
# Optimizing a Universal Mosaic T cell vaccine for HIV-1

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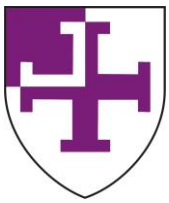

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# Abstract

The development of an effective human immunodeficiency virus type 1 (HIV-1) vaccine is the best solution to the global HIV-1 pandemic. This has proven difficult due to the high mutation rate and genetic diversity of the virus. One way to overcome this is for a T-cell vaccine to target highly functional conserved regions of HIV-1 across all clades, mutations would typically incur a cost on viral fitness. Our first conserved vaccine, HIVconsv, is a clade alternating consensus immunogen comprised of 14 conserved regions across the HIV-1 proteome. HIVconsv showed very promising results in mice and human clinical trials, inducing HIV-1 specific $CD8^+$ T cells of high magnitude and breadth.

In my work, four conserved mosaic immunogens were designed *in-silico*, to maximize the coverage of potential 9-mer T cell epitopes. The aim was to develop a new conserved mosaic vaccine to enhance the breadth and depth of the vaccine elicited responses. The first, tHIVcmo, designed to complement tHIVconsv and tConsv1, tConsv2 and tConsv3 were designed to complement each other in a trivalent vaccine. The immunogens were expressed from a plasmid DNA (D), ChAdV63 (C) and a MVA (M) vector, used in heterologous prime boost regimens DDDCM and CM. HHD transgenic mice, expressing a chimeric human HLA-A*02:01 were used to asses the breadth and depth of vaccine elicited T cells. I assessed whether or not combining tHIVcmo to tHIVconsv had any benefits, and compared this bivalent combination to the trivalent conserved mosaic vaccines. Over 70 known human HLA-A*02:01 epitopes of HIV-1 across major clades where used for analysis. The trivalent mosaic vaccines elicited T cell responses with greater breadth, depth, magnitude and killing efficacy compared to that of the bivalent or HIVconsv elicited responses. These are the first data on mosaic vaccines with regards to known human epitopes, using a model with a human HLA molecule and the first conserved mosaic vaccines tested.

In a separate series of experiments, the effects of the tPA leader sequence were examined, comparing HIVconsv with and without tPA. Experiments in BALB/c mice have revealed that the addition of a tPA leader sequence had no added benefit on our T cell vaccine. Specificity, magnitude, breadth, depth and killing efficacy were all not affected by the addition of tPA to the vaccine.

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# Abbreviations

Ab: Antibody

Ad: Human adenoviruses

Ad5: Human adenovirus serotype 5

ADCC: Antibody dependent cellular cytotoxicity

ADCP: Antibody dependent cellular phagocytosis

ADCVI: Antibody dependent cell mediated virus inhibition

Ag: Antigen

AIDS: Acquired Immune Deficiency Syndrome

APC: Antigen presenting cells

APOBEC3G: Apolipoprotein B mRNA-editing enzyme catalytic polypeptide like 3G

ASK1: Apoptosis signal regulating kinse-1

BGH poly-A: Bovine growth hormone polyadenylation signal

BiP: Binding immunoglobulin protein

bNAbs: Broadly neutralising antibodies

CA: Gag capsid

CAR: Coxsackie and adenovirus receptor

CCL: Chemokine (c-c motif) ligand

CCR: C-C (motif) chemokine receptor

CD4bs: CD4 binding site

CDR: Complementarity determining regions

ChAdV63: Chimpanzee adenovirus serotype 63

CM: ChAdV63-prime MVA-boost regimen

CMV-IE: Cytomegalovirus immediate early promoter

CMV: Cytomegalovirus

COT: Centre-of-tree sequence

CRT: Calreticulin

CTL: Cytotoxic T lymphocytes

CTLA-4: Cytotoxic T-lymphocyte associated protein 4

CXCR4: C-X-C (motif) chemokine receptor 4

DC-SIGN: DC-specific ICAM3-grabbing non-integrin

DC: Dendritic cells

DDDCM: DNA, DNA, DNA-prime, ChAdV63 and MVA-boost regimen

DNA: Deoxyribonucleic acid

DOM: Domain sequence

dsDNA: Double stranded DNA

E.coli: *Escherichia coli*

ER: Endoplasmic reticulum

ERAP: Endoplasmic reticulum aminopeptidase

ESCRT: Endosomal sorting complexes required for transport

FasL: Fas ligand

FcγRs: Fc receptor

FrC: Fragment C of tetanus toxin

FSW: Female sex workers

GA: Golgi apparatus

GALT: Gut-associated lymphoid tissue

GEM: Germline-encoded mycolyl-reactive T cells

GLP: Good Laboratory Practice

GM-CSF: Granulocyte-macrophage colony stimulating factor

gp120: Envelope glycoprotein 120

gp160: Envelope precursor glycoprotein

gp41: Envelope glycoprotein 41

HAART: Highly active antiretroviral therapy

HCV: Hepatitis C virus

HEK-293: Human embryonic kidney-293 cells

HIV: Human immunodeficiency virus

HIVconsv: HIV conserved vaccine immunogen

HLA: Human leukocyte antigen

HTLV-III: Human T-cell leukemia virus III

i.m.: intra-muscular immunisation route

IDO: Indoleamine 2,3-dioxygenase

IDU: Injection drug use

IFN-α: Interferon alpha, a type I interferon cytokine

IFN-γ: Interferon gamma cytokine

Ig: Immunoglobulin

IL: Interleukin cytokines

ILT: Immunoglobulin-like transcript

INI: Integrase inhibitors

iNKT: Invariant natural killer T cells

InSTI: Integrase strand transfer inhibitors

IRAP: Insulin-responsive aminopeptidase

ITAM: Immuno-receptor tyrosine activation motif

ITIM: Immuno-receptor tyrosine inhibition motif

Kan-R: Kanamycin resistance gene

KIR: Killer immunoglobulin-like receptor

LTNP: Long term non-progressors

LTR: Long terminal repeats

M-CSF: Macrophage colony stimulating factor

M: Main group, HIV-1 subtype

M1: Pro-inflammatory activated macrophages

M2: Anti-inflammatory activated macrophages

MA: Gag matrix protein

mAb: Monoclonal antibody

MAIT: Mucosal associated invariant T cells

mDCs: Myeloid dendritic cells

MFI: Median fluorescence intensities

MHC: Major histocompatibility complex

MIC A: MHC class I-related chain

MIP-1α: Macrophage inflammatory protein-1alpha

MIP-1β: Macrophage inflammatory protein-1beta

MOI: Multiplicity of infection

MPER: Membrane proximal external region

MRCA: Most recent common ancestor

mRNA: Messenger RNA

MSM: Men who have sex with men

MVA: Modified vaccine Ankara

N: Non-M non-O group, HIV-1 subtype

NAbs: Neutralising antibodies

NC: Nucleocapsid protein

NF-κB: Nuclear factor kappa B

NK: Natural killer cells

NKG2/CD94: Killer C-type lectin-like receptors

NKT: NK T cells

NLRs: NOD like receptors

NNRTI: Non-nucleoside reverse transcriptase inhibitors

NOD: Nucleotide oligomerization domain

NP: Nucleoprotein

NRTI: Nucleoside/nucleotide analogue reverse transcriptase inhibitor

O: Outlier group, HIV-1 subtype

P: Putative group, HIV-1 subtype

PBMC: Peripheral blood mononuclear cells

PD-L1: Programmed death ligand 1

pDCs: Plasmacytoid dendritic cells

PIC: Preintegration complex

PLC: Peptide loading complex

PR: Protease

Pr160: 160 kDa Gag-Pol precursor polyprotein

Pr55: Gag 55 kDa precursor protein

PTE: Potential T cell epitopes

RIG-1: Retinoic acid-inducible gene 1

RLRs: RIG-1 like receptors

RNA: Ribonucleic acid

RRE: Rev responsive element

SAMHD1: Sterile alpha motif domain and HD (histidine/aspartic acid domain) containing protein 1

SFU: Spots forming units

SHIV: Simian-human immunodeficiency virus

SIV: Simian immunodeficiency virus

SIVcpz*Ptt*: Simian immunodeficiency virus chimpanzee subspecies *Pan troglodytes troglodytes*

SIVgor: Simian immunodeficiency virus Gorilla

SIVmac: Simian immunodeficiency virus Macaques

SIVsmm: Simian immunodeficiency virus Sooty Mangabey

SOCS3: Suppressor of cytokine signaling-3

TAP: Transporter associated pathway

tConsv1/2/3: tPA- Conserved mosaic 1 or 2 or 3

TCR: T cell receptor

$T_{EM}$ cell: Effector memory T cells

Tfh: Follicular helper T cells

TGF-β: Transforming growth factor beta

Th: T helper cells

Th17: T helper 17 cells

tHIVcmo: tPA- HIVconsv mosaic immunogen

TLRs: Toll-like receptors

TNF-α: Tumor necrosis factor alpha cytokine

TNF: Tumor necrosis factor

TNFR-2: TNF receptor type 2

tPA: Tissue plasminogen activator

TRAIL: TNF-related apoptosis-inducing ligand

Tregs: Regulatory T cells

TRIM5α: Tripartite motif-containing protein 5 alpha

ULBP: UL-16-binding proteins

V: Variable loop

γδ T-cells: Gamma delta T cells

# Chapter 1:

## *Introduction*

# 1. Introduction

## 1.1. HIV-1

### 1.1.1 Origin and Epidemiology

Acquired Immune Deficiency Syndrome (AIDS) was first recognised in 1981 in the United States of America. It was discovered in a number of young men who have sex with men (MSM), previously healthy, who have succumbed to opportunistic infections i.e. *Pneumocystis carinii* pneumonia, cytomegalovirus, *Candida albicans;* and rare malignancies such as non-Hodgkin's lymphoma and Kaposi's sarcoma (1-6). Epidemiological studies suggested transmission occurred through sexual encounter, intravenous drug use or blood transfusion (1, 2, 6). It was not till 1983 that a retrovirus, human T-cell leukemia virus III (HTLV-III), was first discovered as the causative agent of AIDS, isolated from the lymph node of a MSM patient with multiple lymphadenopathies (7-10). Subsequently in 1986 the name human immunodeficiency virus (HIV) was coined for the AIDS causative agent (11-13).

HIV-1 is a result of a zoonotic transfer of a virus from chimpanzees, specifically simian immunodeficiency virus chimpanzee subspecies *Pan troglodytes troglodytes* (SIVcpz*Ptt*) (14-16). This most likely occurred due to exposure of bushmeat hunting (17). HIV-1 is classed into four distinct lineage groups, main (M), outlier (O), non-M non-O (N), and putative (P) group, each resulting from an independent cross-species crossover transmission event. HIV-1 group M was the first to be discovered and is the most prevalent group worldwide causing the epidemic (18). Group O discovered in 1990 is less prevalent, found in less than 1% of infections and is largely restricted to Cameroon, Gabon and neighbouring countries (19, 20). Group N first discovered in 1998 in Cameroon is very rare with only 13 documented cases (21, 22). Finally group P, first discovered in 2009 in a

Cameroonian woman living in France, has only been identified in one other person in Cameroon (23, 24). HIV-1 group P has been shown to originate from gorillas (SIVgor) (15, 23). Another genetically distinct virus that causes AIDS, HIV-2, first discovered in 1986 and endemic to West Africa only originated from sooty mangabey (SIVsmm) (25, 26). The discovery of different simian immunodeficiency viruses (SIV) in various different sub-Saharan African primates was a clue that HIV-1 and HIV-2 originated from primates (16, 26). Interestingly, these different SIV's are non-pathogenic in their natural primate hosts. The only other primate species to develop AIDS like disease are macaques (Asian primates) from SIVmac. As with humans, macaques are not natural hosts to SIVmac. SIVmac virus was generated accidentally when macaques in US primate centers were inoculated with blood and/or tissue from naturally infected sooty mangabeys (27, 28).

The first cases of AIDS in Africa were described in early 1980s in heterosexual men in equatorial countries including Zaire (DR Congo), Rwanda and Uganda and spread to southern Africa in the late 1980s (29-32). In Latin America and the Caribbean, HIV-1 pandemic started in the late 1970s and early 1980s and associated with MSM and injection drug use (IDU). By the year 2000, the Caribbean had highest prevalence of HIV-1 infected adults outside sub-Sahara Africa (UNAIDS 2001). In Asia, HIV/AIDS was first reported in the mid to late 1980s in Thailand, India and China (33-35). Since the early 1990s, HIV-1 has been highly prevalent in female sex workers (FSW) and IDUs in countries near the "golden triangle", a heavy use drug region where the borders of Thailand, Myanmar and Laos meet (36-38). The epidemic has spread since to more countries in Asia through IDU, heterosexual transmission, MSM contact and mother to child. The burden of the epidemic globally is mostly on low to middle income countries, due to poor socioeconomics, poor health care, high IDU, gender inequality and other factors.

Since the start of the epidemic, roughly 78 million people have been infected with HIV-1 and 39 million people have died of AIDS related illness. In 2013 there were around 35 million people living with HIV, with 2.1 million people newly infected and 1.5 million death due to AIDS related illness worldwide. Since 2001, the rate of new infections in people has fallen by 38% and by 58% in children under the age of 15 whereby transmission is thought to be *in utero* and via breast milk. Furthermore, the rate of AIDS related death globally has fallen by 35% since 2005 (Table 1.1.) (UNAIDS 2013). The burden of the epidemic is in Sub-Saharan Africa, accounting for 70% of people living with HIV and new infections globally. In 2013, 24.7 million people were living with HIV in Sub-Sahara Africa, woman accounting for 58% of infected people, with 1.5 million new infections and 1.1 million deaths (Fig. 1.1). The rate of new infections and AIDS related death in Sub-Saharan Africa has fallen by 33% and 39% respectively since 2005 (UNAIDS 2013). The global decline in new infections and AIDS death is due to better education and access to anti-retroviral drugs.

**Table 1.1.** A global summary of the HIV-1 epidemic in 2013 (modified from UNAIDS, AIDS epidemic update 2013).

| | | |
|---|---|---|
| **People living with HIV** | Total | 35 million (33.2 - 37.2 million) |
| | Adults | 31.8 million (30.1 - 33.7 million) |
| | Women | 16 million (15.2 - 16.9 million) |
| | Children (<15 years) | 3.2 million (2.9 - 3.5 million) |
| **People newly infected with HIV** | Total | 2.1 million (1.9 - 2.4 million) |
| | Adults | 1.9 million (1.7 - 2.1 million) |
| | Children (<15 years) | 240 000 (210 000 - 280 000) |
| **AIDS death** | Total | 1.5 million (1.4 - 1.7 million) |
| | Adults | 1.3 million (1.2 - 1.5 million) |
| | Children (<15 years) | 190 000 (170 000 - 220 000) |

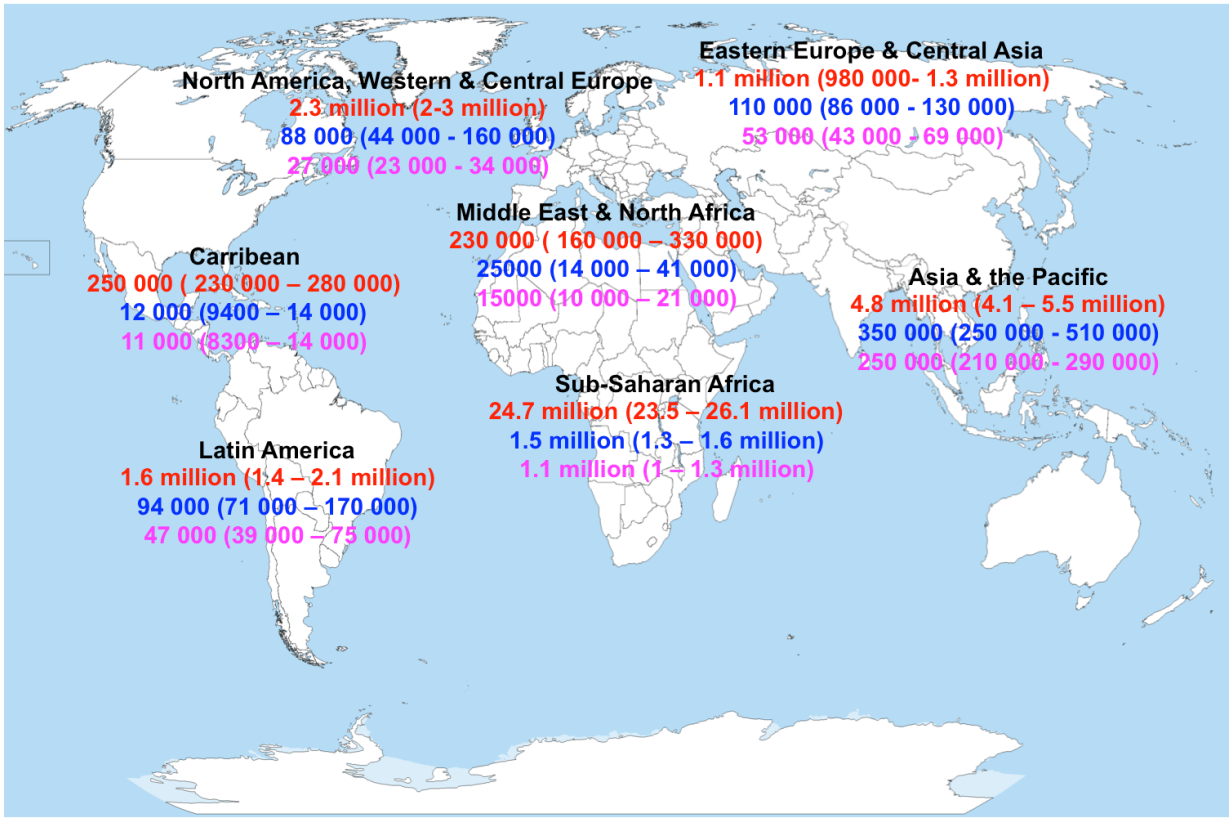



**Figure 1.1. A global summary of the HIV-1 epidemic in 2013 by region.** Global prevalence of people, across all age groups, estimated to be; living with HIV-1 (Red) total 35 million, newly infected with HIV-1 (blue) total 2.1 million and death from AIDS related illness (purple) total 1.5 million people (modified from UNAIDS, AIDS epidemic update 2013).

### 1.1.2 Genome and Structure

HIV-1 is a human retrovirus that belongs to the family *Retroviridea* and the lentivirus subfamily. It is 100 – 120 nm in diameter and has a spherical shape. HIV-1 has two copies of a single stranded ribonucleic acid (RNA) genome, which is reverse transcribed to a double strand deoxyribonucleic acid (DNA) and integrated into the infected cell chromosome. The genome in 9.8 kb in length and has three large open reading frames (Gag, Pol and Env) and six smaller genes (Tat, Rev, Vif, Vpr, Vpu and Nef), encoding nine genes in total (Fig. 1.2) (39-41). The genome is flanked at the 5' and 3' end by long terminal repeats (LTR) containing important regulatory regions for transcription initiation and polyadenelation. The three open reading frames are initially translated as polyprotein precursors encoding structural proteins and enzymes. These polyprotein precursors are processed and cleaved by cellular and viral proteases to produce mature viral proteins (39).

The Gag open reading frame is translated to produce a 55 kDa precursor protein (Pr55) called assemblin, indicative of its role in viral assembly. The Pr55 polyprotein is integrated into the budding virus whereby it is cleaved by viral protease during and after budding of the virus. This produces the myristylated p17 matrix protein, lining the inner envelop of the viral particle; p24 capsid protein, forming the capsid core within the virus containing the RNA genome, enzymes and accessory protein; p7 nucleocapsid, coating the RNA genome within the virus; and p6 protein, involved in the budding of the virus (Fig. 1.2) (39, 40, 42, 43). Pol is translated from a 160 kDa Gag-Pol precursor polyprotein (Pr160), produced by ribosome frame shifting near the 3' end of Gag. The polyprotein is incorporated intact into the virus particle where it is cleaved producing enzymes of the virus. Pol encodes the viral enzymes protease, which cleaves the Gag proteins; reverse transcriptase, which transcribes the double strand DNA from the RNA; RNAse; and integrase, which inserts the double strand DNA into the host cell genome (Fig. 1.2) (39, 40, 43). Both the Gag and Pol genes are highly conserved. The Env open reading frame is

translated into a precursor glycoprotein (gp160), which is processed and cleaved by cellular proteolytic enzymes, producing the gp41 transmembrane protein and gp120 external glycoprotein. The gp120 and gp41 proteins are bound non-covalently and are associated as a trimer on the cell surface. The gp41 transmembrane protein anchors the envelope glycoprotein into the cell membrane via hydrophobic amino acids spanning the lipid membrane. The gp120 glycoprotein contains the recognition and binding sites for the CD4 receptor and the seven transmembrane domain chemokine co-receptors (Fig. 1.2) (39, 40, 43).

Tat and Rev encode regulatory proteins localized in the nucleus and are essential for HIV gene expression. Tat is a transactivator of gene expression regulating transcription. There are two forms of Tat, a minor form Tat 1 exon of 72 amino acids and a major form Tat 2 exon of 86 amino acids. Tat protein binds a hairpin stem-loop structure of a Tat responsive region, TAR RNA, in newly transcribed RNA. Binding TAR RNA activates transcription initiation and elongation from the LTR promoter, preventing the polyadenylation signal in the 5'LTR from terminating transcription prematurely. This increases the synthesis rate of RNA polymerase, amplifying viral RNA generation and amount of viral protein synthesised (39-41, 43, 44). Rev expresses a 19 kDa phosphoprotein promoting the nuclear export and stabilisation of unspliced or partially spliced messenger RNA (mRNA) (Gag, Pol, Env) from the nucleus to the cytoplasm for the development of structural proteins. It does so by binding the Rev responsive element (RRE) in the mRNA. RRE consist of 200 nucleotides, it is encoded within the Env region and is essential for Rev function (39-41, 43). Nef encodes a 27 kDa myristylated protein, is one of the first proteins produced in infected cells and is the most immunogenic accessory protein. Nef down-regulates the expression of CD4 receptor, major histocompatibility complex (MHC) class I and interleukin 2 (IL-2) receptors from the surface of the infected cell. Nef is

essential for viral spread, maintaining high virus load and disease progression to AIDS (Fig. 1.2) (39-41, 43).

