficiency syndrome, HIV/AIDS is a significant medical problem worldwide with nearly 60 million infected people. Therefore, an effective and safe vaccine remains a high priority. To date, most HIV vaccine candidates have failed to elicit effective humoral and cellular immune responses that are necessary to control HIV infection. A promising phase III trial was conducted in Thailand in 2009 using a recombinant canary pox vector vaccine (ALVAC) expressing HIV Gag protein in combination with recombinant HIV Env glycoprotein gp120 (AIDSVAX), and was the first trial to show significant (31.2%) efficacy in humans.

This project aimed to use influenza viruses as a mucosal live vaccine vector to stimulate an effective CD8+ T cell immunity. Reverse genetics technology was used to generate recombinant influenza A viruses H3N2 (HK-X31) and H1N1 (A/PR8/8/34) expressing a defined mouse HIV-CD8+ T cell epitope (H-2Kd Gag197) in the neuraminidase (NA) stalk (X31-NA-Gag197 and PR8-NA-Gag197). This epitope was cloned into a pHW2000 reverse genetics plasmid encoding the appropriate influenza virus NA segment using recombinant PCR. Following co-transfection with the other seven influenza gene segments in a co-culture of 293T and MDCK cells, facilitated the generation of live influenza-HIV vaccine vectors.

Live recombinant influenza viruses were successfully used as HIV vaccine vectors, utilising various mucosal prime-boost vaccination routes in a mouse model, as evidenced by the induction of robust CD8+ T cell responses. This was shown through intracellular cytokine and tetramer staining, which detected HIV and influenza-specific CD8+ T cell immunity. Mucosal CD8+ T cells were also characterized through expression of specific mucosal homing integrins. These HIV-specific CD8+ T cells migrated to mucosal regions following intranasal or intravaginal prime boost administration of the recombinant vaccine.

Following intranasal prime-boost vaccination, tetramer and intracellular cytokine staining showed comparable HIV and endogenous influenza-specific CD8+ T cell responses in the spleen, and broncho-alveolar lavage, the mediastinal and inguinal lymph nodes. These mucosal HIV-specific CD8+ T cells expressed the integrins α4β7, CD103 and CCR9. The mucosal α4β7+CD8+ T cells expressed higher levels of the cytokine IL-15 cytokine and the IL-15Rα receptor compared to α4β7- controls.
Finally, challenge with a rVac-Gag virus revealed that Gag\(_{197}^{\text{a}}\alpha\beta^{7}\text{CD8}^+\) T cells contributed to protection following vaccination. This research highlights the importance of mucosal CD8\(^+\) T cells in viral immunity and emphasizes the need for additional studies to provide key insights to underpin future vaccine development.
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Chapter 1: Literature Review

1. Chapter 1: Literature review

1.1. General introduction

Since its identification in 1983, Human immunodeficiency virus/Acquired immunodeficiency syndrome (HIV/AIDS) has become a serious threat to humanity. More than 60 million individuals have been infected in this epidemic, with 34 million of those currently living with HIV/AIDS. Moreover, more than 2.6 million new cases of infection are reported every year (UNAIDS 2013; UNAIDS 2012). Most cases of infection have occurred in the developing world, with nearly half of those infected succumbing to the disease (Duerr, Wasserheit and Corey, 2006; Mathers and Loncar, 2006; UNAIDS 2010).

The first cases of HIV/AIDS were identified among homosexual men with complicated respiratory distress from unusual pneumonia in mid-1981 in the United States of America (USA). It was later discovered that the disease had been transmitted from central Africa to Haiti and then onto the USA. Several different modes of transmission were identified, and included blood transfusions, mother to-child and sexual transmission (Piot, 1989; Quinn et al., 1986). Many approaches have been used to reduce the risk of HIV transmission from infected persons to healthy individuals. For example, testing of blood and blood products of donors before transfusion, use of condoms during sexual intercourse and immediate use of antiretroviral therapy as a prophylactic treatment in neonates or in adults after exposure. However, none of these interventions have completely prevented the spread of HIV infection (Carvalho et al., 2011; Haile-Selassie, Townsend and Tookey, 2011; Shevkani et al., 2011).

The generation of a safe and effective HIV vaccine remains the most likely approach to eliminate infection (Johnston and Fauci, 2008). Several clinical trials have been conducted to test new HIV vaccine candidates in the last 25 years. Many of these vaccine candidates are still under study. Since 1987, more 200 Phase I/II trials have been used for testing 60 HIV-1 candidate vaccines. These include DNA vaccines and recombinant vector and subunit vaccines. However, only a few candidate vaccines have been tested for efficacy in humans at the phase III level (Esparza and Van Regenmortel, 2014; Esparaza, 2013; Richert et al., 2014). These vaccine candidates were designed to induce HIV-specific T cells alone or both T cell and antibody responses (Chanzu and Ondondo, 2014). All have resulted in poor efficacy with the exception of one vaccine (RV144: phase III trial performed in Thailand 2009) using a heterologous prime-boost vaccine strategy that elicited a moderate level of protection against
HIV (31%) (Nath, 2010; Vaccari, Poonam and Franchini, 2010; Rerks-Ngarm et al., 2009). The need for an alternative, more robust vaccine is the motivating factor for this body of work. The following literature review firstly introduces the *Retroviridae* family, while the second part discusses influenza virus and its use as a vector in HIV vaccine research.

### 1.2. *Retroviridae*

Retroviral diseases have been known for more than two centuries, with the first reported cases being ovine pulmonary adenocarcinoma and bovine leucosis identified in 1825 and 1870, respectively. In the decades following, the causative agent of these two diseases was reported as a retrovirus (Johnson and Kannene, 1992; Voisset, Weiss and Griffiths, 2008). Retroviruses were first isolated from mice and birds between 1908-1910 in the form of oncogenic viruses (cancer-causing viruses), such as the mouse mammary tumor virus (MMTV) and the avian leukosis virus (ALV) (Mathews, 1982). However, other retroviruses were subsequently identified that led to non-malignant diseases characterized by immunological and neurological symptoms, such as HIV-1 and HIV-2 (Burmeister et al., 2007; Voisset, Weiss and Griffiths, 2008).

All Retroviridae family viruses have the ability to invade host cells and incorporate into their genome. Indeed, about 5-10% of the human genome is composed of sequences resulting from reverse transcription of viral-derived genes called retro-sequences (Lander et al., 2001; Levy, 1993). The current classification of retroviruses depends on the genetic variation of the reverse transcriptase protein to differentiate between seven major genera within this family (Coffin, Hughes and Varmus, 1997) (Figure 1.1).

These seven major genera of the retroviridae family included the following:

1) Alpha-retroviruses: simple retroviruses characterized by C-type morphology. These viruses are widespread in avian species, such as an avian leukemia virus (ALV) (Coffin, Hughes and Varmus, 1997).

2) Beta-retroviruses: simple retroviruses characterized by a round eccentric core and B-type morphology. These viruses infect primates, sheep and mice causing tumor diseases, such as the mouse mammary tumor virus (MMTV) (Hardy, 2009).

3) Gamma-retroviruses: simple retroviruses characterized by C-type morphology that cause leukemia in different animals, such as the feline leukemia virus (FLV) (Cohen and Fauci, 2001).
4) Delta-retroviruses: complex retroviruses characterized by C-type morphology. These infect humans and other animals causing leukemia, for example human T-lymphotropic virus (HTLV) (Burmeister, Schwartz and Thiel, 2001).

5) Epsilon-retroviruses: simple retroviruses characterized by C-type morphology. These are found in fish and reptiles, for example, Walleye dermal sarcoma virus (WDSV) (Hardy, 2009).

6) Spumaviruses: complex retroviruses characterized by unique virion morphology with an uncondensed core and foamy pathogenic changes in infected cells, for example human foamy virus (HFV). These viruses originated in chimpanzees and were subsequently transmitted to humans (Buchschacher, 2001; Delelis, Saïb and Sonigo, 2003).

7) Lentiviruses: complex retroviruses distinguished from other retroviruses by unique virion morphology with cylindrical cores. These viruses infect humans, cattle, horses, sheep, primates and rabbits causing malignancies as well as lymphatic and neurological disorders, such as HIV/AIDS (Baig et al., 2008; Gilbert et al., 2009; Katzourakis et al., 2007).
Figure 1.1. Phylogeny of Reteroviruses. Phylogenetic tree showing the seven genera of endogenous retroviruses, including simple and complex genome viruses, with HIV-1 grouped with the Lentiviruses (Weiss, 2006).

**ALV**: Avian Leukemia Virus; **RSV**: Rous Sarcoma Virus; **JRSV**: Jaagsiekte Sheep Retrovirus; **MMTV**: Mouse Mammary Tumor Virus; **SRV**: Squirrel Retrovirus; **HERV-K**: Human Endogenous Retrovirus-K; **HERV-W**: Human Endogenous Retrovirus-W; **PERV**: Porcine Endogenous Retrovirus; **GALV**: Gibbon Ape Leukemia Virus; **MLV**: Murine Leukemia Virus; **FeLV**: Feline Leukemia Virus; **HTLV**: Human T-Lymphotropic Virus; **BLV**: Bovine Leukemia Virus; **WDSV**: Walleye Dermal Sarcoma Virus; **SnRV**: Snakehead Retro-Virus; **FFV**: Feline Foamy Virus; **BFV**: Bovine Foamy Virus; **SFVagm**: Simian Foamy Virus (African green monkey); **SFVcpz**: Simian Foamy Virus (in Chimpanzees); **EIAV**: Equine Infectious Anemia Virus; **FIV**: Feline Immunodeficiency Virus; **MVV**: Maedi-Visna Virus; **SIVmac**: Simian Immunodeficiency Virus (in Macaques); **HIV**: Human Immunodeficiency Virus.
1.2.1. Lentiviruses

Lentiviruses are a genus of viruses that represent one of the largest subgroups of the Retroviridae family (Şenocak et al., 2009). The name of the virus originates from the Latin words, *lent*, *lenta*, *lento* and *lenti*, to indicate the slow nature of disease progression. They are found endemically worldwide (Ross and Craig, 2010). These viruses are characterized by continuous genome evolution in the host cell due to high mutation rates (Hirsch et al., 1995). Lentiviruses include large and ubiquitous viruses that cause highly infectious diseases in humans, such as HIV-1 and HIV-2, as well as other primate lentiviruses, such as simian immunodeficiency virus (SIV), which shares genetic similarity with HIV-1 and 2 (Peeters, Courgnaud and Abela, 2001). In addition to the major structural genes that are represented in all retroviruses (*pol*, *gag* and *env*), lentiviruses encode accessory genes, such as *rev* and *tat*, (Buchschacher, 2001; Kerbs, Goldstein and Kilpatrick, 2010; Liu et al., 2009) (Figure 1.2). HIV is a member of the lentivirinae subfamily within the lentivirus genus that causes significant disease in humans (Chiu et al., 1985).

Figure 1.2. Phylogenic relationships among lentiviruses. Classification of the lentiviruses on the basis of accessory genes, such as *nef*, *vif*, *vpr* and *vpu*, with HIV-1 showing similarity to other lentiviruses within the primate group (Gifford et al., 2008).
1.2.2. Discovery of HIV

Between 1970 and 1980, several cases of unknown infection with symptoms of immunologic dysfunction were recognized as an unusual syndrome among homosexual men in the USA and Europe (Cohen and Fauci, 2001). This syndrome was characterized by several distinctive symptoms, such as generalized lymphadenopathy, cytomegalovirus-associated retinitis, and cryptococcal meningitis (Cohen and Fauci, 2001; Mayer and Bell, 2007).

By June 1981, HIV was proposed as the causative agent of AIDS reported mainly in homosexual males, intravenous drug users and hemophiliacs. This designation was officially confirmed in 1983 (Barré-Sinoussi et al., 1983; Luft, Seme and Poljak, 2004). Indeed, HIV/AIDS has now become one of the largest causes of death around the world, particularly in Sub-Saharan Africa and within the African American population in the USA (Carter and Saunders, 2009; Crampin et al., 2002).
1.2.3. HIV-1 genes

The genome of HIV-1 is 9749 nucleotides in size, consisting of nine genes, which are flanked by two long terminal repeats (Gallo et al., 1988; Varmus, 1988) (Figure 1.4). These genes are classified into three types: i) structural genes, such as \textit{gag}, \textit{pol}, and \textit{env}; ii) regulatory genes including \textit{tat} and \textit{rev}; and iii) accessory genes, such as \textit{vpu}, \textit{vpr}, \textit{vif} and \textit{nef} (Berger et al., 1991). Various HIV genes have been expressed in delivery vectors for induction of HIV-specific immune responses (Yusim et al., 2002).

1.2.3.1. Envelope glycoprotein (Env)

Env is an envelope glycoprotein (Env gp160) trimer that forms the viral envelope and surrounds HIV particles with 72 little spikes. It mediates virus entry into host cells using the trimeric envelope glycoprotein spike in order to attach to the cellular receptors and then fuses to the viral and target cell membrane. It also represents a direct target for antiviral antibodies. The Env glycoprotein trimer is encoded by a 2.5 kilo-base (Kb) gene and contains 850 amino acids in total (Sattentau, 2013; Mao et al., 2013). It is made up of two glycoproteins subunits: (i) surface gp120 (SU), located on the outside of the HIV-1 virus, which plays an important role in initiating attachment to target cells by binding to the CD4 receptor; and (ii) transmembrane gp41 (TM), a hydrophobic protein normally embedded within the viral envelope but exposed after conjugation of gp 120 with the CD4 receptor molecule, which leads to activation of gp 41 for enhancement of viral-cell membrane fusion (Capon and Ward, 2003). Some regions of the Env protein are conserved between different strains of HIV-1, but this protein is generally characterized by alternative glycosylation and divergent sequences. Each conserved region of the Env protein (C1-C5) is separated by highly variable regions (V1-V5), leading to altered immunogenicity of Env glycoproteins that play an important role in the evasion of the immune response. This is because of the high diversity of the HIV-1 Env amino acid sequence among the nine clades of the HIV-1 virus, A, B, C, D, F, G, H, J and K, and also because there is more than 35 circulating recombinant forms that can be mutated to 20% within a clade and to 35% between different clades (Walker and Korber, 2001; Gaschen et al., 2002). Accordingly, the HIV-1 clades are distinguishable on the basis of their Env glycoprotein sequences (Dorn et al., 2000; Go et al., 2009; Ho et al., 2008; Huang et al., 1997). Despite this, Env has also been considered as a potential candidate for HIV-1 vaccines (Ahmad et al., 1994; Betts, Yusim and Koup, 2002).
As a consequence of high variable regions of Env gene sequences of the same HIV-1 virus strain or between different clades that contributes to inducing a less protective CD8$^+$ T cell response (Go et al., 2009; Ho et al., 2008), it is highly recommended that multiple Env peptides or the highly conserved positions of other HIV-1 peptides like Gag and Tat be used in HIV vaccine candidates.

1.2.3.2. Group specific or associated antigen (Gag)

The gag, or group-associated antigen, encodes capsid proteins that influences the physical structure of the virus. Gag is directly associated with the membrane of the host cell and enhances the assembly of new immature virions (Bryant and Ratner, 1990). After virus maturation, gag is cleaved into 4 major specific proteins. The first of these is the matrix protein (MA or P17) that is embedded into the envelope of the virus where it initiates binding of the precursor polyprotein (Pr55 gag) to the host cellular envelope. This is an important step in regulating and controlling assembly and maturation of the virion (Hayakawa et al., 1992). The capsid protein (CA or P24) forms the main shell structure surrounding the viral genome, located in the central core of the viral ribonucleoprotein (Göttlinger, Sodroski and Haseltine, 1989). The nucleocapsid protein (NC or P7) is localized in the virion and has an important role in assembly of RNA proteins into the HIV-1 virion. A fragment of NC (P6) also has an important function in facilitating the release of complete viruses from infected cell membranes (Clavel and Mammano, 2010; Patil, Gautam and Bhattacharya, 2010). Most previous HIV vaccine candidates failed to control HIV infection because a high diversity of the HIV-1 protein sequences between different clades of virus enabled the virus to escape the immune response as the majority of induced CD8$^+$ T cell responses were directed against variable HIV epitopes (Kunwar et al., 2013; Yoshimura et al., 1996). Therefore, the main aim in the development of an effective vaccine against HIV is to induce a strong immune response targeting conserved regions of the HIV-1 virus. This would elicit a breadth of CD8$^+$ T cell responses that recognize the most conserved positions of HIV epitopes that have an important role in controlling HIV viremia (Kunwar et al., 2013; Kulkarni et al., 2013). There is a high degree of functional conservation in the sequence of the whole Gag protein among the 8 major clades of HIV-1, especially in the capsid subunit. This was evident by a low diversity of amino acid sequences throughout the full length of the Gag protein subunit, with 17% between different clades of the HIV-1 virus, and only 9% within the same virus clade (Li et al., 2013). Also, Gag is commonly targeted by CD8$^+$ T cell responses across various HIV-1 clades as a result of a high cross reactivity of Gag peptide sequences among these clades. Gag-specific CD8$^+$ T cells obtained
from HIV-1 clade C infected individuals were able to recognize at least one Gag peptide from different HIV-1 clades. For instance, these CD8\(^+\) T cells recognized the Gag peptide of 92% from African clade CDu422, 90% for the Chinese clade C\(CH\), 77% for clade A, 69% for clade B, and 87% for clade D (Zembe et al., 2011). This was demonstrated by a higher magnitude of functional HIV-1 CD8\(^+\) T cells detected in HIV-1 infected case with 96% against the Gag pool compared to 86% for Pol and Nef. Moreover, the level of Gag-specific CD8\(^+\) T cells were inversely correlated with the HIV-1 virus load in those infected individuals (Edwards et al., 2002). Indeed, highly conserved regions of the HIV-1 Gag protein encode the main HIV-1 cytotoxic CD8\(^+\) T cell epitopes that are used as vaccine targets and induced CD8\(^+\) T lymphocyte responses (Goonetilleke et al., 2009; Nchinda et al., 2010; Tritel et al., 2003). As a result of the highly conserved regions of the HIV-1 Gag, this protein is regarded as one of the best candidates for use in HIV vaccines (Addo et al., 2001; Currier et al., 2006; Yusim et al., 2002). Therefore, the HIV-1 Gag antigen is widely considered as one of the most conserved viral proteins used in CD8\(^+\) T cell based vaccines, and has also induced a breadth of Gag specific CD8\(^+\) T cell responses. For instance, there was a significant level of IFN-\(\gamma\)/CD8\(^+\) T cell response detected in BALB/c mice vaccinated subcutaneously with multiple doses of the HIV-1 Gag protein alone or when combined with the Tat protein. Broad CD8\(^+\) T cell responses were directed to the limited nine amino acids of 197 to 205 of the Gag compared to the other Gag peptides (Cellini et al., 2008).

1.2.3.3. Transcriptional transactivator (Tat)

The HIV-1 Tat gene encodes two specific proteins; the 72 residue “Tat-1 protein” and the 86 residue “Tat-2 protein” (Howoroft et al., 1993; Ma and Nath, 1997). These proteins serve to regulate transcription and translation of polymerase (pol) and transcription of double stranded DNA after incorporation into the host genome. They do so by binding to specific transactivation elements called the Tat activating region (TAR), which stimulate the activity of RNA polymerase II and enhance transcription of viral RNA (Braddock et al., 1993; Jones and Peterlin, 1994; Liang and Wainberg, 2002; Raha et al., 2005). Tat proteins are also released by cells infected by HIV-1 and transmitted to non-infected cells which interfere with normal immune responses. This is achieved through the inhibition of MHC-1 presentation of HIV-1 antigens by dendritic cells and other antigen presenting cells (APCs) (Gavioli et al., 2004; Petersen, Morris and Solheim, 2003; Raha et al., 2005). Tat is also considered one of the main regulatory proteins that is frequently targeted by cytotoxic CD8\(^+\) T cells and is induced
following natural HIV-1 infection; therefore it may be considered an important target for CD8\(^+\) T cell based vaccines to control HIV (Addo et al., 2001).

**Figure 1.4.** Organization of the HIV-1 genome. Specific gene architecture distinguishing HIV-1, including structural genes (*gag*, *pol* and *env*), and each of the regulatory (*tat* and *rev*) and accessory genes (*vpu*, *vpr*, *vif* and *nef*), arranged between the two long terminal repeats (LTR) (Frankel and Young, 1998).

### 1.2.4. HIV-1 pathogenesis

HIV-1 enters the human body through mucosal surfaces and is dependent on the high affinity binding of surface envelope glycoprotein (Env gp120) with the host cell CD4\(^+\) receptor. HIV-1 also needs to bind to specific major co-receptors such as the chemokine receptors CCR5 and CXCR4, which are located on the surface of target cells (Kramer, 2007; Lee and Montaner, 1999; Nguyen and Taub, 2002), in order to facilitate the effective entry of the HIV virus into CD4\(^+\) cells. Indeed, the mechanism of interaction between HIV-1 and these co-receptors is a critical step in the HIV-1 life cycle that influences the progression of the disease (Gorry and Ancuta, 2011). Acute HIV-1 infection generates a high level of viremia, leading to accelerated depletion of CD4\(^+\) cells accompanied by severe acute symptoms. This is followed by a partial recovery of CD4\(^+\) T cell numbers with management of the virus infection, followed by clinical latency that lasts for 3-10 years (Weiss, 2001; Mayer and Bell, 2007) (Figure 1.5). HIV-1 emerges from latency through alteration of the host’s immune responses through ongoing exhaustion of immune cells, high depletion of CD4\(^+\) T cells (<200 cells/\(\mu l\)) and decreased
cytokine activity (Banda et al., 1999; Berger et al., 1998; Gorry and Ancuta, 2011). Most cases of HIV-1 infected individuals are known as chronic progressors, characterized with a high level of virus replication which leads to extreme decline of CD4$^+$ T cells (Blankson, 2010). As a result, opportunistic diseases occur that ultimately lead to death 10-15 years following initial infection (Lü and Jacobson, 2007; Mayer and Bell, 2007; Rodríguez et al., 2006; Weiss, 2001) (Figure 1.5).

Figure 1.5. Stages of HIV infection. Delineation of the different stages of HIV-1 infection with respect to CD4$^+$ T cell count and number of HIV RNA copies. The primary infection, which extends from the initial infection for several months, is accompanied with acute symptoms followed by a long clinical latency period and complete destruction of host immunity (Coffin, Hughes and Varmus, 1997).

1.3. HIV immunity

The immune system defends the human body against a wide range of different pathogenic agents. The recognition of these microorganisms by the immune system is a necessary step in initiating the battle against pathogens. The host immune system includes two major arms: innate and adaptive immunity (Levy, Scott and Mackewicz, 2003; Mogensen et al., 2010; Sui et al., 2010).

1.3.1. Innate immunity

The innate immune response is the first line of immune defense following acute HIV-1 infection. The immune response starts with recognition of the HIV-1 antigen and includes
various parts, such as the epithelial barrier, phagocytic and antigen presenting cells (Mogensen et al., 2010). The innate immune response is characterized by a fast onset and a short period of response (Guatelli, 2010; Lehner et al., 2008). The main aim of the innate immune response against HIV infection is to reduce virus replication by inducing the cascade of inflammatory cytokines like interferons (IFNs) and natural killer (NK) cells, which play a critical role in controlling of infection. The natural killer cells contain specific receptors and killer immunoglobulin-like receptors (KIRs) that recognized the HIV-1 infected cells and then destroy these infected cells (Carrington and Alter, 2012). Activation of innate immunity that is induced directly following early HIV-1 infection includes a contribution by recruited cells, such as granulocytes, macrophages and lymphocytes. Both lymphocytes and macrophages target the HIV-1 virus (Mogensen et al., 2010). There are other antigen-specific immune cells that contain surface Toll-like receptors that recognize molecules from the HIV-1 virus (Bafica et al., 2004; Datta and Raz, 2005; Klotman and Chang, 2006). The dendritic cell (DC) also acts as an early innate immune effector cell that responds rapidly following HIV infection and arms the immune system by recognizing of the HIV-1 antigen and stimulating naïve T lymphocytes (Howie, Ramage and Hewson, 2000). There are two main types of dendritic cells: myeloid dendritic cells (MDCs), which secrete IL-2 to stimulate a proliferation of T lymphocytes, and plasmacytoid dendritic cells (PDCs), which produce interferons (mostly IFN-α) (Colonna, Trinchieri and Liu, 2004; Fitzgerald-Bocarsly and Jacobs, 2010; Hardy et al., 2007). Innate immune response to HIV-1 infection includes trigerring specific Toll-like receptors, such as TLR7 and TLR8, and activating the protective role of dendritic cells (DCs) (Meier et al., 2009). As a result, PDCs are able to directly inhibit replication of HIV in the infected cells, particularly in the spleen and lymph nodes, by releasing both type 1 IFNs and tumor necrosis factor α (TNF-α) (McKenna, Beignon and Bhardwaj, 2005; Nascimbeni et al., 2009). Furthermore, dendritic cells in women induce a higher level of IFN-α compared to the same age group of men, which is influenced by their response to the HIV RNA triggering of TLR7/8. This would lead to a lower level of virus replication, thus enhancing control of a HIV-1 infection, compared to HIV-1 infected individuals (Meier et al., 2009).

Another important component of the innate immune response are neutrophils, which secrete cytokines such as IL-12 into the circulation to stimulate mucosal immune responses, particularly in the genital mucosa (Fahey et al., 2005; Gaudreault and Gosselin, 2009). Additionally, neutrophils release specific proteinase inhibitors such as human neutrophil...
elastase inhibitor (HNE). HNE is one of the serine proteinase released into the mucosa that targets invading microorganisms. HNE also plays an important role in the migration of neutrophils through infected tissues and up-regulates the expression of several types of cytokines, such as IL-6, IL-8 and transforming growth factors (TGF-β). This further stimulates the innate immune response (Fitch et al., 2006; Wiedow and Meyer-Hoffert, 2005).

Innate immunity functions also include serum specific proteins called mannose-binding lectin (MBL), which have the ability to attach to HIV and evoke a host cell response to destroy the invading virus (Alfano and Poli, 2005; Bouwman, Roep and Roos, 2006; Ezekowitz et al., 1989). Depletion of MBL enables a high titer of the virus, leading to a rapid progression of HIV infection (Martin et al., 2003; Rantala et al., 2008; Spear, 1993).

1.3.2. Adaptive Immunity

The adaptive immune response involves contributions from different immune components, and are referred to as the humoral and cellular immune responses (Vieillard et al., 2006; Yamamoto and Matano, 2008). The humoral immune response includes B lymphocytes, responsible for the production of antibodies, while the cellular immune response involves T lymphocytes, including cytotoxic CD8+ T lymphocytes and CD4+ T lymphocytes. Both the humoral and cell mediated immune responses play critical roles in the defense against HIV-1 (Clerici et al., 1993; Weber et al., 2001; Zhu et al., 2001).

1.3.2.1. CD4+ T lymphocytes and HIV immunity

CD4+ T lymphocytes are characterized by a specific receptor located on their surface, CD4. This molecule is the primary target of the HIV-1 envelope glycoprotein, Env (gp120/41) and the HIV-1 infection (Bourgeois et al., 2006; Selig et al., 2001). As such, the CD4+ T cells are specifically targeted by HIV-1 during acute infection, leading to an abrupt depletion of CD4+ cells (Bentwich, 2005; López-Balderas et al., 2007; McKinnon et al., 2010). This is highly significant as CD4+ T lymphocytes play a critical role in the control of HIV/AIDS. Activating B lymphocytes to produce antibodies assists helper T cells to secrete cytokines necessary for the regulation of a productive immune response (Ostrowski et al., 2000). Two types of helper T cells perform this function. The first T helper 1 (TH1) cells, which release the cytokines interferon-γ (IFN-γ) and interleukin-2 (IL-2) necessary for activation of macrophages and cytotoxic CD8+ T lymphocytes (Bandera et al., 2010; Fortna et al., 2004). The second type of helper T (TH2) cells, which produce other interleukins, such as IL-4, IL-5, IL-6 and IL-10,
which are involved in antibody production. These cells collectively act to reduce the spread of HIV by stimulating antibodies and enhancing the immune response, through the recruitment of neutrophils, eosinophils and lymphocytes (Bandera et al., 2010; Fortna et al., 2004; Plana et al., 2004).

1.3.2.2. Cytotoxic CD8\(^+\) T lymphocytes immune response

Cytotoxic CD8\(^+\) T cells also play a key role in controlling HIV infection (Gómez, Smaill and Rosenthal, 1994; Landay et al., 1993; Liu et al., 1998). The protective role of HIV-1 specific CD8\(^+\) T cells can be identified by a rise in the initial HIV-1-specific CD8\(^+\) T cell responses during the first weeks of infection, which are directed against a limited number of HIV-1 epitopes, like Env, Pol and Gag (Streeck and Nixon, 2010). The level of these initial CD8\(^+\) T cells is positively related to the slow progression of the virus and low viremia rate and there is a direct link between the rapid progression of HIV infection and the reduction of HIV-1 specific CD8\(^+\) T cells (Betts et al., 2001; Streeck et al., 2009). CD8\(^+\) T cells are normally stimulated during primary viral infections in humans to support the host immune response and inhibit HIV-1 virus replication (Callan et al., 1998; Reddehase and Koszinowski, 1984; Reusser et al., 1999; Rickinson and Moss, 2003). During acute HIV-1 infection, CD8\(^+\) T cells are involved in the reduction of the viral load through the destruction of HIV-1 infected cells by secreting a high amount of HIV-1 specific-IFN-\(\gamma\) (Cao et al., 2003; Borrow et al., 1994).

Despite the low number of CD8\(^+\) T cells induced following acute HIV infection, they do inhibit the virus titer after an early period of HIV-1 viremia. These CD8\(^+\) T cells induced for chronic HIV infection are also less effective in inhibiting virus replication. In addition, following persistent viremia caused by the rapid progression of the HIV-1 infection, the level and functional activity of specific CD8\(^+\) T cells is impaired and exhausted as a result of certain death factors expressed on HIV tetramer\(^-\)CD8\(^+\) T cells, such as inhibitory receptor programmed death 1 (PD-1 or PDCD1), which down-regulates the activated CD8\(^+\) T cells (Day et al., 2006; Miguele et al., 2002). This evident in the observation that first CD8\(^+\) T cells stimulated in an acute infection has a highly functional role in the suppression of virus replication compared to the impaired suppressive role of equivalent cells that are induced later. This suggests that early CD8\(^+\) T cells directly target a limited number of HIV-1 epitopes (Streeck and Nixon, 2010). For example, there was a high detection level of Env-specific CD8\(^+\) T cells responses in acute HIV-1 infected individuals showing a rapid reduction of viremia compared to a high viremic stage of infection characterized by a low level of CD8\(^+\) T cell response (Borrow et al., 1994). Also, CD8\(^+\) T cell responses directed for HIV-1 antigens like Gag, Pol and Env, detected in most
primary infected individuals show low levels of virus replication. These cells were detected within a period of a few weeks to a few months while no neutralizing antibodies were detected during the same period of infection. This can be regarded as evidence there is a critical role of CD8⁺ T cell response to the primary HIV-1 infection (Koup et al., 1994).

Furthermore, highly active CD8⁺ T cells are also involved in the immune response against HIV infection. These poly-functional CD8⁺ T cells are characterized by high proliferative capacity and the ability to secrete a range of cytokines, including IFNγ, IL-2 and TNF-α, thus resulting in a high level of cytotoxicity (Betts et al., 2006; Harari et al., 2007; Kiepiela et al., 2004). HIV-specific CD8⁺ T cells are highly stimulated by specific HIV antigens, such as Gag, Pol and Env. These cells were detected in HIV-1 infected individuals after 3-6 months following presentation of the HIV-1 virus antigen (Koup et al., 1994; Duvall et al., 2008; Holmgren and Czerwinski, 2005). Human leukocyte antigen-restricted CTLs (HLA-class 1) were also detected, which induced highly protective immune responses that controlled HIV infection through the reduction of viremia (Koup et al., 1994). These cytotoxic CD8⁺ T cells have the ability to eliminate HIV-1 infected cells by secreting soluble factors, such as β-chemokines and specific CD8⁺ T cell antiviral factor (CAF), to combat HIV-1 infection by suppressing the binding and transcription of the virus (Gulzar and Copeland, 2004). For example, HIV-1 specific CD8⁺ T cells have been shown to play an effective role in preventing replication of the virus at mucosal surfaces, such as the genital mucosa, thus resulting in control of HIV infection. Also, sero-negative, highly exposed but resistant Kenyan sex workers have been shown to possess a high level of mucosal cytotoxic CD8⁺ T cells when compared to HIV-1 seropositive sex workers (Kaul et al., 2000). The protective role of HIV-1 specific CD8⁺ T cells includes their ability to clear the low level of infection and prevent HIV-1 virus dissemination (Card, Ball and Fowke 2013). In general, 5-10% of commercial female sex workers in the Pumwani district in Kenya were identified as HIV-exposed seronegative (HESN). These individuals were resistant to HIV infection, characterized by a higher level of HIV-CD8⁺ T cells detected in the genital mucosa compared to the HIV sero-positive female sex workers (Fowke et al., 1996). Furthermore, less than 1% of HIV-1 infected individuals, known as Elite controllers or suppressors (ES), have shown a clinically undetectable infection in addition to a low level of virus replication (<50 RNA copies/ml) without using antiretroviral treatment (ART). These individuals contain a higher significant level of polyfunctional CD8⁺ T cells which enhance suppression of HIV-1 replication compared to chronic progressive HIV-1 infected individuals.
(Blankson, 2011; Buckheit et al., 2012). All of these studies indicate the critical role of highly functional subsets of CD8+ T cells in control of HIV-1 infection in non-progressive HIV-1 chronic cases (Betts et al., 2006).

A high response of polyfunctional CD8+ T cells was also detected in uninfected volunteers in the United Kingdom. These were administered subcutaneously with a prime-boost vectored vaccine expressing highly conserved amino acid positions of Gag, Pol, Vif and Env proteins obtained from the most conserved regions of the HIV-1 clades. This induced CD8+ T cells to target both Gag and Pol conserved epitopes which led to inhibited virus replication (Borthwick et al., 2014). Moreover, HIV-1 resistant individuals were similarly characterized by high levels of granzyme and perforin compared to progressive HIV-1 infected cases. This led to high cytolytic activity of cytotoxic CD8+ T cells. Although both non-progressive and progressive HIV-1 infected individuals were included in HIV-specific CD8+ T cells, only non-progressor individuals characterised with a higher significant proliferation were up-regulated by the highest perforin expression compared to HIV progressors (Migueles et al., 2002). This was also evident by measuring the level of granzymes and perforin expressed on human CD8+ T cells obtained from four groups of chronic-HIV-infected individuals in relation to their stage of progression and viremia. According to the results, the highest level of granzymes and perforin was detected in the elite controllers compared to viremic controllers, chronic progressive and viremic non-progressive individuals. Also, a negative correlation was detected between the HIV-1 RNA copy number and the level of granzymes and perforin expressed by CD8+ T cells. This was due to the highly proliferative capacity of induced CD8+ T cells up-regulated by the highest level of expressed perforin and also because of the higher lytic function of granzyme B granules destroying the HIV-1 infected cells (Migueles et al., 2008; Hersperger et al., 2010).

Furthermore, the protective role of SIV-CD8+ T cells was detected in macaques (macaca mulatta) injected subcutaneously with the recombinant RhCMV/SIV virus expressing multiple SIV proteins, like Gag, Rev, Tat, Nef and Env. Polyfunctional CD8+ T cells were induced that enabled control of the intrarectal challenge of the SIVmac239 virus (Hansen et al., 2009). This protective role was also confirmed in SIVmac-infected macaques (Macaca mulatta) which were administered intravenously for three days with anti-CD8+ T cell monoclonal antibodies (OKT8F). The effectiveness of these anti-CD8+ T cell monoclonal antibodies was firstly apparent with the early depletion of CD8+ T cells following the first hour of first dose, with
99.9% depletion CD8+ T cells, thus leading to an increase in SIV virus replication. Following a complete reduction of these monoclonal antibodies, a reduced SIV virus titer had been detected (Jin et al., 1999). Similar results have also been identified in African green monkeys injected intravenously with anti-CD8+ T cell antibodies (cM-T807) following infection with the SIVagm virus (Gaufin et al., 2010).

### 1.3.2.3. HIV-1 specific binding neutralizing antibodies

HIV-1 specific neutralizing antibodies are directed mainly against the HIV-1 Env proteins and control the primary HIV infection by preventing the viral envelope from binding to CD4+ receptor molecules (Mascola and Montefiori, 2010). In general, the effectiveness of neutralizing antibodies is mainly restricted to halting the progression of chronic HIV infection (Morris, 2002). Their effectiveness has been shown in macaque trials where animals have been vaccinated with recombinant HIV Env proteins and have acquired a high level of anti-Env neutralizing antibodies that protected them against a homologous highly virulent simian-human immunodeficiency virus (SHIV) (Someya et al., 2005).

Moreover, heterologous multi-clade HIV-1 epitopes of HIV Env gp140, were identified from six patients with HIV infection. The antibodies were neutralizing and poly-reactive when tested against HIV-1 virus in vitro (Mouquet et al., 2010). High levels of neutralizing antibodies, such as HIV specific mucosal IgA, have been detected in the genital tract and the plasma of resistant groups of female sex workers (Broliden et al., 2001; Devito et al., 2002; Kaul et al., 1999). However, one limitation of HIV specific antibodies induced for HIV-1 Env (gp120) is the lack of cross clade protection. This is due to the high diversity of HIV envelope glycoprotein sequences between HIV-1 clades (Letvin, 2009; Letvin, 2009a), which results in the escape of virus recognition of the antibody (Anzinger, Olinger and Spear, 2008; McGrath et al., 2001; Montpetit, 1995; Morris, 2002). However, this has been changed in recent years after the 2009 isolation of broadly neutralizing antibodies (Nabs) from chronic HIV-1 infected patients that can bind to different regions of HIV-Env gp120 i.e V1/V2 loop and V3 glycan region to control HIV-1 virus titers in long-term non-progressors (Benjelloun, et al., 2012; Mascola and Haynes, 2013).

