NOVEL RECOMBINANT DNA AND LIVE VIRUS VACCINES TO PREVENT OR CONTROL HIV-1 INFECTION

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Abstract

Background

Vaccination is the most cost effective and long-term solution to the global human immunodeficiency virus (HIV) pandemic. The HIV Gag and Tat proteins are attractive components of a HIV vaccine as immune responses targeting these proteins confer protective benefits against HIV infections in humans. This thesis has developed two innovative candidate HIV vaccines viz. a DNA vaccine encoding oligomerised and secreted Tat (pVAX-sTat-IMX313), and a recombinant live human rhinovirus serotype A1 (HRV-A1)-based vaccine encoding Gag and Tat (rHRV-Gag/Tat).

Methods

To construct pVAX-sTat-IMX313, Tat was fused with the oligomerisation domain of IMX313 to form Tat heptamers and linked to the leader sequence of tissue plasminogen activator to ensure that the bulk of oligomerised protein is secreted. To develop the rHRV-Gag/Tat vaccine, initially, the full length *tat* gene and 5 discrete overlapping fragments corresponding to the full length *gag* gene were individually inserted into the junction between the HRV-A1 genes encoding structural and non-structural proteins (P1/P2 junction) to ensure that the exogenous HIV Gag or Tat proteins were separated from the recombinant polyprotein using the HRV encoded 2Aprotease enzyme. Thus, one recombinant HRV encoding Tat (rHRV-Tat) and 5 rHRVs each encoding a unique Gag fragment (rHRV-Gag1-5) were generated. The individual rHRVs were then mixed into a single cocktail vaccine (rHRV-Gag/Tat), purified and titrated for inoculation in mice.

The immunogenicity of these vaccines was evaluated in female BALB/c mice that received up to five intradermal injections of pVAX-sTat-IMX313 (50 μg per dose) at 2 weekly intervals in one study. In another study, mice were vaccinated intranasally with 2 doses ($5x10^6$

TCID50/dose) of the rHRV-Gag/Tat followed by a single 50 μg booster dose of a cocktail DNA vaccine containing pVAX-sTat-IMX313 and pVAX-Gag-Perforin. Vaccine-induced immune responses were examined 2 weeks after the last dose by antibody ELISA, *in-vitro* Tat transactivation neutralization, IFN-γ ELISpot, $KdGag_{197\text{-}205}$ tetramer staining and intracellular cytokine staining assays.

Results

Data showed that fusing Tat with IMX313 results in complete heptamerisation of Tat. Furthermore, the data suggested that pVAX-sTat-IMX313 vaccination elicited higher titers of serum neutralizing Tat-specific IgG, secretory IgA (sIgA) in the vagina and CMI responses, and showed superior control of ecotropic HIV (EcoHIV) infection, a surrogate murine HIV challenge model, compared with animals vaccinated with other DNA vaccines tested in this study. Human rhinovirus serotype A1 (HRV-A1) was successfully engineered into a replication-competent genetically stable recombinant vector to deliver a mucosally-targeted vaccine, rHRV-Gag/Tat, by inserting exogenous HIV *gag* and *tat* sequences into the HRV-A1 genome. Finally, intranasal administration of 2 doses of rHRV-Gag/Tat followed by a single DNA booster dose induced superior poly-functional Gag-specific CD8 T cell responses in the spleen (systemic) and mesenteric lymph nodes (mucosal), higher Tat-specific serum IgG and sIgA in the vagina, and effective control of EcoHIV infection compared to other vaccination regimens tested in this study.

Conclusion

First, the data support the inclusion of IMX313 as a molecular adjuvant for Tat-based HIV DNA vaccines. Second, the data demonstrated that intranasal vaccination with rHRV-Gag/Tat followed by a single DNA booster dose is effective in eliciting HIV-specific immunity pan-mucosally and systemically. Collectively, the data support further testing of the pVAX-sTat-

IMX313 and rHRV-Gag/Tat vaccines in macaques, preferably in a heterologous prime-boost vaccination strategy, and results from these studies might influence future HIV clinical trials.

Declaration

I certify that this work contains no material which has been accepted for the award of any other degree or diploma in any university or other tertiary institution and, to the best of my knowledge and belief, contains no material previously published or written by another person, except where due reference has been made in the text. In addition, I certify that no part of this work will, in the future, be used in a submission for any other degree or diploma in any university or other tertiary institution without the prior approval of the University of Adelaide and where applicable, any partner institution responsible for the joint-award of this degree.

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List of abbreviations and acronyms

AIDS: acquired immune deficiency syndrome

Ad5: Adenovirus serotype 5

APCs: Antigen presenting cells

ADCVI: Antibody-dependent cell-mediated virus inhibition

ADCC: Antibody-dependent cellular cytotoxicity

ADCP: Antibody-dependent cellular phagocytosis

ADCD: Ab-dependent complement deposition

Ad5: Adenovirus serotype 5

APOBEC-3G: Apolipoprotein B mRNA editing enzyme, catalytic polypeptide-like-type 3G

~: Approximately

cDNA: Complementary DNA

CCR5: Chemokine receptor 5

CCR5 Δ32: CCR delta 32

$CD4^+$: Cluster of differentiation 4 positive

$CD8^+$ Cluster of differentiation 8 positive

Δ *Nef:* delta Nef

DNA: Deoxyribonucleic acid

DCs: Dendritic Cells

DC-SIGN: Dendritic Cell-Specific Intercellular adhesion molecule-3-Grabbing Non-integrin

ELISA: Enzyme-linked immunosorbent assay

ELISpot: Enzyme-linked immunosorbent spot assay

eIF-4GI: Eukaryotic initiation factor 4GII

ESCRTs: Endosomal sorting complexes required for transport

gp120: Glycoprotein 120

gp41: Glycoprotein 41

g: Gram

≥: Equal to or greater than

HIV-1 or HIV-2: Human immunodeficiency virus type 1 or 2

HIV LTR: HIV long terminal repeats

HLA: Human leukocyte antigen

HAART: Highly active anti-retroviral therapy

HeLa cells: Henrietta Lacks cells

HEK cells: Human embryo kidney cells

HCV: Hepatitis C Virus

IRES: Internal ribosome entry site

IN: Intra nasal

IFN-γ: Interferon gamma

IL-2: Interleukin-2

IL4: Interleukin-4

IL-7: Interleukin-7

ISCOMs: Immune stimulating complexes

IAVI: International AIDS Vaccine Initiative

sIgA: Secreted immunoglobulin A

IgG: Immunoglobulin G

IgE: Immunoglobulin E

I.e: That is to say

Kb: kilo base

kDa: kilo dalton

<: Less than

LRAs: Latency reversing agents

LEDGF/p75: Lens epithelium-derived growth factor/p75

MHC-I/II: Major histocompatibility complex class I or II

MHC-E: Major histocompatibility complex class E

MPER: Membrane proximal external region

ml: millilitre

mg: milligram

µl: micro litre

mRNA: messenger RNA

MSM: Men-who have-sex with men

Nabs: Neutralizing antibodies

NF-kB: Nuclear factor NF-κB

NFAT: Nuclear factor of activated T-cells

NK cell: Natural killer cells

%: Percentage

/: Per

RNA: Ribonucleic acid

STDs: Sexually transmitted diseases

SIV: Simian immunodeficiency

SIVmac251: Simian immunodeficiency for macaques strain 251

SIVmac239: Simian immunodeficiency for macaques strain 239

SIVsm E660: Simian immunodeficiency for macaques strain E660

SIVsmm: Simian immunodeficiency virus for sooty mangabeys

Th1 and 2: Type 1 and Type 2 immune responses

TNF-α: Tumor necrosis factor-alpha

UTR: untranslated region

UNAIDS: United Nations Programme on HIV and AIDS

Viz: namely

WHO: World Health Organisation

Chapter 1.0: Literature review

1.1 General introduction

Human Immunodeficiency Virus(HIV) causes Acquired Immunodeficiency Syndrome (AIDS)[1]. HIV has infected over 75 million people globally, and is responsible for about 2.2 million new infections and 1.7 million deaths each year especially in Sub-Saharan Africa and South East Asia[2,3]. The HIV pandemic has been partially contained by Highly Active Antiretroviral Therapy (HAART) but still presents a huge human and financial burden in low- and middle-income countries[2]. Therefore, there is an urgent need for a safe and efficacious vaccine that could represent the best hope to achieve sterilizing immunity and control of HIV infections.

1.2 HIV classification

HIV belongs to the genus *Lentivirus*, which together with genera *Spumaviruses* and *Oncoviruses* form the *Retroviridae* family[4]. Other members of this family include Simian Immunodeficiency Virus (SIV), Feline Immunodeficiency Virus (FIV), Equine Infectious Anaemia Virus (EIAV) and the Maedi-Visna Virus (MVV). There are two types of HIV namely HIV-1 and HIV-2 with similar modes of transmission, replication cycle, primary target cells ($CD4^+$ T cells), nature of infection and origin[5]. HIV-2 is restricted mainly to West Africa, is less virulent compared to HIV-1, associated with limited epidemics, and is divided into 8 subtypes (A through to H)[5]. On the other hand, HIV-1 is more virulent, associated with the global HIV pandemic, and is divided into 4 groups (M, N, O, and P) and several circulating recombinant forms[6,7].

1.3 The structure and organisation of the HIV genome

HIV is a spherical, enveloped, single-stranded- positive-sense RNA virus as shown in figure 1. It possesses a 9.7 Kb diploid genome that is subdivided into major, regulatory and auxiliary genes[8].

1.3.1 The major genes

1.3.1.1 The *gag* gene

The *gag* gene is 1.5 Kb and encodes a 55 kDa precursor polyprotein ($p55^{Gag}$), which is cleaved by viral proteases into the structural proteins namely, p17-(matrix, MA), p24 (capsid, CA), p7 (nucleocapsid, NC), and small core proteins (p1, p2 and p6)[9]. These proteins are involved in facilitating transportation of the HIV pre-integration complex into the nucleus during viral reverse transcription[8], incorporating the envelope into newly formed virus[9], packaging cellular cyclophilin A needed during budding of the virus from the host cell membrane, stabilising the structural integrity of the virus[10] and promoting assembly, maturation and release of progeny viruses[11].

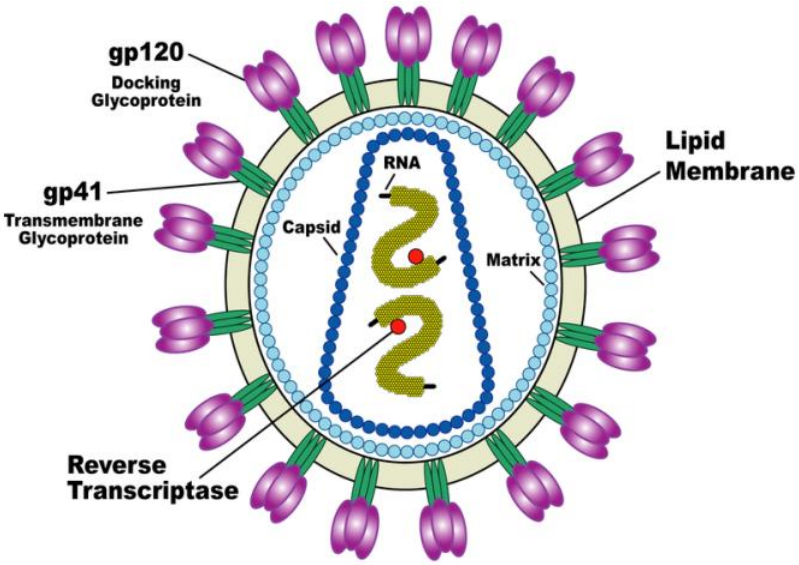

Figure 1: The structure of a HIV virion[12]. *The gp120 and gp41 form the envelope trimers located on the surface of the virus. The viral genome is enclosed in a protective capsid surrounded by the matrix.*

1.3.1.2 The *env* gene

The *env* gene is approximately 2.5 Kb and encodes a precursor protein (gp160) that is cleaved by viral proteases into the gp120 and gp41 proteins[9]. These proteins are required during the

early stages of viral replication to bind and fuse the virus with the host cell and represent major determinants of the host range of HIV[10].

1.3.1.3 The *pol* gene

The *pol* gene is approximately 3Kb and encodes a precursor polyprotein (p160) which is cleaved into several non-structural proteins viz. protease, reverse transcriptase, nuclease and integrase [9]. These proteins regulate viral genome replication and maturation of viral proteins.

1.3.2 Regulatory genes

1.3.2.1 The *tat* gene

The trans-activator gene, *tat*, encodes a protein (Tat) of variable length (86-104 amino acids) and of 14-16 kDa that is expressed early in the virus life cycle and released into the extracellular milieu by HIV infected cells[13,14]. It binds to heparin sulphate proteoglycans and accumulates in tissues from where it performs various activities[15]. Tat is essential for establishing an active HIV infection[16]. Accumulation of Tat in infected cells is followed by up-regulation of viral transcription and protein synthesis[17]. Tat also promotes the expression of viral genes by inhibiting the formation of the transcription terminating poly adenylation signal, facilitates HIV transmission from infected cells to naïve cells via RGD-integrin receptor interaction, activates naïve cells to create additional target cells for HIV infection, modulates host cell gene function and protects infected cells against HIV-specific immunity[14,16,18].

1.3.2.2 The *rev* gene

The *rev* gene encodes a 19 kDa phosphorylated protein (Rev) that regulates the expression of viral structural proteins, facilitates transportation of the pre-integration complex into the and stabilizes viral ribosome-associated mRNA[9].

1.3.3 The auxiliary genes

1.3.3.1 The *nef* gene

The *nef* gene encodes a functionally diverse 27 kDa protein (Nef) which is expressed early in the viral life cycle[19]. Nef is required for the establishment of high viremia, regulation of surface expression of CD4, CD25, CD28, and IL-2 cell activation receptors, down regulation of surface expression of MHC-1 and protection of HIV-infected cells from apoptosis[20,21].

Other auxiliary genes include the *vif*, *vpr*,*vpu* and *Vpx* genes[9]. Proteins encoded by these genes facilitate viral replication especially in non-dividing cells, formation of infectious progeny virus and virus release from the host cell membrane during the late stages of viral replication[22].

1.4 The origin of HIV/AIDS

The HIV/AIDS was first recognised in men-who have sex-with-men (MSM) and later in haemophiliacs and intravenous drug users [23]. Recently, the origin of HIV has been traced back to Kinshasa, the capital of Democratic Republic of Congo[24]. HIV is genetically similar to SIVsmm which infects and causes an AIDS-like infection in captive macaques[5,25,26]. This genetic similarity presumably implies that HIV might have emerged from zoonotic transfer of a primate virus to humans[5]. This transfer might have occurred in several discrete events, which represent the different groups of HIV-1 and HIV-2[5] and resulted from humans hunting chimpanzees, gorillas and monkeys, and sharing the same ecological niche with non-human primates[26]. HIV-1 subtypes M, N and O might have originated from the common chimpanzee, *pan troglodytes troglodytes*[25], HIV-1 subtype P from gorillas[6] and HIV-2 from sooty mangabeys, *Cercocebustorquatus atys*[5].

1.5 Epidemiology of HIV

The current global prevalence of HIV is illustrated in figure 2. According to the WHO/UNAIDS report on global HIV incidence and prevalence (2013), Sub-Saharan Africa

accounts for the majority (about 70%) of the global HIV pandemic, and a majority of new infections and HIV-related mortalities[2,3]. Although several factors have contributed to the high HIV prevalence in Sub-Saharan Africa, poverty is believed to be a major contributing factor. However, improvements in HIV prevention strategies (see section 1.8) in this region have resulted in a significant decline in the incidence (25%), similar to the decline reported in the Caribbean (42%) [2].

Like Sub-Saharan Africa, HIV AIDS is also a major pandemic in the low and middle income countries of South East Asia where it is estimated that over 5million people live with HIV[2]. In this region, poverty has also been reported to be the major contributing factor for the high HIV prevalence [27]. There is a sharp contrast in the HIV prevalence between the developed and developing world. It is estimated that fewer than one million people are infected with HIV in Europe, the Caribbean, the Americas, Oceania and Latin America[2]. This low prevalence might be attributed to improved management of infection, stronger prevention strategies and ease of access to the necessary medical attention, all of which elude the resource-limited settings of Sub-Saharan Africa and South-East Asia.

1.6 HIV distribution and the global epidemic

HIV-1 and HIV-2 are both genetically and geographically diverse[28]. Of the 8 recognised serotypes of HIV-2 discussed in section 1.2, only subtypes A and B have been identified in human epidemics [28]. The other serotypes are rarely isolated in human infections, and probably represent dead-end transmissions[5]. Subtype A and B epidemics are mainly restricted to Guinea Bissau (up to 10% prevalence), but they have been also identified in other West African states, Europe, South America, South Africa and India[29,30].

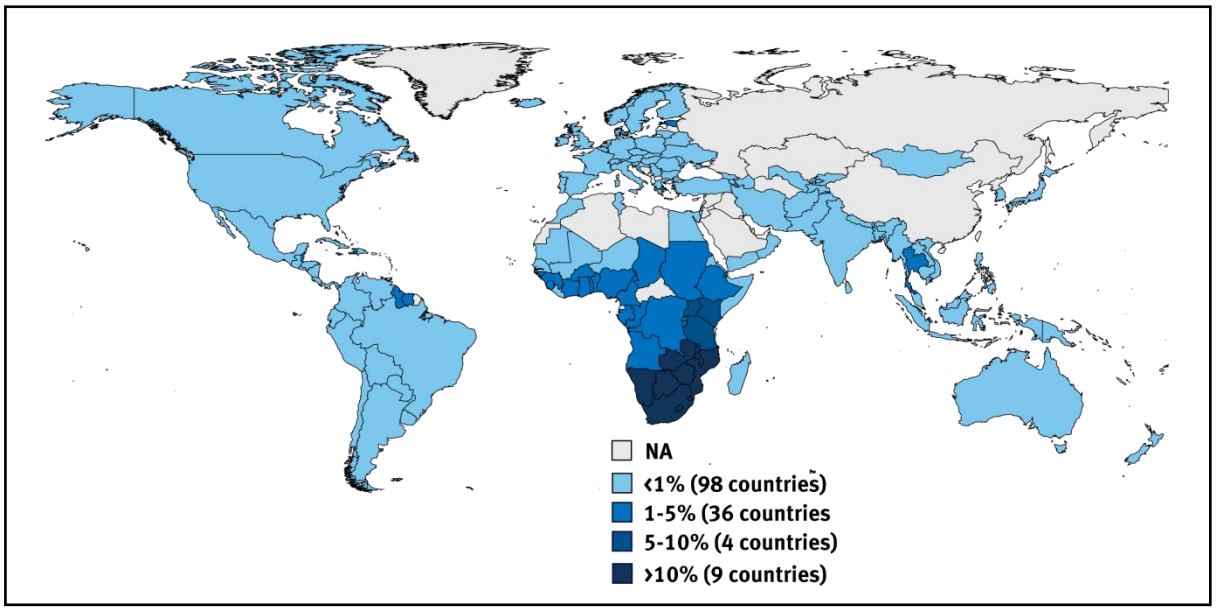

Figure 2: *Global prevalence of HIV*[2]*. Schematic diagram showing the worldwide % prevalence of HIV among adults 15-49 years of age.*

HIV-1 group M is the major group identified in the current global HIV pandemic and exists as subtypes A, B, C, D, F, G, H, K, or circulating recombinant forms, CRFs viz. CRF01AE and CRF04cpx[31]. Generally, all these subtypes are dispersed over the globe in discrete geographical regions and show equal circulation in Central and Western Africa[32]. However, half of the current global pandemic is caused by subtype C viruses[3]. HIV-1 groups N (Non O/Non M), O (Outlier) and P are minor groups and are mainly identified in West Africa[6,25].

1.7 Transmission of HIV

HIV is primarily transmitted sexually via the genital and rectal mucosa either as cell-free or cell-associated virus[33,34] and appears to proceed more favourably from female-to-male than male-to-female[35]. This route of transmission is responsible for more than 85% of all HIV infections [2]. However, in the absence of risk factors such as genital ulcers, HIV transmission across mucosal surfaces is generally inefficient and presents a transmission "bottle neck" that selects a single variant from a donor quasi species to establish a productive infection in the recipient[36].

Other routes of HIV transmission include percutaneous exposure from needle stick injuries and intravenous drug use, blood and organ transfer and mother-to-child transmission[2]. The majority HIV transmission occurs from an infected individual to a non-infected individual when the viral load is ≥1500 copies/ml[37,38]; thus the level of viremia predicts HIV transmission and the rate of disease progression. Other factors associated with a high risk of HIV transmission include unprotected sexual intercourse, commercial sex workers, living in a discordant relationship, migration, incarceration, medical injuries, corrective medical procedures and prior-existing STDs[39].

1.8 Reducing HIV transmission

Several strategies have been put in place to reduce HIV transmission, especially in low and middle-income countries where HIV is debilitating[40,41]. These strategies include HIV counselling, condom use, safe male circumcision, post-exposure prophylaxis, prevention of mother-to-child HIV transmission, proper waste disposal, occupational safety, tissue transplant and blood transfusion safety[40,42,43]. Indeed, in regions where these strategies have been implemented, there has been an appreciable decline in the number of new HIV infections and mortalities[2].

1.9 HIV life cycle

The life cycle of HIV proceeds in a complex, but well co-ordinated manner from binding and entry into the host cell, integration of viral DNA into the host cell chromosome, expression of viral genes and the production of nascent virions[44] as illustrated in figure 3.

1.9.1 Binding and entry into the host cell

Entry of HIV into the host cell is initiated by an interaction between the viral envelope gp120 and the primary HIV receptor, CD4[45] but also requires the expression of the β-chemokine co-receptors (CXCR4 and CCR5 receptors) located on the surface of $CD4^+$T-cells[46]. This interaction results in fusion of the viral envelope with the cell membrane and delivers the viral

nucleocapsid to the cytoplasm. Recently, gene therapy approaches have focussed on developing compounds that can neutralize HIV before it binds to and enters cells, and these appear to be promising in animal models[47]. Other cellular factors that participate in viral entry include, DC-SIGN, syndecan-1 protein, heparin sulphate proteoglycans and nucleolin, although these molecules are not normally found on CD^+ T-cells, but on other cells such as DCs[48,49].

1.9.2 Reverse transcription of viral genome

In the cytoplasm, the viral genome is reverse transcribed into double-stranded linear cDNA by the virus-encoded reverse transcriptase (RT)[50]. The newly synthesised viral DNA remains associated with the reverse transcription complex (RTC) until it is transported into the host cell nucleus as part of the pre-integration complex (PIC)[44].

1.9.3 Integration of viral DNA and translation of viral genes

Once in the nucleus, viral DNA is permanently integrated into the host chromosomal DNA at transcriptionally active domains[51]. This ensures that HIV proteins are produced throughout the cell's lifetime, during an active infection. It has also been reported that viral DNA might alternatively remain in the nucleus as unintegrated DNA either as 1 or 2 LTR circles formed after self-ligation of the HIV LTR at each end of the DNA[9]. The integration process is controlled by the viral integrase and host cell chromosomal DNA repair enzymes[50], and results in the formation of proviral DNA[51]. Other cellular and viral proteins that participate in this process include LEDGF, emerin, IN, Vpr, Tat, MA, DNA flaps, NF-kB, NFAT, importin 7 and importin β proviral DNA represents the template for transcription of viral RNA by cellular RNA polymerase II and the host cell transcription machinery into spliced and unspliced viral RNA transcripts[9]. These RNA transcripts are transported to the cytoplasm where they are translated into the various viral structural and non-structural proteins[52].

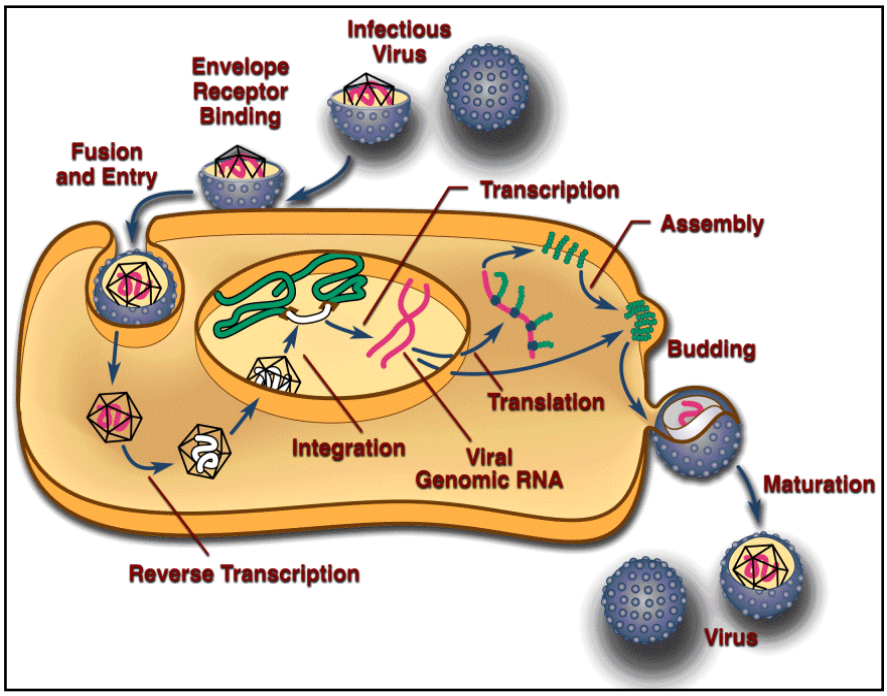

Figure 3: The replication cycle of HIV[53]: *The virion binds to and fuses with the cell membrane to enter the host cell. Inside the host cell cytosol, reverse transcription of the viral genome takes place under the control of virus-encoded reverse transcripatase to form viral cDNA. This DNA is delivered to the nucleaus, in integrates with host chromosome to form proviral DNA which represents the template for viral RNA synthesis prior to translation into viral proteins in the cytosol. The viral proteins are assembled in the cytosoland associate with the cell membrane to form immature virions which will later transform into mature and fully infectious HIV.*

1.9.4 Assembly of viral proteins and release of progeny virus

The translated viral proteins assemble as immature virions at the host cell plasma membrane[9]. At this stage, viral gp160 is cleaved by viral proteases into the gp120 and gp41 resulting in the final maturation of progeny viruses[54]. This process also requires *cis*-elements, ESCRTs, Ψ-site packaging signal, cellular cyclophilin A and the ATP-binding protein[54]. These newly synthesised mature viruses bud from the host cell plasma membrane[55].

1.10 The natural history of HIV infection

HIV infection is life-long[2] and progresses in defined stages viz. acute, chronic and AIDS phases as illustrated in Figure 4. The primary sites for HIV entry and initial virus replication are the gut and genital mucosa and the mesenteric lymph nodes[56] which possess a high concentration of resting memory $CD4^+$T cells expressing the CCR5 co-receptor and the α4β7 gut-homing integrin[57,58]. HIV then spreads systemically from these surfaces to infect other distant organs[59,60]. Normally, the initial infection is caused by a single founder virus that rapidly mutates to give rise to escape mutants forming the HIV 'quasi-species'[36,61]. This results in immunological escape and reversion to wild type viruses upon transfer to uninfected host cell, as mutations often come at a cost of viral fitness[62].

1.10.1 The primary stage of infection

The acute phase of infection is short (about a month)[63] and is mainly characterised by a high viremia (>10^7 RNA copies/ml) that peaks within three weeks in the absence of therapy and extensive depletion of $CD4^+$ memory T-lymphocytes[64]. As the infection continues, the immune system mounts a seemingly potent $CD8^+$T cell response that results in a dramatic rebound of $CD4^+$ T-cell counts to pre-infection levels and a decrease in viremia to a viral set point[63]. The set point also depends on viral virulence and the host HLA type [65] as discussed in section 1.10.3.2.

Acutely infected individuals present with several non-specific and self-limiting symptoms such as fever, fatigue, pharyngitis, myaglia, generalized lymphadenopathy, erythema, maculopapular rash, vomiting, diarrhea, nausea, weight loss, night sweat, neuropathy, mucocutaneous ulcers, headaches, aseptic meningitis, lymphopenia, thrombocytopenia, flu-like symptoms, dermatitis, mononucleosis and lymph node hyperplasia[63,64].

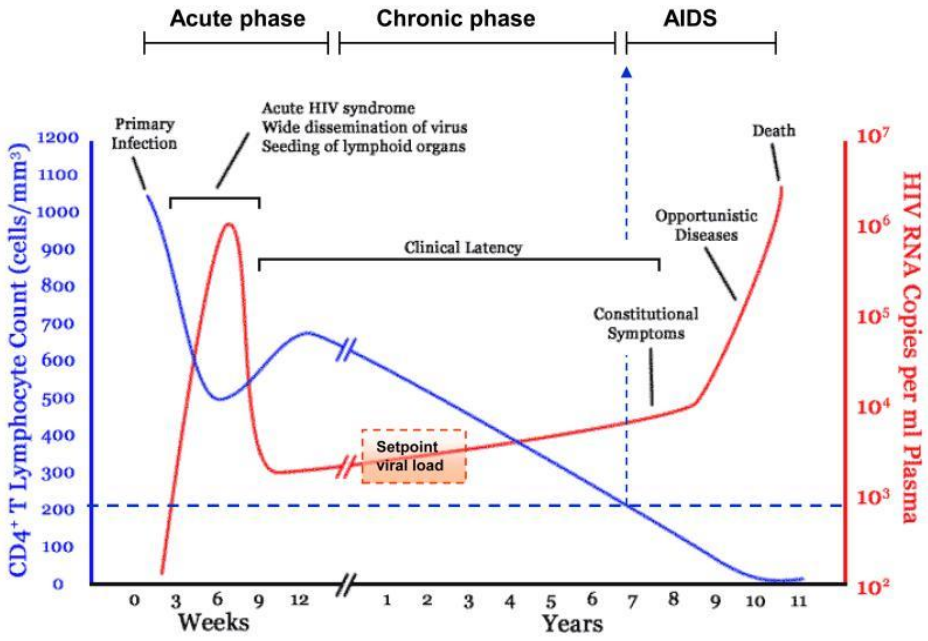

***Figure 4**: **The natural history of HIV infection**[66]. The infection proceeds in three phase viz. primary (acute) phase, chronic (latent) and AIDS (final) phases. These phases occur at different time points during the infection in different individuals.*

1.10.2 The chronic stage of HIV infection

This stage of HIV infection lasts for years in different HIV patients and is mainly characterised by chronic immune activation as demonstrated by increased expression of CD38, HLA-DR, CD71 and Ki67[63]. Chronic immune activation results from continuous virus replication, mucosal dysfunction, presence of opportunistic infections, and bystander activation of B and T cells resulting from increased production of pro-inflammatory cytokines[67,68].

Furthermore, chronic immune activation devastates the immune system by promoting immunological senescence, abnormal regulation of the cell cycle, increased T-lymphocyte proliferation, enhanced production of pro-inflammatory cytokines, severe mucosal and thymic dysfunction and loss of homeostatic control[67,69,70]. The chronic stage of infection is also characterised by the emergence of a new strain of HIV that utilises the CXCR4 receptor (X4

or T- tropic virus) and this is associated with severe cytotoxicity and extensive depletion of $CD8^+$ and $CD4^+$ T cell pools[71]. In some individuals, both X4 and R5 HIV strains co-circulate and this normally results in expedited loss of virologic control[72]. Therefore, in the absence of proper treatment, the infection rapidly progresses and death quickly ensues.

Apart from chronic immune activation and emergence of X4 viruses, the chronic stage of HIV infection is also characterised by establishment of latent HIV reservoirs in central memory $CD4^+$ T cells[73,74]. The mechanism by which latency is established is poorly understood, but it is thought to involve chromatin modification, cell activation, low level production of transcription factors and the critical selection of the integration site[75]. There are several elements that are thought to contribute to the establishment of latency, viz. histone acetylases (HATs), histone diacetylases (HDACs), DNA methylators and chemicals such as bromodomain inhibitors[76-79]. The physiological status of these molecules either enhances or depresses viral gene expression[80].

When activated, latent reservoirs provide a repertoire of replication competent viruses that are released into the circulation to counteract virologic control by the immune system[73,81]. This partly accounts for failure to achieve sustained virologic remission and lack of a HIV vaccine[74,82] and therefore, there is growing research interest in developing antiviral agents that can reactivate latent viruses and reverse latency. Some of these compounds are in advanced stages of clinical testing[75-77,83] and include protein kinase C agonists such as prostratin, bryostatin and diterpene compounds[84-86], HDACs inhibitors such valproic acid, vorinostat, romidespin and panobinostat[83,87], bromol domain inhibitors such as ingenol[88] and IL-2 and IL-7 agonists[89]. Recent evidence indicates that combining these LRAs augments the activity of each individual LRA and thus, more effectively reactivates latent HIV infections[86,90,91]. In addition, there is evidence that combining LRAs with HAART might be an effective strategy

to re-activate HIV infection and kill circulating virus or virus-infected cells, which would at least control viremia or even completely clear the infection[92-94].

However, there are concerns associated with the use of LRAs in re-activating HIV: (1) inefficient viral re-activation, (2) non-specific activity of LRAs, (3) re-activation might create new virus-producing cells that might not be readily recognised and cleared by the immune system, (4) LRAs reactivate a small minority of latently infected cells[76,95] and (5) LRAs are associated with toxic side effect[96]. Therefore, it has been suggested that repressing viral gene expression to maintain latency might be another feasible strategy to contain HIV infections[97,98]. This would require developing antiviral agents that primarily inhibit Tat whose roles in HIV pathogenesis and viral gene expression have been discussed in section 1.3.2.1. Nevertheless, none of these compounds has fully realised its theoretical promise.

1.10.3 The late stage of HIV infection

The final stage of HIV infection, AIDS, occurs several years after primary HIV infection and is mainly characterised by a positive p24 antigen test, a $CD4^+$T cell count of < 200 $CD4^+$ cell/ml, rapid disease progression and $CD4^+$T cell decline, severe immune suppression, emergence of syncytia-forming viral strains and severe opportunistic infections[99]. During this phase, individuals with a higher set-point progress faster to AIDS compared to those with a lower set-point[38] and if proper treatment is not administered, death ensues.

1.10.3.1 Typical progressors, rapid progressors, and long-term survivors

A majority (80%) of HIV-infected individuals normally progress to AIDS within 8-10 years after an asymptomatic infection[100]. Such individuals are referred to as typical progressors[63]. However, 15% of HIV-infected individuals experience extensive depletion of CD4 T cells in less than five years and rapidly progress to AIDS[101]. Such individuals are referred to as rapid progressors[102]. It is not clear what predisposes these individuals to rapid disease progression, but it is thought to be associated with the emergence of X4 viruses[71], viral genetic

polymorphisms[103], increased production of interferon-γ [104], production of poor quality cell-mediated immunity (CMI)[105] and presence of high risk HLA alleles such as B07, B35 and B3503 associated with rapid disease progression[65,101,106]. In contrast, long-term survivors present with a low CD4 T-cell (≤ 500 copies/µl), low viremia (<2000 copies/ml)[107] and therefore, progress slowly to AIDS[108]. It is not clear how long-term survivors are able to maintain their infection stratus, but identifying factors associated with help long-term survival might be useful in constructing a therapeutic vaccine against HIV.

1.10.3.2 Long-term non-progressors and elite controllers

Long-term non-progressors (LTNPs) constitute a small (about 5%) and unique group of HIV positive individuals who consistently maintain a low plasma viremia (≤ 10, 000 copies/ml) and high $CD4^+$ T cell counts (>850 cells/µl) throughout the course of infection, without necessarily requiring highly HAART[109,110]. Furthermore, a much smaller group (about 1% of LTNPs) of HIV infected individuals naturally and consistently maintain undetectable HIV viral load (≤ 50 copies/ml) for years without requiring HAART[111,112]. Such individuals are referred to as elite controllers (ECs)[113,114].

It is not clear how LTNPs and ECs control viremia and $CD4^+$ T cell decline. However, several studies have shown that LTNPs and ECs mount a robust, poly-functional gag-specific CMI response with broad cytotoxic activity[102,115-119], broad and cross-reactive ADCC responses[120] and non-canonical Tat-specific neutralizing antibody responses[121-123] which may explain the unique nature of the infection control. Apart from mounting robust immune responses, there are several other viral and host factors that might be responsible for this control including infection with attenuated and less evolved forms of HIV[124], protective HLA alleles A2, B27, B51, B5701, B5703, B5801 and B57[125,126], CCR5 Δ32 polymorphisms[127] and Δ *Nef* polymorphisms[128].

1.10.3.4 Highly exposed sero-negatives

Highly exposed sero-negatives (HEPs) are a unique group of individuals who are frequently involved in high risk behaviour, but persistently maintain a HIV negative sero-status[129,130]. This group mainly includes commercial sex workers, discordant couples, exposed infants born to HIV positive mothers and haemophiliacs[129,131,132]. HEPs have been studied extensively with the hope of replicating their unique protection in the form of an effective prophylactic HIV vaccine[133-136].

However, like other unique individuals who control HIV infection, the nature of protection against HIV infection is unclear. It is thought that protection might be a result of a more concerted interaction between several host and viral genetic factors[131,137], establishment of potent anti-viral immunity at mucosal surfaces[129] and reduced secretion of pro-inflammatory cytokines that would otherwise increase the risk of HIV acquisition[130].

Generally, HEPs, LTNPs and ECs have provided clues to what might correlate with protection against HIV during a natural infection and have inspired the design of candidate HIV vaccines[100,138]. Unfortunately, clinical trials of vaccines which tried to replicate this level of protection have been largely futile[139-141] or demonstrated modest protection and no effect on the viral load[142].

1.11 Innate protection against HIV infection

The HIV infection invokes innate and adaptive immune responses that are unable to eradicate the virus. The innate immune response is the primary response against an infectious organism[143]. This immune response is rapid, short-lived, non-specific, lacks memory, independent of cell surface immunoglobulins and T cell receptors, and not restricted by MHC haplotype[144]. The innate immune system recognises infectious organisms via pathogen recognition receptors (PRRs) that detect conserved Pathogen Associated Molecular Patterns (PAMPs)[144,145]. These PRRs include C-type lectin receptors (CLRs) (Dectin-1 and 2)[146], Toll-

like-receptor (TLRs)[144], retinoic acid inducible gene-1 (RIG-1)-like receptors (RLRs)[147] and nucleotide-binding domain and leucine rich repeat-containing receptors (NLRs)[148]. The interaction between these PRRs and PAMPs activates innate cells such as NK-cells, macrophages and DCs to secret pro-inflammatory cytokines and chemokines[144,145] which in turn activates the adaptive immune system[149]. Therefore, innate immune cells provide a bridge between the innate and adaptive immune systems[149].

The mucosa provides the first barrier to primary HIV infection[33,150], as previous studies have shown that inflammation of genital mucosa increases the risk of HIV acquisition in humans[151-153]. The mucosa is covered in a layer of epithelial cells which produce several secretions such as antibodies, cytokines, complement, lytic enzymes, surfactant proteins, leucocyte protease inhibitor-1 (SLP-1), APOBEC3G, tetherin and defensins[149,154]. Other secretions produced by epithelial cells include granzyme A, perforin, FasL and granulocyte-macrophage colony stimulating factor[155,156].

Furthermore, DCs, macrophages and monocytes in the epithelium act as professional APCs to enable antigen processing and presentation via MHC[157]. These APCs link the innate immune system to the adaptive immune system and thus, shape the resultant antigen-specific immune response[158]. However, epithelial cells at times facilitate HIV spread through bystander activation of $CD4^+$T cells, enhancing HIV transcytosis across the epithelium, acting as reservoirs of latent virus and secreting various pro-inflammatory cytokines which enhance HIV infection[159,160].

1.12 Adaptive immunity and correlates of protection against HIV

Unfortunately, the correlates of immune protection against HIV are poorly understood since there is no evidence of natural recovery from HIV infection[161,162]. However, evidence accumulated over 3 decades of research suggests that humoral responses might be crucial for

sustained protection against primary HIV[163], whereas cell-mediated immunity might be vital in controlling viremia and disease progression[161,164].

1.12.1 Non-neutralizing antibodies

At mucosal surfaces, the principal antibody present is sIgA[165], but IgG has also been detected[166]. These antibodies utilise their Fab-fragment to bind to the surface of HIV and their Fc-fragment to recruit cells that possess the Fc-receptors such as APCs (DCs and macrophages)[167]. Furthermore, through Fc-receptor function, non-neutralizing antibodies opsonise HIV-infected cells, activate complement, exert ADCC, ADCVI, ADCP and ADCD[168-171] on HIV-infected cells and inhibit HIV transcytosis across the epithelium[165,172]. In fact, establishment of such antibody responses appears to directly correlate with improved virologic control in HIV infected individuals[173,174], SIV infected non-human primates[169,175,176], and recent evidence suggests that antibodies capable of ADCC activity show efficacy against reactivated HIV[177,178]. This evidence suggests that vaccines that induce non-neutralizing antibodies at mucosal surfaces might offer protective benefits against HIV, and thus showed be studied in detail.

1.12.2 Neutralizing antibodies (NAbs)

Approximately 28 days after acute HIV infection, autologous NAbs emerge[63]. These antibodies normally target the variable region of gp120 and are able to neutralize the transmitted HIV strain, but not the quasi-species. As viral evolution continues during HIV infection, broadly neutralizing antibodies (bNAbs) emerge[163,179,180] and are detectable as early as 6 months post-infetcion in some individuals[181]. These antibodies mainly target the conserved regions of gp120 (antibody 2G12)[182], the CD4 binding site (antibodies b12 and VRC01)[183,184], the MPER region of gp41 (antibodies 2F5, 4E10, and m66)[185], the quaternary proteoglycan epitopes (antibodies PG 9 and PG 16)[186] and the glycan outer domains (antibodies PGT 128 and PGT 121)[187,188].

Broadly neutralizing antibodies are more potent and cross-reactive against different HIV strains than autologous neutralizing antibodies. Previous studies have shown that passive or gene transfer of bNAbs protected animals against SHIV infection post challenge[189-194]. It has also been shown that bNAbs VRC01, PGT 128 and PGT 121 efficiently inhibit cell-to-cell transmission of HIV[195]. These antibodies do so by accumulating at virologic synapses to inhibit the formation of clusters between virus-infected and target cells, and transfer of viral genomic material from the virus-infected and target cells[195]. Such observations suggested that bNAbs might prevent primary HIV infection, when induced appropriately. However, due to several challenges encountered in generating broadly neutralizing antibodies (discussed in section 1.12.2.1 below), they are still unable to clear the virus.