Vif, Vpr and Vpu are three small genes in HIV-1 expressing accessory proteins. Vif, viral infectivity factor, expresses a 23 kDa cytoplasmic protein essential for infectivity of the virus but not its production. The lack of Vif renders free viruses defective in infecting cells, but cell to cell transmission is not affected (39-41, 43). Vpr, viral protein R, expresses a 14 kDa protein incorporated into the virion. It interacts with p6 element of Gag and is localised in the nucleus. It is thought that Vpr is involved in cell growth arrest, entry of the virus into the nucleus by targeting the nuclear import of preintegration complexes, transactivation of cellular genes and induction of cellular differentiation (39, 40, 43). Vpu, viral protein U, expresses a 16 kDa type I integral membrane protein that is unique to HIV-1. The Vpu protein degrades CD4 in the endoplasmic reticulum and enhances virion release from infected cells by reducing complex formations between gp120 and CD4 glycoproteins within the cell (Fig. 1.2) (39, 40, 43).

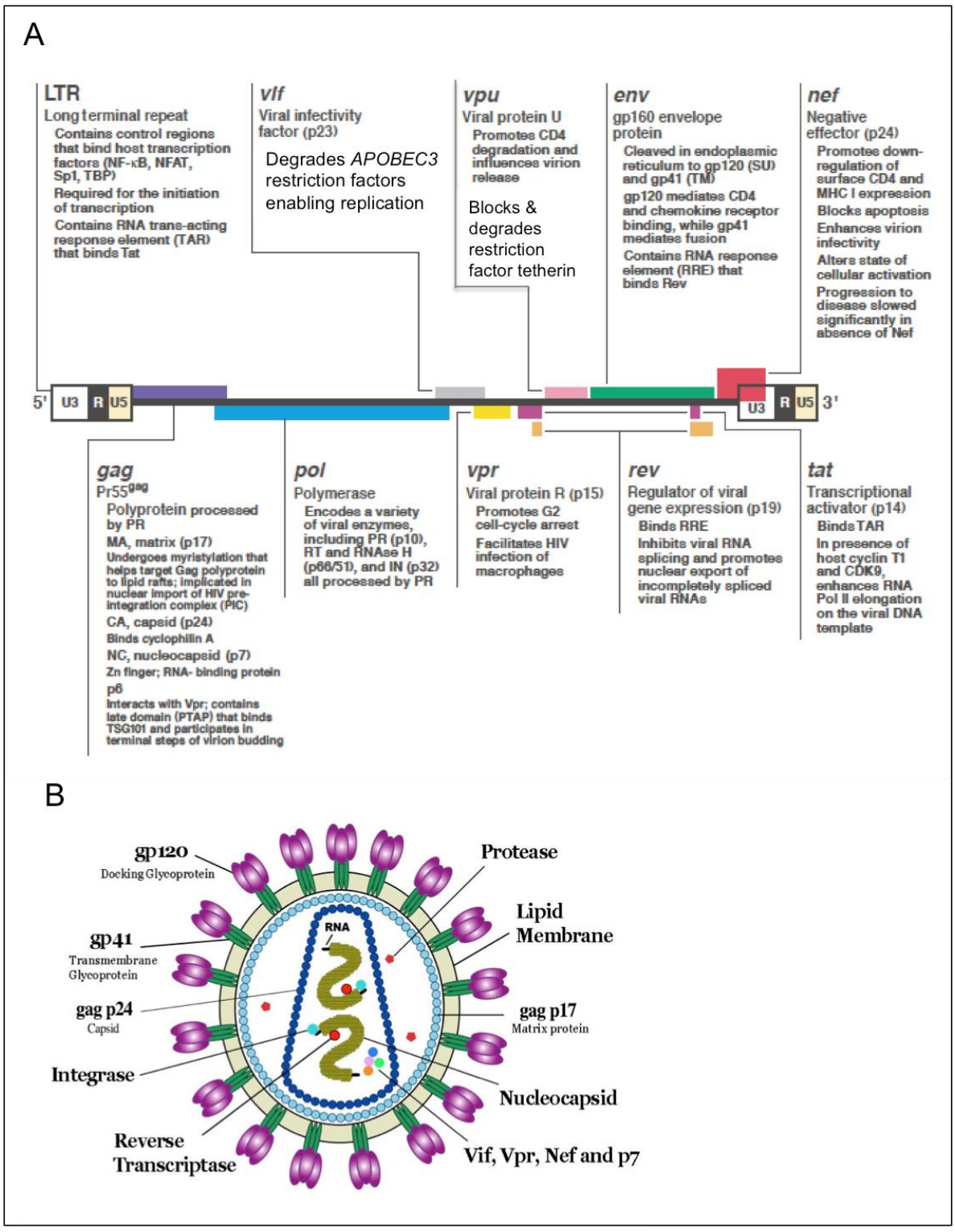



**Figure 1.2. HIV-1 genome and viral structure.** (A) An illustration of the HIV-1 genome and functional description of each gene. (B) Illustration of HIV-1 structure, the membrane, receptors and proteins (Configured from ref 43).

### 1.1.3 Life cycle of HIV-1

Sexual transmission accounts for more than 80% of HIV-1 infection, where the virus gains entry through the mucosal surface (cervicovaginal, rectal, penile). The other 20% of infections occur through percutaneous and intravenous injections or in utero. Early events of HIV-1 transmission through the mucosa and establishing infection are still not well understood in human. Most of our *in vivo* understandings are based on studies of rhesus macaques inoculated with SIV intravaginally and intrarectally (45, 46). It is still not clear if HIV-1 is transmitted as a free or virus infected cell, but SIV can be transmitted in both forms (46). Furthermore, the mechanism by which HIV gains access to the mucosal epithelium has not been fully determined, but several observations have been made that shed some light and complexity on the topic. First, transmission requires multiple exposures in most cases. Also cervicovaginal mucus was shown to reduce HIV diffusion and injury, tissue trauma or the presence of a co-existing genital infection such as human papilloma virus (HPV) increases the rate of transmission (47, 48). However, several mechanism have been proposed as how the virus accesses the mucosal epithelium; (i) crossing the epithelium via transcytosis, (ii) infecting Langerhans cells, a subset of dendritic cells specific to the epithelium (iii) crossing the epithelium wall and infecting mucosal CD4 T cells and Langerhans cells (Fig. 1.3) (47, 49).

Upon transmission of the virus into a new host, HIV-1 binds to a host immune cell to initiate its replication life cycle. The first step of infection occurs through the binding of gp120 Env protein to a conserved binding site in CD4 receptors found on macrophages and $CD4^+$ T helper cells (Th) (43, 49, 50). Upon binding the CD4 receptor, the variable loops (V1, V2 and V3) are rearranged causing a conformational change of gp120 and exposing structural elements than bind chemokine coreceptors, CCR5 or CXCR4 on the cell surface. Binding of the coreceptors is vital for viral entry as it activates membrane fusion of the virus and cell (51-55). The coreceptor binding determines the tropism of the virus. Viruses

that bind CCR5 are called R5 HIV, those that bind CXCR4 are termed X4 HIV and those that bind both are called R5X4 HIV (56). Transmitted and founder viruses are nearly always R5 tropic, also known as macrophage tropic, as the receptor is expressed on CD4 T cells and macrophages (47, 49, 55). Mutations in the CCR5 allele have rendered individuals resistant to HIV-1 R5 tropic infections (57, 58). X4 tropic viruses, also know as T-cell tropic, develop as the disease progresses. The binding of the coreceptors induces the exposure of the hydrophobic gp41 fusion peptide that penetrates the host cell membrane, bringing the virus and cell membrane closer and mediating membrane fusion (Fig. 1.4) (43, 59).

Following the virus binding and fusion with cell, the viral capsid is uncoated and its contents are released into the cytoplasm that include Vpr, reverse transcriptase, integrase and other enzymes that form the reverse transcription complex (RTC). This initiates the reverse transcription of the viral RNA genome into a double strand DNA and transforming the RIC into the preintegration complex (PIC). Vpr transports the PIC from the cytoplasm into the nucleus and the viral DNA gets integrated into the host cell genome by use of integrase enzyme in the PIC. Once integrated the viral genes are treated as cellular genes and are transcribed via the cellular polymerase enzymes. Following integration, the virus either becomes transcriptionally silent, entering a latent phase and activated when cells are activated themselves in response to antigen stimuli; or proceeds to be active and is transcribed by cell machinery producing virions (Fig. 1.4) (43, 60-64).

When transcription is initiated, short spliced mRNA encoding regulatory proteins Tat and Rev are produced. Tat and Rev are translated in the cytoplasm and exported back into the nucleus controlling the rate of transcription and ensuring it proceeds in distinctive phases. Tat activates viral transcription by binding the transactivation responsive region, stimulating the elongation of the viral long terminal repeats which acts as the viral promoter. The Tat protein together with cellular nuclear factor kappa B (NF-κB),

specificity protein 1 (Sp1) transcription transactivating protein and RNA polymerase II induces high level transcription of the viral RNA (65-68). Rev mediates the export of spliced and partially spliced mRNA's encoding structural and enzymatic proteins (Gag, Pol, Env) from the nucleus to the cytoplasm, facilitating the translation of these proteins (69-71).

Translation of the Env protein occurs in the endoplasmic reticulum, travelling through the cellular secretory pathway where it is glycosalted and processed by the cellular protease furin into the transmembrane gp41 and surface domain gp120, and transported to the plasma membrane via vesicular transport (43). Translation of Gag, Gag-Pol and accessory proteins occurs in the cytoplasm. The Gag and Gag-Pol polyprotein itself mediate all the essential events in virion assembly, binding the plasma membrane, creating the spherical particles and packaging the viral RNA genome. The budding process of the virion from the cell is mediated by the cellular machinery ESCRT (endosomal sorting complexes required for transport) (72, 73).

The Gag polyprotein initially assembles to a spherical immature particle and is transported to the cell membrane. The amino terminal Gag domain encodes the matrix protein (MA) that binds to the cell membrane and recruits the Env proteins. The central domain of Gag called capsid (CA), mediates protein-protein interaction for the immature virion assemble and later the capsid. The basic Gag nucleocapsid (NC) domain contains two retroviral zinc finger motifs, capturing viral RNA genome during assembly. The Gag carboxy-terminal p6 region contains binding sites for several proteins, including accessory viral proteins and assembly proteins of the ESCRT pathway. Gag also contains spacer peptides (SP1 and SP2) involved in regulating the conformational change from immature to a mature virion. Linker regions containing a protease (PR) cleavage site separate the Gag domains. PR is activated during the budding of the immature virion, initiating the maturation process by cleaving Gag in a systematic fashion into its components MA, CA,

NC and p6 proteins and releasing the SP1 and SP2 peptides (Fig. 1.4) (43, 73, 74). Two copies of the viral RNA genome are packaged into the virion through interaction between the NC protein and a *cis*-acting sequence within the 5' UTR resulting in the dimerization of the RNA genome prior to packaging (74, 75). The final assembled virion contains all the essential components for infectivity; two copies of positive sense RNA genome, Env proteins, Gag polyprotein, protease, reverse transcriptase and integrase viral enzymes.

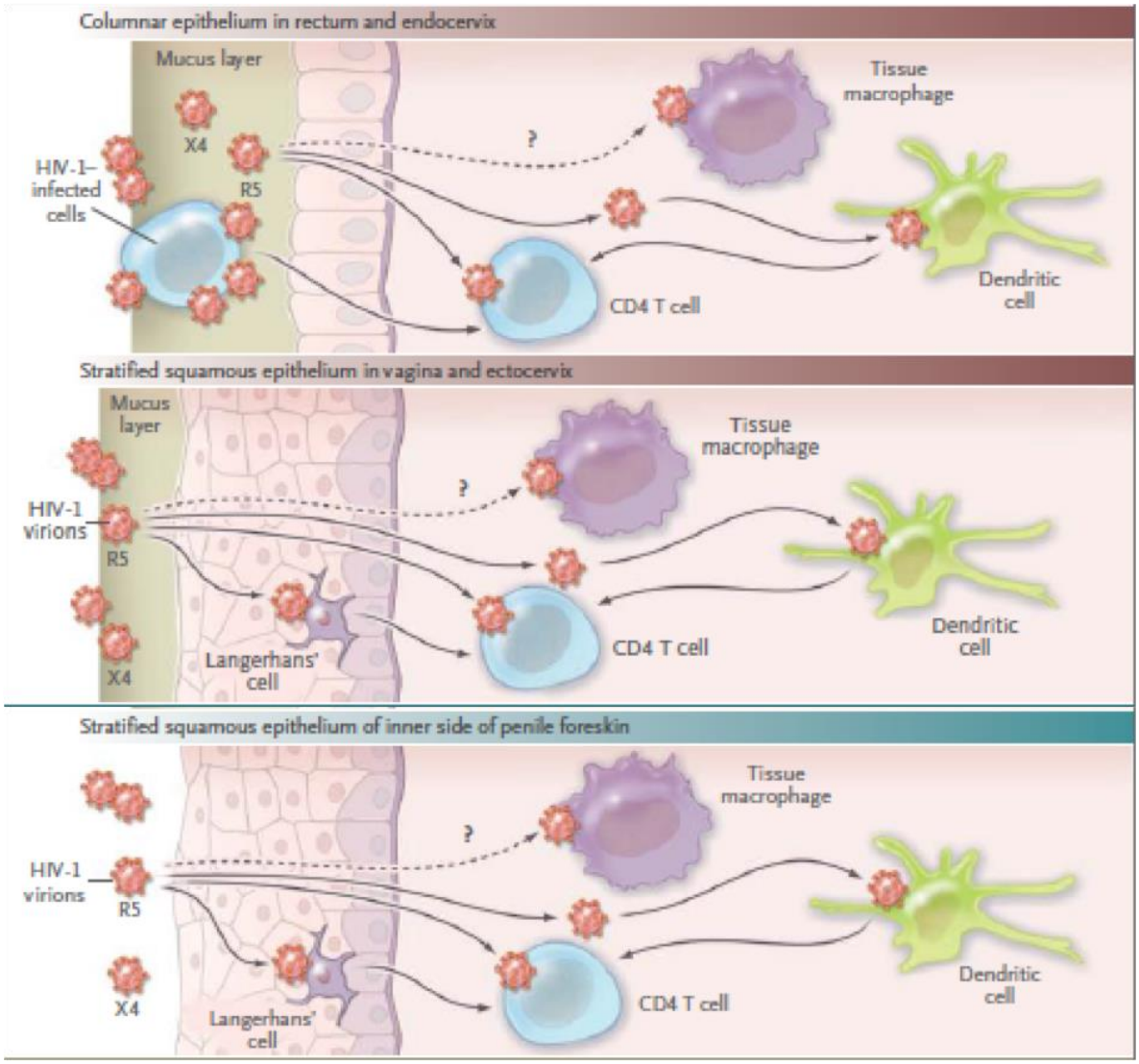



**Figure 1.3. Mechanisms of HIV-1 transmission.** Modes of HIV-1 transmission through the mucosa epithelium in the rectum and endocervix, vagina and ectocervix or the penile foreskin; and the different target cells and modes of infection (49).

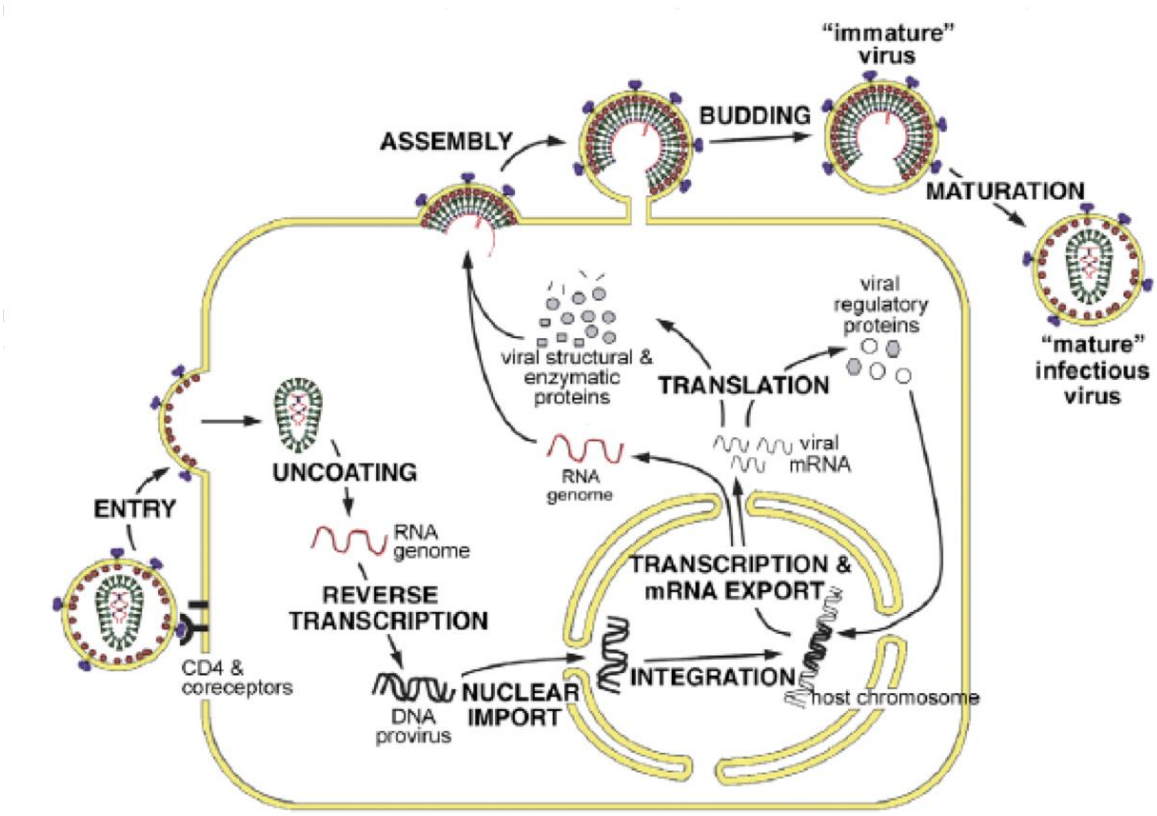



**Figure 1.4. HIV-1 life cycle.** Stages of HIV-1 life cycle, staring with attachment and entry into the cell; uncoating and translocating into the nucleus; integration into the host's genome; transcription and translation of viral proteins; assembly and budding from the target cell (73).

### 1.1.4 Clinical stages of HIV-1 infection

Following an infection of an individual with HIV-1, the disease progresses through three phases: i) primary acute stage; ii) asymptomatic / clinical latency; iii) symptomatic / AIDS defining illness.

**Acute phase**

Once transmission has been established, the virus enters an eclipse phase, lasting 7 to 20 days, during which it is undetectable in the plasma but is replicating in the mucosa and submucosa (49, 76). The establishment of infection, in 80% of mucosally transmitted HIV-1, arises from a single virion and nearly always being of a CD4/CCR5 T cell tropic virus (47, 49, 55). The end of the eclipse phase is characterized by the virus and virus infected cells reaching the draining lymph node, encountering activated $CD4^+$ $CCR5^+$ T cells. Innate immunity further augments the rate of infection through the secretion of cytokines activating T cells. Furthermore, dendritic cells and B cells may also propagate viral migration to activate CD4 cells through DC-specific ICAM3-grabbing non-integrin (DC-SIGN) and complement receptor CD21 respectively (77-79).

The peak viraemia phase is characterized by a drastic increase in replication and spread of the virus through the lymphatic system, particularly gut-associated lymphoid tissue (GALT), home to a large population of $CD4^+$ $CCR5^+$ memory T cells, many of which also express T helper 17 (Th17) cell surface marker (80-82). 20% of the GALT $CD4^+$ T cell population gets infected and up to 60% of the uninfected cell are activated and die by apoptosis, resulting in immune suppression through to the production of tumor necrosis factor (TNF)-related apoptosis-inducing ligand (TRAIL), TNF receptor type 2 (TNFR-2), Fas ligand (FasL) and apoptotic microparticles (80, 81, 83, 84). During this phase whereby CD4 T cells are significantly depleted in the GALT and virus replication is high in lymphoid tissues and the GALT, plasma viraemia reaches a peak 21 to 28 days post infection (80, 81, 83-85). Levels of CD4 T cells in the blood recover to normal levels later,

however the 80% depletion in the GALT does not recover (80, 84, 85). Dysregulation of the immune response is a prominent feature of the acute phase, with activation of innate, cellular and humoral cells. Most striking is the positive correlation between CD8 T cell activation and the rapid increase in viral load and CD4 depletion (86, 87). After reaching the peak, the level of HIV-1 viraemia decreases over 12-20 weeks to a stable level, reaching an equilibrium known as the viral set point, thus entering the asymptomatic stage. Escape mutants are generated and selected for during this phase in response to the pressure of adaptive immune response (49, 88).

**Asymptomatic / Clinical latency**

Once viraemia reaches the viral set point, a population of HIV-1 infected resting memory $CD4^+$ T cells is established, with the number of latently infected cells at 1 in $10^6$ cells. The persistence of the latent reservoir is irrespective of antiretroviral treatment and viral replication and $CD4^+$ T cell depletion, at a rate of 30 to 60 cells/μl per year, continues during this stage although patients are asymptomatic (89, 90). Cell to cell transmission of HIV-1 is common during this stage to circumvent the antibody response (91-93). Infected individuals remain in this stage for around 10 years, during which the viral load is maintained through a dynamic balance between viral clearance and production (90, 94). This period persists until the depletion of $CD4^+$ T cell reaches the level of 200 cells/μl or below, thereafter the patient develops AIDS and is symptomatic.

**Symptomatic / AIDS**

Due to $CD4^+$ T cell depletion, with level below 200 cells/µl, viraemia levels increase again. During this stage, HIV-1 infected individuals become susceptible to opportunistic infections and cancers such as *Pneumocystis carinii* pneumonia, tuberculosis, non-Hodgkin's lymphoma, thus becoming symptomatic. This stage is known as AIDS. Individuals not progressing to AIDS within 10 years are known as long term non-progressors (LTNP); and individuals progressing rapidly, within 2 to 3 years, are know are rapid progressors. LTNP individuals are characterized with a robust immune response, with neutralising antibodies, HIV specific $CD8^+$ T cells and a strong natural killer cell response (95-98). Furthermore, host genetics of human leukocyte antigen (HLA) alleles such as HLA-B*5701, HLA-B*5703, HLA-B*27 play a pivotal role in controlling infection and slowing the progression of the disease (99-102).