### 1.3.3. Mucosal immunity

The first line of defense for the human body against the external environment involves skin and the mucosal system (Jakasa et al., 2010; Proksch et al., 2008). The mucosal system includes nasal, oral, gastrointestinal, respiratory, conjunctival and urogenital mucosal surfaces, and
these are important for mucosal transmitted pathogens, such as influenza virus, Herpes simplex virus and HIV (Kaetzel and Bruno, 2007; MacDonald, 2003). Mucosal defense is characterized by physical barriers (such as epithelial cells) to protect against the invasion of pathogens and immune cells, such as dendritic cells and macrophages stimulated as a result of a bacterial or viral infection. This mode of defense also leads to the induction of both mucosal IgA and cytotoxic T lymphocytes (Kunisawa and Kiyono, 2005). Mucosal CD8+ T lymphocytes are important for providing long term protection against mucosal transmitted viruses, such as HIV-1 (Belyakov and Berzofsky, 2004; Belyakov et al., 2004; Gallichan and Rosenthal, 1996; Suvas et al., 2007). For instance, HIV-resistant female sex workers in Kenya are characterized by persistent mucosal CD8+ T cells in the cervix compared to those at risk of HIV infection (Kaul et al., 2000). Additionally, the humoral immune response also plays an important role in mucosal immunity by stimulation of specific mucosal antibodies, such as IgA, blocking the main portal of HIV virus entry through mucosal surfaces like urogenital mucosa (Mascola et al., 2000).

The main aim of developing HIV vaccines is to induce a strong and stable level of mucosal immune response in addition to systemic immunity. This may be achieved by using the mucosal route for the delivery of HIV vaccines rather than the systematic route. This is not to say that systemic vaccination cannot induce mucosal immunity. For example, Gag-specific CD8+ T cells were shown to migrate to different effector mucosal sites after a prime boost intramuscular vaccination with recombinant adenovirus vectors expressing Gag protein in mice and macaques (Kaufman et al., 2008). It should be noted there is also an important role for HIV-specific neutralizing antibodies in immunity against human immunodeficiency virus type 1 (HIV-1) infection. This antibody immune response may include a local induction of IgA but this role is very limited when compared to more potent role of IgG for controlling HIV-1 at different mucosal sites. This role was highlighted in macaque studies where administered IgG induced protection following vaginal challenge using SHIV virus compared to control macaques (Klein et al., 2013). Mucosal immune responses induced by mucosal HIV vaccines aim to decrease virus transmission and progression (Gardner and Luciw, 2008; Gherardi and Esteban, 2005; Im and Hanke, 2004), by stimulating mucosal IgA and IgG responses, in addition to a cytotoxic CD8+ T cell responses (Bretscher, 1999; Caley et al., 1997). There are several routes of mucosal immunization; however, HIV mucosal vaccines are the most effective when administrated intrarectally or intragastricly (Belyakov et al., 1998; Duerr, 2010). Vaccines administered
intranasally, leading to high mucosal and systemic CD8+ T cell immune responses that have protected BALB/c mice against the challenge virus, and the recombinant vaccinia virus expressing HIV-1IIIB gp160 when compared to the subcutaneous route of vaccination (Belyakov et al., 1998). Also, in macaques there was no significant difference between various mucosal route of vaccination, such as intrarectal, intragastric, oral and intranasal vaccination, that induced robust CD8+ T cells in different mucosal regions following vaccination (Duerr, 2010).

1.3.3.1 Mucosal CD8+ T cells and homing integrin receptors

Integrins are a large family of heterodimeric glycoprotein receptors first identified over 25 years ago (Tamkun et al., 1986). In addition to their direct role in cell adhesion, integrins play also important role in cell migration which is essential in many vital activities, such as embryonic development, tissues repair and regeneration, and immune responses (Ridley et al., 2003). Lymphocytes are continually migrating between the peripheral blood circulation and different tissues. These cells are trafficked from circulation to various organs via lymph nodes and pass through venues high endothelial venules (HEV) (Girard and Nerem, 1995).

In order to detect mucosal CD8+ T cell responses, research groups have been studying various homing receptors, such as LPAM-1, CD103 and CCR9 expressed on antigen-specific CD8+ T cells migrating to different lymphoid organs. These homing CD8+ T cell integrins include α4β7 (LPAM-1), αEβ7 (CD103) and CCR9 (CD199) integrins that are mainly expressed following HIV-1 infection or vaccination to prevent dissemination of the HIV-1 virus (Hogg et al., 2003). Mucosal homing integrins are heterodimeric transmembrane protein receptors that work as bridges for cell-cell and cell-extracellular matrix (ECM) interactions. They participate in many important biological functions, such as migration, differentiation and apoptosis (Gupta et al., 2005; Tang and Rosenthal, 2010). These homing integrins up-regulated trafficking of antigen-specific CD8+ T cells to mucosal compartments. For instance, polyfunctional HIVGag-specific CD8+ T cells obtained from prime boost intramuscular vaccination of BALB/c mice using the recombinant rAd26-Gag and Ad5HVR48-Gag virus, transferred intravenously into naïve mice, led to the migration of these cells to the gastro-intestinal tract with higher expression of homing integrins, such as β7 integrin, CCR9, and CD103 compared to non-transferred mice (Kaufman et al., 2008). Furthermore, transfer of mucosal CD8+ T cells expressing these homing integrin receptors has been shown to be critical for protection against the intranasal A/PR/8/34 influenza infection in BALB/c mice. This included an intravenous adoptive transfer of 10^7 effector CD8+
T cells after one hour of intranasal influenza infection. The influenza virus titer was reduced by the fourth day following infection compared to the negative control (Cerwenka et al., 1999). These mucosal CD8+ T cells were functionally important in the clearance of the rotavirus from intraperitoneal infected mice following the intravenous adoptive transfer of α4β7+ expressed CD8+ T cells (Rose et al., 1998).

1.3.3.2. Basic structure of integrins

Homing T cell integrins include α (alpha) and β (beta) subunits that form the main chain subunits of the homing integrin. Both subunits differ in their nature of expression by lymphocytes and endothelial cells. There are 8β subunits (β1-β8) and 18α chain subunits. Both α and β subunits combine for 24 different integrins with distinct αβ integrin subunit pairs (Hogg et al., 2003). The main structure of integrins consists of two parts. The first part is the extracellular part, which is elongated from stalk and ends with a globular head region. This part includes approximately 700 amino acids in the length of α subunit and 1000 amino acids in the length of the β subunit. The second part is the cytoplasmic tail of less than 75 amino acid in length (Xiong et al., 2001) (Figure 1.6).

![Image of integrin structure](image)

**Figure 1.6.** The basic structure of mucosal homing CD8+ T cell receptors. These integrins are transmembrane receptors consisting of a heterodimer of alpha and beta subunits. They are expressed on mucosal homing T cells and mediate the attachment specific to endothelial cells (Eslami, 2005).
These integrins also mediate cell adhesion through attachment of their globular head region (ligand binding extracellular part) with the extracellular matrix glycoproteins, such as laminins and collagens located on the cellular plasma membrane. The β subunit is shared between different integrin molecules located in different animals. For example, the β1 subunit works as the main active subunit of integrins in chickens, the human fibronectin receptor, the human laminin receptor and VLA proteins (Stroeken, 2002). T cells mainly express the two β7 subunit integrins, α4β7 and αEβ7, that are highly involved in the migration of effector T cells to peripheral lymph nodes (Hogg et al., 2003). About 12 of these integrins are expressed by T cells and are highly involved in T cell mediated immunity. The ability of T cells to express these integrin heterodimers depends on their specific subsets and maturation (Pribila et al., 2004). For example, low levels of these integrins, such as αLβ2 (leukocyte function associated antigen-1: LFA-1), α4β1 (very late antigen-4: VLA-4) and α4β7 (lymphocyte Peyer's patch adhesion molecule integrin: LPAM) are expressed by naïve CD8+ T cells compared to effector or memory CD8+ T cells. In order to enhance migration of T cells to lymphoid effector sites, these integrins are binding specific ligands like intracellular adhesion molecule-1 (ICAM-1), vascular cellular adhesion molecule-1 (VCAM-1), and mucosal addressin cell adhesion molecule-1 (MAdCAM-1) (Egger et al., 2002; Pribila et al., 2004). This facilitates the adhesion of CD8+ T cells to the endothelial layer and their migration into different lymphoid organs like the peripheral lymph nodes (DeNucci, Mitchell and Shimizu, 2009; Hogg et al., 2003; Stroeken, 2002) (Figure 1.7).
Figure 1.7. The multi-steps model of CD8+ T cell migration to mucosal surfaces. Migration of specific CD8+ T cells from the effector sites of infection to mucosal regions includes expression of specific mucosal integrins, such as α4β7 (LPAM-1), αEβ7 (CD103) and CCR9 (CD199) on epithelial cells transmit through perivascular membranes on their way to mucosal surfaces of different lymphoid tissue (Hogg et al., 2003).

In addition, a migration strategy includes a contribution of these integrins with specific secreted chemokines, such as CD44, CD62L and CCR9, which combine with these expressed integrins to facilitate the migration of antigen-specific T cells to the effector sites of infection (Fousteri et al., 2009; Wermers et al., 2011). In general, high expression of beta 1 or 7 integrin subunits facilitates the attachment of high endothelial venules of Peyer’s patches and T cells with adhesion molecules, such as LPAM-2 (α4β1) which only binds the very late antigen 4 (VLA-4). While the beta 7 integrin subunit molecule has a high affinity of adhesion property by binding to specific legand molecules, such as (MAdCAM-1) for LPAM-1(α4β7) and vascular adhesion molecules (VCAM-1) for CD103 (αEβ7) (Strauch et al., 1994; Yang et al., 1998).

1.3.3.2.1. LPAM-1 (α4/β7) integrin

The lymphocytepeyer's patch adhesion molecule (LPAM-1 or α4β7 integrin heterodimer) is one of the homing integrin receptors expressed on T cells characterized by adhesive properties responsible for the recognition of migrated lymphocytes into lymphoid compartments, such as the gut-associated lymphoid tissues (Rosé et al., 1998). LPAM-1 is binding to its ligand called the mucosal addressin cell adhesion molecule (MAdCAM), which is located mainly on high
endothelial venules (HEVs) of mucosal lymphoid organs (Gorfu, Rivera-Nieves and Ley 2009; Petrovic et al., 2004). This enables the antigen-specific T cells to migrate from the main circulatory system to mucosal areas, particularly the endothelium of the gut or other related lymphoid tissues (such as Peyer’s patches and other lymph nodes except the peripheral lymph nodes) (Gorfu, Rivera-Nieves and Ley, 2009). Indeed, the homing integrins are differentially expressed on migrated T cells depending on the status of these cells (Petrovic et al., 2004). For instance, the low level of α4β7 integrin is expressed on naïve T and B cells while it increases following inflammation or infection. The α4β7 integrin is also expressed on different types of cells, such as natural killer (NK) cells, and stimulated monocytes, macrophages and eosinophils (Erle et al., 1994). However, the α4 subunit has the main role to mediate migration of activated CD8+ T cells to the intestinal mucosa. Therefore, α4β7 has been considered the more dependent T cell homing marker which has been highly expressed in the intestinal mucosa following the intravenous injection of C57BL/6J mice using the recombinant vesicular stomatitis virus expressing ovalbumin (VSV-ova) peptide, SIINFEKL. Mucosal CD8+ T cells expressing α4β7 were transferred intravenously into naïve mice. These mice were infected intravenously with a wild type of vesicular stomatitis virus which led to high trafficking of the transferred cells to effector mucosal regions to control virus infection (Lefrançois et al., 1999). Some pathogenic microorganisms that invade mucosal regions, such as rotavirus, herpes simplex virus type-2, HIV and Chlamydia, generate a mucosal CD8+ T cell response that provides protection against the invading pathogens. The overall response is affected by the migration of CD8+ T cells to infected mucosal tissues and is mediated by the α4β7 integrin homing receptor which facilitates attachment to specific lymph nodes in the intestine, respiratory and vaginal mucosal surfaces (Gupta et al., 2005; Tang and Rosenthal, 2010).

There are some differences in the structure and affinity of binding between LPAM-1 and LAPAM-2. For instance, LPAM-2 (α4β1) integrin contains an α subunit which is recognize the specific analogous to α chain of the human specific integrins heterodimer (VLA-4) while LPAM-1 works analogous to other T cell homing heterodimer integrin molecules in humans (Holzmann, McIntyre and Weissman, 1989). Therefore, LPAM-1 also binds other ligands, such as VCAM-1 or the CS-1 subdomain of human fibronectin, but with lower affinity compared to MAdCAM (Rott et al., 1996). The LPAM-1 integrin and other groups of integrins work as the CD8+ T cell homing receptors detect mucosal CD8+ T cell immune responses. These can also be used to distinguish memory and effector T cells in response to a rotavirus infection in mice. For example, functional CD8+ T cells obtained from rotavirus infected mice induced a high
percentage of the α4/β7+ subset of CD8+ T cells compared to naïve T cells that included a subset α4/β7+CD8+ T cell. Therefore, LPAM-1 is mainly expressed on antigen-specific CD8+ T cells and is used for detecting mucosal CD8+ T cell immune responses that are induced following an intraperitoneal (i.p) rotavirus infection in mice. These mucosal cells are highly effective in clearing infection compared to other mucosal homing CD8+ T cell integrin receptors (Rosé et al., 1998).

1.3.3.2.2. CD103 (αEβ7) integrin

CD103 or αEβ7 is a mucosal integrin receptor and is composed of a heterodimer of two subunits, αE and β7. This integrin works mainly as a homing receptor in various cell types, such as epithelial cells that are localized in various mucosal compartments including the gastrointestinal and female reproductive tract, and the important entry sites of mucosal infections, such as herpes simplex and HIV (Gebhardt et al., 2009; Tang and Rosenthal, 2010; Kiravu et al., 2011). It is characterized as one of the T cell homing receptors that have an important role in trafficking of the activated CD8+ T cells from the blood to the genital and gastrointestinal tract mucosa (Kiravu et al., 2011). Also, CD103 mediates an interaction or adhesion of mucosal T cells and it surrounds the epithelial layers through its binding to a specific ligand called E-cadherin or L-CAM, which has a combined function for determining the shape and motility of lymphocyte cells (Kiravu et al., 2011; Schlickum et al., 2008). A high percentage of mucosal HSV-2 specific CD8+ T cells found in genital tract mucosa have been shown to stain positive for the homing integrins CD103 (αEβ7 integrin) and CD49a (α1β7 integrin or VLA-1) one week after HSV-2 infection in a mouse model (Tang and Rosenthal, 2010), thus suggesting an important role for these integrin expressing cells in anti-viral immunity and in the control of these viruses.

The ability of these integrins to be expressed on migrating effector T cells depends not only on the integrin but also on the area of localization. For instance, CD103 (αEβ7) was expressed on 90% of gut intraepithelial lymphocytes (IEL) localized on Peyer’s patches and other villi to mediate their attachment to epithelial cells (Koseki et al., 2001). This was not available for other integrins like L-Selectin. Indeed, it has been confirmed that CD103 is directly involved in trafficking HIV-specific CD8+ T cells to the cervical mucosa, which are mainly included in specific Gag+CD8+ T cells (Kiravu et al., 2011). The highly effective role of the αEβ7 integrin heterodimer is represented by the induction of CD8+ T cells in both peripheral and intestinal epithelium as a response to an infection or tumor. For instance, there is a highly effective role for CD103 chemokine receptors in regulatory functions to stimulate the migration of effector
T cells to the infected foci, such as mesenteric lymph nodes and Peyer’s patches, which are represented by a CD103+CD8+ subset of T cells (Wermers et al., 2011; Apostolaki et al., 2008).

1.3.3.2.3. CD199 (CCR9) integrin

CCR9 or CD199 is a mucosal homing integrin mainly expressed on lymphoid cells following its binding to the specific ligand (CCL25) that is presented on epithelial cells in the gut with enhanced migration of T cells from the periphery and secondary lymphoid organs to the gut mucosa (Johansson-Lindbom and Agace, 2007). Indeed, the CCR9 integrin heterodimer has an important regulatory role in the migration of T cells to the infected regions during a specific inflammatory status, particularly in the intestine, which was represented by an increase in the subset of CCR9+CD8+ T cells found in the spleen, MLN, the lamina propria and the mesenteric lymph nodes in infected mice. This regulatory role of CCR9 has also been confirmed by intraperitoneal administration of the anti-mouse CCR9 monoclonal antibody which led to an increase in the severity of ileitis in B6 mice (Wermers et al., 2011).

It has also been confirmed that mucosal CD8+ T cells are differentiated by expressing CCR9, which mediates the fast recovery of BALB/c mice from acute colitis. This was achieved by the migration of effector CD8+ T cells from blood circulation to Peyer’s patches and the small intestine, compared to a low level of expressed CCR9 in the large intestine (Wurbel et al., 2011). As a result of induced mucosal CD8+ T cells expressing CCR9, wild mice were shown with normal weight after 17 days of infection compared to CCR9 deficient mice who suffered severe lose weight and a high mortality rate (Wurbel et al., 2011). Furthermore, CCR9 was also highly required to control ileitis in mice through its expression on lymphocytes at the last stage of infection that were localised in intestinal lamina propria and mesenteric lymph nodes (Wermers et al., 2011). In addition, a high level of SIV-specific CD8+ T cells expressing both CCR9 and αEβ7 were detected especially in mesentric lymph nodes of intranasally infected monkeys using recombinant modified vaccinia virus expressing SIV-Gag, Pol and Env proteins (rMVTTSIVgpe). The monkeys were then boosted with an intramuscular infection with recombinant adenovirus expressing SIV-Gag, Pol and Env proteins (rAd5SIVgpe). This mucosal T cell response was able to protect monkeys against mucosal infection from the SIVmac239 challenge virus (Tang et al., 2012).

Indeed, there are many factors regulating expression of mucosal homing integrins and stimulating the migration of CD8+ T cells to various mucosal compartments. For instance,
retinoic acid is up-regulating both $\alpha_4\beta_7$ and CCR9 expressed on CD8$^+$ T cells. Also, retinoic acid works through enhancing the signaling receptors of retinoic acid in CD8$^+$ T cells in the spleen and mesenteric lymph nodes by expressing CD103$^+$CCR9$^+$ subset of CD8$^+$ T cells (Svensson et al., 2008). Furthermore, 90% of T cells in the intestinal mucosa express $\alpha_4\beta_7$ and CCR9 as they are trafficked from the peripheral to the gut and this would be impaired in $\alpha_4\beta_7^-$ and CCR9$^-$ deficient mice (Berlin et al., 1993; Wurbel et al., 2001). There are several other factors affecting the migration of effector CD8$^+$ T cells to mucosal surfaces.

1.3.3.2.4. Interleukin 15 (IL-15)

IL-15 was first discovered in 1994 and identified as one of the T cell growth factors. Interleukin-15 (IL-15) is a glycoprotein and is a member of the 4 $\alpha$-helix bundle cytokine family of approximately 15-kDa (Waldmann and Tagaya, 1999). There is a high similarity of IL-15 sequences among different species. For instance, there is about a 97% of identical residues between human and simian IL-15, and 82% identical residues between human and porcine IL-15 (Canals et al., 1997; Anderson et al., 1995). IL-15 is mainly produced by dendritic cells (Kuniyoshi et al., 1999), monocytes, macrophages (Sandau et al., 2004) and T cells (Grabstein et al., 1994; Bamford et al., 1996).

IL-15 is a pleiotropic cytokines highly involved in an innate and adaptive immune response and is mainly active on natural killer (NK) cells (Ma, Koka, and Burkett, 2006). IL-15 utilizes the same common receptors chains as IL-2, $\beta$ (CD122) and $\gamma$ (CD132) chain of their trimeric receptors. Both cytokines also share similar biological properties and particular function enhancements of T cell proliferation, generation of cytotoxic T cells and survival of NK cells (Bodnar et al., 2008; DiSanto et al., 1997). However, IL-15 has little sequence homology with IL-2. Indeed, IL-15 has an important role in the development and function of NK/NKT cells and also in the maintenance of naive and memory CD8$^+$ T cells (Gill and Ashkar, 2009). Furthermore, IL-15 is highly required for the differentiation and persistence of both effector and memory CD8$^+$ T cells (Munger et al., 1995). Also, the regulatory role of cytokine IL-15 for the development and maintenance of different types of immune cells is confirmed by a high decrease of CD8$^+$ T cells, natural killer cells, NKT cells and intraepithelial T cells detected in defective cytokine IL-15 mice (Yajima et al., 2006). IL-15 also stimulates the immunological function of CD8$^+$ T cells by enhancing their cytolytic activity and inducing long lived memory T cells that controlling malaria re-infection in mammalian host as IL-15 gives rise the level of...
IFN-γ producing CD8+ T cells in addition to its role in increasing CD44hiCD62Llo CD8+T cell subset necessary to control malaria re-infection (Krzych et al., 2012).

The main role of cytokine IL-15 is to maintain, survive, proliferate and turnover CD8+ T cells detected with high availability during periods of inflammation in mice. This is necessary to improve the immune response in the infected mice (Rubinstein et al., 2008), with less antigen-specific CD8+ T cells maintained in IL-15-deficient mice. Also, the cytokine IL-15 is considered one of the key cytokines generated in response to mucosal infection. IL-15 enhances the proliferation and migration of effector CD8+ T cells to the infected areas such as the lung following intranasal influenza infection (Verbist et al., 2011), the gut in mice after infection with the lymphocytic choriomeningitis virus (LCMV) (Masopust et al., 2006) and the skin following epicutaneous infection using the vaccinia virus (Jiang et al., 2012). For example, high levels of IL-15 have been identified in the lung airways at day 6 of intranasal influenza infection in mice. This promotes specific CD8+ T cells to migrate throughout the mucosal area of the lung (Rubinstein et al., 2008).

IL-15 also works as an adjuvant used to increase the ability of the antigen to be stimulated for a longer time in order to induce higher immune response and enhance the migration of specific natural killers or CD8+ T cells to effector sites following intranasal influenza infection of mice (Verbist et al., 2011; Verbist et al., 2012). Moreover, the removal of specific dendritic cells that produce cytokine IL-15, or the administration of a specific IL-15 antagonist, inhibits the proportion of influenza specific CD8+ T cells in the lungs and increases the virus load (McGill, Van Rooijen and Legge 2010; McInnes et. al., 1996).

It is also recognized that cytokine IL-15 has a responsibility to stimulate effector influenza specific CD8+ T cells to migrate to the site of infection (lungs airways) in the intranasal infected IL-15-deficient B6 mice using either A/HK-X31 (X31, H3N2) or A/Puerto Rico 8 (PR8, H1N1) influenza viruses and intranasally administered with IL-15 plus IL-15Rα soluble complex (Verbist et al., 2011). Furthermore, IL-15 is necessary to stimulate a protective CD8+ T cell response in HIV infected chimpanzees as a result of the regulatory role of interleukin IL-15 to maintain the level of interferon-gamma (IFN-γ). For instance, a highly significant reduction of both IL-15 and tumor necrosis factor alpha (TNF-α) interferon-gamma (IFN-γ) were detected in progressive HIV-infected chimpanzees compared to a robust maintained level of IL-15 and
interferon-gamma (IFN-γ) in those resistant to infection (Rodriguez et al., 2007). The role of IL-15 is directly controlled by the specific receptor, IL-15Rα, which is mainly expressed mainly on various types of cells, such as dendritic cells, monocytes and macrophages (Steel, Waldmann and Morris 2012; Anderson et al., 1995) and T cells (Rowley et al., 2009). The IL-15Rα receptor is characterized by a high affinity binding to soluble IL-15, and IL-15 acts through its binding to IL-15Rα receptors on CD8+ T cells to enhance the survival and proliferation of CD8+ T cells (Mortier et al., 2006; Mortier et al., 2008). For example, higher levels of expressed IL-15 mRNA on CD8+ T cells were detected in BAL, mediastinal draining lymph nodes and the spleen of intranasally infected BL/6 mice with A/Hong Kong-X31 influenza virus. There was a four fold increase in its expression at day 3 of infection compared to naïve mice, and expression of IL-15 mRNA continued to increase in the lung airways until the day 7 of infection (Verist et al., 2011). Furthermore, IL-15 had an important role for the expansion of primary antigen-specific CD8+ T cells detected indifferent organs, such as the spleen, peripheral lymph nodes, mesenteric lymph nodes, lung, liver, and small intestinal lamina propria (LP) of the intravenous infected C57BL/6J mice with vesicular stomatitis virus (VSV). However, VSV specific CD8+ T cells were reduced significantly (less than 50%) in IL-15−/− and IL-15Rα−/− mice. Also, the level of IL-15Rα expression was correlated to the status of a CD8+ T cell as it was fairly low on naive CD8+ T cells while up-regulated on Ag-specific effector cells. As a result, IL-15 is necessary for the generation and maintenance of specific antiviral CD8+ T cells (Kimberly et al., 2002). Therefore, both IL-15 cytokine and IL-15Rα receptor expression by specific immune cells, such as dendritic and CD8+ T cells are considered as an indicator of highly migrated T cells to mucosal regions to control various mucosal infections, such as an influenza infection and or other inflammatory diseases like rheumatoid arthritis (McGill, Van Rooijen and Legge 2010; McInnes et. al., 1996). In addition, other cytokines have a role in the migration of T cells to mucosal effector sites following mucosal or parenteral vaccination. One of these cytokines is IL-12, which was highly expressed in draining lymphoid tissues, such as mesenteric lymph nodes and Peyer’s patches following oral or intraperitoneal reovirus infection in BALB/c mice (Mathers and Cuff, 2004).

On the other hand, high expression of IL-4 and IL-13 down-regulates HIV-1 specific CD8+ T cells. This was identified in IL-14−/− and IL-13−/− mice primed intranasally with recombinant Fowl Pox Virus expressing the HIV-Gag, Pol and Env proteins. The mice were then inoculated
with an intramuscular dose of recombinant vaccinia virus expressing the Gag and Pol protein. As a result, polyfunctional CD8$^+$ T cells specific for H-2K$^d$ Gag$^{197-205}$ were only detected in the IL-14$^-$ and IL-13$^-$ compared to the wild type of infected mice. Accordingly, it is recognized that higher protective HIV-specific CD8$^+$ T cells induced following mucosal vaccination can be enhanced perfectly by down-regulating expression of IL-4 and IL-13 at mucosal lymphoid regions (Wijesundara et al., 2013). Furthermore, other cytokines were generally suppressed following vaccination. For example, IL-10 is one of the inhibited cytokines and suppressed in lymphoid tissues like Peyer's patches and mesenteric lymph nodes. Also, IL-4 is highly inhibited and suppressed in the mesenteric lymph nodes in oral or intraperitoneal vaccinated mice using reovirus compared to unvaccinated mice (Mathers and Cuff, 2004; Jackson et al., 2014).

1.3.3.2.5. CD62L and CD44 molecules

CD62L or L-Selectin is a cell adhesion molecule or homing receptor expressed on lymphocytes as a member of the Selectin family of protein. It enhances lymphocytes moving to secondary lymphoid tissues and regulates migration of T cells to the peripheral lymph nodes via their rolling through the endothelium of blood vessels (Robbins et al., 1998). CD62L, in addition to IFN-$\gamma$ and TNF-$\alpha$, was highly produced by both CD8$^+$ and CD4$^+$ T cells induced in lymph nodes isolated from vaccinia virus naturally infected individuals after a zoonotic outbreak that occurred in Brazil in 2005 (Medeiros-Silva et al., 2013). It was also detected in 40% of CD4$^+$ and 50% of CD8$^+$ T cells located in cervical draining lymph nodes, or spleen and distal lymph nodes after intranasal immunization of C57BL/6 mice using ovalbumin (OVA) plus CpG oligodeoxynucleotide adjuvant (Ciabattini et al., 2011). CD62L also differentiates effector T cells from naïve T cells. For instance, T cells can be recognized by subsets of CD62L$^{hi}$ CD8$^+$ T lymphocytes in naïve mice compared to CD62L$^{lo}$ specific subset of CD8$^+$ T cells in influenza infected mice, and C57BL/6J using recombinant influenza virus (HKx31-OVA) expressing the ovalbumin (SIINFEKL) (Kedzierska et al., 2007).

CD44 is a polymorphic group of proteins controlled by highly conserved gene, the CD44 gene and binds to various legands, such as osteopontin, collagens, and matrix metalloproteinases (MMP) (Spring et al., 1988). The CD44 is within the size range of 80–200 KDa, which was firstly identified as an antigen recognized by a monoclonal antibody raised against human leukocytes (Screaton et al., 1992). The CD44 is a transmembrane cell surface glycoprotein
molecule working as a homing cell adhesion molecule, which regulates certain changes like differential glycosylation in extracellular matrix composition. This leads to the control of different cellular functions, such as growth, survival and differentiation (Ponta, Sherman and Herrlich, 2003). The CD44 is also involved in several cellular functions, such as cell–cell interactions, and the migration of lymphocytes to effector sites (Screaton et al., 1992). Furthermore, CD44 is the most dominant subset of CD8+ T cells as lymph nodes homing receptors recognize the specific CD8+ T cell induced during primary intranasal influenza infection in mice (Kedzierska et al., 2007).

1.4. HIV-1 Vaccines

Vaccines are still regarded as the most effective way to control the majority of infectious diseases, particularly those diseases that are caused by highly pathogenic viruses (Johnston and Fauci 2008). Since the first identification of HIV/AIDS, more than 50 vaccine candidates have been tested to control HIV infection (Barouch, 2008; Girard, 2006).

All of these trials have failed due to the following key challenges:

(i) Highly variable antigens of HIV-1;

(ii) The high mutation rate of HIV-1 permitting the virus to escape host immunity;

(iii) The lack of efficient immune responses against latently infected cells;

(iv) The absence of synergistic activity between humoral and cellular immunity to control HIV; and

(v) The limitations of standard laboratory animal models used for HIV vaccine trials (Barouch, 2008; Girard, 2006).

The most important function of any HIV-1 vaccine is to produce an immune response that will prevent transmission and replication of the virus at the entry portal, such as the vaginal, rectal, urethral and oral mucosa (Haase, 2010; Haynes and Shattock, 2008; Veazey, 2005). To achieve this, HIV vaccines must be safe, immuno-efficient, highly prophylactic and capable of reducing viral loads to less than 1500 RNA copies per ml in the HIV infected persons, at which point HIV progression can be controlled (Newman et al., 2010; Quinn et al., 2000).

1.4.1. Traditional vaccines strategies

Traditional strategies for HIV vaccines include live-attenuated virus vaccines, whole killed virus vaccines and subunit vaccines (Barouch 2008).
1.4.1.1. Live attenuated vaccines

Live attenuated vaccines have been used to control highly infectious diseases such as smallpox, poliomyelitis and herpes viruses, and are still regarded as potentially the most protective vaccines (Chakrabarti et al., 1996; Plotkin, 1994; Riedel, 2005). Use of live attenuated SIV vaccines in monkeys provided significant protection to macaques against a virulent homologous SIV (Koff et al., 2006; Wyand et al., 1996). Also, there is an inverse correlation between the level of virus attenuation and the level of protective immune response in macaque studies (Wodarz, 2008). The use of attenuated viruses with deleted or truncated genes, such as nef or deletion of both nef and vpr resulted in a strong level of protective immune responses in vaccinated macaques (Johnson and Desrosiers, 1998). Moreover, intravenous vaccination of rhesus monkeys using attenuated SIV with deleted nef and vpr sequences (SIVmac239), or the deleted nef and vpx sequences, or deleted nef, vpr, vpx sequences, then intravenously or intravaginally infected with the SIVmac251 challenge virus. The less attenuated SIV vaccine induced robust SIV specific T cell responses leading to higher protection in the intravaginal challenged group than protection against those intravenously challenged (Johnson et al., 1999).

Furthermore, other trials with macaques used attenuated simian immunodeficiency virus (SIVmac239) constructed with the six deleted accessory genes, nef, vpr, vpx, vif, tat, and rev genes. These were given within multiple doses of intravenously to macaques and then challenged intrarectally using pathogenic type of homologous SIV challenge virus, produced protection in 75% of vaccinated macaques with decreasing viral load to less than 1,000 copies/ml for 6 months, and with a stable count of CD4+ T cell for 2 years following challenge (Guan et al., 2001). However, there was no complete protection for the rhesus monkeys who were vaccinated intravenously with SIVmac239-D-nef (deleted nef), or with SIVmac239-D3 (deleted nef and vpr), and then challenged intravenously using the wild type of SIV (SIVmac251). The result was the most vaccinated monkeys were infected after 18 to 44 weeks of challenge (Wyand et al., 1996). Also, there was a concern with the safety of the SIV attenuated vaccine represented by a persistent rise of viremia in vaccinated neonate macaques because of inability of immune response induced for nef or vpr to decrease the pathogenicity of SIV in neonate macaques (Baba et al., 1999). In another study in macaques vaccinated with highly attenuated SIVmac239-Delta 3 which included large deletions of nef and vpr, and then challenged with a wild type of the SIV virus, saw the level of immune response decline, accompanied with serious adverse reactions. These reactions included recurrent viremia,
thrombocytopenia, wasting, and opportunistic infections after a few years of vaccination in vaccinated macaques compared to non-vaccinated macaques (Baba et al., 1999). Accordingly, a live attenuated vaccine is unlikely to be used in humans, this is because of the risk of reversion to the virulence of virus and the attenuated virus strain could cause HIV infection as a result of a virus mutation which can lead to immune dysregulation in the vaccinated individuals (Baba et al., 1999; Baba et al., 1995; Foster and Garcia, 2008; Gorry et al., 2007).

1.4.1.2. Whole killed and subunit vaccines
Whole killed and subunit vaccines have also been used for immunization against HIV/SIV infection. Preclinical trials of formalin-killed potentiated with respective adjuvants or subunit SIV vaccines have resulted in protection against experimental homologous infection of pathogenic SIV virus in rhesus macaques (Macaca mulatta) (Mascola and Montefiori, 2010; Patterson, Robey and Robert-Guroff, 2000). However, whole killed viruses and protein subunits are recommended in vaccination programs because of their inability to induce broad antibody or CD8+ T cell responses multiple doses potentiated adjuvants (Murphey-Corb et al., 1989; Flynn et al., 2005). The mucosal adjuvants used included combinations of cytokines, such as IL-1α, IL-12, and IL-18 to potentiate an antibody immune response in BALB/c mice intranasally vaccinated with the polyvalent C4/V3 HIV-1 Env peptide as the HIV vaccine candidate (T1SP10 MN) (Bradney et al., 2002). When used to deliver the HIV-1 Env peptide with multiple doses, it resulted in a high level of antibodies involving serum IgG1 and secretory IgA in different sites of mucosal surfaces, such as saliva, and the vaginal lavage in vaccinated mice (Bradney et al., 2002; Staats and Ennism, 1999; Staats et al., 2001). One of the first generations of subunit vaccines represented by the Phase I HIV vaccine trial was conducted in 50 healthy adult participants who were at risk of HIV infection but were sero-negative for HIV-1. This group was given the vaccine composed of recombinant HIV-1 Env proteins (gp 120 or gp160) conjugated with alum adjuvant, which led to the induction of a specific HIV-1 Env antibody response in sera. There were samples of sera obtained from 16 HIV-1 infected persons used to evaluate neutralizing antibody activity against the 5 primary viruses (US 1-US5) that were isolated from the HIV-1 infected volunteers in addition to the heterologous laboratory-adapted HIV strains or (MN, IIB, and SF2) (Frey, 1999; Mascola et. al., 1996; VanCott et al., 1997). However, there was no long term protection against virus infection due to the lack of long active HIV neutralizing antibodies (nAbs) (Goebel et al., 1999; Mascola et. al., 1996).
1.4.2. Novel vaccine strategies

Traditional HIV vaccine strategies have failed to provide significant protection (Foster and Garcia, 2008; Das, Jeeninga and Berkhout, 2010). Accordingly, there is currently no commercially available vaccine to prevent HIV infection (Koup and Douek, 2011; Wahren et al., 2010). Due to safety concerns and the ineffectiveness of traditional HIV candidate vaccines, alternative strategies involving various genetic approaches are being used to develop novel vaccines (Excler, 2007; Letvin, 2004; Voronin, Manrique and Bernstein, 2010). These novel HIV vaccine strategies include DNA vaccines and live recombinant vectors (Dubensky, Liu and Ulmer, 2000; Letvin, 2005).

1.4.2.1. DNA vaccines

DNA vaccines utilize plasmids that encode specific viral proteins as immunogens for the induction of strong immune responses (Casimiro et al., 2003; Graham et al., 2006; Vasan et al., 2010). In general, most DNA candidate vaccines for HIV-1 have been shown to be safe when injected in mice or macaques, although they have failed to induce a protective immune response (Johnston and Flores 2001). The immunogenicity of plasmid DNA vaccines has been improved with adjuvants (Barouch et al., 2000a; Chong et al., 2007; Kutzler et al., 2009; Xu et al., 2008). For example, DNA vaccines have been conjugated with conventional adjuvants, such as aluminum salts, which increase antibody responses 100-fold in mice and 10-fold in macaques compared to non-conjugated DNA vaccines. They also enhance cellular immunity as represented by Th1 and Th2 cytokine responses (Petrovsky and Aguilar, 2004; Ulmer et al., 1999). Recent preclinical trials of DNA plasmid vaccine candidates have expressed genes within the highly conserved regions, such as gag, pol and nef. These have been isolated from multiple HIV-1 clades, and then followed by a recombinant adenovirus vector expressing SIVmac Gag, Pol and Nef proteins alone or combined with the Gag protein. Following these trials, all vaccinated Indian-origin rhesus monkeys were challenged using the simian-human immunodeficiency virus (SHIV-89.6P) with no protection against the virus. These monkeys experience a rapid loss of CD4 T cells, despite of robust IFN-γ ELISPOT responses to the Gag/Pol/Nef and Env plus the high-titer antibodies, especially in the Env vaccinated group (Letvin et al., 2004). The same strategy was also conducted in Russia was used in BALB/c mice who were vaccinated intramuscularly with recombinant plasmids expressing the HIV-1 genes, env, gag, pol and nef, the induced robust cytotoxic T cell and IFN-γ’CD8+ T cell responses for HIV-1 peptides mainly for Env (Murashev et al., 2007). Another trial of a DNA vaccine, expressing recombinant SIV-Gag and HIV-1 Env was used in macaques induced with
a low level of cytotoxic T lymphocytes and CD8\(^+\) T cell responses that resulted in high viremia and rapid loss of CD4\(^+\) T cells following an intravenous challenge infection using the pathogenic SHIV-89.6P virus (Barouch et al., 2000).