1.12.2.1 Challenges in inducing broadly neutralizing Env antibodies by vaccination

Attempts to design an HIV vaccine that can induce bNAbs have been futile so far[140]. This partly results from poor B cell activation by envelope proteins due to the paucity of Env trimers on the surface of HIV[179,196]. Furthermore, the envelope domains that are targeted by bNAbs are hyper glycosylated, variable in structure and occluded by non-immunogenic N-linked glycans[197,198]. These hurdles have complicated the design of stable and immunogenic Env immunogens. Moreover, bNAbs normally take up to four years to emerge after primary HIV infection[163,179] and are characterised by high levels of somatic mutations in the immunoglobulin genes, auto-reactivity and extra-long antibody combining regions (heavy chain determining regions, HCDRs)[199-202] which are difficult to replicate in an immunogen. To address these concerns, several researchers have developed and tested multi-clade or mosaic Env immunogens that optimally present broadly neutralizing epitopes[203,204], Env immunogens that can activate germline B cell receptors to produce bNAbs[205,206] and developed stabilised trimeric forms of gp120 that can be optimally recognised by the immune

system[207-211]. Although these novel immunogen designs are promising, their potential is yet to be realised.

1.12.3 Tat neutralizing antibodies

As discussed in section 1.3.2.1 Tat is crucial in the pathogenesis of the HIV infection. Therefore, targeting Tat might help control viremia and disease progression in HIV-infected individuals. Theoretically, anti-Tat antibodies block the integrin-mediated intake of HIV into monocyte-derived DCs thereby preventing HIV infection of these cells[212]. Therefore, producing these antibodies by vaccination might offer protective benefits against HIV. Furthermore, there are several attributes that support the use of Tat as a component of a candidate HIV vaccine.

Tat possesses several conserved immuno-dominant B cell epitopes that can be targeted to induce cross-clade neutralization antibodies[213,214]. Tat possesses immuno-modulating properties as it can enter DCs and cells of the reticulo-endothelial system via its RGD (arginine-glycine-aspartic acid) and protein transduction (PTD) domain interactions[13,16,17,213]. Tat also modifies the composition of the proteosome to enhance the presentation of cytotoxic T cell epitopes for heterologous antigens[215] and promotes DC maturation and functionality[216]. Although some studies show that the presence of Tat-specific antibodies does not correlate with reduction in viremia[217,218], about 20% of HIV positive individuals who develop Tat-specific antibodies[219] show improved control over loss of $CD4^+$ T cells and progress to disease more slowly[121,122,214]. The reason behind this controversy remains poorly understood.

Nevertheless, previous clinical trials have also demonstrated that Tat-based vaccines are safe, well tolerated and induce humoral and CMI responses[17,123,220,221] and such responses are required to restore immune homeostasis[221,222]. In addition, these responses correlated with improved control over disease progression and reduced viremia in vaccinated

individuals[123,222,223]. It has also been reported that Tat-specific responses protected vaccinated animals against SHIV[16,224-228] and SIV [229,230].

In other studies, the inclusion of Tat in candidate HIV multi-immunogen vaccines enhanced the levels of HIV Gag-and Env-specific immune responses and correlated with improved protection[227,231]. This accumulated evidence support further consideration of Tat as a potential component of an effective HIV. For this reason, therefore, this thesis attempted to generate a candidate HIV vaccine with the ability to induce high titer anti-Tat humoral responses (see chapter 3 of this thesis).

1.12.4 Cell-mediated immunity (CMI)

During acute HIV infection, CMI responses are mainly type 1 (Th1 $CD8^+$T cells) responses and are characterised by potent cytotoxic T lymphocyte (CTL) activity[63]. CTLs recognise HLA-class 1 restricted peptide epitopes that are located on the surface of virus-infected cells to trigger cellular cytotoxicity and apoptosis[232]. CTL responses control viral replication and maintain the viral load at the set-point[63,233]. As mentioned from sections 1.10.3.2 to 1.10.3.4, the establishment of CMI is crucial in protecting HEPs, LTNPs and ECs against HIV, and loss of CTL responses results in rapid SIV disease progression in animals[234-236]. Therefore, it is generally agreed that the early induction of such immune responses by vaccination might clear or at least control HIV replication at the primary site of HIV infection[162,164].

Apart from Th1 responses, type 2 (Th2 $CD4^+$T cell) CMI responses are also crucial for effective HIV infection control[138,237]. Th2 responses are vital in activating B cell differentiation, switching antibody classes, regulating cellular homeostasis and shaping Th1 immune responses[237-239].

Like Th1 responses, the establishment of high avidity and high magnitude Th2 responses (especially Gag-specific) against HIV results in an improved infection control and delays disease progression[238,240,241]. However, HIV-specific $CD4^+$ T cells are susceptible to HIV

infection[45,46] and thus, their frequency and functionality declines as the infection progresses[105,242]. Unfortunately, T cell responses develop too late to prevent primary HIV infection and are unable to contain escape mutants[243]. Furthermore, efforts to replicate T cell immunity observed in LTNPs and ESs were futile[244,245].

1.13 Treatment of HIV infection

To date, HAART is the gold standard treatment against HIV infections[2]. It is a combination therapy (cART) of at least three different classes of antiretroviral drugs (ARVs), usually a nucleoside analogue, a non-nucleoside analogue and a protease inhibitor[246] (see table 1.0). In this way, there is enhanced drug synergism[40,246], reduced drug adverse effects and delayed emergence of drug-associated resistance[247].

Although HAART has failed to cure HIV infection, it delays establishment of latent reservoirs[248-250], results in immune restoration to near pre-infection levels[251], suppresses viremia below detection levels[252,253], reduces the rate of progression to AIDS[254] and reduces the chances of HIV transmission[40].

It is generally accepted that the benefits of HAART are best realised when therapy is initiated early, preferably when the CD4 count is 300-350 ml and before the emergence of opportunistic infections[255]. Indeed, early administration of HAART has been shown to be beneficial in post-treatment controllers[249,252,254,256,257], who make up about 15 % of HIV positive individuals and have the ability to achieve long-term virologic remission even when cART is interrupted[249,258,259].

However, HAART has not fully achieved its potential due to the chronic nature of HIV infection, emergence of drug-resistant mutants, drug-associated adverse effects and the huge long-term financial burden that the treatment imposes on individuals and low-income economies[40,246].

Apart from HAART, allogeneic stem-cell therapy has emerged as another treatment option for HIV infection. This therapy has been associated with enhanced virologic control in HIV patients[260-262]. Previously, Timothy Brown was functionally cured from HIV infection after two rounds of stem-cell therapy from a matched CCR5 Δ32 homozygous donor[263]. Although the mechanism of protection in this patient remains poorly understood, the Δ32 CCR5 mutation has been shown to abolish the ability of HIV to recognise and use the CCR5 co-receptor during the early stages of infection resulting in resistance to HIV infection as observed in this patient[264,265].

However, attempts to replicate this level of protection against HIV have been futile so far[266] . It should be also noted that stem cell therapy is risky and expensive, and thus it might not be adopted globally in the management of HIV infection. In addition, the CCR5 Δ32 allele is extremely rare (< 10% and mainly in Europe and Western Asia)[267,268], thus donors with this allele are equally rare. These pitfalls further highlight the need for an effective vaccine against HIV as alternative to the expensive and inaccessible treatment approaches currently available.

1.14 Vaccine evolution

Vaccination is cheap and affordable, and is generally regarded as the optimum strategy to contain and prevent infections, especially in low and middle-income countries[138,161]. A majority of licensed human vaccines induce neutralising antibodies that correlate with protection[269]. These vaccines are categorised as live-attenuated, killed, sub-unit or virus-like particle (VLP) vaccines.

Antiretroviral drugs class (es)	Drug (s)
Nucleoside and nucleotide reverse transcriptase inhibitors (NRTIs)	Tenofovir, abacavir, zidovudine, stavudine, lamivudine, emtricitabine and didanosine
Non-nucleoside reverse transcriptase inhibitors (NNRTIs)	Efavirenz, nevirapine, etravirine and delavirdine
Integrase inhibitors	Raltegravir
Protease inhibitors	Fosamprenavir, atazanavir, darunavir, lopinavir, saquinavir (ritonavir), nefilnavir, indinavir and tipranvir
CCR5 inhibitors	Maraviroc
Fusion inhibitors	Enfuvirtide
Integrase transfer inhibitors	Raltegravir and dolutegravir

Table 1.0: Summary of ARVs in clinical practice[247,270]*. The majority of these drugs are administered in combination therapy to enhance their synergy and effectiveness.*

1.14.1 Licensed vaccines against human viral infections

1.14.1.1 Live-attenuated vaccines

Live-attenuated virus vaccines (LAVs) are either naturally or artificially attenuated to retain their immunogenicity, but show reduced or no pathogenicity in humans[271]. In addition to inducing robust and long-lasting protection, LAVs do not require adjuvants for effective immune stimulation[272]. LAVs have been developed and used successfully against smallpox,

polio, yellow fever, rotavirus, mumps, rubella, measles, influenza, hepatitis A virus infection and recently against Dengue virus infection[269,273]. However, attenuation to generate LAVs sometimes reduces vaccine efficacy and there are concerns over LAVs reverting to virulence.

1.14.1.2 Killed or inactivated vaccines

Killed or inactivated viral vaccines are derived from viruses that have been inactivated by use of heat, radiation, or chemicals[274]. The inactivation process ensures that the virus is killed, but the immunogenicity is preserved. Therefore, inactivated virus vaccines cannot revert to virulence, are safe, well tolerated and immunogenic. The currently licensed inactivated vaccines available for human use include: the inactivated polio vaccine (IPV), inactivated hepatitis A vaccine and the rabies vaccine[274]. Generally, killed vaccines generate short-term immunity in vaccinated individuals and thus, require multiple booster doses and adjuvants to generate long-term protection[274]. These hurdles have greatly frustrated attempts to use killed forms in HIV vaccine development.

1.14.1.3 Subunit vaccines

Sub unit vaccines contain a component of the virus that is immunogenic, but non-infectious[275,276]. These vaccines are usually safe and immunogenic when administered to humans[277]. The only licensed subunit vaccine is against influenza (the trivalent influenza A vaccine, TIV) in humans[276]. However, the TIV is less stable and induces short-term protection in vaccinated individuals when compared with cold-adapted trivalent influenza vaccine (CAIV-T)[276].

1.14.1.4 Virus-like particles vaccines

Virus-like-particle (VLP) vaccines contain multimeric viral proteins that are similar in organisation and conformation to the native forms of the virus, but they lack the viral genome[278]. These vaccines are replication defective, safe and highly immunogenic in humans,

and thus have been widely considered as candidate HIV vaccines by several research groups[279-281]. Currently, there are three VLP-based vaccines available for human use viz. the human papillomavirus vaccine[282], the hepatitis B vaccine[283] and the hepatitis E vaccine [284].

1.15 HIV vaccine development

There is no effective HIV vaccine and this stems from our poor understanding of markers of convalescence which usually correlate with desirable features of a vaccine[161,285]. Furthermore, there is lack of suitable vaccine adjuvants and animal models that could be used in preclinical testing of candidate HIV vaccines[285]. Moreover, HIV is a "quasi-species" that is globally diverse and continuously evolves within its host and undergoes latency during the early stages of infection[100,161], which further complicates designing a vaccine that can provide global protection against HIV.

As the search continues for an HIV vaccine, it is generally accepted that an ideal HIV vaccine will have to induce robust HIV-specific CMI[164], with a high magnitude and avidity as such high quality responses often result in an improved control over HIV infection and delayed disease progression in humans[119,286,287]. In addition, the vaccine will have to induce potent antibody responses which are thought to be important in preventing primary HIV infection[82,188,288]. It will be particularly important for these responses to be established at mucosal surfaces and systemically for effective prevention and control.

1.15.1 Previous HIV vaccine clinical trials

As most licensed vaccines induce protective neutralizing antibodies[269], initial efforts focussed on developing recombinant vaccines encoding HIV envelope proteins to generate such protective antibodies[140,289-292]. As these efforts failed, attention was switched towards developing an HIV vaccine that would induce CMI[139,141]. However, clinical trials for CMI-based vaccines have also been futile[139,141].

1.15.1.1 The AIDSVAX HIV vaccine trial

The AIDSVAX HIV vaccine trial was a randomised, double-blind, placebo-controlled, phase III trial aimed at assessing the protective efficacy of a bivalent recombinant HIV gp120 clade B/E vaccine[140]. This trial was conducted in intravenous drug users in Bangkok, Thailand, with a primary end-point of controlling HIV-1 infection. This vaccine was safe and well tolerated, but had a low efficacy and did not protect against HIV acquisition in vaccinated individuals[140], and the results were similar to those generated from earlier clinical trials conducted in high risk HIV cohorts of MSM[289,290].

1.15.1.2 The Merck™ STEP and Phambili trials

The STEP and Phambili trials were multi-site phase II double-blinded test-of-concept trials of a trivalent Ad5 Gag/Pol/Nef vaccine[139]. These vaccines were formulated in a 1:1:1 ratio in which each HIV protein was encoded by a single replication-defective recombinant Ad5 vector. The trial tested vaccine safety, tolerability and vaccine efficacy in vaccinated individuals. Preliminary evaluation of the trial results showed that all vaccinated individuals developed modest CMI responses[293]. However, the vaccine failed to protect vaccinated individuals and there was a reported higher risk of HIV acquisition among vaccinated and uncircumcised men who had high levels of pre-existing immunity to Ad5[294,295]. It has been suggested that Ad5 Nabs enhanced systemic immune activation and inhibited activation of an appropriate innate immune response to Ad5[245,295,296] which increased HIV acquisition among Ad5 sero-positive vaccinees. Furthermore, a similar trial (HVTN 505) that tested a DNA prime recombinant Ad5 boost showed no efficacy and was prematurely terminated[141].

The data from these clinical trials suggested that CMI responses were probably not essential in controlling HIV-1 infection, and thus they dealt a huge blow to the development of CMI-based HIV vaccines. However, more recent data from macaques studies conducted by Hansen and colleagues have shown that the establishment of effector memory CD8 T cell responses is

absolutely critical in SIV control[297-300]. These data have re-energised research into the development of novel HIV vaccines capable of inducing robust CMI.

1.15.1.3 The Thai trial: RV144

The RV144 trial was a community-based, randomised, multicentre, double-blinded phase IIb HIV trial that assessed the protective efficacy of priming with recombinant canary pox virus (ALVAC-HIV-vCP1521) vaccine and boosting with the AIDSVAX^{Tm}gp120 B/E vaccine[142]. The vaccine regimen was well tolerated amongst vaccinated individuals, in whom only minor and self-limiting adverse effects were reported. Although the RV144 vaccine regimen did not reduce viremia and prevent $CD4^+$ T cell loss, it resulted in a 31.2% efficacy following a modified-intention-to-treat analysis and generated modest-but-short-lived protection against HIV infection in vaccinated individuals[142]. This protection was mainly attributed to ADCC, infectious virus capture and viral neutralization activity as a result of synergy between antibodies that target the envelope constant region 1 (C1) and variable regions 1 and 2 (V1/V2)[162,171,301,302] and partially due to patients' genetics[303] .

Unfortunately, the trial reported no statistical significant differences in viral load between vaccinated and non-vaccinated individuals[142] to suggest no effect on viremia. However, to date, the RV144 trial remains the only clinical trial to have demonstrated efficacy of a candidate HIV vaccine. This trial highlighted the importance of a prime-boost strategy and the potential of non-neutralizing antibodies and CD4 T cell-mediated responses in HIV vaccine development. In fact, the results from this trial are likely to provide a basis on which future HIV clinical trials will be based in design and conduct.

Recent follow up studies of the RV144 trial[142] showed that the vaccination regimen induced predominantly poly-functional effector memory CD4 T cell-responses that targeted the HIV Env V2-region, produced IL-2 and IFN-γ and were associated with cytotoxicity and vaccine efficacy[241,304]. Section 1.12.4 noted that CD4 T cell responses are crucial for efficient B cell

proliferation and maturation. Indeed, it was postulated that the vaccine-induced CD4 T cell responses in the RV144 trial provided sufficient help for B cell maturation, but were insufficient to control the HIV viral load[241]. Furthermore, several studies have also shown that the establishment of CD4 T cell responses similar in characteristics to those generated by the RV 144 vaccination regimen correlated with improved virologic control[305-308].

1.16 Improving HIV vaccine efficacy

1.16.1 Mucosal vaccination

Providing protection at the mucosa, the site of a majority of primary HIV transmissions[2], will be important in preventing HIV transmission and primary HIV infection. This is because a single "founder" virus is believed to result in an established HIV-1 infection following primary transmission at the mucosa[35,36,309], which gives a window of opportunity to more effectively control or even eradicate the infection at this stage than later on when "quasi-species" emerge. However, previous HIV vaccine development strategies focused on systemic vaccination[139,142,292,310] which induces responses that don't home to the mucosa[311], hence did not provide mucosal protection. Therefore, there has been a switch to mucosal vaccination as a way of generating robust mucosal protection.

Mucosal vaccination involves delivering vaccines directly onto mucosal surfaces via the oral, rectal, vaginal or ocular routes. It is a needle-free procedure, non-invasive, safe, painless, fast, cheap, acceptable and easy to administer even in mass vaccination[312]. Mucosal vaccination induces robust, long-lasting, high avidity antigen-specific immune responses at local and distant mucosal surfaces, and systemically[313,314] and can be used to overcome pre-existing systemic immunity to the vaccine vector[315-318].

Intranasal vaccination is probably the most convenient route to induce robust protection at the mucosa as it induces local immunity at the nasal mucosa, at distant mucosal surfaces of the

vagina, rectum and the oral-pharynx and systemically as reported in various non-human primate studies[314,319-323].

There are several concerns associated with administering vaccines mucosally and these, to some extent, have inhibited its global success. Mucosally administered vaccines can be broken down by mucosal secretions and vaccine uptake can be limited by various mucosal barriers[324]. Therefore, mucosal vaccines are generally administered in a high dose which makes the optimum dose difficult to determine, presents safety concerns and might induce anergic responses[325].

1.16.2 Adjuvants

Adjuvants are substances that enhance vaccine efficacy by preserving the conformational integrity of the vaccine immunogen, activating effector cells, stabilising vaccines during storage and attracting APCs to the point of vaccination for optimum immunogen uptake by APC[326]. There are two groups of adjuvants viz. particulate and non-particulate[327]. Particulate adjuvants target immunogens directly to APCs especially DCs and include ISCOMs, aluminium salts, oil-water emulsions (MF59 and AS03), liposomes, calcium salts, virosomes and immunogen-containing nano-particles[326]. These adjuvants act through the formation of an immunologically inert depot at the site of injection from where immunogens are slowly released and phagocytosed by APCs[326,328].

Particulate adjuvants might also recruit DCs to the site of vaccination through inducing cell death which results in release of danger-associated molecular patterns (DAMPs)[329,330], inducing formation of an Nlrp3 inflammasome complex[331,332] and facilitating the accumulation of uric acid[326]. Alum, MF59 and AS03 are the only licensed adjuvants for use in human vaccines[333], highlighting the need for novel adjuvants particularly for use in HIV vaccines.

On the other hand, non-particulate adjuvants are agonists which either bind to ganglioside receptors (cholera and *E.coli* toxins), PRRs (bacterial LPS, muramyl dipeptide (MDP)), saponins, mannan, cytidine-phosphate-guanosine oligodeoxynucleotides (CpG) or cytokine receptors (IL-1, 5, 6, 12 and IL-15) to exert their activity[326,334]. PRR agonists especially LPS (TLR 4 agonist) and CpG (TLR 9 agonist) are the most widely studied non-particulate adjuvants[328]. Unfortunately, none of the adjuvants has yet been approved for use in humans.

1.16.2.1 The C4-binding protein (C4b-p)

The C4b-p is a soluble protein that regulates the lectin and classical complement activation pathways by controlling the relative levels of c3 convertase[335]. This protein is found mainly in plasma of many organisms including humans, produced by hepatocytes and polymerises in the endoplasmic reticulum[336]. The C4b-p is a 570 kDa polymeric protein that exists in several isoforms (three isoforms in humans and one isoform in mice), the majority of which contain 7 identical α-chains and a single β-chain (α7β1) linked at the C-terminus[335]. Other isoforms consist of 6 α-chains and a single β-chain (α6β1) or are entirely made of α-chains (α7β0)[337].

The C4b-p protein appears as a spider or octopus under the electron microscope with the α-chains forming the extended tentacles[338], as shown in figure 5. Eight and three complement control protein (CCP) domains, respectively are contained within the α-chains and β-chain[335]. In addition, the C-terminus of the α-chain (57 amino acids) contains an amphiphatic α-helix region that is required for polymerisation[336]. These protein oligomers are stabilised by the cysteine residues located within the α-chains[335]. The cysteine residues also form disulphide bonds that hold the α-chains in humans. However in mice, the α-chains are held together by non-covalent interactions[339]. During circulation, C4b-p is associated with vitamin K-dependent protein S, which gives C4b-p the ability to interact with negatively charged phospholipids in the cell membrane[335].

1.16.2.1.1 The C4-bp as an adjuvant

As mentioned in section 1.16.2 above, there are few adjuvants licensed for use in human vaccines. This underlines the need to develop new adjuvants especially for use in candidate HIV vaccines. Earlier animal studied that tested the efficacy of vaccines containing the C4b-p protein as an adjuvant showed promising results[340-345] and these results have been confirmed in more recent animal[346] and human studies[347]. Most importantly, these studies have shown that the addition of C4b-p protein greatly enhances both humoral and CMI, which are thought to be important in preventing and controlling many viral infections, including HIV infections.

Ogun et al, 2008 reported that four of six BALB/c mice that received a protein vaccine consisting of the *Plasmodium yoelii*MSP119merosoite surface protein (MSP)-19 fused to the murine C4b-p developed high titer and protective antibodies against a lethal dose of *Plasmodium yoelii*[343]. All unvaccinated animals had progressively high parastaemia and were culled a week after challenge with *Plasmodium yoelii*-infected RBCs.

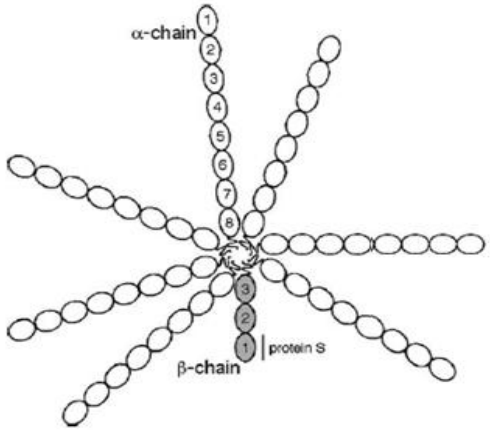

Figure 5: Structure of the C4-bp: Structure of the C4-bp[338]. *Octopus or spider-like appearance of the C4b-p with the 7 identical α-chains and one β-chain extended appearing extended, as seen under an electron microscope.*

However, MSP-19-murine C4b-p vaccinations induced antibodies that also cross-reacted with the circulating native murine C4b-p. Furthermore, the same study also demonstrated that a hybrid C4b-p, IMX313 for example, which was genetically modified to contain a chicken C4b-p motif and < 20% similarity to human and murine C4b-p when fused to MSP-19 generated antibodies with superior titers as compared to vaccination with MSP-19 human or murine C4b-p conjugates. This variation in adjuvant activity has been attributed to variation in oligomer strength and stability[348].

Another study also reported that priming with recombinant adenovirus and boosting with recombinant pox virus encoding *Plasmodium yoelii*MSP119 merosoite surface protein (MSP)-42 fused to murine C4b-p generated higher titer and protective MSP-42-specific IgG than recombinant viruses encoding the non-conjugated MSP-42. These antibodies completely protected 10 of 17 vaccinated animals against a lethal challenge with *Plasmodium yoelii*[349]. In addition to enhancing humoral responses, Forbes and colleagues (2012) reported that addition of IMX313 to a recombinant adenovirus vaccine encoding MSP also enhanced the magnitude of MSP-specific CMI, particularly multi-functional $CD8^+$ and $CD4^+$ T cells that secreted both IL-2, TNF-α and INF-γ in animals following a single vaccination[344]. Spencer et al (2012) has also reported similar results from vaccination of BALB/C mice and rhesus macaques with a recombinant MVA vaccine encoding *Mycobacterium tuberculosis* protein 85A fused to IMX313[345].

Although the mechanism by which C4b-p enhances immunogenicity remains poorly understood[343,344], there is evidence suggesting that the protein acts as a cofactor to the serine protease factor 1 required for the proteolytic activation of c4b to prevent the formation of (c4bc2a) and binds nascent c4b to prevent its assembly (c4bc2a)[338,350].

Furthermore, C4b-p binds B cell CD40, C-reactive proteins, pentraxins and serum amyloid P-complexes resulting in increased Fcγ-receptor-mediated phagocytosis and cytokine

secretion[344]. This interaction also enhances CD54 (ICAM-1) and CD86 expression and IL4-dependent IgE isotype switch[351]. As a result, it enhances immunogenicity, with a dose-sparing effect[344]. It has been also suggested that efficient polymerisation and secretion of C4b-p is essential for adjuvanticity[344,349] and polymerisation increases and prolongs antigen presentation by APCs[345,352], increases serum half-life of antigens[343,353] and amplifies functional domains within immunogens[354].

Collectively, this body of evidence mandates further investigation into the use of C4b-p protein as a molecular adjuvant to augment the choice of adjuvants available for use in human vaccines, especially against HIV infections. Therefore, this thesis attempted to use IMX313 as a molecular adjuvant to enhance the immunogenicity of a candidate Tat- based HIV DNA vaccine (see chapter 3).

1.17 New HIV vaccine development strategies

Although LAVs confer protective benefits against SIV in macaques[355-357], they are not considered extensively in the development of HIV vaccines mainly over safety concerns[285]. There are fears over live attenuated forms of HIV reverting to virulence, recombining with a wild-type strain, inducing uncontrollable immune stimulation, integrating into the host chromosome and producing low vaccine efficacy due to attenuation[285].

1.17.1 HIV-VLP-based vaccines

VLPs possess several attributes as vaccines (described in section 1.14.1.4), and these have spurred in-depth exploitation of VLP-based vaccine designs in HIV vaccine development. HIV VLPs mainly contain the highly conserved and immunogenic Gag protein or its precursor protein pr[55], but may also contain Env proteins[358,359]. HIV VLPs have been produced using the baculovirus expression system, yeast expression, drosophila S2-cells, bacterial plasmids and plant systems[360,361]. Although HIV VLP-based vaccines have been promising in animals[362,363], they are yet to realise their potential in humans.

1.17.2 Recombinant plasmid DNA-based HIV vaccines

DNA vaccines consist of a plasmid backbone, a foreign gene encoding an immunogen, an enhancer and poly-adenylation signal required for efficient gene expression[364]. These vaccines are safe, cheap to construct, relatively stable at high temperatures, non-toxic, reproducible, well tolerated, can be manipulated to encode various immunogens, target key antigen epitopes and induce both humoral immunity and CMI at the mucosa and systemically[365]. Furthermore, plasmid DNA possesses an inherent adjuvant effect arising from the unmethylated CpG motifs, is not affected by pre-existing immunity while vaccine immunogens are processed and presented in a manner similar to infection with a native virus[365,366].

DNA vaccines are licensed for veterinary use against haematopoetic necrosis virus in salmon[367], West Nile virus in horses[368], melanoma in dogs[369] and growth hormone releasing hormone to prevent foetal death in swine[370]. The effectiveness of these vaccines in animals suggests that DNA vaccines can be used in humans.

However, in humans, DNA vaccines suffer from inefficient plasmid delivery, poor distribution in tissues, susceptibility to phagocytic breakdown and low plasmid uptake by APCs, thus require administration of a high dose to evoke an effective immune response[365,371]. Nevertheless, recent studies have shown that a DNA-based vaccine elicits antigen-specific immunity that controls progression of tumor lessions and cervical cancer in humans[372,373] and that a DNA vaccine delivered by electroporation protected 68% of vaccinated rhesus macaques against repeated low dose intravaginal challenge with pathogenic SIVsmE660[374]. These studies provide further evidence that DNA vaccines, especially against HIV, can be effective in humans, if optimally designed. Thus, this thesis attempted to develop a novel DNA vaccine that can provide protective benefits against HIV-1 infection (see chapter 3 of this thesis).

1.17.2.1 Improving DNA vaccine efficacy

Most DNA vaccines are administered intramuscularly, which mainly targets myocytes and a small population of APCs[365]. It is worth mentioning that DCs, the principle APCs, are generally rare in circulation, but more abundant in the skin[375,376]. Thus, targeting a small population of these rare cells after intramuscular vaccination often results in poor immune stimulation[365].

New vaccination strategies are aimed at (1) improving vaccine uptake and (2) improving DC targeting as a way of improving immune stimulation[377-379]. Such strategies include improving immunogen structure and epitope design[209-211,380], using codon-optimised DNA for optimum gene expression in humans[377-379], using molecular adjuvants[346,349,374,378] and using alternative vaccination routes such as intradermal[377-379,381] and mucosal vaccination[313,314,323].

Needle-free vaccination techniques have also been developed to augment vaccine delivery viz. particle bombardment[382], use of micro needles[383], high pressure delivery[384], topical delivery using dermal patches[385] and electroporation[374].

Furthermore, administering the same vaccine several times in a homologous prime-boost or different vaccines containing the same immunogen in a heterologous prime-boost has been shown to improve vaccination outcome[386,387]. Multi-dose homologous vaccination suffers from induced immunity if CMI is desired, but this concern can be overcome by heterologous vaccination[388]. Indeed, studies have reported that administration of DNA vaccines followed by either a recombinant protein vaccine[389-391] or recombinant viral vector vaccine[392-394] generated sub-optimal, but protective humoral and cellular immune responses in primates.

The sub-optimal immune stimulation generated after a DNA prime might be as a result of poor DNA uptake and antigen presentation by APCs at the site of vaccination. As a result, there has been a shift from priming with DNA vaccines to recombinant viral vectors encoding vaccine immunogens and boosting by either a protein vaccine[142,395-397], DNA vaccine[398,399]

and recombinant viruses encoding a similar vaccine immunogen[400,401]. Such vaccination protocols induce high avidity $CD8^+$ T cell responses which can recognise low antigen concentrations, but with high levels of cytotoxicity at the mucosa and systemically[399]. Unfortunately, heterologous prime-boost vaccination has achieved very little in humans as only the RV144 clinical trial has shown some level of protection against HIV although there was no effect on viremia[142]. This emphasises the need for further research into novel DNA-based HIV vaccines and ways of enhancing immunogenicity of the existing candidates.

1.17.3 Recombinant virus vector-based HIV vaccines

In order to mimic the attributes of LAVs, replication-competent and replication-defective viruses have been extensively exploited as vectors to deliver HIV immunogens for *in vivo* expression[402,403]. The choice for a suitable vaccine vector depends on its genetic stability, ease of large-scale production, cloning capacity, safety and levels of pre-existing immunity[271].

Replication-competent vectors establish an infection that mimics the natural viral infection[272], infect several cell types, are easily administered via several routes and do not require the addition of adjuvants[403]. In addition, replication-competent vectors efficiently deliver immunogens and elicit long-lasting humoral immune and CMI responses[403]. In contrast to replication-competent viral vectors, replication-defective viral vectors are safer, but induce short-lived immune protection in vaccinated individuals[271].

Several viruses have been used in HIV vaccine development (summarised in Fig 6); however, adenoviruses, pox viruses and herpes viruses are the most widely used viral vectors[271,402], but their efficacy is reduced by pre-existing anti-vector immunity[271,402].

Vector	Advantages	Limitations
Adenovirus	Efficient transduction of dividing and non-dividing cells High levels of transgene expression Vector genome does not integrate with host genome Suitable for mucosal vaccine delivery	High level of pre-existing immunity in humans Transient delivery of immunogens
HSV	Wide cellular tropisms Large cloning capacity (up to 50 Kb) Multiple cloning sites Persistent high level expression of foreign genes	Transient exogenous gene expression High level of pre-existing immunity in humans
Pox viruses	Large cloning capacity (up to 30 Kb) Multiple cloning sites Vector genome does not integrate with host genome	Transient expression of exogenous genes
Alpha viruses	Low levels of pre-existing immunity Large cloning capacity (up to 30 Kb) Transduce dividing and non-dividing cells Vector genome does not integrate with host genome	Transient delivery of immunogens
Rhabdo viruses	Vector genome does not integrate with host genome Genetically stable Low levels of pre-existing immunity Large cloning capacity (up to 30 Kb)	Neuro-invasiveness
Paramyxo viruses	Suitable for mucosal vaccine delivery Transduce diving and non-dividing cells Stable expression of exogenous genes	High level of pre-existing immunity in humans especially to measles virus
Polio viruses	Induce long-lived immune responses Efficient delivery of immunogens	High level of pre-existing immunity in humans especially to measles virus Poor genetic stability Low cloning capacity
HRV-A1	Extremely rare in humans (< 5% of circulating HRVs) Suitable for intranasal vaccine delivery Infects both mice and humans Efficient delivery of immunogens	Low level of pre-existing immunity in humans especially to measles virus Poor genetic stability Low cloning capacity

Table 2.Summary of virus vectors in vaccine development*. Viruses have different attributes that compel their use as vectors for a candidate HIV vaccine*.

1.17.3.1 Pox viruses as vaccine vectors

Pox viruses, especially Vaccinia virus, were first used to develop the small pox vaccine[404]. The global success of eradicating smallpox by vaccination spurred interest in using Vaccinia virus as a vector for candidate HIV vaccines. The large poxvirus genome can be manipulated to create multiple cloning sites that can be used to deliver a multitude of foreign genes[405] and the pox virus genome does not integrate into the host chromosome[406].

Vaccinia viruses (VV) were the first poxvirus vectors to be developed[407] and candidate VV-based vaccines have been shown to elicit protective antigen-specific immune responses in vaccinated animals[408,409]. However, use of wild type Vaccinia virus as a vaccine vector presents safety concerns, especially in immune-compromised individuals, and therefore, it has been replaced by Tiantan Vaccinia virus (TVV)[410], MVA[411], ALVAC[395] and NYVAC[412].

Modified Vaccinia Ankara (MVA) was developed by multiple serial passage of the original Vaccinia Ankara strain in chicken embryo fibroblasts, resulting in loss of more than 15% of the viral genome[411]. MVA undergoes an incomplete life cycle within the host, thus it does not form infectious viral[406]. MVA-vectored vaccines are safe and well tolerated and stimulate protective immunity at the mucosa and systemically[231,413,414]. Unlike MVA, NYVAC, canary pox (ALVAC) and fowl pox viruses can infect mammalian cells, but are unable to replicate in these cell lines[415,416]. Vaccines delivered by these vectors are also safe and well tolerated, and induce long-lasting immunity against SIV in non-human primates[395,398,417,418].

1.17.3.2 Adenoviruses as vaccine vectors

Adeno virus genome does not integrate into the host chromosome, can infect a multitude of cells, express immunogens at high levels and are transmitted via the respiratory tract, and thus are useful in delivering mucosally-targeted vaccines[419,420]. These attributes have made adenoviruses one of the most exploited viral vectors in HIV vaccine development[419].

Replication-defective adenoviruses have been used more extensively as HIV vaccine vectors than replication-competent adenoviruses partly due to their superior safety levels and packaging capacity[419]. These viruses are attenuated by the deletion of the early regions (E1) genes responsible for viral replication and (E3) genes responsible for lysis of adenovirus-infected cells to release newly synthesised viruses and immune evasion[421,422]. These deletions allow for larger foreign gene fragments to be cloned into adenovirus vectors.

Adeno viruses have been promising as HIV vaccine vectors in animal studies. Xiao et al showed that priming female rhesus macaques with recombinant Ad5 encoding SIV Env/Rev/Gag proteins and boosting with SIVmac251 gp120 stimulated robust CMI and humoral immune responses at the mucosa and systemically[321]. Although vaccinated animals eventually acquired SIV infections, in this study the viral set-point was lower compared with that in unvaccinated controls. In another study, this vaccination regimen stimulated immune responses that fully protected 8 of 12 female macaques against intra-rectal challenge with pathogenic SIVmac251[397] and similar results have been reported by other researchers[400,423].

However in humans, vaccines delivered by adenoviruses have been largely disappointing[139,141,296]. This is due to the high level of pre-existing immunity in humans to adenovirus serotypes 2 and 5 that are commonly used as vectors[424]. Moreover, adenoviruses share conserved T-cell epitopes that are recognized by most adenovirus serotypes[294]; therefore, prior exposure to one adenovirus serotype might result in immune recognition of another serotype. These concerns have been addressed, to some extent, by using adenovirus serotypes that are rare in humans[425-427], use of adenovirus serotypes of non-human primate origin[428-431] and developing hexon chimeric adenovirus vectors that can induce NAbs[432,433].

1.17.3.3 Herpes viruses as vaccine vectors

Replication-competent herpes viruses cause latent and persistent infections in humans[434]. This ensures persistently high level expression of foreign genes when herpes viruses are used as

vaccine vectors. Furthermore, approximately 50% of the herpes virus genome is not essential for viral replication[434], thus multiple cloning sites can be designed to insert multiple large foreign genes by knocking out the redundant genes.

Among herpes viruses, cytomegalovirus (CMV) is probably the most extensively tested viral vector[297-299,435,436]. CMV can be modified to package foreign genomes of up to 6 Kb, is highly attenuated without loss of immunogenicity and appears to be unaffected by prior immunity when used as a vaccine vector[298,436].

Hansen and colleagues have shown that vaccination of male rhesus macaques with recombinant CMV encoding SIV multimeric proteins induced effector memory CTL responses that protected vaccinated animals against pathogenic SIVmac239 challenge[297,298,436]. Although the actual correlates of protection have not been clearly identified, this research group noted that the emergence of effector memory CTL responses that are broadly restricted by MHC-E might have contributed to SIV clearance in half of the vaccinated animals[298,435,437,438]. However, since CMV is associated with long-term infection and disease in humans[439], recombinant CMVs are yet to progress into human trials as candidate vaccine vectors.

Other herpes viruses that have been developed as vaccine vectors include herpes simplex virus (HSV) and varicella zoster virus (VZV). Previous studies have shown that administration of recombinant HSV encoding SIV Gag, Env and SIV Rev-Tat-Nef followed by a recombinant *SIV-gag/env/pol* DNA boost stimulated robust humoral and cellular immune responses which protected vaccinated rhesus macaques against pathogenic SIVmac239 challenge[440,441]. Furthermore, administration of recombinant simian VZV encoding SIV Gag and Env proteins generated modest, but protective SIV-specific immune responses in vaccinated macaques[442] and vervet monkeys[443]. These results demonstrate the potential of

HSVs as candidate vaccine vectors, but this might be complicated by pre-existing immunity to HSVs in humans, and by the potential for recombination with wild type virus.

1.17.3.4 Alphavirus vectors

Alpha viruses are genetically stable and optimally present large immunogens and rarely encountered in humans infections[444]. As a result, extensive research has been conducted on alpha viruses as potential HIV vaccine vectors. In animal studies, mice vaccinated with recombinant Sindbis virus (SIN virus) encoding the HIV Gag protein induced protective HIV gag-specific cellular immune responses at the mucosa and systemically[445]. Similarly, vaccination of macaques with recombinant Venezuelan equine encephalitis virus (VEE) encoding SIV Gag stimulated potent cellular and humoral immune responses at the mucosa and systemically[446]. These immune responses were protective against intravenous SIVsm E660 challenge and reduced the viral load by 750 fold in vaccinated animals compared to the control animals. In two of the protected animals, viremia was reduced to below detectable levels (1500 copies/ml) within 40 weeks post challenge. Moreover, all unvaccinated animals progressed to end stage clinical AIDS.

1.17.3.5 Rhabdovirus vectors

As vaccine vectors, rhabdoviruses are genetically stable, can encode large immunogens, their genome does not integrate into the host genome and prior immunity to these viruses among humans is relatively low[447]. Amongst rhabdoviruses, the rabies virus (RV) and vesicular stomatitis virus (VSV) are the most widely studied vaccine vectors[448,449]. Previously, Egan and colleagues, (2004) showed that vaccination of female macaques with rVSV encoding HIV gp160 and SIV Gag induced robust and potent CMI and humoral immune responses[448]. In addition, it was also noted that intranasally vaccinated animals generated higher $CD4^+$ T cell counts compared with intramuscularly vaccinated animals. Similar immune responses were detected by Rose et al, (2001) that protected vaccinated animals against pathogenic

SHIV for at least a year compared to the unvaccinated controls which progressed to AIDS[449]. Another study showed that vaccination of adult male macaques with recombinant rabies virus encoding either SIV Gag or SHIV Env induced SIV-specific cellular and humoral immune responses that protected 3 of 4 vaccinated animals against SHIV89.6P challenge[450].

1.17.3.6 Paramyxovirus vectors

Paramyxo viruses offer a number of advantages as vaccine vectors. Firstly, paramyxoviruses primarily replicate in the airway epithelial cells[451], thus are potential vectors for candidate vaccines targeted to the mucosa. Secondly, paramyxoviruses replicate in the cytoplasm, are genetically stable and target a broad-spectrum of cells[451,452]. Sendai virus (SeV) is the most extensively studied of this group of viruses for vaccine delivery[453-456]. An advantage of this virus as a vector for human vaccines is that it does not naturally infect humans[457], thus there is a less likelihood of prior immunity to this virus in humans. In fact, a clinical trial is currently underway to test the safety and immunogenicity of recombinant SeV encoding HIV Gag protein in a prime-boost strategy with recombinant Ad35 encoding HIV Gag, integrase, Nef and reverse transcriptase proteins[458].

In animal models, both replication-competent and replication-defective SeV vectors induced robust anti-SIV immune responses[459]. In a heterologous vaccination strategy, administration of *env* and *nef* deleted SHIV DNA followed by recombinant SeV encoding SIV Gag induced persistent SIV-specific cellular immune that protected vaccinated animals against rapid $CD4^+$ T cell depletion after pathogenic SHIV challenge[460]. Furthermore, the same vaccine candidate induced SIV-specific immune responses that protected vaccinated animals against pathogenic SIVmac239 challenge[453]. Tsukamoto and colleagues (2009) detected SIV Gag-specific $CD8^+$ T cellular immune responses that controlled SIVmac239 viremia over a long period in macaques primed with plasmid DNA encoding truncated *gag* and boosted with recombinant SeV encoding SIV Gag/GFP-fusion protein[454]. In other studies, heterologous prime-boost

administration of replication-competent rVV and rSeV encoding the HIV Env generated robust Env-specific CMI and neutralizing antibody responses at the mucosa and systemically[461,462].

Apart from SeV, measles virus (MV) has emerged as another promising vaccine vector[463,464]. This is largely attributed to the worldwide success of the live-attenuated measles vaccine, which induces long-term protection in vaccinated individuals[465]. In animal models, systemic administration of recombinant MV encoding the HIV Env followed by recombinant MV encoding SHIV gp140 stimulated neutralizing antibodies and cellular immune responses against pathogenic SHIV89.6P in macaques and mice[464]. Interestingly, increased levels of MV antibodies were detected in animals with pre-existing immunity to MV, which implied that MV vectors are not necessarily affected by pre-existing immunity. A similar observation has been made in other studies[466,467]. This is particularly important considering the high level of MV immunity within the human population following the administration of the MMR vaccine.