## 1.2. Immune Responses to HIV-1

### 1.2.1 Innate Immunity to HIV-1

Innate immunity is the first line of defence against pathogens and plays a crucial role in the pathogenesis of HIV-1. Unlike the adaptive immune system, which is antigen specific using T cell receptors and is HLA restricted, innate immunity is specific but not adaptive, using pattern recognition receptors to detect pathogen associated molecular patterns, and lacks memory (103). Three classes of pattern recognition receptors have been identified, the toll-like receptors (TLRs), the retinoic acid-inducible gene 1 (RIG-1)-like receptors (RLRs), and the nucleotide oligomerization domain (NOD)-like receptors (NLRs). Activation of different combination of these receptors on different innate cells leads to the induction of an inflammatory response and activation of innate and adaptive immune cells. A wide array of innate cells is involved in the response against HIV-1 including monocytes, macrophages, dendritic cells (DC), natural killer cells (NK), NK T cells (NKT) and gamma delta T cells (γδ T-cells) (103).

Plasma from newly HIV-1 infected individuals has given us an insight to the initial systemic innate immune response to the virus. Elevated levels of serum amyloid A, an acute phase protein, are one of the first innate immunity markers detected during the eclipse phase, a time when viral RNA is first detected prior to the systemic activation of the immune response (104). Levels of cytokines and chemokines in the plasma increase in tandem with viraemia increase. A rapid but transient increase is observed in levels of interleukin-15 (IL-15), type I interferon (IFN-α) and CXC-chemokine ligand 10 (CXCL10). Other cytokines such as IFN-γ, TNF-α, IL-18, inducible protein 10 (IP-10), IL-22 and IL-10, which peaks the latest, also increase rapidly, some at a slower rate, but are sustained at high levels for longer periods of time (Fig. 1.5) (77). Some of these cytokines have antiviral activity, whilst others enhance and initiate the activation of innate and

adaptive immune responses. The definitive cellular sources of these acute phase cytokines have not yet been fully clarified, but are hypothesised to be a combination of innate cells and $CD4^+$ $CCR5^+$ T cells. The magnitude of the cytokine storm observed in the acute phase of HIV-1 is unique, as it is much greater than ones observed in acute hepatitis B and hepatitis C virus infections. This indicates a systemic response of this magnitude is not required for viral clearance and is more likely to promote viral replication and immunopathology (77).

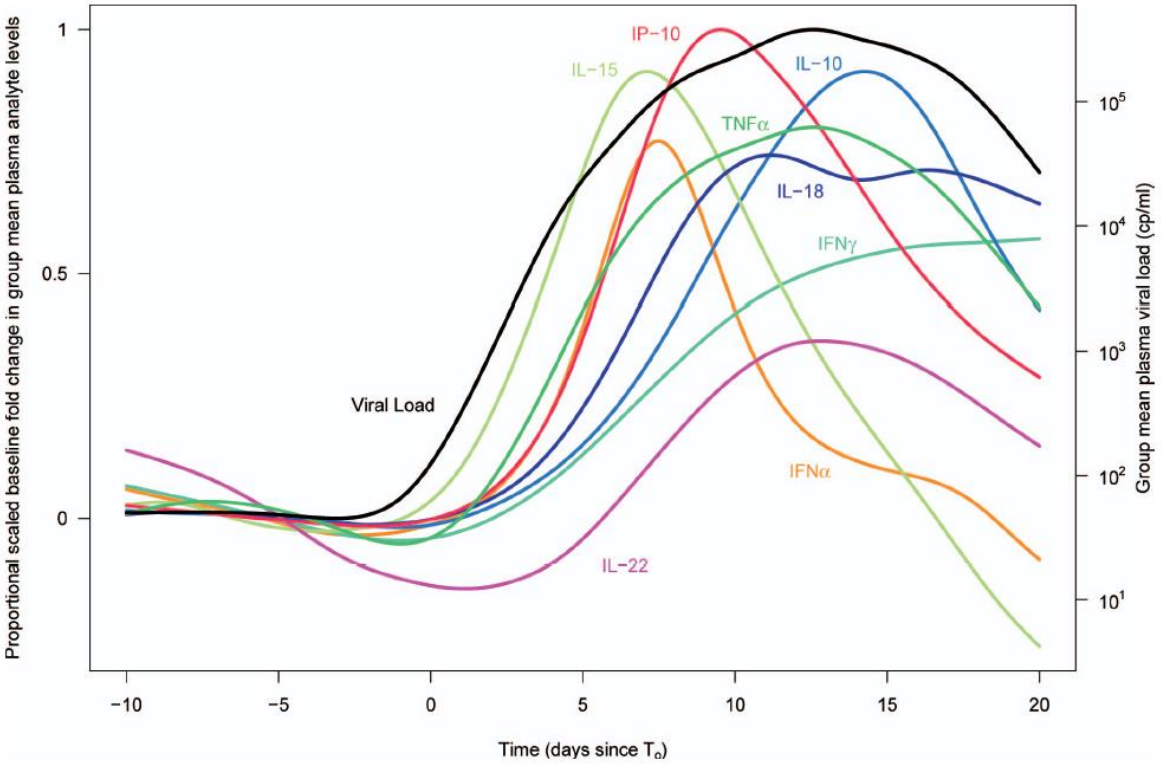



**Figure 1.5. The cytokine storm during acute infection.** Kinetics of the cytokines produced by innate cells in the early phase of the infection causing a cytokine storm (77).

**Natural Killer Cells**

Natural killer (NK) cells form an integral part of innate immunity. They develop and differentiate in the bone marrow then enter into circulation, however they can also develop in the thymus, spleen, tonsils and lymph nodes. They can make up to 10 – 15% of the peripheral blood mononuclear cells (PBMC) (105). NK cells are very effective in killing virus infected cells and humans lacking NK cells are prone to suffer from repeated viral and bacterial infections (106). Unlike $CD8^+$ T cells, NK cells do not express antigen specific receptors, but rather they express activating and inhibitory receptors that bind to different ligands including MHC-I on target cells. There are three major families of NK receptors: i) killer immunoglobulin-like receptor (KIR), ii) immunoglobulin-like transcript (ILT) and iii) killer C-type lectin-like receptors (NKG2/CD94) (107-109). The inhibitory or activating function of the receptors are dictated by the length of their cytoplasmic domain, the long tails contain an immuno-receptor tyrosine inhibition motif (ITIM), while the short tail contains an immuno-receptor tyrosine activation motif (ITAM) (107, 109).

NK cells survey the blood whereby their inhibitory receptors bind the MHC I ligand determining self and inhibiting activation. The absence of the MHC I ligand, as seen in some cancerous and infected cells, alerts NK cells but is not sufficient for activation. Killing of a target cell requires a signal through activation ligands e.g. NKG2D which is typically expressed on infected or tumour-transformed cells (107-110). Upon activation, NK cells can secrete a host of cytokines and chemokines activating and recruiting adaptive immune cells i.e. IFN-$\gamma$, TNF-$\alpha$, IL-15, IL-10, IL-12, transforming growth factor beta (TGF-$\beta$), granulocyte-macrophage colony stimulating factor (GM-CSF), CCL-5, MIP-1$\beta$ (CCL-4) and MIP-1$\alpha$ (CCL-3). Furthermore, NK cells can directly kill a target cell through TRAIL, FasL or the release of cytolytic factors (perforin and granzyme), (107, 109, 111, 112).

NK cells are activated and proliferate rapidly during the acute phase of HIV-1 due to the release of IFN-α and IL-15 during the cytokine storm (113, 114). HIV has evolved to evade detection and lysis by NK cells through Nef proteins. Nef proteins induce the endocytosis of MHC class I molecules in the Golgi, thus down regulating their expression on the surface of infected cells preventing recognition by $CD8^+$ T cells. However, to avoid alerting NK cells, Nef proteins specifically down regulate HLA-A and HLA-B, while sparing HLA-C, HLA-E and the dominant inhibitory receptor KIR2D (115, 116). Furthermore, Nef and Vpu proteins also down regulate the expression of CD155 and NKG2D activating ligands i.e. MIC A, ULBP-1 and ULBP-2 on the surface of infected cells, essential for the activation of NK mediated cell lysis (117, 118).

NK cells have been implicated in slowing the progression of HIV-1 through particular KIR/MHC combinations. An individual co-expressing the activating KIR3DS1 allele in conjunction with Bw4 allele (HLA-Bw4-80I) progression to AIDS is much slower than individuals lacking these alleles. This specific KIR/MHC interaction induces a strong antiviral signal to NK cells enabling the control of HIV infection (119-121). Furthermore, KIR3DS1 allele may also be associated with protection, as HIV-1 negative individuals that are highly exposed have high transcript levels of KIR3DS1 (122). However, there is no evidence yet that NK cells drive escape mutations, although particular amino acid changes do impact the KIR/MHC interaction and in turn effect NK's antiviral effect. Disease progression has also been associated with low NK cell count in the blood. This inverse correlation indicates the importance of NK cells in HIV-1 infection control (109, 114).

**Dendritic Cells**

Dendritic cells (DCs) are antigen presenting cells and form part of the innate immune response. They are crucial in the recognition of a wide range of pathogens, processing and presenting antigens to T cells via the MHC complex on their cell surface. There are two types of DCs, the first is plasmacytoid DCs (pDCs), they circulate in the blood, are not very effective at antigen presentation and are considered a precursor to DCs (123). The second type is myeloid DCs (mDCs), they are found in different peripheral tissues throughout the body, sampling antigens and travelling to lymphoid organs to present to and activate naïve T cells (124). Both pDCs and mDCs are targets and can be infected with HIV-1 as they express the CD4 entry receptor and CCR-5 and CXC4 co-receptors. During acute infection DCs are significantly reduced, particularly pDCs. It is hypothesised that the reduction in circulating DCs is due to activation-induced cell death or migration of activated DCs to the lymph nodes, where an increase in DCs is noted (78, 125).

pDCs are the earliest cells arriving at the mucosal site of infection. They are activated through the recognition of viral RNA via TLR7, leading to the production of copious amounts of type I interferon (IFN-α and IFN-β) (126, 127). Activation requires the binding of the viral envelope protein to a CD4 receptor on the cell surface, mediating viral endocytosis, endosomal acidification and the triggering of TLR7 by viral RNA (127, 128). The activation of pDCs inadvertently promotes HIV-1 survival and mucosal infection by inducing an inflammatory response recruiting and activating target cells for infection (127). Furthermore, HIV-1 stimulated pDCs produce indoleamine 2,3-dioxygenase (IDO) in response to the triggering of TLR9 with unmethylated CpG. The production of IDO inhibits the proliferation of $CD4^+$ T cell and induces the differentiation of naïve $CD4^+$ T cell into regulatory T cells (Tregs) with suppressive functions (129-131).

Immature mDCs residing within the epithelial surface or the submucosa are primary early targets of infection. Following the capture of virions at site of infection, mDCs mature

and travel into the lymph node, transmitting the virus to $CD4^+$ T cells and initiating a viral replication centre. Some virions captured by mDCs are internalised into endosomal compartments protected from proteolytic cleavage, remaining intact and infecting cells later via cell to cell transfer (132, 133). Unlike pDCs, mDCs are more resistant to HIV-1, they are not activated by the virus and do not support viral replication. This is due in large part to the activity of SAMHD1 (sterile alpha motif domain and HD (histidine/aspartic acid domain) containing protein 1), a cellular restriction factor upregulated by IFN-α. SAMHD1 has a dNTPase activity thus reducing the cellular deoxynucleotide (dNTP) pool, which in turn limits the activity and efficiency of HIV-1 reverse transcriptase (133-135). There are several other restriction factors induced by IFN-α with anti-viral activity such as TRIM5α (tripartite motif-containing protein 5 alpha), tetherin and APOBEC3G (136-139). APOBEC3G (apolipoprotein B mRNA-editing enzyme catalytic polypeptide like 3G) is an intracellular antiviral factor packaged into retroviral virions, deaminating viral cystidine to uridine, rendering them non-functional and inhibiting viral replication (136, 140). HIV-1 has evolved several mechanisms to neutralise these restriction factors, including capsid mutations preventing TRIM5α binding, Vif mediated degradation of APOBEC3G and Vpu degradation of tetherin (137-139, 141).

Nonetheless, HIV-1 infection does induce certain response from mDCs contributing to the cytokine storm. One such response is the up-regulation of programmed death ligand 1 (PD-L1) triggered by viral TLR7 and TLR8 ligands, or by HIV-1 Tat protein and TLR4, resulting in the activation and exhaustion of PD-1+ T cells (142-144). PD-L1 correlates inversely with $CD4^+$ T cells and directly with viral load in infected individuals. Increased expression of TNF-α and IL-6, stimulating the proliferation of naïve T cells is a consequence of PD-L1 reverse signalling on mDCs (133, 144). However, since mDCs do not get fully activated when exposed to HIV-1 they are defective in producing IL-12, which

is in accordance with the decrease seen in IL-12 levels during the acute phase of the infection (77).

**Macrophages**

Macrophages comprise an important part of the innate immune system surveillance and antimicrobial defence, and play a crucial role in the transition to adaptive immunity. Present in organs throughout the body to remove apoptotic and necrotic cells to maintain the homeostasis. As with other innate cells, they recognise foreign antigens through pattern recognition receptors, kill microbes through phagocytosis, present antigens through MHC II to $CD4^+$ T cells and secrete cytokines and chemokines to recruit and activate innate and adaptive immune cells (133). There are two types of macrophage states, depending on the cytokine microenvironment. The classically activated macrophages (M1), are pro-inflammatory, being activated by and expresses pro-inflammatory cytokines and chemokines e.g. IL-12, IFN-$\gamma$, TNF-$\alpha$, while the alternatively activated macrophages (M2) are anti-inflammatory expressing IL-4, TGF-$\beta$ and IL-10 (145, 146). Macrophages play a pivotal role in early immunity against HIV-1 but they are also targeted by virus and contribute to the pathogenesis of the disease. The $CD14^{++}CD16^+$ is the most susceptible monocyte phenotype to HIV-1 infection and harbouring. A significant expansion of $CD14^{++}CD16^+$ monocytes associated with increased levels of CCL2 is observed in infected individuals (147).

Unlike activated $CD4^+$ T cells where viral replication is rapid and cytopathic, infected macrophages survive for long periods of time, as viral decay is slower. Macrophages are highly resistant to the viral cytopathic effects, apoptosis and oxidative stress stimuli. Macrophages also have a longer half-life than activated $CD4^+$ T cells, ranging from six weeks to several years. Therefore, macrophages are considered an important reservoir and source of ongoing viral replication in infected individuals (148-

152). Activated $CD4^+$ T cells can account for 99% of plasma viraemia in infected individuals, however, macrophages are thought to be the main source of plasma viraemia in individuals receiving highly active antiretroviral therapy (HAART) as viral replication in T cells is disrupted (152, 153).

The biology and function of macrophages is modulated when infected with HIV-1. HIV-1 infection of macrophages induces the expression of pro-survival cytokine macrophage colony stimulating factor (M-CSF), that down-regulates TRAIL-R1/DR4 and up-regulates anti-apoptotic genes, rendering the cell resistant to TRAIL mediated apoptosis (154). The expression of Nef in macrophages activates several signalling pathways that induce the expression of soluble CD23 and soluble ICAM (155). The release of both proteins renders neighbouring bystander $CD4^+$ T cells more permissive to infection thus expanding the viral reservoir by infecting resting lymphocytes (155). Nef also induces the expression of FasL on the surface of infected cells causing an interaction with Fas (CD95) on neighbouring cells including cytotoxic T cells and leading to the killing of bystander cells (156). But as to avoid apoptosis of virally infected cells expressing the FasL, Nef interacts and inhibits apoptosis signal regulating kinse-1 (ASK1), a signalling intermediate in the Fas and TNF-$\alpha$ death signalling pathways, thus inhibiting both Fas and TNF mediated killing of HIV-1 infected cells. This allows the enhanced apoptosis of bystander cells as a mean of immune evasion while protecting HIV-1 infected cells (156).

Macrophages play a pivotal role in HIV-1 dissemination to $CD4^+$ T cells. They are able to transmit the virus directly to $CD4^+$ T cells by fusing and transmitting the virus through a virological synapse (157). This style of cell to cell transfer is as twice more efficient than free virus infection and evades antibody detection (157). HIV-1 infected macrophages also secrete a wide range of chemokines, recruiting and activating many immune cells. The binding of gp120 to CCR5 on macrophages induces the secretion of

CCL2, CCL3, CCL4 and CCL5, recruiting a variety of immune cells including monocytes, macrophages, dendritic cells and T cells (153, 158, 159).

### 1.2.2 Humoral Immunity to HIV-1

Humoral immunity is one of two arms of the adaptive immune response. It is an antibody (Ab) mediated immunity secreted B-lymphocytes killing foreign microorganisms, preventing the spread of intracellular infection and forming immune memory. Antibodies have multiple potential pathways of blocking HIV-1 replication, exerting pressure on the virus to mutate and escape. They can prevent infection of target cells, thus disrupting replication by binding cell free virions or mediate killing of infected cells through effector cell mechanisms (160, 161).

Antibody responses can be detected 1 week post HIV-1 infection in the form of immunoglobulin (Ig)M and IgG, followed by plasma anti-gp41 antibodies on day 14 and anti-gp120 a couple of weeks later. This initial response is non-neutralising having no effect on viraemia and does not exert any selective immune pressure on the virus (162). Neutralising antibodies (NAbs) against the infecting virus (autologous virus) do not appear till a few month post infection, but are unable to neutralise divergent viruses (heterologous viruses) (163, 164). However, NAbs do exert selective pressure on the virus, generating escape mutants through single amino acid substitutions, insertions, deletions or shifting the glycan shield to prevent access to epitopes (165-167). Autologous NAbs develop within month post infection in approximately 20% of infected individuals, targeting different region of Env with variable loop (V)1 and V2 being major targets (168-170). Some infected individuals, elite neutralisers, after years of viral stimulation they develop broadly NAbs (bNAbs) targeting conserved regions, have greater breadth recognising hundreds of different viral quasi-species from different subtypes (169, 171-173). These bNAbs are of great interest for potential vaccines or treatments. Studies of HIV-1 bNAbs target epitopes

have led to the identification vulnerable sites on HIV-1 Env comprising of four targets: (i) CD4 binding site (CD4bs); (ii-iii) *N*-glycan-associated epitopes on V1/V2 loops and V3 loop; (iv) the membrane proximal external region (MPER) on gp41. A combination of factors can determine the breadth and potency of neutralisation: (i) numerous epitopes targeted by a multitude of NAbs; (ii) clonal variants of the same specificity but with somewhat different neutralisation spectra; (iii) recognition of different epitopes by several potent bNAbs exhibiting complementary neutralising activity.

At the mucosa, high levels of anti-gp41 IgA are produced in the first 2 weeks of infection, in a T cell independent manner by short lived plasma cells (174). A rapid decline in IgA levels is observed soon after, most likely due to early damage of mucosal B cell populations, rapid depletion of $CD4^+$ T cells in the gut, damage to the GALT and germinal centre loss thus hindering T cell induction of long lived plasma cells, would be producers of IgA (175-177). It has been suggested thet HIV-1 Env specific IgAs can neutralise HIV-1 by blocking viral transcytosis (translocation through the epithelial cells) and replication in epithelial cells in infected and highly exposed seronegative individuals (178-180).

Non- or weakly neutralising antibodies also have a role in the control of HIV-1. They utilise a crucial mechanism by which they recruit effector cells such as NK cells, macrophages, dendritic cells neutrophils and γδT cells to induce cytolysis or apoptosis of infected cells they bind. This is achieved through the recognition of viral proteins on the surface of infected cells through the Ab IgG Fab portion and the binding of the Ab Fc portion to an Fc receptor (FcγRs) on an effector cell (161, 181-185). Depending on the FcγRs, the formation of the complex induces either an antibody dependent cellular cytotoxicity (ADCC) response, whereby an infected cell is killed through the release of cytolytic factors granzymes or perforin by the effector cell; or an antibody dependent cell mediated virus inhibition (ADCVI) response, inducing the release of cytolytic factors and

antiviral cytokines inhibiting viral spread; or antibody dependent cellular phagocytosis (ADCP). These Fc-mediated responses have also been attributed to bNAbs (161, 181-185).

HIV-1 infections cause pathological changes in lymphoid tissues supporting B cells such as follicular hyperplasia and germinal centre alteration causing major disruptions to B cells deregulation, differentiation and function, leading to a dysfunctional humoral immune response. Due to this, several abnormalities in B cells arise such as: (i) B cell hyperactivity e.g. hypergammaglobulinemia and polyclonal B cell activation; (ii) increase in immature B cells frequency; (iii) increased plasmablast differentiation; (iv) exhaustion of blood tissue-like memory B cells associated with increased viraemia (177, 186-188). Furthermore, alterations are also caused through direct interactions between viral proteins and B cells. Binding of gp120 to $\alpha 4\beta 7$ integrin on B cells induces the expression of TGF-$\beta$1 cytokine and inhibitory receptor FcRL4, while also decreasing the expression of the co-stimulatory receptor CD80, inhibiting the activation and proliferation of B cells (189).

### 1.2.3 Cell Mediated Immunity to HIV-1

**Antigen processing and presentation**

The major histocompatibility complex class I (MHC-I) and class II (MHC-II) molecules are vital to cellular immunity, presenting endogenous and exogenous peptides to CD8 and CD4 T cells respectively. MHC alleles are polymorphic, with amino acid variations being concentrated in the groove where peptides bind, enabling the binding of a wide range of peptides. MHC-I molecules are expressed on the surface of all nucleated cells and present endogenous peptides originating from the cytosol or nucleus to CD8 T cells via the T cell receptor (TCR) to activate them (190). The MHC-I antigen presentation system enables T cells to recognise infected or transformed cells by presenting modified-self or foreign peptides. For the presentation of peptides on MHC-I molecule, antigens within the cell are degraded by the proteasome, generating peptides that are translocated

into endoplasmic reticulum (ER) via the transporter associated with antigen processing (TAP) whereby they bind the MHC-I molecule and get expressed on the cell surface (190).