1.4.2.2. **Recombinant viral vaccine vectors**

Recombinant live vaccine vectors express HIV antigens or epitopes from a recombinant virus. The most common viral vectors used as HIV vaccine candidates are the adenoviruses, modified vaccinia viruses, canarypox and rabies viruses. It is important that there is a difference in the ability of various HIV genes to induce immune responses (Liu, 2010). For example, \(\text{gag, vif}\) and \(\text{nef}\) expressed in Hela-CD4 cells leads to the stimulation of an immune response sufficient to reduce the viral titers, while the use of other HIV genes, such as \(\text{tat, rev}\) and \(\text{vpu}\) increased the rate of infection compared to no inhibitory effect of HIV \(\text{env, vpr}\) and \(\text{pol}\) expressed genes to control the homologous T-cell-line-adapted HIV-1 strain (D’Aloja et al., 1998). Indeed, viral vector vaccines were considered the most likely used vaccine candidates to induce both a humoral and T cell immune response against infectious diseases in humans and animals (Draper and Heeney, 2010). Recently, viral vector vaccines have been regarded as the most likely vaccines to be used to stimulate both a humoral and cellular immune response (Draper and Heeney, 2010).

1.4.2.2.1. **Adenovirus vectors**

The adenovirus serotype 5 (Ad5) has been used as a vector in many preclinical and clinical trials of recombinant HIV vaccines. For example, one clinical trial of a randomized, double-blind phase 1 trial conducted by the Vaccine Research Center (VRC), utilized 4 rAd5 vectors to express the recombinant HIV-1 Gag-Pol fusion protein (subtype B) and also the Env protein (subtypes A, B, and C) to evaluate safety. The immunogenicity of this recombinant vaccine was administered intramuscularly to 30 healthy non-infected volunteers, with induced T cell responses included in 93.3% of vaccine recipients, IFN-\(\gamma\)-CD8\(^+\) T cells in 60% of vaccine recipients, and Env-specific antibody responses in only 50% with moderate adverse reactions, such as headache, malaise, myalgia, chills or mild fever persisting for 1 day after vaccination (Catanzaro et al., 2006). A trivalent MRK Adenovirus type 5 has also been used in a Merck trial for the expression of HIV-1 antigens, such as Gag, Pol and Nef (MRKAd5-Gag MRKAd5-Pol, and MRKAd5-Nef of clade B), in 259 intramuscular vaccinated recipients. This trivalent vaccine induced cell mediated T cell included CD8\(^+\) T cells immune responses without serious adverse reactions, however this trial was discontinued due to lack of efficacy (Priddy et al., 2008).
Recombinant adenovirus serotype 5 vectors were also used to express whole SIV Gag/Pol and Env proteins in a prime-boost vaccine given to rhesus macaques primed with a plasmid DNA vaccine, including SIVmac239 *gag-pol-nef* genes, that stimulated both cytotoxic CD8+ T cells and antibodies (Santra et al., 2005). Another preclinical trial of the SIV vaccination used replication-deficient Ad5 expressing HIV-1 Tat, Env (gp140), recombinant Env (SHIV-89.6P gp140) and a combination of SIV Gag and Env (gp140) as one of the SHIV challenge studies administered intramuscularly in macaques (Macaca mulatta), and then challenged intravenously using SHIV-89.6P. Each of the vaccines induced significant protective cytotoxic CD8+ T cells targeting Env gp140, and Gag compared to lower induced immune response to Tat (Liang et al., 2005). However, rAd5 vectors used in the phase 2 STEP study to express HIV-1 Gag, Pol, and Nef antigens, induced insufficient T cell immune responses and no env-specific antibody responses which failed to protect vaccinated individuals against HIV-1 infection (Barouch, 2010). However, serious adverse reactions after vaccination including viremia were shown in participants. This was due in part to a high mutation of the virus escaping from the host immune response (Flynn et al., 2005; Patterson and Robert-Gouroff, 2008). Furthermore, the recombinant adenovirus 5 expressing HIV-1 Gag protein (rAd5-gag) was used in BALB/c mice as an intramuscular or intravaginal prime dose of prime boost vaccine following the recombinant *Listeria monocytogenes* expressing HIV-Gag protein (rLm-Gag). This was administered via different mucosal routes, such as orally, intrarectally or intravaginally, and resulted in a robust Gag-specific CD8+ T cell response in the spleen and the vaginal secretion and lamina propria. The best CD8+ T cell response detected in the vagina was induced after the intravaginal route of vaccination, particularly in intravaginal-intravaginal prime boost vaccinated mice that produced higher protection against intravaginal challenge using vaccinia virus expressing the whole HIV-Gag protein compared to other routes of vaccination (Li et al., 2008).

### 1.4.2.2.2. Modified vaccinia virus vectors

Most HIV vaccine studies in humans or animals have utilized modified vaccinia virus as a safe and effective vector for expressing viral proteins and for delivering them to mucosal surfaces to induce a mucosal protective mucosal immune response against respiratory pathogens, such as the influenza virus, respiratory syncytial virus and mycobacterium tuberculosis (Artenstein, 2008; Gherardi and Esteban, 2005; McCurdy et al., 2004). In addition, the recombinant modified vaccinia virus vector (rMVA) has been used to deliver HIV or SIV antigens, induced HIV specific immune responses not only in genital and rectal mucosa in mice or macaques but
also controlled HIV or SIV progression and dissemination of the virus (Gherardi and Esteban, 2005). For example, a modified vaccinia virus vector expressing the HIV-1 Env IIIB protein (MVA-Env) conjugated to cholera toxin was given to BALB/c mice intranasally and intravaginally, which induced mucosal CD8$^+$ T cells with both IgA and IgG1 specific antibodies (Gherardi et al., 2004). Another trial in the UK, using recombinant modified vaccinia viruses expressing HIV-Gag proteins (p24 and p17) from HIV-1 clade A administered intradermally in healthy males and females between 18-60 years of age after an intramuscular priming dose of HIV DNA plasmid (expressing Gag protein of HIV-1 clade A). This vaccine induced specific CD4$^+$ and CD8$^+$ T cell immune responses in 89% of volunteers (Mwau et al., 2004). In a similar clinical trial, DNA containing HIV-Env gp160 (subtypes A, B, and C), Rev (subtype B) and the p17/p24 Gag (subtype A) was injected intradermally or intramuscularly into 40 healthy volunteers as a priming, followed by a recombinant HIV Env, Gag and Pol-vaccinia virus boost. This vaccine trial induced IFN-γ$^+$ from CD8$^+$ and CD4$^+$ T cells in 30% of volunteers following the prime dose of vaccine, which increased to 92 % after boosting (Sandström et al., 2008). Also, SIV proteins, such as Gag, Pol and Env, expressed by the modified vaccinia virus Ankara (MVA) were injected intramuscularly into rhesus macaques and then challenged intravenously using pathogenic SIVsm-E660, thus resulting in a reduction of viremia in the vaccinated macaques (Ourmanov et al., 2000).

1.4.2.2.3. Canarypox virus vectors
Canarypox is a strain of the avian pox virus strain from the Orthopoxvirus family. It is isolated from the foci of the pox infection in canaries and attenuated by multiple passaging (200 serial passages) in chicken embryo fibroblasts to produce recombinant virus vectored vaccines (ALVAC) that express specific genes of pathogenic viruses, such as HIV, influenza, and African horse sickness (Bruyn et al., 2004; Bublot et al., 2006; Guthrie et al., 2009). Recombinant canarypox has also been used as a vector to express retroviral proteins, such as the feline leukemia virus (FeLV) Gag and Env, and when given intranasally induced Gag and Env specific cytotoxic T cell immune responses (Poulet et al., 2003; Tartaglia et al., 1993).

1.4.2.2.4. Rabies virus vectors
The rabies virus is a non-segmented RNA virus of the Rhabdoviridae family. It is used as a safe and immunogenic vectored vaccine inoculated in animals as a result of complete inactivation of the virus (Faber, Dietzschold and Li, 2009). The recombinant rabies virus vector (RV) has the ability to express a large viral genes, sequence of up to 4 Kb, such as the HIV-1 Gag-Pol precursor (Pr160) or 2.2 Kb of the Env protein which is necessary to elicit a broad
host immune response in mice and monkeys (Gomme et al., 2010; McGettigan et al., 2003; Addo et al., 2001). For example, the recombinant rabies virus expressed HIV-1 Gag (SPBN-ΔG-Gag) was given intramuscularly in female BALB/c mice as a priming dose and then intraperitoneally administered with the vaccinia virus expressed HIV-1 Gag (VV-Gag). This induced a significant increase of Gag-specific CD8⁺ T cell responses (Gomme et al., 2010). Recombinant rabies expressing the HIV-1 Gag-Pol precursor protein was given intramuscularly in BALB/c mice, and after 6 weeks they were challenged using an intraperitoneal dose of the vaccinia virus expressing the whole HIV-1 Gag. This induced about 12-14% of CD8⁺ T cells specific for a Gag p24 epitope detected after 5 days of challenge (McGettigan et al., 2003). Recombinant rabies viruses expressing the HIV-1 Env (gp 160) protein were able to induce a robust humoral and T cell immune response targeting Env in BALB/c mice vaccinated with an intramuscular-intraperitoneal prime boost regime (Schnell, 2000). Furthermore, BALB/c mice primed intramuscularly with the recombinant rabies virus expressing the HIV-1 Gag protein induced a significant increase of cell response targeting the HIV-1 Gag. This is in addition to 26.8% of Gag⁺IFN-γ⁺ producing CD8⁺ T cells in the vaccinated mice after an intraperitoneal challenge with a recombinant vaccinia virus expressing a HIV-1 Gag (McGettigan et al., 2001). Also, six adult male macaques (Macaca mulatta) were injected intramuscularly using the rabies virus vector expressed SHIV-89.6P Env and SIVmac239 Gag proteins as a priming dose, which induced seroconversion in vaccinated macaques following their initial vaccination. After 45 weeks, they were given an intranasal or intramuscular boost dose of the same vaccine but there was no immune response induced following the boosting dose. This was as a result of preexisting antibodies for the G protein of the rabies virus. Therefore, when this boosting vaccine was replaced by the G protein of the vesicular stomatitis virus (VSV) expressing SHIV-89.6P Env protein and SIVmac239 Gag protein, strong humoral immune responses and low T cell responses were induced. After four months of the boost dose, all vaccinated macaques were intravenously challenged using the pathogenic SHIV-89.6Pvirus. Induced robust neutralizing antibodies and T cell responses resulted in a reduction of the viral load and maintained a level of CD4⁺ T cells in vaccinated macaques compared to high viremia and rapid loss of CD4⁺ T cells in the non-vaccinated group (McKenna et al., 2007).
1.5. Clinical trials of HIV vaccines

1.5.1. Phase 1 and phase 2 clinical trials

Many HIV-1 candidate vaccines have been tested in Phase I and II clinical trials and found to be safe and immunogenic. Some key examples include:

1) The ALVAC vaccine trial: This was the first clinical trial of HIV-1 vaccines involving 150 high and low behavioral risk HIV-1 uninfected volunteers (18-60 years old), using recombinant canarypox vaccine candidates expressing whole Gag and Pol proteins (ALVAC-HIV vCP205) alone or combined with the recombinant subunit Env protein boosting dose (Envgp120). All volunteers were divided into 8 treated groups and then vaccinated intramuscularly using various schedules of multiple doses of vaccines in addition to four control groups followed for a period of 2 years. At the end of the vaccination period, a significant cytotoxic T cell response (64-89%) was detected in all vaccinated groups depending on the number of doses compared to control groups. A significant response of neutralizing antibodies was also detected in all vaccinated groups compared to controls with no serious diverse reactions which confirmed the vaccine was safe and immunogenic (Gupta et al., 2002). A similar trial was also followed in phase II that involved 330 healthy HIV seronegative volunteers using intramuscular administering canarypox vaccine candidates (vCP1452) expressing Gag and Pol proteins alone or combined with a Env gp 120 subunit vaccine boosting dose. This result was strong neutralizing antibody response in the range of 57-95%, while cytotoxic CD8+ T cell response was only 13-16% in all vaccinated groups (Russel et al., 2007).

2) The HIV Envgp120/Nef-Tat/AS02A vaccine trial: This was a randomized phase 1 trial of the subunit protein vaccine conducted in 20 HIV-1 chronically infected volunteers who were well controlled by highly active antiretroviral therapy (HAART). This vaccine included a plasmid DNA encoding HIV-1 proteins, Nef, Tat and Env gp120 (clade B), and was conjugated with AS02 adjuvant. It also included strong and highly significant HIV Env specific CD4+ cells that produced IL-2 cytokine, with proliferation of both CD4+ and CD8+ T cells following multiple doses of intramuscular immunization compared to controls (Lichterfeld et al., 2012; de Rosa and McElrath, 2008).

3) The EUROVAC 02 trial: This was a randomized phase 1 trial using recombinant attenuated poxvirus (vaccinia virus: NYVAC) expressing genes isolated from the HIV-1 clade C. These genes included gag, env, pol and nef. This clinical trial involved 50 healthy participants (low behavioral risk of HIV infection) who were injected intramuscularly by the recombinant
NYVAC vaccine alone or primed first by intramuscular administration of the recombinant DNA expressing the same HIV-1 proteins. The prime boost vaccine induced a higher T cell response, with 90% of participants compared to only 33% following the NYVAC vaccine only. Also, this vaccine induced polyfunctional CD4+ and CD8+ T cells as IL-2+IFN-γ+TNF-α secreting cells. Furthermore, the highest and most frequent T cell responses targeted Env gp120 (91% of vaccinees) compared to only 48% for Gag-Pol-Nef. However, this vaccine has induced lower antibody response, and was only IgG specific for the Env protein, which was detected in 75% of participants who received the DNA/NYVAC prime boost vaccine compared to only 25% induced for the NYVAC vaccine alone (Harari et al., 2008; Midgley et al., 2008).

4) The RV 172 trial: This was a randomized phase I/II clinical trial that included a mixture of three plasmid DNA expressing HIV Env glycoprotein (isolated from HIV-1 clades A, B, and C) and three plasmids encoding Gag, Pol, and Nef proteins (clade B), in addition to a boosting dose of replication-defective recombinant adenovirus 5(rAd5) expressing the HIV-1 Gag/Pol polyprotein (clade B) and Env glycoprotein (clades A, B, and C). This vaccine was conducted in 324 of HIV-1 non-ninfected African participants from Kenya, Tanzania, and Uganda who were injected intramuscularly with a single dose of rAd5 or intramuscular prime boost vaccine (DNA/rAd5) of multiple doses over 24 weeks. Induced HIV-specific T cells responses were represented by IFN-γ ELISPOT in 63% of the total participants, with no significant difference between T cell responses specific for Env, Gag and Pol induced in groups vaccinated by rAd5 alone (63%) or prime boost DNA/rAd5 (62%). Also, there was a low level of HIV specific antibody responses in all groups and no effect of preexisting Ad5 neutralizing antibodies on T cell responses in prime-boost vaccinated individuals (Kibuuka et al., 2010).

5) The trial (HVTN 041): This was a randomized phase 1 trial conducted in 84 HIV-1 uninfected volunteers who were injected intramuscularly with multiple doses of HIV-1 recombinant proteins included Env gp120, Nef and Tat (HIV-W61D). These were expressed by recombinant *Pichia pastoris* yeast cells, and formulated with AS02A adjuvant. This vaccine induced robust neutralizing antibodies in all vaccine recipients following the second week with third dose of the vaccination. There was also a strong titer of binding antibody responses induced specifically for Env gp120, which was detected by antibody-dependent cellular cytotoxicity assay (ADCC), also with a significant increase of HIV-specific CD4+ T cells. However, no CD8+ T cell response was detected in vaccinated individuals (Goepfert et al., 2007).
1.5.2. Phase 3 clinical trials

1) The VAX004 trial: This was a double-blind and randomized trial held in Canada and the Netherlands in 1998-1999. This included two recombinant HIV-1 Env gp120 proteins, MN-rgp120/HIV-1 and GNE8-rgp120/HIV-1 clade B administered intramuscularly within multiple doses in 5403 healthy volunteers, such as men who had sex with men and women at risk of heterosexual transmission of HIV-1. This vaccine was used as a subunit vaccine with multiple doses to induce specific Env gp120 neutralizing antibodies. This clinical trial of HIV vaccine resulted in low specific binding neutralizing antibodies (nAbs) that led to a low level of immuno-efficacy (6%) but with no significant overall protection in vaccinated individuals (Flynn et al., 2005). A similar trial called the VAX003 trial was also conducted in 2546 healthy volunteers among heavy intravenous drug users at high behavioral risk of HIV-1 infection. This trial also used also two recombinant Env glycoproteins, MN-rgp120/HIV-1 and A244-rgp120/HIV-1 (clade B and E). Volunteers were injected intramuscularly within multiple doses held in Thailand in 1999-2000. However, this trial only resulted only 0.1% efficacy in all vaccinated participants without a significant difference between vaccinated and control individuals following 36 months of vaccination (Pitisuttithu et al., 2006).

2) The STEP trial: This trial started in 2004 and concluded in 2007 with approximately 1500 healthy participants who were at high risk of HIV infection, including homosexual men and female sex workers. This trial included the development of recombinant adenovirus serotype 5 (rAd5) by Merk, which was used as a vector for expressing the HIV-1 whole proteins, Gag, Pol and Nef (MRKAd5-gag/pol/nef). To begin with results were promising as this vaccine induced a CD8⁺ T cell response in intramuscular vaccinated volunteers without a serious adverse reaction. However, the stimulated immune response could not prevent the transmission and progression of a HIV-1 infection and the trial was terminated because of an increased risk of HIV-1 infection represented by a high titer of the virus at the last stage of the trial. 40,000 copies/ml were detected in the vaccinated group and 37,000 copies/ml in the control group. This was as a result of the high prevalence of preexisting Ad5-specific neutralizing antibodies that led to no suitable efficacy after vaccination (O’Brien et al., 2009; Takahashi, Rolling and Owen, 2010; Zak et al., 2012).

3) The RV144 phase 3 trial: This trial used a combination of the recombinant Canarypox live virus vector (ALVAC-HIV,vCP1521) that was genetically modified to express the HIV-1 Gag and Pro protein obtained from the HIV-1 subtype B combined with Env gp120 protein (subtype
E) boost with (AIDSVAX B/E HIV-subtypes) the vaccine containing bivalent Env glycoproteins. This was undertaking during two trials. The first was conducted in Thailand in 1999-2000 with a high risk group including 2546 intravenous drug users using the AIDSVAX B/E HIV (VaxGen) vaccine for 36 months. No protection was induced against HIV-1 infection in vaccinated subjects (Pitisuttithum et al., 2006). The second was also held in Thailand, involved 16395 subjects divided into three groups: female sex workers, drug users and homosexual men, and the results of this trial were concluded in 2009 (Rerks-Ngarm et al., 2009; Vaccari, Poonam and Franchini, 2010). As a result of a HIV-specific response (90.1%) and (49.3%), a T cell proliferation induced in the vaccinated participants targeting both Env and Gag proteins along with the T cells that produced an interferon-γ, were also detected in 19.7% of vaccine recipients by ELISPOT, and 11.1% and 7.6% of IFN-γ CD8+ T cells were detected for Env and Gag by ICS. Also, binding antibody responses induced for Env (98.6%) and Gag (52.1%) were detected by antibody-dependent cellular cytotoxicity assay (ADCC) (Rerks-Ngarm et al., 2009). Overall results demonstrated partial protection (31.2 %) with modest reduction in the rate of HIV-1 infection (Pitisuttithum et al., 2010; Rerks-Ngarm et al., 2009). This provides hope for future work to control HIV/AIDS infection rates (Pantaleo et al., 2010; Vaccari, Poonam and Franchini, 2010).

1.6. Influenza virus

Influenza virus is a single stranded, negative sense segmented RNA virus infecting a wide range of mammals and bird species. It belongs to the Orthomyxoviridae family and causes respiratory illness, ranging from mild respiratory symptoms to severe pneumonia causing death, particularly in infants, elderly and immuno-compromised individuals (Barnard, 2009; Bouvier and Lowen, 2010). Five influenza virus genera have been identified: A, B, C, Thogotovirus and Isavirus (Kawaoka et al., 2005). Influenza virus type A and B are comprised of a genome with eight RNA segments compared to type C viruses, which have seven segments (Lamb and Krug, 1996; Lewis, 2006). The three types differ in their pathogenicity and ability to infect humans (Mateo et al., 2007). The most pathogenic influenza viruses are the type A viruses which are characterized by high virulence causing the most severe disease in humans. For instance, the H1N1 influenza virus infected humans and led to a high mortality rate with the Spanish flu in 1918 (Johnson and Mueller, 2002), the Asian flu of 1957 (the result of a re-assorted H2N2 virus), the Hong Kong flu of 1968 (caused by a re-assorted H3N2 virus), and the swine flu in 2009 (caused by re-assorted H1N1) (Garten et al., 2009; Klenk, 2005). In addition, the avian re-assorted H5N1 influenza virus is considered one of the highest
pathogenic influenza viruses in humans as it has the ability to infect humans leading to severe complications, such as multiple-organ and respiratory failure and ending with death (de Jong et al., 2006; Uyeki et al., 2009; Tran et al, 2004; Beigel et al., 2005).

Influenza A viruses have a spherical or filamentous virion with a diameter of 80-120 nm. These viruses have two surface glycoproteins, hemaglutinin (HA) and neuraminidase (NA), embedded in a lipid envelope surrounding the virus in addition to other viral glycoproteins, such as matrix proteins (M1 and M2), nucleoprotein (NP), polymerase complex proteins PB1, PB2 and PA, and non-structural proteins NS1, NS2 (Palese and Shaw, 2007; Jagger et al., 2012). HA plays a direct role in regulating the infectivity of the influenza virus through attachment to the host cell membrane by binding to sialic acid on specific receptors located at the cell surface. NA is responsible for the assembly and release of new progeny viruses from infected cells. There are 17 subtypes of HA and 9 subtypes of NA amongst influenza A viruses (Kaverin et al., 2000; Pushko et al., 2005; Taubenberger, 2006; Tong et al., 2012). Therefore, antigenic drift caused by gradual accumulated mutations in highly variable regions of HA resulting seasonal influenza epidemic outbreaks, with antigenic shift introducing new subtypes of influenza A viruses in the human population, thus leading to pandemic outbreaks of an influenza virus infection (Smith et al., 2004; Herfst et al., 2012). Both mutation mechanisms enable influenza viruses to evade an immune response through escape recognition by virus specific antibodies because of an absence of neutralizing antibodies to these viruses in the total population (Fouchier et al., 2005; de Jong et al., 2000).

1.6.1. Pathogenesis of the influenza virus

Influenza viruses have a high affinity for replicating at the mucosal surfaces of the upper and lower respiratory tract. The first step of influenza infection is the attachment of the virus to molecules on the surface of the target cells by the haemagglutinin glycoprotein (HA) binding to the epithelial surface receptor (sialic acid) followed by the cleavage of HA into two linked subunits, HA1 and HA2, by membrane-associated proteolytic enzymes. This enables the virus to enter the cytoplasm of the target cells and then replicate in these infected target cells (Taubenberger, 2006; Zhirnov, Ikizler and Wright, 2002). Following the first few hours of initial infection, newly forming viruses are released to infect other cells facilitated by the neuraminidase glycoprotein (NA) (Lamb and Krug, 1996; Murphy and Webster, 1996). The infectivity of the influenza virus depends on the level of HA cleavage. For example, highly pathogenic influenza viruses, such as H1, H5, and H7 subtypes, are characterized by high levels
of HA cleavage compared to low pathogenic or non-pathogenic influenza viruses (Senne et al., 1996; Shortridge et al., 1998). After its replication in respiratory epithelial cells, the influenza virus subsequently infects macrophages and monocytes located in the alveoli, leading to the destruction of these cells and the formation of necrotic areas as a result of damaged and dying cells accumulating in these locations and ending with pneumonia (Fesq et al., 1994; Taubenberger and Morens, 2008).

There are different models of laboratory animals that are characterized with high sensitivity to infectin by influenza viruses and can be used in preclinical experiments to evaluate vaccines produced for influenza viruses. These animal models include animals naturally susceptible to be infected by human influenza viruses like ferrets and guinea pigs, and other animals which need adaptation to be infected by influenza viruses like mice (Bouvier and Lowen, 2010). The susceptibility of mice to be infected by the influenza viruses depends on the strain of mice and the laboratory adapted influenza viruses. For example, BALB/c and C57BL/6 mice are considered the main commonly laboratory strains used in research. These mice are the highest sensitive strain of mice infected intranasally with adapted influenza viruses, such as A/Puerto Rico/8/1934 (H1N1; PR8), A/WSN/1933 (H1N1; WSN) or A/Aichi/2/68 (H3N2; X31). These viruses replicate in the respiratory tract and lead to weight loss or death depending on the pathogenic strain and lethal doses of the influenza viruses used in the two lab strains of mice, with wild mice mainly resistant to these influenza viruses (Staeheli et al., 1988; Haller, 1981). Indeed, the ability of these mice adapt to the influenza viruses that induce the pathogenic effects following primary intranasal infection depends on the dose of infective viruses. For example, BALB/c mice start losing weight on day 2 of intranasal infection of a highly lethal dose (10x the 50% lethal dose, LD$_{50}$) of the PR8 (H1N1: A/PR/8/34) influenza virus strain (2 × 10$^3$ pfu), and died between days 6 to 8 of infection as a result of rapid replication of the virus in the lungs. The X31 (H3N2: A/Aichi/2/68) influenza virus strain resulted in less pathogenic effects detected in BALB/c mice who were infected intranasally using (10x LD$_{50}$) 3.5 × 10$^6$ pfu of X31, with gradual weight loss beginning with day 2 of infection and then death on day 10 (Quan et al., 2008; Yetter et al., 1980; Tamura et al., 1992). This is compared to a sub-lethal dose of the X31 influenza virus of 1 × 10$^4$ pfu which showed a peak of viremia within days 3 to 5 of primary intranasal infection accompanied with gradual weight loss in BALB/c mice. This declined within days 6 to 7 to a complete recovery and clearance of the virus on day 10 of infection (Cukalac et al., 2009).
A similar course of infection was also detected after the intranasal influenza infection in BALB/c mice using a sub-lethal dose of 50 pfu of the PR8 influenza virus but with higher weight loss (Edenborough, Gilbertson and Brown, 2012; Tamura and Karuta, 2004; Tamura et al., 1998). Similar results were also detected in BALB/c mice infected by a sub-lethal dose of the PR8 influenza virus, 10 TCID\textsubscript{50}. The infected mice showed a short period of mild weight loss and then began to recover on day 7 post-infection, with complete recovery on day 9 post-infection. This was compared to a moderate pathogenic effect of a higher dose (100 TCID\textsubscript{50}) of the PR8 (A/PR8/34) influenza virus represented by higher weight loss that started on day 3 of infection, and reached 25\% weight loss. These infected mice began to recover on day 10 and then reached their starting weight within 10-14 days post-infection. While rapid viremia and severe weight loss were detected in mice after intranasal infection using a higher lethal dose, 1000 TCID\textsubscript{50} of the PR8(A/PR8/34) influenza virus saw mice reaching 30\% weight loss on day 8 and then dying on day 15 post-infection (Verhoeven, Teijaro and Farber, 2009).

1.6.2. Influenza immunity

Mucosal surfaces represent not only the main portal of entry for influenza, but also the first line of non-specific defense. This involves the epithelium, mucin layer, non-specific cytokine responses and secretory IgA released by the epithelial cells. Following the infection of epithelial cells, the innate and adaptive immune system play the major role in defense (Lamb and Krug, 1996; Murphy and Webster, 1996; Tamura and Kurata, 2004).

1.6.2.1. Innate immune response

Innate immune response is the first line of defense mechanisms against an influenza virus infection characterized by a fast onset and a short period of effectiveness following primary influenza infection. Innate immunity includes several components facilitated by recognition of influenza antigens that also induce other parts of the immune response to control infection (Banchereau and Steinman, 1998; Guermonprez et al., 2002). Dendritic cells (DCs) are considered the main professional antigen presenting cells (APCs) during early influenza infection by degrading viral proteins into small peptides by proteasomes or phagocytosis. Dendritic cells transport these small peptides to the endoplasmic reticulum and then load to MHC class I molecules to be recognized by the CD8\(^+\) T cells or present them to the MHC class II peptide complexes and then recognized by CD4\(^+\) T helper cells (Bhardwaj et al., 1994; Yewdell et al., 2003). As a result of early recognition of influenza viral antigens, naïve T and B lymphocytes are activated to control infection. Dendritic cells also work as a link between
an innate and adaptive immune response, induced activation and proliferation of cytotoxic CD8$^+$ T cells against influenza viral antigens, and also migrate to various draining lymph nodes, such as the mediastinal lymph node (Braciale, Sun and Kim, 2012; Kim and Braciale, 2009).

Influenza viruses are first detected after a few hours of primary infection in an infected human or in animals by the innate immunity. Viruses are then destroyed as a result of non-antigen specific mechanisms involving macrophages, natural killer cells, and cytokines, such as IFN-α and β interferons. These are not active for a long time during induction, thus giving influenza viruses the opportunity to escape this early mechanism of defense (Murphy and Webster, 1996; Ada and Jones, 1986). High amounts of IFN-α and β, secreted by influenza infected cells, are rapidly detected following influenza infection in both nasal and pulmonary regions and their level is directly related to reducing the replication of influenza viruses in humans and animals (Husseini, Sweet, Collie and Smith, 1982; Wyde, Wilson, and Cate, 1982). Natural killer cells are cytotoxic lymphocytes activated in an innate immune response that is rapidly detected in upper respiratory mucosa and the lungs within 24 hours following primary influenza infection. These cells produce IFN-γ in addition to their direct role in reducing the spread of the virus by the lysis of influenza infected cells enhanced by perforin activity (Hicks et al., 1978; Kopf et al., 2002). IFN-γ acts as a linking factor between the innate and adaptive immune response against influenza infection as it induces the cytotoxic CD8$^+$ T cell response and regulates homeostasis and proliferation of these cells in secondary lymph nodes and their trafficking to the site of infection necessary to control the influenza infection (Turner et al., 2007; Bot, Bot, and Bona, 1998). The early innate immune response for the influenza virus includes some inhibitory factors located in the respiratory tract mucosa, which have an identical structure to acetyl-neuraminic acid binding to the HA glycoprotein and highly reduces its infectivity and spread of influenza viruses in the host cells of BALB/c mice infected nasally with mouse adapted influenza viruses such as the PR8 influenza virus (A/PR/8/34; H1N1) (Yoshikawa et al., 2004; Shugars, 1999). Also, macrophages localized in the alveoli have an important inhibitory role against influenza virus infection by direct lysis of infected cells or influenza virus particles by phagocytosis. Activated macrophages in alveoli also induce a pro-inflammatory response, including IL-6 and TNF-α (van Riel et al., 2011; Becker, Quay and Soukup, 1991), and they regulate an adaptive immune response as there is a direct correlation between the depletion of alveolar macrophages leading to low levels of antibodies and a reduction of CD8$^+$ T cells induced following influenza infection (Kim et al., 2008). There is
also an indirect immune role of macrophages during early influenza infection by activating natural killer cells through their secreting specific cytokines, such as IL-1, IL-6, IL-12 and TNF-\(\alpha\), which are also necessary for decreasing virus shedding following primary influenza infection (Monteiro, Harvey and Trinchieri, 1998).

However, influenza viruses easily escape the early innate immunity defense mechanism but these viruses can be detected and eliminated by the main active components of the adaptive immune mechanism, such as influenza specific antibodies and cytotoxic T cells targeted influenza virus and the infected cells (Tamura and Kurata, 2004). There was a high level of both T and B cells accumulated in the upper respiratory mucosa of BALB/c mice intranasally infected with the A/PR/8/34 influenza virus. The infected mice reached their peak on day 7 post-infection. The first detected antibody response was on day 5 post-infection and reached its peak on day 7 of infection, with IgM being the first antibody detected for the primary influenza infection, followed by IgA and IgG (Tamura et al., 1998). IgM was first detected in a few days of primary influenza infection and then diminished quickly within a few days (Qiu et al., 2011). This is in comparison to IgA and IgG immunoglobulins, which are the only immunoglobulins that detected following secondary influenza infection (Tamura et al., 1998; van de Sandt, Kreijtz and Rimmelzwaan, 2012).

1.6.2.2. Adaptive immune response

Adaptive immune response is the second line of the defense mechanism for influenza virus infection characterized by slow induction against primary infection but with faster onset and stronger effectiveness for the secondary influenza infection (van de Sandt, Kreijtz and Rimmelzwaan, 2012). The adaptive immune response includes two parts, humoral immune response represented by influenza-specific antibodies produced by B cells, and cellular immune response represented by T cell response, which includes both CD4\(^+\) and CD8\(^+\) T cells (Tamura et al., 1998; van de Sandt, Kreijtz and Rimmelzwaan, 2012; Baumgarth et al., 2000).

1.6.2.2.1. Humoral immune response

The humoral immune response represents the main defense against primary influenza infection in humans (Strugnell and Wijburg, 2010; Lamb and Krug, 1996), especially in young infants who are mainly protected against influenza virus infection by maternal antibodies produced following vaccination in humans and mice (Hwang et al., 2010; Zuccotti et al., 2010). The early humoral immune response against influenza virus infection is characterized by the production of specific IgM, IgA and IgG antibodies, while later stages involve IgA and IgG
IgM antibodies are highly active in neutralizing the influenza virus, but only for a short lived period. They also contribute to activating the complement system necessary to inhibit virus replication (Fernandez Gonzalez, Jayasekera and Carroll, 2008; Jayasekera, Moseman, and Carroll, 2007). IgA works as the predominant immunoglobulin necessary for recovery from recent influenza infection in humans (Tamura et al., 1998). On the other hand, IgG antibodies directed for influenza antigens are characterized with a long lived protective role against influenza infection (Koutsonanos et al., 2011; Onodera et al., 2012). Most immunoglobulins mainly target influenza HA, NA, NP and M proteins, although the most predominant antibodies are those induced by HA and NA, which are highly involved with protective immunity against influenza infection. The Ig-molecules play a major role in preventing replication of the influenza virus (Glathe, Bigl and Grosche, 1993; Lamb and Krug, 1996; Murphy and Webster, 1996). For instance, antibodies directed to HA bind to the receptor located on the globular head of HA, and prevent the virus from binding to targeted host cells (de Jong et al., 2003; Knossow and Skehel, 2006), and most HA-specific antibodies are not effective for neutralizing new variants of influenza subtypes produced by antigenic drift mutation (Yu et al., 2008; de Jong et al., 2000). Antibodies produced targeting the NA glycoprotein act either by inhibiting the sialidase activity of NA and preventing the release and spread of newly formed viruses from virus infected cells, or antibodies are actively involved in destroying virus-infected cells by facilitating the ADCC mechanism important for virus clearance (Palese and Shaw, 2007; Hashimoto, Wright and Karzon, 1983; Bosch et al., 2010). Furthermore, antibodies induced against influenza NP are potentially involved in protection against hetero-subtypes of influenza A viruses as it is highly conserved glycoprotein between these viruses, and their protective role may include ADCC mechanism of infected cells and activating T cell responses in human and mice (Lamere et al., 2011; Sambhara et al., 2001; Carragher et al., 2008).

The protective role of influenza specific antibodies was also evident between 1991 and 1992. Using one dose of trivalent influenza vaccine containing A/Singapore/6/86 (HIN1), A/Beijing/353/89 (H3N2) and B/Yamagata/16/88, five different groups of people were vaccinated. The result was an increased level of antibodies directed for HA, with 96-100% protection in the vaccinated groups (Glathe, Bigl and Grosche, 1993). Furthermore, the therapeutic role of antibodies to control influenza infection was proven in B-cell-deficient muMT (-8) mice, who were highly susceptible to the intranasal infection of mouse-adapted
influenza type A virus A/PR/8/34 (H1N1). Around 80%-94% of these infected mice survived by intravenous transferred spleen cells obtained from normal B6 mice and included a higher virus-specific precursor B cell response. This was compared to muMT (-8) mice which did not produce antibodies and were highly sensitive to influenza infection, with BCR-transgenic spleen cells transferred, these cells contained 10 times lower virus-specific precursor B cells, and were not protected from PR8 influenza infection (Mozdzanowska et al., 2005). Also, specific-influenza antibodies have an important role in the control of primary influenza infection in mice using intranasal PR8 infection. This was detected by a higher increase of the virus titer in B cell-deficient (muMT) mice compared to less sensitive IgM-/- and wild-type infected mice (Kopf et al., 2002).