1.17.3.7 Poliovirus as vaccine vectors

The great success of polio vaccines in controlling poliovirus infections globally, has inspired the use of polioviruses as vaccine vectors. As vaccine vectors, polioviruses are safe, efficiently deliver immunogens, and induce protective immunity at the mucosa and systemically[468-470]. Previously, administration of a recombinant Sabin poliovirus vaccine containing the full SIV genome protected female cynomologous monkeys against intra-vaginal challenge with pathogenic SIVmac251[469]. All seven vaccinated monkeys remained healthy 48 weeks post challenge, four of which exhibited strong virologic control and two had undetectable viremia. In contrast, half of the unvaccinated controls developed clinical evidence of SIV infection and progressed to AIDS post-challenge with pathogenic SIVmac251. The level of protection in vaccinated animals was attributed to establishment of

high quality SIV-specific CTL responses[469], which were 10 fold more potent than earlier polio virus constructs[471]. However, like all other replication-competent viruses, progressive testing of replication-competent polioviruses as vectors in humans has been abandoned mainly due to high level of existing immunity to polioviruses[470,472].

1.17.4 Human rhinoviruses (HRVs)

HRVs are the commonest viruses isolated from individuals with acute respiratory illnesses[473] and account for over 23-80% of acute respiratory illnesses every year across all age groups worldwide[474,475]. The common cold associated with HRV is self-limiting and benign, with symptoms resolving within two weeks in immuno-competent individuals[473]. HRVs have been implicated in other infections viz. acute otitis media especially in children, community acquired sinusitis, broncho-pneumonia, exacerbated asthma and chronic obstructive pulmonary disease[476,477]. Most of these infections are mainly transmitted via the mucosa in aerosols or by contact with contaminated surfaces[473].

Upon successful transmission, HRV primarily infects epithelial cells of the nasal mucosa where it replicates in association with cytoplasmic membranous vesicles[478]. However, the actual mechanism of this replication is poorly understood[479]. Viral gene expression takes place in a cap-independent manner and is initiated by the IRES which specifically directs ribosomes to the viral RNA genome which functions as mRNA[476,477]. This process generates a polyprotein of approximately 250 kDa, which is auto-catalytically and co-translationally cleaved by viral proteases into precursor and mature proteins[480]. Generally, HRV RNA can be detected five hours post infection, viral replication is completed approximately 12 hours post-infection[476] and peak viral load is reached 48 hours post-infection[481].

1.17.4.1 HRV genotypes and serotypes

HRVs are closely similar to polioviruses[482], but differ from polioviruses by being acid labile with low temperature culture requirements[473]. HRV are divided into genotypes A and B based

on phylogenetic analysis of the 5'UTR and capsid coding sequences[483,484]. A third genotype, HRV, HRV87 (HRV-C) has been previously identified[485,486]. HRV genotypes A and B are further classified into the major receptor group and minor receptor groups based on receptor recognition[476].

The major receptor group constitutes about 90% of all HRVs (all genotype B and a few genotype A)[487]. These viruses use the intracellular adhesion molecule (ICAM-CD54), a member of the immunoglobulin super family as the cellular receptor[488]. In addition to ICAM-CD54, some major group HRVs utilise heparin sulphate proteoglycans (HSPG) to bind to the host cell membrane[489,490]. In contrast to the major receptor group, the minor receptor group (only genotype A) bind to the host cell membrane via a family of low density lipoprotein receptors (LDLRs) viz. LDLR, VLDLR, LDLR-related proteins and megalin[491]. These receptors are located on the surface of human and murine cells[492], thus most minor group HRVs can infect both human and murine cells. Although receptors for genotypes A and B HRVs have been clearly identified, the receptor used by HRV-C remains poorly understood, but there is evidence that it uses a sialo protein receptor[493].

In addition to receptor usage, HRVs have been further divided into species A (containing 74 serotypes), and B (containing 25 serotypes) based on anti-viral susceptibility (especially to capsid-binding compounds)[473]. Species A viruses are more susceptible to longer capsid-binding compounds such as pleoconaril as compared to species B viruses. Furthermore, HRVs have been also divided into M strains which grow in human and monkey kidney cells and H-strain that initially grew in human cell lines before they were adapted to grow in monkey cells[473].

1.17.4.2 HRV structure and genome

The 5'-end of the HRV genome contains the 5' UTR and the VPg protein, followed by the P1-P3 regions and terminated by the 3' UTR and a poly-A tail at the 3'-end[473], as illustrated

in figure 6. Each of these genomic regions plays unique, but important roles during viral replication. The VPg protein is involved in priming genome replication while the 5' UTR contains the internal ribosomal entry site (IRES) that is important in initiating mRNA translation[487]. The P1 region encodes the capsid (structural) proteins (VP1-VP4, also known as α, β, γ, and δ, respectively)[482]. Up to 60 copies of each of these structural proteins assemble to form the characteristic icosahedral capsid[473]. Furthermore, the VP1 protein possesses a canyon that is involved in attachment to the receptor on the cell membrane[482]. In addition, the VP1, VP2, and VP3 also contain immunogenic regions (NIm-IB, NIm-II, and NIm-III) that are targeted by neutralizing antibody[494]. In proviruses, the VP2 and VP4 proteins exist as VP0 precursor protein[479]. The 3' UTR and a poly-A tail facilitate specific viral RNA selection by the viral RNA-dependent RNA polymerase during viral genome replication[495].

The P2 and P3 regions encode the non-structural proteins (2Apro to 2C and 3A to 3D, respectively)which are produced during viral gene expression[496]. The 2A protease ($2A^{pro}$) self-cleaves from the viral polyprotein to separate the structural from the non-structural proteins and to ensure further processing of the structural and other non-structural proteins [476].

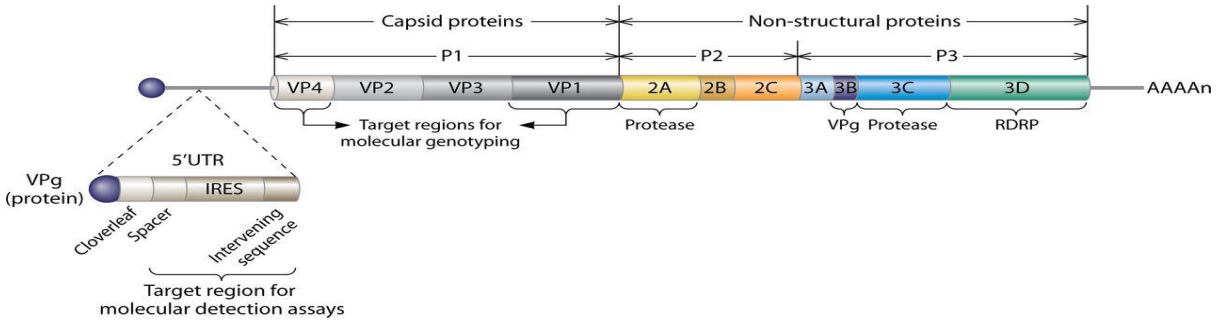

Figure 6: Schematic illustration of the structure of the human rhinovirus genome[473]*. The different gene regions are indicated as P1, P2 and P3.*

The 3C protease ($3C^{pro}$) and its precursor (3CD), are responsible for the catalytic cleavage of the polyprotein to the individual mature VP1, VP3, 2B, 2C, 3A, 3B, and 3D proteins[476,496]. Furthermore, $3C^{pro}$ together with $2A^{pro}$ are also involved in shutting-off 5'cap-dependent host

cellular translation process by inactivating the cap-binding eIF-4GI and eIF-4GII proteins to ensure that all cellular machinery is directed towards viral translational and replication[497]. The roles played by the 2B, 2C, and 3A proteins during viral genome replication are poorly understood, but they might be involved in cell membrane rearrangement, formation of cytoplasmic vesicles and determine the host range for HRVs[476,479]. The 3B protein, also referred to as VPg protein, acts a primer to initiate viral genome replication[479]. The 3D protein is an RNA-dependent RNA polymerase ($3D^{Pol}$) that catalyses viral transcription[476].

1.17.4.3 Human rhinoviruses as vectors for HIV vaccines

HRVs replicate at mucosal surfaces[477,498], thus they can be exploited to deliver mucosally targeted vaccines against HIV. Furthermore, the close similarity between HRVs and PVs suggests that HRV vectors might be equally immunogenic to PV vaccines. Among the first studies to develop and test HRV as candidate vaccine vectors, Resnick et al, (1995) showed that HRV chimeras encoding HIV Env V3 loop sequences elicited potent V3-specific neutralizing antibodies in vaccinated guinea pigs[499]. In addition, these antibodies neutralized pseudo HIV-1 strains *in-vitro*. These results were later confirmed by another study[500]. Furthermore, recombinant HRV encoding the immunogenic membrane surface proteins of RSV induced RSV-specific antibody responses in vaccinated mice[501].

More recently, Arnold and colleagues (2009) vaccinated guinea pigs with recombinant HRV chimeras encoding the HIV gp41 ELDKWA neutralizing antibody epitope with an aim of demonstrating the immunogenic characteristics of HRV chimeras[502]. They detected a modest neutralizing antibody response in all vaccinated animals. Yi et al then confirmed that a similar vaccine elicited ELDKWA-specific antibodies in guinea pigs with broad neutralization characteristics against 10 out of 12 HIV-1 pseudo types that represent the commonest circulating HIV subtypes in the current pandemic[503], and that the immunogenicity of a

recombinant HRV vaccine encoding HIV proteins administered repeatedly in a prime-boost vaccination regimen is not affected by prior HRV-specific antibody responses[504].

Taken together, the above studies show that HRV can be engineered into vaccines that can induce HIV-specific immunity at the mucosa and systemically. However, it should be noted that these studies were conducted in transgenic mice in which the human receptor (ICAM-1) for the major group HRVs has been inserted[505] and not wild type mice models. As a result HRV infection in these models does not closely mimic HRV infections in humans, which casts a doubt over the reproducibility of the results in human clinical trials. Furthermore, the above studies used HRV serotype 14 (HRV-14) as the vaccine vector, but this is among the most encountered serotypes in humans suffering from HRV-associated infections[474,475], thus it would be expected that there is a high level of pre-existing immunity to HRV-14 in humans which would dampen vaccine efficacy.

Therefore, in this thesis, a novel strategy to engineer a genetically stable, replication-competent, recombinant HRV serotype-A1 (HRV-A1) as a vector for HIV immunogens is described (see chapters 4, 5 and 6 of this thesis). As a member of the minor group HRVs, HRV-A1 replicates in and infects murine[492,506] and human epithelial cell lines[507], which allows for evaluating efficacy of HRV-A1 delivered vaccines in a wild type mouse model and then in humans. In addition HRV-A1 comprises about 0.5% of all circulating HRVs[474,508] and is one of the rarest HRV serotypes detected in humans[475,509,510]. Consequently, there is low level of pre-exposure to HRV-A1 in the human population, making it a more suitable and potential vector for HIV vaccines than other HRV serotypes tested previously[502-504] as HIV vaccines.

1.19 Aims of the studies in thesis

The main aim of this thesis was to design and construct novel HIV vaccines viz. a DNA-based Tat vaccine intended to elicit high titer anti-Tat humoral and CMI responses, and a recombinant virus vaccine based on a replication-competent HRV encoding Gag and Tat intended to induce high quality (high magnitude and poly-functional) Gag and Tat-specific CMI and anti-Tat antibodies at the mucosa and systemically. The efficacy of these vaccine candidates was evaluated in mice. This broad aim was subdivided into 3 specific aims, listed below:

1) Generate a highly immunogenic recombinant DNA vaccine encoding an oligomerised, secreted form of HIV Tat, and evaluate the protective efficacy of this vaccine against EcoHIV challenge in mice (see chapter 3).
2) Construct and characterise a series of genetically stable, live recombinant human rhinoviruses encoding HIV Gag or Tat (rHRV-Gag 1-5 and rHRV-Tat) (see chapters 4 and 5).
3) Evaluate the immunogenicity of a cocktail vaccine (HRV-Gag/Tat) containing rHRV-Gag 1-5 and rHRV-Tat and the protective efficacy of HRV-Gag/Tat against EcoHIV challenge when administered in heterologous-prime boost vaccination regimen in mice (see chapter 6).

Chapter 2.0: Materials and Methods

This chapter describes the materials and methods used throughout different chapters of this thesis. Methods that are specific for certain chapters are described within the relevant chapters.

2.1 Vaccines

Codon optimised HIV-1 clade B *tat* and IMX313 genes (Gene Art, Germany) were inserted into pVAX downstream of the CMV promoter. Four plasmids were generated encoding either wild type Tat viz. (1) pVAX-Tat, (2) pVAX-Tat-IMX313 or secreted Tat (sTat) generated by the upstream introduction of the TPA leader sequence[378] (3) pVAX-sTat and (4) pVAX-sTat-IMX313. pET-DEST42, a kind gift from A/Prof David Harrich, (Queensland Institute of Medical Research, Australia) encoding Tat fused to a 6x-His tag was used to synthesize the protein in *Escherichia coli* for ELISA. PCR primers for cloning are listed in the supplementary material and all plasmids were sequenced. DNA vaccines were purified using standard molecular biology and endotoxins removed with the endotoxin removal solution (Sigma-Aldrich, Sydney, Australia). The endotoxin level in each DNA vaccine was <0.1 EU/µg DNA (SA Pathology, Adelaide, Australia).

The design and construction of the pVAX-Gag-PRF DNA vaccine has been described previously [377]. Briefly, a codon-optimised clade B HIV *gag* gene was inserted downstream of a strong CMV promoter, while the gene encoding cytolytic PRF was inserted downstream of a SV40 promoter in the pVAX plasmid backbone. This cloning strategy ensured that the Gag expression was always 10 fold higher than that of cytolytic PRF[377].

A comprehensive methodology for the design, construction and production of replication-competent, genetically stable, live recombinant human rhinovirus vaccine encoding the exogenous HIV Gag and Tat (rHRV-Gag/Tat) is described later in chapter 4 of this thesis.

2.2 Cell culture and DNA transfection

HEK 293T cells were obtained from American Type Culture Collection (ATCC, Manassas, VA, USA) and maintained at 37°C in 5% CO_2 in Dulbecco's modified Eagle's medium (DMEM Gibco, Life Technologies, CA, USA) supplemented with 10% (v/v) FCS and 1% (v/v) penicillin/streptomycin (Life Technologies).DNA was transfected using Lipofectamine-LTX transfection reagent (Life Technologies) as per the manufacturer's recommendations.

2.3 Western blot analysis

To detect Tat expression from the 4 plasmids each encoding a different form of Tat, HEK 293T cells were transiently transfected with 3 ug DNA in 6 well plates and 48h later cell lysates and supernatants were harvested in Laemmli buffer under reducing or non-reducing conditions. The total protein concentration in the sample was determined using the Pierce BCA protein assay kit (Thermo Scientific, MA, USA) and 50 µg of total protein was analysed in 10-12% (v/v) SDS-PAGE under reducing (with the addition of β-mercaptoethanol, β-Me) or non-reducing (without β-Me) conditions. Proteins were blotted onto a 0.45µm PVDF membrane (Immobilon, Merck-Millipore, Germany) under semi-dry transfer conditions (Bio-Rad, CA, USA). To detect Tat expression, a cocktail of Tat monoclonal antibodies 4D5.24, 5A5.3 and $1D9^{511}$ (obtained through the NIH AIDS Reagent Program) and goat-anti mouse horseradish peroxidase (HRP)-conjugated secondary antibody (Antibodies Australia, VIC, Australia) were used. The membrane was developed by extreme sensitivity chemiluminescence substrate (Western Lightning Ultra, Perkin Elmer, MA, USA) and imaged by LAS 4000 digital imaging system (Fujifilm, Japan).

2.4 Animals and immunisations

Female 6-8 week old BALB/c mice (Animal Resource Centre, Australia) were obtained from the University of Adelaide Laboratory Animal Services and were maintained in the Queen Elizabeth Hospital animal house under PC2 conditions in individually ventilated cages fitted

with a HEPA filter. All animal experiments were approved by the University of Adelaide and the South Australia Pathology Animal Ethics Committees. For the Tat DNA vaccines, mice were divided into 5 groups (n=7 animals) and vaccinated with (1) pVAX-Tat DNA; (2) pVAX-Tat-IMX313 DNA; (3) pVAX-sTat DNA; (4) pVAX-sTat-IMX313 DNA or (5) pVAX-only DNA (mock group, n= 3 animals). All animals received 3-5 doses of 50 μg/dose of endotoxin free DNA in saline (final vaccine volume per dose 50 μl) at 2 week intervals, by the intradermal (ID) route as described previously[377,378].

For rHRV-Gag/Tat vaccine experiments, vaccinations were performed after 2% isofluorane anaesthesia via the intranasal (IN) route for the rHRV-Gag-Tat cocktail as described[512,513] or via the intradermal (ID) route for DNA vaccines as described[378,514]. Mice (n=7) received either 2 doses of wt-HRV-A1 (each containing $5x10^6$ $TCID_{50}$ in 50 μl) followed by 50μg pVAX(vaccination control group) at intervals of 2 weeks or 2 doses of rHRV-Gag-Tat (each containing $5x10^6$ $TCID_{50}$ in 50 μl) followed by a single dose of 50μg of a DNA cocktail containing equimolar concentrations of pVAX-sTat-IMX313 and pVAX-Gag-PRF. This vaccination strategy will be referred to as rHRV-DNA prime-boost vaccination in the following sections. Another group of mice were vaccinated with 3 doses of pVAX-Gag-PRF/pVAX-sTat-IMX313 (50μg/dose at 2 weeks intervals) and referred to as pVAX-Gag-Tat prime-boost vaccination. Blood was collected by the sub-mandibular cheek bleed method[515] and cervical vaginal lavage (CVL) samples were collected as described [516]. Serum and CVL samples were taken one day before each vaccination and examined for anti-Tat antibody responses. At 14 days post final boost, the mice were euthanized and splenocytes and lymphocytes harvested from mesenteric lymph nodes (mesenteric lymphocytes) for analysis.

2.5 Enzyme-linked immunosorbent spot assay (ELISpot)

ELISpots were performed to determine the breadth and magnitude of HIV-specific CMI in splenocytes from vaccinated mice. A panel of 11 overlapping amino acids 15-19 mer peptides

spanning the entire Gag or Tat protein was obtained from the NIH AIDS reagent bank (Germantown, MD, USA). Mouse IFN-γ ELISpot was performed on red blood cell (RBC)-depleted splenocytes that were re-stimulated for 36h with 4 μg /ml of 5 Gag peptide pool or a Tat peptide pool as we described previously[377,379]. Briefly, multiscreen-IP HTS plates (Merck Millipore, Germany) were coated with anti-mouse IFN-γ (clone AN18, MabTech, Sweden) and secreted IFN-γ was detected with anti-mouse IFN-γ -biotin (clone R4-6A2, MabTech) followed by streptavidin-AP (Sigma-Aldrich) and SigmaFast BCIP/NBT (Sigma-Aldrich). Phytohemagglutinin (5 μg/ml) was used as a positive control and un-stimulated splenocytes as a negative (background) control. Developed spots were counted automatically using an ELISpot reader (AID GmbH, Germany). The number of spots in unstimulated splenocytes was subtracted from the number in peptide-stimulated cells to generate the net number of Tat-specific spot forming units (SFUs).

2.6 Fluorescent target array (FTA) assay

The FTA assay was used to examine the magnitude and quality of T-cell responses generated after vaccination in an *in vivo* assay[517]. Splenocytes from naïve BALB/c mice were co-labelled with combinations of four different concentrations of cell trace violet (CTV) and carboxyfluoresceinsuccinimidyl ester (CFSE) dyes to generate a 16 parameter FTA, as described previously[517,518]. The fluorescent tagged cells were then pulsed with 0.01–10 μg/ml of Tat peptides for 4h at 37 °C in 5% CO_2. The peptides were divided into pools 1-3 in which pool 1 contained peptides representing the complete Tat protein, whereas pool 2 lacked the theoretical $CD8^+$ T cell immunodominant epitopes and pool 3 contained only the theoretical $CD8^+$ T cell immunodominant epitopes (HIV molecular immunology database, http://www.hiv.lanl.gov/). The peptide-pulsed dye-labelled cells were pooled and about 40 $x10^6$ cells were adoptively transferred intravenously via the lateral tail vein into each mouse, 13 days after the final vaccine dose. Splenocytes were then harvested 18h post-FTA transfer,

RBC-depleted and stained with antibodies against mouse antigens (allophycocyanin conjugated anti-CD69 (eBioscience #561240) and Alexa Fluor 700 conjugated anti-B220 (eBioscience #56-0452-80)). The percentage specific killing of FTA cells was calculated using the formula described previously[517,519]. The magnitude of $CD4^+$ T-cell help provided to naïve B cells in vaccinated mice was determined by measuring the geometric mean fluorescence intensity (GMFI) of CD69 on gated $B220^+$ cells within the FTA. Samples from each vaccinated mouse were analysed on a BD FACS Canto (Becton Dickinson, NJ, USA) and FACS plots and GMFI analyses were performed using the FlowJo software (version 8.8.7).

2.7 Intracellular cytokine staining (ICS) and flow cytometry

Multi-colour ICS was performed on RBC-depleted splenocytes and mesenteric lymphocytes to determine IFN-γ, interleukin (IL)-2, and tumor necrosis factor (TNF)-α production from Gag-specific $CD8^+$ T cells as described previously[377,378]. Briefly, the cells were stimulated for 1 h with 5 µg/ml of the H-$2K^d$-restricted $Gag_{197\text{-}205}$ (AMQMLKETI) immunodominant peptide[520] (China peptides, China) then cultured in the presence of protein transport inhibitor (Brefeldin A, eBiosciences) for a further 6 h. Staining was performed using fluorescence-activated cell sorting (FACS) Cytofix/Cytoperm buffer (BD Biosciences) with antibodies specific to mouse antigens CD8α (allophycocyanin (APC)-efluor780 conjugated), CD44α (APC conjugated), IL-2 (peridinin chlorophyll 5.5 conjugated), IFN-γ (fluorescein isothiocyanate (FITC) conjugated) and TNF-α (phycoerythrin conjugated) (BD Biosciences). The cells were analysed on a BD FACS Canto based on the post-acquisition gating strategy and including fluorescent minus one (FMO) control for spectral overlap for each fluorochrome described in the Appendix. The results analysed with FlowJo X.0.7 software (Ashland, OR). The number of cells producing cytokines without prior stimulation was subtracted from the number in peptide-stimulated cells to generate the net Gag response.

2.8 H-2Kd- $Gag_{197-205}$ tetramer and antibody staining

RBC-depleted splenocytes were initially stained with the APC-conjugated H-$2K^d$-restricted $Gag_{197-205}$ (AMQMLKETI) immunodominant peptide tetramer (Biomolecular Resource Facility, John Curtin School of Medical Research, Australian National University, Canberra, Australia) for 1 h at room temperature, washed twice in phosphate-buffered saline (PBS) and then re-stained with FITC-conjugated anti-mouse CD8α antibody (BD Biosciences). The cells were analysed on a BD FACS Canto using the gating strategy described in Appendix and the results analysed with FlowJo X.0.7 software. The number of unstained tetramer positive cells was subtracted from the number in tetramer-stained cells to generate the net tetramer-positive cells.

2.9 Enzyme-linked immunosorbent assay (ELISA)

Serum and CVL samples from mice were analysed for anti-Tat antibodies by indirect ELISA. The Tat protein used as the ELISA antigen was produced by transforming competent *Escherichia coli* BL21 (DE3) cells (Life Technologies) with pET-DEST42 DNA[521] and induced with 1 mM Isopropyl β-D-1-thiogalactopyranoside (IPTG) and purified using a Ni-NTA slurry (Qiagen). The His-tagged Tat protein was eluted with 250 mM imidazole and the Ni-NTA beads pelleted by centrifugation at 10,000 rpm at 4°C. The total proteins in the supernatant were quantified in a BCA assay, stored at -20 °C until required and the Tat protein detected in a Western blot assay using the NIH anti-Tat antibodies noted above. Maxisorp plates (Sigma-Aldrich) were coated with 500 ng of purified Tat protein in carbonate-bicarbonate buffer at 4° C overnight. Serum and CVL samples were serially diluted in phosphate buffered saline (PBS) containing 0.5% (v/v) FCS and analysed in duplicate. Bound antibodies were detected using HRP-conjugated goat anti-mouse IgG (Amersham, GE Healthcare Life sciences, USA) or anti-mouse IgA (Life Technologies) diluted in 0.5% (v/v) FCS/PBS. Plates were developed by adding hydrogen-peroxide and o-

Phenylenediaminedihydrochloride peroxidase substrate (Sigma-Aldrich), and the optical density (OD) read at 492nm (OD492) on a FLUOstar OPTIMA plate reader (BMG LabTech, Germany). Endpoint titers were determined as the reciprocal of the highest serum or CVL sample dilution with an OD reading above the cut-off. The cut-off was set as two standard deviations above the mean OD of that of serum samples from naïve mice or of serum samples taken before the first vaccine dose.

2.10 Anti-Tat neutralization assay

The neutralizing activity of anti-Tat antibodies was assessed in an *in vitro* transactivation assay using Cf2-Luc cells that stably express the luciferase gene under the control of the HIV-1 long terminal repeat (LTR)[522], as described previously[523,524]. Cf2 cells were maintained in DMEM supplemented with 10% (v/v) FCS, 100 µg/ml penicillin/streptomycin and 0.7 mg/ml of G418. 50 ng/ml of purified Tat protein (NIH Reagent Bank), previously incubated for 1h in PBS or a 1/25 dilution of serum samples from the different vaccinated groups, was added to the cells and 48h later cell lysates were prepared in 1% (v/v) Triton X-100 (Sigma-Aldrich) in PBS containing protease inhibitors (Sigma-Aldrich) as described [525,526]. The lysates were assayed for luciferase activity using the dual luciferase assay system (Promega) in the FLUOstar OPTIMA plate reader (BMG LabTech) at 492 nm relative to the untransfected cell control, and the data expressed as mean relative luminescence units (RLUs) ± S.E.M. The percentage neutralization was calculated using the formula: % specific neutralization = [(RLUs in the absence of test sera- RLUs in the presence of test sera)/ RLUs in the absence of test sera] x 100.

2.11 EcoHIV/NL4-3 challenge

EcoHIV was prepared as described previously[377,378,527]. Briefly, EcoHIV plasmid DNA was transfected into HEK 293T cells following the calcium phosphate transfection protocol and 48h later, EcoHIV was concentrated from the supernatant using Amicon 100 kDa centrifugal

filters (Sigma-Aldrich,) and titrated using the ZeptoMetrix p24 antigen ELISA kit (ZeptoMetrix, NewYork, USA,) as per the manufacturer's protocol. Vaccinated mice were challenged via the intra-peritoneal route with EcoHIV equivalent to 1.5 µg p24 as we described previously[377,378]. The mice were culled, and spleen and peritoneal exudate cells (PECs) were collected 7 days post challenge. RNA was isolated using Trizol (Invitrogen) as directed by the manufacturer. Briefly, the RT reaction was set up using the Superscript II RT-kit (Life Technology) in a 20µl volume that contains 5µg of RNA, 500µg of primer, 10Mm dNTPs, and incubated at 42°C for 2 min, then chilled for not more than 5 min. A mixture containing 1µl 0.1M DTT, 4µl 1X first-strand transfer buffer, 200U of Superscript II RT and 40U/µl of RNase-out was added to the RT-reaction, and then incubated further at 42°C for 1 hr before the reaction was terminated by heating at 70°C for 15 min. The qRT-PCR was performed as described[377,378] using the QuantiFast kit (Qiagen) following the manufacturer's instructions in a reaction containing 2.5 mM MgCl2, 1X QuantiFast SYBR Green PCR Master mix, 1 µl cDNA and 1 µM of the forward and reverse primers. The PCR consisted of a 2-step cycling program: 35 cycles for 95 °C for 5 min, followed by 95 °C for 5 min and 60 °C for 10 s.Each reaction was set up in duplicateand the primers used for qRT-PCR are shown inAppendix I. Results were normalised to RPL13a mRNA levels after examining primer efficiency using the ΔΔCT (threshold cycle) quantification method[528,529].

2.12 Statistical analysis

Data presented as mean ± standard error of the mean (SEM) were generated with GraphPad Prism version 6 (GraphPad Software, La Jolla, CA, USA). Non-parametric Kruskal-Wallis test was used to compare the difference between multiple vaccine groups. If this showed significant differences, then the Mann-Whitney U test was performed to compare differences between each vaccine group, independently. Statistical significance was determined using the

Mann-Whitney U test; $p < 0.05$ was considered significant and $p > 0.05$ was considered non-significant.

Chapter 3.0: A novel candidate HIV-Tat based DNA vaccine

(pdf version of the manuscript SREP-16-03591A, accepted for publication in Scientific Reports on 15/06/2016)

Statement of Authorship

Title of Paper	**A HIV-Tat/C4-binding protein chimera encoded by a DNA vaccine is highly immunogenic and contains acute EcoHIV infection in mice**
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Publication Details	**A HIV-Tat/C4-binding protein chimera encoded by a DNA vaccine is highly immunogenic and contains acute EcoHIV infection in mice** KhamisTomusange, Danushka Wijesundara, Jason Gummow, Tamsin Garrod, Yanrui Li, Lachlan Gray, Melissa Churchill, Branka Grubor-Bauk and Eric J. Gowans **In press: Nature Scientific Reports 18th June 2016**

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Overall percentage (%)	80%
Certification:	This paper reports on original research I conducted during the period of my Higher Degree by Research candidature and is not subject to any obligations or contractual agreements with a third party that would constrain its inclusion in this thesis. I am the primary author of this paper.
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Co-Author Contributions

By signing the Statement of Authorship, each author certifies that:

the candidate's stated contribution to the publication is accurate (as detailed above);

permission is granted for the candidate in include the publication in the thesis; and

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A HIV-Tat/C4-binding protein chimera encoded by a DNA vaccine is highly immunogenic and contains acute EcoHIV infection in mice

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DNA vaccines are cost-effective to manufacture on a global scale and Tat-based DNA vaccines have yielded protective outcomes in preclinical and clinical models of human immunodeficiency virus (HIV), highlighting the potential of such vaccines. However, Tat-based DNA vaccines have been poorly immunogenic, and despite the administration of multiple doses and/or the addition of adjuvants, these vaccines are not in general use. In this study, we improved Tat immunogenicity by fusing it with the oligomerisation domain of a chimeric C4-binding protein (C4b-p), termed IMX313, resulting in Tat heptamerisation and linked Tat to the leader sequence of tissue plasminogen activator (TPA) to ensure that the bulk of heptamerised Tat is secreted. Mice vaccinated with secreted Tat fused to IMX313 (pVAX-sTat-IMX313) developed higher titres of Tat-specific serum IgG, mucosal sIgA and cell-mediated immune (CMI) responses, and showed superior control of EcoHIV infection, a surrogate murine HIV challenge model, compared with animals vaccinated with other test vaccines. Given the crucial contribution of Tat to HIV-1 pathogenesis and the precedent of Tat-based DNA vaccines in conferring some level of protection in animal models, we believe that the virologic control demonstrated with this novel multimerised Tat vaccine highlights the promise of this vaccine candidate for humans.

The HIV Tat protein is required for efficient virus replication[1,2] and is released into the extracellular milieu by infected cells where it can be taken up by other cells to increase transcription from the HIV long terminal repeat (LTR)[1]. The *tat* gene is more genetically stable than the *env* gene and immunogenic Tat epitopes are conserved across HIV-1 subtypes in group M[3]. Thus, a Tat vaccine may provide cross protection across all group M viruses, which account for a majority of HIV infections globally[4]. These attributes make Tat a potential component of an HIV vaccine. As it has been difficult to generate canonical Env-specific neutralising antibodies (NAb)[5], the production of high titre anti-Tat NAb might be a feasible alternative to control HIV replication and delay disease onset. Tat-based HIV vaccines are safe and generate high titre Tat-specific humoral and cell mediated immunity (CMI) that correlate with asymptomatic infection or slower disease progression in humans[6,7] and animals. However, native Tat is poorly immunogenic, easily oxidised and degraded by proteolysis[1]. Thus, protecting Tat from these detrimental processes improves the immunogenicity of Tat-based vaccines[8,9]. We now report a novel strategy that incorporates Tat into heptamers by fusing Tat with the oligomerisation domain of the C4 binding protein (C4b-p), an inhibitor of the classical and lectin pathways of complement[10]. Previously the most effective form, termed IMX313, a hybrid derived from the oligomerisation domains of human and murine C4b-p α-chains[11–13] induced oligomerisation of *P. falciparum* or *M. tuberculosis* antigens and enhanced the immunogenicity and protective efficacy of the resultant vaccines[11–14]. The IMX313 amino acid sequence is $<$20% identical to the murine or human C4b-p oligomerisation domains, thus IMX313 does not induce antibodies[12], and has recently been shown to be safe for use in humans[15] making it a practical molecular adjuvant. However, as the adjuvanticity of IMX313 requires efficient secretion of the oligomerised protein[14], a TPA leader sequence was introduced upstream of the

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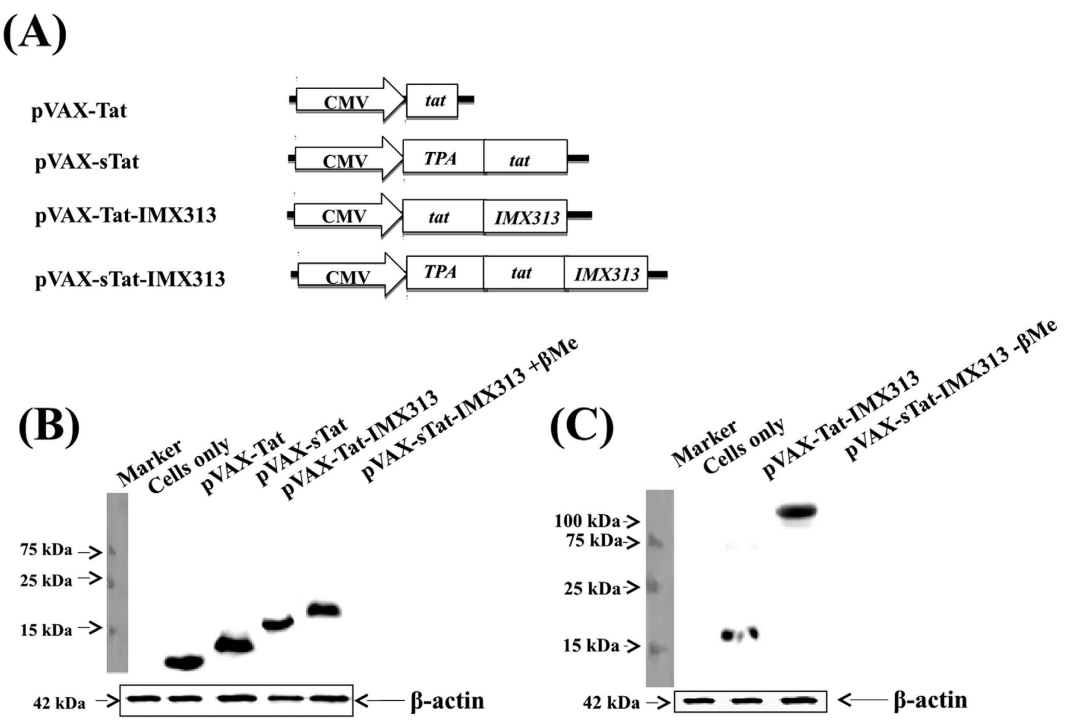

Figure 1. Vaccine constructs and Tat expression. (**A**) Schematic representation of the vaccine constructs; (**B**) reducing Western blot analysis of Tat in cell lysates from HEK293T cells transfected with plasmid DNA encoding the different forms of Tat (tracks 2–5) and (**C**) non-reducing Western blot analysis of Tat in supernatant fluids of HEK293T cells transfected with pVAX-Tat-IMX313 (track 2) or pVAX-sTat-IMX313 (track 3) DNA. The 42 kDa β-actin protein was used as loading control for the Western blot. Blots in Fig. 1B,C are cropped for clarity and conciseness; full length blots of these figures are presented in supplementary figure S1A,B.

sequences encoding Tat and IMX313 to drive expression of the oligomerised protein into the secretory pathway. Indeed, DNA and recombinant viral vaccines encoding IMX313 are in advanced clinical testing[16]. DNA vaccines are stable, readily manufactured, induce humoral and CMI, and are licensed for veterinary use[17], emphasising their potential in developing human vaccines. To improve the immunogenicity of Tat-based DNA vaccines, we fused Tat to IMX313, and compared the immunogenicity and protective efficacy of this vaccine with that of the corresponding vaccines lacking IMX313 with a major thrust to elicit robust humoral responses.

Results

Tat oligomerisation. Four plasmids, each encoding a different form of HIV Tat were constructed (Fig. 1A). In this study, the DNA vaccine encoding the native form of Tat protein was used as the benchmark against which the efficacy of plasmids encoding secreted and/or oligomerised forms of Tat were compared. Native Tat antigen, was not used as a control , as others have shown that its immunogenicity is affected due to its susceptibility to oxidation and proteolysis[1]. To confirm expression of Tat, HEK293T cells were transfected with plasmid DNA, and Western blot analysis performed on all four cell lysates (Fig. 1B) and supernatant fluids from pVAX-Tat-IMX313 or pVAX-sTat-IMX313-transfected cells (Fig. 1C). Oligomers of protein-IMX313 fusions can only be detected in non-reducing conditions[12,14] and consequently in a reducing Western blot, 11, 14, 18 and 20 kDa bands corresponding to proteins expressed from pVAX-Tat, pVAX-sTat, pVAX-Tat-IMX313 and pVAX-sTat-IMX313, respectively, were detected (Fig. 1B) and supplementary figure S1A (for full size blot).The assay was repeated in the absence of β-Me and bands of ~18 kDa from pVAX-Tat-IMX313-and ~140 kDa from pVAX-sTat-IMX313-transfected cells detected (Fig. 1C) and supplementary figure S1B (for full length blot). Thus the different forms of Tat were detected as predicted and fusion of IMX313 to sTat resulted in Tat oligomerisation. However, as oligomerisation requires secretion of the fusion protein[14], expression from pVAX-Tat-IMX313 probably failed to result in an oligomer of~126 kDa under non-reducing condition (Fig. 1C), due to cytoplasmic retention of the protein.

DNA vaccines encoding Tat generate T-cell immune responses *in vivo*. Tat-specific CMI is associated with improved virological control in humans[1] and non-human primates[18,19]. Initially, we evaluated the ability of the DNA vaccines to induce CMI using IFN-γ ELISpot as a surrogate indicator of Tat-specific CMI in vaccinated animals. The animals received 3 vaccine doses, and splenocytes harvested 14 days later were re-stimulated for 36 h with Tat peptides which included the Tat epitopes targeted by CTL in BALB/C mice[20]. Responses were significantly higher in splenocytes from pVAX-Tat-IMX313 (mean SFU of 105, $p = 0.0332$), pVAX-sTat (133, $p = 0.0239$) and pVAX-sTat-IMX313 (160, $p = 0.0006$)-vaccinated mice than pVAX-Tat-vaccinated animals (23) (Fig. 2A). pVAX-sTat-IMX313 generated slightly higher responses than pVAX-Tat-IMX313 ($p = 0.197$) or

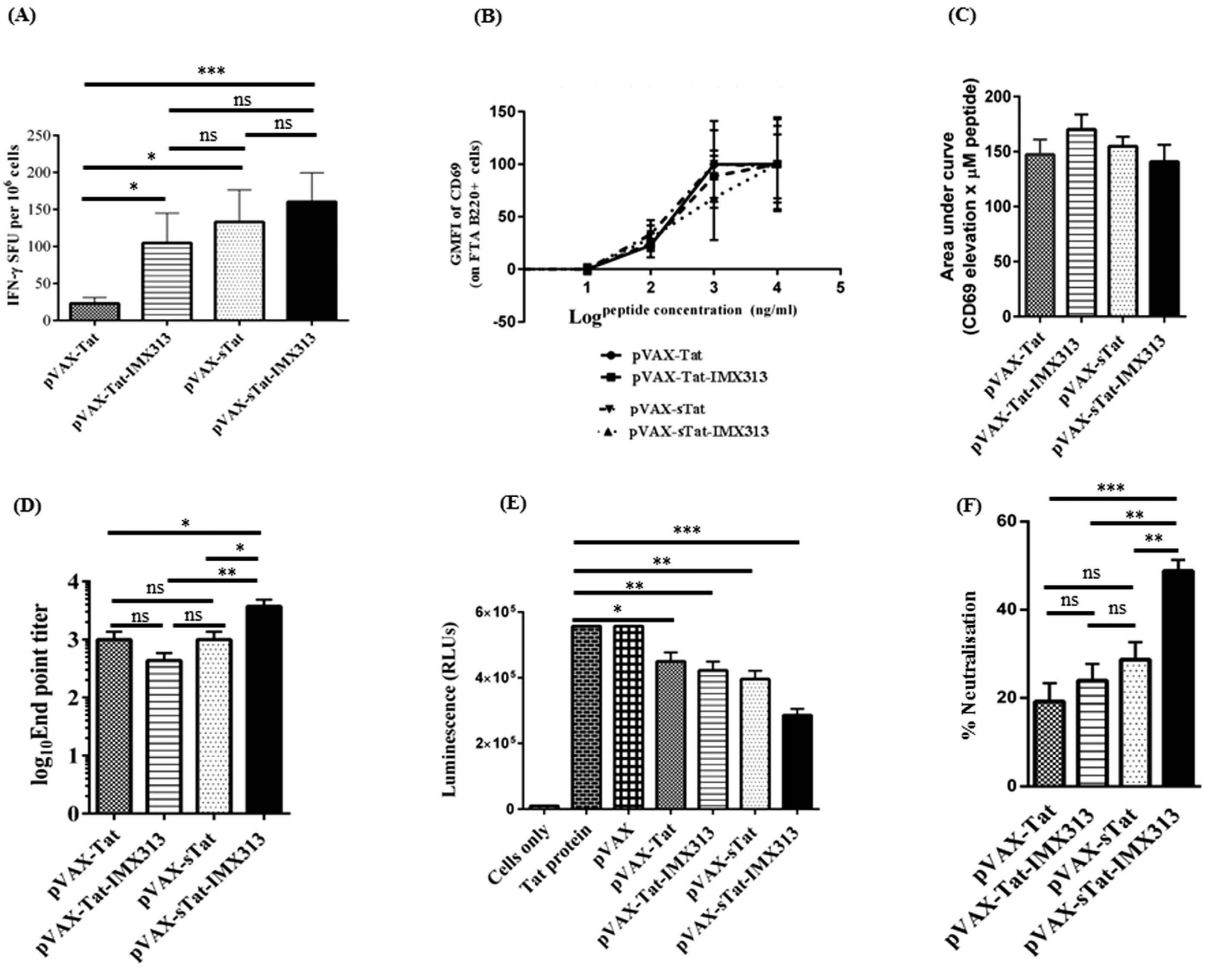

Figure 2. Tat DNA vaccination induces Th cell responses and humoral immunity. Mice (n = 7 per group) were vaccinated ID with 3 doses of 50 μg of pVAX, pVAX-Tat, pVAX-Tat-IMX313, pVAX-sTat or pVAX-sTat-IMX313 at 2 week intervals. Splenocytes were collected 14 days after the last dose. Blood was collected one day before each vaccination and on the day of euthanasia. (**A**) IFN-γ ELIspot assay to examine Tat-specific CMI in splenocytes stimulated with Tat peptides for 36 h. (**B**) FTA assay to examine Th cell responses from pVAX-Tat, pVAX-Tat-IMX313, pVAX-sTat or pVAX-sTat-IMX313 vaccinated mice above the GMFI of CD69 on FTA $B220^+$ cells from naïve mice. The graph is representative of the FTA with cells pulsed with Tat peptide pools 1, 2 or 3. (**C**) AUC values for Th cell responses depicted in (**B**). (**D**) The results of an indirect ELISA to detect Tat-specific serum IgG. Titres are expressed as the reciprocal of the serum dilution and plotted as log10 IgG endpoint titre. The data are representative of 2 independent experiments in which each serum sample was analysed in duplicate. (**E**) Tat transactivation activity in Cf2-Luc cells after the addition of Tat pre-incubated in PBS or a 1/25 dilution of serum samples from mice vaccinated with the respective vaccines. Graphs show mean RLUs (±SEM) relative to untransfected control cells and are representative of 3 independent experiments in which each sample was analysed in triplicate. (**F**) Percentage decline in luminescence (an indicator of the neutralisation of Tat transactivation activity) depicted in (**E**). Data shown in the entire figure depict the mean (n = 7) ± SEM; an unpaired non-parametric Mann–Whitney U test was used to analyse the statistical significance of the data; $*p < 0.05$, $**p \leq 0.01$, $***p \leq 0.001$ and $p \geq 0.005$ = non-significant (ns).

pVAX-sTat ($p = 0.05134$). These results suggest that Tat immunogenicity was enhanced when Tat was secreted and fused to IMX313.