The proteasome generates the bulk of peptides for MHC-I binding through proteolysis and defines their carboxyl terminus. The 26S proteasome consists of a 20S core barrel shape structure with protease activity; and two 19S caps with deubiquitinase and unfoldase activity facilitating target proteins entering the barrel (191-194). Immune cells also express immunoproteosome, they are more active than 26S proteasomes and are able to degrade proteins under immune stress preventing the aggregation of proteins (195). Peptides in the cytosol are then transported into the ER lumen via TAP. For the nuclear peptides to reach TAP, they must diffuse into the cytosol (196).

TAP is a heterodimer protein composed of two subunits, TAP1 and TAP2, encoded by closely linked genes in the MHC, and mediates translocation via ATP hydrolysis through the nucleotide binding domain (197, 198). TAP is also a platform for MHC-I folding, interacting with a number of chaperone proteins (Tapasin, calreticulin (CRT) and ERp57) forming the peptide loading complex (PLC). Calrecticulin stabilizes the interaction between the MHC-I molecule and tapasin (199, 200). This leads to the binding of ERp57 protein assisting in the folding of newly synthesized glycoproteins in the ER (201). Tapasin also has a peptide-editing role, mediating the binding of high-affinity peptides to MHC-I (202). Thus the peptide loading complex ensures proper folding and stability of MHC-I and the binding of peptides.

For peptides to bind the MHC-I molecule they need to be 8-10 amino acid long. When transported into the ER they are generally longer, 8-16 amino acid long. In the ER, peptides are trimmed by two ER aminopeptidase, ERAP1 and ERAP2 to yield peptides of the correct length (203). The aminopeptidase are not part of the PLC, therefore, peptides are trimmed in the ER lumen then re-enter the PLC to bind the MHC-I molecule (204). Aminopeptidase are essential, as studies in ERAAP knockout mice have shown peptides

bound to MHC-I molecules are elongated and the complex is unstable (205). Once peptides are bound to the MHC-I molecule, they are transported from the ER through the golgi apparatus to the cell surface to present the epitopes to CD8 T cells.

*Cross-priming*

Naïve CD8 T cells need to be activated by professional antigen presenting cells (APC) to become cytotoxic T cells. However when APC's are not infected with intracellular pathogen or are not transformed into tumour cells, they need to acquire antigens exogenously and present them on MHC-I to activate CD8 T cells. The presentation of exogenous antigens on MHC-I is known as cross-priming or cross-presentation and is mainly done by dendritic cells (206-208). There are two pathways for cross-presentation, a cytosolic and a vacuolar pathway. The cytosolic pathway is sensitive to proteasome inhibitors, suggesting phagocytosed antigens are degraded by the proteasome, and resulting peptides processed through the classical MHC-I antigen processing pathway (209). As TAP is recruited onto the phagosome and endosome, peptides may also be loaded onto MHC-I molecules within these compartments (210, 211). In addition to ERAP, a second aminopeptidase, insulin-responsive aminopeptidase (IRAP), located in the endosomal compartment has been shown to trim peptides during the process of cross-presentation (212). The vacuolar pathway is resistant to proteasome inhibitors and independent of TAP, however it is sensitive to inhibitors of lysosomal proteolysis (cathespin S inhibitor), suggesting both the processing and loading of peptides onto MHC-I molecules occurs in the phagosome and endosome compartments (213, 214). Cross presentation is vital in immunity against viruses and tumours and essential for vaccinations.

**The T cell receptor**

The T cell receptor (TCR) orchestrates cellular immunity, as it recognizes antigenic peptides to initiate a response. According to their TCR heterodimer, T cells are divided into two main categories, those expressing an αβ TCR or a γδ TCR (215). Those expressing the αβ make up the majority of T cells, mostly recognising peptides bound to an MHC molecule (216). However, as with γδ T cells, there are a few unconventional αβ T cells types that are not MHC restricted, including: (i) mucosal associated invariant T cells (MAIT); (ii) invariant NK T cells (iNKT); (iii) germline-encoded mycolyl-reactive T cells (GEM) recognising glycolipids as do iNKT through CD1b and CD1d respectively (217-219). There are also hybrid T cells e.g. δ/αβ T cells as a result of recombination.

The αβ heterodimer TCR are generated somatically via gene rearrangement, enabling great diversity in the receptors produced from the few sets of genes. The TCR α locus consists of variable (V), junctional (J) and constant (C) gene segments, whereby the V and J segment recombine and are juxtaposed to a C segment. The TCR β locus has a further diversity (D) gene segment, producing recombinant of V-D-J (215, 220). Variability is restricted to three hairpin loops, know as complementarity determining regions (CDR) on each TCR chains forming the antigen binding site (221). The V αβ gene segments encode the variable CDR1 and CDR2 in the germline, whereas the hypervariable CDR3 loops are the product of nucleotide addition and deletion at the junction between recombining V-D-J gene segments (220). This variability is essential for the ability of TCR to recognise the various antigens. To ensure T cells recognise self MHC-molecule and are not autoreactive, they undergo thymic selection during development. During positive selection, T cells receive a survival signal if they express TCR that recognise self peptides as self MHC. T cells that do not recognise self peptides die by neglect. In negative selection, T cells that bind too strongly to self peptides i.e. autoreactive are culled through an apoptotic signal.

This ensures that only αβ T cells recognising self MHC with low affinity populate the periphery (222-224).

T cells are activated through signals induced by the TCR and several co-receptors. CD4 and CD8 are two co-receptors that assist the recognition of peptide bound MHC molecules and bind MHC together with the TCR. This initiates a signal through their cytoplasmic tail which is associated with the tyrosine kinase Lck that leads to the phosphorylation of the CD3 immunoreceptor tyrosine based activation motif (ITAM) (225, 226). The phosphorylation of ITAM recruits and activates cytosolic tyrosine kinase Zap70 that in turn phosphorylates Lat, leading to an intracellular signaling cascade that activate the cell (227, 228). Furthermore, there are other receptors that have a costimulatory or inhibitory function and play a role in T cell activation in tandem with TCR. CD28 is a costimulatory receptor that binds CD80 or CD86 ligands, activating transcription factor NF-κB promoting proliferation, while CTLA-4 is a coinhibitory receptor inhibiting activation (229, 230). These are some, but not all, of the proteins and receptors that are associated with the TCR and T cell activation, as more is still being discovered.

**$CD4^+$ T cells**

$CD4^+$ T cells form part of cell mediated immunity, the second arm of the adaptive immune response. They are critical to the adaptive immune response whereby they recruit and activate other immune cells such as $CD8^+$ T cells, macrophages, B cells, neutrophils and eosinophils through the release of cytokines. Activation of $CD4^+$ T cells is achieved via two signals from antigen presenting cells (APC). The first signal is achieved through the binding of the T cell antigen receptor (TCR) and the major histocompatibility complex (MHC II) molecule on APC presenting a peptide antigen. The second signal is through the engagement of a co-stimulatory receptor on T cells to a co-stimulatory ligand on APCs (231, 232). The expression and release of different transcription factors and cytokines

respectively, define the function of the $CD4^+$ T cell, based on which they are divided into different subsets i.e. T helper 1 (Th1), Th2, Th17, follicular helper T cells (Tfh) and regulatory T cells (Tregs). Differentiation of naïve $CD4^+$ T cells is driven by the cytokine microenvironment into one of the subsets is: Th1 (IL-12 and IFN-γ); Th2 (IL-2, IL-4, IL-7, IL-13); Th17 (IL-6, IL-21, IL-23 and TGF$\beta$); Tfh (IL-6, IL-21, IL-27); and Tregs (TGF$\beta$). These cytokines lead to the upregulation of specific transcription factors to develop the different subsets: Th1 – Tbet and Stat4; Th2 – GATA 3 and Stat6; Th17 - RORγt and Stat3; Tfh – Bcl-6, Stat3 and Stat5; Treg – FoxP3 and Stat5 (Fig. 1.6) (231-240).

Th1 cells are essential in the protection against intracellular pathogens, producing high amounts of IFN-γ, TNF-α and IL-12 to recruit and activated macrophages and $CD8^+$ T cells. Th2 cells are activated in response to parasites and allergens, producing IL-4, IL-5, IL-9, IL-10 and IL-13, recruiting eosinophils and induce IgG Ab production (231, 232). Th17 cells are essential at maintaining immune homeostasis at mucosal sites, playing a critical role in the response against extracellular bacteria and fungi through the release of IL-17a, IL-17f, IL-21 and IL-22 cytokines; and are also implicated in several autoimmune diseases including asthma and inflammatory bowl disease (241, 242). Treg cells are anti-inflammatory, they maintain immune homeostasis by limiting the magnitude of the effector response and establishing immune tolerance. They suppress and down regulate immune cells e.g. Th1, Th17 and CD8 either by cell-to-cell contact or through the release of inhibitory cytokines IL-10 and TGF$\beta$ (Fig. 1.6) (239, 240). Tfh cells are crucial for the formation of germinal centres and help B cell in germinal centres of secondary lymphoid organs. They are crucial in the differentiation of B cells into long-lived memory B cells and plasma cells. They also assist B cells in the production of high affinity neutralising and non-neutralising Ab, Ig class switching and somatic hypermutation (243, 244). Tfh cells are characterised by the high level expression of surface marker programme death-1 (PD-1), CXCR5 and transcription factor B-cell lymphoma 6 (Bcl-6) (244).

HIV-1 primary target are $CD4^+$ T cells. During the acute phase of HIV-1 infection, activated and memory $CD4^+$ T cells are significantly depleted, most notably from the gut-associated lymphoid tissue. As much as 60% of the $CD4^+$ population is destroyed in the early phase of the infection as a result of infection by virions or bystander activation induced cell death (80, 85, 245). The virus preferentially targets HIV-1 specific CD4+ T cells, however the majority of these cells remain un-infected even in the presence of high viraemia (245).

Th17 cells in the GALT mucosa express high levels of CCR5 are depleted significantly, with the rate of depletion correlating with progression and contributing to the pathogenesis of HIV-1 infection (246, 247). The loss of Th17 cell is though to be a major cause of microbial translocation across the intestinal mucosa into circulation leading to chronic immune activation and disease progression (80, 248). Although Th17 promoting cytokines IL-6 and TGF$\beta$ are highly expressed in HIV-1 infections, Th17 cells induction is suppressed via other pathways (249). HIV-1 infected individuals have been shown to express a number of proteins inhibiting the JAK/STAT3 signalling pathway, which is essential in the development of Th17 cells. Suppressor of cytokine signaling-3 (SOCS3) a STAT3 suppressor that inhibits the development of Th17 cells and supports HIV-1 replication is found at elevated levels in infected individuals (250, 251). Another suppressor, protein tyrosine phosphatase (SHP-2), interfering with the IL-6 signalling mediated activation of STAT3 is also recruited in HIV-1 infected CD4+ T cells in response to gp120 binding to the cell (252, 253).

The number of Tregs in HIV-1 infected individual's increases as the disease progresses (254). Several mechanisms can explain this: (i) increased regression of Tregs in the thymus (255); (ii) enhanced proliferation and resistance to apoptosis, due to decreased sensitivity to TCR-stimulation (256). Also, exposure to gp120 up-regulates the anti-apoptotic protein Bcl-2 (257); (iii) accumulation in lymphoid tissue (258); (iv) conversion

of naïve CD4+ T cells into Tregs by pDC through IDO (131). The role of Treg in HIV-1 infection has two conflicting arguments. On the one hand of the argument, some data supports the hypothesis that Tregs have a beneficiary role by reducing chronic immune activation of $CD4^+$ T cells, therefore decreasing cell loss and immune cell recruitment, thus slowing disease progression (259-261). However, on the contrary, other data support the hypothesis that Tregs may have a detrimental effect by suppressing HIV-1 specific effector T cell responses, thus enhancing viral persistence and causing immune exhaustion; and may also be targets for infection and viral replication themselves (262, 263). This hypothesis is also supported by data from LTNP individuals with lower Tregs in peripheral blood and mucosal tissue (264, 265). Another study also showed Tregs from LTNP were effective in suppressing cytotoxic T lymphocytes (CTLs) restricted by non-protective alleles but were unable to suppress CTLs restricted by the protective alleles HLA-B*27 or HLA-B*57 within the same individual (266). Furthermore, Tregs in HIV-1 infected individuals express high levels of CD39, which correlate positively with plasma viral load and $CD4^+$ T cell depletion (254, 267). A polymorphism associated with down regulation of CD39 was associated with slower disease progression (267, 268).

Until recently Tfh cells were thought to be protected from HIV-1 infection as they lack expression of the CCR5 co-receptor on their surface. Now it is established that they support viral replication and are infected early on in the infection at an equivalent level or even higher than memory $CD4^+$ T cells (269). However, despite being infected, levels of Tfh cells increase during the acute phase of the infection in correlation with viral load, suggesting they might be a source of viraemia (269, 270). Tfh expansion also correlates with germinal centre B cell populations and antibody production. Also this irregular expansion is associated with B cell abnormalities such as hypergammaglobulinemia, B cell driven lymphadenopathy and poly-clonal B cell activation (269-271). Production of IL-21 by Tfh cells, essential for B cell survival and differentiation into long lived plasma cells, is

hindered, due to the chronic ligation of PD-1 to its ligand PD-L1 on Tfh and germinal centre B cells respectively (271).

Although $CD4^+$ T cells are primarily targets of HIV-1 they do have an effector function in the control of HIV, especially in maintaining a $CD8^+$ T cell response. A strong $CD4^+$ T cell response emerges earlier than $CD8^+$ T cells during the primary infection but is lost rapidly as the disease progresses (272, 273). Although depleted, $CD4^+$ T cells are not entirely absent. However, this depletion is the most likely the cause for the abnormalities noted in the development and differentiation of $CD8^+$ T cells, as an effective $CD8^+$ T cell response is maintained by T helper cells (274, 275). The production of IL-21 by $CD4^+$ T cells is particularly important in maintain CTL responses and prevent cellular exhaustion (276, 277). Furthermore, a robust and effective CTL response in elite controllers has been correlated with a strong preserved $CD4^+$ T cell response. Most likely they are preserved through an aggressive active retroviral therapy in the primary phase of the infection (278-281).

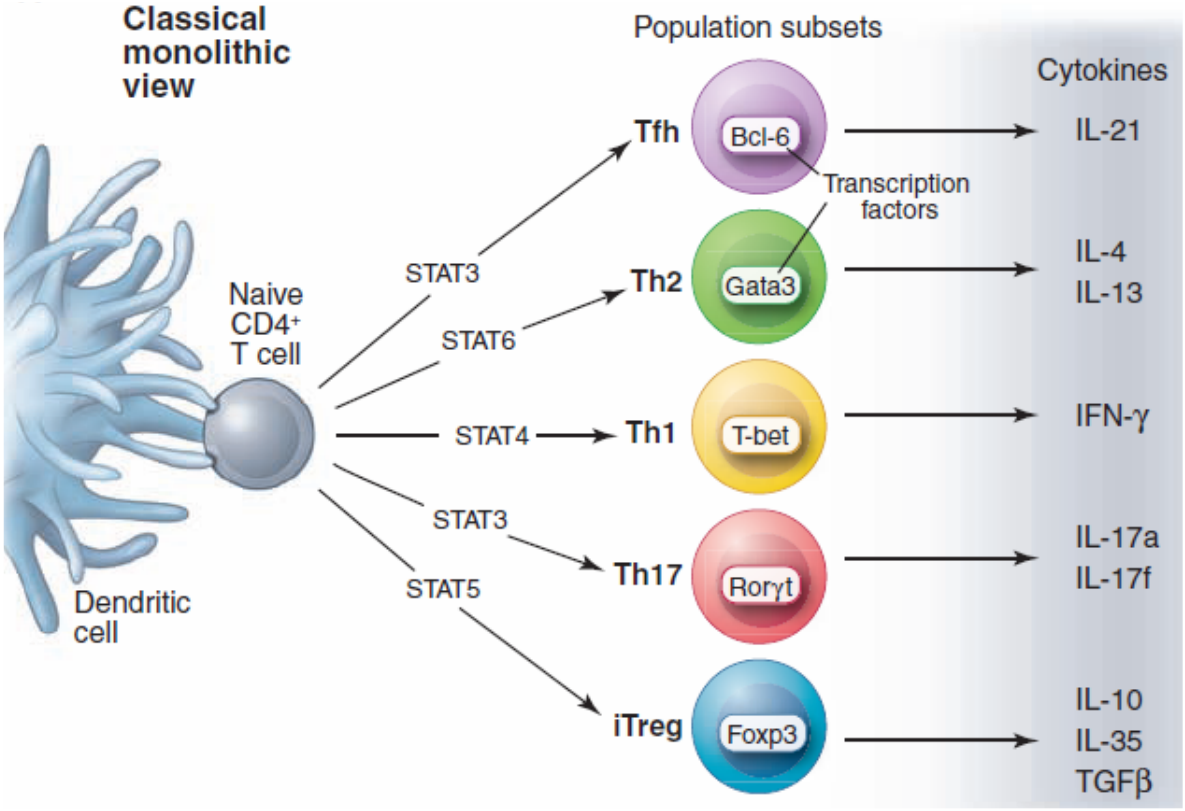



**Figure 1.6. CD4$^+$ T cell lineages and subsets.** Differentiation of naïve CD4$^+$ T cells into the different effector subsets and the specific signalling pathway and transcription factor associated with each subset, and the hallmark cytokines produced by each CD4$^+$ T cell subset (232).

**$CD8^+$ T cells**

$CD8^+$ T cells of the cell mediated immunity response are integral to the adaptive immunity against intracellular pathogens. $CD8^+$ T cells, also know as cytotoxic T lymphocytes (CTL), have both a cytotoxic and non-cytotoxic response. The cytotoxic response is achieved through the secretion of cytolytic factors perforin/granzymes or inducing apoptosis of target cells via Fas/FasL complex (282, 283). The non-cytotoxic response involves the release of antiviral cytokines and chemokines i.e. IFNγ, TNF-α, IL-2, CCL3, CCL4 and CCL5, which also enhance the response by activating and recruiting other immune cells (284, 285). The CTL response is generated through the recognition of epitopes bound to the MHC class I complex on target cells, by the T cell receptor (286, 287). Despite the depletion and damage to $CD4^+$ T cells in HIV-1 infected individuals, $CD8^+$ T cells produce a robust and effective immune response controlling disease progression.

The first HIV-1 specific $CD8^+$ T cell responses are detected in the early phases of acute infection, after viraemia reaches its peak, and before the onset of an antibody response (288-290). The lack of mutations in the founding viruses population as viraemia reaches its peak implies there are no $CD8^+$ T cell responses driving mutation in this period of increasing viraemia (76, 291). The initial CTL responses, until viraemia reaches its viral set point, are very narrowly directed, targeting one to three epitopes of higher entropy, predominantly from the Env and Nef regions of HIV-1 (272, 292-295). Nonetheless, these initial narrow CTL responses are effective, causing a significant decrease in viraemia and driving escape mutations. Viral mutations, mostly selected by the CTL response, begin soon after viraemia reaches it peak level and continues as viraemia declines to the viral set point. Within 10 days, these mutations were found to replace the original sequence, allowing them to escape recognition by CTLs. This pattern continues throughout the course of the infection, with mutations at different epitopes developing, generating new escape

mutants (76, 94, 272, 296). Mathematical models estimate CTLs of a single specificity can kill 15-35% of infected cells prematurely per day, thus reducing viral production during acute infection (272). Mutations in the sequence of the virus can occur within CTL epitopes, or in regions flanking epitopes, affecting antigen processing and recognition (297, 298). Responses to more conserved epitopes from Gag and Pol arise in later waves of CTL responses. These immunodominant responses can lower the level of viraemia at the set point and can incur a fitness cost on escape mutant viruses. Therefore, the level of set point viraemia is influenced by the fitness of the transmitting virus and the specificity of the $CD8^+$ T cell response (290).

During chronic infection $CD8^+$ T cell responses have greater breadth, targeting an average of 14 epitopes simultaneously as they maintain the viral set point. However, the killing rate of target cells declines significantly from that seen in acute infection, with the majority of infected cell death being caused by viral cytopathicity or cell activation. The magnitude and breadth of $CD8^+$ T cell responses are higher in individuals with a progressive infection (286, 299-302). The specificity of $CD8^+$ T cell responses in the chronic phase has been shown to influence viral load, as individuals with broad responses specific to Gag epitopes have lower viral loads than individuals with Env specific responses (303-306). The types of HLA molecule determine the specificity of the response and are strongly associated with responses either being protective or non-protective.

Several HLA types have been identified to confer strong immune responses and control of HIV-1 i.e. HLA molecules B57, B27, B58, B81 and B14. They mainly target Gag epitopes that are relatively conserved. Specific T cell responses against these conserved epitopes are more favourable to the host as they are efficient in viral control or forcing mutations that incur fitness cost i.e. loss of virulence in escape mutants (304, 307-311). This strong link between HLA class I alleles and viral fitness is well documented and can be seen in LTNP and elite controllers. Therefore, efficacy of CTL responses is due to

both, inhibition of viral replication through the killing of infected cells and blocking viral infections, and inducing mutations with fitness cost. Studies of elite controllers whom express these protective alleles are able to maintain viral load at low levels without therapy. Also these studies have revealed the viruses are considerably less fit and these mutations impairing fitness were selected early on in the infection (304, 307, 309, 311-313). Compensatory mutations do arise later on to restore viral fitness, but this can be a slow process. However these compensatory mutations can lead to the deletion of an epitope without loss of function, and these new viruses can be transmitted and would escape recognition. Therefore these specific protective CTL responses can drive the evolution of more efficient escape mutants, which has been noted in certain populations (314-319).

During the acute infection phase, $CD8^+$ T cell antiviral function is mostly cytolytic. They do not produce high amounts of cytokines or chemokines, but rather they are high perforin expressors (94, 320-323). In the chronic stage of infection CTL responses are more polyfunctional and produce cytokines and chemokines along with cytolytic factors in elite controllers. It is hypothesised the prolonged antigen stimulation and the lack of excess activation and immune exhaustion enables $CD8^+$ T cells to produce these cytokines. Effective control of viraemia in the acute phase through CTL responses or therapy seems to enable the emergences of polyfunctional $CD8^+$ T cells producing IL-2, IFN-$\gamma$, TNF-$\alpha$, CCL3, CCL4, CCL5 and CD107a (324, 325).