1.6.2.2.2. Cellular immune response

Cellular immune response for influenza A viruses consist of CD4+ and CD8+ T cells as they have a regulatory role for influenza specific immune response and recovery from infection (van de Sandt, Kreijtz and Rimmelzwaan, 2012). Indeed, the cellular immune response is more efficient than the humoral arm in the protection against influenza virus infection because the humoral response is induced specifically for each virus subtype and is less efficient in recognizing newly mutant influenza A subtypes resulting from antigenic drift. This includes altered HA or NA compared to high cross activity of cytotoxic T cells in recognition of hetero-subtypes of influenza A viruses, thus giving the cellular immune response a higher efficient role in the clearance of influenza virus infection in humans (Biddison, Shaw and Nelson, 1979; Shaw and Biddison, 1979; McMichael and Askonas, 1978). For instance, CD4+ and CD8+ T cells are mainly involved in broad cross-protection for hetero-subtypic influenza viruses. This was recognized by impaired hetero-subtypic protection represented by no efficient control of virus replication in acute CD4 and CD8 depleted mice infected intranasally using a prime dose of H1N1, H2N2 or H3N2. The mice were then challenged intranasaly using H1N1 or N3N2 influenza viruses, represented by higher virus titer in the lungs and a higher mortality rate compared to depleted Ig-/- immunoglobulin infected mice (Benton et al., 2001). This protective role was also recognized by intranasal inoculation of the live influenza virus, A/Munich/1/79 virus (H1N1) into 63 volunteers conducted in the United Kingdom to check their levels of Hemagglutination-inhibition titer (HAI). An anti-neuraminidase titer and cytotoxic T cell response in infected individuals, with induced cytotoxic T-cell responses higher than 10% with no shedding of the virus following three weeks of infection was compared to lower effective antibodies in the clearance of the influenza virus (McMichael et al., 1983).
As the first component of cellular response in regulating immune responses to influenza infection, CD4+ T cells are activated directly after recognition of influenza viral epitopes loaded with MHC class II molecules. They are then differentiated into two functional types, T helper 1 cells (Th1) or Th2 CD4+ T cells based on the cytokine they produce (Zhu and Paul, 2010). The T helper 1 lymphocyte secretes IFN-γ, TNF-α and IL-2 is also directly involved in CTL responses and induction of memory CD8+ T cells (Mosmann et al., 1986; Deliyannis et al., 2002). The CD4+ T helper 1 cell also mediates delayed types of hypersensitivity (DTH) reactions inhibiting the replication of the influenza virus by secreting IFN-γ (Cher and Mosmann, 1987; Tamura et al., 1996). In addition, robust IgG and IgA antibodies are induced following intranasal influenza infection in mice produced by the CD4 T helper 1 lymphocyte (Topham et al., 1996; Roman et al., 2002). While the CD4+ T helper 2 lymphocyte plays an important role in producing IL-4, IL-5 and IL-13, and in activating B cells to produce antibodies, such as IgG1 and IgE in BALB/c mice (Gerhard et al., 1997). In addition, it also acts as a bridge between CD4+ and CD8+ T lymphocytes that are required for a protective immune response against hetero-subtypic influenza A viruses, which share the same internal proteins but differ in HA and NA subtypes (Okoye and Wilson, 2011; Lamb et al., 1982; Kamperschroer et al., 2006). Furthermore, the CD4+ T cell has another protective role in the cell mediated immune response following intranasal PR8 influenza infection in BALB/c mice. These mice were transferred intravenously with the CD4+ effectors harvested from TCR Tg BALB/c mice directly with the HA126-138 peptide from influenza A/Puerto Rico/8/34 (PR8) CD4+ T cells 18-24 hours previous to an influenza challenge. The function of these CD4+ T cells in the transferred mice was represented by enhanced perforin and granzymes to lyse influenza-infected cells, inducing high amounts of anti-influenza antibodies, thus leading to clearance of the influenza virus and significant survival of the mice compared to controls (Brown et al., 2006). Also, memory CD8+ T cells were mainly induced following primary influenza infection and then localized in the respiratory airways compartments especially in the lungs, to provide faster control of secondary influenza infection (Teijaro et al., 2011; Strutt et al., 2010).

The protective role of CD4+ T cells in clearance of the influenza virus infection in mice was also evident in CD4- depleted C57BL/6J (B6) (Ig-/- mMT) mice infected intranasally using a prime or prime boosting dose of HK-X31 (H3N2) influenza A virus. Results obtained from this study indicated a significant delay of influenza virus clearance from lungs harvested from CD4-
depleted mice for a period extending from one to three months of infection. A significantly low percentage of survival for the CD4+ depleted (-CD4mMT) challenged mice was detected and a significantly diminished number of recruitment CD8+ T cells directed to Db NP366, which were detected in BAL and the spleen compared to the control group of mice (Riberdy et al., 2000). Also, there was highly significant reduction of secreted cytokines, such as IL-2, IFN-γ, IL-4 and IL-5, detected in draining lymph nodes, mediastinal LN and BAL of the CD4-deficient mice following intranasal influenza infection compared to the wild type of mice (Tripp, Sarawar and Doherty, 1995). This was highlighted the very important role of CD4+ T cell in clearing influenza virus by those secreted cytokines at the site of the infection. Furthermore, the μMT mice generated from BALB/c back-cross mice, and then maintained as homozygous CD4+ depleted mice (-CD4 μMT−/−) by treating them with anti-CD4 depleting antibodies, did present with a high mortality rate (80%) when they were infected intranasally using a small dose of a virulent strain of the PR8 influenza virus. This was in comparison to only 15% in normal μMT mice (Mozdzanowska, Maiiese and Gerhard, 2000).

The CD8+ T cell is the second major component of cellular immune response induced to control primary or secondary influenza virus infection (Benton et al., 2001; Hemann, Kang and Legge, 2013). After influenza virus antigen has presented by the MHC-I molecule and loaded on antigen presenting cells (APCs), a T cell receptor (TCR) of a naïve CD8+ T cell binds to a specific influenza peptide for activation (Norton et al., 1992). The activated CD8+ T cells undergo high differentiation into cytotoxic CD8+ T cells localised in respiratory lymphoid organs, especially in draining lymph nodes. These cells then migrate to the site of the infection and destroy infected cells through lytic activity of perforin and granzymes, and inhibit the replication and the spread of newly formed viruses by producing pro-inflammatory cytokines, such as IFN-γ and TNF-α (Nakanishi et al., 2009; Topham, Tripp and Doherty, 1997). Influenza specific CD8+ T cells are characterized by high cross-reactivity against heterosubtypes of influenza A viruses in humans when they directed to the highly conserved internal viral proteins, such as NP, PA, PB2 and M, among various influenza virus subtypes (Assarsson et al., 2008; Kreijtz et al., 2008).

The protective role of CD8+ T cells, response directed for a heterosubtypic immune response was also detected in mice following influenza A virus infections (Grebe, Yewdell, and Bennink, 2008). For instance, C57BL/6 mice primed intranasally with the influenza virus X-
31 (H3N2) and then challenged intraperitoneally following 4 weeks with a virulent strain of the A/PR/8/34 (H1N1) virus, presented a higher significant level of specific CD8\(^+\) T cells detected for the H-2D\(^b\) restricted NP\(_{366-374}\) epitope, with fast clearance of the A/PR/8/34 virus from the lungs and increased survival rates of mice compared to controls which were not primed with the X31 virus. Furthermore, it was evident that there were no neutralizing antibodies directed to the HA or NA of the PR8 challenge virus, allowed the heterosubtypic CD8\(^+\) T cell response to be protective against the virulent challenge virus (Kreijtz et al., 2007). The hetero-subtypic protective CD8\(^+\) T response detected in C57BL/6J mice primed intranasally with the human influenza virus, A/Hong Kong/2/68 (H3N2), which was characterized by the ability to replicate in mice, actually protected mice from the intranasal challenge with the highly pathogenic influenza virus A/Indonesia/5/05 (H5N1). This robust hetero-subtypic CD8\(^+\) T cell response was mainly directed to the highly conserved influenza epitopes, such as NP\(_{366-374}\) and PA\(_{224-232}\), and inhibit replication of the virus in the lungs and significantly reduce body weight loss and mortality rates compared to unvaccinated infected mice with the highly pathogenic avian influenza virus (HPAI A/Indonesia/5/05) (Kreijtz et al., 2009).

Indeed, the majority of cross-reactive immune response against influenza A viruses included CD8\(^+\) cytotoxic T lymphocytes that were directed toward the highly conserved viral proteins, such as NP and M1. These CD8\(^+\) T cells were detected in the spleen of the CBA/J strain of mice after 5 days of intraperitoneal administration of hetero-subtypes of influenza A viruses, such as PR8 (A/PR/8/34: H0N1), and AA (A/Ann Arbor/23/57: H2N2) (Effros et al., 1977). Therefore, the effective vaccine used for protection against various subtypes of influenza viruses needs to induce the heterosubtypic immunity (HIS) included specific CD8\(^+\) T cells that recognize influenza specific epitopes located in the highly conserved six shared internal viral proteins (Grebe, Yewdell, and Bennink, 2008; Hillaire, Osterhaus and Rimmelzwaan, 2011). Several studies related to influenza vaccines in humans and mice have shown the most commonly detected proportion of the CD8\(^+\) T cell response has been that directed toward the relatively conserved NP and M1 protein, which allows cross-protection against various subtypes of influenza viruses (Yewdell et al., 1985; Kees and Krammer, 1984; Lee et al., 2008).

The protective role of the CD8\(^+\) T cell in controlling influenza virus infection was also associated by a significant delay with virus clearance in CD8\(^+\) T cell-deficient mice (Thomas
et al., 2006). As recognised in transgenic homozygous B2-M mice (−/−), lack of MHC-I molecules and being unable to produce mature CD8+ T cells showed a significant delay in the clearance of the virus from the lungs until day 15 after intranasal administration of the mice using influenza viruses, such as A/Port Chalmers/1/73 (H3N2) or A/Puerto Rico/8/34 (H1N1). This was in comparison to complete clearance of the virus between day 6 and 8 in normal mice or heterozygous B2-M (+/−) mice who were able to express MHC-I molecules (Bender et al., 1992). The CD8+ T cell response induced following both primary and secondary influenza virus infection in mice was detected within a highly protective role in humans and mice (Flynn et al., 1998; McMichael et al., 1983). The CD8+ T cells were induced firstly in lungs and BAL, and later detected in MLN of the C57BL/6J (B6) in significant numbers after seven days following the intranasal primary infection using a sub-lethal dose of A/HKX31 (H3N2) which was higher than that induced for primary intranasal infection using the B/HK influenza virus.

The percentage of NP366-374 specific CD8+ T cell responses induced robust influenza specific CD8+ T cell responses in the BAL of X31 infected mice with 7.3% compared to less than 0.1% for the CD8+ T cell induced for the primary infection the B/HK influenza virus (Flynn et al., 1998). This was because the virus did not include the NP366-374 peptide (Allan et al., 1993). However, influenza specific CD8+ T cells that produced IFN-γ+ were first detected after five days of the secondary intranasal challenge using the PR8 influenza virus, and the detected response in BAL was 5-fold higher than that detected in the primary response at the peak of response on day 10 of infection (Flynn et al., 1998). The CD8+ T cells were also detected expressing IFN-γ+ directed to endogenous influenza H2-Kd NP147 in BALB/c mice following secondary intranasal infection using a prime boosting dose of recombinant X31-NA-Env311 plus PR8-NA-Env311. This was higher than the primary response of 9-fold in the spleen and 4-fold in the BAL (Cukalac et al., 2009).

Moreover, it was also detected that the highly important protective function of CD8+ T cells in B cell–deficient mice promoted their recovery from lethal dose influenza virus infection. The B cell deficient mice (μKO mice; H-2b) were firstly intranasally inoculated using 10x LD50 influenza A/Japan/57 (H2N2) virus and 30 minutes later they were intravenously administered with 10⁷ clone cells (including CD8+ T cells) obtained from spleen cells. The cells were harvested from μKO and C57Bl/6 mice who had received a sub-lethal intranasal dose of A/Japan/57 and stimulated in vitro with influenza A/Japan/57 virus-infected cells. This induced complete protection of CD8+ T cell transferred mice represented by the fast clearance of the
virus from the lungs compared to non-transferred mice who were more than 100-fold more susceptible to a lethal virus infection than the wild-type C57Bl/6 mice. On the other hand, there was only a partial protective role of CD4$^+$ cells adoptively transferred in B cell-deficient or antibody-deficient mice (Graham and Braciale, 1997; Epstein et al., 1998).

1.6.3. Influenza virus as a vaccine vector

Influenza virus type A has been used as an efficient viral vector for the expression of recombinant viral antigens, particularly those targeting various infectious diseases, especially mucosally transmitted diseases (Marsh and Tannock, 2005; Satterlee, 2008; Smith, 2007). For instance, HIV, tuberculosis and malaria antigens have been inserted into influenza virus vectors for use for vaccination (Martínez-Sobrido and García-Sastre, 2007). This is due to the ability of this virus to infect through mucosal portals, resulting in broad and long term humoral immune responses (Mueller et al., 2010; Yao and Wang, 2008).

It is also relatively easy to manipulate gene segments of the influenza virus by reverse genetics to generate novel recombinant viruses for use as vaccine vectors (Steinhauer and Skehel, 2002; Steinhauer et al., 2009; Hoffmann et al., 2002). Furthermore, influenza virus vectors are not integrated in the host chromosomal sequences, and so will not induce any adverse mutation in the host DNA, and there is no ability for influenza viruses to progress into a persistent or chronic infection in humans, which avoids neutralizing antibodies affected by immune response induced by the virus vector (Martínez-Sobrido and García-Sastre, 2007). Most of the recombinant influenza viruses generated by reverse genetics have involved the manipulation of HA and NA genes (Hwang et al., 2000; Kobayashi et al., 1992; Poon et al., 1998). The strategy of reverse genetics has been used for developing many live vaccine vectors generated from different segmented and non-segmented viruses, such as the influenza virus (Neumann, Whitt and Kawaoka, 2002). The first attempt to express foreign sequences into the modified genome of the influenza virus as a negative-sense RNA virus was in 1989. At this time, it was used a recombinant influenza virus bearing recombinant RNA containing the coding sequence of the chloramphenicol acetyl-transferase gene that replaced the NS gene for re-assorting the influenza helper virus but this attempt failed as the generated virus rapidly lost this gene following few passages (Luytjes et al., 1989). However, the first successful recombinant influenza virus was generated in 1999 by using the plasmid based technique which resulted in 12 modified plasmid recombinant influenza viruses, such as A/WSN/33 (H1N1) and A/PR/8/34 (H1N1) that have generated from the clone cDNA (Neumann et al., 1999).
Subsequently, various foreign epitopes from the infectious agents have been inserted into HA, NA and NS gene segments in order to generate a live influenza virus vector using the reverse genetics strategy (Li et al., 2005; Nakaya et al., 2004; Takasuka et al., 2002). In this way, influenza viruses have been used as vector expressed malaria-specific CD8^+ T and B cell epitopes which induced robust CD8^+ T cell and antibody responses in intraperitoneally-vaccinated BALB/c mice. For instance, the SYVPSAEQI CD8^+ T cell epitope sequence of \textit{Plasmodium yoelii} was inserted in the NA protein of the influenza virus A/HIQ8/68 (H3N2; HK virus), while the B cell epitope, QGPGAPGPGAP was inserted in the HA protein of the A/WSN/33 virus (WSN virus) (Rodrigues et al., 1994). Another attempt in BALB/c involved intraperitoneally or intranasally infected using the recombinant HIV-1/MN-A/WSN/33 influenza virus (HK-WSN) including a modified HA protein, expressing HIV-Env gp120 peptide (IHIGPGRAFYTT), induced neutralizing antibodies and cytotoxic CD8^+ T cells in the vaccinated mice (Li et al., 1993). A similar study in BALB/c mice primed using recombinant influenza virus with HK-WSN expressing the Env protein on the HA segment. A boosting dose of the recombinant vaccinia virus expressing the same peptide, which induced robust CD8^+ T cells (Gonzalo et al., 1999) was then given. Furthermore, a robust CD8^+ T cell response was induced in macaques following multiple doses of a prime boosting dose of intratracheal and intranasal administration of influenza viruses like X31, (H3N2, A/HKx31) and PR8, (H1N1, A/Puerto Rico/8/1934). Expressed SIV-CD8^+ T cell epitopes, such as SIV Gag146-172, SIV Tat5-7 and SIV Tat114-123 inserted in the manipulated NA gene segment. (Sexton et al., 2009). Also, both X31 and PR8 influenza viruses expressed the CD8^+ T cell Env311 (RGPGRAFVTI) epitope that was also inserted in the NA segment. These were used in BALB/c mice to induce a protective CD8^+ T cell response (Cukalac et al., 2009). The methodology is discussed for the whole procedure used in generating our influenza virus vectors in Chapter 2, Section (2.2.8), and more details about their results are mentioned in Chapter 3, Section (3.1.1).

1.6.3.1. Influenza virus vectors in HIV vaccine candidates

Live recombinant influenza viruses have been used as viable HIV vaccine vectors because of their ability to stimulate immune responses in the respiratory and urogenital tracts to control various mucosal infectious diseases (Holmgren and Czerkinsky, 2005; Johansson et al., 2001). Indeed, recombinant influenza viruses manipulated in this way have been used to express HIV or SIV-CD8^+ T cell epitopes to stimulate cytotoxic T cell responses (Liu et al., 2009; Pachler, Mayr and Vlasak, 2010; Sexton et al., 2009). For example, a recombinant influenza-HIV vaccine (rFlu-p17) involved the PR8 (A/PR/8/34, H1N1) influenza virus expressing the HIV
Gag protein (P17) which was inserted into the NA segment to stimulate both the humoral and CD8\(^+\) T cell responses in prime boost intranasal or intraperitoneal vaccinated BALB/c and C57BL/6 mice. Both the specific p17 neutralizing antibody and the CD8\(^+\) T cell responses were highly induced in the two groups of mice vaccinated with the rFlu-p17 vaccine compared to those groups given the rFlu-Rev vaccine as a negative controls (de Goede et al., 2009). Furthermore, the HIV-1 matrix Gag p17 protein is considered a good vaccine candidate. It regulates most stages of HIV-1 virus replication and reduces HIV-1 pathogenesis by inducing specific CD8\(^+\) T cell and neutralizing antibody responses directed to the HIV-Gag p17 protein (Fiorentini et al., 2010).

Furthermore, both the core/matrix proteins (p24 and p17) and the Env glycoproteins (gp120 and gp41) which are considered the most frequent HIV proteins targeted by high levels of neutralizing antibodies (Robey et al., 1985). These are regarded as the most frequent HIV protein recognized by specific cytotoxic T cells in HIV/AIDS patients (Addo et al., 2003). The HIV-H-2D\(^d\)Env\(_{311}\) epitope has been inserted into the NA stalk of recombinant influenza viruses using reverse genetics and also used in prime boost experiments to stimulate immunity (Cukalac et al., 2009). In this instance, vaccination involved a primary dose using mouse-adapted (A/HKx31), X31-NA-Env\(_{311}\) as the intranasal primary infection in BALB/c mice. This was compared with secondary infection in another group of BALB/c mice primed with a dose of virulent (A/Puerto Rico/8/34, H1N1), PR8-NA-Env\(_{311}\) administered intraperitonealy and then challenged with an intranasal dose of X31-NA-Env\(_{311}\). This vaccination regime induced a robust polyfunctional Env\(_{311}\)^+CD8\(^+\) T cell with IFN-\(\gamma\), IL-2 and a TNF-\(\alpha\) subset of HIV-Env\(_{311}\) CD8\(^+\) T cells detected in the spleen and BAL following secondary infection compared to a lower response detected in primary infected BALB/c mice (Cukalac et al., 2009). Also, recombinant influenza viruses expressing CD8\(^+\) T cell epitopes (SIV Gag\(_{164-172}\)), (SIV Tat\(_{87-96}\)), and (SIV Tat\(_{114-123}\)), inserted separately into the NA stalks of viral vectors were generated for using in the vaccination regimen of macaques (Rollman et al., 2008). These two lab strains of the influenza viruses were also used to induce SIV-mucosal CD8\(^+\) T cell response in macaques. The macaque study included an inoculation of naïve and chronic SIV\(_{mac251}\) infected macaques with a prime dose of initial intranasal X31-SIV, boosted with PR8-SIV, and then challenged intravenously using infective doses of SIV\(_{mac251}\). Data from this study indicated the ability of this vaccination regimen to maintain high levels of SIV mucosal CD8\(^+\) T cells. However, there was no control of viremia in all infected vaccinated macaques (Sexton et al., 2009) because of
the ability of the virus to escape the narrow immune response directed to highly variable SIV-CD8\(^+\) T cell epitopes. Recombinant influenza virus vectors (H1N1 and H3N2) expressing CD8\(^+\) T cell epitopes have also been used previously in mouse model trials as a vector of the HIV vaccine because they are mouse adapted strains of influenza viruses and are serologically distinct containing the same six internal gene segments, and differ in the expression of HA and NA genes. This is important to avoid neutralizing antibodies that inhibit the secondary T cell response (Cukalac et al., 2009).

1.7. Project hypotheses

The project is based on two hypotheses:

1- Vaccination with recombinant influenza viruses expressing HIV epitopes will enhance the specific and mucosal CD8\(^+\) T cell immune response in mice.

2- Adoptive transfer of induced mucosal CD8\(^+\) T cells protected naïve mice against the challenge virus (recombinant Vaccinia virus expressing the whole HIV-Gag protein).

1.8. Project aims

In order to investigate these hypotheses, the followingspecific aims will be explored:

1- To generate live influenza vaccine vectors using a reverse genetics strategy expressing HIV-CD8\(^+\) T cell epitopes.

2- To compare the ability of different mucosal routes of vaccination inducing CD8\(^+\) T cell immune responses.

3- To study both the quality and characteristics of induced mucosal CD8\(^+\) T cell responses.

4- To transfer induced mucosal CD8\(^+\) T cells into naïve mice for protection against the challenge rVac expressing the whole HIV-Gag protein.
2. Chapter 2: Materials and Methods

2.1. Materials

A detailed list of materials is provided in Appendix A.

2.2. Methods

2.2.1. Cloning strategy to generate X31-NA and PR8-NA with HIV-CD8+ T cell epitopes

The 8 plasmid reverse genetics system was used to generate and rescue recombinant influenza viruses, X31 or PR8, expressing a modified NA gene segment inserted with the exogenous CD8+ T cell epitopes, H2Kd Gag197-205 or H-2d Tat17-25 as described by Hoffmann et al., (2000). Generation and rescue of recombinant viruses involved in all viral RNAs and mRNAs were generated through bi-directional transcription and translation from the specific single DNA template (Andreansky et al., 2005). In order to generate these recombinant influenza viruses, nucleotide sequences of H-2Kd Gag197 (AMQMLKETI: 5’-GCC ATG CAA ATGTTA AAA GAG ACC ATC-3’) or of H-2d Tat17 (QPKTACTNC: 5’-CAG CCT AAA ACT GCT TGT ACC AAT TGC-3’) were inserted into the sequence corresponding to position 44-45 of the NA stalk of X31 (H3N2) or PR8 (H1N1) influenza laboratory strains, using two PCR reactions. Standard PCR was performed to generate two overlapping NA fragments inserted with Gag197 or Tat17 epitopes. The first DNA fragment with a 200 base pair (bp) size and the second DNA fragment was with a 1.2 kilobase (Kb) size. In order to form the whole NA fragment inserted with the HIV-CD8+ T cell epitopes, recombinant PCR was performed to combine the two NA fragments using specific forward primers, such as Bm-NA-1 and reverse primers like X31-NA197-R. All primers used are presented in Table 2.1, and PCR reactions conditions are provided in Table 2.2.
## Materials and Methods

### Table 2.1. Nucleotide sequences of primers used for PCR amplification. Shown are forward and reverse primer sequences used in the amplification of modified NA segments expressing HIV CD8+ T cell epitopes (Gag<sup>197-205</sup> and Tat<sup>17-25</sup>).

<table>
<thead>
<tr>
<th>HIV epitope</th>
<th>Primer nucleotide sequences (5’-3’)</th>
<th>Primer name</th>
<th>Range of Nucleotides</th>
</tr>
</thead>
</table>
| Gag<sup>197-205</sup> AMQMLKETI (5’-GCC ATG CAA ATGTAA AAA GAG ACC ATC-3’) into X31-NA. | Forward (short fragment) 5’TAT TCG TCT CAG GGA GCA AAA GCA GGA GT-3’. Reverse (short fragment) 5’-GAT GGT CTC TTT TAA CAT TTT CAT GGC GGA GTC GCA GTC ATATG CTT AAA ATG CAA TG-3’. Forward (large fragment) 5’-GCC ATG CAA ATG TTA AAA GAG ACC ATC CCC GCG AGC AAC CAA GTA ATG CCG TGT GAA-3’. Reverse (large fragment) 5’ATA TCG TCT CGT ATT AGT AGA AAC AAG GAG TTT TTT-3’. | Bm-NA-1- F  
X31-NA<sub>197</sub>  
X31-NA<sub>197</sub> F  
Bm-NA-1413- R | 924-950 |

| Tat<sup>17-25</sup> QPKTACTNC (5’-CAG CCT AAA ACT GCT TGT ACC AAT TGC-3’) into X31-NA. | Forward (short fragment) 5’TAT TCG TCT CAG GGA GCA AAA GCA GGA GT-3’. Reverse (short fragment) 5’-GCA ATT GGT ACA AGC AGT TTT AGG CTG GGA GTC GCA CTC ATA TTG CTT AAA ATG CAA TG-3’. Forward (large fragment) 5’-CAG CCT AAA ACT GCT TGT ACC AAT TGC CCC GCG AGC AAC CAA GTA ATG CCG TGT GAA-3’. Reverse (large fragment) Reverse (large fragment) 5’ATA TCG TCT CGT ATT AGT AGA AAC AAG GAG TTT TTT-3’. | Bm-NA-1-F  
X31-Tat<sub>17</sub>  
X31-Tat<sub>17</sub> F  
Bm-NA-1413- R | 5425-5451 |

| Gag<sup>197-205</sup> AMQMLKETI (5’-GCC ATG CAA ATG TTA AAA GAG ACC ATC-3’) into PR8-NA. | Forward primer (short fragment) 5’TAT TGG TCTCAG CGA GCA AAA GCA GGA GT-3’. Reverse (short fragment) 5’-GAT GGT CTC TTT TAA CAT TTT CAT GGG TTG AAT TGA ATG GCT AAT CC-3’. Forward (large fragment) 5’-GCC ATG CAA ATG TTA AAA GAG ACC ATC AAC CAT ACT GGA ATA TGC AAC AAC ATC-3’. Reverse Primer (large fragment) 5’ATA TGG TCT CGT ATT AGT AGA AAC AAG GAG TTT TTT-3’. | Ba-NA-1- F  
PR8- NA<sub>197</sub>  
PR8- NA<sub>197</sub> F  
Bm-NA-1413- R | 924-950 |

| Tat<sup>17-25</sup> QPKTACTNC (5’-CAG CCT AAA ACT GCT TGT ACC AAT TGC-3’) into PR8-NA | Forward primer (short fragment) 5’TAT TGG TCTCAG CGA GCA AAA GCA GGA GT-3’. Reverse primer (large fragment) 5’-GCA ATT GGT ACA AGC AGT TTT AGG CTG TTG ACT TCC AGT TGG AAT TGA ATG GCT AAT CC-3’. Forward primer (large fragment) 5’-CAG CCT AAA ACT GCT TGT ACC AAT TGCAAC CAT ACT GGA ATA TGC AAC AAC AA ATC-3’. Reverse Primer (large fragment) 5’ATA TGG TCT CGT ATT AGT AGA AAC AAG GAG TTT TTT-3’. | Ba-NA-1-F  
PR8-NA-Tat<sub>17</sub>  
PR8-NA-Tat<sub>17</sub> F  
Bm-NA-1413- R | 5425-5451 |
Table 2.2. Conditions for PCR reactions. A) Typical PCR conditions used in cloning work, B) Conditions for the recombinant PCR reactions to generate the modified NA gene segments.

**A) Standard PCR conditions.**

<table>
<thead>
<tr>
<th>Step of reaction</th>
<th>Temp.</th>
<th>Time</th>
<th>Cycle number</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>94°C</td>
<td>4 min</td>
<td>1</td>
</tr>
<tr>
<td>2</td>
<td>94°C</td>
<td>20 sec</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>57°C</td>
<td>30 sec</td>
<td>30</td>
</tr>
<tr>
<td>4</td>
<td>72°C</td>
<td>2 min</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>95°C</td>
<td>1 min</td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>57°C</td>
<td>1 min</td>
<td>1</td>
</tr>
<tr>
<td>7</td>
<td>72°C</td>
<td>7 min</td>
<td></td>
</tr>
<tr>
<td>8</td>
<td>4°C</td>
<td>Hold</td>
<td></td>
</tr>
</tbody>
</table>

**B) Recombinant PCR conditions.**

<table>
<thead>
<tr>
<th>Step of reaction</th>
<th>Temp.</th>
<th>Time</th>
<th>Cycle number</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>94°C</td>
<td>2 min</td>
<td>2</td>
</tr>
<tr>
<td>2</td>
<td>55°C</td>
<td>30 sec</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>72°C</td>
<td>2 min</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>94°C</td>
<td>30 sec</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>59°C</td>
<td>30 sec</td>
<td>33</td>
</tr>
<tr>
<td>6</td>
<td>72°C</td>
<td>2 min</td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>72°C</td>
<td>7 min</td>
<td>1</td>
</tr>
<tr>
<td>8</td>
<td>4°C</td>
<td>Hold</td>
<td></td>
</tr>
</tbody>
</table>
2.2.2. Gel electrophoresis

PCR products and restriction digests were examined by gel electrophoresis using 0.8% (w/v) Agarose (Analytical grade, Promega) in 1x TAE buffer (Tris-acetate EDTA, Promega) containing SYBR safe DNA gel stain (Invitrogen). DNA samples were mixed with a 6x loading dye (Bromophenol blue, Xylene cyanol, glycerol and water) (see Appendix A), and loaded onto the gel using agarose solution (0.8%) which was electrophoresed at 100 V for 40 min. A 1 Kb-Plus DNA Ladder (Invitrogen) was used as a size marker for estimation of fragment sizes.

2.2.3. DNA gel extraction and purification

Gel slices containing recombinant NA-H-2d Gag197 or NA-Tat17 PCR products were extracted using a QIAquick Gel Extraction Kit (manufacturer) using the manufacturer’s instructions and quantified using gel electrophoresis with pBR322 standards (New England Biolabs) (NEB).

2.2.4. Ligation

Recombinant NA segments containing the H-2d Gag197 or Tat17 epitopes were ligated into plasmid pHW2000 using a ligation mixture including 1 μl of T4 DNA ligase, 20 ng of the digested and purified plasmid pHW2000 treated with CIAP (Alkaline phosphatase, calf intestinal; Promega M182A: 1 u/μl), 19 ng of recombinant NA-Gag197, 2 μl of 10x DNA ligase buffer. The total volume of this mixture was adjusted to 20 μl by adding DNAase free H2O, and then incubated at 16°C O/N in the PCR machine. The ligation products were then examined by gel electrophoresis.

2.2.5. Transformation

Ligation products were transformed into E. coli, JM109 or DH5α competent cells (Life Technologies). Frozen competent cells were removed from storage at -70°C and placed on ice for thawing. 30 μl of the competent cells were then mixed with 10 μl of the DNA ligation mixture. The mixture of DNA and competent cells was incubated on ice for 30 minutes prior to heat shock for 45 seconds at 42°C without shaking. The cells were immediately returned to ice for 2 minutes, and 900 μl of LB broth (RT) was added to each transformation reaction, prior to incubation at 37°C for 1 hour with shaking at 225 rpm. The transformation culture was centrifuged for 5 minutes at 2000 RPM and the supernatant poured off. The cell pellet was then suspended in the residual LB broth medium (~100 μl) and spread onto a LB agar medium containing 100 μg/ml Ampicillin, and incubated O/N at 37°C.
2.2.6. **Plasmid purification**

Single colonies were placed into 2 ml LB broth containing Ampicillin (100 μg/ml) and incubated at 37°C with shaking at approximately 250 RPM for 8 hours. Purification of the recombinant plasmid DNA from these cells was achieved by using the PureYield™ Plasmid Mini Prep-protocol (Promega, USA). For larger cultures, the PureYield™ Plasmid Maxiprep protocol (Promega, USA) was used.

2.2.7. **Restriction analysis**

The purified recombinant plasmid DNA was digested with the specific restriction endonucleases enzymes, PvuI and PstI, to allow for NA cloning (Hoffmann et al., 2002; Webby et al., 2003), using appropriate buffers and temperatures. It was then subjected to electrophoresis.

2.2.8. **Sequencing**

Plasmids containing the inserted HIV-1 epitopes in the NA segment, were prepared for whole sequencing by mixing each with 1 μl template ds DNA (300 ng), 1 μl of 2.5x Big-Dye terminator Premix (v3.1), 3.5 μl of 5x Reaction buffer, 1 μl of Primer 3.2 pmol (5 mM), with the total volume brought up to 20 μl by nuclease free water (Table 2.3). Samples were then sent to the DSR Sequencing laboratory at CSIRO/AAHL for sequencing using the dideoxy chain termination method (Sanger et al., 1980).

2.2.9. **Rescue of recombinant influenza virus by reverse genetics**

2.2.9.1. **MDCK and HEK 293 T co-culture**

Plasmids containing modified NA genes were combined with the 7 plasmids encoding each of the other 7 influenza virus gene segments (pHW2000 PB1, PB2, PA, NP, M, NS and HA) from A/HK/X31 (H3N2) as both influenza laboratory strains (X31 and PR8) contained the same 6 internal influenza viral genes, with different pHW2000 HA plasmids corresponding to the X31 or PR8 influenza lab strains. All 8 plasmids were collectively transfected into HEK 293 T cells and then amplified in MDCK cells (as described in Chapter 3, Section 3.1.2 and shown in Figure 3.11). Two 75 cm² flasks containing confluent MDCK and HEK cells were maintained in a 5% FCS-OPTI-MEM medium. Cells were harvested with 5 ml of (1x) Trypsin-Versene® (Trypsin-EDTA) and incubated at 37°C for 5 min. They were then centrifuged at 1600 rpm for 5 min with 10 ml of DMEM medium for washing added and then re-suspended in 10 ml Easy Flu-medium-Invitrogen (OPTI-MEM-1-Reduced-Serum-medium; GibCO), supplemented with hypoxanthine, thymidine, sodium pyruvate, L-glutamine, trace elements, and growth
factors (but no fetal calf serum, FCS). The MDCK and HEK 293T cells were then mixed in a 1:2 ratio (1 ml of MDCK cells + 2 ml of HEK 293 T cells) in a 50 ml tube to seed a co-culture of cells. 15 ml of Easy Flu medium was then added to 3 ml of the cell mixture and aliquoted into individual wells of a 6-well plate. This was followed by incubation at 37°C, and 10% CO₂ for 18-24 hours to attain confluency.

2.2.9.2. Transfection
Transfections involved mixing 1 μg of each plasmid (NP, NS, PB1, PB2, M, PA, HA and NA-Gag₁⁹⁷ or NA-Tat₁⁷) into a 1.5 ml tube, with 10 μl of each plasmid taken total volume of 80 μl of the mixture of 8 plasmids, followed by incubation at room temperature for 45 min. In a second tube, 3 μl of Fugene 6 (Roche Transfection Reagent) for each 1 μg of plasmid DNA was added (total amount = 24 μl). The total volume of the transfection reagent was brought up to 200 μl by adding 176 μl of the OPTI-MEM medium (no FCS and no antibiotics) per transfection reaction and incubated for 5 min at room temperature. Next, the plasmid solution was added to the (Fugene 6 reagent + Opti-MEM) and incubated at room temperature for 45 min, after which the total volume of the mixture was increased to 1000 μl by adding 720 μl of Opti-MEM. Media was removed from each MDCK/HEK 293 T cell co-culture well and replaced with 1 ml of transfection mixture for 6 hours at 37°C and 10% CO₂. The transfection mix was then carefully removed and replaced with 1 ml of Easy flu medium, followed by incubation of the 6 well plates for 18-30 hours at 37°C and 10% CO₂. To assist replication, 1 ml of the Easy flu medium containing 1 μg of TPCK-trypsin in PBS (Trypsin stock 1μg/μl) was added to the co-culture to give a final volume of 2 ml in each well, with cells incubated for a total time of 72 hours at 37°C followed by collection of virus-containing supernatants.

2.2.9.3. Hemagglutination assay
The presence of the influenza virus in reverse genetics transfection supernatants was detected by hemagglutination activity with the use of chicken red blood cells (RBCs). Firstly, chicken red blood cells (RBCs) were washed twice with PBS and then diluted with PBS to 0.5% packed cell volume (PCV). Two-fold dilutions of the virus were made and chicken RBCs (0.5%) were then added to all wells followed by mixing and incubation for 30 min at room temperature after which agglutination was scored as either positive or negative.
2.2.9.4. Amplification of modified viruses in 10 day old embryonated chicken eggs

Recombinant influenza viruses were amplified in 10 day old fertilized eggs. The transfection supernatants (100 μl) were added directly to 10 day old embryonated chicken eggs and incubated for 2 days in a 35°C incubator. Allantoic fluids were then harvested into 50 ml Falcon tubes on ice and then at -80°C after being aliquoted into small volumes into cryogenic vials (Nunc®CryoTubes®1.8 ml).