We also measured the vaccine-induced CMI by CTL activity and T-helper (Th) cell responses *in vivo* using the fluorescent target array (FTA) assay[21]. The FTA target cells were injected into vaccinated animals 13 days after the third vaccine dose, the splenocytes harvested 18h later and analysed by multi-parameter flow cytometry for CTL and Th responses *in vivo*. Initially, CTL activity was examined and showed <1% killing of peptide-pulsed FTA-target cells, a level of killing that was similar across all vaccinated groups (Supplementary Fig. S2B). This was unexpected and contrasts with previous studies reporting potent CTL activity post Tat vaccination[1,22].

Second, we examined Th responses mediated by $CD4^+$Th cells required for efficient maturation and activation of B cells[23]. The $CD4^+$Th cell response was determined by measuring the up-regulation of CD69 on peptide-pulsed B cells in the FTA of challenged mice, relative to controls. The data showed that there was a dose-dependent elevation of CD69 expression on FTA B cells in all vaccinated animals (Fig. 2B) but no significant

difference between the different groups. To assess the cumulative magnitude of Th cell responses induced following vaccination, we calculated the area under the curve (AUC) for the data analysed in Fig. 2B, but this analysis also revealed similar Th cell responses in all vaccinated groups (Fig. 2C). Collectively, these results suggest that pVAX-Tat, pVAX-Tat-IMX313, pVAX-sTat and pVAX-sTat-IMX313 elicited Th cell responses capable of activating naïve B cells, but were ineffective in generating detectable CTL responses.

DNA vaccines encoding Tat induce anti-Tat NAb. As Tat-specific serum IgG capable of neutralizing Tat activity is desirable for an effective Tat-based vaccine[7,8,24], we analysed serum samples from vaccinated mice for Tat-specific antibodies by ELISA. Tat-specific IgG was detected in serum with mean titres of 433 for pVAX-Tat-IMX313, 1000 for pVAX-Tat, 1029 for pVAX-sTat and 3728 for pVAX-sTat-IMX313 (Fig. 2D). Thus, pVAX-sTat-IMX313 elicited an antibody titre 9-fold higher than that elicited by pVAX-Tat-IMX313 and ~3.6-fold higher than that from pVAX-Tat or pVAX-sTat. These results also demonstrated that Tat immunogenicity was enhanced when the DNA vaccine encoded a secreted and oligomerised version of Tat (pVAXsTat-IMX313).

We next examined whether anti-Tat-specific antibodies could neutralize Tat transactivation activity *in vitro*. Tat protein pre-incubated in PBS or in a set dilution of serum (1/25) from vaccinated mice was added to Cf2 cells. It is expected that once the Tat protein is added to Cf2 cells, it will be taken-up by the cells and up-regulate transcription from the HIV LTR resulting in expression of luciferase and thus luminescence. However, Tat-specific antibodies that are present in the serum of vaccinated animals will bind and neutralize Tat thereby blocking its uptake into cells and inhibiting its transactivation activity, resulting in reduced or no luminescence. The mean RLU in cells incubated with Tat in PBS was 557345 (Fig. 2E). A similar result was obtained from cells to which the Tat protein pre-incubated with diluted serum from pVAX-vaccinated mice was added. Importantly pre-incubation of Tat protein with diluted serum samples from the Tat-vaccinated mice, resulted in a clear reduction of mean RLUs. Mice vaccinated with pVAX-Tat showed a 19% decline (mean RLU 451449), pVAX-Tat-IMX313 a 24% decline (mean 423582), pVAX-sTat a 29% decline (RLU 395715) while pVAX-sTat-IMX313 vaccinated mice showed the most significant reduction of 49% (mean 285758) (Fig. 2E,F). This level of neutralisation appeared to correlate with the previously determined anti-Tat antibody titre. As the pVAX-sTat-IMX313 and pVAX-sTat vaccines induced the strongest anti-Tat NAb, they were selected for further analysis.

Higher titre anti-Tat responses were induced by multiple vaccine doses. High titre anti-Tat antibodies develop after multiple vaccine doses[7,18,19,25] thus, we determined if an increased number of doses of pVAX-sTat or pVAX-sTat-IMX313 would the antibody titres. Consequently, mice were vaccinated with a total of 5 doses of 50 μg DNA, and serum and CVL samples examined for anti-Tat IgG and mucosal IgA, respectively, in samples collected after the 3rd, 4th and 5th doses. In addition, splenocytes were harvested and analysed by ELISpot. After the 4th dose, serum anti-Tat IgG titres increased by ~4.7 fold in all pVAX-sTat vaccinated mice, from a mean endpoint titre of 1800 to 8486 ($p = 0.0262$) and by ~8.16 fold in pVAX-sTat-IMX313, from a mean endpoint 5526 to 45129 ($p = 0.0006$) (Fig. 3A). Significantly, inclusion of IMX313 resulted in an increase of ~5-fold in the titres from pVAX-sTat-IMX313 mice compared to pVAX-sTat-vaccinated mice ($p = 0.0029$). Administration of a 5th dose increased the serum IgG titres by ~4 fold (8486 to 35871, $p = 0.0152$) in pVAX-sTat- and by ~1.5 fold (45129 to 65957, $p = 0.2657$) in pVAX-sTat-IMX313-vaccinated animals (Fig. 3A). At this time point, the difference in serum titres between the two groups was no longer significant ($p = 0.0862$).

To assess if the higher titres also resulted in increased NAb activity, we repeated the Tat transcription assay using serum collected 2 weeks after the 5th vaccine dose. This showed an increase in Tat neutralisation activity of 4.09 fold (i.e. reduced RLUs from 488049 to 119040 RLUs, 27% to 76% neutralisation, $p = 0.0043$) for pVAX-sTat and by 2.68 fold (from 341062 to 127144, 49% to 81% neutralisation, $p = 0.0022$) for pVAX-sTat-IMX313 relative to the samples taken after 3 doses (compare Fig. 3B,C with 2E,F). However, the difference in percentage neutralisation between the two vaccines at this time point was not significant ($p = 0.7879$).

The Tat DNA vaccine induced sIgA responses in CVL. We tested CVL samples for sIgA as anti-Tat sIgA could potentially block HIV activity at the mucosa[26,27]. Low levels of sIgA were detected in 42% (3/7) pVAX-sTat- and 57% (4/7)pVAX-sTat-IMX313-vaccinated mice after the 3rd dose (mean IgA titres of 4 and 7, respectively) (Fig. 3D,E). After administration of the 4th dose, sIgA was detected in 57% (4/7) pVAX-sTat and 85% (6/7) pVAX-sTat-IMX313 mice, while the mean titres increased by ~3.5-($p = 0.054$) and 4-fold ($p = 0.0431$) to 14 and 28 respectively (Fig. 3D,E). Importantly, after the 5th dose, sIgA was detected in 100% (7/7) of pVAX-sTat-IMX313 mice, while it had no effect on pVAX-sTat vaccinated mice (57%; 4/7). At this time point, the mean sIgA titres increased by ~3 fold for each vaccine and reached 47 ($p = 0.554$) and 84 ($p = 0.554$) in samples from pVAX-sTat and pVAX-sTat-IMX313 vaccinated animals, respectively (Fig. 3D,E). The difference in titres was not significant, although only pVAX-sTat-IMX313 induced Tat-specific sIgA in all vaccinated animals.

Increasing vaccine doses enhances CMI to Tat. We then examined CMI to Tat by ELIspot 2 weeks after 5 doses of pVAX-sTat or pVAX-sTat-IMX313. Lower responses were detected in splenocytes from pVAX-sTat than pVAX-sTat-IMX313-vaccinated mice (mean SFUs 498 vs 601, $p = 0.7768$), but this difference was not statistically significant (Fig. 3F). These responses were ~4 fold higher than those generated after 3 doses of pVAX-sTat-IMX313 (total mean IFN-γ SFUs 601 vs 160, $p = 0.0163$) or pVAX-sTat (total mean IFN-γ SFUs 498 vs 133, $p = 0.0163$) suggesting that administration of 2 extra booster doses enhanced CMI. However, a caveat for these results is that the comparison between CMI generated after the 3rd and 5th doses is based on results from 2 different experiments.

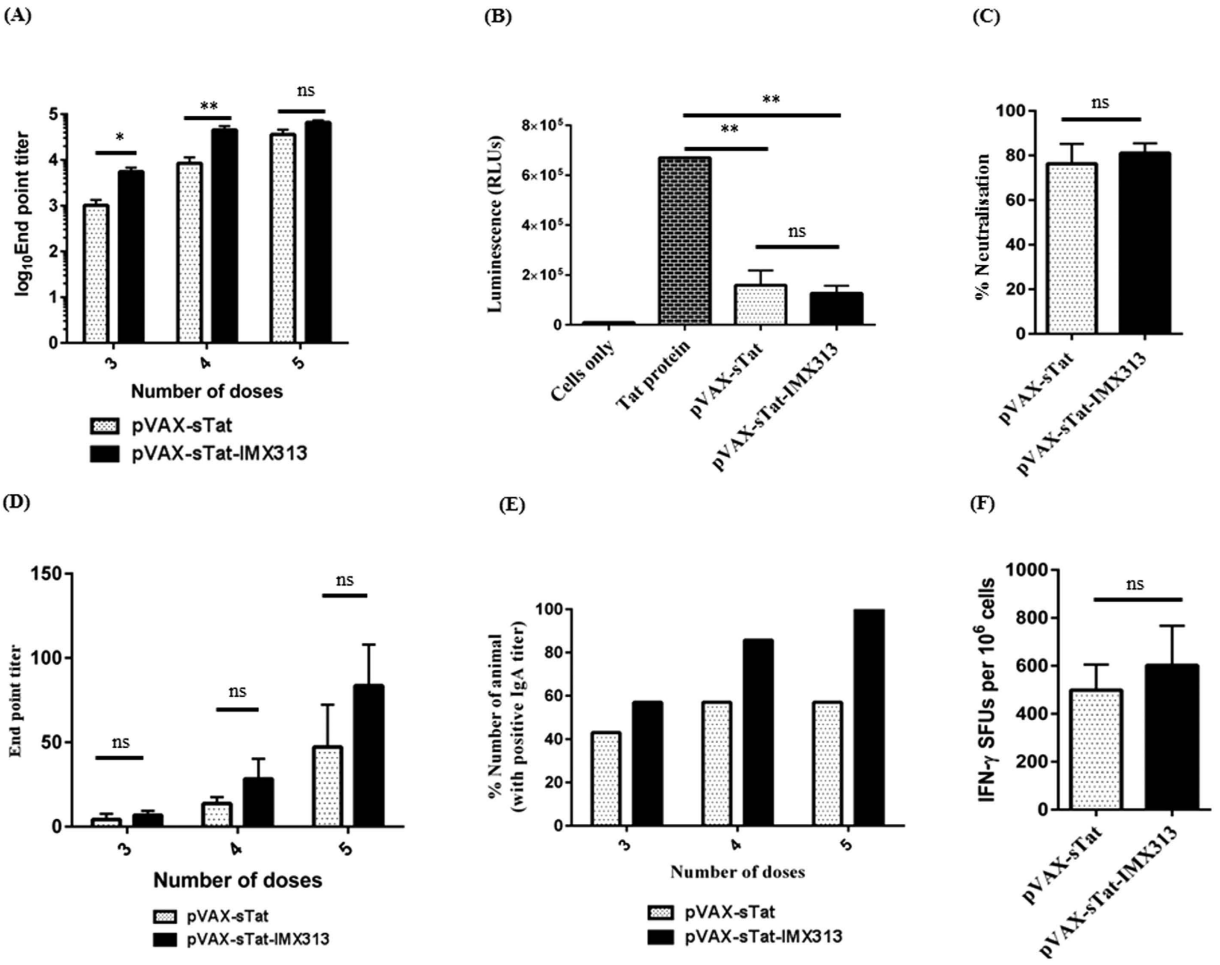

Figure 3. Humoral responses and CMI are increased after 5 doses of Tat DNA vaccine. Animals were vaccinated ID with 5 doses of 50 μg of pVAX-sTat or pVAX-sTat-IMX313 at 2 week intervals. Splenocytes were collected 14 days after the last dose. Blood and CVLs were collected one day before each vaccination and on the day of euthanasia. (**A**) ELISA results showing serum anti-Tat IgG titres. (**B**) Serum neutralisation of Tat transactivation activity, (**C**) Percentage decline in luminescence (an indicator of the neutralisation of Tat transactivation activity) that is depicted in (**B,D**) ELISA results showing anti-Tat sIgA titres in CVLs from pVAX-sTat or pVAX-sTat-IMX313 vaccinated mice. Data are representative of 2 independent experiments. (**E**) Percentage of vaccinated animals in which sIgA was detected after 3–5 vaccine doses in the experiment shown in 3D. (**F**) IFN-γ ELIspot assay to examine Tat-specific CMI responses following administration of 5 doses of pVAX-sTat or pVAX-sTat-IMX313. Data shown in the entire figure depict the mean (n = 7) ± SEM; an unpaired non-parametric Mann–Whitney U test was used to analyse the statistical significance of the data; $*p < 0.05$, $**p \leq 0.01$, $***p \leq 0.001$ and $p \geq 0.005$ = non-significant (ns).

DNA vaccines encoding Tat control EcoHIV viral load post-challenge. To the best of our knowledge, EcoHIV is the only surrogate murine HIV challenge model and has been used previously to successfully evaluate the efficacy of candidate HIV vaccines[28,29]. There is no documented evidence that EcoHIV can infect mice via the intravaginal route which accounts for a majority of HIV transmission[30] and thus the intraperitoneal route has been used in our laboratory and by other research groups as a convenient route to successfully deliver the virus[28,29,31,32]. Furthermore, mice are infected following a single EcoHIV challenge resulting in spread of the infection from the primary infection site to the spleen and the brain in a manner reminiscent of HIV infection[33]. EcoHIV encodes the complete range of HIV proteins, including Tat, from the HIV-1 Clade B NL4-3 strain, except for the HIV gp120 which was replaced with gp80 from the murine leukemia virus[33]. Consequently, as EcoHIV infects murine lymphocytes[33], we examined the protective efficacy of vaccination with pVAX-sTat or pVAX-sTat-IMX313 against EcoHIV challenge in mice which received 5 doses of the vaccines. The level of EcoHIV infection was determined by measuring the viral load in PECs and splenocytes from challenged animals by RT-qPCR targeting the MLV *env* gene and the results were normalised to the RPL13a house-keeping gene as described previously[28,29]. However, no clinical data eg. $CD4^+$ cell counts, were gathered as the primary site of infection is peritoneal cells and because the virus causes no overt disease[33]. Both groups of vaccinated mice showed significantly reduced levels of EcoHIV RNA in PECS and splenocytes compared with unvaccinated mice. The viral load in pVAX-sTat-IMX313 vaccinated mice was reduced by ~11 fold in PECS ($p = 0.0022$) and ~7 fold

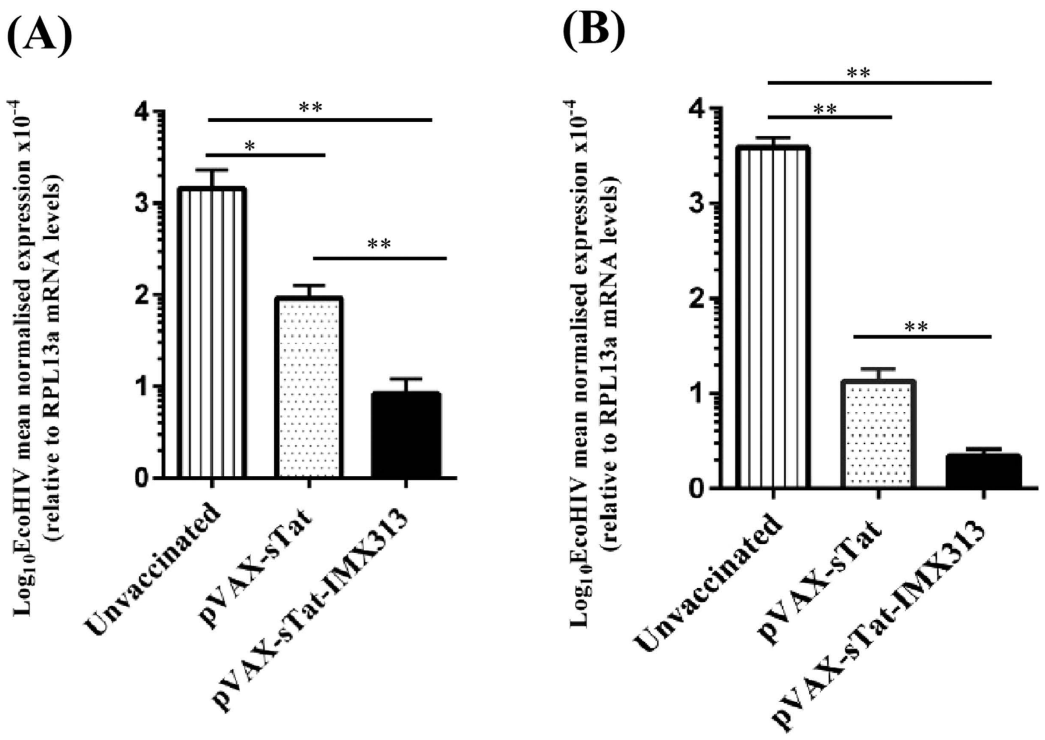

Figure 4. pVAX-sTat-IMX313 vaccinated mice exhibit superior control against EcoHIV challenge. Unvaccinated mice or vaccinated mice which received 5 doses of 50 μg of either pVAX-sTat or pVAX-sTat-IMX313 were challenged with 1.5 μg p24 of EcoHIV/NL4-3 10 days post final vaccination. The viral load in PECs and splenocytes was determined by qRT-PCR; EcoHIV RNA levels in (**A**) PECs and (**B**) splenocytes 7 days post challenge. EcoHIV mRNA levels were normalised to RPL13a mRNA and the data represent the mean (n = 7) ± SEM. $^{**}p < 0.05$ and $^{**}p \leq 0.01$(Mann–Whitney U test).

in splenocytes ($p = 0.0022$) compared with pVAX-sTat vaccinated mice (Fig. 4A,B) although pVAX-sTat- and pVAX-sTat-IMX313-vaccinated mice limited EcoHIV infection significantly compared to unvaccinated control mice ($p = 0.0012$ and $p = 0.0022$, respectively). Overall, the data show that oligomerisation of a secreted form of Tat can significantly augment anti-Tat mucosal and systemic antibody responses, and importantly increases the protective efficacy of Tat DNA vaccines.

Discussion

In this study, we exploited a novel strategy to generate high titre Tat-specific serum IgG with potent anti-Tat neutralisation activity following administration of a DNA vaccine encoding sTat fused to the oligomerisation domain of C4b-p (pVAX-sTat-IMX313). The vaccines were administered via the ID route as this site is rich in antigen presenting cells[34,35]. pVAX-sTat-IMX313 elicited high titre Tat-specific systemic IgG and mucosal sIgA in all vaccinated mice and significantly reduced the EcoHIV viral load compared to unvaccinated controls, but surprisingly only resulted in a modest cell-mediated response. Although the mechanism of C4b-p induced protein oligomerisation is not well defined[12,14], our results suggest that oligomerisation requires efficient secretion of the fusion protein (Fig. 1B,C), consistent with previous studies[11,12,14].

Although oligomerised proteins are more stable, resistant to oxidation and proteolysis[8,9], and more immunogenic compared to their monomeric counterparts, canonical Tat protein-based vaccines are poorly immunogenic, likely due to the poor stability of native Tat protein[1,2]. A few studies suggest that an increase in Tat stability and protection from proteolysis is required to enhance Tat immunogenicity[8,9]. Consequently, our studies were restricted to a comparison of different DNA constructs. Indeed, pVAX-sTat-IMX313 was superior in inducing anti-Tat NAb responses compared to the other DNA vaccines tested. The appearance of anti-Tat antibodies appears to correlate with protection in humans[6,7,24,36,37] and vaccination with DNA encoding Tat induced protection in non-human primates[18,22,25] thus, inducing high titre Tat-specific antibodies is an important component of future HIV vaccines as these antibodies are expected to neutralise Tat activities thereby blocking the early stages of virus replication.

Tat-based vaccines may not be expected to elicit sterilizing immunity, but to control HIV replication and disease onset[1], and control of virus burden normally requires potent CTL responses[18,38]. Current assays which evaluate T cell immunity (eg. ELISpot, ICS, and ^{51}Cr release) are performed *ex vivo* after *in vitro* stimulation of T cells. Whilst these assays provide useful information, they involve extensive (several hours to days) manipulation of T cells *in vitro* and are not directly indicative of T cell killing function *in vivo*. The FTA is currently the most sensitive and versatile assay available to evaluate $CD4^+$ and $CD8^+$ T cell effector functions *in vivo* in

preclinical models[21]. The assay essentially involves 'barcoding' targets with a combination of cell tracking dyes (eg. CFSE, Cell Proliferation Dye eFluor670 (CPD) and Cell Trace Violet (CTV)) to generate up to 252 unique cell clusters as targets that can be pulsed with various peptides prior to FTA challenge of vaccinated animals[21]. This technique allows for sensitive and direct measurement of *in vivo* killing in vaccinated animals, as well as for the measurement of $CD4^+$ T helper cell activity based on their ability to up-regulate CD69 expression. Thus, we examined *in vivo* whether the Tat DNA vaccines induced a CTL response in vaccinated animals using this assay. Surprisingly, the FTA killing assay revealed <1% killing of target cells. This result was in clear contrast to previous studies which reported potent CTL responses after Tat vaccination[18,22]. It is likely that CTL responses are affected by the route and dose of each vaccine, as suggested in other previous studies[18,25]. However, using the same assay we observed an equally potent Th response *in vivo* in all Tat DNA vaccinated mice (Fig. 2C). Based on these results, we anticipated that similar levels of anti-Tat antibodies should be generated following vaccination with pVAX-Tat, pVAX-Tat-IMX313, pVAX-sTat or pVAX-sTat-IMX313. Interestingly, the highest antibody titre and serum neutralisation was detected in serum from pVAX-sTat-IMX313 vaccinated mice suggesting that the generation of these antibodies was not exclusively dependent on the magnitude of T-cell help. Whether the improved stability and oligomerisation of secreted Tat resulted in greater antigen presentation to B cells independently of $CD4^+$ T cells or potent cross-linking of the B-cell receptor was not explored in this study and warrants further investigation.

Tat vaccines often require >3 doses to induce robust immune responses[7,18,22,39] and our data showed that two additional doses significantly enhanced Tat IgG titres and NAb responses, to neutralise 76 and 81% Tat activity activity for pVAX-sTat and pVAX-sTat-IMX313, respectively (Fig. 3C), although the differences were not statistically significant. As the concentration of Tat protein (50 ng/ml) added to the transactivation assay is similar to that of soluble Tat detected in serum from HIV-infected individuals[40] and was largely neutralised by a 1/25 dilution of mouse serum, a similar or greater level of neutralisation of Tat transcription activity would be expected following administration of pVAX-sTat-IMX313 in humans.

Furthermore, 100% of pVAX-sTat-IMX313-vaccinated mice but only 57% of pVAX-sTat-vaccinated mice (Fig. 3E) developed mucosal sIgA proving that pVAX-sTat-IMX313 generated mucosal humoral responses more effectively. The presence of sIgA at the mucosa and in blood appears to correlate with protection against HIV in exposed, persistently seronegative individuals[41–43] and in non-human primates[44,45]. Since the mucosa is the site for primary HIV infection[46], and humoral responses at the mucosa are mainly mediated by sIgA[47], we expect the presence of Tat-specific sIgA at mucosal site to confer similar protective benefits in pVAX-sTat-IMX313-vaccinated individuals. In addition to boosting humoral responses, increasing the number of doses to five significantly increased Tat-specific CMI, with pVAX-sTat-IMX313eliciting slightly higher CMI than pVAX-sTat (Fig. 3). We were unable to perform the FTA on animals which received five doses to determine if this would result in increased CTL killing and ongoing experiments are planned to address this issue.

Robust immune responses are not necessarily indicative of protection against virus challenge[48,49]. Therefore, an effective Tat-based HIV vaccine should demonstrate efficacy against viral replication and control disease progression. To investigate this aspect, we challenged pVAX-sTat-IMX313 and pVAX-sTat vaccinated mice with EcoHIV. The results from this experiment showed that the pVAX-sTat-IMX313 vaccine elicited superior control of virus replication at the site of infection (peritoneal space) and control over EcoHIV spread to the spleen compared with pVAX-sTat vaccination (Fig. 4A,B). However, we did not investigate the longevity/durability of EcoHIV control in vaccinated animal. Experiments to address these aspects will require a non-human primate model. It is unclear if humoral immunity and/or CMI was responsible for the observed control of EcoHIV infection as we were unable to conduct a correlation analysis owing to the limited number of animals studied per vaccination group. As there is no evidence for an interaction between the MLV envelope and HIV Tat, anti-Tat antibodies are unable to bind to- or inhibit uptake of-EcoHIV. Therefore, based on the *in-vitro* Tat neutralisation data presented here, we are tempted to speculate that the control of the EcoHIV viral load was associated with the presence of anti-Tat neutralising antibodies as reported in previous animal studies[50–52], but we cannot discount the potential of CMI to control the viremia and an additional study to address this is warranted. Although we did not determine if the mice generated anti-IMX313 antibodies, a previous[12] noted that such antibodies were not detected. Irrespective of this, the Tat antigen used in the transactivation assay did not contain the IMX313 sequence and thus the neutralisation was specific for Tat. In addition, although anti-Tat antibodies may contribute to ADCC[53], we did not investigate whether the antibodies had ADCC activity.

As there was no statistical difference in antibody titres after 5 doses of pVAX-sTat-IMX313 or pVAX-sTat vaccination (Fig. 3A), the EcoHIV challenge data also suggest that pVAX-sTAT-IMX313 elicited antibodies of superior quality compared with pVAX-sTat.

Collectively, the results show that pVAX-sTat-IMX313 was most immunogenic and compared favourably with previous Tat-based vaccines[7,18,22]. The ability of pVAX-sTat-IMX313 to induce high titre anti-Tat responses can most likely be attributed to Tat oligomerisation and the secretion of Tat heptamers as particulate antigens such as oligomerised proteins, lead to greater uptake by phagocytosis and result in enhanced antibody production[12,14]. Previously, the formation of plasmodium merozoite surface proteins and *Mycobacterium tuberculosis* antigen 85A heptamers enhanced the immunogenicity of candidate malaria[11,12,14] and tuberculosis vaccines[13] respectively. Although the mechanism has not been defined, it has been suggested that IMX313 binds to B-cell CD40, C-reactive proteins, pentraxins and serum amyloid P-complexes resulting in increased Fcγ-receptor-mediated phagocytosis and cytokine secretion[14]. This interaction also enhances CD54 (ICAM-1), CD86 expression and IL4-dependent IgE isotype switch[54] resulting in increased antigen presentation by APCs[13,55] and prolongation of serum half-life of the immunogens[12,56,57]. Consequently, these interactions enhance adaptive immune responses[14] which might explain why the pVAX-sTat-IMX313 vaccine induced high titre anti-Tat responses. Thus, IMX313 acts as a molecular adjuvant when included in DNA vaccine formulations.

For optimal efficacy, the pVAX-sTat-IMX313 vaccine could be combined with other DNA vaccines, and as we previously reported that a unique cytolytic DNA-gag vaccine also elicits significant control of EcoHIV infection post-challenge[28], experiments to address this are planned. Our results support further testing of pVAX-sTat-IMX313 as a candidate HIV vaccine in a larger animal model and avoid the need to purify Tat, thus circumventing the disadvantages associated with proteolytically degraded and oxidised Tat protein.

Materials and Methods

DNA plasmids. Codon optimised HIV-1 clade B *tat* and IMX313 genes (Gene Art, Germany) were inserted into pVAX downstream of the CMV promoter. Four plasmids were generated encoding either wild type Tat viz. (1) pVAX-Tat, (2) pVAX-Tat-IMX313 or secreted Tat (sTat) generated by the upstream introduction of the TPA leader sequence[29] (3) pVAX-sTat and (4) pVAX-sTat-IMX313. pET-DEST42, encoding Tat fused to a 6x-His tag was used to synthesize the protein in *Escherichia coli* for ELISA. PCR primers for cloning are listed in the supplementary material and all plasmids were sequenced. DNA vaccines were purified using standard molecular biology and endotoxins removed with the endotoxin removal solution (Sigma-Aldrich, Sydney, Australia). The endotoxin level in each DNA vaccine was <0.1 EU/μg DNA (SA Pathology, Adelaide, Australia).

Western blot analysis. To detect Tat expression, cell lysates and supernatants were harvested from HEK293T cells transfected with DNA and 50 μg of protein was analysed in 10–12% (v/v) SDS-PAGE under reducing (with β-mercaptoethanol, β-Me) or non-reducing (without β-Me) conditions as described[12,14]. A cocktail of Tat monoclonal antibodies, 4D5.24, 5A5.3 and 1D9 (NIH AIDS Reagent Program) and goat-anti mouse HRP-conjugated secondary antibody (Antibodies Australia, VIC, Australia) were used to detect Tat expression essentially as described previously[29].

Animal immunisations. Female 6–8 week old BALB/c mice purchased from the University of Adelaide Animal Services were maintained in the Queen Elizabeth Hospital animal house under PC2 conditions in individually ventilated cages fitted with a HEPA filter. All experiments were approved by and conducted in accordance with guidelines and protocols approved by the University of Adelaide and the South Australia Pathology Animal Ethics Committees. Groups (n = 7) were vaccinated with the different vaccines or pVAX (n = 3) with 3–5 doses of 50 μg in saline at 2 week intervals, by the ID route as described[28,29]. Blood and cervical vaginal lavage (CVL) samples were collected[28] and examined before and after each vaccine dose for anti-Tat antibody responses; 14 days post vaccination, the mice were euthanized and splenocytes prepared for analysis.

Enzyme-linked immunosorbent spot assay (ELISpot). Splenocytes were stimulated with a panel of 11 overlapping 15–19 mer peptides (NIHAIDS reagent program) spanning the entire Tat proteinas we described[28,29]. Briefly, multiscreen-IP HTS plates (Merck Millipore, Germany) were coated with anti-mouse IFN-γ (clone AN18, MabTech, Sweden) and secreted IFN-γ detected with anti-mouse IFN-γ -biotin (clone R4-6A2, MabTech). Phytohemagglutinin and un-stimulated splenocytes were used as positive and negative controls, respectively. Spots were counted using an ELISpot reader (AID GmbH, Germany). The number of spots in unstimulated splenocytes was subtracted from the number in peptide-stimulated cells to generate the net number of Tat-specific spot forming units (SFUs).

Fluorescent target array (FTA) assay. The FTA assay was used to examine the magnitude and quality of T-cell responses generated after vaccination in an *in vivo* assay[21]. Splenocytes from naïve BALB/c mice were co-labelled with combinations of four different concentrations of cell trace violet (CTV) and carboxyfluoresceinsuccinimidyl ester (CFSE) dyes to generate a 16 parameter FTA (Supplementary Fig. S2A), as described previously[21].

The fluorescent tagged cells were then pulsed with 0.01–10 μg/ml of Tat peptides for 4 h at 37 °C in 5% CO_2. The peptides were divided into pools 1–3 in which pool 1 contained peptides representing the complete Tat protein, whereas pool 2 lacked the theoretical $CD8^+$ T cell immunodominant epitopes and pool 3 contained only the theoretical $CD8^+$ T cell immunodominant epitopes (HIV molecular immunology database, http://www.hiv.lanl.gov/). The peptide-pulsed dye-labelled cells were pooled and about 40×10^6 cells were adoptively transferred intravenously via the lateral tail vein into each mouse, 13 days after the final vaccine dose. Splenocytes were then harvested 18h post-FTA transfer, RBC-depleted and stained with antibodies against mouse antigens (allophycocyanin conjugated anti-CD69 (eBioscience) and Alexa Fluor 700 conjugated anti-B220 (eBioscience)). The percentage specific killing of FTA cells was calculated using the formula described previously[24]. The magnitude of $CD4^+$ T cell help provided to naïve B cells in vaccinated mice was determined by measuring the geometric mean fluorescence intensity (GMFI) of CD69 on gated $B220^+$ cells within the FTA. Samples from each vaccinated mouse were analysed on a BD FACS Canto (Becton Dickinson, NJ, USA) and FACS plots and GMFI analyses were performed using the FlowJo software (version 8.8.7).

Enzyme-linked immunosorbent assay (ELISA). Serum and CVL samples were analysed for anti-Tat antibodies by indirect ELISA. Briefly, Maxisorp plates (Corning Sigma-Aldrich) were coated with 500 ng of Tat, then serum and CVL samples (diluted in PBS/0.5% FCS) added. Bound antibodies were detected using HRP-conjugated goat anti-mouse IgG (GE Healthcare Life Sciences, USA) or anti-mouse IgA (LifeTechnologies) and the OD read at 492 nm. Endpoint titres were determined as the reciprocal of the highest serum or CVL sample dilution with an OD reading above the cut-off, set as 2SD above the mean OD of serum samples from pre-vaccinated- or naïve- mice.

Anti-Tat neutralisation assay. The NAb activity of anti-Tat antibodies was assessed in an *in vitro* transactivation assay using Cf2-Luc cells[3,58]. 50 ng/ml of purified Tat protein (NIH Reagent Bank), previously incubated for 1h in PBS or serum diluted 1/25, was added to the cells, and cell lysates prepared 48 h later as described[58,59] then

assayed for luciferase activity using the dual luciferase assay system (Promega). The data are expressed as mean relative luminescence units (RLUs) ± SEM. The percentage neutralisation was calculated using the formula: % specific neutralisation = [(RLUs in the absence of test sera- RLUs in the presence of test sera)/ RLUs in the absence of test sera] x100.

EcoHIV/NL4-3 challenge. EcoHIV was prepared and titrated as described[28,29] and vaccinated mice challenged via the intra-peritoneal route with EcoHIV equivalent to 1.5 μg p24[28,29]. The mice were culled, and spleen and peritoneal exudate cells (PECs) collected 7 days post challenge then examined by qRT-PCR[28,29].

Statistical analysis. Data presented as mean ± SEM were generated with GraphPad Prism version 6 (GraphPad Software, La Jolla, CA, USA). Non-parametric Kruskal-Wallis test was used to compare the difference between multiple vaccine groups. If this showed significant differences, then the Mann-Whitney U test was performed to compare differences between each vaccine group, independently. Statistical significance was determined using the Mann-Whitney U test; $p < 0.05$ was considered significant and $p > 0.05$ was considered non-significant.

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Author Contributions

K.T. performed all the experiments and wrote the manuscript; D.W., J.G., T.G. and Y.L. helped in designing experiments, data analysis and reviewing the manuscript; L.G. and M.C. helped in setting up the Tat transactivation and neutralisation assays and reviewed the manuscript; B.G.-B. and E.J.G. conceived the study, helped in data analysis and reviewing the manuscript.

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Chapter 4.0: Human rhinovirus-A1 as an expression vector

(Word version of the manuscript, accepted for publication as a book chapter in Methods in Molecular Biology)

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Contribution to the Paper	Performed all experiments, analysed data and wrote the manuscript.
Overall percentage (%)	90%
Certification:	This paper reports on original research I conducted during the period of my Higher Degree by Research candidature and is not subject to any obligations or contractual agreements with a third party that would constrain its inclusion in this thesis. I am the primary author of this paper.
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Co-Author Contributions

By signing the Statement of Authorship, each author certifies that:

the candidate's stated contribution to the publication is accurate (as detailed above);

permission is granted for the candidate in include the publication in the thesis; and

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Human rhinovirus-A1 as an expression vector

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4.1 Summary

Expression vectors that are based on live human rhinoviruses (HRVs) are attractive, yet overlooked in vaccine development; mainly due to their limited capacity for foreign gene inserts and poor genetic stability. This article describes a novel methodology to engineer a replication-competent genetically stable recombinant HRV (rHRV) without affecting viral replication capability. We have previously used these methods to generate live, genetically stable recombinant HRVs encoding HIV Gag and Tat proteins (rHRV-Gag-Tat), a potential mucosally-targeted HIV vaccine.

Keywords: HRV-A1, live vaccine vector, HIV-Gag and HIV-Tat

4.2 Introduction

4.2.1 Classification of human rhinoviruses (HRVs)

HRVs are single-stranded, positive-sense RNA viruses belonging tothe genus Enteroviruses of the *Picornaviridae* family[1]. Other genera within this family are the Hepatoviruses[2], Kobu viruses[3] and Parechoviruses[4] all of which cause diseases in humans and animals. Perhaps the most important species among the picornaviruses are the polioviruses and human rhinoviruses (HRVs) owing to their historical clinical associations. HRVs are closely similar to polioviruses, but differ by being acid labile with low temperature culture requirements[5]. Polioviruses cause poliomyelitis in humans, while HRVs are mainly isolated from individuals with acute and chronic respiratory illnesses[6]**.** Infections with polioviruses have been reduced and the viruses are nearing global eradication as a result of the global vaccination campaign. HRV infections tend to be seasonal, peaking during the winter months and are usually self-limiting.

4.2.2 Virion structure and genome organisation

HRVs are icosahedral viruses with a diameter of ~ 30nm with a RNA genomeof ~7.4 kb enclosed in a capsid[7]. The 5'-end of the genome contains a 5' UTR encompassing an internal ribosomal entry site and the VPg protein which primes genome replication, followed by the P1 region that encodes the capsid (VP1-VP4) proteins, and the P2 and P3 regions that encode the non-structural proteins (2Apro to 2C and 3A to 3D, respectively). The genome is terminated by a 3' UTR and a poly-A tail which are required for efficient genome replication. The VP1 protein possesses a canyon required during cell entry to bind the cellular receptor, the VP2, VP3 and VP4 proteins contain the neutralizing antibody immunogenic regions (NIm-IB, NIm-II, and NIm-III) and VP4 also anchors the RNA into the capsid. The 2A protease ($2A^{pro}$) cleaves the viral polyprotein to separate the structural from the non-structural proteins. The 3C protease ($3C^{pro}$) cleaves the polyprotein into the individual mature VP1,

VP3, 2B, 2C, 3A, 3B, and 3D proteins. Together with $2A^{pro}$, 3Cproshuts-off 5'cap-dependent host cellular translation to ensure that the cellular machinery is directed towards viral translational and replication. The 2B, 2C, and 3A proteins are involved in cell membrane rearrangement and the formation of cytoplasmic vesicles, and also influence the host range for HRVs, while the 3D protein acts as a RNA-dependent RNA polymerase ($3D^{Pol}$). The 3B protein is the VPg protein.

4.2.3 HRV genotypes and serotypes

HRVs are classified as genotype A, B or C (HRV87 is the only currently identified C strain) based on phylogenetic analysis of the 5'UTR and capsid coding sequences[8,9]. HRV genotypes A and B are further classified into the major and minor receptor groups based on receptor recognition[10]. The major receptor group HRVs use the intracellular adhesion molecule (ICAM-CD5) or heparin sulphate proteoglycans (HSPG) for cell entry[11,12]. They constitute about 90% of all HRVs (all genotype B and a few genotype A strains)[1]. The minor receptor group HRVs (genotype A only) use a family of low density lipoprotein receptors (LDLRs) viz. LDLR, VLDLR, LDLR-related proteins and megalin[13], which are located on the surface of human and murine cells[7]. Therefore, most minor group HRVs can infect both human and murine cells[14,15]. The receptor used by HRV-C remains poorly understood, but previous evidence shows that a sialoprotein is used [16]. HRVs have been also further divided into species A (74 serotypes) and B (25 serotypes) based on susceptibility toanti-viral agents such as capsid-binding compounds, for example pleoconaril[7]. They have also been divided into M-strains which grow in human and monkey kidney cells and H-strains that initially grew in human cell lines before they were adapted to grow in monkey cells[5].

4.2.4 Viral replication

The actual mechanism of HRV replication is poorly understood. However, upon successful attachment to the membrane, viruses are either endocytosed or micro-pinocytosed into the

cell. This is followed by conformational changes in the virion induced by the endosomal micro-environment, resulting in the formation of hydrophobic virus particles[5]. The viral RNA exits the endosome via membrane pores into the cytosol where it is translated in a cap-independent manner in association with cytoplasmic membranous vesicles into a polyprotein of ~250 kDa, which is auto-catalytically and co-translationally cleaved by the viral proteases into precursor and mature proteins. Generally, HRV RNA can be detected five hours post infection, viral replication is completed ~12 hours post-infection and the peak viral load is reached 48 hours post-infection in permissive cell lines.