The importance of $CD8^+$ T cells in the control of HIV-1 infection is evident based on: (i) decline of viraemia during acute infection to the set point level. (ii) Immunological pressure exerted by $CD8^+$ T cells leading to the development of escape mutants during the acute and chronic stages of the infection. (iii) Correlation between HLA class I alleles and viral control in elite controllers and LTNP (iv) $CD8^+$ T cell depletion studies in rhesus macaques results in complete loss of viral control.

## 1.3. HIV-1 Vaccine Development

Despite the great strides made in HIV-1 research the past 30 years, there is still no cure or prophylactic vaccine developed. Antiretroviral drugs are the only treatments available in combating HIV-1, controlling infection and slowing disease progression by interfering with viral replication (326, 327). Antiretroviral drugs are administered in a cocktail combination, known as highly active antiretroviral therapy (HAART), targeting different mechanisms of HIV-1 replication (328-330). These drugs are classed into six groups; (i) nucleoside/nucleotide analogue reverse transcriptase inhibitor (NRTI) block viral reverse transcriptase activity by competing with natural deoxy-nucleotides, and being incorporated into the viral DNA, terminating the growing of the viral DNA chain (331); (ii) non-nucleoside reverse transcriptase inhibitors (NNRTI) bind directly to the reverse transcriptase protein, inducing conformational changes to the substrate binding site and reducing the activity of the DNA polymerase (332, 333); (iii) integrase inhibitors (INI), also known as integrase strand transfer inhibitors (InSTI), bind exclusively to the complex between the viral DNA and integrase, and interact with magnesium in the active site of integrase and the viral DNA (334, 335); (iv) protease inhibitors (PI); (v) fusion inhibitor disrupt the binding of homologous proteins in the gp41 protein and prevent fusion of the virion to the cell membrane (336); (vi) co-receptor antagonist, are small molecule CCR5 antagonists inducing conformation of the co-receptor and inhibit Env binding (337). As for preventative measures, educating the public and the use of condoms have been the two most effective tools. The use of condoms is arguably the single most important invention in the fight against HIV-1 infection, reducing the risk of transmission by 95% (338). Yet also the use of pre-exposure prophylaxix (PrEP) or treatment as preventative (Tasp) and the used of microbicides are also emerging as an effective method of HIV control.

### 1.3.1 Challenges in developing an effective HIV-1 vaccine

The development of an effective prophylactic vaccine would be the best solution to the AIDS pandemic. The most effective way of preventing disease transmission is vaccination. Vaccination is also a more economically viable solution to anti-retroviral and would be more accessible to people, specifically is poorer countries and would have less harmful side effects than life time drug treatments. Several key challenges have hindered the development of such vaccine for HIV-1, and they are; (i) viral clade and sequence diversity; (ii) viral mutation and evasion of humoral and cellular immune responses; (iii) viral latency; (iv) infection of immune cells and compromising the immune system (v) lack of clear immune correlates of protection; (vi) ineffectiveness of eliciting broadly neutralising antibodies; (vii) dangers of using attenuated viruses in humans; (viii) lack of small-animal model. Some of these challenges posed in developing have been discussed earlier in depth.

The enormous genetic diversity within the HIV-1 sequence is due to several factors; (i) mutations introduced during replication due to the high error rate of the reverse transcriptase enzyme, yielding at least $3.5x10^{-5}$ per base pare per replication cycle (339, 340); (ii) high viral turnover rate, generating $10^9$ virions per day (341); (iii) retroviral recombination within and between different subtypes (342); (iv) immune pressure selecting for mutations (94, 165). Based on the genetic diversity, HIV-1 is classed into three groups, M, O, N and P (discussed earlier). The M group of HIV-1, responsible for 95% of the world infection can be further subdivided into nine groups or clades (A, B, C, D, F, G, H, J, K) distributed geographically (343, 344). Variation between clades can be as high as 30% in the variable Env region and 15% in the more conserved Gag region (343, 344). This genetic diversity is the biggest challenge in the development of a vaccine.

When HIV-1 virus particles infect a cell, part of their replication cycle is integrating their DNA into the hosts' genome. When target cells survive virus cytopathy, the virus can

remain within the cell in a latent state, establishing a latent reservoir (63, 89). In cells such as macrophages, more resistant to the cytopathic effects of the virus, infection is permanent and can persist the life span of the cell, and as the cell is activated more virions are produced (63, 148, 152). These latent reservoirs cannot be eradicated by HAART and persist throughout the infection (89).

An effective HIV-1 vaccine would ideally induce both humoral and cellular mediated immunity. However, this has been an impossible task for HIV-1, therefore, research and has been divided into developing two types of vaccines inducing either of the two immune arms.

### 1.3.2 Antibody Inducing Vaccines

Historically, effective prophylactic vaccines such as those against smallpox, measles, polio and hepatitis A, all induce potent antibody responses, targeting invariant molecular components that block infection. As for HIV-1, vaccine induced antibodies target the Env glycoprotein, the most variable part of the virus, whereby sequences from circulating strains can differ by 35% (344). Besides the high mutation and diversity of the virus, the envelope glycoprotein displays a small number of spikes on its surface, with a high density of shifting host derived N-glycans shielding the vulnerable sites. These glycans do not induce a response as they are recognised as self by the humoral immune response (167, 345, 346). Binding of gp120 to CD4 on the target cells induces substantial conformational changes to the glycan shield, exposing more glycan free viral spikes. However, these potential target viral spikes are protected by the close proximity of the virus bound to the target cell, restricting access of antibodies (347). Therefore, any vaccine developed would need to induce high affinity antibodies that can bind effectively to sparsely and diversified spikes and able to penetrate the glycan shield.

High-throughput screening of sera antibodies from a large cohort of infected individuals has revealed that 10 – 30% of infected individuals produced highly effective cross-reactive NAb. Amongst that population, 1% are elite neutralisers, producing bNAb with high potency and cross-clade activity (169, 348, 349). Passive infusion of HIV-1 NAbs have been shown to protect rhesus macaques from infection by a chimeric simian-human immunodeficiency virus (SHIV) inoculated orally or intravaginal (350-352). This has ushered the identification of new bNAbs and their targets "sites of vulnerability" to develop new vaccines. Vulnerable sites (epitopes) on the glycoprotein of HIV-1 have been identified as potential targets; (i) CD4 binding site (CD4bs), a conserved region of gp120; (ii) N160 glycan dependent sites associated with variable loops of gp120 (V1, V2); (iii) N332 glycan dependent sites associated with V3 of gp120; (iv) membrane-proximal external region (MPER) of gp41; (v) outer domain glycan (352-359). A number of new potent bNAbs have also been identified, targeting these vulnerable sites with neutralisation breadth of 70-90% of circulating strains and have been shown to block infection in rhesus macaques (table 1.2) (352-355, 357, 360, 361). A key feature of all these bNAbs, regardless of the target, is a high degree of somatic hypermutation, a result of multiple rounds of germinal centre selection. These somatic mutations are essential to their binding breadth and potency of their neutralisation activity (361, 362).

Based on these findings, new vaccines are being developed to induce potent cross-reactive bNAbs. However, there are still a lot of uncertainties about how these bNAbs develop. One issue concerns why these antibodies only develop in a few and not all infected individuals? This revealed that the development of bNAbs may not be determined by host genetic factors, but rather are determined by the Env sequence of the transmitting virus (359, 363). A study in rhesus macaques support this hypothesis, as two groups of macaques infected with two strains of SHIV, having different Env sequences, develop different NAb responses, whereby one group elicited cross reactive NAb while the other

group did not (363). In humans, observations have been noted in superinfected individuals who developed bNAbs to only one of the two viruses present, thus suggesting the sequence of the virus was the mitigating factor in eliciting the response (359). Also, superinfected patients have higher chances of developing bNAbs due to the larger number of Env sequences they encounter, thus increasing the chances of encountering the catalyst sequence that initiates there production (364).

Two approaches proposed to develop bNAbs inducing vaccines are; design computational immunogens based on the sequence and structure of the bNAbs and their ligands to specifically activate B cells expressing the germline precursor of bNAbs (365-367). The second approach is to use HIV-1 Env proteins from elite neutralisers, and try to reproduce the natural pathway that led to the development of these bNAbs with neutralising breadth and potency. This can be achieved by developing a sequence of vaccines that would simulate Env evolution, which would activate first B cells, then guide the ones expressing the correct germline antibody to evolve into bNAbs (368-371).

**Table 1.2. bNAbs targeting HIV-1 Env epitopes (319)**

| Epitope | Antibody | Year reported | Comments | *In vitro* efficacy* | *In vivo* efficacy |
|---|---|---|---|---|---|
| CD4bs | b12 | 1991 | First reported HIV-1bnAb targeting the CD4bs | Neutralizes 41% of 190 HIV-1 isolates tested [24] | Protects macaques against SHIV vaginal challenge |
| | VRC01-03 | 2010 | VRC01-02closely mimic CD4 and enhance 17b binding to gp120 | VRC01-02 and VRC03 neutralize about 91% and 57% of 190 HIV-1 isolates tested, respectively [24] | VRC01 protects macaques against SHIV mucosal challenge |
| | NIH45-46 | 2011 | A clonal variant of VRC01 | Neutralizes 96% of 118 HIV-1isolates tested [25] | NA |
| | 3BNC117 | 2011 | | Neutralizes 96% of 118 HIV-1 isolates tested [25] | NA |
| | VRC-PG04 | 2011 | | Neutralizes 76% of 178 HIV-1 isolates tested [27] | NA |
| | A12 | 2008 | First reported llama VHH targeting the CD4bs | Neutralizes 42% of 65 HIV-1 isolates tested [34] | NA |
| | J3 | 2012 | Llama VHH | Neutralizes 96% of 100 HIV-1 Isolates tested [35] | NA |
| CoRbs | m36 | 2008 | First reported human VH against HIV-1 | Neutralizes 10 of 11 HIV-1 isolates tested with IC50s < 10 μg/ml [33] | m36.4, an affinity-matured version of m36, protects four of six humanized mice against high-titer HIV-1 intrasplenic challenge |
| Carbohydrate | PGT125-128, 130-131 | 2011 | | Neutralize 40 - 72% of 162 HIV-1 isolates tested [28] | NA |
| | 2G12 | 1996 | First reported HIV-1 bnAb targeting carbohydrate | Neutralizes 32% of 162 HIV-1 isolates tested [28] | In combination with 2F5, reduces viral loads and increases CD4+ T cell counts in HIV-1-infected individuals |
| Quaternary structure | PG9, PG16 | 2009 | | Neutralize 79% and 73% of 190 HIV-1 isolates tested [23] | NA |
| | PGT141-145 | 2011 | | Neutralize 38 - 78% of 162 HIV-1 isolates tested [28] | NA |
| MPER | 2F5 | 1992 | First reported HIV-1 bnAb targeting gp41 | Neutralize 57% of 177 HIV-1 isolates tested [29] | See 2G12 *in vivo* efficacy |
| | 4E10 | 2001 | | Neutralizes 98% of 181 HIV-1 isolates tested [29] | In combination with 2F5 and 4E10, delays viral rebound in HIV-1-infected individuals who stopped HAART before antibody infusion |
| | 10E8 | 2012 | Non-autoreactive | Neutralizes 98% of 180 HIV-1 isolates tested [29] | NA |
| | m66.6 | 2011 | Less mutated than other bnAbs targeting MPER | Neutralizes 24% of 164 HIV-1 isolates tested [30] | NA |

### 1.3.3 T-cell Based Vaccines

Although T cells cannot prevent infection, an effective T cell vaccine can control HIV-1 progression, as evident in elite controllers and the mutational escape pressure they exert on the virus. As with antibody inducing vaccines, the primary challenge is the high mutation rate and diversity of HIV-1. An effective T cell vaccine needs to induce responses to HIV-1 of different clades and needs to counteract escape mutations. Several approaches have been tested to overcome this challenge.

The simplest and most common approach initially utilised was using a natural protein isolate, from a single clade, with a sequence closest to other isolates. The aim was to develop a vaccine for a certain subtype and induce sufficient cross-reactive T-cell response against epitope variants of the same and other clades. This was supported by cross-reactive T cell responses seen in infected individuals (372-375). In a large international cohort study, individuals infected with HIV-1 of different clades were able to induce cross-reactive T cell responses to HIV-1 Gag and Nef from clade A, B and C (374). This was also noted in another study in Botswana and Cameroon, where infected individuals mounted cross reactive T cell responses to HIV-1 Gag, Pol and Nef. Anti-Nef responses were the most cross-reactive of all three in both studies (374, 375). Furthermore, individuals expressing HLA-B*57 had cross-reactive $CD8^+$ T-cells against a p24 epitope (KAFSPEVIPMF), inducing efficient responses to the different variants of the epitope (373). However, these cross reactive responses were observed after long periods of infections and will not necessarily be mimicked by vaccination in terms of magnitude, breadth and depth. Even if these vaccines where able to induce some cross-reactive T cell responses, it will most likely be partial and sufficient. Furthermore, single amino acid mismatches in epitopes are sufficient for viral escape and evasion of highly specific CD8 T cell responses (344, 376-378).

A second approach is delivering a vaccine with a cocktail of antigens from multiple clades. In mice, a polyfunctional, cross-clade cellular response against Env was noted in the group vaccinated with an Env protein from two clades, in a prime boost regimen (379). Results from several human clinical trials have also been encouraging with cross-clade responses to each antigen observed (380-384). A multi clade DNA prime, recombinant adenovirus 5 boost vaccines, encoding Gag, Pol and Nef from clade B, and Env from clade A, B and C, evaluated in healthy individuals from multiple countries, was shown to induce cellular response against all antigens, based on epitopes from group M (380-382, 384). Although results have been encouraging using these types of immunogens, immune interference and epitope antagonism may be limiting factors. This could arise between different peptide sequences in the vaccine that are closely related. Antagonism occurs when a response induced to an agonist epitope, is simultaneously exposed to an antagonist epitope variant, thus interfering with the induction of a T-cell response to the agonist epitope and leading to a defective response (385-387).

A third approach is utilising centralised sequences for immunogen design to overcome diversity. This can be done using one of three strategies. The first type is a phylogenetically reconstructed sequence known the most recent common ancestor (MRCA). Using ancestral sequences to predict the most likely base in each position in an alignment (388, 389). The second strategy is centre-of-tree sequence (COT), whereby the average evolutionary distance of each tip of a phylogeny tree is minimised (390, 391). The third type is a consensus sequence, where the most common amino acid at a given position in sequence is predicted to a common clade or group. This approach aims to minimise the sequence differences between circulating viruses and the vaccine immunogen (344, 392-394). This approach has been utilised by our group when designing HIVconsv immunogen (discussed later) (395).

Another approach being utilised is targeting cellular immune responses to conserved regions of HIV-1, such as Gag and Pol. Targeting these regions would increase the chance of inducing cross-reactive T cell response, as these regions are conserved among the different clades. Furthermore, mutations within these conserved regions would incur a fitness cost to the virus, therefore, targeting them would either induce efficient viral control or force escape mutants that are less virally fit (308, 395, 396). As noted in elite controllers and LTNP, their effective control of the virus is due to HLA alleles targeting CTL response to conserved regions of HIV-1 (99, 314, 397). Furthermore, natural responses are directed predominantly to variable regions of the virus, which are immunodominant, thus permitting escape and disease progression (294). Also, trials of HIV-1 vaccines using natural isolates have noted responses directed to more variable regions and therefore were less effective. Thus by designing immunogens targeting conserved regions, the vaccine induced immune response can shift the immune-dominance profile and focus the initial response on conserved regions (395, 396, 398). This approach has been employed by our group in the design of the HIVconsv vaccines and the vaccines that I have developed throughout this project (discussed later). Finally, there are polyvalent mosaic vaccines, a computationally designed centralised protein, engineered to induce broad T cell response with greater breadth and depth. The vaccines discussed in my thesis are conserved mosaic vaccines and will be discussed further in the results chapters.

Another aspect of vaccine design is the vector used, as it directs the type and magnitude of the response induced. There are many vectors employed in vaccine designs, ranging from plasmid DNA to viral vectors (discussed in results). But one particular new and very exciting vector worth mentioning is cytomegalovirus (CMV). Unlike other vectors, CMVs persist in the host and are able to maintain high frequency differentiated antigen specific T cell circulating and in tissue (399-401). CMV, when used as a vector has been shown to induce effector memory T cells ($T_{EM}$ cell), which unlike other T cells, they

are localised in effector sites and can immediately mediate antiviral activity; lacking the need for phase expansion, effector differentiation and travel to site of infection for activation to mediating antiviral activity (399-403). $T_{EM}$ cell long term maintenance is associated with persistent antigen presentation and T cell response to chronic infection, particularly CMV. A recent study tested their efficacy in rhesus macaques, whereby they were vaccinated with RhCMV encoding SIV Gag, Pol, Rev, Nef, Tat and Env and challenged with a highly pathogenic SIV intrarectally or intravaginally. Half of the challenged monkeys illustrated unprecedented effective control of viraemia to undetectable levels, in contrast to the control group which all manifested high viral load and fast disease progression (399, 400). Tissue analysis were able to detect low levels of SIV RNA in tissue up to 20 weeks post infection only, thereafter detection was very difficult, even after $CD8^+$ T cell depletion. Furthermore, vaccinated monkeys that mediated control were indistinguishable from uninfected animals 1.5-3 years post infection, suggesting complete immune clearance of the virus (400). These data strongly support the use of CMV as vectors and maintaining a $T_{EM}$ cell population would induce a strong and effective control of HIV-1, potentially leading to complete clearance.

### 1.3.4 Animal models for HIV-1 research

One of the major obstacles in HIV-1 research and vaccine development is the lack of animal models that can recapitulate all of the features of an infection. African chimpanzees are the only animals available that host a similar virus, SIVcpz, the ancestral virus of HIV-1. However, they are not a practical model for AIDS as they are an endangered species, high cost and maintenance and do not develop disease like humans from SIVcpz. There are however a few alternative models that have been developed over the years facilitating HIV-1 research, although not perfect.

For non-human primates, Asian monkeys, in contrast to African monkeys, are not natural hosts to SIV, and infection with different strains of SIV causes the development of disease like AIDS. Several species of Asian macaques are used as models for HIV/AIDS i.e. rhesus macaques, pig-tailed macaques and cynomolgus macagues. Rhesus macaques are the most used and best characterised non-human primate model for AIDS. They are most commonly infected with either SIVmac251 or SIVmac239, resulting in a high viral load, $CD4^+$ T cell depletion in the GALT and progression to AIDS within 1-2 years (27, 84, 404). They can also be infected with SHIV expressing proteins from HIV-1, most commonly Env but Pol has also been expressed (405, 406). Rhesus macaques have two types of MHC, *Mamu-A* encoded by four genes (correspond to HLA-A) and *Mamu-B* encoded by an undefined number and variable of genes (correspond to HLA-B) (407). As with humans, their MHC alleles are associated with viral control e.g. *Mamu-A1*001, Mamu-B*008 and Mamu-B*017* (408-410). Rhesus macaques have offered us great insight and better understanding of HIV-1, and they have been used extensively in vaccine development as a challenge model. However, since SIVsmm and SIVmac differ by 47% in nucleotide identify and in the ORF organisation from that of HIV-1, results noted in challenge studies do not translate directly to humans (411, 412).

As for small animals models such as mice, rats and rabbits, there are no strains of HIV-1 or other related viruses causing AIDS that can infect them. Cats are the only small animals that naturally host a similar virus, feline immunodeficiency virus (FIV), however, they have limitation i.e. lack of accessory gene found in HIV-1 and containing ORF absent from primate lentiviruses, uses CD134 rather than CD4 receptor thus infecting B cells also, and its chronic phase period differs from that of HIV-1, and are therefore not widely used (413). The advancement of genetic manipulation has facilitated the development of different transgenic mice that can be used for HIV-1 research. One such example are transgenic mice expressing human HLA's (414-417). Transgenic mice have been engineered to express chimeric forms of HLA-A*02:01, A*24-02, B*08:01, B*27:05, B*35:01, B*07:02 and many more (415-417). These transgenic mice can be used in vaccine development, testing responses to known human epitopes. This can be vital in determining what type of response the vaccine are inducing in relation to the HLA's and epitopes (414).

Humanised mice, are a genetically immunocompromised mouse engrafted with human tissues to reconstitute the human immune system. There are several types of humanised mice, differing by the tissue they have been engrafted with. For vaccine development, the best model is BLT mouse. These are, non-obese diabetic (NOD) severe combined immunodeficiency (scid) mice (NOD scid) or NOD scid gamma mice, implanted with human fetal thymus, liver cells and bone marrow transplant with human $CD34^+$ cells (BLT: bone marrow-liver-thymus) (418). They can be infected with HIV-1 through inoculation of mucosal tissue, resulting in T cell depletion. They induce both viral specific cellular and humoral immune responses. Also, viral escape was noted in BLT mice in response to certain HLA's, demonstrating that CTL responses in BLT mice are functional and exert selection pressure on HIV-1 (419-421). BLT mice offer a good model HIV-1 research and vaccine development.

### 1.3.5 HIV-1 Vaccine Clinical Trials

The first clinical trial against HIV-1/AIDS was initiated in 1986 by Zagury *et.al* (422, 423). Their vaccine was developed used a vaccinia vector encoding gp160 protein of HIV-1 and was tested in Zaire (422, 423). Since then, over 200 vaccine trials have been conducted world wide against HIV-1, trying to induce T cell responses, antibody responses or both. A few vaccine candidates have shown some immunogenicity in the initial stages and have progressed to efficacy trials (table 1.3) (424).

The world's first HIV-1 vaccine phase III efficacy trials were conducted in 1999-2003, Vax003 and Vax004 (425-427). Both studies tested the efficacy of AIDSVAX B/E and AIDSVAX B/B (VaxGen), a monovalent subtype B, bivalent subtype B/E or subtype B/B recombinant gp120 (rgp120) vaccines (425-427). The Vax003 trial was conducted in Thailand on 2527 HIV-1 uninfected injection drug users, using the AIDSVAX B/E vaccine (425). The Vax004 trial was conducted in North America and The Netherlands on 5403 HIV-1 uninfected individuals, including 5095 non-injecting drug using men who have sex with men (MSM) and 308 women at high risk of heterosexual transmission (426). Both vaccines induced a robust humoral immune response, producing binding and neutralising antibodies against gp120, but no reduction in HIV-1 transmission and thus had no efficacy (425-427).