2.2.9.5. RNA extraction of harvested allantoic fluids for sequencing

RNA was extracted using the RNeasy Mini Kit (Qiagen) as follows. First, 150 μl of harvested allantoic fluid was mixed well with 500 μl of 70% (v/v) ethanol and then 500 μl of RLT buffer was added. Next, 750 μl aliquots of the mixture were transferred to a purification column, followed by centrifugation for 30 seconds at 1500×g. The eluate was discarded and the previous step repeated with the remaining mixture. 500 μl of the RW1 buffer was then added to a wash column with centrifugation at 1500×g for 30 seconds and the eluate discarded. The column was washed twice using a 500 μl RPE buffer in a similar manner. The column was then dried by centrifugation for 1 min at 1500×g, followed by elution of RNA with 20 μl of nuclease-free water. This eluate was left at room temperature for 1 min to dissolve the extracted RNA and followed by centrifugation for 30 seconds at 1500×g to obtain the purified RNA product.

2.2.9.6. Reverse transcription of RNA to cDNA for NA sequencing

Purified RNA (1 μg) was added to an RNase-free PCR tube in a 10 μl volume. To this, 2 μl of a 10× reverse transcriptase buffer (RT buffer), 2 μl dNTP mix (5 mM each dNTP), 2 μl of 10 μM oligo-dT primer (or BmNA-1 forward primer), 1μl of Omniscript reverse transcriptase, and 3 μl RNA (up to 2μg per 20 μl reaction), were added and mixed well before incubation for 60 min at 37°C. An aliquot of the resultant cDNA (5 μl) was mixed with 10 μl of 1× phusion master mix (PhusionTM Flash High-fidelity PCR master mix, Finnzymes #F-548S), 2 μl of forward and reverse neuraminidase primers (BmNA-1 and BmNA-1413), and 1 μl of nuclease free water. PCR conditions (Table 2.2 B) were used to amplify DNA. The PCR product was electrophoresed on a 0.8% agarose gel, as previously described to check the accurate size of the amplified DNA. After that, the sequencing PCR reaction was performed on the amplified DNA samples using the reaction mixture included in the 1 μl template ds DNA (300 ng), 1 μl of 2.5x Big-Dye terminator Premix (v3.1), 3.5 μl of 5x Reaction buffer, and 1 μl primer 3.2 pmol (5 mM). The total volume was brought up to 20 μl by nuclease free water. The sequencing
reaction mixture followed the reaction cycle conditions (Table 2.3). After this, all samples were sent to the DSR Sequencing laboratory in the CSIRO/AAHL for sequencing.

**Table 2.3.** Conditions of preparation DNA samples for sequencing reaction. This table shows typical cycles of sequencing reaction performed on the amplified DNA samples.

<table>
<thead>
<tr>
<th>Step of reaction</th>
<th>Temperature</th>
<th>Time</th>
<th>Cycle</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>96 °C</td>
<td>1 min</td>
<td>1</td>
</tr>
<tr>
<td>2</td>
<td>96 °C</td>
<td>10 sec</td>
<td>30</td>
</tr>
<tr>
<td>3</td>
<td>50 °C</td>
<td>5 sec</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>60 °C</td>
<td>4 min</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>4 °C</td>
<td>Hold</td>
<td></td>
</tr>
</tbody>
</table>

**2.2.9.7. Plaque assay**

To measure the titer of newly generated recombinant influenza viruses, a plaque assay was performed on Madin-Darby Canine Kidney (MDCK) cell monolayers, using the viral titer of pfu/ml for each virus (Tannock, Paul and Barry, 1984; Thomas et al., 2006). A monolayer of MDCK cells was grown on 6 well tissue culture plates. The MDCK cell growth media was removed and duplicate wells were inoculated with 150 μl of ten-fold serially diluted virus stock (10⁻¹ - 10⁻⁷). The plates were then incubated at 37°C, 5% CO₂ for 45 min with shaking every 15 minutes. A 200 μl aliquot of 0.1% Trypsin Worthington TW (TPCK Trypsin Worthington from Scima-R; Cat. No. #LS003740; (stock 1μg/ml)) was added to 2 × L15 medium (Invitrogen) and mixed with 1.8% agarose solution (A-6013 Agarose, Sigma-Aldrich) to generate the agarose overlay. 3 ml volume of the agarose overlay was added to the infected cell monolayer, followed by incubation at 37°C, with 5% CO₂ for 3 days. Plaques in each well were counted and plaque forming units per ml calculated (Cukalac et al., 2009).

**2.2.10. Vaccination of mice**

Six week old BALB/c mice were purchased from the Animal Resources Centre (ARC) and inoculated with the recombinant influenza vaccine strains following environmental adaptation for 2 weeks. All animal experiments were completed according to protocols approved by the CSIRO/AAHL Animal Ethic Committee.
2.2.10.1. Procedures for intravaginal and intranasal vaccination of mice

The vaccination program followed six different vaccine strategies involving primary and secondary vaccination to stimulate CD8\(^{+}\) T cell responses.

2.2.10.1.1. Primary vaccination

In order to recognize the ability of each of the generated HIV-CD8\(^{+}\) T cell epitopes (H-2\(^{K}\)\(^{d}\) Gag\(_{197}\) and H-2\(^{d}\) Tat\(_{17}\)) to induce a specific HIV-CD8\(^{+}\) T cell response, two groups of BALB/c mice were used for primary infection using influenza virus vectors expressing Gag\(_{197}\) or Tat\(_{17}\) epitopes. The first group of BALB/c mice were primed intranasally with 30 μl (1×10\(^{4}\) pfu) of the recombinant influenza vaccine (X31-NA-Gag\(_{197}\)). Also, there is another group of mice were vaccinated with a primary intranasal infection of 30 μl (1×10\(^{4}\) pfu) recombinant influenza vaccine (X31-NA-Tat\(_{17}\)). Both groups were rested for 10 days following infection and then euthanased to harvest their spleen to detect CD8\(^{+}\) T cell responses.

2.2.10.1.2. Prime boost vaccination

There were four different procedures of prime boost vaccination used in BALB/c mice as follows: i) Intranasal administration of 30 μl (1×10\(^{4}\) pfu) of the recombinant influenza vaccine (X31-NA-Gag\(_{197}\)) as a prime dose, and then after six weeks by intranasal infection with 30μl (50 pfu) of PR8-NA-Gag\(_{197}\); ii) Intravaginal administration of 30 μl (1×10\(^{4}\) pfu) recombinant influenza vaccine (X31-NA-Gag\(_{197}\)) as a prime dose followed 6 weeks later by intravaginal infection with 30μl (50 pfu) of PR8-NA-Gag\(_{197}\); iii) Intranasal administration of 30 μl (1×10\(^{4}\) pfu) of the recombinant influenza vaccine (X31-NA-Gag\(_{197}\)) as a prime dose followed 6 weeks later by intravaginal infection with 30 μl (50 pfu) of PR8-NA-Gag\(_{197}\) and iv) Intravaginal administration of 30 μl (50 pfu) of PR8-NA-Gag\(_{197}\) as a prime dose followed 6 weeks later by intranasal immunization with 30 μl (1×10\(^{4}\) pfu) of the recombinant influenza vaccine (X31-NA-Gag\(_{197}\)). To obtain CD8\(^{+}\) T cell population, all groups of infected mice were euthanased after 7 days of the boost dose of the infection for harvesting different organs, such as the spleen, bronchoalveolar lavage (BAL), mediastinal (MedLN) and inguinal (ILN) lymph nodes.

2.2.10.1.3. Lymphocyte isolation and preparation

After 10 days of primary infection or 7 days of prime boost infection, groups of infected mice were euthanased by CO\(_{2}\) asphyxiation. After that, spleens, inguinal (ILN) and mediastinal (MLN) lymph nodes were harvested from the infected mice. These organs were added to 10 ml of Hank’s buffered salt solution (HBSS) for the preparation of single cell suspensions of lymphocytes. Two frosted slides were used to gently disrupt solid organs (spleen and lymph
nodes) before filtration of cell suspensions through 70 μm cell strainer. Enriched T cells were obtained from the spleen by the panning of B cells using 15 ml anti-mouse IgM + IgG immunoglobulins (5 vials + 200 mL of PBS, H + L; Jackson Immuno-Research Laboratories) in a petri dish for 45 min. The non-adherent cell suspension was harvested and then centrifuged for 6 min at 1600 RPM and re-suspended in 3 ml of HANKS solution. The supernatant was then gently discarded before adding 3 ml of Ammonium Tris-Chloride buffer (see in Appendix A) to each tube and incubating for 5 min at room temperature to lyse RBCs. The reaction was stopped by adding 7 ml of HBSS to each tube before centrifugation for another 6 min. Following removal of the supernatant cells, they were washed twice using 10 ml of HBSS and re-suspended in 3 ml of complete RPMI for intracellular cytokine staining assay preparation and tetramer staining. To obtain T cells from broncho-alveolar lavage (BAL), 3 ml (1x) of harvested BAL samples (included PBS as a diluent) were plated in 6 well plates and incubated for 1 hour at 37ºC to remove adherent cells (macrophages). The non-adherent cells were collected into 10 ml tubes and centrifuged at 1600 rpm for 6 min at 4ºC. The cell pellet was re-suspended in 2 ml of complete RPMI for intracellular cytokine staining assay preparation and tetramer staining. Lymphocytes from all samples were counted using a hemocytometer.

### 2.2.10.2. Phenotype and surface marker staining

T cells were phenotyped using rat-anti-mouse antibodies CD4-FITC (BD-Pharmingen, Clone RM-4.4) and CD8-α-PerCP5.5 (BD-Pharmingen, Clone 53-6.7). A 100 μl aliquot of lymphocyte suspension was added into a 96 well culture plate (BD Falcon) followed by 50μl of an antibody cocktail of rat anti-mouse CD4-FITC (1/400) and CD8-α-PerCP5.5 (1/200) antibodies (Appendix A). This was then incubated on ice for 30 min and the cells washed twice with FACS buffer (Reagent section, Appendix A). Samples were then analyzed on FACS BD-FACS LSR II using BD DIVA software.

### 2.2.10.3. Intracellular cytokine staining

For intracellular cytokine staining, 100 μl of enriched CD8+ T cells was added to a 96 well round bottom plate (Corning® Costar®), followed by the addition of a 100 μl stimulation mixture (Table 2.4). This stimulation mixture of 1 ml included 50 μl of IL-2 (50 U/ml), 2 μl of Golgi-plug (Brefeldin) (10 μg/ml) and 20 μl of the H-2Kd Gag197 or H-2Kd NP147 peptides (2 μg/ml). It was then made up for 1000 μl by adding c-RPMI. After that, mixture of 100 μl CD8+ T cells and 100 μl of stimulation mixture (including Gag197 or NP147 peptide or no-peptide stimulation mix) were incubated for 5 hours at 37ºC with 5% CO2. The plate was then centrifuged at 1600 rpm for 4 min at 4ºC. The supernatant was discarded and cells were washed
twice with a 150 μl FACS buffer. Cells were then stained in a 50 μl volume of FACS buffer that included anti-CD8-α-PerCP5.5 (1/200).

These samples were then incubated on ice for 20 minutes, washed and centrifugation performed at 1600 rpm for 4 min at 4°C. Cells were then re-suspended in 100 μl of Golgi-plug solution to keep the stimulated intracellular cytokines inside cells and not escaping out of cells (see Appendix A). One hundred μl of 0.5% paraformaldehyde was added before incubation for 15 min at room temperature to fix the treated cells without undergoing any changes. The plate was then covered in foil and incubated overnight at 4°C. Following centrifugation, cells were re-suspended in 150 μl of Perm wash solution (10%) and incubated for 20 min at 4°C prior to the addition of a cytokine antibody cocktail (anti-IFNγ-FITC, anti-IL2-PE and anti TNF-α-APC, 1/100, in a 50 μl volume. The plate was incubated on ice for 30 min and the cells washed twice with 150 μl of a 10% Perm Wash buffer. Finally, cells were re-suspended in a final volume of 200 μl FACS buffer for analysis on the BD-FACS LSR II.

**Table 2.4.** Stimulation mixture solutions used in intracellular cytokine staining (ICS). The three types of stimulation mixture are shown, including the H-2K\textsuperscript{d}NP\textsubscript{147}, H-2K\textsuperscript{d}Gag\textsubscript{197} peptide. No peptide stimulation mixture used as a negative control.

<table>
<thead>
<tr>
<th>Item used</th>
<th>NP\textsubscript{147} peptide</th>
<th>Gag\textsubscript{197} peptide</th>
<th>No peptide</th>
</tr>
</thead>
<tbody>
<tr>
<td>Peptide</td>
<td>20 μl</td>
<td>20 μl</td>
<td>-</td>
</tr>
<tr>
<td>rIL-2</td>
<td>50 μl</td>
<td>50 μl</td>
<td>50 μl</td>
</tr>
<tr>
<td>Golgi (Brefeldin)</td>
<td>2 μl</td>
<td>2 μl</td>
<td>2 μl</td>
</tr>
<tr>
<td>C-RPMI</td>
<td>928 μl</td>
<td>928 μl</td>
<td>948 μl</td>
</tr>
<tr>
<td>Total volume</td>
<td>1000 μl</td>
<td>1000 μl</td>
<td>1000 μl</td>
</tr>
</tbody>
</table>
2.2.10.4. Tetramer staining

Tetramers were prepared by complexing MHC-1 molecules with the specific CD8+ T cell receptor, conjugated with specific peptides (glycoprotein H-2K\textsuperscript{d} NP\textsubscript{147} or H-2K\textsuperscript{d} Gag\textsubscript{197}) and then treated with Streptavidin-Phycoerythrin. The Allophycocyanin conjugated H-2K\textsuperscript{d} NP\textsubscript{147} or H-2K\textsuperscript{d} Gag\textsubscript{197} tetramers were kindly synthesized by the Biomolecular Resource Facility, John Curtin School of Medical Research. Aliquots (100 \(\mu\)l) of enriched CD8+ T cells were added to a 96 well round bottom plate, followed by centrifugation at 1600 rpm for 4 min at 4°C. The cell pellet was re-suspended in 50 \(\mu\)l of appropriate tetramer, H-2K\textsuperscript{d} NP\textsubscript{147}-APC (TYQRTRALV) or H-2K\textsuperscript{d} Gag\textsubscript{197}-APC (AMQMLKETI) at 1/100 dilution. The 96 well plate was then covered with foil and incubated at room temperature for 1 hr, followed by washing (two times) with 150 \(\mu\)l of FACS buffer. An antibody cocktail (anti-CD8-PerCP (1/200), anti-CD62L-FITC (1/400), anti-CD44-PE (1/400), anti-LPAM-1-PE (1/400), anti-CD103-FITC (1/400), anti-CD69-PE (1/1400) and anti-CCR9-FITC (1/400) was added in a 50 \(\mu\)l volume using appropriate combinations. The plate of stained cells was incubated for 30 min on ice samples washed with 150 \(\mu\)l of FACS buffer and cells re-suspended in a final volume of 200 \(\mu\)l FACS buffer before FACS analysis (BD-FACS-LSR II).

2.2.10.5. Cell sorting of HIV Gag\textsuperscript{+}LPAM-1\textsuperscript{+}CD8\textsuperscript{+} T cell populations

The total HIV Gag\textsubscript{197}+CD8\textsuperscript{+} T cells were harvested from the spleen, mediastinal and bronchoalveolar lavage from vaccinated BALB/C mice. CD8\textsuperscript{+} T cells (from the spleen and MLN) were enriched as per section (2.2.14.2) and then stained with a H-2K\textsuperscript{d} Gag\textsubscript{197} tetramer for 1 hr at room temperature as described above. Two populations of CD8\textsuperscript{+} T cells were stained with a specific purified H-2K\textsuperscript{d} Gag\textsubscript{197} tetramer. Both were HIV-H-2K\textsuperscript{d} Gag\textsuperscript{+}CD8\textsuperscript{+} T cells but with differentially expressed the mucosal homing integrin, \(\alpha 4\beta 7\)\textsuperscript{+} (LPAM-1\textsuperscript{+}) or \(\alpha 4\beta 7\)\textsuperscript{-} (LPAM-1\textsuperscript{-}). The HIV H-2K\textsuperscript{d} Gag\textsuperscript{+}CD8\textsuperscript{+} T cells were then stained with anti-mouse LPAM-1(\(\alpha 4\beta 7\)) (1/400) and anti-mouse CD8\textalpha PerCP (1/200 dilution) in 2 ml of a sort buffer (0.1% BSA) on ice for 30 min to sort using the sorter (BDFACS Aria\textsuperscript{TM} II). RNAs were extracted from the HIV-Gag\textsubscript{197}\textsuperscript{+}\(\alpha 4\beta 7\)\textsuperscript{-}CD8\textsuperscript{+} T cell or HIV-Gag\textsubscript{197}\textsuperscript{+}\(\alpha 4\beta 7\)\textsuperscript{+}CD8\textsuperscript{+} T cell populations using an RNeasy Mini Kit (Qiagen), as described in the previous Section (2.2.11). RNA was then reverse transcribed to cDNA using a SuperScript kit as previously described (Section 2.2.12). (Superscript III First-Strand Synthesis Supermix).
2.2.10.6. Real time PCR (RT-PCR) for IL-15 and IL-15Rα expression

Real time PCR (RT-PCR) was performed on the corresponding cDNA synthesized from the Gag197+α4β7+ and Gag197+α4β7−CD8+ T cells using the clear optical 96-well plate. This assay used ABI-Taq-Mn MGB primer/probes specific for IL-15 or IL-15Rα supplied with ABI Taqman gene expression assays (Applied Biosystem), the Taqman gene expression assay mix (including probe and primers), (mouse IL-15-FAM (#Mm00434210-ml) (20×) or (#Mm04336046-ml). The mouse IL-15Rα was then combined with the ABI Universal PCR Master Mix of Taqman gene expression (2×) for standard PCR amplification. The Applied Biosystems-StepOne™/Real-Time PCR protocol of duplicate wells for each cDNA sample was used. Firstly, 5 μl of template cDNA synthesized from RNAs were extracted from Gag197+α4β7+ or Gag197+α4β7−CD8+ T cells, and added to each well of a clear optical 96-well plate to 20 μl of the PCR Master-Mix added and mixed gently. After that, a clear film was used to cover the plate and then spin down the plate at 1600 rpm for 1 min to be ready for turning on the iCycler machine. The cycle conditions used were 2 min at 50ºC and 10 min at 95ºC, followed by a denaturation step of 40 cycles, 15 sec at 95ºC, and an annealing step with 60 sec at 95ºC.

All samples were normalized to the house-keeping gene, GAPDH (#Mm99999915-gl) as an internal standard control of genes used in each run to act as a normalizer, and the experiment was repeated three times. Data from the IL-15 cytokine or IL-15Rα expression detected by real-time PCR used StepOne™-Software v2.0. The average CT was calculated for both α4β7+ or α4β7− samples and GAPDH and then the ΔCT (CT, α4β7+ - CT, GAPDH) and ΔCT (CT, α4β7− - CT, GAPDH) were determined. After that, the absolute value of the slope was made up to zero and efficiencies of relative quantification for expressed IL-15 cytokine or IL-15Rα were detected by calculating the average ΔΔCT (ΔC, α4β7+ - ΔCT, α4β7−).

2.2.10.7. Adoptive transfer and challenge of BALB/c mice

Gag197+α4β7+ and Gag197+α4β7− CD8+ T cells were sorted (as described in Section 2.2.17.5) from intranasal-intranasal prime-boosted BALB/c mice. Gag197+α4β7+ or Gag197+α4β7−CD8+ T cells, 5 × 10^4 cells/200 μl were transferred intravenously (tail vein) into two groups of naïve BALB/c mice, in addition to a negative group of mice given PBS only (Mock group) and then rested for 6 days. These mice were then challenged with a pseudo-virus, a recombinant vaccinia
virus expressing the whole HIV Gag protein (rVac-Gag) (see Section 2.2.17.8) to test the protective capacity of these populations.

2.2.10.8. Pseudo-challenge of vaccinated mice with a recombinant Vaccinia virus expressing whole Gag protein.
Six days following the adoptive transfer, BALB/c mice were infected intranasally with $1 \times 10^7$ pfu recombinant vaccinia 633-Gag (rVac-gag) virus expressing the whole HIV-Gag protein. Five days after the pseudo-challenge, lungs and ovaries were harvested from individual mice and placed on ice before complete homogenization with a clean probe for 1 min. Lung homogenates were placed into a 10 ml sealed screw cup tube and centrifuged at 2000 rpm for 7 minutes, with supernatants tested by a plaque assay to determine the titer of rVac-gag in all three groups of infected mice. Both the spleen and mediastinal lymph nodes were harvested from the three infected groups of mice at this time point in order to determine HIV-H-2K\textsuperscript{d} Gag\textsubscript{197}\textsuperscript{+}CD8\textsuperscript{+} T cell responses using an ICS and tetramer staining assay.

2.2.10.9. Statistical analysis
Means and standard deviation (SD) were used to calculate weight change and CD8\textsuperscript{+} T cell immune responses amongst the different groups of mice infected with recombinant influenza-HIV viruses. ANOVA with Tukey’s test were used to make statistical comparisons of immune responses induced in different routes of infection. P Values were considered statistically significant when levels were P<0.05. In addition, the two sided t-test was used as the statistical analysis to compare the two groups of vaccinated animals in some laboratory experiments.
3. Chapter 3: Generation of influenza viruses expressing HIV-CD8$^+$ T cell epitopes

3.1. Overview

This Chapter describes the generation of recombinant influenza viruses that express the H-2K$^d$ HIV Gag$_{197}$ or H-2$^d$ Tat$_{17}$ CD8$^+$ T cell epitopes. Two laboratory strains of mouse-adapted influenza viruses, X31 (H3N2) and PR8 (H1N1), were generated using established reverse genetics systems. Reverse genetics systems is the novel molecular strategy discovered in 1978 to generate newly modified live viruses that belong to various families like Rhabdoviridae, Paramyxoviridae, Filoviridae, Bunyaviridae and Orthomyxoviridae through a modification of their genome resulting from the cloned cDNA (Neumann, Whitt, and Kawaoka, 2002). Reverse genetics systems were first used to modify non-segmented positive stranded RNA viruses by transfecting a full length of the RNA genome into appropriate cells that permitted expression of viral proteins and generation of attenuated vaccine viruses able to induce an immune response to control infectious diseases (Taniguchi, Palmieri and Weissmann, 1978; Racaniello and Baltimore, 1981). In 1994, modified rabies virus was the first example of a non-segmented negative stranded RNA virus that was successfully generated by a reverse genetics strategy (Neumann, Whitt, and Kawaoka, 2002; Schnell, Mebatsion and Conzelmann, 1994). However, this was more difficult with segmented negative stranded RNA viruses as there was a big challenge to generate the segmented negative-sense RNA viruses because of a complication related to treating the segmented RNA genome. In addition, there was also the need to co-express the viral polymerase complex and vRNAs to initiate transfection of viral RNA segments and generate modified viruses (Neumann, Whitt, and Kawaoka, 2002). Fortunately, all of these challenges have been overcome using a plasmid based technique. The first segmented negative stranded RNA virus, generated in 1996, was a member of the Bunyviridae family and included three RNA segments (Bridgen and Elliott, 1996). This was followed by the successful generation of an eight segmented negative stranded RNA virus, the influenza A virus (Neumann, Whitt, and Kawaoka, 2002; Neumann et al., 1999; Fodor et al., 1999).

The plasmid-based technique used to generate the genetically modified recombinant influenza virus contained a total of 12 plasmids, including eight plasmids transfected into eukaryotic cells. The virus also included Vero cells to encode precise copies of eight viral RNA gene segments (PB2, PB1, PA, NP, HA, NA, M and NS) (Zobel, Neumann and Hobom, 1993) combined to the other four expressing plasmids to encode the four viral polymerase complex.
proteins, PB1, PB2, PA and NP necessary for the transcription and replication of newly forming virus (Hoffmann et al., 2000; Neumann et al., 1999). After two years, a more developed reverse genetics technique has been used to generate recombinant genetically engineered influenza viruses entirely from cloned cDNA by the eight plasmids reverse genetics system. This system uses only eight plasmids encoding the eight viral protein segments transfected into eukaryotic cells, such as HEK-293T cells, thus resulting in expression of influenza viral proteins and newly formed virus replication that is mainly used as attenuated viral vaccines (Hoffmann et al., 2000; Neumann et al., 2005). In this Chapter, live influenza virus vectors were generated using a cloned viral genome recombining the eight segments of the virus including modified NA segment in which was inserted with HIV-H-2K\textsuperscript{d} Gag\textsubscript{197} or H-2\textsuperscript{d} Tat\textsubscript{17} CD8\textsuperscript{T} T cell epitopes. This step included the generation of plasmids containing NA segments from the two influenza viruses, X31 (H3N2) and PR8 (H1N1) into HIV-epitopes, H-2K\textsuperscript{d} Gag\textsubscript{197} and H-2\textsuperscript{d} Tat\textsubscript{17}. HIV-CD8\textsuperscript{T} T cell epitopes were amplified by providing all negative sense of the RNA converted from the cloned cDNA, and utilizing specific enzymes, such as RNA polymerase I. The recombinant plasmid combined with the additional seven influenza plasmids were transfected into HEK-293T cells and further amplification of the virus was carried out via MDCK cells to generate the recombinant influenza viruses. Following this, Hemagglutination (HA) assay and sequencing were used to confirm the generated viruses.

3.1.1. Generation of a recombinant X31(H3N2) or PR8 (H1N1) influenza viruses expressing HIV-CD8\textsuperscript{T} T cell epitopes

HIV-H-2K\textsuperscript{d} Gag\textsubscript{197} (AMQMLKETI) and H-2\textsuperscript{d} Tat\textsubscript{17} (QPKTACTNC) CD8\textsuperscript{T} T cell epitopes were cloned into the NA segments of X31 and PR8 viruses after amino acid residue 44 (Figure 3.1). A recombinant PCR based strategy was used to introduce these epitopes. The first set of PCR reactions generated 2 NA fragments (200 bp and 1200 bp) that were then recombined to generate a modified NA gene segment expressing the introduced epitopes as depicted in the cloning strategy (Figure 3.2). The two amplified fragments totaling 1.4 Kb were then recombined using PCR to generate recombinant X31-NA-Gag\textsubscript{197-205}, X31-NA-Tat\textsubscript{17} and PR8-NA-Tat\textsubscript{17} gene segments. These would then be cloned into an established influenza reverse genetics backbone plasmid pHW2000 (Figure 3.2) for use in virus rescue experiments to generate a modified virus expressing exogenous HIV-CD8\textsuperscript{T} T cell epitopes (Figure 3.3).
Figure 3.1. Amino acid sequences of the X31-NA (A) and PR8-NA (B) genes used for the generation of recombinant HIV-influenza vaccine vectors. The position into which the H-2K<sup>d</sup> Gag<sub>197</sub> (AMQMLKETI) or H-2<sup>d</sup> Tat<sub>17</sub> (QPKTACTNC) epitopes were inserted are shown with an asterisk (*).
Chapter 3: Generation of influenza viruses expressing HIV-CD8\(^+\) T cell epitopes

Figure 3.2. Generation of a recombinant neuraminidase (NA) gene segment containing HIV-H-2\(^{Kd}\) Gag\(_{197-205}\) or H-2\(^{d}\) Tat\(_{17-25}\) T cell epitopes. The schematic diagram depicts the stepwise PCR strategy used to generate recombinant reverse genetics plasmids.
**Figure 3.3.** Generation of a recombinant influenza virus expressing HIV-CD8\(^+\) T cell epitopes. This schematic structure shows the full length modified neuraminidase segment of PR8-NA and X31-NA, where HIV-CD8\(^+\) T cell epitopes, H-2K\(^d\) Gag\(_{197}\) and H-2\(^d\) Tat\(_{17}\) were inserted.
Chapter 3: Generation of influenza viruses expressing HIV-CD8+ T cell epitopes

PCR reactions were performed to generate the two fragments noted in Figure 3.2 which expressed the HIV-H-2Kd Gag197 CD8+ T cell epitope. A DNA fragment of band size 200 bp and a second DNA fragment (1.2 Kb) were amplified as predicted with BsmB1 restriction sites (Figure 3.4 A). A second recombinant PCR reaction generated a single recombinant modified NA gene segment (1.4 Kb) with BsmB1 restriction sites at both the 5’ and 3’ ends (Figure 3.4 B). Data shown for the generation of a modified X31-NA-Gag197 gene segment was representative of two other manipulated gene NA segments, X31-NA-Tat17 and PR8-NA-Tat17. The PR8-NA-Gag197 recombinant virus was generated by the Stambas group at the University of Melbourne and was not cloned by the candidate.

3.1.2. Cloning of modified NA plasmids into the pHW2000 reverse genetic plasmid system

Recombinant influenza NA gene segments (X31-NA-Gag197, X31-NA-Tat17, and PR8-NA-Tat17) with BsmB1 restriction sites were then cloned into the pHW2000 plasmid for use in downstream virus rescue experiments. The ligated products were transformed into competent cells to generate new plasmids pHW2000-X31-NA-Gag197, pHW2000-X31-NA-Tat17 and pHW2000-PR8-NA-Tat17. These were extracted and purified using the PureYield™ Plasmid Miniprep system and run on a 0.8% agarose gel (Figure 3.5). Three products were visualised on gel electrophoresis. The largest ligation product (4.4 Kb) contained both the pHW2000 vector (3 Kb) and the recombinant NA with inserted H-2Kd Gag197 sequence (1.4 Kb). To confirm the insertion of the NA segment containing the H-2Kd Gag197 or H-2d Tat17 CD8+ T cell epitopes, the recombinant pHW2000 plasmids were digested with PvuI (which cuts within the vector), and PstI (which cuts within the insert). Clones that contained the appropriate modified NA gene segment generated two products (1.9 Kb and 2.2 Kb), as shown in lane 3, Figure 3.6). Lane 2 shows the undigested plasmid (including different forms of DNA) and lane 4 shows the pHW2000-X31-NA-Gag197 cut with PvuI alone resulting in a single 4.4 Kb band (Figure 3.6). To further confirm insertion of HIV-CD8+ T cell epitopes, sequencing of three plasmids, pHW2000-X31-NA-Gag197, pHW2000-X31-NA-Tat17 and pHW2000-PR8-NA-Tat17 was carried out in full (Figures 3.7, 3.8 and 3.9). We also confirmed the sequence of pHW2000-PR8-NA-Gag197 previously generated by the Stambas laboratory (Figure 3.10).
Figure 3.4. Gel electrophoresis of PCR products used a 0.8% of agarose gel. Panel A shows the predicted PCR products contained the HIV-H-2K\textsuperscript{d} Gag\textsubscript{197} CD8\textsuperscript{+} T cell epitope. Panel B shows the recombined PCR product representing the full length modified NA gene segment expressing H-2K\textsuperscript{d} Gag\textsubscript{197}. Data shown in this figure is representative of other PCR products generated for the rescue of X31 or PR8 influenza viruses expressing HIV-H-2K\textsuperscript{d} Gag\textsubscript{197} or H-2\textsuperscript{d} Tat\textsubscript{17} CD8\textsuperscript{+} T cell epitopes.
Figure 3.5. Gel electrophoresis of ligated PCR products into the pHW2000 reverse genetics plasmid used a 0.8% agarose gel. Lane 1 shows the use of 1 Kb plus a DNA ladder ranging from 100 bp to 12 Kb. Lane 2 shows the ligation reaction with three products. These bands represent the non-ligated plasmid (pHW2000) at 3 Kb and the recombinant modified NA at 1.4 Kb. The ligation band size at 4.4 Kb represents the recombined pHW2000-NA-Gag197 plasmid.
Figure 3.6. Gel electrophoresis of restriction enzyme digestion of the pHW2000-X31-NA-Gag_{197} plasmid using endonucleases PvuI and PstI. Lane 1 contains the 1 Kb plus DNA ladder with band sizes ranging from 100 bp to 12 Kb. Lane 2 shows undigested plasmid and lane 4 shows the pHW2000-X31-NA-Gag_{197} plasmid cut with PvuI alone. The pHW2000-X31-NA-Gag_{197} plasmid was digested with both PvuI and PstI in lane 3. Similar results were shown for the other generated plasmids, pHW2000-X31-NA-Tat_{17} and pHW2000-PR8-NA-Tat_{17}. 

1Kb 2Kb 3Kb 4.4Kb 2.2Kb 1.9Kb

500bp 100bp DNA ladder Undigested product Digested PvuI & PstI Digested PvuI
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Figure 3.7. Nucleotide sequence of X31-NA-Gag. The HIV-H-2Kd Gag197 epitope inserted into the NA gene segment is highlighted in bold letters (AMQMLKETI-protein and GCCATGCAAATATGTCAAAGAGACCACATC-DNA sequence).

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Figure 3.8. Nucleotide sequence of pHW2000-PR8-NA-Tat17. The HIV-H-2d Tat17 epitope inserted into the NA gene segment is highlighted in bold letters (QPKTACTNC-protein and CAGCCTAAAACCTGTTGATCAATTTGC-DNA sequence).
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Figure 3.9. Nucleotide sequence of pHW2000-X31-NA-Tat17. The HIV-H-2d Tat17 epitope inserted into the NA gene segment is highlighted in bold letters (QPKTACTNC-protein and CAGCCTAAAACCTGTGCTTGGACAAATGGACDNA sequence).
Figure 3.10. Nucleotide sequence of pH2000-PR8-NA-Gag197. The HIV H-2K<sup>d</sup> Gag<sup>197</sup> in the NA gene segment is highlighted in bold letters (AMQMLKET1-protein and nucleotides GCCATGCAAATGTAAAAAGAGACCATC-DNA sequence).
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3.1.3. Rescue of recombinant influenza viruses using influenza reverse genetics

Once the sequence of the recombinant plasmids was confirmed, the recombinant influenza viruses containing the modified NA genes were generated using reverse genetics. This technique is summarized in Figure 3.11 and involved the transfection of the eight pHW2000 plasmids (pHW2000-NP, pHW2000-PA, pHW2000-PB1, pHW2000-PB2, pHW2000-M, pHW2000-NS, pHW2000-HA and the pHW2000-NA-Gag197 or pHW2000-NA-Tat17), each containing one of the influenza gene segments into a co-culture of HEK-293T and MDCK cells. Supernatant containing the recombinant virus was then further amplified in 10 day old embryonated chicken eggs (Figure 3.11).

3.1.4. Amplification of generated recombinant influenza viruses

Following reverse genetics, three rescued influenza viruses were further amplified by inoculation of the 10 day old embryonated eggs (Methods, Section 2.2.12) and the supernatant collected. The allantoic fluids containing the amplified viruses were then harvested and kept at -80ºC. All allantoic fluid samples were subjected to a semi-quantitative HA assay to confirm the rescued viruses. HA titers were then determined for each virus. X31-NA-Gag197 was 1/256, PR8-NA-Tat17 was 1/256, and X31-NA-Tat17 was 1/128 (Figure 3.12). Following confirmation of the viral titers, the rescued viruses were determined by plaque assay (see Methods Section 2.2.14). The virus titer for X31-NA-Gag197 was $6.0 \times 10^7$ pfu/ml, X31-NA-Tat17 was $6.7 \times 10^7$ pfu/ml and PR8-NA-Tat17 was $8.2 \times 10^7$ pfu/ml. The titer of the fourth virus already generated in the Stambas laboratory for PR8-NA-Gag197 was $4.5 \times 10^8$ pfu/ml (Table 3.1).

3.1.5. Sequencing of recombinant viruses amplified in allantoic fluid

RNA was extracted from the allantoic fluid and PCR was amplified using NA primers, forward primer, Bm-NA-1-F ([TAT TCG TCT CAG GCA AAA GCA GGA GT-3']) and reverse primer, Bm-NA-1413-R ([ATA TCG TCT CGT ATT AGT AGA AAC AAG GAG TTT TTTTT-3']). This PCR product was run on an agarose gel electrophoresis, excised, purified and sequenced. It was at the expected 1.4 Kb site as previously described in Figure 3.4 B. Sequencing confirmed the NA gene contained our inserted HIV-CD8+ T cell epitopes as described previously in Figure 3.7. The confirmed construct was appropriately labelled X31-NA-Gag197. In addition, the sequencing results confirmed the NA gene segment with inserted H-2d epitopes, Gag197 and Tat17, had been faithfully maintained in the amplification process of all rescued viruses as shown previously when plasmids were sequenced in Figures 3.8, 3.9 and 3.10.
Figure 3.11. Reverse genetics strategy for the rescue of recombinant influenza viruses expressing HIV-CD8+ T cell epitopes. This schematic diagram shows the procedure used to rescue recombinant influenza viruses. The manipulated pHW2000-X31-NA-Gag_{197-205} or PR8-NA-Tat_{17} or X31-NA-Tat_{17} plasmid was co-transfected with 7 other pHW2000 encoding influenza gene segments (pHW2000-PB1, PB2, PA, HA, NP, M and NS) in a co-culture of HEK-293T and MDCK cells (Hoffmann et al., 2000).
Figure 3.12. Hemagglutination assay of three rescued recombinant influenza viruses. Supernatants were harvested from the reverse genetic cultures of three viruses, PR8-NA-Tat17, X31-NA-Gag197 and X31-NA-Tat17. They were diluted two fold in a Hemagglutination assay (HA) in combination with 0.5% chicken RBCs. This figure shows the positive results of hemagglutination applied on a round bottom plate using two serial dilution presented for three rescued viruses (A) anda graph that presents the HA titers of these rescued viruses (B).
Table 3.1. Titration of rescued recombinant influenza viruses by plaque assay. Allantoic fluids harvested following 10 day old embryonated chicken eggs were used in a plaque assay on MDCK cell monolayers to determine viral titers.