4.2.5 Human rhinoviruses serotype A1 (HRV-A1) as vaccine vectors

Owing to the global success of the poliovirus vaccines (especially the live-attenuated oral polio vaccine) to the near eradication of poliovirus infections, interest was generated in utilising live polioviruses as effective recombinant expression vectors[17-19]. This was further supported by the mucosal transmission of polioviruses, which meant that vectors based on these viruses could potentially be used to deliver mucosally-targeted vaccines against diverse viral infections. Live poliovirus vectors have been mainly evaluated in animal models as candidate vaccines against human immunodeficiency virus (HIV) infections. These vaccines have been shown to induce simian immunodeficiency virus (SIV)-specific immune responses that protect vaccinated macaques against intravaginal challenge with a pathogenic SIVmac251[19-21], a surrogate macaque HIV challenge model. However, the high level of prior immunity to polioviruses in humans prevented further testing of vaccines delivered by live poliovirus vectors. Prior immunity to a vaccine vector resulted in poor responses in a large-scale clinical trial evaluating a potential HIV vaccine[22].

On the other hand, the close similarity between polioviruses and HRVs implied that vaccines delivered by live HRV vectors might be equally immunogenic relative to live poliovirus vaccines. Indeed, early evidence showed that HIV vaccines delivered by HRV vectors were

highly immunogenic in animals[17,18,23-26] and this was further supported by data from a more recent study[27]. Unfortunately, the same reasons (i.e. pre-existing immunity) that hampered testing vaccines delivered by a live poliovirus vector have affected the use of live HRV vectors in humans.

Nevertheless, HRV-A1 a member of the minor group HRVs has emerged as a potential suitable expression vector. This serotype is considered the rarest among all HRVs circulating in the human population[28,29] which suggests that prior immunity to this serotype among humans is uncommon. Furthermore, HRV-A1 can infect mice and humans[14,15], permitting experiments to evaluate the efficacy of recombinant HRV-A1 vaccines in a mouse model prior to human clinical trials. We have recently produced the first evidence that HRV-A1 can be engineered into a genetically stable, replication-competent expression vector[30]. We inserted the HIV *gag* or *tat* genes, as outlined in section 4.4, to generate a live recombinant HRV vaccine encoding HIV Gag or Tat proteins (rHRV-Gag/Tat) as a potential mucosally-targeted HIV vaccine.

4.3. Materials

4.3.1 Cells

1. H1-HeLa cells (only this cell strain was used in all experiments unless otherwise stated).
2. *E.coli* DH5α cells

4.3.2 Enzymes

1. Restriction endonucleases viz. Apa1, Mlu1 and Xho12.4DNA ligase (NEB™, MA, USA)
2. Antarctic phosphatase (NEB™)

4.3.3 Growth Media

1. LB-broth: 1% w/v Tryptone, 0.5% w/v Yeast extract and 200 mMNaCl.(Sterilise by autoclaving)
2. LB-Agar: 1% w/v Tryptone, 0.5% w/v Yeast extract, 1.5% w/v Agar and 200 mMNaCl. (Sterilise by autoclaving)
3. SOC medium1% w/v Tryptone, 0.5% w/v Yeast extract, 5M NaCl, 1M KCl, 1M $MgCl_2$, 1M $MgS0_4$ and1M glucose. (Sterilise by autoclaving)
4. Cell culture media: 10%w/v FCS, 1% w/v Pen/Strep in 500ml DMEM
5. Virus growth media: 10%w/v FCS, 1% w/v Penicillin/Streptomycin, 30 mM $MgCl_2$ in 500ml DMEM
6. Virus harvest media: PBS (1X), 30 mM $MgCl_2$ and30 mM $CaCl_2$.(Filter sterilised)
7. Opti-MEM® I reduced serum media

4.3.4 Preparative Kits

1. KAPA™HiFi PCR kit (KAPA Biosystems, SA, Australia)
2. KAPA™Taq PCR kit (KAPA Biosystems)
3. PureLink® Quick Plasmid Miniprep Kit (Invitrogen,VIC, Australia)
4. PureLink® Quick Gel Extraction Kit (Invitrogen)

5. PureLink® PCR Purification Kit (Invitrogen)

6. RNeasy Mini Kit (Qiagen, CA, USA)

7. TransIT®-mRNA Transfection Kit (Mirus, WI, USA)

8. MEGAscript® T7 Transcription Kit (Invitrogen)

9. MEGAclear™ Transcription Clean-Up Kit (Invitrogen)

10. QIAGEN OneStep RT-PCR Kit (Qiagen)

4.3.5 Reagents, General Materials and Instrumentation

1. RNase AWAY™ Decontamination Reagent

2. 70 and 100% Ethanol

3. 10 and 50 ml Falcon tubes

4. Corning® cell scrapers

5. Nalgene™ 0.2 and 0.45 μm syringe filters

6. Amicon Ultra-15 Centrifugal Filter Units, NMWL 100 kDa (Millipore, MA, USA).

7. Polyethylene Glycol 6000

8. 5M Sodium chloride (filter sterilized). Store at room temperature.

9. Syringes (2 ml to 20 ml)

10. Dry ice

11. Sterile, cotton plugged pipette tips

12. Sterile single-wrapped transfer pipettes

13. Sterile 6 well tissue culture plates

14. Sterile T75 and T175 tissue culture flasks

15. DNA Engine PCR machine (BioRad, CA, USA)

16. NanoDrop 2000c (ThermoFischerScientific, WI, USA)

17. V5 mouse monoclonal antibody (Thermo Fisher Scientific)

18. Goat anti-mouse IgG (H+L) secondary antibody, Alexa Fluor 488 conjugate (Thermo Fisher Scientific)

4.4 Methods

4.4.1 General procedure to generate recombinant HRV (rHRV)

By analogy with the polioviruses, there are several potential sites within the HRV genome into which exogenous genes may be inserted without abrogating viral replication, including the P1/P2 junction, the VPg/VP1 junction, the 2C/3A junction and the VPg/3C^{pro} junction[17]. Exogenous HIV *gag* or *tat* genes are inserted into the P1/P2 junction of the HRV genome (Fig. 1A) to ensure that the resultant recombinant HRVs remained replication-competent by utilising the autocatalytic activity of the HRV-2A^{pro} to cleave the Gag/Tat proteins from the recombinant viral polyprotein[30].

Several technical issues must be addressed when inserting exogenous genes into the HRV genome to ensure expression of the corresponding proteins in cells infected with the recombinant viruses:

1. Like all picornaviruses, HRVs have a limited capacity for the insertion of foreign genes to ensure that the final genome is ≤115% that of wild type HRV RNA. Thus, exogenous genes of around 1100 bases may be considered. Inserts which exceed this limit will be naturally lost and the recombinant virus will probably revert to wild type upon serial passage.
2. The P1/P2 junction of the cDNA encoding the HRV-A1genome contains a unique Apa1restriction endonuclease site. Therefore, this site can be used to insert exogenous genes. Apa1 restriction enzyme sites in foreign genes must therefore be mutated or a linker containing a multiple cloning site (MCS) with various restriction endonuclease sites can be introduced into the P1/P2 junction.
3. To ensure that the protein encoded by the inserted gene is cleaved from the recombinant polyprotein, nucleic acid sequences which encode the 2A^{pro} cleavage sequence should be introduced at the 5'and 3'ends of the exogenous gene insert.

4. To enhance the genetic stability of recombinant viruses, it is necessary to reduce the sequence homology of the two $2A^{pro}$ cleavage sites and thus inhibit detrimental homologous recombination.

These concerns were addressed as follows:

1. We divided the full length *gag* gene (~1500 bp) into five discrete overlapping fragments (*gag-1* to *gag-5*) ranging from 393 to 513 bp, within the putative capacity of HRVs, and then individually cloned each fragment into a separate HRV plasmid to generate 5 rHRVs each encoding an adjacent region of Gag (rHRV-Gag-1 to rHRV-Gag-5). Although up to 1100 bp of exogenous genes could theoretically be inserted into the HRV genome, we preferred to insert gag fragments of up to 513 bp to closely mimic the previous construction of highly immunogenic recombinant Sabin poliovirus vectors[19] as shown in table 1.

Insert	First (amino acid)	Last (Amino acid)	Size (Amin acids)
Gag-1	1	131	132
Gag-2	92	262	171
Gag-3	129	286	158
Gag-4	264	431	168
Gag-5	361	497	137

Table 1. HIV Gag-1 to Gag-5 fragment length. *The full length gag gene was divided into 5 discrete overlapping fragments which were individually cloned into separate HRVs to generate 5 rHRV each encoding a different Gag protein (rHRV-Gag-1-5).*

2. Each of the *gag* fragments contains a unique Apa1 site, thus a MCS containing a Xho1 site can be inserted to aid the insertion of these gene fragments into the P1/P2

junction. However, the full length *tat* gene (303 bp) is within the insertion limit, thus the full length gene can be cloned into HRV-A1 to generate a single rHRV encoding full length Tat protein (rHRV-Tat).

3. To ensure that the Gag and Tat proteins are cleaved from the recombinant viral polyprotein, PCR is employed to introduce a second $2A^{pro}$ cleavage site upstream of all *gag* and *tat* inserts (Fig. 1B).
4. To enhance the genetic stability of the rHRV-Gag/Tat, several silent mutations can be introduced into the coding sequences of both flanking $2A^{pro}$ cleavage sites to reduce the homology between the $2A^{pro}$ cleavage sequences, of these sites from 100% to 77% (Fig. 1C).

   The PCR primers used to generate the HIV *gag* and *tat* inserts have been described previously[30] and consist of (i) sequences corresponding to fragments of the 2Apro auto cleavage site, (ii) sequences corresponding to either Apa1 (for rHRV*gag* 1-2) or Xho1 (for rHRV*gag* 3-5 and *tat*) restriction enzyme site, (iii) non-specific sequences (2-6 bp) required for efficient restriction enzyme digestion and (iv) sequences (22 bp) corresponding to either *gag* or *tat*. A V5 tag coding sequence[31] is placed at the 3′ends of each insert to facilitate detection of the truncated Gag proteins, which contain few B cell epitopes[32], and the Tat protein. To generate rHRVs, we used a cDNA encoding a live, infectious clone of HRV-A1[33] obtained from Dr William Jackson (Research Medical College of Wisconsin, Milwaukee, Wisconsin, USA). This plasmid contains an inherent site for the Mlu1 restriction endonuclease and an ampicillin resistance gene. All inserts are digested with the restriction endonuclease similar to those used to linearize the HRV plasmid cDNA.

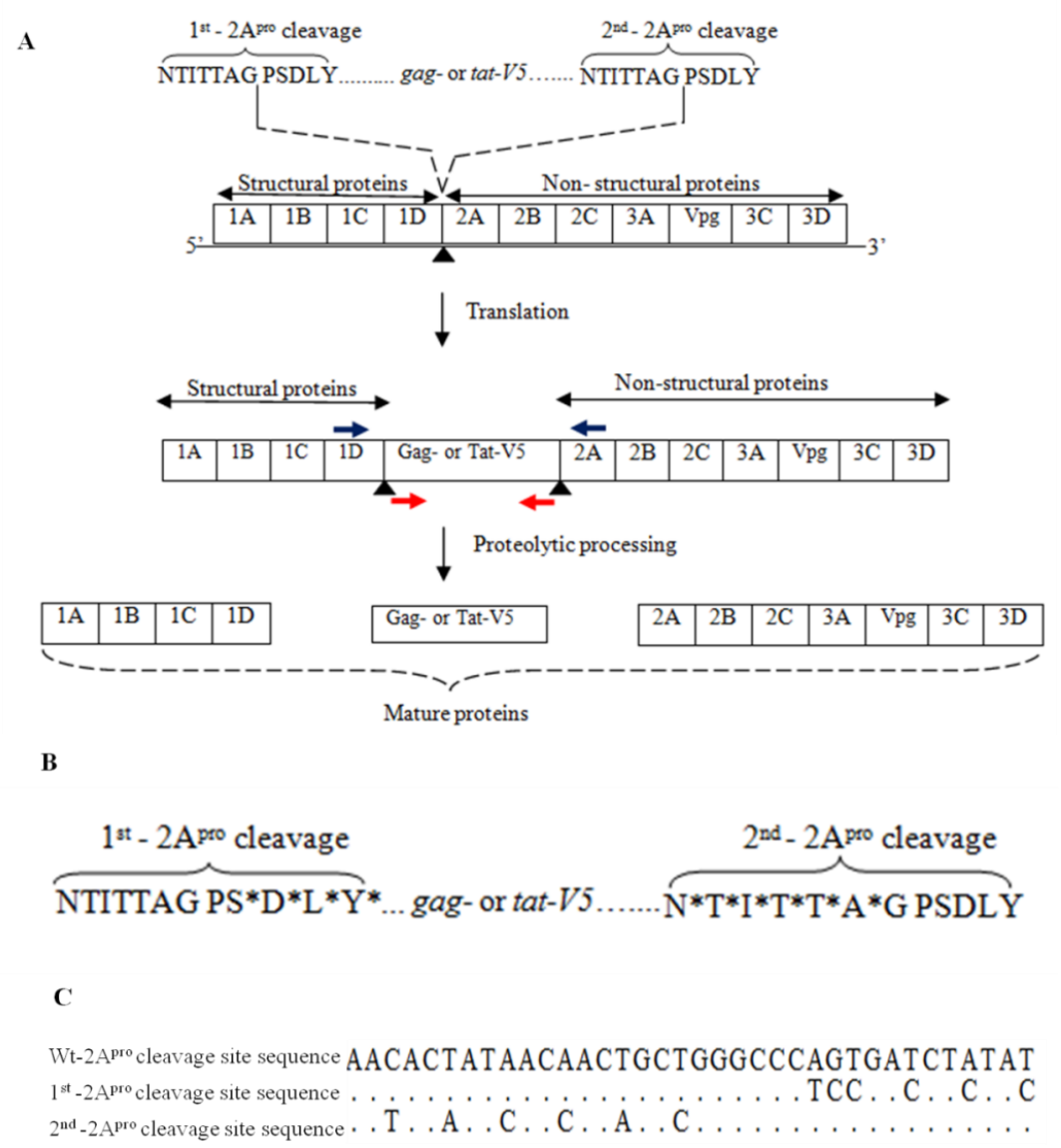

Fig. 1: Cloning strategy and production of replication-competent recombinant HRVs. *(A) Schematic presentation of the construction of the replication-competent, recombinant HRV-A1 vector and the expression strategy for the recombinant viral proteins. HRV genes and proteins are presented in boxes. 2A^{pro} cleavage at points indicated by solid triangles separates HRV structural from non–structural proteins and releases HIV proteins. Solid red arrows indicate the binding sites for insert-specific primers and solid blue arrows indicate binding sites for primers that anneal to HRV-A1 sequences flanking the inserts. (B) Amino acid sequence of the 1st and 2nd 2A^{pro} auto-cleavage sites. * indicate silently mutated codons for these amino acids.(C)*

RNA sequences encoding the 2A^{pro} auto-cleavage site of wt-HRV-1A (top line) and the sequences of the mutated sites; the 1st-2A^{pro} auto-cleavage site (middle line) and the 2nd- 2A^{pro} auto-cleavage site (bottom line). Dots correspond to nucleotides in the 1st and 2nd-2^{Apro} auto-cleavage sites that are homologous to the wt-HRV-A1 2A^{pro} auto-cleavage site sequence. This figure is reproduced from Virus Research, 2015 with permission from Elsevier Limited, License number 3841340715142, issued on 03/04/2016.

The linearized, dephosphorylated plasmid and the digested insert are then ligated and in turn used to transform competent E.coli cells. Positive transformants are screened using directional colony PCR using primers described previously[30]. Plasmid mini-preparations of positively transformed colonies are prepared and the authenticity of these plasmids confirmed by sequencing. This plasmid DNA is linearized using Mlu1 and then used in an *in vitro* transcription assay to generate rHRV mRNA. The purified mRNA is used to transfect H1-HeLa cells from which rHRVs are produced, harvested and purified. The recombinant viruses are stored in small volume aliquots at -80°C.

4.4.2 PCR amplification of HIV Gag or Tat inserts

All reactions to amplify HIV *gag* or *tat* inserts are carried out using the KAPA^{TM}HiFi PCR kit.

1. In a sterile PCR tube, mix 1X KAPA HiFi Buffer, 0.3 mM of each dNTP, 0.3 μM forward primer, 0.3 μM reverse primer, 100 ng DNA template and 0.5 U KAPA HiFi DNA polymerase. Include a negative control for the reaction (use sterile distilled water instead of the DNA template).
2. Run the reaction in the DNA Engine PCR machine using thermocycler conditions summarised in table 2.

3. Load the PCR products on a 1 % w/v agarose gel in TBE buffer (0.22M trizma-base, 180 mM boric acid, 5 mM EDTA, pH 8.3, with 5% v/v Gel red^{TM}) in parallel with a suitable marker.
4. Run gel at 120 kVA for approximately 1.5 hours.
5. Cut-out the band corresponding to the predicted product size from the gel using a sterile scalpel blade.
6. Extract and purify from the gel DNA using PureLink® Quick Gel Extraction Kit following the manufacturer's instruction.
7. Quantify the harvested DNA using the NanoDrop 2000c spectrophotometer at 280nm absorbance.
8. Store DNA at +4-8°C for short term only or use immediately.

Number of cycles	1	30	30	30	1
Temperature (°C)	95	98	Tm*	72	72
Time (minutes)	2	0.5	0.5	60/Kb	5

Table 2. Generating gag/tat inserts. *Summary of PCR thermo cycling conditions to amplify the required DNA for the different HIV gag or tat inserts. * Tm, the primer annealing temperature, depended on the length and guanine-cytosine (G-C) composition of the primer (>50-65 °C).*

4.4.3 Preparing the HRV vector

It is important to completely linearize the plasmid backbone before ligating it with the insert as this improves the cloning efficiency and reduces the number of false positive bacterial colonies formed as a result of uncut plasmid backbone.

1. Cut ~2µg of HRV-A1 plasmid cDNA with Apa1 or Xho1 in 1X enzyme buffer with 2U enzyme for 3-4 hours at 25°C (for Apa1) or 37°C (for Xho1).

2. Heat at 65°C for 20 minutes to inactivate the enzyme.
3. Load on to a 1 % w/v agarose gel and run as stated in section 4.4.2 steps 3-4 above.
4. Cut out the desired band corresponding to the linearized plasmid.
5. Extract and purify DNA from the gel as stated in section 4.4.2, step 6 above.

4.4.4 Dephosphorylating the vector

Since restriction endonuclease digestion of plasmid DNA is performed with a single restriction endonuclease, the chances of plasmid re-ligation are high, thus dephosphorylating a digested plasmid minimises this possibility.

1. Dephosphorylate ~500 ng of linearized plasmid DNA with 1U of antarctic phosphatase in 1X enzyme buffer.
2. Incubated the reaction for 1 hour at 37°C.
3. Inactivate the enzyme by incubating the reaction at 65°C for 10 minutes.
4. Purify the linearized, dephosphorylated plasmid DNA as stated in section 4.4.2, step 6.

4.4.5 Preparing the HIV inserts

1. A restriction enzyme site for Apa1 endonuclease flanks the HIV *gag-1* and *gag-2* inserts, whereas a restriction enzyme site for Xho1 flanks *gag 3-5* and *tat*, thus *gag-1* and *gag-2* are digested with Apa1 while *gag 3-5* and *tat* are digested with Xho1. About 1 µg of gel-extracted PCR-amplified DNA was digested as stated in section 4.4.3, step 1 above.
2. Load all products onto a 1 %w/v agarose gel and run as stated in section 4.4.2.
3. Extract and purify DNA as stated in section 4.4.2.

4.4.6 Ligating the vector and insert

Ligation reactions are setup to ligate the HIV inserts with the HRV-A1 vector digested with the same restriction endonucleases. The relative amounts of insert and vector are calculated

using the equation: *Insert [ng] = ((170[ng] vector x insert size [kb])/ vector size [kb]))/ insert [ng]*. (*See* **Note 1**). In an Eppendorf tube, mix the appropriate volume of insert and vector in a reaction containing 1U of DNA ligase enzyme and 1X enzyme buffer.

1. Incubate the mixture at 4°C overnight.

4.4.7 Transforming bacterial cells

The heat shock method is used to transform competent DH5α *E.coli* cells with the ligated *HRV-gag* or *tat* DNA.

1. Thaw a vial of competent DH5α cells (50µl per vial) on ice for 3-5 minutes.
2. Pipette 5µl of the ligation mixture (from a 10µl reaction) and mix with thawed DH5α cells on ice.
3. Incubate the cells on ice for 30 minutes.
4. Incubate the cells at 42°C for 45 seconds.
5. Place the cells back on ice for 2 minutes.
6. Add 500 µl of sterile SOC media to cell and close the vial tightly.
7. Rock the cells in the vial at 225 rpm for 1 hour at 37°C.
8. Centrifuge the vial at 15000 g to pellet cells.
9. Resuspend the pellet in ~50 µl LB broth.
10. Spread the cells on LB-agar plates containing 0.1% v/v ampicillin.
11. Incubate the plates at 37°C for at least 16 hours.
12. Count the number of colonies formed on each plate. Ideally, no colonies should appear on the vector control plate but some colonies may appear if the vector was not completely dephosphorylated. However, this number should be 5-10 fold lower than that from plates inoculated with the ligated insert and vector.

4.4.8 Colony screening

Screening for colonies transformed with the plasmid/insert is performed using the rapid colony PCR method (*see* **Note 2**).

1. Randomly select and pick up several colonies from each inoculated plate and emulsify each in 10 µl of sterile water in a separate sterile PCR tube. The number of colonies picked from each plate depends on the number of colonies present on the "vector only" plate. Fewer colonies on this plate indicate that fewer colonies need to be screened from the "insert plus vector" plates and vice versa.
2. Setup the PCR using the KAPA^{TM}Taq PCR kit in a reaction containing 0.25 U KAPA^{TM}Taq polymerase (0.08µl), 1X KAPA Taq buffer, 0.4 mM of each dNTP, 0.8 µM forward primer, 0.8 µM reverse primer and 5µl of emulsified bacteria colony.
3. Run the reaction under conditions summarised in table 3.
4. Load the PCR products on to a 1 % w/v agarose gel and run gel as stated in section 4.4.2, step 6.
5. Select colonies which show the expected band sizes on the gel and use these to generate plasmid DNA by the mini-preparation method.

Number of Cycles	1	30	30	30	1
Temperature (°C)	95	95	55°C	72	72
Time (min)	2	0.5	0.5	1 min/ Kb	2

Table 3. Colony PCR. *Summary of thermo cycling conditions used to screen correctly transformed E.coli colonies.*

4.4.9 Preparing rHRV-*gag/tat* plasmid DNA mini-preps

Generally, ~1 µg of plasmid DNA is required for transcription and transfection experiments, thus it is necessary to prepare plasmid DNA mini-preps from transformed bacterial cells to yield the required amount of DNA.

1. Into a clean, sterile 10 ml centrifuge tube and in a safety hood, dispense 5ml of LB-broth supplemented with 0.1% v/v ampicillin.
2. Inoculate the LB-broth with ~5 µl of bacterial suspension.
3. Close the tube and place on a rack.
4. Incubate tube at 37°C overnight in a rocking incubator at 220 rpm.
5. Take ~500 µl of the bacterial culture and add it to the same volume of sterile 100% glycerol to make a glycerol stock that can be used in the future to culture the same bacterial clone. Store this at -80°C.
6. Pellet the remaining bacterial culture at 3000 g for 5 minutes at room temperature.
7. Discard the supernatant and use the pellet to extract plasmid DNA.
8. Extract and purify plasmid DNA using the PureLink® Quick Plasmid Miniprep Kit following the manufacturer's instructions.
9. Quantify the harvested DNA as stated in section 4.4.2, step 7.
10. Store DNA at +4°C for short-term storage, at -80°C for long-term storage or use immediately in subsequent experiments.
11. Validate the authenticity of plasmid constructs. It is important to authenticate plasmid DNA before using it in any experiments to ensure the validity of the experimental results. Only colonies with the correct plasmid and insert sequences should be selected for further use.

4.4.10 Linearizing plasmid HRV-gag/tat plasmid DNA

Since HRVs are positive sense RNA viruses, to cultivate these viruses *in vitro* requires transfection of susceptible cell lines with *in vitro* transcribed mRNA generated from a linearized plasmid DNA template (*see* **Note 3**).

1. Linearize the recombinant HRV plasmid DNAs using Mlu1 in a reaction consisting of 1X enzyme buffer, 3 µg plasmid DNA and 10 U of Mlu1.

2. Incubate the mixture at 37°C for at least 10 hours to ensure total digestion of the template. Inactivate the enzyme by incubating the mixture at 65°C for 20 minutes.
3. Analyse the reaction products on agarose gel as stated in section 4.4.2, steps 3-4.
4. Cut-out the band corresponding to linearized plasmid from the gel.
5. Extract and purify DNA as stated in section 4.4.2, step 6.
6. Determine the DNA yield as stated in section 4.4.2, step 7.
7. Use the harvested plasmid DNA immediately in a transcription reaction. Do not keep this DNA for future use as the probability of re-annealing and therefore re-circularising are extremely high.

4.4.11 Transcription of rHRV-Gag/Tat mRNA

All experiments should be setup in a clean RNA preparation room. It is always a good habit to wipe down every apparatus and workspace with RNase AWAY™ to remove any RNases that might compromise RNA yield. Always wear fresh gloves.

1. Use the MEGAscript^{TM} transcription kit and follow the manufacturer's recommendation.
2. In a clean, sterile PCR tube, add ~200 ng of linear plasmid DNA, 1X recommended buffer, 150 mM of each dNTPs and 2 U of the enzyme mix.
3. Incubate the reaction at 37°C for at least 6 hours.
4. Terminate the reaction by adding 2 U of DNase and incubate the reaction at 37°C for 15 minutes to remove any traces of template DNA.
5. Purify the RNA transcripts using the MEGAclear^{TM} Kit following the manufacturer's instructions.
6. Quantify the RNA yield using the Nanodrop 2000c spectrophotometer at 260 nm absorbance.

7. As RNA is very unstable, store the harvested mRNA at -80°C or use immediately in a transfection experiment.

4.4.12 Transfecting H1-HeLa cells with HRV-Gag/Tat mRNA

All experiments are to be performed in a clean, sterile cell culture hood using aseptic techniques. Wipe down the hood and all other apparatus with RNase AWAY™ to remove any RNases that might compromise RNA quality.

1. One day prior to transfection, seed ~1 x 10^5 H1-HeLa cells per well of a 6-well plate in 500 µl of cell culture media ensuring that the cells reach ~80% confluence before transfection.
2. On the day of transfection, replace the cell culture media with ~ 500µl of fresh virus growth media ~3 hours prior to transfection.
3. Setup the transfection solution using the TransIT®-mRNA Transfection Kit following the manufacturer's instructions in a reaction containing 3 µg of transcribed RNA, 6µl of mRNA boost reagent, 6 µl of TransIT-mRNA reagent and 250 µl of Opti-MEM per well. Include a transfection control i.e. containing all reagents except *in vitro* transcribed RNA and a cell only control (no transfection mixture added).
4. Incubate the transfection mixture at room temperature for not more than 5 minutes
5. Add the mixture drop-wise to the cells.
6. Incubate the cells between 32-33 °C rather than 37°C (*see* **Note 4**) in 5% CO_2 and monitor daily for evidence of virus-induced cytopathic effect (CPE).

4.4.13 Harvesting rHRVs

1. Using a sterile cell scrapper, scrap the cell monolayer infected with rHRV into the culture media when 70-80% of cells show evidence of infection. Avoid extended incubation as this can result in low virus yields, due to the release of rHRV into the cell culture supernatant.

2. Transfer the cells and supernatant to a sterile 50 ml falcon tube.
3. Pellet the cells by centrifuging at 500 g for 10 minutes at 4°C.
4. Discard the supernatant and keep the cell pellet.
5. Resuspend the pellet in ~500 µl of virus harvest media.
6. Lyse the cells and release the virus by freeze-thawing three times. Freeze in a dry ice-ethanol bath and thaw in a 37°C water bath.
7. Centrifuge at 3000 g for 15 minutes at 4°C to remove cell debris.
8. Harvest the virus-rich supernatant into a sterile 1.5 ml sterile Eppendorf tube. This represents passage zero (P_o virus).
9. Store viruses at ⁻80°C until required or subsequently passage it in fresh cells.

4.4.14 Large scale preparation of rHRVs

Reserve an aliquot (~200 µl) of the P_o virus as a backup and for future reference (*see* **Note 5**) or to confirm the presence of *gag* or *tat* inserts (see section 4.4.15) and the expression of Gag or Tat proteins (see sections 4.4.16). An appropriate volume of virus should be reserved after each virus passage for the same purposes. Store these at -80°C.

1. Seed ~1 x 10^6 H1-HeLa cells in one T75 flask with 15 ml of cell culture media to ensure 80% confluent at the time of infection.
2. Dilute ~250 µl of P_o virus in 10-15 ml of fresh virus growth media.
3. Prior to infection, wash the cells with sterile PBS and replace the culture media with virus growth media prepared in step 2 above. Ensure that the whole H1-HeLa monolayer is covered with medium by gently rocking the flask side-to-side.
4. Incubate the flask as stated in section 4.4.12 until 70-80% CPE is observed.
5. Harvest the virus (P_1virus) in ~1 ml of virus harvest media as stated in section 4.4.13 above.

6. Passage ~300-500 µl of P_1 rHRV in one T175 flask with cells at 80% confluence and maintained in ~20-25 ml of fresh virus growth media.
7. Continue harvesting and passaging virus using a 1:4 ratio i.e. from 1x T175 flask to 4x T175 flasks until the desired virus volume is reached. This process can also be referred to as scaling-up virus volume and titer (*see* **Note 6**). Assuming 35x T175 flasks are infected, harvest infected H1-HeLa cells as stated in section 4.4.13. Pool the pellets from 3x T175 flasks to facilitate freeze-thawing. Resuspend the cell pellet in 10 ml of medium. This yields ~100 ml of crude virus preparation that needs to be concentrated and titrated.

4.4.15 RT-PCR

RT-PCR is performed to confirm the retention of the exogenous HIV gag/tat inserts upon serial passage of rHRVs as an indication of the genetic stability of the rHRVs.

1. Plate H1-HeLa cells at 3x 10^5 per well of a 6 well cell culture plate and incubate as stated in section 4.4.13
2. Wash the cells with sterile 1x PBS and then inoculate with virus multiplicity of infection, M.O.I, = 5 at a particular passage number in virus growth media. See section 4.4.17 for virus titration and determining M.O.I. Include a cell only (no virus) control.
3. Incubate the cells at 32-33 °C in 5 % CO2 until 70-80% of cells show evidence of infection.
4. Using a sterile cell scraper, scrape the cells into the culture media and pellet at 800 g for 10 minutes at 4°C.
5. Discard the supernatant and wash the cells twice with ice cold 1x PBS.
6. Extract rHRV RNA from the cell pellet using the RNeasy kit following the manufacturer's instructions.

7. Quantify harvested RNA as stated in section 4.4.11.
8. Use ~1 µg of this RNA in a reverse transcription reaction using the QIAGEN OneStep RT-PCR Kit as per manufacturer's protocol to generate rHRV cDNA.
9. Prepare two separate PCR mixtures using the KAPA HiFi kit as stated in section 4.4.2, with 1 µl of rHRV cDNA generated in step 8 above and using either insert-specific primers (in PCR-1) or primers flanking the insert (in PCR-2).
10. Load the PCR products in 2 separate 1% w/v agarose gels and run gels as stated in section 4.4.2.
11. Analyse the banding patterns on each gels for the presence of exogenous gene inserts i.e. in PCR-1, only a band corresponding to the exogenous gene insert is observed (Fig. 2A) , whereas in PCR-2 a larger band corresponding to the inserted exogenous gene along with HRV gene sequences flanking the insert is observed (Fig. 2B). PCR-1 yields no band, if the exogenous gene insert is deleted.

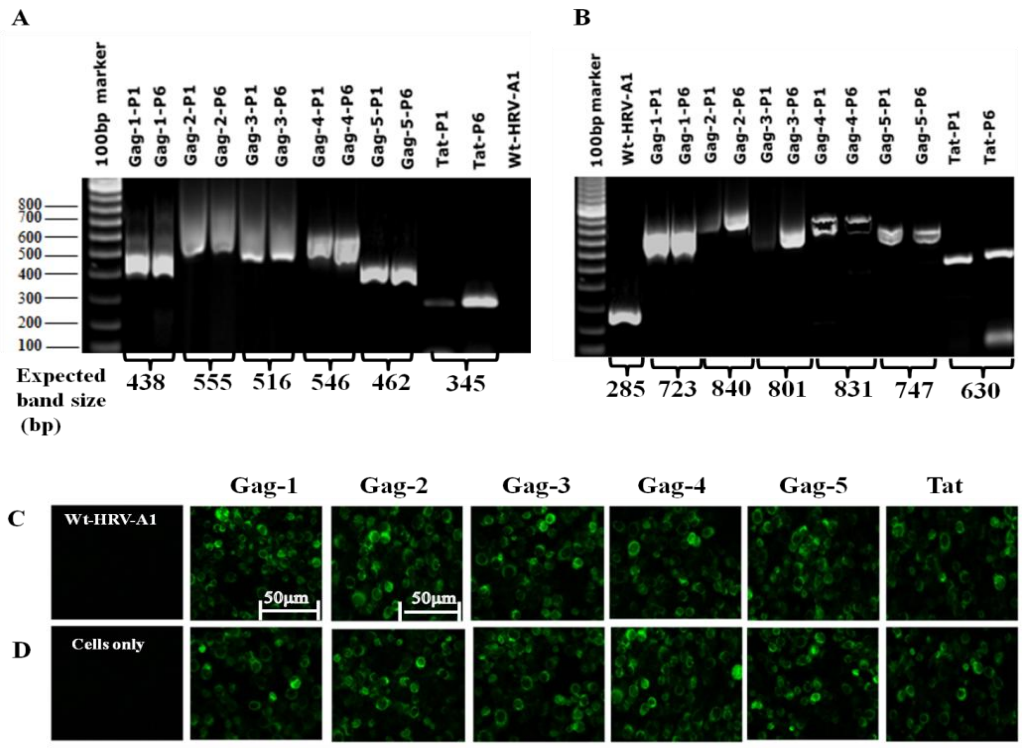

Fig. 2: Agarose gel electrophoresis of RT-PCR products to examine the genetic stability of rHRVs and immunofluorescence to detect expression of Gag-1 to Gag-

and Tat. *(A) gel electrophoresis of PCR products generated using insert-specific primers and (B), using primers which anneal to HRV sequences flanking the HIV inserts. In A, fragments of 438bp, 555bp, 516bp, 546bp, 462bp and 345bp corresponding to Gag-1, Gag-2, Gag-3, Gag-4, Gag-5 and Tat, respectively were amplified. No band was amplified corresponding to wt-HRV-A1. In B, fragments of 285bp, 723bp, 840bp, 801 bp, 831bp, 747bp and 630bp corresponding to wt-HRV-A1, rHRV-Gag-1, rHRV-Gag-2, rHRV-Gag-3, rHRV-Gag-4, rHRV-Gag-5 and rHRV-Tat, respectively were amplified. Expression of Gag and Tat proteins in H1-HeLa cells infected with C, P1 viruses (M.O.I=5) and D, cells infected with P6 viruses (M.O.I=5) at 48 hours post-infection. This figure is reproduced from Virus Research, 2015 with permission from Elsevier Limited, License number 3841340715142, issued on 03/04/2016.*

4.4.16 Immunofluorescence

Immunofluorescence is performed to confirm the expression of Gag and Tat proteins in cells infected with rHRVs at a particular passage number.

1. Plate cells at 1x 10^5 per well of a 24 well cell culture plate and incubate as stated in section 4.4.12.
2. Wash the cells with sterile 1x PBS and then re-feed with fresh virus culture media containing virus at a M.O.I = 5 for a particular passage number. Remember to include a cell only (no virus) control.
3. Incubate cells as stated in section 4.4.12 until 70-80% of cells show evidence of infection.
4. Carefully remove virus culture media, wash the cells with ice-cold 1x PBS and then add 100 µl of 2% paraformaldehyde to each well.
5. Cover the plate and incubate at 4^{o}C for 10 minutes to fix cells.

6. Wash the plate 2x with ice-cold 1x PBS whilst taking extra care not to lift the cells off the surface.
7. Block the wells with ice-cold 1% w/v BSA at room temperature for 30-45 minutes.
8. Wash the plates 2x with ice-cold 1x PBS.
9. Add 100 µl per well of primary antibody diluted in 1% w/v BSA (V5 mouse monoclonal antibody diluted at 1:5000).
10. Incubate the plate for 1 hour at 37ºC.
11. Wash 2x with 1x PBS.
12. Add 100 µl per well of fluorescently labelled secondary antibody diluted in 1% w/v BSA (Alexa 488 conjugated anti-mouse, use at 1:10,000).
13. Incubate at 37ºC for 1 hour.
14. Wash 2x with 1x PBS.
15. View the plates under a fluorescence microscope to detect the expression of exogenous V5-tagged HIV Gag or Tat proteins. See Fig. 2C and 2D for the expected fluorescence.

4.4.17 Concentrating, titrating and storing rHRVs

The rHRVs intended for animal vaccinations to evaluate vaccine immunogenicity should be concentrated and titrated before use (*see* **Note7**).

1. Filter the virus-containing supernatant through a 0.2 µm syringe filter.
2. To each 10 ml of clarified virus, add 2.8 g of polyethylene glycol (PEG)-6000 and 4 ml of 5 M NaCl.
3. Mix on a roller at room temperature for 5 minutes to dissolve PEG, followed by incubation on ice for 1 hour with gentle mixing by hand inversion every 20 minutes to precipitate the virus.

4. Recover the precipitated virus by centrifugation at 3000 g for 1 hour and dissolve the virus pellet in 15 ml of 1x PBS, mix on a roller for 5 minutes and then centrifuge at 3000 g for 15 minutes to remove insoluble debris.
5. Filter the virus using a 0.2 μm syringe filter into an Amicon ultra centrifugal device.
6. Centrifuge the Amicon ultra centrifugal device at 3000 g until the supernatant (containing virus) is concentrated to ~0.5 ml in the Amicon device. Add 10 ml of PBS, pipette thoroughly to mix the concentrated virus in the filtration device (be careful not to damage the filter with the pipette tip) and repeat the process. Harvest the concentrate and wash out the device with additional 2x 1 ml of PBS. Combine washes with 0.5 ml virus to yield 2.5 ml total. Aliquot into 2x 250 μl and 4x 500 μl or as required and store at -80^{o}C. A small volume (~50-100 μl) of concentrated virus should be saved for titration. Avoid freeze-thawing the virus stocks as this reduces the titer. Titrated viruses should be thawed and used only once for vaccination experiments.
7. Measure the infectious titer of rHRVs using H1-HeLa cells. Serially dilute purified rHRV stock to yield dilutions from 10^{-1} to 10^{-8} and add 50 μl of each dilution to 8 replicate wells of HeLa cells in a 96 well plate. Include a "no virus" or cell only control.
8. Incubate the plate at 32-33^{0}C in 5% CO_2 for 4 days.
9. On day 4 post-infection, determine the highest dilution where CPE is still visible using a light microscope.
10. Determine the tissue culture infective dose 50% ($TCID_{50}$) /ml value by scoring the sum of positive wells. The highest dilution where CPE is still visible is determined by counting the number of wells showing CPE. A Spearman Karber formula can be used to estimate the TCID50. Alternatively, the Reed and Muench end point assay can be

used with the formula: *TCID50 = log_{10} dilution factor (% of infection at next above 50%) –50%/(% of infection at next above 50%) – (% of infection at next below 50%).*

4.5 Notes

1. The volume (µl) of insert or vector used in the ligation reaction depends on the corresponding DNA concentration. A "no insert" or "vector only" control must be included as this control accounts for any colonies resulting from vector re-ligation.
2. Directional colony PCR is used to screen inoculated agar plates for correctly transformed *E.coli* colonies. This method allows rapid screening of multiple transformants (within 2 hours) and simultaneously determines the orientation of the inserts. The forward primers used in this PCR anneal to sequences in the plasmid upstream of the insert, whereas the reverse primers anneal to sequences at the 3'end of the insert. Alternatively, digestion of plasmid DNA prepared from selected colonies with restriction endonucleases can also be used. In this case, the banding pattern obtained after resolving the digested DNA can provide information to select positive colonies. However, this method is labour intensive, uses more reagents and results are normally obtained after 2 days.
3. Plasmid DNA must be completely linearized with Mlu1 before it is used in an *in vitro* transcription assay because the presence of circular plasmid templates will generate long, heterogeneous RNA transcripts. It is extremely important to confirm complete linearization of the plasmid by agarose gel electrophoresis as the presence of even small traces of uncut circular plasmid will generate a large proportion of transcripts.
4. For efficient *in vitro* cultivation of HRV-A1, it is desirable that the cultures are maintained between 32-33°C because HRV-A1 is particularly sensitive to high temperatures, and thus its growth is affected when cultures are incubated at temperatures >33°C. Efficient packaging of HRV-A1 requires Mg^{2+} and Ca^{2+};

therefore, the virus will not grow in cell culture unless these salts are added to the growth media.

5. After every passage, it is important to demonstrate the presence of the gene inserts by RT-PCR and protein expression from these genes by immunofluorescence as stated in sections 4.4.15 and 4.4.16. This is absolutely vital as it confirms the genetic stability of the rHRVs before they are passaged or used for animal vaccination experiments. Note the passage number to ensure that viruses from the same stock are always used in any experiment, and in the event of any change in growth characteristics or stability of the rHRV at a given passage, then trouble shooting is easier.
6. Normally, considerable virus is lost during purification and concentration, which necessitates starting with a high volume of high titer virus. In this case, it may be necessary to scale-up the virus to 25-35x T175 flasks.
7. For mouse vaccination experiments, it is important that the viruses are purified to remove cellular debris that might elicit undesired immunological responses. These non-specific immune responses might obscure the desired antigen-specific immune response which impacts vaccine efficacy. Furthermore, a small volume of virus (<100 µl) with a high titer is desired for vaccination as this is more likely to be well tolerated.

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Chapter 5.0: Engineering human rhinovirus serotype-A1 as a vaccine vector

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Overall percentage (%)	80%
Certification:	This paper reports on original research I conducted during the period of my Higher Degree by Research candidature and is not subject to any obligations or contractual agreements with a third party that would constrain its inclusion in this thesis. I am the primary author of this paper.
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permission is granted for the candidate in include the publication in the thesis; and

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Short communication

Engineering human rhinovirus serotype-A1 as a vaccine vector

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ABSTRACT

Herein we describe the construction of recombinant human rhinoviruses (rHRVs) encoding HIV Gag or Tat by inserting the full length *tat* gene or regions of the *gag* gene flanked by sequences encoding the HRV 2A protease cleavage site into the junction between HRV genes encoding structural (P1) and non-structural (P2) proteins. Most recombinants were unstable, but this was corrected by mutation of the flanking cleavage sites. Thereafter, all rHRV constructs retained the inserts throughout six passages. Such constructs may find utility as vaccine vectors to generate mucosal immunity.