The STEP (HVTN 502 / Merk 023) and Phambili (HVTN 503) studies were the first phase IIb efficacy vaccine trials of a HIV-1 prophylactic T cell based vaccine (428, 429). The aim was to test whether a prophylactic T cell vaccine could induce potent CTL response preventing infection or controlling infection and reducing viraemia post infection (428-430). The MRKAD5 were a recombinant non-replicating human adenovirus serotype 5 (rAd5) vectored vaccines, encoding one of Gag, Pol and Nef HIV-1 genes of clade B. The STEP trial was conducted in North and South America, Caribbean and Australia, enrolling 3000 HIV-1 negative individuals but of high risk of infection including MSM and

heterosexual women and men at high risk (428). The Phambili study was conducted in South Africa enrolling 801 of a scheduled 3000 HIV-1 negative heterosexual men and woman (429). The STEP trial was terminated prematurely on futility grounds due to lack of efficacy, after 30 individuals became infected with HIV-1, with the rate of infection being higher in the vaccinated group than in placebo (428-430). As a result the Phambili trial was also halted for safety concerns. Although preclinical data were very promising in monkeys challenged with SHIV, the vaccine was shown to have no affect on viral transmission or viral control in comparison to the placebo group (431). A similar outcome occurred once again in another phase IIb efficacy study, the HVTN 505 clinical trial (384, 432). The clinical trial employed a prime boost regimen using two vectors. The prime vaccines were six plasmid DNA vectors encoding Gag, Pol and Nef of HIV-1 clade B, and Env protein from HIV-1 clade A, B and C; and a boost using four rAd5 vectors encoding a Gag-Pol fusion protein of HIV-1 clade B, and Env protein of HIV-1 clade A, B and C, aimed at inducing an antibody and T cell response (384, 432). The trial was conducted in the United States using HIV-1 negative circumcised men and transgender woman who are at high risk of infection. As with the previous studies, the HVTN505 trial was terminated prematurely due to the high number of infected individuals, 41 participants in the vaccine group and 31 in the placebo group, thus showing lack of efficacy (432). The termination of these trials cast a doubt on T cell vaccines and more so on adenovirus vectors.

The only HIV-1 vaccine clinical trial to show any efficacy was the RV144 phase III study. The RV144 trial consisted two vaccines, ALVAC-HIV and AIDSVAX B/E, used in a prime regimen boost. The prime was four injections of the ALVAC-HIV vaccine, a recombinant canarypox vector expressing Gag-Pro and gp41 transmembrane domain from HIV-1 clade B, and Env gp120 protein from HIV-1 CRF01_AE clade E; and two booster injections of AIDSVAX B/E vaccine, a bivalent vaccine expressing rgp120 (described above) (433, 434). The trial was conducted in Thailand on 16,402 HIV-1 negative

volunteers of both sexes, heterosexuals of low risk of infection. The vaccine was designed to induce both antibody and cell-mediated immune response. The trial achieved a 31.2% efficacy, with 74 HIV-1 infections in the placebo group compared to 51 infections in the vaccinee group. Protection was achieved through antibody responses against V1/V2 loop in Env by non-neutralising antibodies and ADCC (433, 434). To date, this is the first vaccine showing any sign of efficacy or protection, thus giving hope that a protective vaccine against HIV-1 could be developed.

**Table 1.3. Phase II and III HIV-1 vaccine efficacy trials (382)**

| Category | Study protocol | Candidate vaccine | Phase | Volunteers | Location | Result |
|---|---|---|---|---|---|---|
| Pox-protein | RV144 | ALVAC-HIV vCP1521/AIDSVAX MN-CM244 rgp120 (CRF01_AE, B) | III | 16,403 | Thailand | 31% efficacy |
| Pox-protein | HVTN203 | ALVAC vCP1452/MN-GNE8 rgp120 (B) | II | 330 | US | Interferon-γ ELISpot in 16% of volunteers |
| Pox-protein | HIVNET 026 | ALVAC vCP1452/MN rgp120 (B) | II | 200 | Multinational | Not immunogenic |
| Pox-protein | AVEG 202/ HIVNET 014 | ALVAC-HIV vCP205/SF2 rgp120 (B) | II | 420 | US | $CD8^+$ T cells in 33% of volunteers |
| DNA-pox | IAVI 010 | DNA-HIVA/MVA-HIVA (A) | IIa | 115 | East Africa | Rare interferon-γ ELISpot responses |
| DNA-pox | HVTN 205 | GeoVax JS7 DNA/MVA HIV62 (B) | II | 225 | US, Peru, RSA | Ongoing |
| DNA-Ad5 | RV 172 | DNA (VRC-HIVDNA016-00-VP)/ rAd5(VRC-HIVADV014-00-VP (A, B, and C) | I/IIa | 324 | East Africa | Interferon-γ ELISpot in 63% of volunteers |
| DNA-Ad5 | HVTN 204 | DNA (VRC-HIVDNA016-00-VP)/ rAd5(VRC-HIVADV014-00-VP (A, B, and C) | IIa | 480 | Americas, RSA | Interferon-γ ELISpot in >60% of volunteers |
| DNA-Ad5 | HVTN 505 | DNA (VRC-HIVDNA016-00-VP)/ rAd5(VRC-HIVADV014-00-VP (A, B, and C) | IIb | 1350 | US | Ongoing |
| DNA-Ad5 | HVTN 502/ Merck 023 | MRKAd5 HIV-1 gag/pol/nef (B) | IIb | 3000 | US | No efficacy; transient infection risk |
| DNA-Ad5 | HVTN 503 | MRKAd5 HIV-1 gag/pol/nef | IIb | 3000 | RSA | No efficacy |
| Pox-protein | ACTG 326; PACTG 326 | ALVAC vCP1452/AIDSVAX B/B | I/II | 48 | US | Safe, poorly immunogenic |
| Peptide | ANRS VAC 18 | LIPO-5 | II | 156 | France | $CD8^+$ responses in >60% |
| Protein-protein | AVEG 201 | rgp120/HIV-1 SF-2/MN rgp120 | II | 296 | US | NAb in 87%, DTH to gp120 in 59% of volunteers |
| AAV | IAVI A002 | tgAAC09 | II | 84 | RSA, Uganda, Zambia | Interferon-γ ELISpot in 25% of volunteers |
| Protein | VAX 003 | AIDSVAX B/E | III | 2500 | Thailand | No efficacy |
| Protein | VAX 004 | AIDSVAX B/B | III | 5400 | US | No efficacy |

## 1.4. HIVconsv Vaccine

In efforts to develop a universal HIV-1 vaccine, Tomas Hanke's group have developed a new chimeric protein immunogen termed HIVconsv (conserved). HIVconsv is a T cell inducing immunogen, it was assembled from 14 of the most highly conserved regions in HIV-1 group M, clades A, B, C and D, irrespective of whether they contained known T-cell epitopes or not (Fig. 1.7). Nonetheless, HIVconsv contains 270 known T cell epitopes, with 192 (71%) epitopes having an identical amino acid sequence to known epitopes and 59 (22%) epitopes differ by a single amino acid (395). HIVconsv gene is 2.5 kbp in size with each protein is based on the centralised consensus sequence from one of the four clades. Because the regions are so highly conserved, often the employed consensus sequence of one clade would match the consensus sequence of other clades, enhancing the probability of cross-clade T-cell responses. Furthermore, the chimeric protein is assembled from protein regions rather than epitopes, enabling broader coverage of overlapping epitopes presented by multiple HLAs (395). The use of alternating clades for each segment sequentially in a string enables a broader T cell response, covering different geographical regions, also deals with intra-clade variability and avoids immune interference such as epitope antagonism (Fig 1.7) (395). HIVconsv is expressed using three different vectors; a plasmid DNA.HIVconsv (D), MVA.HIVconsv (modified vaccine Ankara) (M), and ChAdV63.HIVconsv (C) (chimpanzee adenovirus serotype 63).

In natural infection, epitopes expressed by HIVconsv are subdominant and have a low entropy value in comparison to the dominant variable epitopes. Although subdominant, T cell responses to some HIVconsv epitopes were noted in natural infection (395). Nonetheless, this is an advantage, as the vaccine will alter the natural immune-dominance and prime T-cells to low entropy conserved epitopes, whereby viral mutations to escape these responses will incur a cost on the viruses fitness (292, 297). This has been noted in

studies where responses against sub-dominant epitopes correlate with good virological control (435).

Results from a current phase I/IIa trial, HIV-CORE 002, showed HIVconsv to be safe in human, and elicited strong $CD4^+$ and $CD8^+$ T cell responses (396, 436). The trial was conducted in Oxford in HIV-1 negative volunteers using one of three prime boost regimens, CM, DDDCM and DDDMC. All vaccinees induced responses to all three regimens with unprecedented high levels of HIV-1 specific T cell responses. These responses were not detected in any of the placebo volunteers (396, 436). Responses were induced to 6 pools of peptides and to estimated 10 epitopes per individual, whereby 60% of the responses were to known $CD8^+$ epitopes. These results show that HIVconsv was able to induce responses of great magnitude and with good breadth, which is a main objective of this vaccine (396). More importantly, results from a virus inhibition assay, whereby vaccine induced $CD8^+$ T cells were tested against infected $CD4^+$ T cells revealed viral inhibition from the vaccine recipients. Viral inhibition was mostly driven by $CD8^+$ T cell responses against Gag but more so against Pol proteins (396). Although protective responses are predominant targeted at Gag in infected individuals and Pol less so, these results reveal that Pol specific $CD8^+$ T cell responses are sufficient in recognising infected cells and inhibiting replication (303, 396, 437). These results are unprecedented for a HIV-1 T-cell based vaccine and are very encouraging as a prophylactic or therapeutic vaccine.

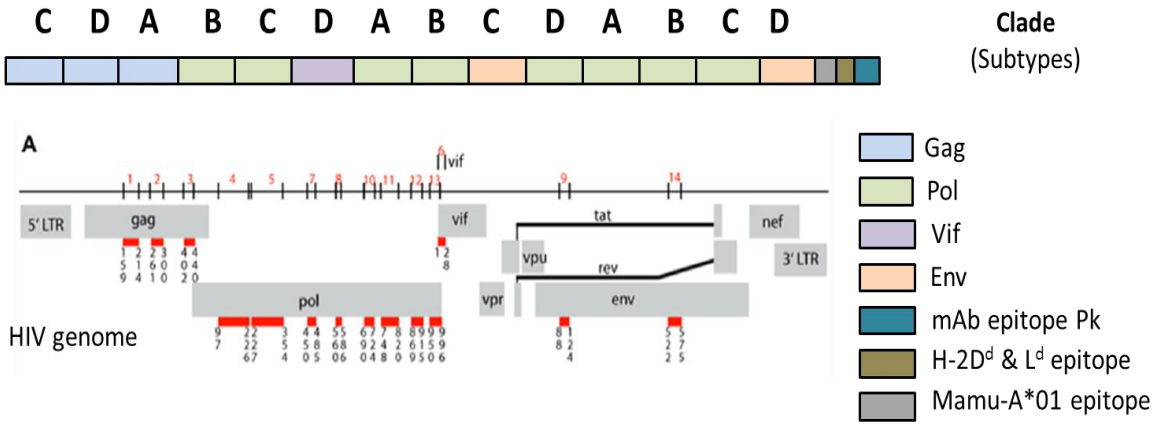



**Figure 2.7. The HIVconsv immunogen** gene and the 14 most conserved regions in HIV-1 proteome used to construct it. The letters A, B, C & D above each segment represent the clades each segment was selected from (modified from Ref 354).

## 1.5. Candidate Vaccines / Aims and Objectives of My Study

A set of conserved mosaic immunogens have been developed for us by Bette Korber (Los Alamos National Laboratory, USA). The objective of my project was to determine whether using conserved mosaic polyvalent vaccines or combining a single mosaic vaccine with HIVconsv would enhance the breadth and depth of vaccine elicited T cells compared to that of the conserved consensus HIVconsv vaccine. The following aims were set out to answer my objective.

Aim 1: Construct the mosaic vaccines using a plasmid DNA, MVA and ChAdV63 vectors for each of the four mosaic immunogens.

Aim 2: Compare different vaccine combinations; HIVconsv vs HIVconsv + 1 mosaic vaccine vs trivalent mosaic vaccine cocktail in HHD transgenic mice and determine whether mosaic vaccines increase the breadth and depth of the response recognition of HLA-A2 epitopes.

Aim 3: Assess whether mosaic vaccines induce stronger responses with greater magnitude and cytolytic function (*ex-vivo* killing of peptide-pulsed target cells).

Aim 4: Determine whether the addition of the tissue plasminogen activator (tPA) leader sequence in a T cell based vaccines enhances the magnitude and specificity of the response.

# Chapter 2:

## *Materials & Methods*

# 2. Materials and Methods

## 2.1 Material2.1.1 Reagents

**Table 2.1** Commercially available reagents used in this study

| **Material** | **Supplier** | **Catalogue Number** |
|---|---|---|
| 0.2µm bottle top filter (Nalgene) | Fisher | TKV-240-015F |
| 0.2µm syringe filter | Sartorius | 16534 |
| 15ml centrifuge tubes | Fisher, Inc. | FB55950 |
| 2-β Mercaptoethanol [50µm] | Gibco | 31350-010 |
| 24-well tissue culture plates | Fisher, Inc. | TKV123011R |
| 2ml syringes | Nu-Care Products | M0185 |
| 50ml centrifuge tubes | Greiner | 227261 |
| 6-well tissue culture plates | Fisher, Inc. | TKT-520-030T |
| 60mm petri dishes | Corning | 430166 |
| 8-well microscope slides | VWR International | 631-0451 |
| 96-well U bottom plates | VWR International | 734-0027 |
| 96-well V bottom plates | Fisher Scientific | DIS-210-150L |
| Agar | Sigma Aldrich | A6686 |
| Agarose | Sigma Aldrich | A9539 |
| Ammonium chloride (NH4Cl) [0.15M] | Sigma Aldrich | A4514 |
| Ampicillin | Sigma Aldrich | A5354 |
| Bovine Serum Albumin (BSA) | PAA Laboratories | K41-001 |
| Caesium Chloride | Sigma Aldrich | 203025 |
| Carbonate-bicarbonate buffer | Sigma Aldrich | C3041 |
| Cell lifter | Fisher | FB55160 |
| Cell Scraper, sterile, 32cm | Nalge Nunc International Corp | 179707 |
| Cell strainer (70µm) | Falcon | 352350 |
| Cluster tubes | Corning | 4401 |

| | | |
|---|---|---|
| Corning conical tubes, 250 ml PP Centrifuge tube, plug seal cap | Fisher | CFT-900-021Y |
| DAPI (4',6-diamidino-2-phenylindole) | Sigma Aldrich | 101101353 |
| DH5α maximum efficiency cells | Invitrogen | 12297-016 |
| Diethanolamine buffer | Pierce | 34064 |
| Dimethyl sulphoxide (DMSO) | Sigma Aldrich | D2650 |
| Disposable Counting Slides | Immune Systems Ltd | BVS100 |
| DMEM Medium | Sigma Aldrich | D6546 |
| Dulbecco's Modified Eagle's Medium (DMEM) | Sigma Aldrich | D6546 |
| Dulbecco's PBS (D-PBS) | Sigma Aldrich | D8537 |
| ELIspot colour development kit | BioRad | 170-6432 |
| Endotoxin Free water | Sigma Aldrich | W3500 |
| Ethanol | Sigma Aldrich | 32221 |
| Ethidium bromide solution | Sigma Aldrich | E1510 |
| Ethylenediaminetetraacetic acid (EDTA) | Sigma Aldrich | E7889 |
| Fetal Calf Serum (FCS) | Sigma Aldrich | F-2442 |
| Gateway® LR Clonase® II enzyme mix | Invitrogen | 11791-020 |
| Glycerol | Sigma Aldrich | G5516-1L |
| GolgiPlug | Becton Dickinson Ltd. | 555028 |
| Human IFNγ ELISpot kit | Mabtech | 3420-2A |
| Hydrochloric Acid (HCl) [1M] | Sigma Aldrich | S-7653 |
| Immersion Oil | Sigma-Aldrich | 10890 |
| Intracellular cytokine staining antibodies | eBioscience | N/A |
| Isofluorane | Oxford University Veterinary Services | N/A |
| Kanamycin | Sigma Aldrich | K0254 |
| L-glutamine [4mM] | Sigma Aldrich | G-7513 |
| LB agar tablet | Sigma Aldrich | L7025-500TAB |

| | | |
|---|---|---|
| LB broth tablet | Sigma Aldrich | L7275-500TAB |
| Lipofectamine 3000™ | Invitrogen | L3000-015 |
| Magnesium chloride (MgCl2) | Sigma Aldrich | M2393 |
| MAIP ELIspot Plates | Millipore | MAIPS4510 |
| MEM α-modification | Sigma Aldrich | M-4526 |
| Methanol | Sigma Aldrich | 32213 |
| Microcentrifuge tubes | Fisher Scientific | FB74031 |
| Microscope slides | Fisher Scientific | MNJ-200-010H |
| MinElute gel extraction kit | QIAgen | 28606 |
| Minimum Essential Medium Eagle (MEM) α-modification | Sigma Aldrich | M4526 |
| Mouse anti c-Myc Tag - FITC | Acris Antibodies | SM1863F |
| Mouse anti His-Tag:Alexa Fluor 488 | AbD Serotec | MCA1396A488 |
| Mouse anti V5-Tag:Alexa Fluor 488 | AbD Serotec | MCA1360A488 |
| Mouse IFN-γ ELISPOT kit (ALP) | Mabtech | 3321-2A |
| Mr. Frosty cryocontainer | Fisher Scientific | CRY-120-010T |
| NH4Cl [0.15M] | Sigma Aldrich | A-4514 |
| PCR primers | Sigma Aldrich | N/A |
| Pen/strep [100U penicillin / 100μg strep] | Sigma Aldrich | P-0781 |
| Permeabilization Buffer (10X)□™ | eBioscience | 00-8333 |
| Phosphate Buffered Saline (PBS) | Sigma Aldrich | D-8537 |
| Phusion® High-Fidelity DNA Polymerase | NEB | M0530L |
| Qiagen plasmid Giga kit | Qiagen | 12191 |
| Qiagen plasmid maxi kit | Qiagen | 12165 |
| Qiagen plasmid midi kit | Qiagen | 12143 |
| QIAprep spin miniprep kit | Qiagen | 27106 |
| Quick Ligation™ Kit | NEB | M2200L |
| Restriction and Ligation Enzymes | NEB | Various |
| Ribonuclease A | Sigma Aldrich | R6513 |

| | | |
|---|---|---|
| RPMI-1640 Medium | Sigma Aldrich | R0883 |
| S.O.C. Medium | Invitrogen | 15544-034 |
| Smart Ladder DNA marker | EurogenTec | MW-1700-02 |
| Sodium Azide | Sigma Aldrich | S2002 |
| Sodium bicarbonate solution 7.5% | Life Technologies | 1423629 |
| SYBR Safe in DMSO | Invitrogen | S33102 |
| T-REx™ 293 cells | Life Technologies | R710-07 |
| T175 Cell culture Flask Corning | Fisher, Inc. | TKV123061C |
| T25 Cell culture Flask Corning | Fisher, Inc. | TKV123011R |
| T75 Cell culture Flask Corning | Fisher, Inc. | TKV123031L |
| Terrific Broth | Sigma Aldrich | T0918 |
| Tetracycline hydrochloride | Sigma Aldrich | T7660-5G |
| Transfer pipettes | Fisher Scientific | FB55348 |
| Tris-acetate-EDTA (TAE) | Fisher Scientific | BP1332-1 |
| Triton™ X-100 | Sigma Aldrich | X100 |
| Trypan Blue | Sigma Aldrich | T8154 |
| VECTASHIELD Hardset mounting media with DAPI | Vector Laboratories | H-1500 |

## 2.1.2 Solutions and Buffers

Solutions, buffers and cell culture media prepared in the Jenner institute as described below. Solutions are referred to accordingly in the methods section and later chapters.

- **Ammonium Chloride Potassium (ACK) Lysis Buffer:** 8.29 g NH4Cl (0.15 M), 1 g KHCO3 (1 mM), and 37.2 mg Na2EDTA, made up to 0.8 L in water. *Used in processing splenocytes.*
- **Coating Buffer:** 15 mM sodium carbonate and 35 mM sodium bicarbonate capsules were dissolved in $dH_2O$ and autoclaved.
- **Complete DMEM:** 500 ml Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 50 ml foetal calf serum (FCS) (10%), 5 ml L-glutamine (2mM) (1%), 5 ml pen/strep (100 U penicillin, 100 µg streptomycin) (1%) and 5 ml non-essential amino acid (NEAA) (1%).
- **FACS buffer:** PBS + 0.5% BSA + 0.05% Sodium Azide in PBS
- **LB Agar/Broth**: Tablets were dissolved in $dH_2O$ (1 tablet per 50 ml). Antibiotics were added at the following working concentrations: ampicillin 100 µg/ml, kanamycin 25 µg/ml.
- **Loading Dye (for DNA):** This was used, sometimes, in replacement of to the 6X loading dye described in the commercial kits table, above. It is made of 0.4% orange G, 15% Ficoll® 400, 10 mM Tris-HCl (pH 8.0), 50 mM EDTA (pH 8.0) made up with dH2O. *Used in Gel electrophoresis.*
- **Perm/Wash:** 10x Perm/Wash buffer was diluted in $dH_2O$ prior to use.
- **Phosphate Buffer Saline (PBS) – Tablet – 0.01M:** NaCl 0.138 M, KCl 0.0027 M, pH 7.4: made by dissolving tablets in dH2O.
- **RPMI-1640 Complete Medium (R10)**: 500 ml RPMI-1640 was supplemented with 5 ml L-glutamine (2 mM), 5 ml pen/strep (100 U penicillin, 100 µg streptomycin), 500 µl 2-mercaptoethanol (50 µm) and 50 ml of heat inactivated FCS or FBS (10%).
- **Triton X-100 in PBS (0.2%):** 100 ul Triton X-100 dissolved in 50 ml sterile PBS. Cut of end of pipette tip to dispense this viscous detergent.