<table>
<thead>
<tr>
<th>Recombinant virus</th>
<th>Titer by plaque forming unit (pfu)/ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>X31-NA-Gag$_{197}$</td>
<td>$6.0 \times 10^7$ pfu/ml</td>
</tr>
<tr>
<td>X31-NA-Tat$_{17}$</td>
<td>$6.7 \times 10^7$ pfu/ml</td>
</tr>
<tr>
<td>PR8-NA-Tat$_{17}$</td>
<td>$8.2 \times 10^7$ pfu/ml</td>
</tr>
<tr>
<td>PR8-NA-Gag$_{197}$</td>
<td>$4.5 \times 10^8$ pfu/ml (Stambas group)</td>
</tr>
</tbody>
</table>
3.2. Discussion

As a result of the failure of most traditional vaccines to induce appropriate immune responses for protection against viral diseases, such as HIV/AIDS, new strategies including live virus/bacterial vectors are currently being investigated to use for expressing specific antigens or peptides of infectious viruses or bacteria as an effective vaccine control infection (Dudek, 2006; Fitzgerald et al., 2003; Izumi et al., 2003). These vectors can be generated by a number of methodologies, one of which is reverse genetics. Reverse genetics is a powerful strategy used in a range of different applications such as gene therapy and vaccine development. This involves the manipulation of plasmid DNA in order to create novel modified features in viral or bacterial agents when rescued as whole organisms (Jalilian et al., 2010; Stoff-Khalili, Dall, and Curiel 2006; Tung et al., 2007). In order to generate these vectors, it is necessary to place antigens, polypeptides or epitopes into genes for further manipulation. Given the availability of a mouse model for live vaccination (influenza based strategy), well defined HIV-CD8+ T cell epitopes derived in the H-2d BALB/c mouse model and influenza reverse genetics plasmids can be used to generate recombinant vectors for use in vaccination studies. Influenza virus type A has been considered a strong candidate for live viral vector delivery, given its ability to infect via mucosal surfaces such as the respiratory tract (Marsh and Tannock, 2005; Satterlee, 2008; Smith, 2007). Negative stranded RNA viruses, such as influenza are characterized by their ability to infect humans and laboratory animals through mucosal portals of entry resulting in broad and long term humoral and cellular immunity (Fodor et al., 1999; Mueller et al., 2010; Neumann and Kawaoka, 2001; Yao and Wang, 2008). In addition, influenza viruses are especially amenable for use as recombinant vaccine vectors due to the presence of a segmented genome and the availability of plasmids that allow manipulation and rescue of novel recombinant viruses (Fodor et al., 1999; Palese et al., 1996). As such, influenza viruses have been used as vectors to express antigens from HIV, SIV, tuberculosis, malaria and lympho- cytic-chorio-meningitis virus as a part of the HA or NA gene segments to generate novel vaccines (Jalilian et al., 2010; Rodrigues et al., 1994; Sexton et al., 2009). Influenza A viruses have also been recombinant vaccine vectors that express HIV antigens or CD8+ T cell epitopes that have been inserted into the NA stalk PR8 (H1N1) and X31 (H3N2) (Pleschka et al., 1996). Early work in particular focused on the expression of HIV envelope antigens or epitopes (Kramer, 2007; Letvin, 2009; Zolla-Pazner, 2004).
Various recombinant influenza viruses expressing HIV-CD8+ T cell epitopes have been successfully generated to use as HIV or SIV vaccines. For instance, A/PR/8/34 (H1N1) and A/Aichi/2/1968 (H3N2) were manipulated by a reverse genetics strategy in order to use in a preclinical trial of vaccination in mice or macaques. The result was stimulation of a mucosal CD8+ T cell response. These stimulated mucosal CD8+ T cells were critical to decrease viral copies and control of HIV or the SIV infection depending on the breadth of stimulated CD8+ T cell responses (Casimiro et al., 2005; Hoffmann et al., 2002; Liu et al., 2009; Pachler, Mayr and Vlasak, 2010; Rimmelzwaan et al., 2007). Some preclinical and clinical trials of HIV-1 vaccine candidates have used HIV-1 Env proteins or HIV-Env CD8+ T cell epitopes which stimulated strong humoral and cellular immune responses (Sattentau, 2013; Cukalac et al., 2009; Rerks-Ngarm et al., 2009).

Historically, most HIV-1 candidate vaccines using Env protein/epitopes have been unable to elicit protective responses (Cho et al., 2001; McBurney and Ross, 2008; Plotkin, 2009). This is because of high variability in envelope glycoprotein amino acid sequences amongst different HIV-1 clades (Sreepian et al., 2009; Stamatatos et al., 2009; Zolla-Pazner and Cardozo, 2010). This study has focused on the generation of strong mucosal cytotoxic CD8+ T cell responses, and thus have revolved around the use of HIV-Gag+CD8+ T cell epitopes. Gag is a structural protein necessary for the assembly of newly generated virions during the life cycle of HIV-1 in infected individuals and is highly conserved making it an ideal candidate for CTL based vaccines (Bryant and Ratner, 1990; Currier et al., 2006). Tat epitopes were also incorporated into our vaccine constructs. Tat is also a frequently targeted by cytotoxic CD8+ T cells as a highly conserved regulatory protein with defined CD8+ T cell epitopes (Addo et al., 2001; Gavioli et al., 2008; Gavioli et al., 2004; Scriba et al., 2005).

In this project, three recombinant live influenza viruses expressing HIV-CD8+ T cell epitopes, H-2Kd Gag197 or H-2d Tat17 epitopes have been successfully generated. These two candidate HIV-1 epitopes were inserted individually into the NA stalk of A/Puerto Rico/8/34 (H1N1) or A/Aichi/2/1968 (H3N2) influenza viruses and then rescued using reverse genetics. These virus strains were used as they were adapted in mice. Three rescued influenza viruses, X31-NA-Gag197, X31-NA-Tat17 and PR8-NA-Tat17 were obtained with similar titers tested by HA and plaque assay. However, they were different from the titer of PR8-NA-Gag197 generated previously by the Stambas group. This was probably because the ability of three newly
generated viruses to modify and replicate in allantoic fluids was similar as they were transfected and modified into a similar batch of co-culture. The viruses were also inoculated as supernatant into the same batch of embryonated chicken eggs, which was completely different from the co-culture inoculated with the PR8-NA-Gag\textsubscript{197}. This may also be the result of the different batches of embryonated eggs inoculated with supernatants of these generated viruses compared to the fourth previously generated virus which might be adapted to grow on different types of chicken eggs. There was also a highly significant increase in the affinity of egg-adapted variants of influenza A and B viruses to replicate compared to the non-adapted. This occurred by increased binding of the plasma membranes of the chorio-allantoic cells of the chicken eggs and also by increasing their multiplication (Gambaryan, Robertson and Matrosovich, 1999). It was anticipated that targeting two different epitopes would broaden the overall CD8\textsuperscript{+} T cell response and decrease opportunities for the generation of CTL escape in the future if the virus was further studied in pre-clinical trials in humans or macaques. Given the success of initial studies with X31 and PR8-NA-Gag\textsubscript{197} recombinant viruses, analysis of X31 and PR8-NA-Tat\textsubscript{17} viruses was not taken further but could be explored at a later point in time.

However, there are several limitations that can affect highly efficient influenza reverse genetics systems in HIV vaccine production. For instance, a limited number of mammalian cells are likely to be infected to amplify the eight plasmids to generate influenza viruses (Palache et al., 1999; Halperin et al., 2002; Brühl et al., 2000). Although MDCK and Vero cells are mainly used to produce the recombinant influenza virus, there is no high efficiency to produce a large amount of the virus (Fodor et al., 1999; Nicolson et al., 2005). Also, some modified influenza viruses are unable to grow with high titer which leads to delay in virus production (Neumann et al., 1999; Hoffmann et al., 2002). Another limitation of using HA or NA in the influenza reverse genetics system is due to their small size. For instance, there is space for approximately 10 amino acids in a foreign amino acid sequence to be inserted into the HA molecule (Garcia-Sastre and Palese, 1995; Palese et al., 1997; Staczek et al., 1998). Although the NA stalk tolerates a longer foreign peptide sequence to be introduced into the stalk region, the maximum inserted length would be less than 82 amino acids with high opportunity for poor presentation of these peptides to the immune system. Another study using mice recognized that the neuraminidase (NA) stalk can tolerate as many as 41 amino acid peptide insertions expressed on the rescued influenza virus A/WSN/33 (H1N1), which was well replicated in intranasal infected BALB/c mice (Castrucci and Kawaoka, 1993; Luo, Chung and Palese, 1993).
Furthermore, it was clearly detected that the maximum length of a foreign peptide is 58 amino acids can be introduced into the neuraminidase stalk without the loss of viral function. The peptide was introduced with the H-2\(^d\) LCMV nucleoprotein that expressed on the A/WSN/33 (H1N1) influenza virus and was used intranasally and intraperitoneally in BALB/c mice. This induced CD8\(^+\) T cell protected vaccinated mice for the intracerebral challenge CA 1371 strain of LCMV (Casstrucci et al., 1994). In addition, a limited size of the NA gene segment inserted with efficient amount of the HIV-1 protein was noted with loss of its functionality. This was detected by the insertion of the HIV-1 p17 Gag protein (rFlu-p17) gene that included 1-132 amino acids inserted into the NA segment of the PR8 (A/PR/8/34) influenza virus. Insertion of the amino acids was not possible until complete removal of the start codon of NA and most of the NA coding sequence was excised by MluI and SpeI endonucleases and replaced by the HIV-1 p17 Gag protein, or HIV-1 Rev protein for use as a HIV vaccine in C57BL/6 and BALB/c mice (de Goede et al., 2009; van Baalen et al., 2005). Furthermore, recombinant influenza viruses generated from this transgenic process were replication deficient viruses because they lacked functional NA, despite the inducement of a detectable immunity response as they were depended on the exogenous source of NA (Sigma-Aldrich) added to enhance the replication (De Wit et al., 2004; Rimmelzwaan et al., 2007). As a result, the strategy for this project was to use only a limited number of amino acids as specific epitopes inserted into the NA stalk, which requires knowledge of the MHC haplotype of specific peptides or epitopes to generate a vaccine. An alternative strategy to rescue recombinant influenza viruses expressing full HIV polypeptide sequences is possible. For example, the residue of 137 amino acids from the HIV-1 Nef protein that contains nucleotides 210-618 inserted into the NS1 segment of PR8 (A/PR/8/34: H1N1), or the A/Aichi/1/168 (H3N2) influenza virus that uses an intranasal vaccine in BALB/c mice to induce robust antibodies and CD8\(^+\) T cell responses in the systemic and mucosal compartments, especially in the urogenital tract (Ferko et al., 2001). This problem can be avoided by using the NS gene segment with an inserted whole green fluorescent protein (GFP) containing 125 amino acids in the NS1 only (consisted of 230 amino acids), that maintains the fully functional non-structural proteins of the recombinant influenza viruses (Kittel et al., 2004). This provides an opportunity to introduce large sequences of HIV-1 proteins into the NS segment (Takasuka et al., 2002; Kittel et al., 2005). Therefore, it is highly recommended any future work involve expressing full length HIV proteins using the NS as this would overcome the need to know the MHC haplotypes.
Live influenza vaccines are currently in use and licensed by MedImmune in the USA. Using seasonal vaccines as a backbone or uncommon HA and NA combination will avoid issues with neutralizing antibodies.
4. Chapter 4: Induction of CD8\(^+\) T cell responses using recombinant influenza viruses.

4.1. Overview

Chapter 3 described the generation of recombinant influenza viruses expressing the HIV-H-2\(^d\) CD8\(^+\) T cell epitopes, Gag197 or Tat17. This chapter tests these recombinant vaccines in vivo in a mouse study using different mucosal routes of vaccination in order to elucidate the optimal approach for inducing HIV-specific CD8\(^+\) T cell immune responses in various lymphoid organs and regional lymph nodes distributed in different parts of the body, especially in the urogenital region which would provide a good opportunity to control HIV-1 infection (Garulli, Kawaoka and Castrucci, 2004). For this reason, different mucosal routes were used, specifically intranasal and intravaginal routes. This is because influenza virus naturally infects various mucosa, especially pulmonary mucosa. The intranasal route of vaccination is commonly used to deliver recombinant influenza A viruses in order to generate antigen specific mucosal responses in the spleen and respiratory tract mucosa that are represented by CD8\(^+\) T cells and neutralizing antibody responses targeting endogenous influenza antigens and exogenous HIV-1 peptides (Byron et al., 2011; Takasuka et al., 2002). A study using a modified A/WSN/33 expressing HIV-Env protein (HIV-1 IIIB gp160) in BALB/c mice resulted in a 4 fold increase of CD8\(^+\) T cell responses in the spleen and less frequent but detectable response in the genital lymph nodes (Gherardi et al., 2003).

In contrast the intravaginal route of vaccination is rarely used in women (Johansson et al., 2001). It is not common for the intravaginal route to be used to induce a mucosal immune response following influenza infection in mice given the lack of trypsin like enzymes to assist replication. There are a couple of examples however to note. Intravaginal using the recombinant influenza A virus vector expressing the HIV-Env protein produced strong durable mucosal CD8\(^+\) T cells specific for the influenza H-2K\(^d\) NP\(_{147}\) (TYQRTRALV) and HIV- H-2 D\(^d\) Env (RGPGRAFVTI) peptide in regional vaginal draining lymph nodes, the inguinal lymph nodes and the spleen when compared to intranasally vaccinated mice that produced robust CD8\(^+\) T cell responses in the distant urogenital lymph nodes and the respiratory lymphoid tissues (Garulli, Kawaoka and Castrucci, 2004). In addition recombinant adenovirus (AdC6) expressing HIV-H-2K\(^d\) Gag\(_{197}\) (AMQMLKETI) viruses have been used in BALB/c mice via intranasal and intravaginal, vaccination to induce large numbers of CD8\(^+\) T cells in the spleen,
iliac lymph nodes and e vaginal tract (IELs). Higher vaginal \( \text{Gag}_{197}^{+} \text{CD8}^{+} \) T cell responses were mainly induced in the intravaginayly primed mice (de Souza et al., 2007). The responses induced through intravaginal vaccination have also been shown to be protective following pseudo-challenge (Li et al., 2008).

The work in this chapter extends previous studies through an analysis of HIV-H-2\(^d\) \( \text{CD8}^{+} \) T cell epitopes, \( \text{Gag}_{197} \) or \( \text{Tat}_{17} \). A direct comparison between intranasal and intravaginal vaccination, and a combination of intranasal and intravaginal routes in eliciting \( \text{CD8}^{+} \) T cell responses will be undertaken. To characterize induction of HIV-specific and mucosal immune responses in a mouse model, two laboratory strains of the influenza virus, (X31, H3N2 and PR8, H1N1) were used as vectors expressing \( \text{CD8}^{+} \) T cell epitopes (as described in Chapter 3). CD8\(^+\) T cell responses from different effector sites including the broncho-alveolar lavage (BAL), the spleen, mediastinal lymph nodes (MedLN) and inguinal lymph nodes (ILN) were characterized using intracellular cytokine and tetramer staining in combination with staining for specific mucosal phenotypic markers. Finally, CD8\(^+\) T cells expressing mucosal surface markers were sorted using flow cytometry and RNA extracted to detect expression of cytokine IL-15 and its surface receptor IL-15R\(\alpha\), all of which are known to be involved in the migration of CD8\(^+\) T cells into effector mucosal sites.

### 4.1.1. Characterization of CD8\(^+\) T cell responses following primary infection with recombinant X31-NA-Gag\(197\) influenza virus

To evaluate the immunogenicity of the recombinant viruses and their ability to induce immune responses against the exogenous HIV-H-2\(^d\) \( \text{Gag}_{197} \) epitope and the endogenous influenza virus H2-\( \text{K}^{d} \) NP\(_{147}\) epitope, various mucosal routes (i.n and i.v) of vaccination were tested in mice to determine which route provided robust HIV specific and mucosal immune responses, and whether these recombinant influenza viruses were safe to use in mice. Six to eight week old BALB/c mice purchased from the Animal Resources Centre (ARC) were housed in a conventional animal house for 7 days for environmental adaptation before inoculation with the recombinant influenza virus. All animal experiments were conducted according to protocols approved by the CSIRO AAHL and Deakin University’s Animal Ethics Committees.

BALB/c mice were primed intranasally or intravaginally with \( 1 \times 10^{4} \) pfu X31-NA-Gag\(197\) influenza viruses and their body weight monitored daily for ten days following infection.
Following the first two days of intranasal infection, mice maintained normal weight but started to lose weight gradually on the third day. The weight loss continued into the fourth, fifth and sixth days, dropping approximately (7.8 ± 1.0 %) from their original starting weight. This weight loss was significant and consistent with results in the literature (Cukalac et al., 2009). Mice gradually began regaining weight on day 7 with complete recovery of original weight occurring by day 10, thus indicating clearance of the influenza virus (Figure 4.1A). However, mice in the intravaginal infected group showed no signs of weight loss throughout the monitoring period following primary infection with the recombinant influenza virus, X31-NA-Gag197 (Figure 4.1B). Weight loss for intranasally infected mice was expected as influenza viruses replicate in the respiratory tract due to the presence of tryptase Clara an enzyme necessary for cleavage of hemagglutinin (HA) (Garulli, Kawaoka and Castrucci, 2004).

In contrast, the absence of protease enzyme activity in the vaginal mucosa results in abortive replication (Garulli, Kawaoka and Castrucci, 2004; Böttcher-Friebertshäuser et al., 2010; Zhirmov, Ovcharenko and Bukrinskaya, 1984). This expected lack of replication in the genital tract mucosa translated to a lack of weight loss. However, there was an immune response induced in the inguinal lymph nodes following intravaginal influenza infection due to this abortive infection. This is because influenza antigens were presented by the regional antigen presenting cells to the immune system in these nodes. Further studies such as a quantitative PCR or immuno-histochemistry could be used to determine antigen levels at these sites. Importantly, these observations serve as evidence that there was no cross contamination between the vagina and respiratory tract during experimental infection as there was no influenza specific CD8⁺ T cell response detected in the pulmonary mucosal compartments, such as BAL or Mediastinal lymph nodes following intravaginal vaccination.
Figure 4.1. Weight loss measured in BALB/c mice following primary infection with recombinant X31-NA-Gag197 influenza virus. BALB/c mice (each group n = 5) were infected intranasally (A) or intravaginally (B) with $1 \times 10^4$ pfu of X31-NA-Gag197 influenza virus and were monitored for changes in body weight over a ten day period following infection. Also, change in the actual weight of individual BALB/c mice infected intranasally (C) or intravaginally (D). All data represents the mean ± SD of three experiments, using the Student t-test **p<0.01 and ***p<0.001 compares drop in weight of the infected mice with the second day following infection.
Next, the HIV-Gag\textsuperscript{197} and influenza NP\textsubscript{147} specific CD8\textsuperscript{+} T cell responses were analyzed to determine the efficacy of the newly generated recombinant viruses. As mentioned above two groups of six to eight week old BALB/c mice were infected intranasally or intravaginally with $1 \times 10^4$ pfu/30 $\mu$l recombinant influenza viruses X31-NA-Gag\textsubscript{197}. After 10 days of infection, the spleens of infected mice were harvested to determine responses at the known peak of adaptive immunity (Figure 4.2). A single dose of X31-NA-Gag\textsubscript{197} given intranasally to BALB/c mice induced a detectable IFN-\(\gamma\)^+CD8\textsuperscript{+} T cell response against HIV-Gag\textsubscript{197} peptide using ICS of the harvested spleen. We also saw a co-dominant influenza NP\textsubscript{147}-specific response (2.56 $\times$ 10\textsuperscript{5} $\pm$ 2.29 $\times$ 10\textsuperscript{4}) IFN-\(\gamma\)^+CD8\textsuperscript{+} T cells for influenza NP\textsubscript{147} compared to (2.32 $\times$ 10\textsuperscript{5} $\pm$ 8.77 $\times$ 10\textsuperscript{3}) IFN-\(\gamma\)^+CD8\textsuperscript{+} T cells for Gag\textsubscript{197} (Figure 4.3).

This is unusual for exogenous CD8\textsuperscript{+} T cell responses induced using a live vaccine vector, where responses are often directed towards the vector. An example of this is the Step/Phambili HIV trial vaccine that used a recombinant adenovirus (rAd5) to express multiple HIV-1 proteins, Gag/Pol/Nef or Env. These epitopes induced both CD8\textsuperscript{+} and CD4\textsuperscript{+} T cell responses in vaccinated groups of homosexual men who were at risk of HIV infection but the majority of the immune responses were directed against the Ad5 vector (Koup and Douek, 2011; Koup et al., 2010). In addition our results are similar to those observed by Cukalac and colleagues who found co-dominance of K\textsuperscript{d} NP\textsubscript{147} and exogenous HIV-D\textsuperscript{d} Env\textsubscript{311} in the spleen and BAL of BALB/c mice following a single intranasal dose of the X31-NA-Env\textsubscript{311} influenza virus (Cukalac et al., 2009). We were unable to detect IFN-\(\gamma\)^+CD8\textsuperscript{+} T cell responses against HIV-H-2\textsuperscript{d} Tat\textsubscript{17} following primary intranasal infection of mice using the X31-NA-Tat\textsubscript{17} virus (data not shown) and so all subsequent experiments have focused on the HIV Gag\textsubscript{197} CD8\textsuperscript{+} T cell epitope. We believe this may have been a processing issue but given time constraints we were unable to analyse this further.

We further analysed the quality of the CD8\textsuperscript{+} T cell responses by analysing the proportion of TNF-\(\alpha\)^+IFN-\(\gamma\)^+CD8\textsuperscript{+} and IL-2^+IFN-\(\gamma\)^+CD8\textsuperscript{+} T cells for the endogenous H-2K\textsuperscript{d} NP\textsubscript{147} and the exogenous H-2K\textsuperscript{d} Gag\textsubscript{197} epitopes as described previously (La Gruta, Turner and Doherty, 2004). No significant difference in TNF-\(\alpha\)^+IFN-\(\gamma\)^+CD8\textsuperscript{+} T cells specific for H-2K\textsuperscript{d} NP\textsubscript{147}^+ (74.8 $\pm$ 3.0331\%) and H-2K\textsuperscript{d} Gag\textsubscript{197}^+ (72 $\pm$ 3.4641\%), (p<0.210) responses were observed. This was
also the case for IL-2\(^+\)TNF-\(\alpha\)IFN-\(\gamma\)CD8\(^+\)T cells were the H-2K\(^d\) NP\(_{147}\) epitope (33.2 ± 3.4205\%) and H-2K\(^d\) Gag\(_{197}\) epitope (30.8 ± 3.9623\%) were equivalent (Figure 4.4).
Figure 4.2. Cytokine staining profiles for CD8+ T cells during a primary response following intranasal influenza virus infection in BALB/c mice. After panning of B cells, FACS dot plots depicting intracellular cytokine staining for CD8+ T cell responses were gated to firstly identify T lymphocytes (A), CD8+ T cells (B) and then IFN-γ+CD8+ T cells specific for HIV-H-2K\textsuperscript{d} Gag\textsubscript{197} (C), influenza H-2K\textsuperscript{d} NP\textsubscript{147} (D) and No peptide negative control (E). Lymphocytes were obtained from the spleens of BALB/c mice (n = 5 mice) at 10 days following primary intranasal influenza infection.
Figure 4.3. Intracellular Cytokine staining assay used for comparison of functional IFN-\(\gamma^+\) CD8\(^+\) T cells responses targeting the influenza K\(^d\) NP\(_{147}\) and HIV-K\(^d\) Gag\(_{197}\) epitopes. Naïve BALB/c mice (number of mice used for each group (n = 5)) were intranasally infected with the HK-X31-NA-Gag\(_{197}\) influenza virus and lymphocytes obtained from spleens and stimulated with the H-2K\(^d\) Gag\(_{197}\) or NP\(_{147}\) peptide. The data shown was statistically analyzed to compare the responses between the Gag\(_{197}\) and NP\(_{147}\) IFN-\(\gamma^+\)CD8\(^+\)T cell response using the Student t-test. All data represents the mean ± SD representative of three experiments, p > 0.05 is non-significant (ns).
Figure 4.4. Quality of antigen-specific CD8+ T cell subsets detected by cytokine staining assay (ICS). Naïve BALB/c mice (number of mice used for each group (n = 5)) were intranasally infected with the HK-X31-NA-Gag197 influenza virus and lymphocytes obtained from spleens and stimulated with the H-2Kd Gag197 or NP147 peptide. The proportion of TNF-α+IFN-γ+CD8+ (A) and IL-2+TNF-α+IFN-γ+CD8+ T cells (B) are depicted. All data represents the mean ± SD of three experiments, using the Student t-test *p<0.05 and **p<0.01.
4.1.2. Comparison of CD8+ T cell responses following various prime-boost vaccination regimes detected by ICS.

In order to compare different mucosal routes for prime-boost infection in BALB/c mice, lymphocytes harvested from spleen, bronchoalveolar lavage, respiratory draining lymph nodes (mediastinal) and genital draining lymph nodes (inguinal) lymph nodes of various mucosal routes of immunized BALB/c mice were assayed for production of cytokines, such as IFN-γ, TNF-α and IL-2 specific for HIV-Gag197-205 and influenza-NP147-155 peptides.

Four groups of BALB/c mice were administered different prime-boost vaccination combinations. Mice were primed intranasally using $1 \times 10^4$ pfu/mouse of X31-NA-Gag197. This was followed six weeks later with an intranasal boost using 50 pfu of PR8-NA-Gag197. PR8-NA-Gag197 and X31-NA-Gag197 are serologically distinct and so neutralizing antibodies are avoided allowing boosting of CD8+ T cell responses. The second vaccination group followed the same regimen but viruses were administered via an intravaginal-intravaginal route. The intent here was to prime immunity in the regional draining node at a site of known viral entry. The third vaccination group used a intranasal-intravaginal route and the fourth vaccination group a intravaginal-intranasal route. Seven days after boosting, lymphocytes were harvested from the spleen, broncho-alveolar lavage, respiratory draining lymph nodes (mediastinal, MedLN) and genital draining lymph nodes (inguinal, ILN). These were analyzed for CD8+ T cell ex vivo responses, particularly for the production of cytokines, such as IFN-γ, TNF-α and IL-2 in response to the HIV-Gag197 and influenza-NP147 peptides. These polyfunctional cells are present in non-progressive HIV-infected individuals compared to those people characterized with progressive HIV infection (Betts, et al., 2006; de Keersmaecker et al., 2012).

Our results demonstrated the ability of various mucosal routes of vaccination to induce CD8+ IFN-γ+ T cells in different lymphoid tissues that are directed against the exogenous HIV-H-2Kd Gag197 and endogenous influenza H-2Kd NP147 peptides in BALB/c mice (Figure 4.5). In addition, it was clearly demonstrated that an intranasal-intranasal prime boost vaccination strategy with recombinant influenza viruses expressing HIVGag197 induced a significantly higher proportion of IFN-γ+CD8+ T cells in the spleen, BAL and MedLN ($p< 0.001$) compared to other vaccination strategies like intravaginal-intravaginal, intranasal-intravaginal or
intravaginal-intranasal). In contrast, in ILN the highest proportion of IFN-γ⁺CD8⁺ T cell responses were detected in the intravaginal-intravaginal prime boost vaccinated group of mice (Figure 4.5).
Figure 4.5. Representative dot plots depicting cytokine production for CD8$^+$ T cells specific for HIV-H-2K$^d$Gag$_{197}$ following prime-boost vaccination by various mucosal routes in BALB/c mice. ICS was used to detect cytokine production in the spleen (A), MedLN (B), BAL (C) and ILN (D).
Overall our data shows a significant increase in HIV-Gag\textsubscript{197}+IFN-\textgamma+CD8\textsuperscript{+} T cells following intranasal-intranasal prime boost vaccination in the spleen, BAL and MedLN (Figure 4.6). In total, $(9.51 \times 10^5 \pm 1.12 \times 10^5)$ cells were detected following i.n/i.n prime boost infection in the spleen, compared to $(1.49 \times 10^5 \pm 1.31 \times 10^4)$ for i.v/i.v, $(4.58 \times 10^5 \pm 4.41 \times 10^4)$ for i.v/i.n and $(1.16 \times 10^5 \pm 3.87 \times 10^4)$ cells for i.n/i.v (Figure 4.6A). Similar trends were observed in the BAL (Figure 4.6B) and MedLN (Figure 4.6C). This can be explained by the fact that influenza viruses replicate in the lung and respiratory system due to the presence of a protease required for the cleavage of haemagglutinin. The higher levels viral antigen are likely to drive increased CD8\textsuperscript{+} T cell responses (Garulli, Kawaoka and Castrucci, 2004; Lu et al., 2012, Lu et al., 2013). However, results were different in the ILN as the intravaginal-intravaginal prime boost strategy induced the largest increase in HIVGag\textsubscript{197} specific IFN-\textgamma+CD8\textsuperscript{+} T cells given it is the draining lymph node of the genital tract (Figure 4.6 D). Moreover, the intravaginal-intravaginal route induced responses in the spleen and ILN while there were no obvious antigen specific CD8\textsuperscript{+} T cells detected in either the BAL or MedLN of the respiratory compartments.

The secondary NP\textsubscript{147}+IFN-\textgamma+CD8\textsuperscript{+} T cell immune response following intranasal-intranasal prime boost vaccination elicited a higher response in the spleen, BAL and MedLN compared to the other routes of mucosal vaccination. In the spleen $(9.31 \times 10^5 \pm 1.86 \times 10^5)$ IFN-\textgamma+CD8\textsuperscript{+} T cells were detected following i.n/i.n prime boost infection, compared to the $(1.44 \times 10^5 \pm 2.21 \times 10^4)$ cells for i.v/i.v, $(4.17 \times 10^5 \pm 6.64 \times 10^4)$ cells for i.v/i.n and $(1.27 \times 10^5 \pm 4.99 \times 10^4)$ cells for i.n/i.v (Figure 4.7A). Similar trends were observed for the BAL and MedLN. Intravaginal-intravaginal prime boost vaccination again led to the greatest increase in NP\textsubscript{147}+ CD8\textsuperscript{+} T cells in the ILN, the draining lymph node of the urogenital tract (Figure 7D). Moreover H-2K\textsuperscript{d} Gag\textsubscript{197} and NP\textsubscript{147} CD8\textsuperscript{+} T cell responses were co-dominant following i.n/i.n, i.v/i.v, iv/i.n and i.n/i.v prime boost vaccination in spleen, BAL and MedLN (Figure 4.6 and Figure 4.7).
Figure 4.6. Number of HIV-H-2K\textsuperscript{d} Gag\textsubscript{197} IFN-\(\gamma\)\textsuperscript{+}CD8\textsuperscript{+} T cells in different organs following different routes of vaccination in BALB/c mice. Four groups of BALB/c mice were infected i.n/i.n, i.n/i.v, i.v/i.v and i.v/i.n with X31-NA-Gag\textsubscript{197} and PR8-NA-Gag\textsubscript{197} influenza viruses and ICS use to determine the number of HIV-Gag\textsubscript{197}\textsuperscript{+}CD8\textsuperscript{+} T cells in the spleen (A), BAL (B) MedLN (C) and ILN (D). Lymphocyte populations were harvested and stimulated for five hours with 1\(\mu\)M HIV-H-2K\textsuperscript{d} Gag\textsubscript{197} peptide before staining with anti-mouse CD8\(\alpha\) and anti-IFN-\(\gamma\) antibodies. Data were normalized using a no-peptide control. Data represent mean ± SD from three repeated experiments, and statistical differences were determined using a one way of variance (ANOVA) and Tukey's multiple comparison test, ***p<0.001.
Figure 4.7. Number of H-2KdNP147+IFN-γ+CD8+ T cells in different organs following different routes of vaccination in BALB/c mice. Four groups of BALB/c mice were infected i.n/i.n, i.n/i.v, i.v/i.v and i.v/i.n prime boost infection with X31-NA-Gag197 and PR8-NA-Gag197 influenza viruses and ICS use to determine the number of NP147+CD8+ T cells responses in the spleen (A), BAL (B) MedLN (C) and ILN (D). Lymphocyte populations were harvested and treated for five hours with 1μM influenza H-2Kd NP147 peptide before staining with anti-mouse CD8α and anti-IFN-γ antibodies. Data represents the mean ± SD from three repeated experiments, and statistical differences determined using a one way of variance (ANOVA) and Tukey's multiple comparison test, **p<0.01 and ***p<0.001.
Further analyses focused on poly-functional subsets of the antigen specific CD8+ T cells (Figure 4.8). There was no significant difference in the proportion of TNF-α+IFN-γ+CD8+ T cells for NP147+ (64 ± 3.8%) and Gag197+ (62 ± 2.8%) (p<0.373) populations induced following intranasal-intranasal vaccination. The i.n-i.n prime boost vaccination induced the highest proportion of TNF-α+IFN-γ+CD8+ T cell compared to other routes of vaccination (p<0.001) (Figure 4.9). Similar results and trends were observed for the IL-2+TNF-α+IFN-γ+CD8+ T cell subsets following intranasal-intranasal prime boost vaccination for NP147 (15.8 ± 1.9%) and Gag197 (13.2 ± 2.7%) antigen specific CD8+ T cells in the spleen (Figure 4.10).

**Figure 4.8.** Cytokine profiles of poly-functional CD8+ T cells. Schematic figure shows three subsets of CD8+ T cells including IFN-γ+CD8+ T cells (A), IFN-γ+ and TNF-α+ CD8+ T cells (B), and CD8+ T cells producing IL-2, TNF-α and IFN-γ (C).
Figure 4.9. Proportion of TNF-α⁺IFN-γ⁺CD8⁺ T cells in the spleen detected by ICS following different routes of vaccination in BALB/c mice. This figure compares HIV-H-2K\(^d\) Gag\(_{197}\) (A) and influenza H-2K\(^d\) NP\(_{147}\) (B) CD8⁺ T cell responses. Data represents the mean ± SD from three individual experiments, and statistical significance was determined using the one way of variance (ANOVA) and Tukey's multiple comparison test, ***p<0.001.
Figure 4.10. Proportion of IL-2$^+$TNF-$\alpha^+$IFN-$\gamma^+$CD8$^+$ T cells in the spleen detected by ICS following different routes of vaccination in BALB/c mice. This figure compares HIV-H-2K$^d$ Gag$_{197}$ (A) and influenza H-2K$^d$ NP$_{147}$ (B) CD8$^+$ T cell responses. Data represents the mean $\pm$ SD from three individual experiments, and the statistical significance was determined using the one way of variance (ANOVA) and Tukey's multiple comparison test, ***p<0.001.
4.1.3. Induction of HIV-specific CD8+ T cells detected by tetramer staining assay

Previous mice studies have demonstrated that the number of antigen specific CD8+ T cells producing IFN-γ in an ICS assay are comparable to the number of antigen specific tetramer+CD8+ cells detected following primary and secondary influenza A virus infection (Flynn et al., 1998). To confirm this, the number of tetramer+CD8+ T cells induced against the HIV-H2-Kd Gag197 epitope (Figure 4.11) and H-2Kd NP147 epitope (Figure 4.12) were analysed. Harvested CD8+ T cells from the spleen, BAL, MedLN and ILN of prime-boost vaccinated BALB/c mice were stained with H-2Kd Gag197 and NP147 labelled tetramers. No significant difference was detected between the total number of IFN-γ+ and tetramer+ CD8+ T cells in the spleen following i.n/i.n, i.v/i.v, iv/i.n and i.n/i.v prime boost vaccination for both antigen-specific cell populations in all tissues tested.