1. Introduction

HRVs are transmitted mucosally (Hayden, 2004), making them potential vectors for mucosally targeted human vaccines. However, these viruses have had limited development as vaccine vectors (Arnold et al., 2009; Smith et al., 1998) mainly due to concerns of pre-existing immunity to HRV in humans, lack of a suitable small animal model to test HRV-vectored vaccines, limited insertion capacity and poor genetic stability (Andino et al., 1994). However, a virus closely related to HRVs, viz. poliovirus, was used successfully as a vaccine vector to elicit protective immunity in macaques to challenge with SIVmac251 (Crotty et al., 2001). Moreover, vaccination of guinea pigs with recombinant HRV encoding the highly conserved membrane proximal external region ELDKWA epitope of HIV-1 induced broadly neutralising antibodies against several HIV pseudotypes (Arnold et al., 2009). Consequently, we wished to address the problems associated with HRVs as potential vaccine vectors. HRVs contain a 7.3 kb genome that encodes a 250 kDa polyprotein, which is cleaved by viral proteases $2A^{pro}$ and $3C^{pro}$ into precursor and mature proteins (Palmenberg et al., 2010). The HRV $2A^{pro}$ protease recognises and cleaves the amino acid sequence NTITTAG*PSDLY at the junction of the viral structural (P1) and non-structural proteins (P2/P3) (Palmenberg et al., 2010).

Minor group HRVs such as HRV-A1 replicate in murine (Tuthill et al., 2003) and human cells (Mosser et al., 2002) and infect mice and humans (Reithmayer et al., 2002), providing an opportunity to test HRV-vectored vaccines in a small animal model. HRV-A1 was reported some time ago to result in ~0.5% of HRV-related diseases (Monto et al., 1987) and this prevalence has been confirmed in more recent studies (Harvala et al., 2012; Martin et al., 2015). Furthermore, HVR-A1 is the rarest HRV serotype detected in humans (Gwaltney et al., 1968). Thus, it is unlikely that rHRV-A1, if used as a vaccine vector, will be significantly affected by pre-existing anti-HRV-A1 immunity. Furthermore, as there is no cross-neutralisation between different serotypes (Jacobs et al., 2013), HRV-A1 has the potential to deliver vaccines in humans who have been infected previously with other HRV serotypes. We thus describe the generation of a new replication-competent recombinant HRV-A1 for potential use as a vaccine vector.

2. Methods

2.1. Constructing rHRVs

To accommodate the limited packaging capacity of HRV, the HIV *gag* gene (Garrod et al., 2014) was divided into five discrete

Abbreviations: HIV, human immunodeficiency virus; AIDS, acquired immunodeficiency syndrome; HRVs, human rhinoviruses; wt-HRV-A1, wild type human rhinovirus serotype A1; rHRV-A1, recombinant human rhinovirus serotype-A1; CPE, cytopathic effect; UTR, untranslated region; NH-2, amino group; NIH, National Institutes of Health; M.O.I., multiplicity of infection.

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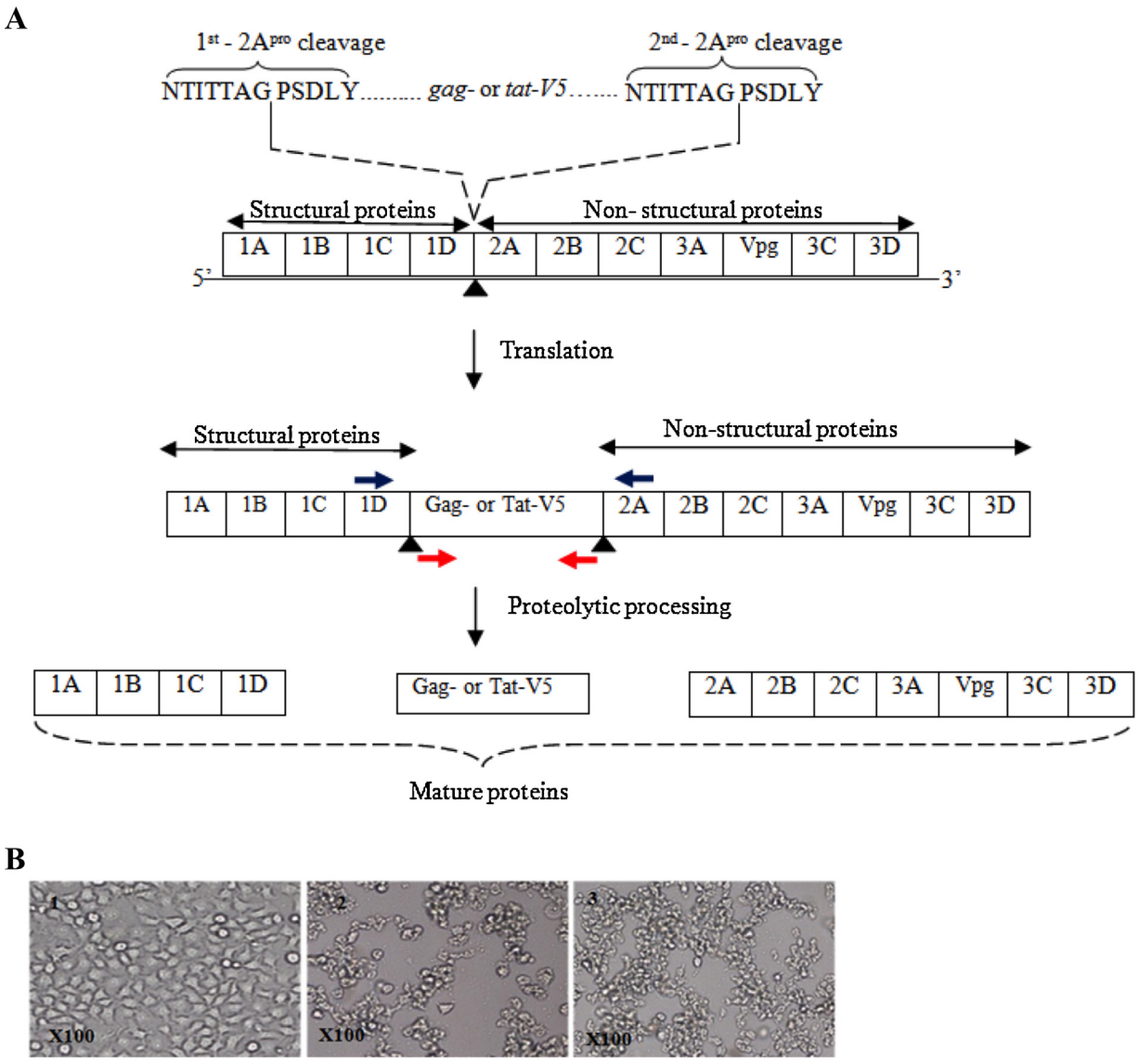

Fig. 1. Cloning strategy and production of replication-competent recombinant HRVs. (A) Schematic presentation of the construction of the replication-competent-recombinant HRV-A1 vector and expression strategy for the recombinant viral proteins. HRV genes and proteins are presented in boxes. $2A^{pro}$ cleavages at points indicated by solid triangles separates HRV structural from non-structural proteins and releases HIV proteins. Solid red arrows indicate binding sites for insert-specific primers and solid blue arrows indicate binding sites for primers that anneal to HRV-A1 sequences flanking the inserts. (B) Appearance of HI-HeLa cells 48 h post transfection with HRV RNA. 1, cell control (no virus or viral RNA); 2, wild type HRV and 3, rHRV gag-1. All other rHRVs showed similar effects in H1-HeLa cells.

overlapping fragments (*gag*-1 to *gag*-5), ranging from 393 to 513 bp. A sequence encoding a V5 tag (Garrod et al., 2014) was placed at the 3′ ends to facilitate detection of the truncated *gag* proteins, which contain few B cell epitopes (French et al., 2013). The *gag* fragments and the 303 bp full length *tat* gene (NIH AIDS Reagent Bank), flanked by the sequence encoding the viral $2A^{pro}$ cleavage site, were amplified by PCR. The PCR products, which contained Apa1 or Xho1 restriction enzyme cleavage sites on the 5′ or 3′ ends, and a cDNA plasmid encoding a replication-competent HRV 1A (Quiner and Jackson, 2010) were digested with the appropriate restriction enzymes, ligated (Fig. 1A) and transformed into *E.coli* (DH5-α), as described previously for a poliovirus vector (Andino et al., 1994). The resultant clones were sequenced to confirm the correct orientation of the respective inserts. The presence of the $2A^{pro}$ cleavage sites should result in release of the HIV proteins from the viral polyprotein via the action of the HRV-encoded protease. To generate genetically stable rHRV *gag-2* to *gag-5* and *tat*, we modified our cloning strategy by introducing several silent mutations into the coding sequences of both flanking $2A^{pro}$ cleavage sites, as described in results.

2.2. Virus culture

Full length viral RNA was generated using the MEGAScript T7 transcription kit (Life Technologies) and was used to transfect H1-HeLa cells using the TransIT®-mRNA transfection kit (Mirus) to produce replication-competent rHRVs. To confirm that the rHRVs retained the inserts through a series of passages, cDNA from total cellular RNA was extracted from virus-infected cells using the RNeasy-RT kit (Qiagen) and amplified by standard RT-PCR (KAPATaq, Biosystems) using (1) *gag* or *tat*-specific primers and (2) primers that annealed to HRV sequences flanking the insert (Fig. 1A). A complete list of all primers used in RT-PCR is presented in Fig. 1 in the supplementary data. The expression of Gag-1 was also confirmed in rHRV-*gag-1* by Western blot analysis using anti-V5 mouse monoclonal primary antibody (Life Technologies™)

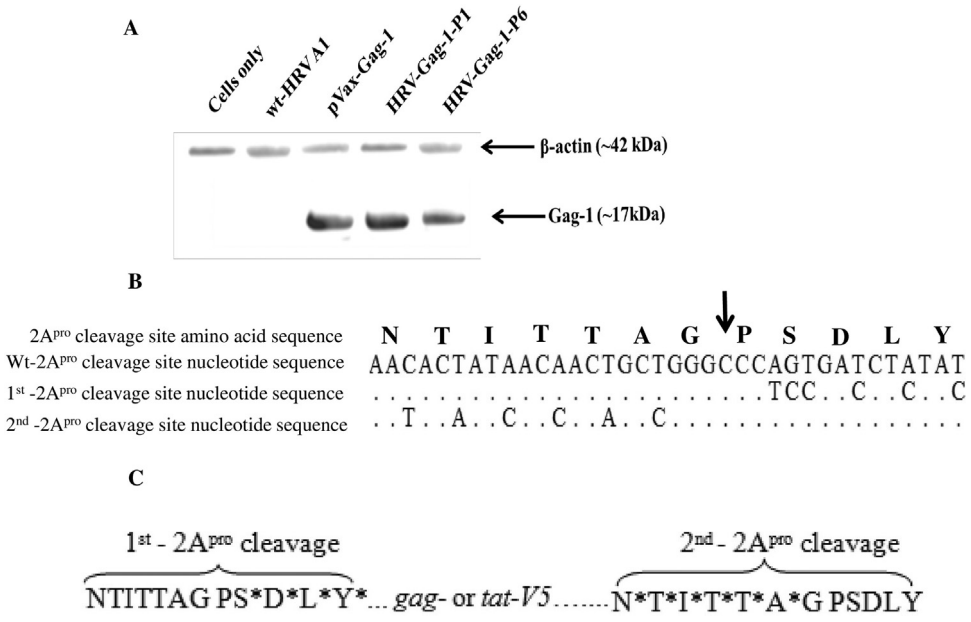

Fig. 2. HIV protein expression and 2A^{pro} auto-cleavage site sequences. (A) Western blot assay to detect the expression of Gag-1. (B) RNA sequences encoding the 2A^{pro} auto-cleavage site of wt-HRV-1A (centre line) and the sequences of the mutated sites; the Ist–2A^{pro} auto-cleavage site (middle line) and the 2nd–2A^{pro} auto-cleavage site (bottom line). The 2A^{pro} auto-cleavage site amino acid (top line); the solid arrow indicates the 2A^{pro} cleavage site. Dots correspond to nucleotides in the 1st and 2nd–2A^{pro} auto-cleavage sites that are homologous to the wt-HRV-A1 2A^{pro} auto-cleavage site sequence. (C) Amino acid sequence for the 1st and 2nd 2A^{pro} auto-cleavage sites. *Indicate silently mutated codons for these amino acids.

and goat anti-mouse-HRP-conjugated secondary antibody (Antibodies Australia™). The expression of the other Gag fragments and Tat was confirmed by immunofluorescence using anti-V5 mouse monoclonal primary antibody and goat anti-mouse-IgG secondary antibody (Alexa Fluor® 488 conjugate) in all rHRV-infected cells from passage 1 (P1) to passage 6 (P6).

3. Results

We transfected H1-HeLa cells with rHRV RNA to generate rHRVs and the characteristic HRV CPE was observed in transfected cells 24–48 h post transfection (Fig. 1B). As the complete replication cycle for picornaviruses is around 8–10 h, the level of CPE shown in Fig. 1B reflects the result of a second or even third round of infection. Analysis showed that the one-step growth curves of wt-HRV-A1 and rHRV-A1 were similar (data not shown). All viruses were harvested when the CPE reached ~80%. Initially, rHRV-*gag*-1 was used as a model to test rHRV genetic stability; RT-PCR products analysed by agarose gel electrophoresis showed that the predicted PCR products were generated (data not shown), and western blot analysis of rHRV-*gag-1*-infected cell lysates also confirmed expression of Gag-1 protein (Fig. 2A). In contrast to rHRV-*gag*-1, analysis of the remaining rHRV *gag*-2 to *gag*-5 and *tat* recombinant viruses showed that culture of these rHRVs resulted in reversion to wild-type within 1–2 passages (data not shown) (although the initial constructs contained the correct inserts). Thus, insertion of the *gag-2* to *gag-5* and *tat* sequences resulted in viruses with unstable genomes. This was corrected following the introduction of silent mutations into the coding sequences of both 2A^{pro} cleavage sites flanking the inserts (Fig. 2B and C). The mutations reduced the homology between the sequences of the flanking 2A^{pro} cleavage sites from 100% to 77% and enhanced the genetic stability of rHRV *gag-2* to *gag-5* and *tat*, permitting passage of these viruses to P6 without loss of insert concomitant with confirmed Gag/Tat expression as revealed by RT-PCR (Fig. 3A and B) and immunofluorescence (Fig. 3C and D), respectively. RT-PCR and immunofluorescence were performed in preference to Western blot because we wished to confirm that the Gag and Tat-specific RNA expressed in cells matched the calculated sizes, and that the corresponding proteins were expressed in the same cytoplasmic location for all constructs. The low molecular weight band in Tat-P6 (Fig. 3B) is likely to represent a minor deletion described previously for polioviruses (Crotty et al., 2001). Only rHRV-infected cells were stained in this immunofluorescence assay (Fig. 3C and D) to confirm Gag and Tat protein expression from P1 to P6. The expression of all Gag and Tat proteins was confirmed in all rHRV passages.

4. Discussion

Although HRVs have had limited use as vaccine vectors (Arnold et al., 2009; Smith et al., 1998), the HRV-A1 has several attributes (Gwaltney et al., 1968; Jacobs et al., 2013; Monto et al., 1987; Mosser et al., 2002; Tuthill et al., 2003) to be considered as a potential vaccine vector. The results presented herein illustrate that rHRV-A1s can retain foreign gene inserts provided that the foreign genes are inserted into the P1 and P2 junction and that repeat sequences are avoided in the genome. We included the HIV *gag* and *tat* genes to generate rHRVs because they are relatively conserved, are vital for viral replication and survival (Freed, 2001), and encode immunogenic proteins that are considered to be important components of a HIV vaccine (Bellino et al., 2014; Betts et al., 2006).

The rHRV-*gag-1* was stable upon serial passage, but this stability could not be replicated in other rHRVs. Since none of the *gag-2* to *gag-5* and *tat* inserts increased the genome sizes of the corresponding rHRVs to >115% that of wt-HRV-A1, the poor genetic stability of these recombinant viruses may have resulted from genetic recombination between the flanking 2A^{pro} auto-cleavage site sequences, as was suggested previously for polioviruses (Lee et al., 2002). Thus, the introduction of several silent mutations in the 2A^{pro} sequences that flank the *gag* and *tat* inserts prevented homologous recombination and subsequently enhanced the genetic stability of the rHRVs to permit passage to P6, although we have no reason to believe that further passages will not be possible. The relative

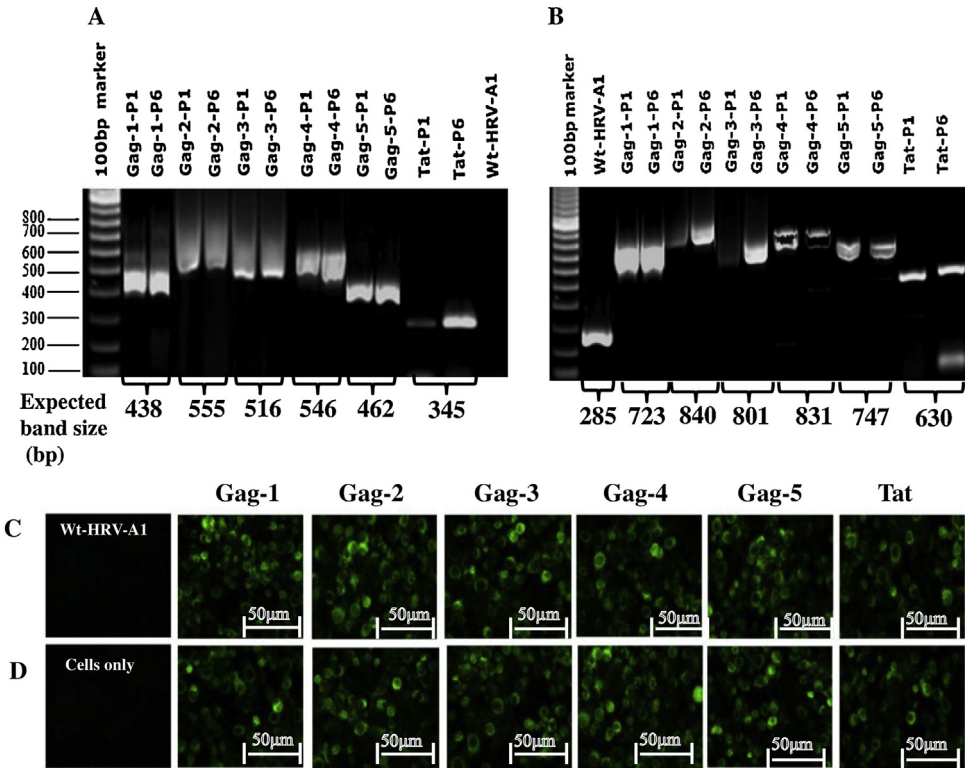

Fig. 3. Agarose gel electrophoresis of RT-PCR products to examine the genetic stability of rHRVs and immunofluorescence to detect expression of Gag-1 to Gag-5 and Tat. (A) Gel electrophoresis of PCR products generated using insert-specific primers and B, using primers which anneal to HRV sequences flanking the HIV inserts. In (A), fragments of 438 bp, 555 bp, 516 bp, 546 bp, 462 bp and 345 bp corresponding to Gag-1, Gag-2, Gag-3, Gag-4, Gag-5 and Tat, respectively were amplified. No band was amplified corresponding to wt-HRV-A1. In B, fragments of 285 bp, 723 bp, 840 bp, 801 bp, 831 bp, 747 bp and 630 bp corresponding to wt-HRV-A1, rHRV-Gag-1, rHRV-Gag-2, rHRV-Gag-3, rHRV-Gag-4, rHRV-Gag-5 and rHRV-Tat, respectively were amplified. In (C), H1-HeLa cells infected with P1 viruses (M.O.I. = 5) and D, cells infected with P6 viruses (M.O.I. = 5) at 48 h post-infection.

stability of rHRV-*gag-1* may be due to other RNA structural features that inhibited homologous recombination between the HRV genome and *gag-1* (Anraku et al., 2008).

Recombinant rHRV-A1s are similar to replication-competent recombinant polioviruses described previously (Andino et al., 1994), and intranasal vaccination of macaques with the latter that encoded a range of simian immunodeficiency virus (SIV) proteins (i) generated immune responses to all the SIV proteins and (ii) protected vaccinated animals against intravaginal SIVmac251 challenge (Crotty et al., 2001). Unfortunately, poliovirus vectors have not progressed to clinical trials due to pre-existing immunity in humans. Pre-existing immunity to adenovirus serotype-5 vaccine vector was the major reason for the disappointing results in a previous HIV vaccine clinical trial (Zak et al., 2012). As polioviruses and HRVs are closely related (Palmenberg et al., 2010), similar protective immunity to that achieved by recombinant poliovirus might be expected with replication-competent HRVs. The latter has the advantage of minimal pre-existing immunity in humans.

To our knowledge, this study describes the first replication-competent HRV-A1 vector. As a majority of HIV transmissions occur via mucosal surfaces (Hladik and McElrath, 2008), we propose that HRV-A1 vectors may find utility as mucosal vaccine vectors for HIV. Furthermore rHRV-A1 may provide an alternative to the humanised mouse (Bartlett et al., 2012, 2015; Singanayagam et al., 2015; Traub et al., 2013) and rat models (Rosenthal et al., 2009) infected with HRV-14 to study sustained HRV-induced inflammation and airway dysfunction. An understanding of the pathogenesis of these diseases might identify novel therapeutic targets and vaccine strategies against asthma and chronic obstructive pulmonary dysfunction.

Conflict of interest

The authors declare no financial or commercial conflict of interest.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.virusres.2015.04.002

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Chapter 6.0: An innovative HIV-1 vaccination regimen

(Word version of the manuscript, submitted to the Journal of Mucosal Immunology)

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Principal Author

Name of Principal Author (Candidate)	Khamis Tomusange
Contribution to the Paper	Performed all experiments, analysed data and wrote the manuscript.
Overall percentage (%)	80%
Certification:	This paper reports on original research I conducted during the period of my Higher Degree by Research candidature and is not subject to any obligations or contractual agreements with a third party that would constrain its inclusion in this thesis. I am the primary author of this paper.
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Co-Author Contributions

By signing the Statement of Authorship, each author certifies that:

the candidate's stated contribution to the publication is accurate (as detailed above);

permission is granted for the candidate in include the publication in the thesis; and

the sum of all co-author contributions is equal to 100% less the candidate's stated contribution.

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Contribution to the Paper	Conceived the original idea and reviewed the manuscript		
Signature		Date	7/6/16

Please cut and paste additional co-author panels here as required.

Intranasal vaccination with a live recombinant rhinovirus and intradermal DNA elicits potent immune responses and contains acute EcoHIV infection in mice

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Running title

Live rHRV-Gag/Tat as a HIV mucosal vaccine

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6.1 Abstract

Mucosal immunity is deemed crucial to control sexual transmission of human immunodeficiency virus (HIV). Herein we report the efficacy of a novel mucosal HIV vaccine strategy comprising initial intranasal (IN) vaccination with a cocktail of six live recombinant human rhinoviruses (HRVs) encoding overlapping fragments of HIV Gag and full length Tat (rHRV-Gag/Tat) followed by intradermal (ID) vaccination with a two plasmid DNA vaccines encoding HIV Gag and Tat (pVAX-Gag-Tat). This heterologous prime-boost strategy will be referred to hereafter as rHRV-DNA. As a control, IN vaccination with wild type (wt)-HRV-A1 followed by a single ID dose of pVAX (wt-HRV-A1/pVAX vaccination) was also included. rHRV-DNA vaccination elicited superior multi-functional $CD8^+$T cell responses in lymphocytes harvested from mesenteric lymph nodes and spleens, and higher titers of Tat-specific antibodies in blood and vaginal lavages compared with wt-HRV-A1/pVAX vaccination. Furthermore, these responses were significantly higher than those generated after the administration of 3 ID doses of pVAX-Gag-Tat in a homologous prime-boost regimen (3X pVAX-Gag-Tat vaccination). The rHRV-DNA strategy also reduced the viral load more effectively after challenge with EcoHIV, a murine HIV challenge model, in peritoneal macrophages, splenocytes and blood compared with wt-HRV-pVAX or 3X pVAX-Gag-Tat vaccination. These data provide the first evidence that a rHRV-DNA vaccination regimen can induce HIV-specific immune responses not only in the gut and vaginal mucosa but also systemically, and supports further testing of this regimen in the development of an effective mucosally-targeted HIV-1 vaccine.

6.2 Importance

Vaccination arguably remains the most cost-effective and enduring solution to control HIV infection, especially in resource-poor regions of the world. Vaccines that elicit robust mucosal immunity are deemed desirable as the vast majority of HIV transmissions occur through genito-urinary and genito-rectal mucosal routes. Furthermore, infection often results in extensive $CD4^+$T cell depletion in the gut, highlighting the need to develop vaccines that also elicit mucosal immunity at the gut mucosa. Here, we report that intranasal administration of a rHRV-Gag/Tat vaccine in a heterologous prime-boost vaccination regimen elicited robust mucosal and systemic HIV-specific immunity, and controlled acute EcoHIV infection in mice. Thus, rHRV-Gag/Tat represents a promising mucosal vaccine candidate against HIV.

6.3 Introduction

A potential reason why previous HIV vaccine trials were ineffective[1-3] or modestly effective[4] is the failure to generate effective mucosal immunity[5]. A majority of HIV transmissions occur via genito-urinary or genito-rectal mucosal surfaces[6] and extensive $CD4^+$ T cell depletion occurs in the gastrointestinal mucosa after infection[7]. This highlights the need to develop vaccines capable of eliciting protective HIV-specific immune responses at these surfaces. Moreover, previous HIV vaccine strategies focused on systemic vaccination, which induced little or no protective immune responses at the mucosa[5]. Therefore, mucosal vaccination strategies able to elicit HIV-specific immunity both systemically and at mucosal surfaces are being actively pursued[8].

Ideally, mucosal HIV vaccines should generate broadly neutralizing antibodies (bNAbs) against the envelope proteins to prevent primary HIV infection as has been observed in animal studies involving passive transfer of broadly neutralizing antibodies[9,10]. However, due to the recognised difficulties in designing vaccines capable of eliciting Env-specific bNAbs[11], a vaccine capable of eliciting high titer anti-Tat NAb might be a feasible alternative to controlling HIV replication and delaying disease onset[12,13]. An effective HIV vaccine should also induce robust poly-functional T cell mediated immunity (CMI) against the relatively conserved Gag proteins, viz. responses similar to those seen in long-term non-progressors[14,15].

Among the several strategies that have been developed to generate mucosal HIV vaccines is the use of mucosally transmitted replication-competent viral vectors[16]. These vectors usually establish an infection that mimics a natural viral infection, efficiently deliver immunogens to mucosal antigen presenting cells (APCs), and facilitate the development of long-lasting humoral and CMI[17] without the need for addition of adjuvants[16]. Several replication-competent viral vectors including adenovirus, poliovirus, influenza virus, poxvirus and cytomegalovirus vectors have been developed and tested as HIV vaccines, with promising

results in large animal models[16,18], particularly when used in heterologous prime-boost vaccination regimens.

Previously, polioviruses, which are transmitted via mucosal surfaces[19], were studied as potential viral vectors for HIV vaccine development[19,20] and vaccination with a cocktail of live recombinant polioviruses generated protective immunity against intravaginal challenge with SIVmac251 in 4/7 (57%) vaccinated macaques[20]. However, replication-competent poliovirus vectors have not advanced to human clinical trials mainly due to the high level of pre-existing vaccine-induced immunity in the community[21] which has been shown to limit the efficacy of virus-vectored vaccines[1]. Like polioviruses, human rhinoviruses (HRVs) are classified in the *Picornaviridae* and share many characteristics, including their genome organisation and mode of transmission[22]. HRVs are transmitted via the nasal mucosa [23] making them potential vaccine vectors to elicit mucosal immunity.

We have previously developed a series of replication-competent, genetically stable recombinant HRV serotype-A1 viruses (rHRV-Gag 1-5 and rHRV-Tat) by inserting discrete overlapping fragments of the HIV *gag* gene or the full length *tat* gene, into the junction of the genes encoding the structural proteins and the non-structural proteins (the P1/P2 junction)[24]. We have now mixed these rHRVs into a single cocktail vaccine suitable for intranasal (IN) administration, a route that has been shown to generate pan-mucosal and systemic immunity[8,25]. Furthermore, IN vaccination is considered to be safe, well tolerated, easily administered and inexpensive[25], and consequently, the rHRV-Gag/Tat vaccine represents a potential cost-effective HIV vaccine candidate for use in low-income countries. Herein we describe the immune responses against Gag and Tat after vaccination of mice IN with rHRV-Gag/Tat followed by ID delivery of DNA encoding Gag and Tat, and evaluate the efficacy of this regimen after challenge with ecotropic murine leukaemia HIV (EcoHIV).

6.4 Material and Methods

6.4.1 Recombinant HRV-Gag/Tat production and purification

We have previously described the production of rHRV-Gag/Tat[24]. Briefly, five overlapping fragments representing the full length HIV *gag* or the *tat* gene were individually inserted into the P1/P2 junction in a replication-competent HRV A1 cDNA[26] to generate 5 rHRVs each encoding a discrete, overlapping fragment of the Gag protein and one rHRV encoding the full length Tat protein. Each recombinant virus was produced by transfecting H1-Hela cells with *in vitro* transcribed viral RNA generated with the TransIT®-mRNA transfection kit (Mirus) following the manufacturer's instructions. The individual rHRVs were purified and titrated as described previously[24] then combined into a cocktail (rHRV-Gag/Tat) and re-titrated to ensure that the vaccination dose contained a uniform virus concentration.

6.4.2 DNA vaccines

Plasmids encoding HIV Gag and perforin (PRF) (pVAX-Gag-PRF) or HIV Tat fused to the oligomerisation domain of the C4b-p (pVAX-sTat-IMX313) have been described recently[27] (Tomusange et al, submitted). Briefly, the HIV *gag* and PRF genes were inserted downstream of the CMV and SV40 promoters, respectively, to generate pVAX-Gag-PRF[27]. To generate pVAX-sTat-IMX313, the human tissue plasminogen activator leader sequence and the oligomerisation domain of C4b-p (IMX313) were introduced at the N- and C-termini respectively of the HIV *tat* gene (Tomusange et al, submitted).

6.4.3 Animals and immunisations

Female 6-8 week old BALB/c mice were obtained from the University of Adelaide Laboratory Animal Services and maintained under PC2 conditions in individually ventilated cages fitted with a HEPA filter. All animal experiments were approved by the University of Adelaide and the South Australia Pathology Animal Ethics Committees. Vaccinations were performed at two week intervals via the IN route after 2% isofluorane anaesthesia for the rHRV-Gag/Tat cocktail and wild-type (wt)-HRV-A1 as described[28] or via the intradermal

(ID) route for the DNA vaccines as described[27]. Mice (n=7) received either 2 doses (containing $5x10^6$ $TCID_{50}$ per dose per animal) of wt-HRV-A1 followed by 50 µg pVAX (vaccination control group, referred to hereafter as wt-HRV-A1/pVax) or 2 doses of the rHRV-Gag/Tat cocktail (containing $5x10^6$ $TCID_{50}$ per dose per animal) followed by a single ID dose of 50 µg of a DNA cocktail (pVAX-Gag-Tat), containing equimolar concentrations of pVAX-sTat-IMX313 and pVAX-Gag-PRF. The latter will be referred to hereafter as rHRV-DNA prime-boost vaccination. Another group of mice received 3 ID doses (50 µg of a DNA cocktail per dose) of pVAX-Gag-Tat and this homologous prime-boost vaccination strategy will be referred to hereafter as 3X pVAX-Gag-Tat vaccination. Blood and cervical vaginal lavage (CVL) samples were collected one day prior to each vaccination and 14 days after the final vaccination, when the mice were euthanized, and examined for anti-Tat antibody responses. Splenocytes and lymphocytes from the mesenteric lymph nodes (mesenteric lymphocytes) were also harvested at this time point for analysis of CMI.

6.4.4 Enzyme-linked immunosorbent spot assay (ELISpot)

ELISpots were performed to determine the breadth and magnitude of HIV-specific CMI in splenocytes from vaccinated mice. A panel of 15-19 mer overlapping peptide pools spanning the entire Gag and Tat proteins was obtained from the NIH AIDS reagent bank (Germantown, MD, USA). Mouse interferon (IFN)-γ ELISpot was performed on RBC-depleted splenocytes that were re-stimulated for 36 h with 4 µg /ml of 5 Gag peptide pools or a Tat peptide pool as we described previously[27,29]. Briefly, multiscreen-IP HTS plates (Millipore) were coated with anti-mouse IFNγ (clone AN18, MabTech) and secreted IFNγ detected with anti-mouse IFNγ-biotin (clone R4-6A2, MabTech). Developed spots were counted automatically using an ELISpot reader (AID GmbH, Germany). The number of spots (spot forming units-SFUs) in unstimulated splenocytes was subtracted from the number in peptide-stimulated cells to generate the net Gag or Tat response.

6.4.5 Intracellular cytokine staining (ICS) and flow cytometry

Multi-colour ICS was performed on RBC-depleted splenocytes and mesenteric lymphocytes to determine IFN-γ, interleukin (IL)-2, and tumor necrosis factor (TNF)-α production from Gag-specific $CD8^+$ T cells as described previously[27,29]. Briefly, the cells were stimulated for 1 h with 5 µg/ml of the H-$2K^d$-restricted $Gag_{197\text{-}205}$ (AMQMLKETI) immunodominant peptide [30] (China peptides, China) then cultured in the presence of protein transport inhibitor (Brefeldin A, eBiosciences) for a further 6 h. Staining was performed using fluorescence-activated cell sorting (FACS) Cytofix/Cytoperm buffer (BD Biosciences) with antibodies specific to mouse antigens CD8α (allophycocyanin (APC)-efluor780 conjugated), CD44α (APC conjugated), IL-2 (peridinin chlorophyll 5.5 conjugated), IFN-γ (fluorescein isothiocyanate (FITC) conjugated) and TNF-α (phycoerythrin conjugated) (BD Biosciences). The cells were analysed on a BD FACS Canto and the results analysed with FlowJo X.0.7 software (Ashland, OR). The number of cells producing cytokines without prior stimulation was subtracted from the number in peptide-stimulated cells to generate the net Gag response. Although a more complex statistical analysis has been used by others to interpret ICS and multi-colour flow cytometric data[31], the statistical analysis described in this thesis was performed as approved by our departmental statistician.

6.4.6 H-$2K^d$- $Gag_{197\text{-}205}$ tetramer and antibody staining

RBC-depleted splenocytes were initially stained with the APC-conjugated H-$2K^d$-restricted $Gag_{197\text{-}205}$ (AMQMLKETI) immunodominant peptide tetramer (Biomolecular Resource Facility, John Curtin School of Medical Research, Australian National University, Canberra, Australia) for 1 h at room temperature, washed twice in phosphate-buffered saline (PBS) and then re-stained with FITC-conjugated anti-mouse CD8α antibody (BD Biosciences). The cells were analysed on a BD FACS Canto and the results analysed with FlowJo X.0.7 software.

The number of unstained tetramer positive cells was subtracted from the number in tetramer-stained cells to generate the net tetramer positive cells.

6.4.7 Enzyme-linked immunosorbent assay (ELISA)

Serum and CVL samples from mice were analyzed for anti-Tat antibodies by indirect ELISA as we described previously (Tomusange et al, submitted). As BALB/c mice do not recognise Gag-specific B cell epitopes (http://www.hiv.lanl.gov/content/immunology/), Gag-specific antibody responses were not evaluated. Briefly, Maxisorp plates (Corning Sigma-Aldrich) were coated with 500 ng of purified Tat protein in carbonate-bicarbonate buffer, blocked with 2 % w/v bovine serum albumin (BSA) in PBS and then serial dilutions of serum and CVL samples added. Bound antibodies were detected using horseradish peroxidase (HRP)-conjugated goat anti-mouse immunoglobulin (Ig)G (GE Healthcare Life Sciences, USA) or anti-mouse IgA (Life Technologies) and the optical density (OD) read at 492 nm. Endpoint titers were determined as the reciprocal of the highest serum or CVL sample dilution with an OD reading above the cut-off, set as twice the standard deviation (SD) above the mean OD of serum samples from naïve mice.

6.4.8 EcoHIV/NL4-3 challenge

EcoHIV preparation, titration and challenge have been described previously[27,29,32]. Vaccinated mice were challenged via the intra-peritoneal (IP) route with EcoHIV equivalent to 1.5 μg p24, then spleen, blood and peritoneal exudate cells (PECs) collected 7 days post challenge and examined by qRT-PCR as described previously[27,33]. Results were normalised to RPL13a mRNA levels after examining primer efficiency using the ΔΔCT (threshold cycle) quantification method[34,35].

6.4.9 Statistical analysis

Data presented as mean ± standard error of the mean (SEM) were generated with GraphPad Prism version 6 (GraphPad Software, La Jolla, CA, USA). Non-parametric Kruskal-Wallis

test was used to compare the difference between multiple vaccine groups. If this showed significant differences, then the Mann-Whitney U test was performed to compare differences between each vaccine group, independently. Statistical significance was determined using the Mann-Whitney U test; $p < 0.05$ was considered significant and $p > 0.05$ was considered non-significant.

6.5 Results

6.5.1 rHRV-DNA prime-boost vaccination elicits robust CMI

A robust CMI to HIV Gag appears to correlate with control of HIV infection in humans[14,15] and non-human primates[36,37]. We previously showed that vaccination of mice with 3 doses of pVAX-Gag-PRF elicited broad, poly-functional Gag-specific CMI able to control EcoHIV after challenge[27], and that vaccination with pVAX-sTat-IMX313 elicited high titer anti-Tat responses that also controlled EcoHIV infection post challenge (Tomusange et al, submitted). Therefore, we wished to compare the efficacy of rHRV-DNA vaccination with that of wt-HRV-A1/pVAX or 3X pVAX-Gag-Tat vaccination. Initially, we used the IFN-γ ELISpot assay (and Gag or Tat peptide pools) to compare CMI responses in splenocytes harvested 14 days after the final vaccine inoculation. This experiment showed that the Gag-specific responses to the different peptide pools ranged from 169 to 427 and 72 to 278 (mean SFU/10^6 cells), in animals vaccinated with rHRV-DNA and 3X pVAX-Gag-Tat, respectively (Figs 1A-D). These responses were detected in splenocytes stimulated with all 5 Gag peptide pools suggesting that each of the 5 Gag fragments encoded in the rHRV-Gag/Tat cocktail vaccine was expressed *in vivo* and thus contributed to the resultant Gag-specific responses. Splenocytes from the wt-HRV/pVAX vaccination control group showed responses which were considerably lower (<22 mean IFN-γ SFU) than those from unstimulated cells from vaccinated mice (mean IFN-γ SFU =25), thus the net IFN-γ responses in this vaccination group is considered zero. The combined Gag response was ~2 fold higher after vaccination with rHRV-DNA compared to 3X pVAX-Gag-Tat vaccination (1147 and 554, respectively, p=0.0175) as shown in Fig 1E. However, it is possible that summating the Gag peptide responses can be misleading due to competition between responses and the fact that errors within each measurement are not taken into account. As a result, stimulating with the entire peptide pool could be considered to overcome these limitations. Nevertheless, a standard

method was used to enumerate the overall Gag-specific T cell response detected in ELISpot assays. It is also important to note that tetramer staining is more sensitive and specific in detecting T cell-specific responses than ICS or ELISpot assays. Tetramer staining measures the overall T cell response irrespective of the cytokines produced, whereas ICS and ELISpot cytokine levels (IFN-γ, IL-2 or TNF-α) are indirect markers of T cell-mediated responses. Stimulation-associated cell death is common with ICS and ELISpot assays[38], but not with tetramer staining, as the latter does not involve cell stimulation prior to analysis. Furthermore, in ICS and ELISpot assays, the absolute number of cells producing or secreting IFN-γ will largely depend on the length of stimulation and perhaps the stimulus i.e. peptide pools or a peptide containing immuno-dominant epitopes, as different cells will respond at different times during stimulation. Therefore, it is possible that the number of cells staining positive with the tetramer will differ to the number of cells producing IFN-γ from ICS or the number of cells secreting the same cytokine in the ELISpot assay.

Similarly, wt-HRV/pVAX vaccination failed to induce Tat-specific CMI responses above background (mean IFN-γ SFU=0), but high magnitude Tat-specific CMI responses could be readily detected after rHRV-DNA vaccination (mean IFN-γ SFU=190) as shown in Fig 1F. Furthermore, the Tat-specific CMI responses elicited by rHRV-DNA were ~3.9 fold higher than those elicited by 3X pVAX-Gag-Tat (190 compared to 49 SFU, respectively, p=0.0041) as shown in Fig 1F.