### 2.1.3 Sequencing Primers

**Table 2.2** Sequencing primers designed using Primer 3 software for the sequencing of the various immunogens. Primers were designed to overlap each other to ensure correct sequencing at the ends of each primer.

| Primer Name | Sequence | Target Immunogen |
|---|---|---|
| Con1.1 | CACTATTAATGGTGGTGATG | tConsv1 |
| Con1.2 | GCCATCTGCACGGCGGTCTTC | tConsv1 |
| Con1.3 | GTTCACGAACTCCCAGTCGG | tConsv1 |
| Con1.4 | GTAGGCGTCGCCCACGTCCAG | tConsv1 |
| Con1.5 | CACGTAGTCGCGGAAGGAC | tConsv1 |
| Con1.6 | GAGAATGCCTTCTCCTCGCG | tConsv1 |
| Con1.7 | GAAGCTGACCGTGTGGGGCATC | tConsv1 |
| Con1.8 | AAGATGCAGGCAGCTGAGTT | tConsv1 |
| Con1.9 | CACAACTTCAAGCGCAAGG | tConsv1 |
| Con2.1 | GTCCAGGCCCAGCAGGGGGTTG | tConsv2 |
| Con2.2 | GCAGGATCTCGCGGTTCTCG | tConsv2 |
| Con2.3 | GTTGAAGCCCTTCTCCTCGC | tConsv2 |
| Con2.4 | GAAGATCATCCGCGACTACG | tConsv2 |
| Con2.5 | GGAAAGGACAGTGGGAGTG | tConsv2 |
| Con2.6 | AAGAGGCCACGATCTCCTTG | tConsv2 |
| Con2.7 | GGTCGTACTGCTTGACCTTGA | tConsv2 |
| Con3.1 | CATGGAGGACTGGAAGATG | tConsv3 |
| Con3.2 | CTCGGACAGGGCGGTGAAC | tConsv3 |
| Con3.3 | GACAAGTGCCAGCTGAAGG | tConsv3 |
| Con3.4 | CAGGACAACTCCGAGATCAAG | tConsv3 |

| | | |
|---|---|---|
| Con3.5 | AGGAAAGGACAGTGGGAGTG | tConsv3 |
| Con3.6 | CTTCTCTCCCGAGGTGATCC | tConsv3 |
| Con3.7 | TCCGCTACTGCTACAACGTG | tConsv3 |
| Con3.8 | GGTGTTCACGAACTCCCACT | tConsv3 |
| Con3.9 | ATGGGGATCTGGTCGTACTG | tConsv3 |
| Cmo4.1 | AGTACACCGCCTTCACCATC | tHIVcmo |
| Cmo4.2 | GGTGTTCACGAACTCCCAGT | tHIVcmo |
| Cmo4.3 | CCTGGGTCTTCTTGTTCAGC | tHIVcmo |
| Cmo4.4 | GTGAAGGTGGTGGAGGAGAA | tHIVcmo |
| Cmo4.5 | GACCGCCTACTTCATCCTGA | tHIVcmo |
| Cmo4.6 | CCTTGATCTCGGAGTTGTCC | tHIVcmo |
| Cmo4.7 | GTGGGGCATCAAGCAGTC | tHIVcmo |
| Cmo4.8 | GCGAGCTCTAGCATTTAGGTG | tHIVcmo |
| Cmo4.9 | CAACGGGACTTTCCAAAATG | tHIVcmo |
| Cmo4.10 | CTGCCGCACTTCCAACAG | tHIVcmo |

### 2.1.4 Peptides

**Table 2.3** List of epitope peptides used to stimulate responses in ELISPOT, ICS and killing assays.

| ID | Sequence | Molecular Weight | Stock Weight | Stock Conc | Dilution 1 Conc | Dilution 2 Conc |
|---|---|---|---|---|---|---|
| CH-800 | TLNAWVKVI | 1043.28 | 5mg | 40mg/ml | 4mg/ml | 40µg/ml |
| CH-801 | TLNAWVAVV | 972.16 | 5mg | 40mg/ml | 4mg/ml | 40µg/ml |
| CH-802 | EPFRDYVDRF | 1343.48 | 5mg | 40mg/ml | 4mg/ml | 40µg/ml |
| CH-803 | EPFREYVDRF | 1357.5 | 5mg | 40mg/ml | 4mg/ml | 40µg/ml |
| CH-804 | FLGKIWPS | 947.15 | 5mg | 40mg/ml | 4mg/ml | 40µg/ml |
| CH-805 | FLGRIWPS | 975.17 | 5mg | 40mg/ml | 4mg/ml | 40µg/ml |
| CH-806 | KMIGGIGGFI | 992.26 | 5mg | 40mg/ml | 4mg/ml | 40µg/ml |
| CH-807 | VLIGPTPVNII | 1135.42 | 5mg | 40mg/ml | 4mg/ml | 40µg/ml |
| CH-808 | VLIGPTPVNI | 1022.26 | 5mg | 40mg/ml | 4mg/ml | 40µg/ml |

| | | | | | | |
|---|---|---|---|---|---|---|
| CH-809 | VLVGPTPINI | 1022.26 | 5mg | 40mg/ml | 4mg/ml | 40μg/ml |
| CH-810 | VLVGPTPANI | 980.18 | 5mg | 40mg/ml | 4mg/ml | 40μg/ml |
| CH-811 | LIGPTPVNI | 923.13 | 5mg | 40mg/ml | 4mg/ml | 40μg/ml |
| CH-812 | LVGPTPINI | 923.13 | 5mg | 40mg/ml | 4mg/ml | 40μg/ml |
| CH-813 | LVGPTPANI | 881.05 | 5mg | 40mg/ml | 4mg/ml | 40μg/ml |
| CH-814 | ALVEICTEM | 1008.22 | 5mg | 40mg/ml | 4mg/ml | 40μg/ml |
| CH-815 | ALTAICEEM | 980.17 | 5mg | 40mg/ml | 4mg/ml | 40μg/ml |
| CH-816 | VLDVGDAYFSV | 1184.32 | 5mg | 40mg/ml | 4mg/ml | 40μg/ml |
| CH-817 | ILDVGDAYFSV | 1198.35 | 5mg | 40mg/ml | 4mg/ml | 40μg/ml |
| CH-818 | TAFTIPST | 836.95 | 5mg | 40mg/ml | 4mg/ml | 40μg/ml |
| CH-819 | TAFTIPSV | 834.98 | 5mg | 40mg/ml | 4mg/ml | 40μg/ml |
| CH-820 | TAFTIPSL | 849 | 5mg | 40mg/ml | 4mg/ml | 40μg/ml |
| CH-821 | TAFTIPSR | 892.03 | 5mg | 40mg/ml | 4mg/ml | 40μg/ml |
| CH-822 | IIYQYMDDL | 1173.36 | 5mg | 40mg/ml | 4mg/ml | 40μg/ml |
| CH-823 | VIYQYVDDL | 1127.27 | 5mg | 40mg/ml | 4mg/ml | 40μg/ml |
| CH-824 | EICQYMDDL | 1129.27 | 5mg | 40mg/ml | 4mg/ml | 40μg/ml |
| CH-825 | IYQYMDDLYV | 1322.51 | 5mg | 40mg/ml | 4mg/ml | 40μg/ml |
| CH-826 | ICQYMDDLYV | 1262.47 | 5mg | 40mg/ml | 4mg/ml | 40μg/ml |
| CH-827 | YQYVDDLYV | 1177.29 | 5mg | 40mg/ml | 4mg/ml | 40μg/ml |
| CH-828 | CQYMDDLYV | 1149.31 | 5mg | 40mg/ml | 4mg/ml | 40μg/ml |
| CH-829 | EILKDPVHGV | 1106.3 | 5mg | 40mg/ml | 4mg/ml | 40μg/ml |
| CH-830 | EILKEPVHGV | 1120.32 | 5mg | 40mg/ml | 4mg/ml | 40μg/ml |
| CH-831 | ILREPVHGV | 1019.22 | 5mg | 40mg/ml | 4mg/ml | 40μg/ml |
| CH-832 | ILKEPVHGA | 963.15 | 5mg | 40mg/ml | 4mg/ml | 40μg/ml |
| CH-833 | ILKDPVHGV | 977.18 | 5mg | 40mg/ml | 4mg/ml | 40μg/ml |
| CH-834 | ILKTPVHGV | 963.2 | 5mg | 40mg/ml | 4mg/ml | 40μg/ml |
| CH-835 | ILKNPVHGV | 976.2 | 5mg | 40mg/ml | 4mg/ml | 40μg/ml |
| CH-836 | PLVKLWYQL | 1159.45 | 5mg | 40mg/ml | 4mg/ml | 40μg/ml |
| CH-837 | LLWKGEGAV | 972.16 | 5mg | 40mg/ml | 4mg/ml | 40μg/ml |
| CH-838 | SLVKHHLTEE | 1192.35 | 5mg | 40mg/ml | 4mg/ml | 40μg/ml |
| CH-839 | SLVKHHLTK | 1062.29 | 5mg | 40mg/ml | 4mg/ml | 40μg/ml |
| CH-840 | QMHEDIISL | 1085.25 | 5mg | 40mg/ml | 4mg/ml | 40μg/ml |
| CH-841 | IISLWDQSL | 1074.25 | 5mg | 40mg/ml | 4mg/ml | 40μg/ml |
| CH-842 | IISLWDESL | 1075.24 | 5mg | 40mg/ml | 4mg/ml | 40μg/ml |
| CH-843 | TLNAWVKVV | 1029.26 | 10MG | 40mg/ml | 4mg/ml | 40μg/ml |
| CH-844 | VLVGPTPVNI | 1008.24 | 10MG | 40mg/ml | 4mg/ml | 40μg/ml |
| CH-845 | LVGPTPVNI | 909.1 | 10MG | 40mg/ml | 4mg/ml | 40μg/ml |
| CH-846 | TAFTIPSI | 849 | 10MG | 40mg/ml | 4mg/ml | 40μg/ml |
| CH-847 | VIYQYMDDL | 1159.33 | 10MG | 40mg/ml | 4mg/ml | 40μg/ml |
| CH-848 | IYQYVDDLYV | 1290.45 | 10MG | 40mg/ml | 4mg/ml | 40μg/ml |
| CH-849 | YQYMDDLYV | 1209.35 | 10MG | 40mg/ml | 4mg/ml | 40μg/ml |
| CH-850 | ILKEPVHGV | 991.21 | 10MG | 40mg/ml | 4mg/ml | 40μg/ml |
| CH-851 | RGPGRAFVTI | 1073.27 | 10MG | 40mg/ml | 4mg/ml | 40μg/ml |
| CH-852 | TSTLQEQIGW | 1162.27 | 5mg | 40mg/ml | 4mg/ml | 40μg/ml |
| CH-853 | TSTLQEQIAW | 1176.3 | 5mg | 40mg/ml | 4mg/ml | 40μg/ml |
| CH-854 | TSNLQEQIAW | 1189.3 | 5mg | 40mg/ml | 4mg/ml | 40μg/ml |
| CH-855 | TSNLQEQIGW | 1175.27 | 5mg | 40mg/ml | 4mg/ml | 40μg/ml |

| | | | | | | |
|---|---|---|---|---|---|---|
| CH-856 | TSTPQEQIGW | 1146.23 | 5mg | 40mg/ml | 4mg/ml | 40μg/ml |
| CH-857 | KAFSPEVIPMF | 1265.55 | 5mg | 40mg/ml | 4mg/ml | 40μg/ml |
| CH-858 | KGFNPEVIPMF | 1278.55 | 5mg | 40mg/ml | 4mg/ml | 40μg/ml |
| CH-859 | KAFSPEIIPMF | 1279.58 | 5mg | 40mg/ml | 4mg/ml | 40μg/ml |
| CH-860 | KAFNPEVIPMF | 1292.57 | 5mg | 40mg/ml | 4mg/ml | 40μg/ml |
| CH-861 | RAFSPEVIPMF | 1293.56 | 5mg | 40mg/ml | 4mg/ml | 40μg/ml |
| CH-862 | KRWIILGLNK | 1240.57 | 5mg | 40mg/ml | 4mg/ml | 40μg/ml |
| CH-863 | KRWIIMGLNK | 1258.61 | 5mg | 40mg/ml | 4mg/ml | 40μg/ml |
| CH-864 | KRWIVLGLNK | 1226.54 | 5mg | 40mg/ml | 4mg/ml | 40μg/ml |
| CH-865 | KKWIIMGLNK | 1230.59 | 5mg | 40mg/ml | 4mg/ml | 40μg/ml |
| CH-866 | RRWIILGLNK | 1268.58 | 5mg | 40mg/ml | 4mg/ml | 40μg/ml |
| CH-867 | ILGLNKIV | 869.12 | 5mg | 40mg/ml | 4mg/ml | 40μg/ml |
| CH-868 | IMGLNKIV | 887.16 | 5mg | 40mg/ml | 4mg/ml | 40μg/ml |
| CH-869 | VLGLNKIV | 855.1 | 5mg | 40mg/ml | 4mg/ml | 40μg/ml |
| CH-870 | IIGLNKIV | 869.12 | 5mg | 40mg/ml | 4mg/ml | 40μg/ml |
| CH-871 | ESFRDYVDRF | 1333.43 | 5mg | 40mg/ml | 4mg/ml | 40μg/ml |
| CH-872 | EPFTDYVDRF | 1288.39 | 5mg | 40mg/ml | 4mg/ml | 40μg/ml |
| CH-873 | ALTEICKEM | 1037.26 | 5mg | 40mg/ml | 4mg/ml | 40μg/ml |
| CH-874 | ALTEICTEM | 1010.19 | 5mg | 40mg/ml | 4mg/ml | 40μg/ml |
| CH-875 | ALIEICTEM | 1022.25 | 5mg | 40mg/ml | 4mg/ml | 40μg/ml |
| CH-876 | ALTEICEEM | 1038.2 | 5mg | 40mg/ml | 4mg/ml | 40μg/ml |
| CH-877 | VLDVGDAYFSI | 1198.35 | 5mg | 40mg/ml | 4mg/ml | 40μg/ml |
| CH-878 | VLDIGDAYFSV | 1198.35 | 5mg | 40mg/ml | 4mg/ml | 40μg/ml |
| CH-879 | YQYMDDLYI | 1223.38 | 5mg | 40mg/ml | 4mg/ml | 40μg/ml |
| CH-880 | IYQYMDDLYI | 1336.54 | 5mg | 40mg/ml | 4mg/ml | 40μg/ml |
| CH-881 | IYSYMDDLYV | 1281.46 | 5mg | 40mg/ml | 4mg/ml | 40μg/ml |
| CH-882 | SLVKHHLTE | 1063.23 | 5mg | 40mg/ml | 4mg/ml | 40μg/ml |
| CH-883 | SLVKHHLTK | 1062.29 | 5mg | 40mg/ml | 4mg/ml | 40μg/ml |
| CH-884 | SLVKHHMYI | 1127.38 | 5mg | 40mg/ml | 4mg/ml | 40μg/ml |
| CH-885 | SLVKHHMHI | 1101.35 | 5mg | 40mg/ml | 4mg/ml | 40μg/ml |
| CH-886 | RMIGGIGGFI | 1020.27 | 5mg | 40mg/ml | 4mg/ml | 40μg/ml |
| CH-887 | KMMGGIGGFI | 1010.29 | 5mg | 40mg/ml | 4mg/ml | 40μg/ml |
| CH-888 | KMVGGIGGFI | 978.23 | 5mg | 40mg/ml | 4mg/ml | 40μg/ml |
| CH-889 | FLGKLWPS | 947.15 | 5mg | 40mg/ml | 4mg/ml | 40μg/ml |
| CH-890 | FLGRLWPS | 975.17 | 5mg | 40mg/ml | 4mg/ml | 40μg/ml |
| CH-891 | FLGKVWPS | 933.13 | 5mg | 40mg/ml | 4mg/ml | 40μg/ml |
| CH-892 | FLGKIWSS | 937.12 | 5mg | 40mg/ml | 4mg/ml | 40μg/ml |
| CH-893 | QMQEDIISL | 1076.24 | 5mg | 40mg/ml | 4mg/ml | 40μg/ml |
| CH-894 | QMHEDVISL | 1071.23 | 5mg | 40mg/ml | 4mg/ml | 40μg/ml |
| CH-895 | QMHTDIISL | 1057.24 | 5mg | 40mg/ml | 4mg/ml | 40μg/ml |
| CH-896 | QMHEDIISI | 1085.25 | 5mg | 40mg/ml | 4mg/ml | 40μg/ml |
| CH-897 | QMQTDIISL | 1048.23 | 5mg | 40mg/ml | 4mg/ml | 40μg/ml |
| CH-898 | QMHADIISL | 1027.22 | 5mg | 40mg/ml | 4mg/ml | 40μg/ml |
| CH-899 | VISLWDQSL | 1060.22 | 5mg | 40mg/ml | 4mg/ml | 40μg/ml |
| CH-900 | IISLWDESL | 1075.24 | 5mg | 40mg/ml | 4mg/ml | 40μg/ml |
| CH-901 | IINLWDQSL | 1101.28 | 5mg | 40mg/ml | 4mg/ml | 40μg/ml |
| CH-902 | IISLWEQSL | 1088.28 | 5mg | 40mg/ml | 4mg/ml | 40μg/ml |

## 2.2 Methods: General Molecular Biology

Methods described in this section were used to construct the various vaccine constructs. The immunogens and the plasmids used for the various vaccines are described in the results chapter 3.

### 2.2.1 DNA Restriction Enzyme Digest

DNA restriction digest were done using restriction endonucleases enzymes, obtained from New England Biolabs (NEB). Several enzymes were used depending on the construct and in most cases multiple enzymes were used per reaction. Reactions were done using one of four NEB buffers, dependent upon the compatibility with the restriction endonuclease enzyme. Enzyme concentration was x3 units in ratio to the DNA i.e. 3 Units of enzyme for 1 μg DNA. The NEB buffer and bovine serum albumin (BSA) at x10, were both used at 1/10 of the total reaction i.e. 5 μl of buffer and BSA was used in a 50 μl reaction. The total of the reaction must be at least 10x that of the enzyme. The reactions were incubated at 37 $^{O}$C for 2 hours (h). For basic cloning, a standard 20 - 50 μl digest was performed:

| | |
|---|---|
| Plasmid DNA | - 2 μg |
| NEB Restriction Endonuclease | - 6 Units |
| NEB Buffer (1-4) x10 | - 5 μl |
| BSA x10 | - 5 μl |
| $dH_2O$ | - To total volume 50 μl |
| Total | - 50 μl |

### 2.2.2 Phosphatase

To prevent the re-ligation of the digested product, the 5' phosphate is removed using Antarctic phosphatase. The reaction is mixed or spun gently, incubated at 37 $^{O}$C for 30 minutes (min) then at 65 $^{O}$C for 5 min to inactivate the Antarctic phosphatase reaction. A standard 60 µl reaction example:

| | |
|---|---|
| DNA restriction digest mix | - 50 µl |
| Antarctic Phosphatase | - 2 µl |
| Antarctic Phosphatase Reaction Buffer (10X) | - 6 µl |
| $dH_2O$ | - 2 µl |
| Total | - 60 µl |

### 2.2.3 Agarose Gel Electrophoresis

DNA can be visualised by agarose gel electrophoresis according to their size. The digested reaction products were separated by 0.8 – 2 % agarose gel electrophoresis (80 - 100 volts, 50 – 70 min) in 1x Tris-acetate-EDTA (TAE) buffer and stained using SYBR© Safe dye (at a 1:10,000 concentration), which binds to double stranded DNA and was visualised on a UV-transilluminator (Biospectrum AC Imaging System, USA). The size of the DNA fragments was estimated by comparison to the DNA marker smart ladder (10 K GeneRuler DNA ladder, Thermo Scientific).

### 2.2.4 Gel Extraction of DNA

The gel was placed carefully on a clean cling film on the transilluminator to excise the DNA fragment of interest from the restriction digest and separate it from unwanted DNA. The fragment was cut out using a clean/new scalpel and the DNA/agarose slice was

placed into a 1.5 ml eppendorf tube. The DNA was purified from the agarose gel using the QIAGEN MinnElute Gel Extraction kit according to manufacturer's protocol.

### 2.2.5 DNA Ligation

This protocol was used to ligate/join double stranded DNA together i.e. ligate a DNA of interest into a vector. T4 DNA ligase (NEB, UK) catalyses the formation of a phosphodiester bonds between the 3' hydroxyl end of one nucleotide with the 5' phosphate end of another. The T4 DNA ligase enzyme will join blunt end and cohesive end termini in DNA. The first step was to acquire an accurate concentration reading, using a nanodrop, of both the insert and vector, and calculate the insert concentration to a 1:1 ratio to the vector.

$$\frac{vector\ concentration\ \times\ inser\ size\ (bp)}{vector\ size\ (bp)}$$

For the ligation reaction, the DNA insert was used at 4X and 8X of the calculated concentration. The vector was used at a concentration of 20 ng. $dH_2O$ was used to total the volume required. The ligation reaction was placed either at 25 °C (room temperature) for 2 - 3 h or incubated at 16 $^{O}$C overnight. For a typical 20 µl ligation:

| | | Vector 20 ng | Insert | T4 ligase | T4 buffer | $dH_2O$ | Total |
|---|---|---|---|---|---|---|---|
| Control | Vector | 1 µl | - | - | 2 µl | X µl | 20 µl |
| Control | Vector +T4 | 1 µl | - | 1 µl | 2 µl | X µl | 20 µl |
| | Insert (4X) | 1 µl | X µl | 1 µl | 2 µl | X µl | 20 µl |
| | Insert (8X) | 1 µl | X µl | 1 µl | 2 µl | X µl | 20 µl |

### 2.2.6 Plasmid Transformation into *E.coli*

Plasmid DNA (ligation product) are introduced and grown up in *E.coli* via heat shock. 50 µl of maximum efficiency or sub-cloning efficiency DH5α *E. coli* were thawed on ice, before adding 10 µl of DNA ligation mix and incubated on ice for 30 min. Following this, cells were heat-shocked at 42 °C for 40 s then kept on ice for 5 min. SOC medium was then added (250 µl) before incubating it at 37 °C for 1 h in a shaking incubator (220 rpm – innova 44) to allow for bacteria growth. The solution mix containing the transformed bacteria was then spread onto LB (Luria-Bertani) agar containing an antibiotic, either ampicillin or kanamycin, depending on the resistance gene encoded in the plasmid. Plates were then incubated at 37 °C overnight (minimum 12 h) then stored at 4 °C. Colonies viability were checked and counted.