The highest numbers of tetramer+CD8+ T cells were induced following i.n/i.n prime boost vaccination for HIV Gag197 (Figure 4.11), and influenza NP147 (Figure 12) in the spleen, BAL and MedLN as per results obtained using intracellular cytokine staining. In the ILN the highest tetramer response was detected following intravaginal-intravaginal prime boost vaccination (Figure 4.11D and Figure 4.12D). HIV-H-2Kd Gag197+ and NP147+CD8+ T cell responses were detected in the urogenital draining lymph nodes following intranasal infection. Previous studies have shown that CD8+ T cell responses can be induced in the vaginal regional lymph nodes of mice infected intranasally with recombinant influenza A virus vectors expressing the HIV-Env311 peptide (Garulli, Kawaoka and Castrucci 2004; Lu et al., 2012). Moreover, an increase of HIV-H-2Kd Nef (EWRFDSRLAFHHVAREL) and H-2Kd NP147 (TYQRTRALV) specific CD8+ T cell responses have been detected in the urogenital draining lymph nodes, in addition to the spleen and respiratory regional lymph nodes, of mice infected intranasally with live recombinant influenza A viruses expressing the Nef protein (Ferko et al., 2001).
Figure 4.11. Number of H-2K^d Gag_{197} tetramer^+CD8^+ T cells in different tissues following different routes of vaccination in BALB/C mice. Four groups of BALB/c mice (each group n=5) were infected with 1 \times 10^4 pfu X31-NA-Gag_{197} influenza virus and then challenged after six weeks with 50 pfu PR8-NA-Gag_{197} influenza virus. Seven days following the final boost, the spleen (A), BAL (B), MedLN (C) and ILN (D) lymphocyte populations were sampled for analysis of tetramer^+CD8^+ T cells. Data shows a representative example of 3 experiments and indicates the mean ± SD. Statistical significance was ascertained using the one way of variance (ANOVA), Tukey's multiple comparison test, ***p<0.001.
Figure 4.12. Number of H-2K<sup>d</sup> NP<sub>147</sub> tetramer<sup>+</sup>CD8<sup>+</sup> T cells in different tissues following different routes of vaccination in BALB/c mice. Four groups of BALB/c mice (each group n=5) were infected with 1 × 10<sup>4</sup> pfu X31-NA-Gag<sub>197</sub> influenza virus and then challenged after six weeks with 50 pfu PR8-NA-Gag<sub>197</sub> influenza virus. Seven days following the final boost, the spleen (A), BAL (B), MedLN (C) and ILN (D) lymphocyte populations were sampled for analysis of tetramer<sup>+</sup>CD8<sup>+</sup> T cells using i.n-i.n, i.v-i.v, i.v-i.n, i.n-i.v and other vaccination strategies. Data shows a representative example of 3 experiments and indicates the mean ± SD. Statistical significance was ascertained using the one way of variance (ANOVA), Tukey's multiple comparison test, ***p<0.001.
4.1.4. Detection of mucosal surface markers (LPAM-1; α4β7 and CD103; αEβ7) and other markers, CD44, CD62L, CCR9 and CD69 on antigen specific CD8+ T cells using flow cytometry

In order to further characterize antigen-specific CD8+ T cells in vaccinated mice, cells were examined for the expression of homing CD8+ T cell integrins, LPAM-1(α4β7) and CD103 (αEβ7) which are considered important for the trafficking of mucosal lymphocytes into lymphoid tissues. These mucosal markers were detected using species-specific anti-mouse mucosal integrin antibodies, and the gating used is shown in Figure 4.13. Cells were first gated on total lymphocytes, then on the CD8+ T cell subset before identification of the HIV-Gag197+CD8+T cell population using tetramer staining. Cells were then analyzed for expression of the mucosal markers LPAM-1 and CD103 in addition to other markers, such as CD44, CD62L, CCR9 and CD69 in samples derived from the spleen, MedLN, ILN and BAL. The intranasal-intranasal prime boost vaccination strategy produced the highest proportion of α4β7'HIVGag+CD8+T cells following various routes of vaccination in the spleen, BAL and MedLN (Figure 4.14A, 4.14B and 4.14C). The intravaginal-intravaginal prime boost strategy induced the highest proportion of α4β7'HIVGag+CD8+ T cells in the ILN (Figure 4.14D). The levels of CD103+HIVGag+CD8+ T cells were similar to those observed for α4β7+ in the spleen, BAL and to a lesser extent the MedLN (Figure 4.16A, 4.16B and 4.16C). However, there was little correlation between the two integrins for the ILN (Figure 4.16D). Moreover similar patterns LPAM-1+ and CD103+ expression on CD8+ T cells were observed for influenza-specific NP147 CD8+ T cells (Figure 4.15 and 4.17).
Figure 4.13. Gating strategy used to analyze mucosal α4β7+Gag+tetramer+CD8+ T cells. Isolated cells were first gated for total lymphocytes (A) followed by CD8+ T cells using an anti-mouse CD8α-PercP antibody (B). HIV-specific CD8+ T cells were gated on the basis of binding to a specific H-2K\(^d\) Gag\(^{197-205}\)-APC tetramer (C). Cells were then interrogated for their expression of the mucosal marker α4β7+ using anti-mouse LPAM-1 (α4β7)-PE antibody (D).
Figure 4.14. Proportion of HIV-Gag<sub>197</sub>α4β7<sup>+</sup>CD8<sup>+</sup> T cells detected in tissues following different routes of mucosal vaccination in BALB/c mice. T lymphocytes were obtained from different lymphoid organs and then stained with H-2K<sup>d</sup> Gag<sub>197</sub>-APC tetramer, anti-mouse CD8α PerCP and anti-α4β7 (LPAM-1)-PE antibodies. α4β7<sup>+</sup> Gag<sub>197</sub><sup>+</sup>CD8<sup>+</sup> T cell numbers were determined (as described in Figure 4.13) with data representative of three repeated experiments. Data is shown as mean ± SD and significance determined using a one way of variance (ANOVA) and Tukey's multiple comparison test, ***p<0.001.
Figure 4.15. Proportion of influenza NP\textsubscript{147} α\textsubscript{4}β\textsubscript{7} CD8\textsuperscript{+} T cells detected in tissues following different routes of mucosal vaccination in BALB/c mice. T lymphocytes were obtained from different lymphoid organs and then stained with H-2K\textsuperscript{d}NP\textsubscript{147}-APC tetramer, anti-mouse CD8\textalpha PerCP and anti-α\textsubscript{4}β\textsubscript{7} (LPAM-1)-PE antibodies. α\textsubscript{4}β\textsubscript{7}NP\textsubscript{147} CD8\textsuperscript{+} T cell numbers were determined (as described in Figure 4.13) with data representative of three repeated experiments. Data is shown as mean ± SD and significance determined using a one way of variance (ANOVA) and Tukey's multiple comparison test, *p<0.05 and **p<0.001.
Figure 4.16. Proportion of CD103⁺HIV-H-2KdGag₁₉₇⁺CD8⁺ T cells detected in tissues following different routes of mucosal vaccination in BALB/c mice. T lymphocytes were obtained from different lymphoid organs and then stained with H-2Kd Gag₁₉₇-APC tetramer, anti-mouse CD8α PerCP and anti-CD103-FITC antibodies. CD103⁺CD8⁺ T cell numbers were determined (as described in Figure 4.13) with data representative of three repeated experiments. Data is shown as mean ± SD and significance determined using a one way of variance (ANOVA) and Tukey's multiple comparison test; ***p<0.001.
Figure 4.17. Proportion of CD103^+NP147^+CD8^+ T cells detected in tissues following different routes of mucosal vaccination in BALB/c mice. T lymphocytes were obtained from different lymphoid organs and then stained with H-2K^d NP\textsubscript{147}-APC tetramer, anti-mouse CD8\textalpha PerCP and anti-CD103 FITC antibodies. CD103^+NP\textsubscript{147}^+CD8^+ T cell numbers were determined (as described in Figure 4.13) with data representative of three repeated experiments. Data is shown as mean ± SD and significance determined using a one way of variance (ANOVA) and Tukey's multiple comparison test, ***p<0.001.
Following the analysis of mucosal markers, we checked the level of expression of as the marker of antigen experience, CD44, and the marker for effector function/memory CD62L, in the spleen, BAL, MedLN and ILN harvested from all groups of vaccinated mice. In general, CD44 and CD62L were differentially expressed on the CD8+ T cell population in naïve, acute effector or memory cells as has been previously described by Kedzierska and colleagues (2007) and also by Kaufman and colleagues, (2008). CD44CD62L−CD8+ T cells were isolated from different lymphoid tissues, such as Peyer’s patches, inguinal, mesenteric, peripheral lymph nodes and the spleen in naïve mice, while effector CD44+CD62L−CD8+ T cells were obtained following vaccination of mice with recombinant adenovirus expressing the SIV-Gag′CD8+ T cell epitope (Kaufman et al., 2008). Furthermore, in response to a novel vaccine containing recombinant Bacillus subtilis or Listeria monocytogenes as a vector expressing HIV-Gag in mice, vaginal CD62L+CD44+CD8+ memory CD8+ T cells were identified five weeks after vaccination (Li et al., 2008).

Following our vaccination regimen, we identified the highest proportion of HIV-Gag197+CD44+CD8+ T cells and influenza NP147+CD44+CD8+ T cells following intranasal-intranasal prime boost vaccination in the spleen, MedLN and BAL. In the ILN the highest proportion of antigen experienced cells was found after intravaginal-intravaginal vaccination (Figures 4.18 and 4.19). As expected after acute infection only a small proportion of antigen specific CD8+ T cells cells were CD62L+(Figures 4.20 and 4.21).

Two final cell surface markers where characterized following vaccination. These included the T cell activation marker CD69 and the T cell migration marker CCR9 (Norment et al., 2000; Kathuria et al., 2012). Previous studies have shown that expression of CCR9 and α4β7 on CD8+ T cells was a characteristic of homing/mucosal T cells (Mavigner et al., 2012). Intranasal-intranasal vaccination induced the highest proportion of CD69+ antigen specific CD8+ T cells (Figures 4.22 and 4.23) whilst differences in CCR9 expression on antigen specific cells were only detected in the spleen Figures 4.24 and 4.25).
Figure 4.18. Percentage of CD44\(^+\)Gag\(_{197}\)\(^+\)CD8\(^+\) T cells detected in different organs following mucosal vaccination in BALB/c mice. T cells obtained from different lymphoid organs were stained with the H-2K\(^d\) Gag\(_{197}\)-APC tetramer, in addition to anti-mouse CD8\(\alpha\) PerCP and CD44-PE antibodies. Data shows a representative example of three experiments. Results are expressed as mean ± SD and statistical significance assessed using the one way of variance (ANOVA) and Tukey's multiple comparison test; *p<0.05 and ***p<0.001.
Figure 4.19. Percentage of CD44\textsuperscript{+}NP\textsubscript{147}\textsuperscript{+}CD8\textsuperscript{+} T cells detected in different organs following mucosal vaccination in BALB/c mice. T cells obtained from different lymphoid organs were stained with the H-2K\textsuperscript{d} NP\textsubscript{147}-APC tetramer, in addition to anti-mouse CD8\textalpha PerCP and CD44-PE antibodies. Data shows a representative example of three experiments. Results are expressed as mean ± SD and statistical significance assessed using the one way of variance (ANOVA) and Tukey's multiple comparison test; *p<0.05 and ***p<0.001.
Figure 4.20. Percentage of CD62L$^+$Gag$_{197}^+$CD8$^+$ T cells detected following mucosal vaccination in BALB/c mice. T cells obtained from different lymphoid organs were stained with the H-2K$^d$ Gag$_{197}$-APC tetramer, in addition to anti-mouse CD8$\alpha$ PerCP and CD62L-FITC antibodies. Data shows a representative example of three experiments. Results are expressed as mean ± SD and statistical significance assessed using the one way of variance (ANOVA) and Tukey's multiple comparison test; **p<0.01 and ***p<0.001.
Figure 4.21. Percentage of CD62L$^+$NP\textsubscript{147}$^+$CD8$^+$ T cells detected following mucosal vaccination in BALB/c mice. T cells obtained from different lymphoid organs were stained with H-2K$^d$ NP\textsubscript{147}-APC tetramer, in addition to anti-mouse CD8$\alpha$-PerCP and CD62L-FITC antibodies. Data shows a representative example of three experiments. Results are expressed as mean ± SD and statistical significance assessed using the one way of variance (ANOVA) and Tukey's multiple comparison test; ***p<0.001.
Figure 4.22. Percentage of CD69<sup>+</sup> Gag<sub>197</sub> CD8<sup>+</sup> T cells detected following mucosal vaccination in BALB/c mice. T cells obtained from different lymphoid organs were stained with the H-2K<sup>d</sup> Gag<sub>197</sub>-APC tetramer, in addition to anti-mouse CD8α PerCP and CD69-PE antibodies. Data shows a representative example of three experiments. Results are expressed as mean ± SD and statistical significance assessed using the one way of variance (ANOVA) and Tukey's multiple comparison test; **p<0.01 and ***p<0.001.
Figure 4.23. Percentage of CD69$^+$NP$_{147}^+$CD8$^+$ T cells detected following mucosal vaccination in BALB/c mice. T cells obtained from different lymphoid organs were stained with the H-2K$^d$ NP$_{147}^+$-APC tetramer, in addition to anti-mouse CD8$\alpha$-PerCP and CD69-PE antibodies. Data shows a representative example of three experiments. Results are expressed as mean ± SD and statistical significance assessed using the one way of variance (ANOVA) and Tukey's multiple comparison test; *p<0.05, **p<0.01 and ***p<0.001.
Figure 4.24. Percentage of CCR9⁺Gag₁₉⁷⁺CD₈⁺ T cells detected following mucosal vaccination in BALB/c mice. T cells obtained from different lymphoid organs were stained with the H-2Kᵈ Gag₁₉⁷-APC tetramer, in addition to anti-mouse CD₈α PerCP and CCR9-FITC antibodies. Data shows a representative example of three experiments. Results are expressed as mean ± SD and statistical significance assessed using the one way of variance (ANOVA) and Tukey's multiple comparison test; *p<0.05 and ***p<0.001.
Figure 4.25. Percentage of CCR9^{+}NP_{147}^{+}CD8^{+} T cells detected following mucosal vaccination in BALB/c mice. T cells obtained from different lymphoid organs were stained with the H-2K^{d} NP_{147}-APC tetramer, in addition to anti-mouse CD8α-PerCP and CCR9-FITC antibodies. Data shows a representative example of three experiments. Results are expressed as mean ± SD and statistical significance assessed using the one way of variance (ANOVA) Tukey's multiple comparison test; *p<0.05, **p<0.01 and ***p<0.001.
4.1.5. Detection of IL-15 and IL-15Rα expression on mucosal HIV-Gag$^+$CD8$^+$ T cells by RT-PCR

Interleukin 15 (IL-15) is a T cell-stimulating cytokine and has an important role in regulating survival, proliferation and development of CD8$^+$ T cells (Zhang et al., 1998). The IL-15Rα receptor binds to IL-15 cytokine on T cells with high affinity (Anderson et al., 1995; Lodolce et al., 1998). Both IL-15 and IL-15Rα have an important role in generating and maintaining antigen-specific CD8$^+$ T cell responses and are necessary for primary expansion of CD8$^+$ T cells following viral infections (Schluns et al., 2002). IL-15Rα is expressed on dendritic, natural killer and lymphocytic cells (Anderson et al., 1995). IL-15 also regulates the maintenance of lymphoid homeostasis by supporting lymphocyte homing and proliferation (Lodolce et al., 1998). IL-15 has been shown to regulate the migration of effector CD8$^+$ T cells to pulmonary airways and as such has been used as an adjuvant to induce influenza-specific CD8$^+$ T cells to facilitate clearance of virus (Verbist et al., 2011). Its role in migration of antigen-specific CD8$^+$ T cells has been demonstrated using trans-well chemotaxis chambers through experiments that show 10 fold increases in movement of influenza-specific NP$^+$CD8$^+$ T cell when compared to media alone controls (Verbist et al., 2011).

We set out to determine if IL-15 and/or the IL-15R could be detected in vaccinated mice. We chose to focus our attention on HIV Gag$_{197}^+$CD8$^+$ T cells that had shown higher expression levels of the $α4β7^+$ mucosal marker. We prepared two HIV Gag$_{197}^+$CD8$^+$ T cell populations in the spleen, BAL and MedLN ($α4β7^+$ and $α4β7^−$). These populations were sorted following intranasal-intranasal prime-boost infection of BALB/c mice with the X31-NA-Gag$_{197}$ and PR8-NA-Gag$_{197}$ recombinant influenza viruses using the gating strategy shown in Figure 4.26. High purity populations (98.5%) of Gag$_{197}^+$α4β7$^+$ and Gag$_{197}^+$α4β7$^−$CD8$^+$ T cells were obtained. RNA was then extracted from HIV-Gag$_{197}^+$α4β7$^+$ and HIV-Gag$_{197}^+$α4β7$^−$CD8$^+$ T cells to determine the mRNA levels of IL-15-Rα and IL-15 by RT-PCR. The HIV-Gag$_{197}^+$α4β7$^+$CD8$^+$ T cell population expressed higher levels of IL-15-Rα when compared to α4β7$^−$CD8$^+$ T cells in spleen cells (23 fold), MedLN (18 fold) and BAL (11 fold) (Figure 4.27). There was also increased expression of the IL-15 cytokine on the mucosal α4β7$^+$CD8$^+$ T cell population in the spleen (10.5 fold), MedLN (9.6 fold) and in BAL (4.8 fold) compared to Gag$_{197}^+$α4β7$^+$CD8$^+$T cells (Figure 4.28).
Figure 4.26. Gating strategy used to sort and analyze HIV-Gag$^{\alpha 4\beta 7^+}$ and HIV-Gag$^{\alpha 4\beta 7^-}$ CD8$^+$ T cell populations. Total T lymphocytes were gated (A), followed by CD8$^+$ T cells (B), H-2K$^d$ Gag$^{197-APC}$ tetramer$^+$CD8$^+$ T cells (C), and finally $\alpha 4\beta 7^+$CD8$^+$ T cells (D) to identify the Gag$^{\alpha 4\beta 7^+}$ and Gag$^{\alpha 4\beta 7^-}$CD8$^+$ T cell populations.
Figure 4.27. IL-15Rα gene expression in α4β7+Gag197+CD8+ T cells following intranasal-intranasal prime boost vaccination in the spleen, MedLN and BAL by quantitative real time PCR (RT-PCR). Data is presented as the fold change expression compared to α4β7+ Gag197+CD8+ T cells, and normalized to 1 using the Δ cycle threshold method. All values are representative of three repeated experiments with a mean ± SD, with different significance by one way of variance (ANOVA) and Tukey's multiple comparison test; *p<0.05, **p<0.01 and ***p<0.001.
Figure 4.28. IL-15 cytokine expression in α4β7$^+$Gag$^{197}_-$CD8$^+$ T cells following intranasal-intranasal prime boost vaccination in the spleen, MedLN and BAL by quantitative real time PCR (RT-PCR). Data is presented as the fold change expression compared to the α4β7$^+$Gag$^{197}_-$CD8$^+$ T cells, and normalized to 1 using the Δ cycle threshold method. All values are represented as a mean ± SD of three repeated experiments, with different significance by one way of variance (ANOVA) and Tukey's multiple comparison test; *p<0.05, **p<0.01 and ***p<0.001.
4.2. Discussion

Similar to other infectious diseases, the need for an effective vaccine to control HIV infection is of prime importance in the efforts of global HIV programs. A number of CD8+ T cell properties are thought to be important in a vaccine-induced response. Mucosal immune responses stimulated by HIV/AIDS vaccines are considered highly desirable for blocking the main portal of HIV-1 entry which is through mucosal surfaces (Shattock et al., 2008), particularly gastrointestinal, vaginal, or oral surfaces (Haynes and Shattock, 2008; Challacombe and Sweet, 2002; Clerici et al., 2001; Demberg and Robert-Guroff, 2009; Jespers et al., 2010).

As the main portal of entry for most infectious agents is through the mucosal surfaces, the best way to control their transmission is by strengthening the mucosal immune responses to ensure that infections do not pass local draining lymph nodes and disseminate throughout the body. Previous studies that looked at mucosal immune responses in HIV infected or high risk individuals have noted high interferon-gamma (IFN\(\gamma\)) and interleukin-2 (IL-2) expression in CD8+ T cells and IgA production in the cervix and blood of resistant HIV female sex workers in Nairobi. There was also a direct correlation between the level of induced mucosal HIV-CD8+ T cells and control of the HIV infection (Orga, Faden and Welliver 2001; Stevceva et al., 2002). This chapter describes the use of different routes of vaccination to determine which strategy generates not only the highest immune response but also has the ability to stimulate a high proportion of antigen specific cells that express mucosal markers.

Many routes of vaccination are thought to induce mucosal immune responses. Vaccines administered intrarectally, intranasally or orally in BALB/c mice using the synthetic HIV peptide PCLUS3–18IIB and combined with a cholera toxin were effective in stimulating long lived HIV-specific CD8+ T cells in regional and distant lymph nodes in addition to the spleen when compared to subcutaneous vaccinated mice (Belyakov et al., 1998). The intranasal and intravaginal route of vaccination is also thought to facilitate the induction of strong mucosal CD8+ T cell immunity as a strong link has been established between the mucosa of the upper respiratory tract and the urogenital tract following administration of antigen on these surfaces (Holmgren and Czerkinsky 2005). Influenza viruses have been used as mucosal vectors to induce mucosal CD8+ T cell immunity following both intranasal and intravaginal vaccination.
Intravaginal infection of BALB/c mice with recombinant influenza viruses expressing the H-2 D\(^d\) HIV-Env\(^+\)CD8\(^+\) T cell epitope (P18IIIB) stimulated CD8\(^+\) T cell responses in both the inguinal lymph node and the spleen (Garulli, Kawaoka and Castrucci, 2004). Recombinant influenza viruses expressing the HIV-1 Env\(^{311}\) epitope have been shown to stimulate IFN-γ\(^+\) and tetramer specific CD8\(^+\) T cell responses in the spleen following a prime boost vaccination (Cukalac et al., 2009).

Previous studies using HIV vaccines in animal models have involved the use of numerous vaccine vectors to deliver specific HIV/SIV epitopes or proteins, i.e., adenovirus, vaccinia virus and fowl pox virus. All of these viral vectors were capable of inducing strong immune responses (Gonzalo et al., 1999; Sexton et al., 2009; Ranasinghe et al., 2011). Use of these recombinant HIV vaccines has induced mucosal CD8\(^+\) T cell immune responses that have led to decreased RNA copies of SIV virus and controlled the viremia in non-human primate models (Casimiro et al., 2005; Hoffmann et al., 2002; Liu et al., 2009; Pachler, Mayr and Vlasak, 2010; Rimmelzwaan et al., 2007; Sexton et al., 2009; Wit et al., 2004). Moreover recombinant influenzaA viruses (H3N2, A/HKx31 and PR8 H1N1, A/Puerto Rico/8/1934) have been used as candidate SIV vaccine vectors to express SIV-CD8\(^+\) T cell epitopes, SIV-Gag\(_{164-172}\), SIV-Tat\(_{87-96}\), and SIV-Tat\(_{114-123}\). Importantly intranasal or intramuscular vaccination of macaques with the HIV-1SF162 envelope protein facilitated the development of mucosal immune response in the vaginal mucosa of macaques, and protected them against virulent SHIV (SHIVSF162P4) challenge (Barnett et al., 2008). This result indicates the intranasal route of vaccination can induce mucosal immune responses at distant urogenital lymph nodes to enable vaccinated macaques to resist SIV challenge.

Our main aim was to stimulate mucosal Gag\(_{197}\)CD8\(^+\) T cells however we did also assess the magnitude and quality of the endogenous influenza NP\(_{147}\) specific CD8\(^+\) T cell response. Similar levels of endogenous influenza NP\(_{147}\) specific CD8\(^+\) T cells were induced in our experiment following primary intranasal vaccination to those results achieved from the study performed by Cukalac and colleagues (Cukalac et al., 2009). The two main integrin molecules α4 and β7, presented in as α4β7, α4β1 and αEβ7, are components regulating the migration of lymphocytes into mucosal areas (Kang et al., 2011). Expression of β7 integrin has previously been associated with the use of recombinant influenza viruses. SIV-specific CD8\(^+\) T cell responses following vaccination and challenge with SIVmac251 (Sexton, et al., 2009). Use of
other live vaccine vectors has also been associated with an increases in α4β7 mucosal marker expression on CD8+ T cells following oral vaccination in macaques. Recombinant Salmonella expressing the SIV-Gag protein and vaccinia virus expressing the SIV-Gag protein were both used in these experiments to stimulate SIV-Gag\textsubscript{181-189} specific CD8+ T cells (Evans et al., 2003). The importance of the α4β7 mucosal homing integrin in virus control has also been observed in other model systems. Rotavirus specific α4β7+CD44+CD8+ T cells has been very effective in the clearance of rotavirus infection. Adoptive transfer of 2 \times 10^4 α4β7+CD44+ CD8+ T cells showed that mice were protected from rotavirus (Rosé et al., 1998). Moreover, the transfer of increased numbers of α4β7+CD44+CD8+ T cells improved protective efficacy. Finally blocking of both CD62L and α4β7 has been shown to impair the migration of CD8+ T cells to mesenteric lymph nodes (Ciabattini et al., 2011).

We noted high levels of mRNA expression of interleukin IL-15R\textalpha and IL-15 in HIV-H-2K\textsuperscript{d} Gag\textsubscript{197}+α4β7+CD8+ T cells compared to the HIV-H-2K\textsuperscript{d} Gag\textsubscript{197}+α4β7-CD8+ T cells following intranasal-intransal vaccination in multiple tissues (spleen, BAL and mediastinal lymph nodes) as seen in Figures 4.19 and 4.20. Removal of dendritic cells that produce IL-15 or blocking of IL-15R has been previously shown to inhibit the migration of influenza specific CD8+ T cells to the site of infection (Verbist et al., 2011; Brincks and Woodland, 2010; McGill, Van Rooijen and Legge, 2010; McInnes et. al., 1996). This may be a direct result of th use of live influenza virus as similar results (high IL-15 R\textalpha mRNA expression) were detected in the BAL, mediastinal lymph nodes (MedLNs) and spleens harvested of intranasally infected (X31 (A/Hong Kong x31)) C57BL/6 mice. These mRNA levels were detected for the first time on day 3 and continued increasing until day 7 with a 4 fold change compared to naïve mice (Verbist et al., 2011). Therefore, increased expression of IL-15 R\alpha in mucosal CD8+ T cells might provide evidence for the important role of this cytokine in regulating survival of HIV-H-2K\textsuperscript{d} Gag\textsubscript{197}+CD8+ T cells at mucosal sites. This is further supported by the fact that expression of IL-15 R\alpha correlated with a high level of IFN-\gamma expression in CD8+ T cells obtained from HIV positive chimpanzees (Rodriguez et al., 2007). An increase of IL-15 R\alpha was also expressed in response to intranasal influenza infection in mice which was highly effective in facilitating the trafficking of CD8+ and natural killer cells into mucosal sites of infection and assist in the control of virus replication (Verbist et al., 2012).
4.3. Conclusion

According to the results of our mice studies, we conclude that intranasal prime boost vaccination in BALB/c mice has an important role in stimulating antigen specific CD8⁺ T cells that express known mucosal markers such as α4β7 and CD103. In addition, these mucosal HIV-Gag₁₉⁷⁺α4β7⁺CD8⁺ T cells stimulated following intranasal prime boost vaccination expressed high levels of IL-15R and also produced small but detectable amounts of expressed IL-15 compared to HIVGag₁₉⁷⁺α4β7⁻CD8⁺ T cells.

The next chapter will test the ability of these cells to protect against a pseudo challenge infection, recombinant vaccinia virus expressing the whole HIV-Gag protein.
Chapter 5 Pseudo-Challenge study

5.1. Overview

Human immunodeficiency virus (HIV) naturally uses mucosal surfaces, such as those of the intestinal and urogenital regions as the preferential sites for rapid replication. Therefore, these mucosal compartments are considered the most important in which to stimulate immune protection in order to control HIV infection and transmission. Previous studies have used various HIV-1 proteins to induce mucosal immunity. For example, HIV-1 Gag protein delivered orally using a recombinant Listeria monocytogenes vector given as prime-boost vaccine induced HIV-Gag-specific cytotoxic CD8+ T cell immunity in a mouse model. HIV Gag-specific CD8+ T cells producing both interferon gamma (IFN-γ+) and tumour necrosis factor (TNF-α+) were induced in the spleen and mucosal-associated lymphoid tissue (MALT) of vaccinated BALB/c mice. These CD8+ T cell responses completely protected mice against a intravaginal or intranasal pseudo-challenge using recombinant vaccinia virus expressing the whole Gag protein (rVac-HIVGag) (Zhao et al., 2006). An additional pseudo-challenge study in mice also indicated a protective role of HIV-1 Env-PCLUS3–18 IIIB (RIQRGPGRAFVTIGK) specific CD8+ T cells in BALB/c mice. These mice were initially primed intrarectally, intranasally, intragastrically or subcutaneous with HIV-1 Env-PCLUS3–18 IIIB. CD8 T cells were then intravenously transferred into naïve mice 5–6 days prior to intrarectal pseudo-challenge with 2.5 × 10^7 pfu rVac virus expressing HIV-1 Env gp160 protein (Belyakov et. al., 1998). Recombinant *Listeria monocytogenes* expressing HIV-1 Gag protein has also been used in pseudo challenge experiments following vaccination. HIV Gag specific CD8+ T cells protected BALB/c mice against an intraperitoneal pseudo-challenge using 2.5 × 10^7 pfu recombinant vaccinia virus expressing whole HIV-1 Gag protein (vVK1) (Rayevskaya and Frankel, 2001).

Our results from Chapter 4 indicate the presence of a large number of HIV-Gag197+α4β7+CD8+ T cells following i.n-i.n prime boost vaccination. These cells expressed higher levels of IFN-γ+ and IL-15R when compared to HIV Gag197+α4β7+CD8+ T cells. This chapter will assess the protective efficacy of this population using adoptive transfer experiments using a pseudo-challenge mouse model.
5.2. Results

5.2.1. Adoptive transfer of mucosal CD8+ T cells into naive mice

To assess the effectiveness of our recombinant influenza HIV vaccine, a pseudo-challenge experiment was performed in BALB/c mice using the recombinant vaccinia virus expressing the whole Gag protein (Rosé et al., 1998; Kaufman et al., 2008). This involved the adoptive transfer of mucosal HIV-1 Gag197+ CD8+ T cells induced in response to the vaccination of BALB/c mice as described in Chapter 4. Seven days after the booster vaccination, spleens and mediastinal lymph nodes were harvested from mice and used for the isolation of mucosal HIVGag197+α4β7+CD8+ T cells. These HIV-Gag197+α4β7+CD8+T cells along with HIVGag+α4β7CD8-T cell controls were sorted (as previously described in Chapter 4, Section 4.1.5 and Methods, Section 2.2.15.6) and enumerated. A total of 5 × 10^4 cells/ 200μl PBS of HIV-Gag197+α4β7+CD8+ T cells, HIV-Gag+α4β7+CD8+ T cells or PBS (Mock control) were adoptively transferred via the intravenous route into separate groups of naïve BALB/c mice and six days later used in challenge studies as per previous studies (Rose et al., 1998) (Kaufman et al., 2008).

5.2.2. Pseudo-challenge of BALB/c mice with recombinant vaccinia virus expressing HIV Gag (rVac633-gag)

Three groups of BALB/c mice containing adoptively transferred cells (HIV-Gag+α4β7+CD8+ T cells, HIVGag+α4β7CD8+ T cells and Mock) were challenged intranasally with 1 × 10^7 plaque forming units (pfu) of recombinant vaccinia virus-633 expressing the whole Gag protein (rVac-gag) to test the protective efficacy of these cells. Five days of challenge, the mice were euthanized and the lungs and ovaries harvested and homogenized to ascertain the titer of the challenge virus (rVac-Gag) as determined by plaque assay (Belyakov et. al., 1998). Analysis of our three vaccinated groups revealed lower number of plaques in the lungs of mice following adoptive transfer (Figure 5.1) of Gag+α4β7+CD8+ T cells (7.82×10^5 pfu/lung) in comparison to those in which Gag+α4β7CD8+ T cells were transferred (1.36×10^6 pfu/lung) or those Mock treated BALB/c mice (1.80 ×10^6 pfu/lung) infected with the rVac-Gag challenge virus (Figure 5.3). Moreover, none of the harvested ovaries showed any plaques (Figure 5.2). The data was analysed using a one way of variance test (ANOVA), which determined a significant difference among the three groups of infected mice. Our results demonstrate an important role for mucosal Gag+α4β7+CD8+ T cells in the control and dissemination of the rVac-Gag virus in this BALB/c mouse model when compared to both Gag+α4β7+CD8+ T cells and the Mock challenged group (Figure 5.3).
Figure 5.1. Plaque assay on infected lungs when challenged with the rVac-gag challenge virus. Lungs were harvested from three groups of r-Vac-Gag-challenged BALB/c mice, homogenised and then diluted to $10^{-2}$, $10^{-3}$ and $10^{-4}$, as indicated. These dilutions were used to infect 143B cells and after two days of infection, the supernatant was removed and plaques visualised by staining with 0.1% crystal violet. Data shown are representative assay for Gag$^{α4β7^+}$ transferred T cells (A), Gag$^{α4β7^-}$ transferred T cells (B) and Mock (C).
**Figure 5.2.** Plaque assay on ovaries of challenged BALB/c mice. Ovaries were harvested from the three groups of rVac-Gag-challenged BALB/c mice, homogenized and then diluted to ten fold dilutions, $10^{-2}$, $10^{-3}$ and $10^{-4}$ and used to infect 143B cells as described in Figure 1. Groups containing Gag$^+\alpha 4\beta 7^+$CD8$^+$ transferred T cells (A), Gag$^+\alpha 4\beta 7^-$CD8$^-$ transferred T cells (B) or Mock (C) as displayed.
Figure 5.3. Graph of a virus titer in the lungs of r-Vac-Gag-challenged BALB/c mice. All data shows the mean ± SD from two repeated experiments with significance determined by a one way of variance test (ANOVA) and Tukey's multiple comparison test, *p<0.05 and ***p<0.001, which compares Gag$^{197+\alpha4\beta7+}$CD8$^+$ T cell intravenous recipient mice to both Gag$^{197+\alpha4\beta7-}$CD8$^+$ T cell transferred mice and Mock controls. It also compares reduction of the challenge virus titer between the group of mice that received the Gag$^{197+\alpha4\beta7+}$CD8$^+$ T cells and mock controls.
5.2.3. CD8$^+$ T cell immune responses following the r-Vac-gag challenge mice trial

Our three groups of BALB/c mice were also assayed for CD8$^+$ T cell responses 5 days following the intranasal r-Vac-gag challenge. Both spleens and mediastinal lymph nodes were harvested for detection of HIVGag$^{197+}$CD8$^+$ T cell responses using ICS and tetramer staining assays.

5.2.3.1. CD8$^+$ T cell immune response detected by tetramer staining

Spleen and MedLN samples were assayed by tetramer staining to determine the percentage of HIV-H-2K$^d$ Gag$^{197+}$CD8$^+$ T cells. In summary, there was a significant increase in the percentage of tetramer HIV-H-2K$^d$Gag$^{197+}$CD8$^+$ T cells in both the spleen and MedLN of the group of mice that received adoptively transferred Gag$^{197+}\alpha4\beta7+CD8^+$ T cells when compared to both Gag$^{197+}\alpha4\beta7^-CD8^+$ T cells and Mock as detected by flow cytometry in Figure 5.4.

Analysis of the numbers followed a similar pattern. The total number of tetramer$^+$HIV-K$^d$ Gag$^{197+}$CD8$^+$ splenic T cells was $(2.87 \times 10^5 \pm 5.03 \times 10^4)$ in the group of mice that received adoptively transferred Gag$^{197+}\alpha4\beta7^+CD8^+$ T cells ($p<0.01$) compared to $(1.38 \times 10^5 \pm 2.65 \times 10^4)$ total cells for the group of mice that received adoptively transferred Gag$^{197+}\alpha4\beta7^-CD8^+$ T cells and $(2.53 \times 10^4 \pm 7.30 \times 10^3)$ HIV-H-2 K$^d$Gag$^{197+}$CD8$^+$ T cells ($p<0.001$) in Mock controls (Figure 5.5 A). A similar pattern was observed in the MedLN (Figure 5.5 B) Significant differences were also found in the Gag$^+\alpha4\beta7^+CD8^+$ T cell transfer group when compared to Mock controls in both the spleen ($p<0.05$) and MedLN ($p<0.01$) (Figure 5.5).
Figure 5.4. Tetramer staining of spleen cells from following r-Vac-gag-challenge. Flow cytometry of single cell spleen suspensions was used to detect the percentage of HIV-K^d Gag_{197}^+CD8^+ T cells in three groups of mice: mice that received adoptively transferred Gag_{197}^+α4β7^+CD8^+ T cells (A), mice that received adoptively transferred Gag_{197}^+α4β7^+CD8^+ T cells (B) and PBS control only (Mock) (C). A lymphocyte CD8^+ T cell and tetramer^+ gated were used for analysis.
Figure 5.5. The number of tetramer\(^+\)HIV-\(\text{H}^2\text{Kd}\) \(\text{Gag}_{197}^+\)CD\(8^+\) T cells following a pseudo-challenge. Flow cytometry of single cell spleen suspension was used to detect the percentage of HIV-\(\text{H}^2\text{Kd}\) \(\text{Gag}_{197}^+\)CD\(8^+\) T cells in three groups of mice: mice that received adoptively transferred \(\text{Gag}_{197}^+\alpha\beta^7\)CD\(8^+\) T cells, mice that received adoptively transferred \(\text{Gag}_{197}^+\alpha\beta^7\)CD\(8^+\) T cells and PBS only (Mock) in spleen (A) and MedLN (B). Data are represent the mean ± SD of two repeated experiments. Statistical differences were analysed using one way of variance (ANOVA), *\(p<0.05\), **\(p<0.01\) and ***\(p<0.001\) comparing \(\text{Gag}_{197}^+\alpha\beta^7\)CD\(8^+\) T cells to \(\text{Gag}_{197}^+\alpha\beta^7\)CD\(8^+\) T cells. In addition, there was a comparison between results of \(\text{Gag}_{197}^+\alpha\beta^7\)CD\(8^+\) T cells transfer group and Mock controls.
5.2.3.2. Functional analysis of CD8+ T cell immune response detected by ICS following the pseudo-challenge

The spleen and MedLN samples were also assayed for functional cytokine responses using the intracellular cytokine assay to measure the number of IFN-γ^HIVGag_{197}^+CD8^+ T cells. T cells harvested from the spleen and individual MedLN were stimulated for five hours with the stimulation mixture containing HIV-K^dGag_{197} peptide, IL-2 and Golgi-plug (Brefeldin). The number and function of specific CD8^+ T lymphocytes was then detected by flow cytometry. This revealed a statistically higher number of specific IFN-γ^HIVK^dGag_{197}^+CD8^+ T cells in the group of mice that received adoptively transferred Gag_{197}^+α4β7^+CD8^+ T cells, (3.05 × 10^5 ± 2.64 × 10^4) cells (p< 0.001) compared to the group of mice that received adoptively transferred Gag_{197}^+α4β7^−CD8^+ T cells (1.07 × 10^5 ± 2.07 × 10^4) cells, and this was also statistically higher in the group of mice that received adoptively transferred Gag_{197}^+α4β7^+CD8^+ T cells (p< 0.001) compared to Mock controls (2.42 × 10^4 ± 8.53 × 10^3) cells.