As IFN-γ ELISpot assays involve extensive *in vitro* stimulation of effector cells, which can cause activation-induced cell death[39], splenocytes from immunized mice were stained with the H-2K^{d}-restricted $Gag_{197\text{-}205}$ tetramer to directly enumerate the Gag-specific CD8^{+} T cells present *in vivo*. The mean number of tetramer-positive CD8^{+}T cells following wt-HRV-

pVAX vaccination was less than background from unstained cells, thus the net number of tetramer positive cells in this vaccination group is considered zero

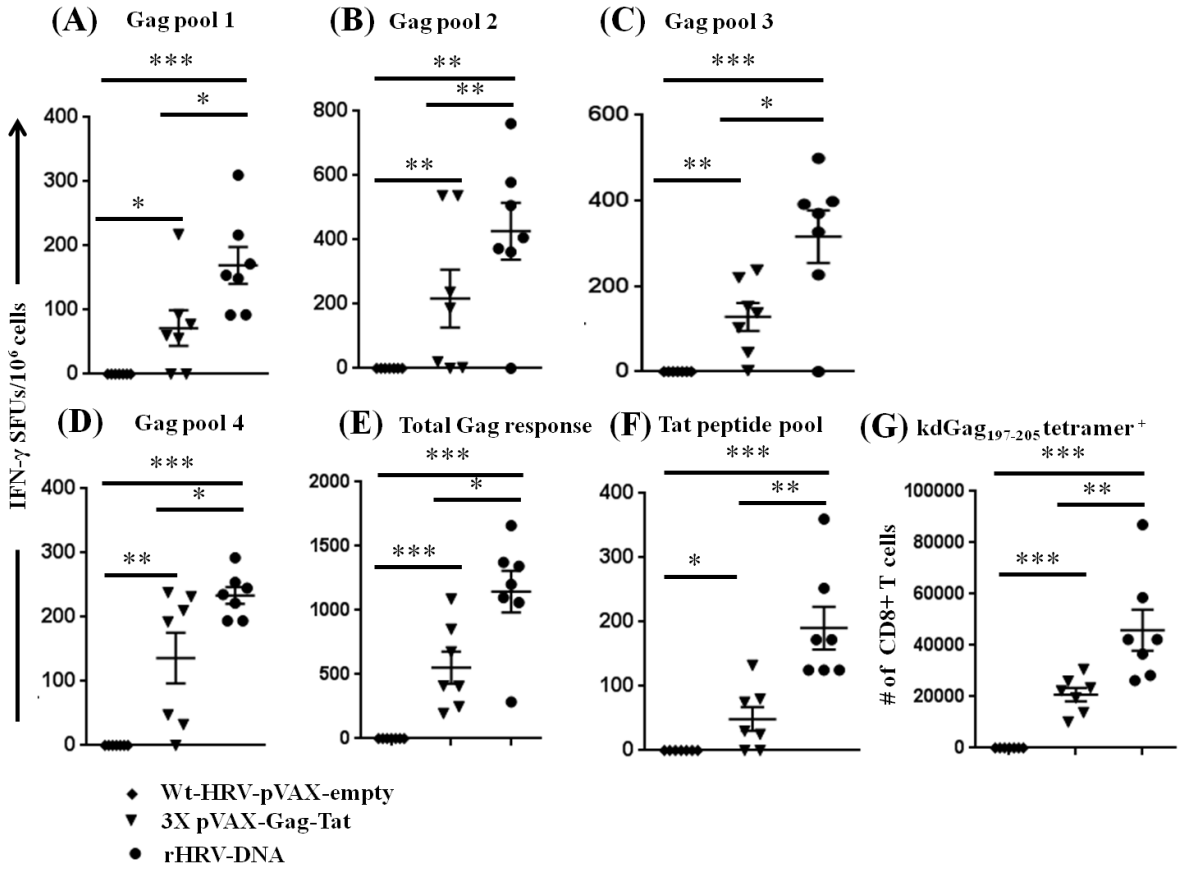

Figure 1. rHRV-DNA vaccination elicits robust CMI in the spleen. *Mice (n=7) received either 2 doses (containing 5x10^6 TCID$_{50}$ per dose per animal) of wild-type (wt)-HRV-A1 followed by 50 µg pVAX (vaccination control group) or 2 doses of rHRV-Gag/Tat (containing 5x10^6 TCID$_{50}$ per dose per animal) followed by a single ID dose of 50 µg of a DNA cocktail (pVAX-Gag-Tat) containing equimolar concentrations of pVAX-sTat-IMX313 and pVAX-Gag-PRF. This vaccination regimen is referred to as rHRV-DNA prime-boost vaccination. Other mice were vaccinated with 3 ID doses (50 µg/dose per animal) of pVAX-Gag-PRF/pVAX-sTat-IMX313 and referred to as 3X pVAX-Gag-Tat vaccination. Splenocytes were collected 14 days after the final dose and restimulated for 36 h in duplicate with overlapping peptides representing the enitre Gag or Tat protein. (A) Gag peptide pool 1, (B) Gag peptide pool 2, (C) Gag peptide pool 3 and (D) Gag peptide pool 4 in an IFN-γ ELIspot. (E) Total*

*Gag responses depicted in pools 1-4. (F) Tat-specific responses from cells stimulated with the complete Tat peptide pool. Splenocytes were also stained with the H-2K^d-Gag$_{197-205}$ tetramer for 1h at room temperature and the number of tetramer-positibe CD8$^+$ T cells was analyzed by flow cytometry (G). The data are representative of 2 independent experiments (n = 7) and are plotted as mean SFU per 10^6 splenocytes (± SEM). Each symbol represents an individual mouse. * p < 0.05, ** p < 0.01 and *** p < 0.001 (Mann–Whitney U test). Note: The scale for the Y-axis may differ from panel to panel.*

Moreover, tetramer binding CD8$^+$ T cells (mean of tetramer positive CD8$^+$T cells=45921) could be readily detected after rHRV-DNA vaccination and these were also significantly higher than those generated following 3X pVAX-Gag-Tat vaccination (45921 vs 20814, P=0.0023) as shown in Fig 1G. Collectively, these results illustrate that rHRV-DNA is more effective at inducing Gag and Tat-specific CMI than wt-HRV-pVAX or 3X pVAX-Gag-Tat vaccination.

6.5.2 rHRV-DNA elicits superior systemic poly-functional CMI

Although other marker such as CD107a. granzyme and perforin were not assessed in this study, T cells capable of producing multiple anti-viral cytokines (eg. IFN-γ and TNF-α) and/or survival/proliferative cytokines (eg. IL-2) i.e. poly-functional T cells, are desirable markers for HIV-1 vaccine development as the presence of these cells appears to correlate with improved control of HIV infection in humans[14,15,40] and SIV infection in non-human primates[36,37]. The data showed that K^{d}Gag$_{197-205}$-specific CD8$^+$T cells from all rHRV-DNA vaccinated animals responded to vaccination by producing IL-2, IFN-γ or TNF-α, whereas responses from wt-HRV-A1/pVAX vaccinated animals did not surpass background from unstimulated cells (Figs 2A-C). Furthermore, the number of CD8$^+$T cells producing IFN-γ, IL-2 or TNF-α was ~2 fold (mean 46318 vs 22679, *P*=0.0006), ~5 fold (38928 vs 7712,

P=0.0006) and ~3 fold (39007 vs 12738, *P*=0.0012) higher following rHRV-DNA than after 3X pVAX-Gag-Tat vaccination, respectively (Figs 2A-C).

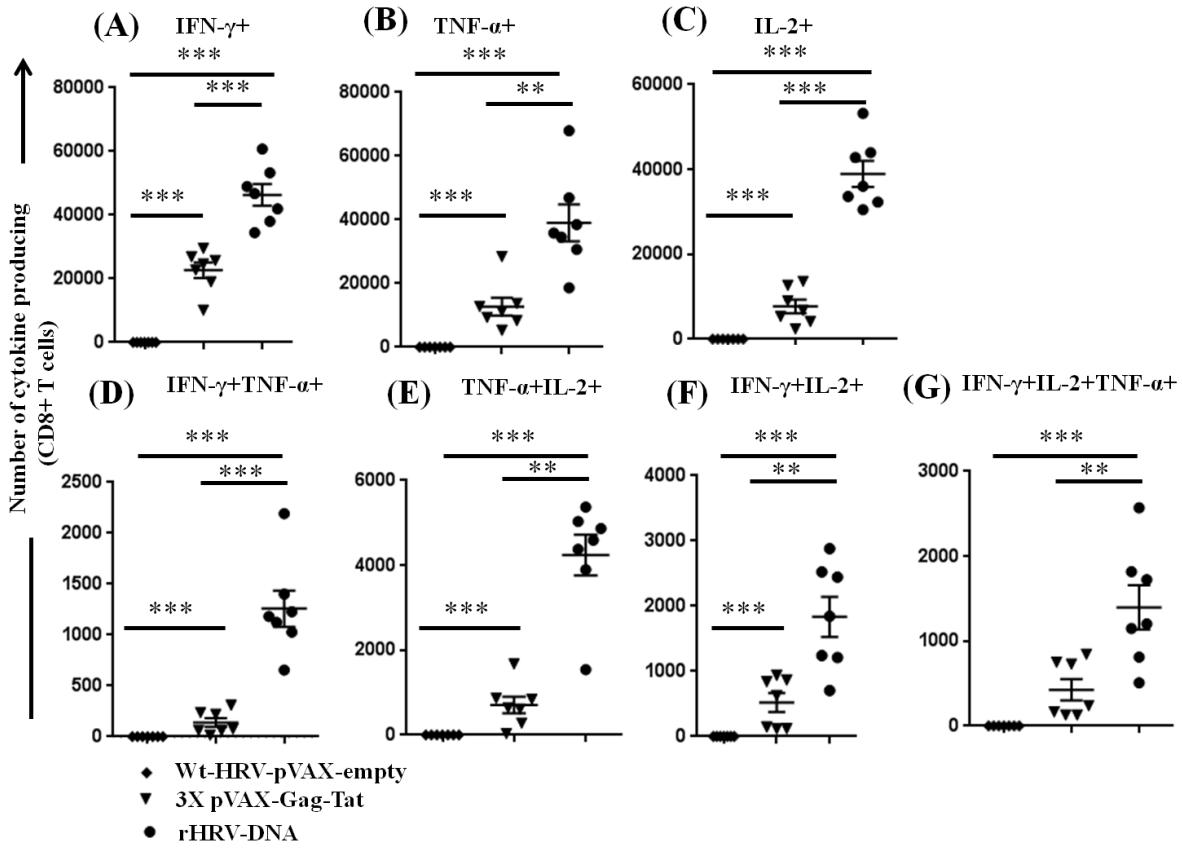

Figure 2. rHRV-DNA elicits superior systemic poly-functional CMI. *Mice were vaccinated as described in the legend to figure 1 and splenocytes harvested 14 days after the final dose. The cells were stimulated for 1 h with 5 µg/ml of the H-2K^d-restricted Gag$_{197-205}$ immuno-dominant peptide, in the presence of protein transport inhibitor (Brefeldin A, eBiosciences) for a further 6h and cytokine production then analyzed by flow cytometry. We gated on CD8$^+$ T cells to assess the number of CD8$^+$ T cells producing (A) IFN-γ, (B) TNF-α, (C) IL-2, (D) IFN-γ and TNF-α, (E) TNF-α and IL-2, (F) IFN-γ and IL-2 and (G) IFN-γ, TNF-α and IL-2 after stimulation. The data are representative of 2 independent experiments, plotted as mean (n = 7) ± SEM and each symbol represents an individual mouse. * p < 0.05, ** p < 0.01, *** p <0.001 and ns, p > 0.05 (Mann–Whitney U test).*

The number of Gag-specific $CD8^+$ T cells that concurrently produced at least 2 cytokines (poly-functional $CD8^+$ T cells) following $K^dGag_{197-205}$ peptide stimulation was also determined. Vaccination with rHRV-DNA resulted in ~ 9-fold more cells producing IFN-γ and TNF-α than 3X pVAX-Gag-Tat vaccination (1259 vs 140, P=0.0006) as shown in Fig 2D. Similarly, the number of cells that produced IL-2 and TNF-α was ~6 fold higher in rHRV-DNA vaccinated mice (4240 vs 707, P=0.0012) as shown in Fig 2E, while the number of cells producing IFN-γ and IL-2 was ~2.5 fold (1836 vs 523, P=0.0041) higher (Fig 2F). The number of triple cytokine- (IFN-γ, IL-2 and TNF-α) producing $CD8^+$T cells was ~3.3 fold higher after rHRV-DNA vaccination (1398 vs 427, P=0.0070) as shown in Fig 2G. We then examined the number of $CD8^+$ cells with an effector memory T cell (T_{EM}) phenotype. Effector memory is vital to establish antigen-specific recall responses[36,41] and is required for an effective HIV vaccine. As T_{EM} are marked by CD44 expression[42], we determined the number of $CD44^{hi}CD8^+$T cells which synthesised cytokines after splenocytes were stimulated with the $K^dGag_{197-205}$ peptide. The number of $CD44^{hi}CD8^+$T cells that produced IFN-γ, TNF-α or IL-2 was 3-4 fold higher in the rHRV-DNA-than in the 3X pVAX-Gag-Tat vaccination group (Supplementary Figs. S1A-C). Although the number of double and triple positive $CD44^{hi}CD8^+$T cells was modest after rHRV-DNA or 3X pVAX-Gag-Tat vaccination, it was significantly higher in the rHRV-DNA group (Supplementary Figs. S1 D-G, respectively).

In summary, the data also illustrate that rHRV-DNA is more effective at inducing poly-functional Gag-specific $CD8^+$T cell responses in the spleen than wt-HRV-pVAX or 3X pVAX-Gag-Tat vaccination.

6.5.3 rHRV-DNA vaccination elicits superior poly-functional CMI in the mesenteric lymph nodes

The vast majority of $CD4^+$ T cell death during acute HIV-1 infection occurs in the gut and thus poly-functional CMI responses at the gut mucosa are likely to significantly reduce $CD4^+$ T cell death[43]. Vaccination with wt-HRV-A1/pVAX failed to induce $K^dGag_{197-205}$-specific

$CD8^+$T cell responses above background from unstimulated lymphocytes prepared from mesenteric lymph nodes, whereas high numbers of Gag-specific cells were readily detected following rHRV-DNA vaccination (Figs 3A-G).

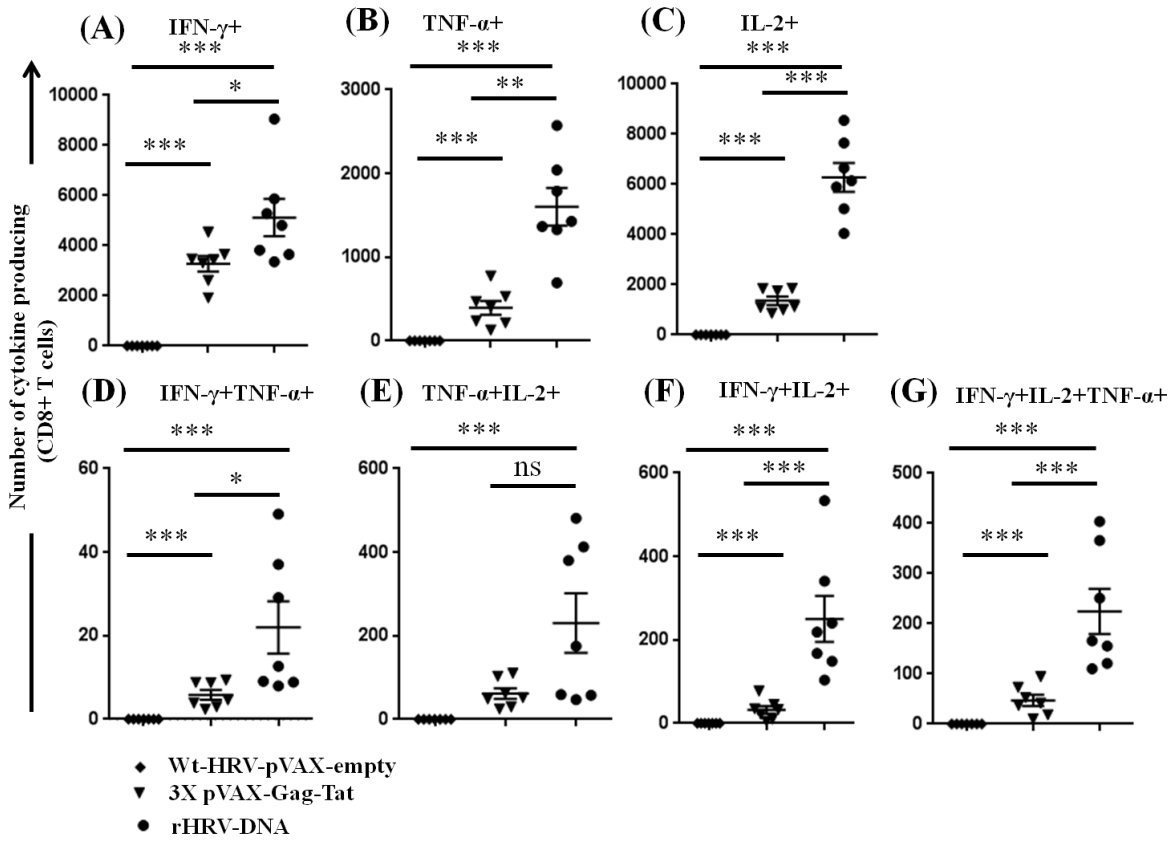

Figure 3. rHRV-DNA vaccination elicits superior poly-functional CMI in the mesenteric lymph nodes. *Mice were vaccinated as described in the legend to figure 1 and lymphocytes prepared from mesenteric lymph nodes harvested 14 days after the final dose. The lymphocytes were stimulated for 1h with 5 µg/ml of the H-2K^d-restricted Gag$_{197-205}$ immuno-dominant peptide and then cultured in the presence of Brefeldin A for a further 6h. Cytokine production was analyzed by flow cytometry. We gated on CD8$^+$ T cells to assess the number of CD8$^+$ T cells producing (A) IFN-γ, (B) TNF-α, (C) IL-2, (D) IFN-γ and TNF-α, (E) TNF-α and IL-2, (F) IFN-γ and IL-2 and (G) IFN-γ, TNF-α and IL-2 after stimulation. The data are representative of 2 independent experiments, plotted as mean (n = 7) ± SEM and each symbol*

*represents an individual mouse. * $p \leq 0.05$, ** $p \leq 0.01$ and *** $p \leq 0.001$ (Mann–Whitney U test).*

Furthermore, the number of mono-functional (Figs 3A-C) and poly-functional cells (Figs 3D-G) generated after rHRV-DNA was significantly higher than that after 3X pVAX-Gag-Tat vaccination. The number of poly-functional T_{EM} cells derived from the mesenteric lymph nodes produced following rHRV-DNA or 3X pVAX-Gag-Tat vaccination was generally modest, although rHRV-DNA appeared to be more effective than 3X pVAX-Gag-Tat (Supplementary Fig S2). Overall, consistent with the splenocyte data, rHRV-DNA induced poly-functional Gag-specific $CD8^+$T cell responses in the gut more effectively than wt-HRV-pVAX or 3X pVAX-Gag-Tat vaccination.

6.5.4 rHRV-DNA vaccination elicits superior Tat-specific humoral responses

High titer Tat-specific serum IgG appears to correlate with control of HIV infection in humans[12,13] and SIV in macaques[44,45]. Therefore, we compared the titers of Tat-specific IgG generated after vaccination with wt-HRV-A1/pVAX, rHRV-DNA or 3X pVAX-Gag-Tat. Tat-specific IgG was detected in serum samples from all vaccinated mice (except in the wt-HRV-A1/pVAX group) with mean reciprocal endpoint titers ranging from 270 to 65610 in the rHRV-DNA vaccinated group and from 30 to 810 in the 3X pVAX-Gag-Tat vaccinated group (Fig 4A). In the rHRV-DNA vaccinated group, anti-Tat IgG was detectable in samples after the first rHRV dose with a mean titer of 236, but was undetectable in the 3X pVAX-Gag-Tat vaccinated group at this time point. After the 2^{nd} dose, the mean anti-Tat antibody titer in serum from the rHRV-DNA vaccinated group increased by ~65 fold (from 236 to 15312) and was considerably higher than that in serum from the 3X pVAX-Gag-Tat group (mean titer=9). Administration of a single DNA booster dose increased the IgG titers by ~ 1.2 fold (from

15312 to 17704) in the rHRV-DNA group and by ~38 fold (from 9 to 338) in the 3X pVAX-Gag-Tat vaccinated group.

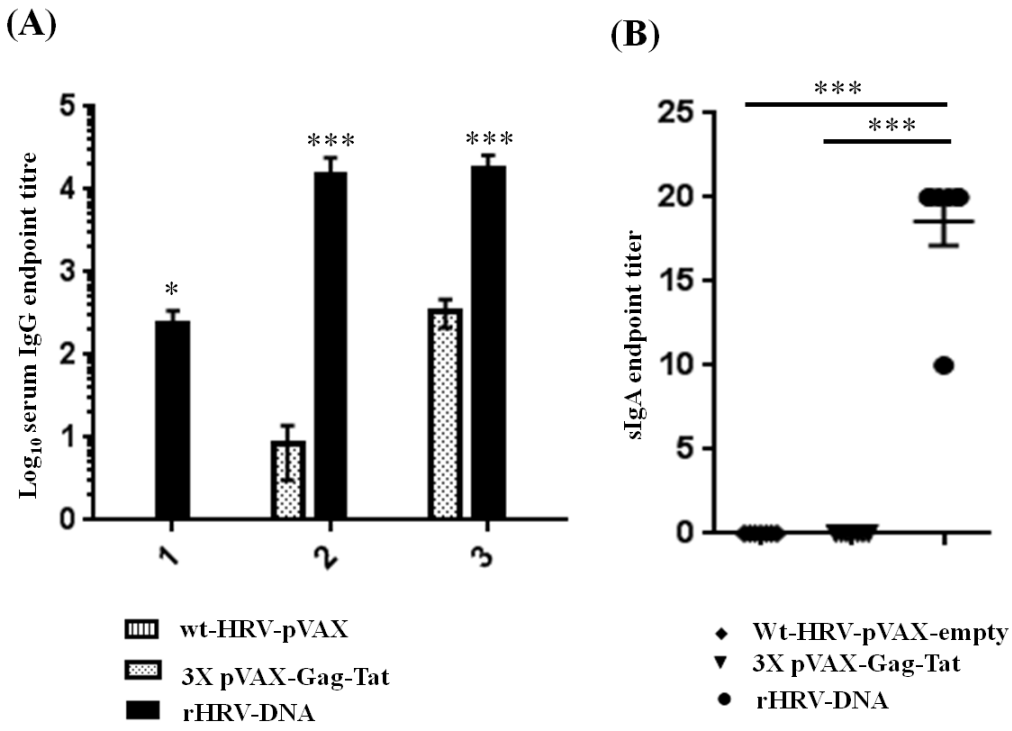

Figure 4. rHRV-DNA vaccination elicits superior Tat-specific humoral responses. *Mice were vaccinated as described in the legend to Figure 1. Blood and CVL samples were collected 1 day before each vaccination and 14 days after the final dose. (A) ELISA results showing serum log_{10} anti-Tat IgG titers. (B) ELISA results showing anti-Tat sIgA titers. Data are plotted as mean (n = 7) ± SEM and are representative of 2 independent experiments. * $p \leq 0.05$ and *** $p \leq 0.001$ (Mann–Whitney U test).*

The differences in serum IgG titers were statistically significant at each time point between the two vaccinated groups (Fig 4A). The OD generated by serum from wt-HRV/pVAX vaccinated animals was lower than the cut-off, thus the antibody titer in this vaccination group was considered to be zero.

We also examined CVL samples from vaccinated animals for Tat-specific mucosal sIgA, as the presence of antigen-specific sIgA in blood or at the mucosa appears to correlate with

protection in humans[46,47]. We detected modest levels of sIgA (mean titer 20) in CVL samples collected after the DNA boost from all mice in the rHRV-DNA group, but not from mice in the wt-HRV/pVAX or 3X pVAX-Gag-Tat vaccinated group (Fig 4B). Most surprisingly, sIgA was not detected in samples prior to the DNA boost, indicating that this was required for the rHRV-Gag/Tat vaccine to induce detectable mucosal sIgA. On the whole, the results described above indicate that rHRV-DNA is more effective at inducing robust Gag and Tat-specific CMI as well as Tat-specific humoral immune responses at the mucosa than wt-HRV-pVAX or 3X pVAX-Gag-Tat vaccination.

6.5.5 rHRV-DNA controls the EcoHIV viral load post-challenge

EcoHIV is a murine HIV challenge model and has been used previously to evaluate the efficacy of candidate HIV vaccines[27,29]. However, there is no documented evidence that EcoHIV can infect mice via the intravaginal route which accounts for a majority of HIV transmissions[6] , and thus the IP route is a convenient route to successfully deliver the virus[27,29,48,49]. EcoHIV encodes the complete range of HIV proteins, including Tat, from the HIV-1 Clade B NL4-3 strain, except for the HIV gp120 which was replaced with gp80 from the murine leukaemia virus[32]. The virus spreads from the primary site of infection to the spleen and the brain in a manner reminiscent of HIV infection[32].

Consequently, we examined the protective efficacy of wt-HRV/pVAX, rHRV-DNA or 3X pVAX-Gag-Tat vaccination against EcoHIV challenge. All animals were challenged 10 days after the final vaccination, then culled 7 days later and splenocytes, PECs and peripheral blood samples collected to quantify the viral load (VL) by qRT PCR. The results were normalised to the RPL13a house-keeping gene as described previously[27,29]. Vaccination with rHRV-DNA or 3X pVAX-Gag-Tat significantly reduced the EcoHIV VL by ~204 fold (p= 0.0006) and ~8 fold (p= 0.0006) in PECs (Fig 5A), ~62 fold (p= 0.0006) and ~11 fold (p= 0.0006) in splenocytes (Fig 5B), and by ~40 fold (p= 0.0006) and ~7.5 (p= 0.0006) in blood

(Fig 5C), respectively, compared to vaccination with wt-HRV/pVAX. Furthermore, rHRV-DNA vaccination showed superior control of EcoHIV infection compared to 3X pVAX-Gag-Tat vaccination and reduced the VL by ~25 fold (p= 0.0006) in PECs, ~5.5 fold (p= 0.0286) in splenocytes and by ~5.4 fold (p= 0.0035) in peripheral blood as shown in figures 5A-C. Taken together, the data suggest that the rHRV-DNA regimen can also be used to achieve effective virologic control of EcoHIV-1 infection.

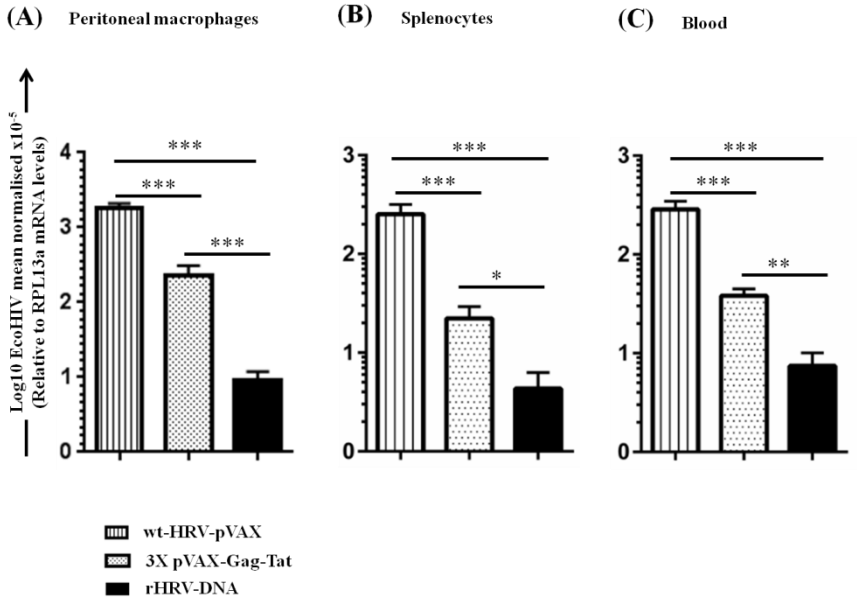

Figure 5. rHRV-DNA vaccination controls EcoHIV viral load post-challenge. *Mice were vaccinated with rHRV-DNA, wt-HRV- pVAX or 3X pVAX-Gag-Tat at 2 week intervals and then challenged with 1.5 µg p24 of EcoHIV/NL4-3 10 days post final vaccination. The viral load in PECs, splenocytes and blood was determined by qRT-PCR; EcoHIV RNA levels in (A) PECs, (B) splenocytes and (C) blood 7 days post challenge. EcoHIV mRNA levels were normalised to RPL13a mRNA and the data represent the mean (n=7) ± SEM. * $p \leq 0.05$, ** $p \leq 0.01$ and *** $p \leq 0.001$ (Mann–Whitney U test).*

6.6 Discussion

In this study, we report that a vaccination regimen comprised of a cocktail of replication-competent rHRVs encoding HIV Gag and Tat (rHRV-Gag/Tat) followed by a DNA vaccine encoding the same proteins (pVAX-Gag-Tat), elicited robust Gag-specific CMI in the spleen and gut mucosa, and high titer anti-Tat serum IgG and sIgA in the vagina of vaccinated mice. Furthermore, the vaccine-elicited immune responses controlled infection in a mouse EcoHIV challenge model. Intranasal (IN) vaccination generates pan-mucosal and systemic immunity as described previously[8,25], and our data provide new support to consider IN vaccination as a method to induce anti-HIV immunity at the mucosa and systemically. It is not possible to directly compare the immunogenicity and efficacy of rHRV-Gag/Tat vaccination to that reported previously by Crotty et al using the recombinant Sabin poliovirus-based HIV vaccines in macaques due to differences in animal models and challenge viruses used[20]. However, our data confirm that, similar to Sabin poliovirus-based vaccines, IN administration of the rHRV-Gag/Tat vaccine in a heterologous prime-boost strategy (rHRV-DNA) elicited systemic and mucosal HIV-specific immune responses that significantly controlled EcoHIV infection.

Heterologous prime-boost vaccination has been shown to generate superior CMI and humoral immunity compared with homologous prime-boost vaccination[50-52]. Similarly, our data showed that the rHRV-DNA regimen elicited poly-functional Gag-specific $CD8^+$ T cells more effectively than wtHRV-A1/pVax or 3X pVAX-Gag-Tat vaccination at the mucosa (Fig 3 and Supplementary Fig S2) and systemically (Figs 1- 2 and Supplementary Fig S1). The appearance of high quality CMI responses appears to correlate with long-term HIV control in humans[14,15] and SIV in non-human primates[36,53], thus our data suggest that the rHRV-DNA regimen might control SIV when tested in macaques or HIV-1 infections in humans. In addition to robust CMI, rHRV-DNA induced higher Tat-specific serum IgG titers than wt-

HRV-A1/pVax or 3X pVAX-Gag-Tat vaccination (Fig 4A), an additional marker which appears to correlate with control of HIV in humans[12,13] and SIV in animals[45,54]. Surprisingly, Tat-specific sIgA was detected in CVL samples from rHRV-DNA vaccinated animals only after the DNA boost (Fig 4B). The reason for this unexpected result remains unclear. However, sIgA was not detected in any animals vaccinated 3X pVAX-Gag-Tat at any time point; this was not surprising as we have previously shown that a minimum of 5 doses is required for a Tat DNA vaccine to elicit detectable mucosal sIgA (Tomusange et al, submitted).

Although other studies generally administer recombinant viruses as a boost following a DNA prime[50,55], our results are consistent with studies which have shown that administering a recombinant virus followed by a DNA [56] or protein vaccine[57] in a heterologous prime-boost vaccination strategy elicits high titer immune responses. Therefore, a boost with a DNA vaccine following rHRV-Gag/Tat prime is likely to elicit robust HIV-specific immune responses at the mucosa and systemically in vaccinated humans. Regular boosting with DNA may also be feasible and offers the advantage that anti-vector responses are unlikely to accumulate over time and prevent further boosting, as would be the case for a recombinant virus-based vaccine.

As robust immune responses do not necessarily indicate protection against virus challenge[58,59], we evaluated the efficacy of rHRV-DNA vaccination using a murine HIV challenge model, EcoHIV[32]. The rHRV-DNA regimen resulted in effective control of the EcoHIV infection (Figs 5A-B). However, it is unclear if humoral immunity and/or CMI were responsible for this control and additional experiments will be necessary to address this issue. Nevertheless, the robust Gag-specific CMI and high titer anti-Tat responses are thought to offer protective benefits against HIV in long-term non-progressors[12,14] and non-human primates[37,44] , and thus it is possible that both Gag-CMI and anti-Tat responses contributed.

In conclusion, we demonstrate for the first time that a live recombinant HRV vaccine encoding HIV proteins when administered in a heterologous vaccination regimen involving priming with virus followed by a single DNA boost is highly immunogenic in mice and that the vaccine-induced immune responses control EcoHIV infection post challenge. The data presented in this study have important ramifications for the development of effective mucosal HIV-1 vaccines in the future.

6.7 Conflict of interest

The authors declare no financial or commercial conflict of interest.

6.8 Acknowledgements

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Chapter 7.0: General Discussion

An effective vaccine is the best long-term and cost-effective solution to the current global HIV pandemic[82,164,285,530]. Although macaque[297,435] and human studies[531,532] have provided insights into the possible correlates of protection against HIV, the actual correlates remain poorly understood[533]. However, it is generally accepted that an effective HIV vaccine will have to induce bNAbs targeting the viral envelope proteins to prevent primary HIV infection as well as robust Gag-specific CMI to keep virus replication "in check"[161,164]. However, in light of the recognised difficulties associated with inducing Env-specific bNAbs by vaccination[163,179] and with the recognised potential of high titer anti-Tat responses in controlling HIV infection[17,213], Tat-based vaccines have been developed as an alternative to Env-based HIV vaccines.

This thesis reports the efficacy of two innovative vaccination strategies aimed at eliciting non-canonical NAb responses to Tat and CMI responses to Gag and Tat at the mucosa and systemically in vaccinated mice. The Tat-based DNA vaccination strategy described in chapter 3 highlighted that pVAX-sTat-IMX313 was the most effective DNA vaccine in evoking high titer Tat-specific NAb responses with the capacity to neutralize Tat activity *in vitro*. pVAX-sTat-IMX313 was then exploited in a mucosal prime-boost vaccination regimen (chapter 6) which successfully elicited mucosal immunity using rHRV vaccines (chapters 4-5) developed during this PhD project. As will be discussed, mucosal priming with a cocktail of rHRVs and systemic boosting with a combination of pVAX-sTat-IMX313 and pVAX-Gag-PRF (rHRV-DNA vaccination) not only elicited the expected Gag- and Tat-specific immunity, but also effectively controlled an acute infection with EcoHIV in vaccinated animals. Thus, data generated in this PhD thesis and the insight they provide show considerable potential for future HIV vaccine development.

7.1. A novel highly immunogenic Tat DNA-based HIV vaccine

Vaccines designed using the recombinant plasmid DNA platform offer several advantages (discussed in section 1.18.2 of this thesis). Although a majority of previous HIV DNA vaccines have been disappointing in clinical trials[366,534-536], two recent studies have shown that DNA-based vaccines were well tolerated, elicited antigen-specific immunity that controlled the HPV viral load, and inhibited the progression of tumor lesions and cervical cancer in humans[372,373]. These studies provide evidence that DNA vaccines can be effective in humans, if optimally designed, despite the disappointing outcomes in the past.

Upon the administration of pVAX-sTat-IMX313, mice developed high titer anti-Tat antibodies (serum IgG and secreted IgA in the vaginal mucosa). Other studies have shown that Tat-based vaccines induce high titer anti-Tat antibodies (serum IgG and secreted IgA in the vaginal mucosa) that appeared to correlate with SIV control in vaccinated macaques[16,228], and with the establishment of the long-term non-progression status during natural HIV infection in humans[121,122]. The presence of these antibodies in the vaginal mucosa, the site of a majority of HIV transmissions[2], is particularly apposite as the presence of such antibodies appears to offer protective benefits in humans[537] and non-human primates[169]. Thus, the ability to induce equally high titer anti-Tat antibodies raises the potential for pVAX-sTat-IMX313 to control SIV or HIV in a similar manner.

Furthermore, previous studies have shown that multiple doses of Tat-based DNA vaccines induced high avidity, robust Tat-specific CTL responses that appeared to correlate with SIV control in vaccinated macaques[538,539]. In contrast, a robust T cell response characterised by secretion of high levels of IFN-γ from peptide-stimulated splenocytes lacking CTL activity was induced after pVAX-sTat-IMX313 vaccination. The lack of CTL responses might be attributed to the route and vaccination dose, as suggested in previous studies[538,540], or the possibility that pVAX-sTat-IMX313 vaccination polarized the immune response towards Th2

responses (resulting in enhanced CD4 Th activity on B-cell maturation, see chapter 3, Fig 2C), without potent CTL Activity. Nevertheless, one would imagine that optimising the dose and route of vaccination would result in the induction of the desired CTL response. Indeed, increasing the number of doses from 3 to 5 enhanced anti-Tat NAb titers. Unfortunately due to resource and time limitations, examination of the *in vivo* CTL activity after the 5th dose was not repeated. Clearly, further investigation is warranted to comprehensively address this crucial aspect of a candidate HIV vaccine. As the primary objective of the study was to induce high titer anti-Tat humoral immunity rather than CMI, the ability of pVAX-sTat-IMX313 to induce a T cell response with potent CD4 Th activity is fitting, as such responses support the induction of high titer antibody responses[541].

It is worth noting that vaccine efficacy increased with the number of doses, suggesting that a similar vaccination regimen might be required for pVAX-sTat-IMX313 to be effective against SIV/SHIV or HIV-1. Particularly in humans, this would create compliance issues. Therefore, there is need to optimise pVAX-sTat-IMX313 delivery to require fewer doses to obviate this problem. Thus, optimising the dosage, vaccination strategy (homologous or heterologous prime-boost vaccination) and the route of vaccination might be worthwhile.

Taken together, the data reported in this thesis indicate that fusing Tat to IMX313 resulted in secretion of the oligomerised protein and significantly enhanced the efficacy of a Tat-based HIV DNA vaccine, similar to data reported previously for candidate malaria and tuberculosis vaccines[343-347,349]. The data also support the inclusion of IMX313 as a molecular adjuvant in DNA vaccines and the use of IMX313 is an attractive strategy that might be considered in developing an effective Tat-based HIV DNA vaccine. Unlike native Tat protein that is highly susceptible to oxidation and proteolysis[17], pVAX-sTat-IMX313 is relatively stable, easy to work with, and obviates the need to purify the Tat protein, which will most likely make the vaccine cheaper to produce on a large scale.

7.2 Vaccination strategies to induce mucosal immunity against HIV

A majority of HIV transmissions occur at the vaginal-rectal mucosa[2] and extensive $CD4^+$ T cell depletion during an acute HIV infection also occurs at the gut mucosa[154,311], highlighting the need to generate robust protective HIV immunity at these mucosal surfaces. Furthermore, HIV transmission especially at the genital mucosa represents a "virologic bottle neck" that restricts HIV-1 infection to only one virus strain (founder virus)[36,311], which presents an opportunity to clear the infection at the mucosa before viral quasi-species emerge significantly and disseminate systemically.

Mucosal vaccination has been used successfully to generate systemic and mucosal immunity against mucosally-transmitted viruses such as poliovirus[542], influenza virus[276,543] and rotavirus[544,545] and recently to elicit HIV-specific immunity at the mucosa[323,546], raising optimism that a rationally designed mucosal vaccine can equally be effective against HIV-1 infections.

Preferably, mucosally-targeted HIV vaccines will be delivered via the IN route. This vaccination route offers several benefits (discussed in chapter one of this thesis), but most importantly, it results in protection at the local nasal mucosa and the distant vaginal, rectal and oral-pharyngeal mucosa as well as systemically[313,314]. Moreover, it has been established that intranasal delivery of viral vector vaccines overcomes pre-existing systemic vector-specific immunity[318]. Of note, intranasal vaccination is a needle-free procedure than can be performed without necessarily requiring prior medical training. This attribute, together with low cost, makes intranasal vaccination an attractive vaccination strategy for a HIV vaccine that will most likely be universally well accepted and easily accessed even among individuals in low income countries where the HIV pandemic is most rampant. However, strategies to develop a HIV vaccine capable of eliciting robust mucosal protection are strictly limited,

highlighting the need to investigate other potential HIV vaccines that can provide protective benefits at the mucosa.

Recently, Singh and colleagues showed that intranasal administration of ovalbumin (OVA) together with galactosylceramide adjuvant induced OVA-specific CD8 T cell responses in the reproductive tract of female mice[546]. Similarly, it has been shown previously that intranasal administration of a non-replicating gp120-depleted HIV-1 (HZ321) encoding clade G HIV Gag in the presence of CpG oligodeoxynucleotide as an adjuvant elicited HIV-specific T cell responses that protected mice against intra-vaginal challenge with a recombinant vaccine virus encoding HIV Gag[547]. Furthermore, Barnett et al then showed that sequential intramuscular- followed by intranasal-administration of HIV-1 SF162 envelope protein together with MF59 induced anti-Env antibodies that protected macaques against intra-vaginal challenge with $SHIV_{SF162P4}$[208]. However, intranasal delivery of protein or DNA-based HIV vaccines requires adjuvanting which at times present additional safety concerns. In contrast, a single dose of a live recombinant virus vaccine, delivered mucosally, is normally sufficient to elicit long-lived immunity without the need for adjuvanting.

Ranasinghe and colleagues demonstrated previously that intranasal priming followed by intramuscular boost with recombinant pox viruses encoding HIV immunogens elicited high avidity cytotoxic CD8 T cell responses at the genito-rectal mucosa[399]. Furthermore, Barouch et al showed impressively that a prime-boost vaccination with a mixture of recombinant Adeno virus serotype serotypes 26/35 together with recombinant MVA encoding SIVGag/Pol/Env elicited robust CMI and humoral immunity at the mucosa that protected macaques against repetitive intra-rectal challenge with SIVmac251[548]. Similarly, vaccination of macaques with recombinant polio viruses encoding HIV proteins via the intranasal route remarkably resulted in sterilizing protection against intra-vaginal challenge with pathogenic SIVmac251[469]. Overall, this body of evidence suggests that viral vectors, when used in

optimised heterologous prime-boost vaccination combinations that involve mucosal delivery, can afford complete protection against mucosal challenge with SIV and might be equally effective in preventing HIV acquisition or at least delay progress to disease.

Although the viral vectors discussed above show impressive efficacy against SIV, fears over pre-existing vector immunity in the human population have greatly derailed their extensive use in HIV vaccine development. As stated throughout this thesis, the HRV-A1 is the rarest of HRV serotypes, allaying fears that pre-existing HRV-A1 vector-specific immunity would compromise the efficacy of vaccines delivered by a live HRV-A1 vector in humans. Furthermore, a candidate HIV vaccine, rHRV-Gag/Tat, delivered by replication-competent HRV-A1 intranasally in a heterologous prime-boost (rHRV-DNA) vaccination regimen has the potential to induce robust mucosal immune responses. Differences in challenge models (murine EcoHIV Vs macaque SIV/SHIV models) used in this study and those in the literature, cannot allow an accurate comparison of the efficacy of rHRV-Gag/Tat with that of other viral vector-based vaccines. Such comparisons can only be drawn in the future if rHRV-Gag/Tat is tested in macaques. Nevertheless, based on the data presented in Chapter 6, it is likely that rHRV-Gag/Tat will show similar levels of protection against SIV/SHIV/HIV acquisition or delay progress to disease.

7.2.1 Human rhinovirus serotype A1 as a HIV vaccine vector

Unlike DNA-based vaccines, replication-competent viruses have been used successfully to vaccinate humans against infections with, for example, poliovirus, yellow fever virus and influenza virus (discussed in section 1.15.1.1 of this thesis). Normally, attenuated viruses are used in the development of live attenuated vaccines (see chapter 1.15.1), although these viruses retain their lytic property (e.g live attenuated poliovirus used in the oral poliovirus vaccine). During necrosis of a virus-infected cell, several DAMPs are released into the extracellular milieu from where they bind to PRRs, including TLRs, located on professional

APCs, particularly DCs, and this results in a robust stimulation of the immune system[549]. Thus, vaccines developed using live virus platforms generally elicit a robust, long-lived immune response without the need for any adjuvant or administration of multiple vaccine doses.