It is important to note that the group of mice that received adoptively transferred Gag_{197}^+α4β7^−CD8^+ T cells had significantly higher numbers of HIVK^dGag_{197}^+CD8^+ T cells than Mock controls (p< 0.001). Similar results to tetramer staining of the spleen were observed (Figure 5.6 A). The MedLN data was consistent with the observations noted via tetramer staining (Figure 5.6 B) when compared to mice that received adoptively transferred Gag_{197}^+α4β7^+CD8^+ T cells. Statistical significance was also established when compared to mock controls (Figure 5.6).
Figure 5.6. Number of IFN-γ⁺HIV-K⁺Gag⁺CD8⁺ T cells following pseudo-challenge. Functional CD8⁺ T cell populations were analyzed for their ability to express IFN-γ⁺ in the spleen (A) and MedLN (B). Data is representative of the mean ± SD from two repeated experiments. Statistical significance was determined using a one way of variance (ANOVA), *p<0.05, **p<0.01 and ***p<0.001 when comparing the Gag⁺αβ⁺⁺⁺CD8⁺ T cell transfer mouse group to both Gag⁺αβ⁺⁻CD8⁺ T cells and Mock controls. There was also a comparison of results between the Gag⁺αβ⁺⁻CD8⁺ T cell transfer mouse group and Mock controls.
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5.2.3.3. Analysis of α4β7+ and CD103+ surface markers on CD8+ T cells following a pseudo-challenge

Mucosal integrins, such as α4β7+ and CD103+ were assessed on HIV-Kd Gag197+tetramer+ CD8+ T cells following pseudochallenge. We detected an increase in the percentage of mucosal markers on HIV-Kd Gag197α4β7+ CD8+ T cells following challenge in both spleen and mediastinal lymph nodes in the group of mice that received adoptively transferred Gag197α4β7+CD8+ T cells (12.95 ± 0.78%), compared to the group of mice that received adoptively transferred Gag197α4β7+CD8+ T cells (9.18 ± 0.13%) and Mock controls (5.1 ± 2.12%) (Figure 5.7). Similar trends were observed in the MedLN (Figure 5.7 B).

CD103 has been used in previous studies as a mucosal marker expressed on specific Gag+CD8+ T cells detected in the vaginal and gut mucosa of mice or macaques (Kiravu et al., 2011; Cerf-Bensussan et al., 1987; Schon et al., 1999; Stevceva et al., 2002). A similar percentage of HIVGag197+CD103+CD8+ T cells was detected in the group of mice that received adoptively transferred Gag197α4β7+CD8+ T cells compared to the group of mice that received adoptively transferred Gag197α4β7+CD8+ T cells and Mock controls suggesting there may be co-expression of these molecules on mucosal cells (Figure 5.8).
Figure 5.7. Percentage of HIV-H-2K^d\ Gag_{197}^+\beta^7^+\ CD8^+\ T\ cells\ following\ a\ pseudo-challenge. The percentage of HIV-K^d\ Gag_{197}^+\alpha\beta^7^+\ CD8^+\ T\ cells\ in\ the\ spleen\ (A)\ and\ MedLN\ (B)\ were\ compared\ in\ three\ groups\ of\ mice\ that\ received\ adoptively\ transferred\ cells.\ Data\ is\ representative\ of\ the\ mean \pm \ SD\ from\ two\ repeated\ experiments.\ Statistical\ significant\ difference\ was\ analyzed\ using\ a\ one\ way\ of\ variance\ test\ (ANOVA),\ **p<0.01\ and\ ***p<0.001.\ This\ was\ then\ used\ to\ compare\ Gag_{197}^+\alpha\beta^7^+\ CD8^+\ T\ cells\ to\ Gag_{197}^+\alpha\beta^7^+\ CD8^+\ T\ cells\ and\ Mock\ controls.\ There\ was\ also\ a\ comparison\ between\ the Gag_{197}^+\alpha\beta^7^+\ CD8^+\ T\ cells\ transfer\ group\ and\ Mock\ controls.
Figure 5.8. Percentage of CD103+ HIV-H2K\textsuperscript{d} Gag\textsubscript{197}+CD8\textsuperscript{+} T cells following challenge. The percentage of HIV-K\textsuperscript{d}Gag\textsubscript{197}+CD103+CD8\textsuperscript{+} T cells in spleen (A) and MedLN (B) were compared in three groups of mice that received adoptively transferred cells. The data represents the mean ± SD from two repeated experiments. Statistically significant differences were analyzed using a one way of variance test (ANOVA), **p<0.01 and ***p<0.001 to compare the Gag\textsubscript{197}+αβ7+CD8\textsuperscript{+} T cell transfer mouse group to the Gag\textsubscript{197}−αβ7−CD8\textsuperscript{+} T cell transfer group and Mock transfer group. A statistical analysis was also performed between the Gag\textsubscript{197}+αβ7−CD8\textsuperscript{+} T cell transfer group and Mock controls.
5.3. Discussion

The ultimate aim of a HIV-1 vaccine is to successfully control HIV-1 infection by decreasing HIV-1 virus progression and dissemination via the mucosal points of entry. Therefore, the aim of this chapter was to investigate the protective capabilities of a subset of HIV-specific mucosal CD8+ T cells (α4β7\(^+\)Gag\(_{197}^+\)CD8\(^+\) T cells) induced by an intranasal prime boost strategy using a recombinant influenza-HIV vaccine (see results from Chapter 4) to control naïve mice challenged with the recombinant vaccinia virus expressing the whole Gag protein. This involved the adoptive transfer of α4β7\(^+\) or α4β7\(^+\)Gag\(_{197}^+\)CD8\(^+\) T cells that were also shown to express high levels of IL-15Ra and IL-15 cytokine as described in chapter 4.

The use of adoptive transfer has been utilized in experimental human trials. HIV-specific CD8\(^+\) T cells sorted from a non-infected identical twin brother of a HIV infected patient following vaccination with recombinant vaccinia virus expressing Env glycoprotein, and then transferred into the HIV-infected individual, led to a decrease in the titer of the HIV-1 virus (Bex et al., 1994). This showed that adoptive transfer of HIV-specific CD8\(^+\) T cells could control virus in a limited capacity as the cells were able to reduce virus dissemination through mucosal compartments (Jespers et al., 2010; Shattock et al., 2008).

Indeed, mucosal CD8\(^+\) T cells expressing HIV-interferon-gamma (IFN\(\gamma\)), tumor necrotic factor (TNF-\(\alpha\)) and interleukin-2 (IL-2) have been detected at high levels in the cervix and blood of female sex workers in Nairobi, thus indicating that mucosal CD8\(^+\) T cells represent a key important line of defence against HIV-1 transmission and progression (Kaul et al., 2000; Trivedi et al., 2001; Steers et al., 2009). Moreover, strong induction of a mucosal immune responses represented by CD8\(^+\) T cells that secrete soluble proteins called alpha or beta defensins in the oral cavity may contribute to HIV immunity (Weinberg et al., 2006; Tomescu, Abdulhaqq and Montaner, 2011; Furci et al., 2007; Zhang et al., 2002). These protective immune responses were also detected in rhesus macaques that were administered enteric coated capsules containing an adenovirus vector expressing Gag protein via the oral route. The rhesus macaques were then given an intranasal dose of adenovirus expressing Env polypeptide that was shown to induce robust mucosal CD8\(^-\) and CD4\(^+\) T cells in the oral cavity, vaginal mucosa and intestine in addition to a Gag and Env-specific antibody response (Mercier et al., 2007). The main aim of this chapter was to test the protective efficacy of mucosal Gag\(_{197}^+\)α4β7\(^+\)CD8\(^+\) T cells against a rVac-Gag pseudo-challenge.
Our results clearly demonstrate that significant partial protection can be achieved with a sorted Gag\textsuperscript{197+α4β7+CD8+} T cell population. Clearly the responses aren’t fully protective, and therefore a combination of other mucosal subsets (CD103\textsuperscript+ or CCR9\textsuperscript+) K\textsuperscriptd Gag\textsuperscript{197+CD8+} T cells or those specific for other HIV antigens may be required to improve efficacy. Previous studies in BALB/c mice have examined the protective role of mucosal CD8\textsuperscript+ T cells induced against the Env 18 IIIB (RIQRGPGRAFVTIGK) peptide. CD8\textsuperscript+ T cell responses were detected in the spleen, peyer’s patches and lamina propria following intrarectal immunisation in mice. Mice were then challenged intrarectally with $2.5 \times 10^7$ plaque-forming units of recombinant vaccinia virus expressing the HIV Env protein (gp160IIIB) (Belyakov et al., 1998) and 6 days later a 4.5 log reduction in challenge virus titer was detected in the ovaries of vaccinated mice compared to mock controls. Furthermore studies in both rhesus macaques and C57BL/6 mice following recombinant adenovirus (rAd5) SIVmac239 Gag protein vaccination and adoptive transfer showed upregulation of mucosal homing integrins, (CD103 and CCR9). The transferred mucosal CD8\textsuperscript+ T cells facilitated protection in mice and macaques following recombinant vaccinia-Gag challenge (Kaufman et al., 2008).

Other studies have used an alternative delivery system, such as Listeria monocytogenes as a live recombinant vector vaccine to induce Gag-specific cytotoxic CD8\textsuperscript+ T cell immune responses in mice following vaccination. These Gag specific cytotoxic CD8\textsuperscript+ T cells produced interferon gamma (IFN-\textgamma) and tumour necrosis factor (TNF-\textalpha) in various mucosal lymphoid tissues, such as the spleen and MALT and protected mice against a recombinant vaccinia virus (rVac-Gag) vaginal challenge (Zhao et al., 2006). BALB/c mice primed intramuscularly using either a rabies virus expressing HIV Gag protein (RABV) and boosted with either RABV-Gag, a vesicular stomatitis virus (VSV-Gag), or a Newcastle disease virus expressing Gag protein (NDV-Gag), induced a robust functional HIV Gag\textsuperscript+CD8\textsuperscript+ T cell responses (Lawrence et al., 2013).

Our pseudo-challenge experiments demonstrated a 1/2 log reduction in the titer of challenge virus by Gag\textsuperscript{197+α4β7+CD8+} T cells. Complete protection was not achieved by Gag\textsuperscript{197+α4β7+CD8+} T cells. This is in contrast to Belyakov and colleagues, (1998) where a 4.5 log reduction in the titer of pseudo-challenge virus (rVac-Env) was observed in the ovaries following 4 doses of HIV Env peptide IIIB in combination with cholera toxin (Belyakov et al., 1998).
Our experimental results are in line with a recent study in BALB/c mice where less than one log reduction was observed following an adenovirus 5 Gag protein (Ad5-Gag) vaccination regimen (Kanagavelu et al., 2014). In summary, the reduction in challenge virus titer in our experiments is promising and provides opportunities to test the protective efficacy of the other mucosal markers that could be used in combination with Gag$_{197}^{+}$α4β7$^{+}$ CD8$^{+}$ T cells. These markers include CD103$^{+}$, CCR9$^{+}$ or a combination of both (CD103$^{+}$CCR9$^{+}$).

Pseudo-challenge studies assessing efficacy of HIV vaccines in mice have focused on intraperitoneal, rectal, vaginal or intranasal administration of recombinant vaccinia virus expressing recombinant antigens (Belyakov et al., 1998; Kaufman et al., 2008; Zhao et al., 2006; Nakaya et al., 2004). These vaccines were able to induce protection against challenge virus in ovaries, spleen and regional lymph nodes as rVV is capable of disseminating systemically. (Belyakov et al., 1998; Zhao et al., 2006).

Previous studies have shown a critical role for mucosal CD8$^{+}$ T lymphocytes induced following intranasal influenza infection. Mucosal surface markers such as α4β7, have been identified in the lymph nodes of infected mice (Schnorrer et al., 2006; Sallusto, 1999; Dieli et al., 2003). Furthermore, transfer of mucosal influenza-specific CD8$^{+}$ T cells expressing homing integrin receptors has been shown to be critical for protection against intranasal A/PR/8/34 influenza infection in BALB/c mice. This involved intravenous adoptive transfer of 10$^7$ CD8$^{+}$ T cells and challenge using 10 times the lethal dose of PR8 (A/PR/8/34: H1N1). Results showed a significant reduction of influenza virus titer 4 days following infection compared to control mice (Cerwenka et al., 1999). As such we can conclude that the ability of mucosal CD8$^{+}$ T cells to control virus infection can be associated with the expression of α4β7 integrin in our study and may also involve CCR9 and CD103 and (Tan et al., 2011; Rudraraju et al., 2012; Chattha et al., 2013). The cytotoxic potential of the Gag$_{197}^{+}$α4β7$^{+}$ CD8$^{+}$ T cells generated following vaccination was not determined in these studies. A comparison with Gag$_{197}^{+}$α4β7$^{-}$ CD8$^{+}$ T cells may offer further insights.
6. Chapter 6: General Discussion
6.1. Overall discussion

HIV/AIDS remains a serious threat to humanity with more than 1.8 million HIV-1 infected individuals dying world-wide every year because of the global spread of the HIV epidemic. According to World Health Organization reports, the mortality rate associated with HIV/AIDS infection will remain at current levels until 2030 (McElrath and Haynes, 2010; Boily et al., 2009; Brown et al., 2008; Bongaarts et al., 2008). Therefore, international efforts to uncover effective solutions to control infection remain vital (Duerr, Wasserheit and Corey, 2006; Mathers and Loncar, 2006). For this purpose, development of an effective HIV vaccine is still regarded as the most appropriate way to control this epidemic (Johnston and Fauci, 2008).

Since 1983, more than 60 vaccine candidates have been generated to control HIV infection (Barouch, 2008; Girard, 2006). A variety of approaches have been trialled, which include DNA vaccines, recombinant vector vaccines and subunit vaccines, most of which have been administered to non-human primates. A very limited number of candidate vaccines have been tested in humans, with most of these unable to elicit significant efficacy. The exception, however, was a vaccine tested in Thailand in 2009 (RV144: Phase III). For the first time, this vaccine elicited a moderate level of protection against HIV-1 (31%). Antibody responses were induced for HIV Env and Gag, as detected by antibody dependent cellular cytotoxicity assay (ADCC) and also a cell mediated response represented by CD8+ T cell proliferation directed against Gag and Env proteins (Rerks-Ngarm et al., 2009). CD8+ T lymphocytes play a major role in controlling HIV infection and may contribute to mucosal immune responses (Gómez, Smaill, and Rosenthal, 1994; Levy, 2003; Liu et al., 1998). CD8+ T lymphocytes are normally stimulated during primary viral infections in humans to support the host immune response (Callan et al., 1998; Reddahse, 1984; Reusser et al., 1999; Rickinson and Moss, 2003). Mucosal CD8+ T lymphocytes are thought to be important for providing long-term protection against HIV-1 virus which is transmitted by mucosally, through blood transfusion and by mother-to-child transmission (Belyakovet al., 1998; Gallichan and Rosenthal, 1996; Suvas et al., 2007). HIV-resistant female sex workers in Kenya possessed persistent mucosal HIV-1-specific CD8+ T cells in the cervix (Kaul et al., 2000). In addition, but not specifically covered in this thesis, the humoral immune response also plays an important role through the stimulation of HIV-specific mucosal antibodies, such as IgA, which acts to directly block virus entry through mucosal surfaces in the urogenital mucosa. This has been shown following
vaginal challenge with SHIV virus in macaques treated with passive IgA or in humanized mice given anti-HIV-polymeric IgA inhibited HIV mucosal transmission. However, the role IgA plays in controlling mucosal transmission of HIV-1 is not completely characterized in human at this point in time (Mascola et al., 1996; Mascola et al., 2000; Hur et al., 2012; Wolbank et al., 2003). Poly-functional CD8+ T cells are stimulated by specific HIV peptides, such as those derived from the Gag protein (Duvall et al., 2008; Holmgren and Czerkinsky, 2005; Gillespie, 2005). During acute and chronic HIV-1 infection, these polyfunctional CD8+ T cells have been shown to be involved in the reduction and control of the viral load. Firstly, they control virus replication by producing high levels of multiple cytokines, such as IFN\textgamma, TNF-\alpha and IL-2 (Kobayashi et al., 2004; Viganò et al., 2008). Secondly, CD8+ T lymphocytes can destroy HIV-1 infected cells through cytolytic action involving granzymes and perforin (Froelich, Dixit and Yang, 1998), with HIV-resistant individuals shown to express high levels of both granzymes and perforin. Moreover, there is a negative correlation between HIV-1 RNA copy number and the level of granzymes and perforin expressed by CD8+ T cells (Kaul et al., 2000; Hersperger, 2010).

The studies described in this dissertation explored a reverse genetics strategy for generating a vaccine that stimulates mucosal HIV-specific immunity. This strategy involved the use of recombinant DNA technology and reverse genetics to generate influenza viruses that expressed HIV-1 specific CD8+ T cell epitopes in order to induce mucosal CD8+ T cell immunity. Two H-2d HIV-1 epitopes, Gag_{197} (AMQMLKETI) and Tat_{17} (QPKTACTNC), were inserted into the NA gene segment of influenza virus strains PR8 (HIN1) and X31 (H3N2) using reverse genetics and mice were immunized via the mucosal route using a prime-boost strategy. Previous HIV vaccine studies in animal models have used a range of vectors (including adenovirus, vaccinia virus and fowl pox virus) to deliver HIV epitopes or proteins. All of these vectors were able to induce HIV-specific immune responses but many did not focus on stimulation of mucosal immunity. The influenza virus was chosen as a live vaccine vector candidate as it efficiently expresses viral epitopes in the respiratory and urogenital compartments (Marsh and Tannock, 2005; Satterlee, 2008; Smith, 2007) and has the ability to generate broad and durable long-term immunity at different mucosal surfaces (Fodor et al., 1999; Mueller et al., 2010; Neumann and Kawaoka, 2001; Yao and Wang, 2008). Moreover, there is a documented strong connection between the mucosa of the upper respiratory tract and of the urogenital tract (Holmgren and Czerkinsky, 2005). This was detected using nasal Cholera
toxin B subunit immunization in humans to induce both IgA and IgG antibody responses in cervicovaginal mucosa in addition to the upper respiratory mucosa and regional secretions (saliva, nasal secretions) (Holmgren and Czerkinsky, 2005; Kozlowski et al., 1997; Johansson et al., 2004). Another advantage of this approach include the fact that the gene segments of the influenza virus could be easily manipulated using reverse genetics technology which allowed the generation of recombinant viruses as vaccine vectors (Palese et al., 1996; Steinhauer and Skehel, 2002; Steinhauer et al., 2009).

Recombinant influenza viruses manipulated in this way have been used as vaccine vectors expressing HIV-CD8+ T cell epitopes to successfully stimulate cytotoxic CD8+ T cell responses (Casimiro et al., 2005; Hoffmann et al., 2002; Pachler, Mayr and Vlasak, 2010; Sexton et al., 2009). Intranasal vaccination of macaques with recombinant HIV vaccines has led to stimulation of a strong mucosal immune response in vaginal mucosa when challenged intravaginally with a virulent SHIV virus (Barnett et al., 2008). Other mucosal HIV-1 vaccination trials were highly effective in inducing both systematic and mucosal immune responses against HIV-1. Examples of this include intranasal immunisation with a recombinant influenza vector expressing HIV-1 Env (gp41) that stimulated strong humoral immune responses, as shown by HIV-1 specific antibodies in the lung, spleen and urogenital mucosa of mice (Ferko et al., 1998). Importantly, the two strains of laboratory influenza viruses associated with our study had previously been used to express the HIV-H-2Dd Env311 CD8+ T cell epitope in mice (Cukalac et al., 2009).

The H-2Kd HIV-Gag197 epitope was used as an antigen for this project for several reasons. Firstly, this Gag epitope is highly conserved, and so should offer protection against diverse viral strains (Mata et al., 1998; Lorgeoux, Guo and Liang, 2012). Indeed, the nine amino acids of Gag from 197 to 205 were the most dominant epitope targeted by a breadth of HIV-CD8+ T cell responses when compared to other Gag peptides (Cellini et al., 2008). Moreover, polyfunctional HIV-specific CD8+ T cells obtained from HIV-elite controllers have been shown to be Gag-specific (Ndhlovu et al., 2012; Berger et al., 2011). Secondly, HIV Gag protein can stimulate specific HIV-CD8+ T cell immune responses with the mostly dominant HIV-1 restricted Gag H-2Kd CD8+ T cell in mouse models (Faul et al., 2009; Lawrence et al., 2013; McGettigan et al., 2001; McGettigan et al., 2003; McGettigan et al., 2003a). This includes conserved regions of the HIV-Gag197 (AMQMLKETI) epitope detected in the wild
type virus genome which induced a robust HIV-1 CD8+ T cell immune response in vaccinated mice (Lawrence et al., 2013; Wallace et al., 2013). Also, strong HIV-1 Gag H-2K^d (AMQMLKETI) restricted CD8+ T cell responses have previously been identified following vaccination of BALB/c mice using a DNA vaccine expressing the HIV-1 Gag protein (Wallace et al., 2013). Also, robust HIV-Gag+CD8+ T cell responses were detected in intramuscularly vaccinated BALB/c mice using recombinant virus vectors, such as the rabies virus (RABV), vesicular stomatitis virus (VSV) and Newcastle disease virus expressing the Gag protein (Lawrence et al., 2013). In addition, Gag-specific CD8+ T lymphocytes have been shown to be highly migratory following intramuscular infection with a recombinant adenovirus vector expressing the whole Gag protein in mice and macaques (Kaufman et al., 2008). These HIV-Gag-specific CD8+ T cells dominant in the Gag D^b-restricted epitope (AAVKNWMTQTL) were found mainly in the spleen and different mucosal sites, Peyer’s patches, the inguinal and mesenteric lymph nodes, and vaginal tract mucosa (Kaufman et al., 2008). It is also important to note that the dominant CD8+ T cell specificities induced in macaques following intratracheal and intranasal recombinant influenza viruses, X31 (H3N2, A/HKx31) and PR8 (H1N1, A/Puerto Rico/8/1934) expressing SIV-CD8+ T cell epitopes and NA stalks were derived from the SIV-Tat_87–96, SIV-Tat_114–123 and SIV- Gag_164–172 epitopes that were separately inserted into the NA stalk of influenza virus vectors (Sexton et al., 2009).

Our study clearly demonstrate the ability of the influenza virus to replicate in the upper respiratory tract of mice when infected intranasally. Replication in the genital mucosa is normally non-productive due to the lack of trypsin-like enzymes required for the cleavage of HA in order to facilitate entry of the viral genome into the cell cytoplasm (Klenk, Rott, Orlich, and Blödorn, 1975; Garulli, Kawaoka and Castrucci, 2004; Garulli et al., 2007). As was detected in this project, using the recombinant X31-NA-Gag_197 influenza virus, intranasally infected BALB/c mice experience significant weight loss compared to intravaginally infected mice in which no weight loss was detected. This can be explained by the ability of influenza viruses to replicate in the respiratory tract mucosa as a result of appropriate protease activity in the pulmonary mucosal area, thus increasing the pathogenicity of influenza virus infection because this enzyme is lacking in the vaginal mucosa (Garulli, Kawaoka and Castrucci, 2004). However, this does not mean that no immune response was induced in the urogenital mucosa following influenza infection. Moreover, methods have been developed to improve the replication rates of viruses in the urogenital area. For example, subcutaneous administration of
progesterone has been shown to improve sensitivity of mice to intravaginal infection with viruses such as herpes simplex virus type 2 (Parr, Bozzola and Parr, 1998; Parr et al., 1994) or influenza virus (Garulli, Kawaoka and Castrucci, 2004).

Experiments by Garulli and colleagues, (2004) demonstrated that the most highly induced CD8$^+$ T cells were specific for HIV-Env epitope (RGPGRAFVTI) of the gp160 envelope protein and the immuno-dominant restricted H-2D$^d$ NP$_{147-155}$ epitope (TYQRTRALV) from the HIV-1 IIIB isolate. Most of these CD8$^+$ T cells were detected in the spleen, iliac lymph nodes and in the genital mucosa of progesterone treated BALB/c mice that were then infected with a high dose of recombinant influenza viruses either A/WSN/33, A/PR/8/34 (PR8) or X-31 viruses expressing the HIVEnv$_{311}$ epitope (Flu/P18IIIB; Env gp160). In addition, HIV-specific antibody responses were detected in both sera and vaginal secretions of these infected mice. However, the group of mice that received a lower dose of the recombinant influenza virus vector showed low or undetectable virus titres following intravaginal infection (Garulli, Kawaoka and Castrucci, 2004). According to this study, influenza A viruses could induce acute infection in the vaginal mucosa of the progesterone-treated BALB/c mice. This means vaginal replication could be enhanced by treatments that mimic protease function thus increasing pathogenicity of the influenza virus. This was also observed in the female genital organs of mice during the estrous cycle on progesterone and resulted in high susceptibility of adult BALB/c mice to vaginal infection with herpes simplex virus 2 compared to non-estrous mice (Parr, Bozzola and Parr, 1998; Parr et al., 1994).

Mucosal immune responses are characterized by markers such as $\alpha$4$\beta$7, CD103 and CCR9. An important role for LPAM-1 ($\alpha$4$\beta$7) as a mucosal homing CD8$^+$ T cell integrin in protective immune responses has been suggested in various virus infections in mice studies. (Holzmann, McIntyre and Weissman, 1989; Rudraraju et al., 2012; Tan et al., 2011). When comparing different mucosal routes of prime-boost infection in BALB/c mice, the results in this project indicate that intranasal vaccination with recombinant influenza virus vaccine vectors induces the best polyfunctional HIVGag$_{197}^+$CD8$^+$ T cell response

The main aim of mucosal vaccines used in this project was to induce HIV specific mucosal CD8$^+$ T cells that enable control of a pseudovirus challenge. Therefore, we conducted experiments to find out whether the induced mucosal HIV-Gag$_{197}^+$4$\beta$7$^+$CD8$^+$ T cells were functionally protective. HIV-Gag$_{197}^+$4$\beta$7$^+$CD8$^+$ T cells were sorted for adoptive transfer into
naïve mice prior to pseudochallenge with the recombinant vaccinia virus expressing HIV-Gag (rVac). Importantly this transferred population was partially protective.

6.2. Research outcomes

This project has successfully investigated a novel approach to stimulate, detect and characterize mucosal CD8⁺ T cells responses using recombinant influenza viruses as a vaccine vector. The key research outcomes of this work were:

1. The generation of recombinant influenza viruses expressing the H-2K<sup>d</sup> restricted HIV-Gag<sub>197</sub> or H-2<sup>d</sup> HIV-Tat<sub>17</sub> CD8⁺ T cell epitopes using reverse genetics technology.
2. Demonstration that recombinant influenza viruses expressing the HIV-H-2K<sup>d</sup>Gag<sub>197</sub> epitope stimulated strong CD8⁺ T cell immunity at multiple anatomical sites as seen through the use of tetramer staining and ICS.
3. Intranasal-intranasal prime-boost vaccination induced the highest proportion and numbers of HIV and influenza-specific mucosal CD8⁺ T cells in the spleen, draining lymph nodes and distal mucosal sites.
4. Adoptive-transfer of Gag<sub>197</sub>α<sub>4</sub>β<sub>7</sub>CD8⁺ T cells into naïve mice enabled partial control of whole Gag protein expressing vaccinia virus challenge.

6.3. Future directions

Due to time limitations, a generic adoptive transfer regime was adopted for protection experiments. As such, the most critical experiments to perform are ones that involve fine-tuning the adoptive transfer conditions given, since only partial protection was achieved. Experiments would therefore involve repeating the intranasal prime-boost vaccination strategy, and transferring a higher number of mucosal α<sub>4</sub>β<sub>7</sub>Gag<sup>+</sup>CD8⁺ T cells to naïve mice to determine if recombinant vaccinia virus challenge could be fully controlled. In addition, these studies could be expended by transferring HIVGag<sub>197</sub> specific CD8⁺ T cells that express more than one mucosal integrin, such as Gag<sub>197</sub>CD103⁺CD8⁺ T cells, to improve protection from a pseudo-challenge virus. This work could also be extended to vaccination that could induce both a mucosal and systematic CD8⁺ T cell immune responses necessary to control challenge virus.

If successful, the transfer experiments could be repeated in macaques. These experiments would involve the use of SIV-Gag<sub>197</sub>CD8⁺ T cell epitopes to initially prove the ability of the
intranasal prime boost immunisation to induce mucosal CD8$^+$ T cells prior to commencement of the adoptive transfer experiments. These experiments are costly and must be carefully considered given the ethic issues around the use of non-human primates. Availability and/or cross reactivity of mucosal markers in this non-human primate model must also be verified prior to commencement. In conclusion, studies that enhance our understanding of mucosal HIV immunity are of utmost importance given the portal of entry for most HIV infections in humans is the mucosa. Development of vaccines that can stop dissemination of the virus, halt its progress at the local draining lymph nodes and therefore avoid latency may prove the most effective in bringing this worldwide epidemic under control.

Finally it would be important to test if influenza-specific $\alpha 4\beta 7^+$CD8$^+$ T cells could behave similarly in protection experiments against intranasal influenza virus challenge when adoptively transferred to mice to establish this mucosal subset as a universal protective marker.
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A. Appendix A

A.1. Solutions, buffers and reagents

A.1.1. Agarose solution (0.8%) for gel electrophoresis

0.8 g agarose (Sigma-Aldrich)
Add 1x TAE buffer (100 ml)
Dissolve by microwave
Add 3 μl SYBR safe DNA gel stain (Invitrogen)

A.1.2. Agarose solution (1.8%) for plaque assay

0.9 g Agarose (Analytical grade, Promega)
Dissolve in Baxter water (50 ml)
Autoclave for 20 min at 121 °C
Keep in a 56 °C water bath for 2 hours
Transfer to a 45 °C water bath to be ready for the assay

A.1.3. FACS buffer (1%BSA; 0.02% sodium azide)

500 ml phosphate-buffered solution (PBS)
50 ml 10% (v/v) bovine serum albumin (BSA)
500 μl 20% (v/v) sodium azide

A.1.4. TAE Buffer (40x) (Sambrook et al., 1989)

1.6 M Tris (Tris: hydroxymethyl methane (Merk)
40 mM EDTA (Ethylene Diamine Tetra Acetic acid) (Merk)
Dissolve in 800 ml of deionized water
pH (8.2-8.4) in a 25°C with 1 M Acetic acid (Merk)
Bring volume to 1000 ml
Store at 22-25 °C and use in 1-2 weeks

A.1.5. Red cell lysis buffer (Ammonium-Tris Chloride buffer)

0.14 M NH₄CL (7.47 g) (BDH, Australia)
0.017 M Tris hydroxymethyl methane (2.06 g) (BDH, Australia)
980 ml Baxter water
Adjust pH to 7.2 with 1 M HCL
Bring volume to 1 L with Baxter water
Store at 22-25 °C and use in 1-2 weeks
A.1.6. RNeasy Mini Kit for RNA extraction (Qiagen)
Including the following steps:
500 μl 70% Ethanol
500 μl RLT buffer
500 μl RW1 buffer
500 μl RPE buffer
Elution buffer (Nuclease free water) dH2O

A.1.7. Plasmids
pHW2000
pBR322 plasmid cloning vector (New England Biolabs (NEB)
pUC19 DNA (0.01 μg/ml)

A.1.8. Competent cells
A.1.8.1. MAX Efficiency® DH5α™ Competent Cells (Invitrogen)
DH5α™ Competent Cells
A.1.8.2. High efficiency JM109 Competent cells (Promega)
E.coli JM109 competent cells >108 cfu/μg

A.1.9. Reverse transcription kit for conversion RNA to cDNA
2μl of 10× Reverse transcriptase buffer (RT buffer)
2μl dNTP mix buffer
2μl 10 μM oligo-dT primer
1 μl Omniscript reverse transcriptase

A.1.10. Permeabilizing solution
One part Perm/washTM buffer (Becton Dickinson, UAS)
Nine part deionized distilled water

A.1.11. Restriction endonuclease enzymes
Specific restriction endonuclease enzyme, PstI (Cat# R6325)
Specific restriction endonuclease enzyme, PvuI (Cat# R611A)
Appendix A

A.1.12. QIAquick PCR Purification Kit protocol
For one volume of the PCR sample:
Add 5 volumes Buffer PB
Apply the sample to the QIAquick column
Centrifugation for 30–60 seconds and Discard flow through
Add 0.75 ml Buffer PE and centrifuge for 30–60 seconds
Discard flow-through and Place QIAquick column in 1.5 ml microcentrifuge tube
Add 30 μl elution buffer and then centrifuge

A.1.13. QIAquick Gel Extraction Kit protocol
Add 3 volumes Buffer QG to 1 volume of gel
Incubate at 50°C for 10 min
Mix by vortexing the tube every 2–3 min to dissolve the gel
Add 1 volume of isopropanol
Apply the sample to the QIAquick column
Discard flow-through.
Add 0.5 ml Buffer QG and centrifuge for 1 min
Add 0.75 ml Buffer PE and centrifuge for 1 min
Discard the flow-through and Place QIAquick column into microcentrifuge tube
Add 20 μl Buffer EB (10 mM Tris·Cl, pH 8.5) and centrifuge for 1 min

A.1.14. PureYield™ Plasmid Miniprep protocol (Promega, USA)
Add 600 μl transformed bacterial culture to 1.5 ml centrifuge tube
Add 100 μl cell lysis buffer and mix well
Add 350 μl cold neutralization solution and mix well
Centrifugation at 13000 rpm for 3 minutes
Transfer the supernatant to the PureYield™ Minicolumn into collection tube
Centrifuge at 13000 rpm for 15 seconds and discard the flow through
Wash by add 200 μl of Endotoxin removal wash (ERB)
Centrifugation at 13000 rpm at 15 seconds
Add 400 μl of column wash solution (CWS) and centrifuge for 30 seconds
Transfer the Minicolumn to 1.5 ml centrifuge tube
Elute the plasmid DNA by adding 30 μl of elution buffer and centrifuge for 15 seconds

A.1.15. Cell lines
Madin-Darby Canine Kidney cells (MDCK cells)
Human embryonic kidney cells (HEK 293T cells)
A.1.16. **PureYield™ Maxiprep System protocol (Promega, USA)**

Grow up 100–250 ml of transformed E. coli bacterial cell culture for overnight

Pellet the cells by centrifugation at 5,000 × g for 10 minutes at room temperature

Discard supernatant and mix the pellet with 12 ml Cell Resuspension Solution

Add 12 ml Cell Lysis Solution, mix well and incubation for 3 minutes at room temperature

Add 12 ml Neutralization Solution and mix well

Centrifuge the lysate at 14,000 × g for 20 minutes at room temperature

Pour the lysate into the blue PureYield™ Clearing Column

Apply maximum vacuum for passing the lysate debris through the PureYield™ Clearing Column

Wash the DNA by add 5 ml of Endotoxin Removal Wash and apply the vacuum

Add 20 ml of Column Wash and apply the vacuum

Dry the membrane by applying a vacuum for 5 minutes

Place a 1.5 ml microcentrifuge tube in the base of the Eluator™ Vacuum

Elute the DNA by 1 ml of Nuclease-Free Water and apply maximum vacuum for 1 minute

A.1.17. **OPTI-MEM 1 medium (Gibco)**

OPTI-MEM® I modification of MEM (Eagle's)

A.1.18. **Easy-flu medium (Invitrogen)**

500 ml OPTI-MEM 1 medium (Gibco)

5 ml Penicillin (10,000 U/ml) and Streptomycin (10,000 μg/ml)

A.1.19. **Fugene® 6 Transfection Reagent (Roche)**

Lipid blends in 80% ethanol, sterile-filtered, packaged in glass vials

A.1.20. **RPMI-antimedia**

500 ml Gibco RPMI 1640

300 μl Gentamycin (40 mg/ml)

5 ml Penicillin 10,000 U/ml (final concentration 100 U/ml)

Streptomycin 40 mg/ml (final concentration 24 μg/ml)

A.1.21. **2x L15 (Leibovitz’s) medium**

2 packets of L15 powder media (2x 13.7 g) (Invitrogen)

Add 900 ml Baxter water

Stir for 1 hour

Adjust pH to 6.8 (1 M Hydrochloric acid)

Add 8 ml of 7% NaHCO₃

Add 800 μl of Hepes buffer (pH 6.8, 1M)
Appendix A

Add 20 ml of Pen/Strep (10,000 U/ml)
Add 1.2 ml Gentamycin (40 mg/ml)

**A.1.22. LB agar culture media**

200 ml LB agar
Dissolve by microwave for 1-2 min
Add 0.2 ml ampicillin 100 μg/ml (Sigma-Aldrich)
Pour 15 ml agar in petri-dish plates
Wait for 5 min and cover with parafilm
Keep at 4 oC

**A.1.23. 1% TPCK-trypsin Worthington (1 μg/ μl)**

10 mg of TPCK-trypsin Worthington (ScimaR)
Dissolve in 10 ml of PBS
Filtering by 0.45 μm Minisart filter
Aliquot into 250 μl volume
Keep at -80 oC

**A.1.24. Trizol kit for RNA extraction**

1 ml Trizol reagent (GibcoBRL) per 5-10x10^6 cells
200 μl chloroform (AnalR)
0.5 ml Propan-2-ol (isopropanol: AnalR)
1 ml 75% ethanol (AnalR)
30 μl HPLC water
Re-dissolve RNA for 10 minutes at 60°C (heat block)

**A.1.25. Plaque assay medium**

500 ml Gibco RPMI 1640 (Invitrogen)
300 μl Gentamycin (40 mg/ml)
5 ml Penicillin /Streptomycin (10,000 U/ml)
5 ml L-glutamine 2 mM (200 mM)
5 ml Sodium pyruvate (100 mM).
45 ml Heat inactivated FCS

**A.1.26. Hanks’ Balanced Salt Solution (HBSS)**

Gibco Hanks’ Balanced Salt Solution (HBSS) (1X) (Invitrogen)

**A.1.27. DNA size ladder**

1 Kb plus DNA ladder (Invitrogen 1μg/μl)

**A.1.28. C-RPMI medium**
Appendix A

500 ml RPMI media (JRH)
2 ml Penicillin/Streptomycin (10,000 U/ml)
40 ml Heat inactivated FCS
25 ml Hepes buffer

**A.1.29. 4 % Paraformaldehyde**
100 ml Phosphate Buffered Saline (PBS)
Pour into the conical flask containing 4g of paraformaldehyde (AnalR)
Stir inside the fume hood
Aliquot in 1 ml and store at 4°C

**A.1.30. 6x DNA Loading Dye**
0.25% (w/v) Bromophenol blue
0.25% (w/v) Xylene cyanol FF
30% (v/v) glycerol in distilled water
### Table A.1. Fluorochrome conjugated Anti-mouse monoclonal antibodies

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