Therefore, it can be expected that a vaccine based on rationally-designed replication-competent recombinant virus vectors will be equally immunogenic and effective against HIV-1 infection. Indeed, replication-competent recombinant viruses have been pursued extensively in developing an effective vaccine against HIV[272,403], with pre-clinical data showing that such vaccines can protect against intra-vaginal challenge with highly pathogenic SIV[297,436,469].

Chapters 4 and 5 of this thesis described a novel strategy to engineer a replication-competent, genetically stable, recombinant human rhinovirus vaccine vector (rHRV) encoding the HIV Gag and Tat proteins, similar to recombinant Sabin polioviruses described previously[468,469].

The strategy involved inserting the exogenous HIV *gag/tat* genes into the P1/P2 junction of the HRV genome to ensure that the proteins encoded by the exogenous HIV genes were cleaved from the recombinant viral polyprotein by the HRV-A1 2Apro enzyme, and that the resultant recombinant viruses (rHRV-Gag1-5 or rHRV-Tat) were genetically stable and retained replication capability. In an effort to recapitulate findings from Crotty et al [469], albeit in a different animal model, the *gag* gene was subsequently divided into 5 discrete overlapping fragments as described in chapters 4 and 5. Aside from this cloning strategy, full length Gag could have been divided into 3 discrete fragments corresponding to P17/24/6 using the natural cleavage sites within this protein. Thereafter, each of these fragments would be separately cloned into HRV-A1 genome as described in Chapters 4 and 5. This strategy was not considered over fears that dividing the full length Gag protein into P17/24/6 would generate gene fragments whose sizes exceeded the cloning capacity of HRV-A1. As a result, rHRVs encoding these individual proteins would be genetically unstable. Nevertheless, this

strategy and that recently described by Tan et al.[323] might be investigated in future for the production of rHRV-Gag/Tat.

HRV serotype A1 (HRV-A1) used in the study is considered to be the rarest HRV serotype detected in humans[475,509], and thus unlikely to be affected by pre-existing immunity, when used as a vaccine vector, a major concern for a number of viral vectors summarised in Table 2. Pre-existing vector immunity resulted in the ineffectiveness of two large scale HIV vaccine clinical trials reported previously[139]. HRV-A1 is transmitted via the nasal mucosa[477], which implies that it can be used to deliver mucosally-targeted vaccines via the IN route. Since there is no cross-neutralization between different serotypes[473], vaccines delivered by HRV-A1 would still be effective in individuals with immunity to other HRV serotypes.

Beyond use as a vaccine vector, HRV-A1 can be engineered following the methodology described in chapter 5 of this thesis to encode a fluorescent protein such as luciferase that might enable the identification of HRV-infected bronchial epithelial cells when studying upper respiratory infections such as chronic obstructive pulmonary disease and asthma in a natural (non-genetically modified) mouse model. Traditionally, these debilitating infections have been studied using humanised mouse models[505,550] which are unable to support chronic HRV infection. The use of HRV-A1 which naturally infects and replicates in mice[506] might lead to identifying novel treatments targeting COPD and asthma. Alternatively, one might take advantage of the Tat transcription activity by infecting immortalised bronchial epithelial cells encoding a HIV LTR-LUC/GFP with rHRV-Tat. The expression of luciferase or GFP is under the control of the HIV LTR and regulated by Tat. This system would allow detailed investigation of rhinovirus infection in differentiated epithelial cells.

7.2.2 A live recombinant human rhinovirus-based HIV vaccine

The work described in chapter 6 of this thesis attempted to develop a HIV vaccine that can be administered via the intranasal route to induce pan-mucosal and systemic HIV-specific immunity. After generating a series of rHRVs encoding fragments of the HIV *gag* gene or the full length *tat* gene as described in chapters 4 and 5 of this thesis, these were mixed into a single cocktail vaccine (rHRV-Gag/Tat). The immunogenicity of the rHRV-Gag/Tat vaccine was tested in mice using a heterologous prime-boost strategy (rHRV-DNA) which was shown previously to elicit superior immune responses (in terms of magnitude and poly-functionality) compared to homologous prime-boost vaccination[551].

The data confirmed that robust HIV-specific mucosal and systemic immunity can be induced following IN vaccination, as suggested previously[314,323], providing further support to consider IN as a cost-effective, convenient and highly efficient strategy to elicit high quality anti-HIV immunity at the mucosa and systemically. It is also possible that the rHRV-Gag/Tat vaccine administered in a rHRV-DNA regimen may be superior to the Sabin poliovirus-based HIV vaccines [469] in terms of immunogenicity and HIV control. However, this premise can only be confirmed once the rHRV-Gag/Tat vaccine is tested in macaque models of HIV infection (SIV or SHIV). These studies are planned for the future.

7.3 Pre-clinical evaluation of potential HIV vaccine efficacy using the EcoHIV challenge model

Macaques are considered the optimum animal model to test HIV vaccine efficacy[285,552] as SIV or SHIV challenge can be conducted via the vaginal-rectal- as well as systemic- routes to result in an infection reminiscent of a natural HIV infection[553,554]. However, macaques are expensive and are subject to stringent ethical considerations which greatly limit their use in pre-clinical trials for candidate HIV vaccines. This necessitates testing these vaccines in a small animal model before using macaques. Moreover, small animal models for HIV infection are limited.

Recombinant vaccinia virus[555] and humanised mice susceptible to HIV infection[556] have been used previously to test HIV vaccine efficacy. However, these models do not accurately mimic natural HIV replication and infection, and can only be handled in a PC-3 laboratory[557,558]. Pigs represent an alternative large animal model to evaluate the immunogenicity of HIV vaccines. The pig's organ function and immune system is similar to humans and their skin is suitable for intradermal vaccination[559], making the pig an attractive model for pre-clinical evaluation of HIV vaccines. Previously, Grubor-Bauk et al used the pig model to evaluate the immunogenicity of a candidate HCV vaccine[381]. Therefore, it would be interesting to examine the immunogenicity of the innovative candidate HIV vaccines described in this thesis in an out-bred pig model. Results from these experiments might influence the use of pigs as an alternative cost-effective model to evaluate the immunogenicity of candidate HIV vaccines.

The data reported in chapters 3-6 of this thesis were generated in mice, a cheap and readily accessible small animal model. Unfortunately, HIV does not infect mice, thus murine experiments generally rely on a surrogate murine HIV challenge model, EcoHIV[377,378,527,560,561], to assess vaccine efficacy. Since a majority of HIV transmission result from heterosexual transmission[2], it is preferable that the EcoHIV challenge is conducted via the intravaginal (IV) route to closely mimic a natural HIV infection, although this route has not been described in the literature. Consequently, the intraperitoneal (IP) was preferred to the IV route for convenience and the former has been used successfully by different research groups[377,378,560,561] to deliver EcoHIV. Furthermore, a single IP challenge results in an EcoHIV infection that spreads from the primary infection site to the spleen, the brain and in peripheral blood in a manner reminiscent of HIV infection[527].

Unfortunately, neither pVAX-sTat-IMX313 nor rHRV-Gag/Tat completely blocked or cleared EcoHIV infection. It is plausible that the vaccine-induced immunity might have been overwhelmed by the high dose of EcoHIV used for challenge. The challenge dose is

traditionally based on the amount of p24, which is considered a poor predictor of infectious titer at least with HIV infection[562]. To the best of our knowledge, there is no published correlation between P24 levels and EcoHIV titer, thus it is possible that the challenge dose used (1.5 μg of p24) might represent an extremely high EcoHIV titer (infectious units). In which case, it is encouraging to note that pVAX-sTat-IMX313 and rHRV-Gag/Tat showed efficacy against this unknown, but likely high dose challenge. Along the same vein, the EcoHIV titer (infectious units) that directly corresponds to that of HIV is currently unknown. Thus, it might not be surprising that the unknown EcoHIV challenge dose used in these studies is higher than that of HIV in humans and therefore, one might expect to observe a similar level of virologic control, if pVAX-sTat-IMX313 and/or rHRV-Gag/Tat are tested against SIV/SHIV in macaques or HIV in humans.

Furthermore, recent evidence suggests that EcoHIV infection is transmissible from an infected female mouse to a healthy male following sexual contact[563], as seen in a natural HIV infection. This evidence of heterosexual EcoHIV transmission highlights an additional benefit of using EcoHIV during pre-clinical testing of candidate HIV vaccines in a cost-effective mouse models before probably using the more expensive macaques. Furthermore, EcoHIV is simple to produce and infects only murine lymphocytes[527]. Therefore, it can be handled in a PC2 facility without concerns over human-related infections.

7.4 Limitations and future studies

The data presented in this thesis support further testing of the pVAX-sTat-IMX313 and rHRV-Gag/Tat vaccines, preferably in a heterologous prime-boost vaccination strategy in macaques. The results from these proposed studies will represent a watershed for the future evaluation of these vaccines in human clinical trials. However, due to unavoidable constraints, certain key aspects related to the use of and the resultant immunity generated after rHRV-Gag/Tat or pVAX-sTat-IMX313 vaccination were not investigated in this thesis.

Owing to time constraints, long-term EcoHIV challenge experiments involving measuring the EcoHIV viral load 6-8 weeks post challenge were not conducted. These would have provided valuable information on the longevity of the vaccine-induced immunity and virologic control. Similarly, vaccine-induced mucosal and systemic immune responses 3-4 months post vaccination were not evaluated, although such information would have demonstrated that the immunogenicity and efficacy of the vaccines developed in this thesis did not wane overtime. Therefore, it will be important to demonstrate that this desirable immunological characteristic is achieved in future studies.

It would be expected that intranasal administration of 2 doses of rHRV-Gag/Tat would induce HRV-specific immune responses which might reduce the immunogenicity of dose 2. However, the data suggested that a second rHRV-Gag/Tat dose, rather than being detrimental, was actually beneficial as it improved the overall Gag/Tat-specific humoral and CMI responses (Chapter 6 section 6.5). In fact, Crotty et al. 2001 previously showed that administering up to 6 doses of recombinant Sabin poliovirus encoding SIV proteins via the intranasal route enhanced vaccine immunogenicity and resulted in sterilizing protection against intra-rectal challenge with SIVmac251[469]. It is possible that anti-HRV-specific antibodies were indeed induced by the first dose, but it is likely that the rHRV-Gag/Tat dose ($5x10^6$TCID50/dose) was sufficiently high to overwhelm HRV-specific immunity, without the effectiveness of the second dose, as noted previously with Adenovirus-based vaccines delivered via the intranasal route[318]. Nonetheless, an experiment involving a single administration of rHRV-Gag/Tat to clearly address this concern, which was not done due to time and resource limitations, should be performed as a priority.

Data presented in Chapter 6 demonstrated that rHRV-Gag/Tat induced Gag-specific CMI in mesenteric lymph nodes and Tat-specific sIgA in the vagina using a rHRV-DNA vaccination regimen suggesting that this strategy has the potential to elicit anti-HIV immunity at the

mucosa. Experiments which were not possible within the time frame of this thesis, would have been necessary to examine tissue-specific Gag/Tat CMI in the rectum, iliac lymph nodes draining the female reproductive system and Peyer's patches in the gut, to comprehensively interrogate mucosal immunity upon rHRV-Gag/Tat vaccination. Similarly, it is desired that anti-HIV immunity at the mucosa is sustained over time for effective HIV prevention. However, due to time constraints, long-term vaccination studies to unravel the longevity of vaccine-induced mucosal responses would not be performed.

As the correlates of protection or virologic control against HIV-1 infection remain poorly understood[161], it is vital to clearly identify these correlates in animals or humans showing protection from virus challenge or sustained virologic control post-infection. Therefore, there is need to fully understand the correlates that were responsible for the reduction of the EcoHIV viral load post-challenge in vaccinated animals. Unfortunately, owing to the small number of animals used in the challenge experiments, meaningful analysis to identify these correlates was not possible. However, the data appeared to suggest that humoral immunity as well as CMI might have contributed to EcoHIV control. Future studies involving the depletion of $CD4^+$ and/or $CD8^+$ T and B cells using anti-CD4, anti-CD8 (singly or in combination) and anti-CD20 antibodies prior to and after EcoHIV challenge may define the correlates that were associated with virologic control.

It is also important to investigate in detail the functionality of anti-Tat antibodies in terms of inhibiting HIV-1 replication, considered vital to control the viral load and slow disease progress[17,222]. The *in vitro* luciferase-based Tat transactivation neutralization assay was used to indirectly demonstrate this desired antibody activity. To complement the *in vitro* Tat transactivation neutralization assay, future studies might consider using an assay that directly quantifies inhibition of virus replication in the presence of Tat-specific antibodies, as described previously[564]. This assay was not performed in the studies described in this thesis

due to limited blood volume that could be safely collected from mice. Similarly, it is important to investigate whether the Tat-specific antibodies elicited by the rHRV-Gag/Tat or pVAX-sTat-IMX313 vaccine possessed ADCC activity, as reported in other studies that tested a Tat-based candidate HIV vaccine previously[169,565]. Antibodies with ADCC activity are thought to have contributed to HIV control in vaccinated individuals in the RV144 trial[142,173], suggesting that the presence of such antibodies might be beneficial in HIV control.

A major caveat for using HRV-A1 as a vaccine vector for mucosally-targeted vaccines is the fact that the natural receptors for a replication-competent HRV-A1 are located in the nasal epithelium, which implies that vaccines, such as rHRV-Gag/Tat, based on this live vector can only be delivered intranasally. Although this thesis provides the first proof-of-concept that a live HRV-A1 can effectively deliver a mucosal vaccine when administered intranasally, it would have been useful, time permitting, to explore other mucosal and systemic routes of administration and different heterologous prime-boost vaccination regimens which might have enhanced its mucosal efficacy. In light of this caveat, alternative mucosal vaccination routes such as oral, rectal and intra-vaginal as well as systemic routes such as intra-muscular or intradermal vaccination were not be performed. To the best of my knowledge, there is no literature indicating that a live HRV-A1 vector can be administered via routes other than intranasal. Overall, these above concerns and points can be investigated conclusively and more comprehensively in macaque studies planned in the future.

7.5 Conclusions

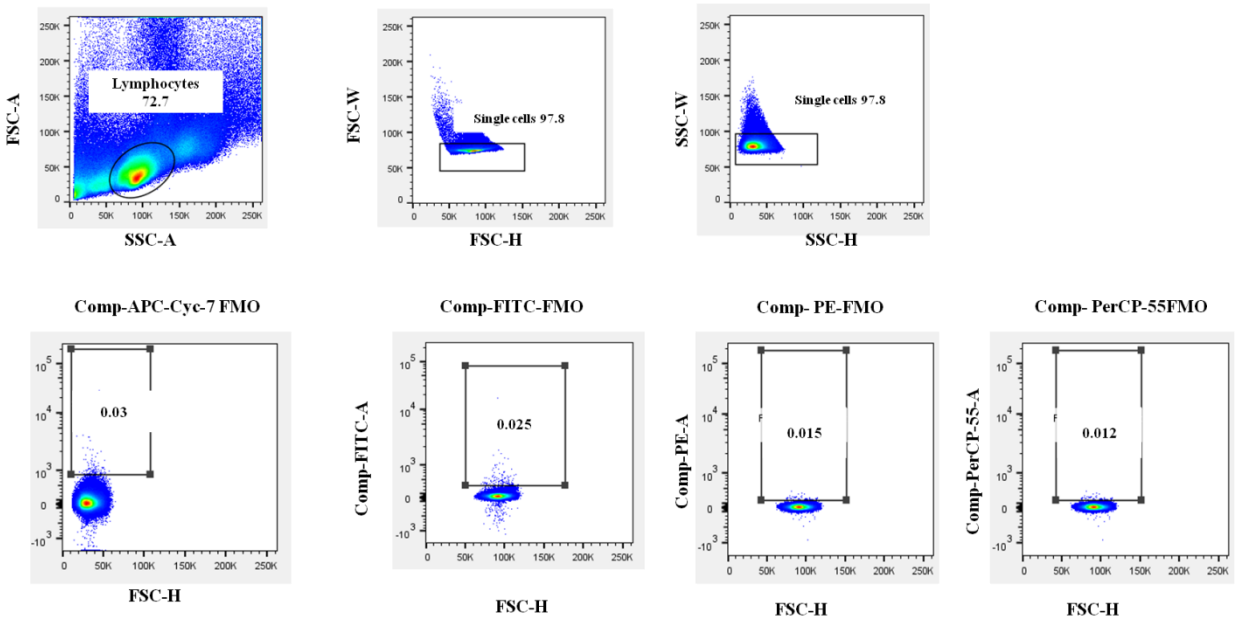
To the best of my knowledge, this thesis provided the first evidence of the use of IMX313 as a molecular adjuvant to enhance the immunogenicity of a Tat-based HIV DNA vaccine. The thesis also provided the first report of engineering HRV-A1 into a live vaccine vector and using recombinant HRVs as candidate HIV vaccines that can induce pan-mucosal and systemic immunity following intranasal vaccination. In summary, the DNA and live virus-

based HIV vaccines described in this thesis have the potential to be considered for future development of a cost-effective, safe and highly effective vaccine against HIV-1 that can be accessed globally, but most importantly in the resource-limited Sub Saharan Africa and South East Asia, where HIV is endemic.

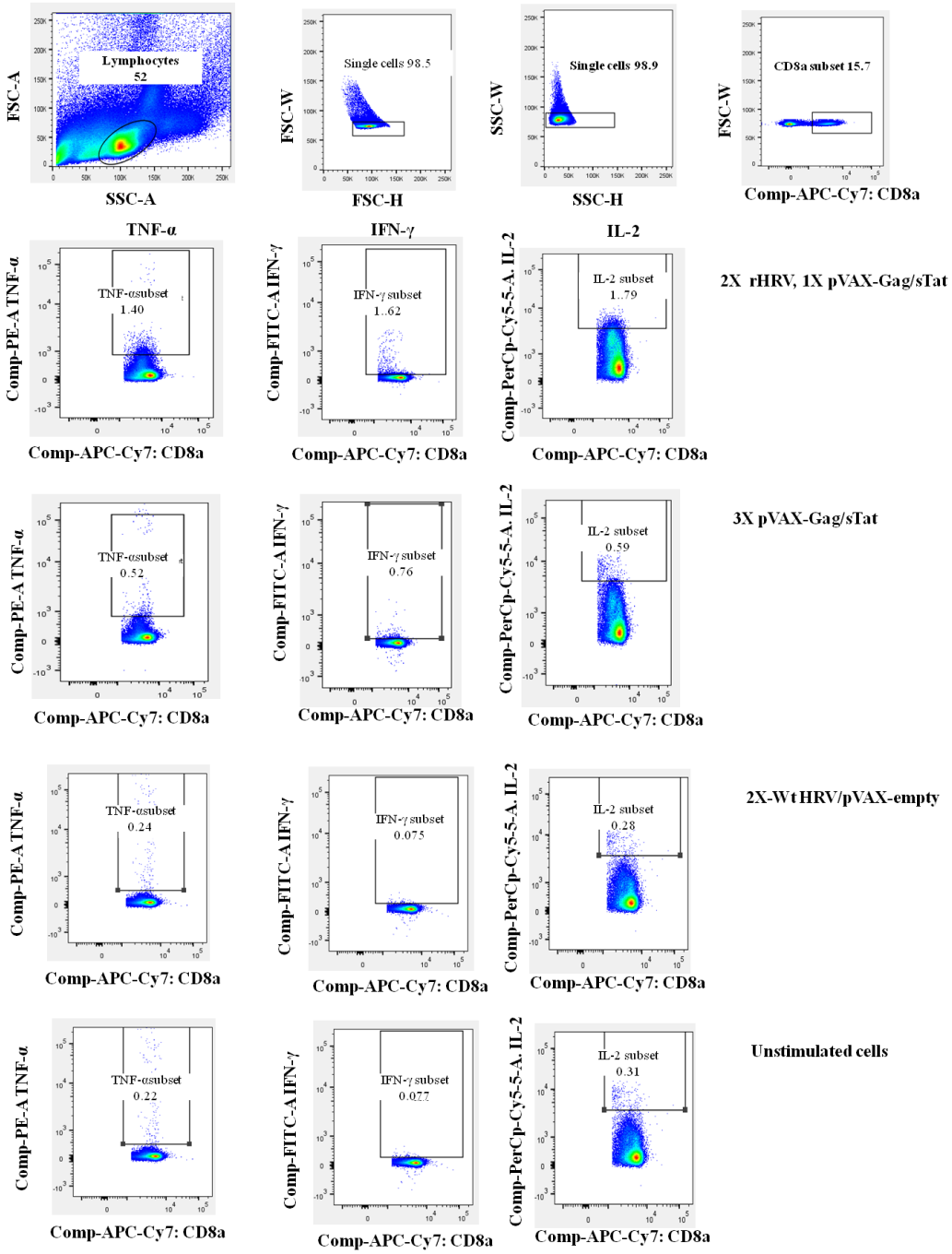
8.0: Appendices

1. Gating strategy to identify various cell population producing target cytokines in the Intracellular cytokines staining assay or staining positive with H-2K^{d}-restricted Gag$_{197\text{-}205}$ peptide. *For results presented in chapter 6.*

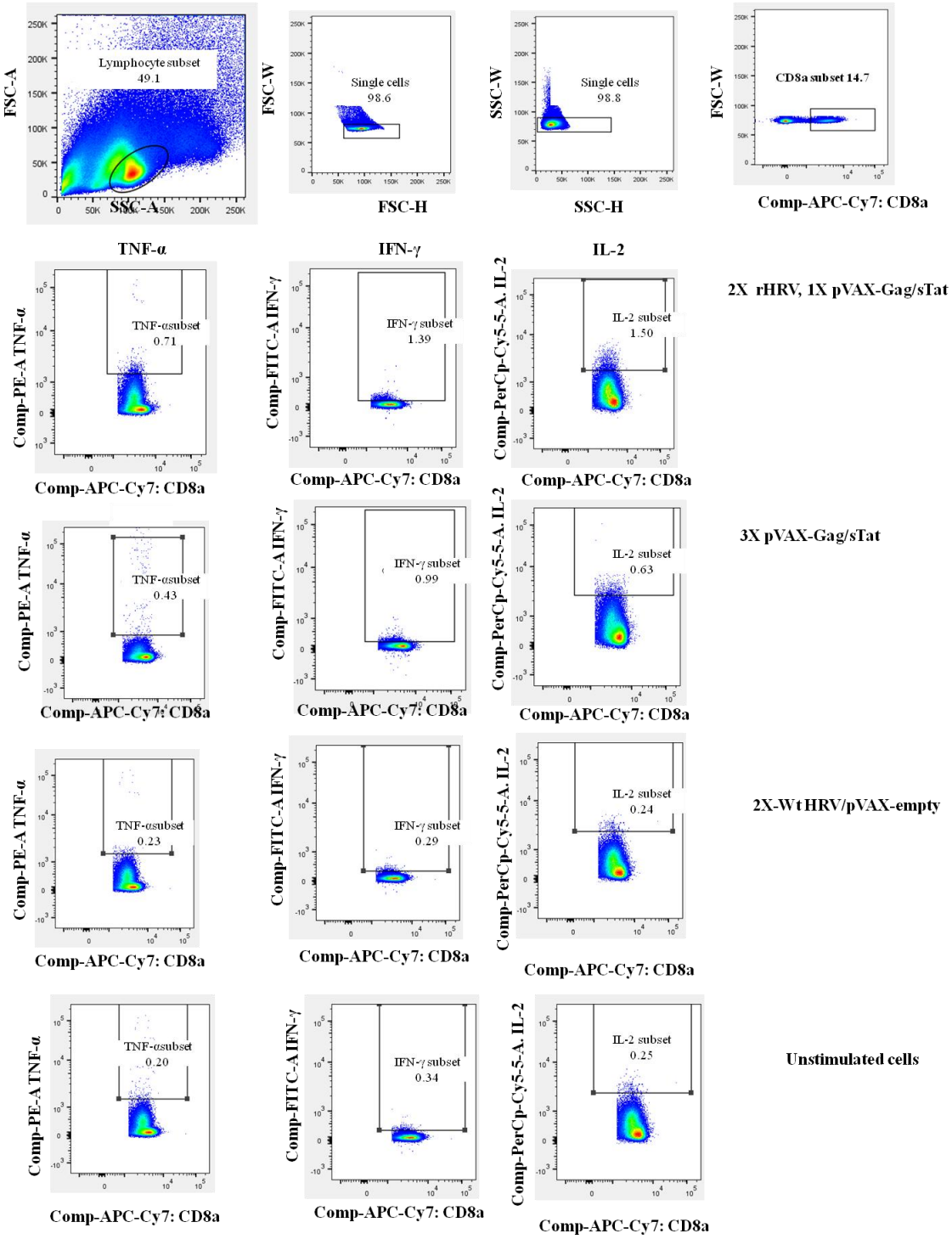
A)


Lymphocytes
72.7
FSC-A
SSC-A
Single cells 97.8
FSC-W
FSC-H
Single cells 97.8
SSC-W
SSC-H
Comp-APC-Cyc-7 FMO
0.03
FSC-H
Comp-FITC-FMO
0.025
Comp-FITC-A
FSC-H
Comp- PE-FMO
0.015
Comp-PE-A
FSC-H
Comp- PerCP-55FMO
0.012
Comp-PerCP-55-A
FSC-H

B)


FSC-A
SSC-A
Lymphocytes
52
FSC-W
FSC-H
Single cells 98.5
SSC-W
SSC-H
Single cells 98.9
FSC-W
Comp-APC-Cy7: CD8a
CD8a subset 15.7
TNF-α
IFN-γ
IL-2
Comp-PE-A TNF-α
Comp-FITC-A IFN-γ
Comp-PerCp-Cy5-5-A. IL-2
TNF-αsubset
1.40
IFN-γ subset
1..62
IL-2 subset
1..79
2X rHRV, 1X pVAX-Gag/sTat
TNF-αsubset
0.52
IFN-γ subset
0.76
IL-2 subset
0.59
3X pVAX-Gag/sTat
TNF-αsubset
0.24
IFN-γ subset
0.075
IL-2 subset
0.28
2X-Wt HRV/pVAX-empty
TNF-αsubset
0.22
IFN-γ subset
0.077
IL-2 subset
0.31
Unstimulated cells

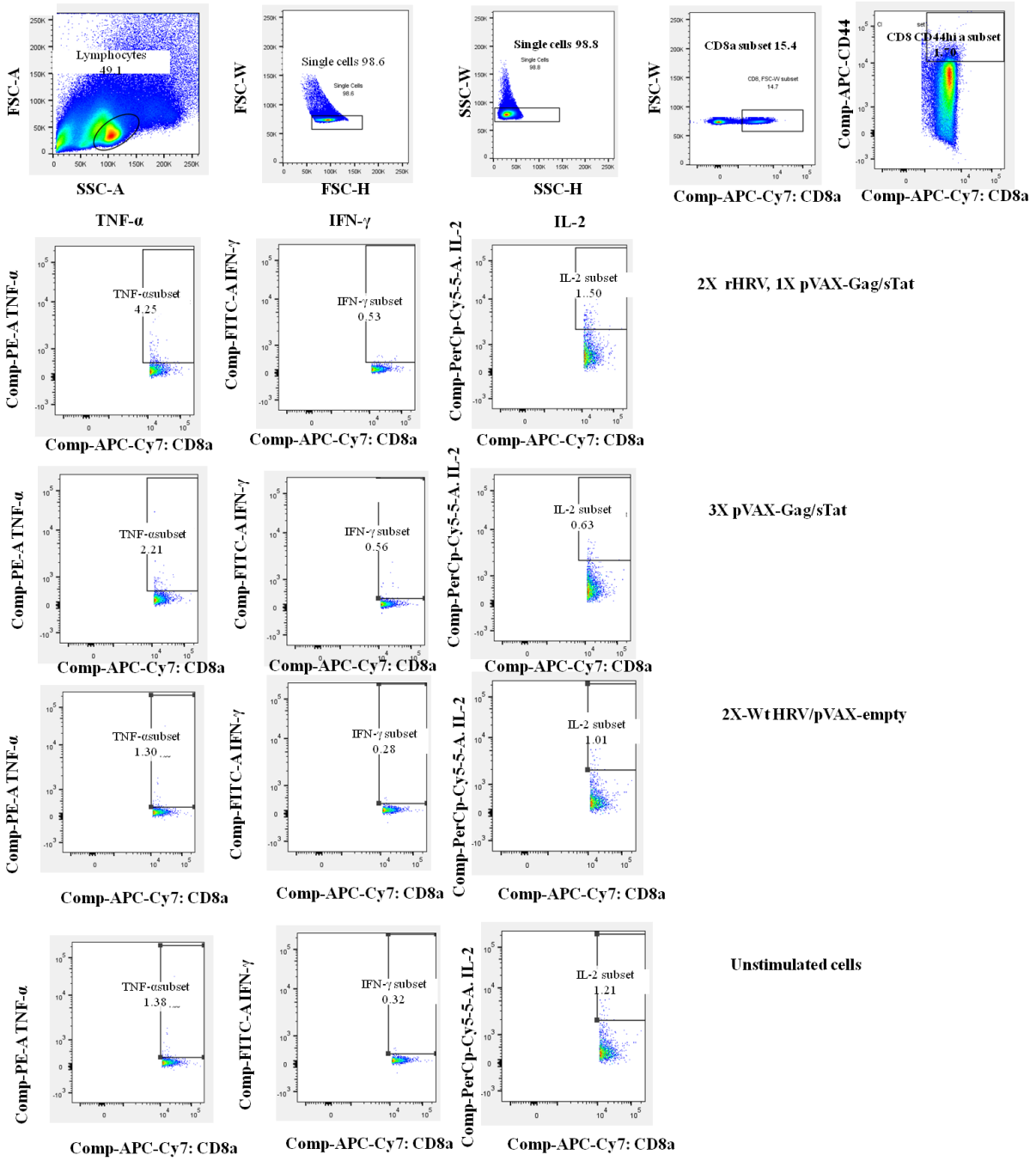
C)

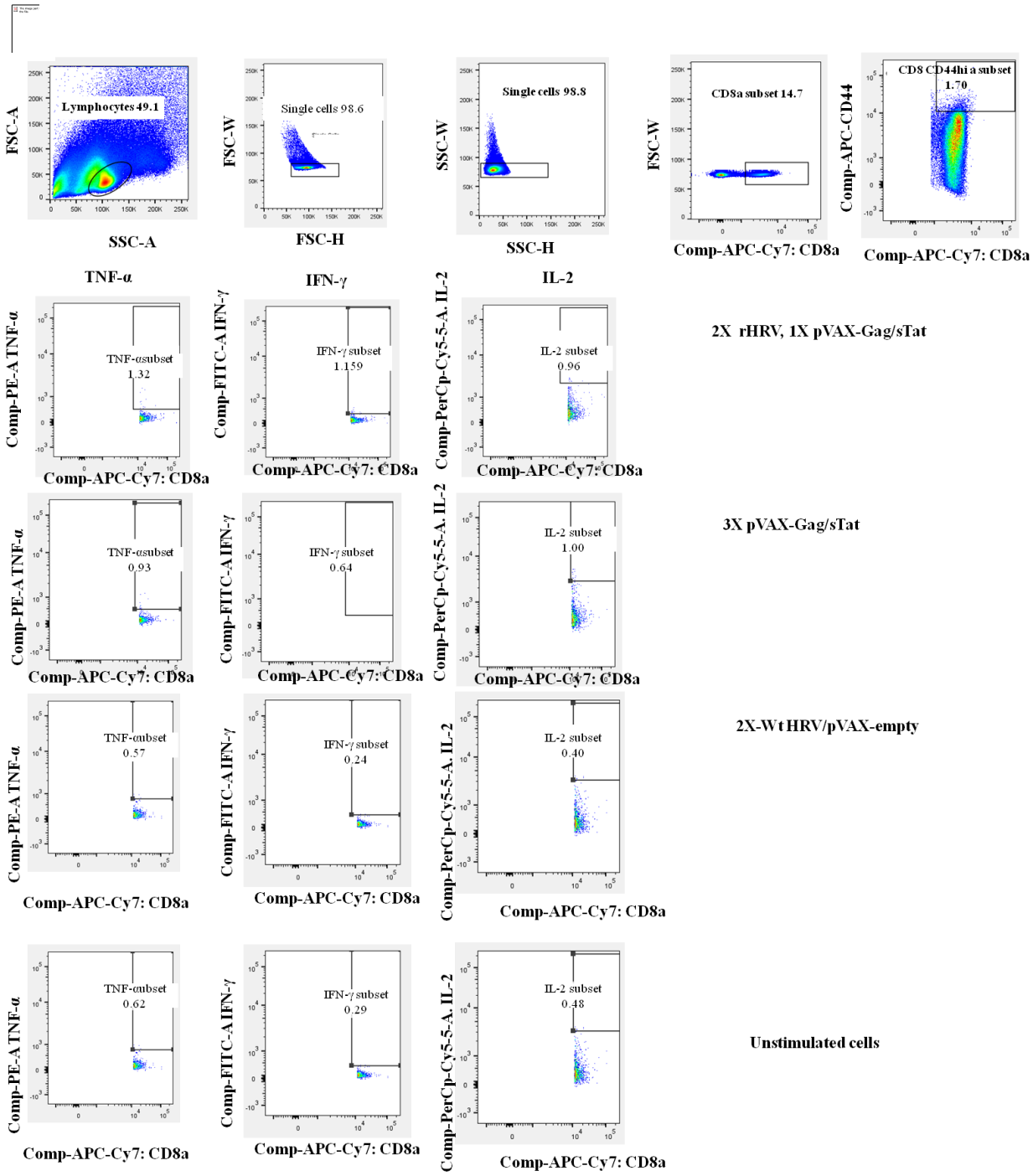


Appendix Figure 1. Fluorochrome minus one (FMO) control for each flourochrome (platform A) and gating strategy used to detect $CD8^{+}$T cells producing multiple cytokines in the splenocyte (platform B) and mesenteric lymphocyte (platform C) populations of mice vaccinated as described in the legend for Figure 1, chapter 6. Splenocytes and mesenteric lymphocytes were harvested on day 14 after the final vaccination for ICS. Cytokine profiles

were determined by using flow cytometry. Splenocytes and mesenteric lymphocytes were gated on the lymphocyte population, followed by doublet discrimination, and then gated on $CD8^+$ T cells to assess the frequency of IFN-γ, TNF-α and IL-2. Representative plots for IFN-γ, TNF-α and IL-2 positive cells are shown.

A)

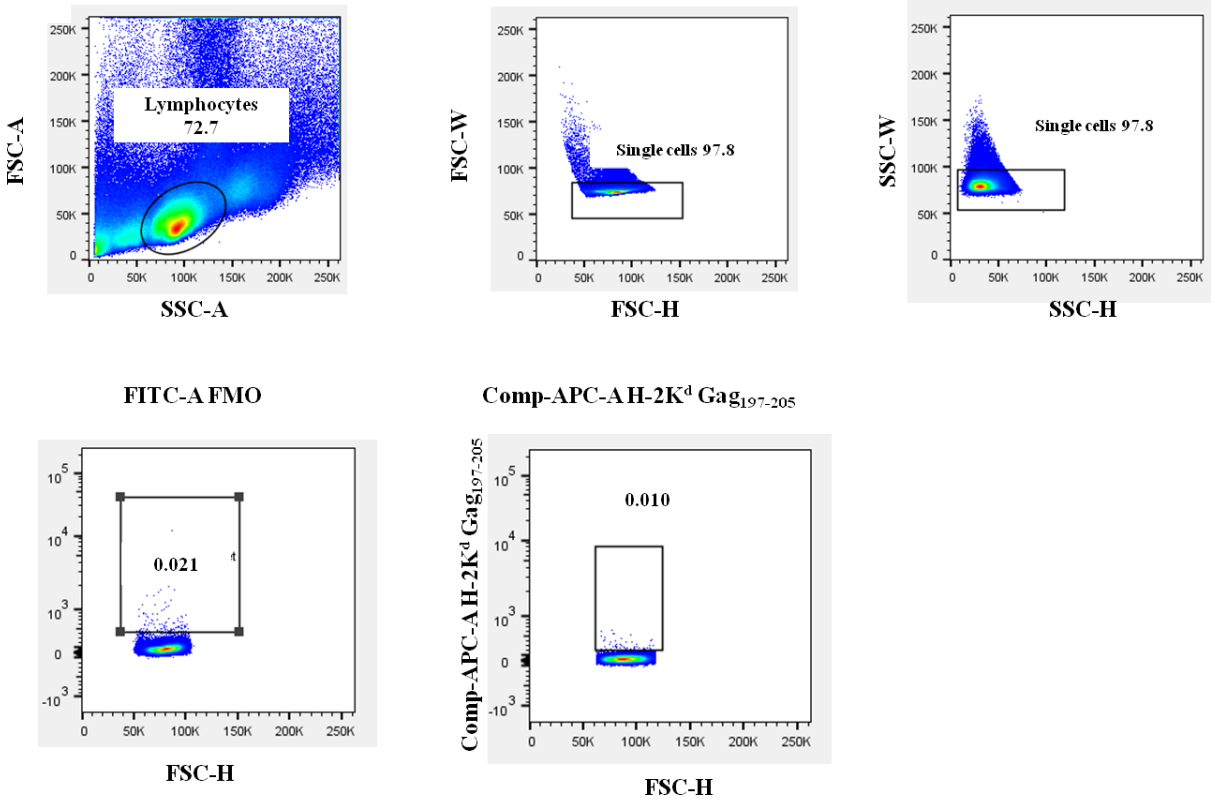




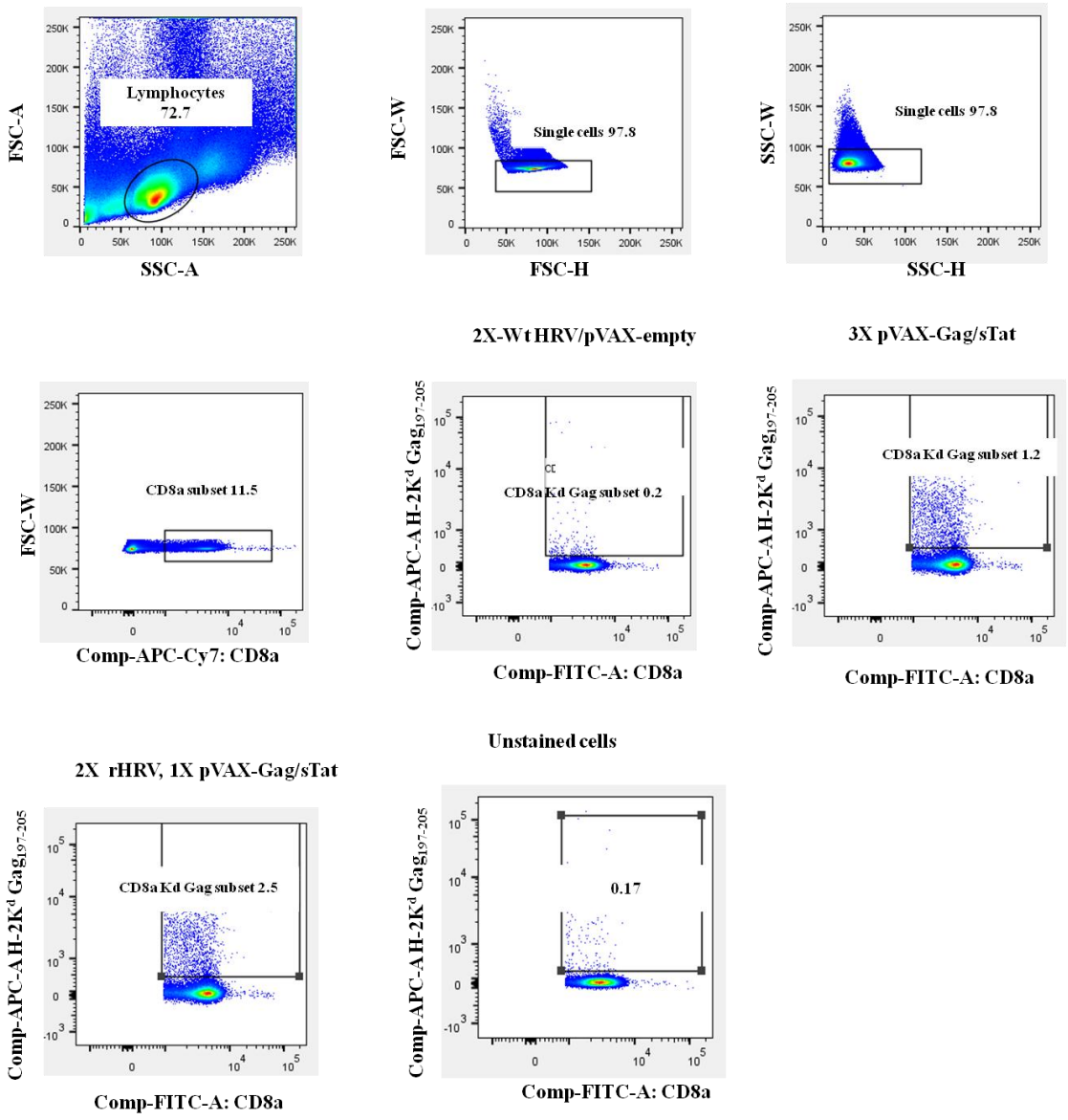
Appendix Figure 2. Gating strategy used to detect effector memory multifunctional $CD8^+$ T cells in the splenocyte (platform A) and mesenteric lymphocyte (platform B) populations of mice vaccinated as described in the legend for Figure 1, chapter 6. Splenocytes and mesenteric lymphocytes were harvested on day 14 after the final vaccination for ICS. Cytokine profiles were determined by using flow cytometry. Splenocytes and mesenteric lymphocytes were gated on the lymphocyte population, followed by doublet discrimination,

and then gated on $CD44^{hi}CD8^{+}$T cells to assess the frequency of IFN-γ, TNF-α and IL-2. Representative plots for IFN-γ, TNF-α and IL-2 positive cells are shown.

A)



B)



Appendix Figure 3. Gating strategy used to detect $CD8^+$ T cells staining positive with the H-$2K^d$-restricted $Gag_{197\text{-}205}$ peptide in the splenocyte populations of mice vaccinated as described in the legend for Figure 1, chapter 6. Splenocytes were harvested on day 14 after the final vaccination for tetramer staining. Tetramer positive cells were determined by using flow cytometry. Splenocytes were gated on the lymphocyte population, followed by doublet discrimination, and then gated on $CD8^+$T to assess the frequency of H-$2K^d$-restricted $Gag_{197\text{-}205}$ positive cells. A) Fluorescent minus one (FMO) control for each fluorochrome. B) representative plots for H-$2K^d$-restricted $Gag_{197\text{-}205}$ positive cells is shown.

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