### 2.2.7 Growing and Bulking-up of Plasmid DNA

Cultures of bacteria are grown to screen plasmid DNA and to prepare large volumes of DNA. Initially small volume cultures are prepared to identify the bacteria with the correct DNA, then after larger volumes are prepared from it. For the initial starter cultures, 5 ml of LB broth media and antibiotic are added to a 15 ml falcon tube. Ampicillin was used at 1/1000 concentration and kanamycin at 1/2000 concentration. A pipette tip is then used to pick a colony, placed in the culture and incubated overnight (12 – 16 h) at 37 °C. Following transformation of ligated product, 6 individual colonies were picked and grown for screening via restriction digest.

Form 5 ml starter cultures, larger cultures are prepared to acquire large volumes of DNA. Volumes between 25 ml - 2.5 l of cultures are prepared to purify 250 µg – 10 mg of DNA. Larger cultures were prepared using LB broth, antibiotics at given concentration above and bacteria culture at a 1/1000 concentration i.e. 25 µl of bacterial culture was dispensed into a 25 ml culture.

### 2.2.8 Plasmid DNA Purification

Plasmid DNA was purified from cultures using endofree QIAGEN kits, following manufacturers protocol. Cultures are spun down and supernatant was discarded prior to purification. Depending on the volume of the culture, different kits were used to purify different quantities of DNA. QIAGEN Spin Miniprep kit or QIAGEN Mini kit (tip 20) were used for 5 ml cultures, yielding up to 20 μg of DNA. QIAGEN Plus Midi kit was used for 25 ml cultures, yielding up to 250 μg of DNA. QIAGEN Plus Maxi kit was used for 100 ml cultures, yielding up to 1000 μg of DNA. QIAGEN Giga kit was used for 2.5 l cultures, yielding up to 10 mg of DNA.

### 2.2.9 Cloning and Generating Recombinant Adenovirus Vectors using Gateway™ Technology.

Gateway™ (Invitrogen) is a universal cloning technology that utilizes site specific recombination properties of bacteriophage lambda, providing a rapid and efficient way of transferring a sequence of interest into multiple vector system. This technology was utilized is the construction of the ChAdV63 vectored vaccines. It was used to generate recombinant adenovirus clones prior to transfection of the genomic DNA into mammalian cells to generate recombinant virus. Transgene of interest, including the CMV promoter and BGH poly A region, were cloned into the ChAd63 entry vector pENTR™4-Mono (p2022), between specific phage lambda *attL* sites. Cloning into the entry vector was confirmed via restriction enzyme digest. The LR Clonase II enzyme was used in the Gateway reaction to perform a site specific recombination between *attL* sites in the Entry vector and *attR* sites in the Destination vector (ChAd63 destination plasmid vector, p1916) according to manufacturer protocol. The recombination product were then transformed and plated for screening. True expression clones will be chloramphenicol sensitive but ampicillin

resistant. Following confirmation and DNA purification, the adenovirus shittle plasmid was linearized with Pme1 as per J004 protocol and stored at -20 ℃ till transfection.

### 2.2.10 Preparation of Oligos via PCR

Various oligos were used in the cloning and development of the various vaccine contructs. Some oligos were cloned into plasmids introducing new multi-cloning sites, others were single epitope antigens (mini-gene). Oligos were provided by eurofins mwg|operon. To anneal two strands of oligo nucleotides, they were resuspended to a concentration of 100 pmol is $dH_2O$ and mixed together and annealed using a PCR machine. They were heated and remained at 95 ℃ for 2 min, then cooled to 25 ℃ over a period of 45 min and stored at 4 ℃ or -20 ℃ for long term storage. Dilutions for the annealed oligos at 1/10, 1/100 and 1/1000 were prepared prior to ligation.

### 2.2.11 PCR Screening of Recombinant MVA

MVA vectored vaccines were prepared by the vector core facility. However, I myself performed the screenings during the development. Screening was done to ensure the presence of antigen and no viral contamination. This was achieved via three PCR screening methods: 1) Identity PCR (ID-PCR) 2) Flank ID-PCR 3) Purity PCR. The ID-PCR and Flank ID-PCR use antigen specific primers and TKL/mH5 primers to confirm the presence of the antigen and promoter. The purity PCR is to check the purity of the clones and no contamination from wild type parental MVA using specific primers. PCR was done using KAPA LongRange HotStart DNA polymerase (KAPA Biosystems) and prepared according to manufacturer protocol.

The programs for the PCR's were as follow:

ID/Purity PCR:

| | | |
|---|---|---|
| 1. Primary Denature | 95 °C | 3 min |
| 2. Denature | 95 °C | 40 s |
| 3. Anneal | 55 °C | 15 s |
| 4. Extension | 68 °C | 1 min/kb |
| 5. Final Extension | 72 °C | 3 min |
| 6. End | | |

Repeat steps 2 – 4 35 times

Flank ID PCR:

| | | |
|---|---|---|
| 1. Primary Denature | 95 °C | 3 min |
| 2. Denature | 95 °C | 40 s |
| 3. Anneal | 55 °C | 15 s |
| 4. Extension | 68 °C | 3 min |
| 5. Final Extension | 72 °C | 5 min |
| 6. End | | |

Repeat steps 2 – 4 35 times

### 2.2.12 Sequencing Confirmation

All cloned plasmids for DNA, ChAdV63 and MVA vaccines were sequenced to confirm the transgenes were cloned. Sequencing was done by Source Bioscience Sequencing (Cambridge, UK) and the samples were prepared according to their protocol. Primers were designed using Primer 3 program and are provided in table 2.2.

## 2.3 Methods: Immunofluorescence Microscopy

Methods described in this section were used to check the expression of the various immunogens from the three vectors (DNA, ChAdV63 and MVA).

### 2.3.1 Preparation of HeLa Cells

A stock of HeLa cells was retrieved from liquid nitrogen storage to start initial culture. A cryovial containing the cells was thawed in a 37 °C water bath quickly, thereafter the cells were transferred into a 50 ml falcon tube containing 25 ml of pre-warmed complete DMEM media. Once mixed, the cell suspension mix was transferred to a T75 conical flask and incubated at 37 °C, 5% $CO_2$ for 24 h. Media was changed after 24 h to remove any residual DMSO.

Cell cultures were split every few days, to maintain fresh cultures at all times. To split cells in a T25 flask, first media was removed from the culture; cells were then washed with 5 ml DMEM neat, swerving the flask gently before removing the media again. To detach cells from the flask, 1 ml of trypsin EDTA was added and incubated at 37 °C, 5% $CO_2$ for 5 min, before adding 5 ml of complete DMEM media to neutralise the trypsin. Cells were transferred to a 15 ml falcon tube and spun for 5 min at 2000 rpm (Sorvel Legend RT), discarding the supernatant afterwards and re-suspending the cells in 5 ml complete DMEM media. Cells were then transferred into new T25 flasks in a 1/5 or 1/10 ratio i.e. 1 ml of cell suspension added onto 4 ml of media (1/5) or 0.5 ml of cell suspension added onto 4.5 ml of media (1/10). For microscopy, cells were cultured in 6 well plates containing sterile glass coverslips. Cells were first counted and an exact equal number of cells were added to all the wells (300,000 – 500,000 cells). A hemocytometer was used to count cells, stained with trypan blue (50 µl + 50 µl dye), using a light microscope.

### 2.3.2 DNA Transfection of HeLa Cells

HeLa cells, in 6 well plates, were transfected with each of the plasmid DNA vaccines individually to check for immunogen expression. Transfection was done using Lipofectamine 3000 Reagent (Invitrogen) following manufacturer protocol.

### 2.3.3 Infecting HeLa Cells

HeLa cells were infected with the various ChAdV63 and MVA vaccines individually to check for immunogen expression. Infection was done on cells at 70% confluency. First, media was aspirated from the 6 well plate cell cultures and cells were washed with PBS gently. Hela cells were then infected with recombinant MVA at a multiplicity of infection (MOI) of 5 (600 µl) for 2 h at 37 $^{O}$C 5% $CO_2$ in serum free DMEM

media; or infected with recombinant ChAdV63 at an MOI of 10 (600 µl) for 1 h at 37 $^{O}$C 5% $CO_2$ in serum free DMEM media. Following infections, viral media was aspirated and cells were washed with PBS and incubated in complete DMEM media for 24 hr. Multiplicity of infection (MOI) is the ratio of virus particles to the number of target cells.

### 2.3.4 Immunofluoresecent Staining of HeLa Cells

Following the 24 h incubation post infection, media was aspirated and cells were washed (x2) with ice cold PBS and fixed with formalin solution, neutral buffered 10% (containing 4% formaldehyde) (sigma) for 10 min on ice and then 20 min at room temperature. Cells were washed again with large volumes of PBS (x3), permeabilized with 0.2% Triton X-100 (TX-100) (sigma) in PBS for 5 min, washed again with PBS (x3), blocked with 1% BSA in PBS for 30 min and stained for 3 – 4 h at room temperature with a 1:100 dilution of the Alexa Fluor 488 conjugated primary mouse anti-Tag monoclonal antibody (mAb). Immunogens had one of three Tag epitope sequences at the end of their proteome: 1) PK Tag – V5 (IPNPLLGLD) 2) Histidine – His 6 Tag (HHHHHH) 3) C-myc Tag (EQKLISEEDL) (AbD Serotec). All three anti-Tag primary mAb were conjugated to Alexa fluor 488 (FITC) thus negating the need for a secondary conjugated antibody. Cells were washed with PBS (x3) for 15 min shaking then mounted on microscope slides with Vectashield 4',6-diamidino-2-phenylindole (DAPI) nuclear stain mounting medium (Vector laboratories) and examined on a fluorescence microscope (DMI 3000B; Leica). Slides were wrapped in tinfoil and stored at 4 $^{O}$C. Images were taken within 36 h of staining. Microscopy images were processed using Image J software.

## 2.4 Methods: General Immunological Assays

Methods described in this section were used to test the immunogenicity of the various vaccines in mice

### 2.4.1 Animal Immunisation

Mice were immunised via the intra-muscular (i.m.) route under anesthesia in accordance with the Home Office guidelines. All work was done in laminar flow hoods ensuring sterility. The concentrations and regimens of the various vaccines are described in the results chapters. Following immunisation, mice were sacrificed in accordance to Schedule 1 protocol, neck dislocation. Spleens were surgically removed from mice for immunological analysis.

### 2.4.2 Mouse Splenocytes Isolation

Spleens isolated from culled mice were transferred to a 50 ml falcon tube containing 20 ml of warm R10 media. Working in a category II laminar flow hood, cell strainers were placed in 50 ml falcon tubes labeled specifically for each mouse. Splenocytes were obtained by pressing the spleen through the strainer using the base of a 2 ml syringe and washed through with 10 ml of pre-warmed R10 media. Cells were spun at 1700 rpm (Sorvel Legend RT) for 5 min and the supernatant was discarded gently without disturbing the cell pellet. The pellet was flicked to loosen the cells before resuspending them in 1 ml of ACK lysis buffer, to lysis red blood cells. Lysis was left to occur for 3 min, after which 5 ml of R10 media was added and cells were spun at 1700 rpm (Sorvel Legend RT) for 5 min. The supernatant was removed, pellet loosened, resuspended in 5 ml of R10 and placed in a 37 $^{O}$C 5% $CO_2$ incubator the cap loose.

### 2.4.3 Splenocytes Cell Count

Splenocytes cell count was obtained using a CASY cell counter (Schärfe Systems, Germany). Cells were diluted 1/2000 in casyton buffer (5 µl/10 ml) to obtain the cells count. Cells were then diluted accordingly use in different immunological assays.

### 2.4.4 Peptides Preparation

ChinaPeptides manufactured our epitope peptides, totaling 105 peptides, with purity >95%, a stock weight of 5 mg, and supplied as lyophilised powder (Table 2.3). Working in a category II laminar flow hood, peptides were reconstituted in 100% DMSO, with a volume of 125 µl, yielding a final concentration of 40 mg/ml of stock peptide. After resuspension, the peptides were vortexed at high speed for 5 – 20 s and sonicated for 4 min at 50 $^{O}$C in a water bath. A peptide stock with a concentration of 4 mg/ml was then prepared using PBS buffer as the following: 25 µl from the 40 mg/ml stock was diluted in 225 µl PBS. A working stock with a concentration of 40 µg/ml was prepared as the following: 20 µl from the 4 mg/ml stock was diluted in 1980 µl of PBS. The 40 and 4 mg/ml stocks were stored at -80 $^{O}$C and the 40 µg/ml working stock was stored at -20 $^{O}$C.

### 2.4.5 *Ex-vivo* IFN-γ Enzyme Linked ImmunoSpot (ELISPOT) Assay

All ELISPOT reagents were supplied from Mabtech, UK in a mouse IFN-γ ELISPOT kit. On day 1, twenty four hours before culling of mice, MAIP ELISPOT plates were coated overnight at 4 °C or at 37 °C for 2 h with anti-mouse-IFN-γ mAb AN18 diluted to 5 µg/ml in coating buffer (carbonate-bicarbonate) dispensed at a volume of 100 µl. On day 2, prior to the processing of spleens, the ELISPOT plates were washed five times with sterile PBS (200 µl per well) and subsequently blocked with R10 media (200 µl per well) for 1 h at room temperature or 3 h at 4 °C. Blocking media was then removed and

100 µl of peptides diluted in R10 to a concentration of 4 µg/ml were then added. Positive control wells had 100 µl of PMA and ionomycin at 10 ng and 500 ng respectively added. For the negative control wells, 100 µl of R10 media was added alone. Diluted splenocytes were then added to the plates, each well containing $2\times10^5$ cells in 100 µl volume ($1\times10^5$ and $1.5\times10^5$ cells/well were also used for some experiment). After adding the cells, the final concentration of the peptides in each well is 2 µg/ml. The pates were then placed in 37 $^{O}$C 5% $CO_2$ incubator for 18-20 h. All tests were done in duplicates.

ELISPOT plates were developed on the third day. Supernatant was removed and the plates were washed 6 times with PBS and incubated for 2 h at room temperature with 100 µl per well of biotinylated rat anti-mouse-IFN-γ mAb R46A2 diluted to 1µg/ml (1/1000) in PBS containing 0.5% foetal calf serum (PBS-0.5% FCS). Plates were then washed 6 times with PBS and subsequently incubated for 1 h at room temperature with 100 µl per well of streptavidin alkaline phosphatase polymer diluted to 1 µg/ml (1/1000) in PBS-0.5% FCS. Plates were washed 6 times with PBS prior to the development of spots by adding 100µl of BioRad AP conjugate development buffer. Spot development was terminated by washing the plates vigorously with tap water. Plates were then air-dried in the dark overnight. Spots were counted using the AID ELISPOT reader system and AID software (AID: Autoimmun Diagnostika, Germany). Results are expressed as spot forming cells (SFC) per million cells. Background responses from the negative controls were subtracted from those measured in the peptide stimulated wells during analysis.

### 2.4.6 Intracellular Cytokine Staining (ICS)

Production of different cytokines by T cells was examined using intracellular cytokine staining. Single cell suspension of splenocytes was prepared as described above. One million cells were added to each well of a U-bottom 96-well plate. Peptides or peptide pools were then added onto the cells to stimulate them at a final concentration of 2 µg/ml

and incubated at 37°C, 5% $CO_2$ for 90 min. If required, anti-CD107a was added at the start of stimulation at a 1:100 dilution. During this time the cytokine trapping media containing protein transport inhibitors was prepared using GolgiStop (containing monensin) and GolgiPlug (containing brefeldin A) (BD bioscience), at a concentration of 0.7 µl/ml and 2 µl/ml respectively (diluted in R10). Following incubation, GolgiStop/GolgiPlug were added onto the cells and incubated for 5 h. Following the incubation, the plate was placed at 4 °C overnight.

Cells were then spun down at 2000 rpm (Sorvel Legend RT) for 5 min and the supernatant discarded, allowing the washing of the cells (x2) with FACS buffer (PBS, 1% FCS, 0.01% Azide), discarding the supernatant after each spin in the wash. Cells were then blocked for 20 min at 4°C with anti-CD16/32 antibodies (Fc block) (eBioscience) diluted at 1:50 in FACS buffer, then spun and washed twice with FACS buffer. Cell were then stained for 30 min at 4 °C with surface markers anti-CD8, anti-CD4 mAb and a live/dead aqua stain (eBioscience), all mixed in FACS buffer at a dilution of 1:100 per well. The wells were then spun and washed twice with FACS buffer, then permeabilized with 100 µl of 1 × BD Cytofix/Cytoperm kit (BD Biosciences) at 4 °C for 30 min. The plate was then spun and washed twice with 200 µl of BD Perm/Wash (BD Biosciences). Cells were then stained for intracellular cytokines: anti-TNFα, anti-IFNγ and anti-IL-2 mAb (eBioscience) diluted 1:100 in BD Perm/Wash and incubated at 4 °C for 30 min. Cells were then washed again twice with BD Perm/Wash and subsequently fixed with 200 µl of 1% paraformaldehyde. Cells were then kept at 4 °C ready for data acquisition through LSRII (BD) flow cytometer and analysis with Flowjo (Tree Star) and SPICE software. The fluorochromes of the antibodies have some time varied between experiments due to availability.

### 2.4.7 *Ex.vivo* Killing Assay

This assay was used to determine the killing efficacy of T cells from vaccinated mice against peptide pulsed target cells. The method I describe here uses splenocytes as targets; nonetheless, the protocol is the same with other cells such as P815 cells, which were also used as targets in some experiments.

Naïve mice were sacrificed, there spleen extracted and splenocytes isolated as described above. The first step was preparing dilutions of the dye CFSE. CFSE dye (Molecular probes) was reconstituted to according to manufacturer's specifications yielding a 5 mM stock. A working stock of CFSE, comprising of 20 µl of 5 mM CFSE added onto 180 µl PBS was then prepared. Form the working stock, three CFSE dilutions were then prepared to be used for staining:

1) 800 nm CFSE dilution: 10 ml PBS + 16 µl of working stock CFSE

2) 160 nm CFSE dilution: 8 ml PBS + 2 ml of 800 nm CFSE

3) 32 nm CFSE dilution: 8 ml PBS + 2 ml of 160 nm CFSE

The 800 nm (CFSE-Hi) and 32 nm (CFSE-Lo) dilutions were used to stain two populations of splenocytes. The naïve splenocytes cell suspension was divided equally into two 50 ml falcon tubes, where an equal volume of the CFSE dilutions were added separately to each tube and incubated at 37°C, 5% $CO_2$ for 10 min. Each of the falcon tubes was then topped up with FCS at 5x volume, to quench the reaction, and placed on ice in the dark for 5 min. The cells were then spun at 1500 rpm (Sorvel Legend RT) for 10 min to remove the FCS and were subsequently washed three times with 50 ml of R10 media, each time spinning and discarding the supernatant. This resulted in two populations of splenocytes labeled with either CFSE-Hi or CFSE-Lo.

Splenocytes labeled with CFSE-Hi were split into the relevant number of 15 ml falcon tubes for peptide pulsing. The cells were spun down at 1500 rpm (Sorvel Legend

RT) for 10 min, supernatant discarded and resuspended in 100 μl residual volume. Peptides at 4 μg/ml concentration were added at a volume of 100 μl onto the cells, resulting in a final concentration of 2 μg/ml in the mix, and incubated at 37°C, 5% $CO_2$ for 2 h with lids loosened. Cells were subsequently spun (1500 rpm for 10 min) (Sorvel Legend RT) and washed three times with R10 media. They were resuspended in 10 ml of R10 media, counted and diluted to $1\times10^6$ cells/ml. The unpulsed CFSE-Lo labeled cells were also diluted to the same concentration.

Splenocytes from immunized mice, prepared as described above, were mixed with the differentially CFSE-labeled splenocytes target cells at an effector-to-target (ET) ratio of 10:1 or 5:1 and incubated overnight at 37°C, 5% $CO_2$. For example, using the 10:1 ET ratio, $1\times10^6$ effector cells (100 μl) were mixed with $1\times10^5$ pulsed target cells (50 μl) and $1\times10^5$ un-pulsed target cells (50 μl).

The following day, cells were spun (1500 rpm for 10 min) (Sorvel Legend RT) to discard the supernatant and washed twice with 200 μl of FACS buffer. A live/dead violet stain was added into the cells at a 1:100 dilution in FACS buffer and incubated at room temperature in the dark for 20 min. The cells were washed again with FACS buffer twice and subsequently fixed with 200 μl of 1% paraformaldehyde. Killing data was acquired using flow cytometry. For analysis, cytotoxicity was calculated using the following formula: % specific lysis = 100 x (# of un-pulsed control cells – # of peptide-pulsed cells)/ # of un-pulsed control cells.

# Chapter 3:

## *Constructing the Mosaic Vaccines*

# 3. Development and Construction of the Mosaic Vaccines

## 3.1 Introduction

The conventional strategy of vaccination was using live attenuated and killed pathogens to induce protective response. These have not been successful or feasible in the fight against more complex variable human diseases such as malaria, tuberculosis and HIV. Hence the focus of vaccine development has been more on use of non-pathogenic vectored subunit genetic vaccines (438). These vaccines are defined by two components. First, an immunogen, a protein from the pathogen used in its natural or chimeric form that determines the specificity of the vaccine-elicited immune response. Our group has focused on developing a T-cell immunogen based on the most conserved regions of HIV-1 (discussed in main introduction). The work discussed here is based on vaccines using mosaic conserved immunogens designed by Bette Korber. Mosaic immunogens are designed *In-silico* (computationally) through a genetic algorithm that generates a set of sequences resembling natural sequences but in combination maximize the coverage of 9-mers potential T cell epitopes (PTE) (344, 439). The algorithm generates a set of sequence populations (parental) via random two-point recombination of natural HIV-1 sequences. Based on their 9-mer coverage, they are scored and rated to exclude sequences with rare or unnatural 9-mers. Further recombination of the parental sequences occurs, generating a set of child sequences. The child sequence is then rated and scored against the parental sequence and the one with better coverage is included into the population to further optimise the coverage in the population (Fig. 3.1) (439). Boundary regions spanning recombination breakpoints are embedded to avoid artificial junctional epitopes and ensuring these mosaic align natural proteins (344, 439). Studies on coverage efficacy and immunogenicity of mosaic vaccines are discussed in results chapter 4.

Choice of a delivery vector is the second vital component of the vaccine, as the type of vector and route of immunisation have a critical influence on the innate and adaptive immune response. They influence the magnitude, phenotype and functional quality of the vaccine elicited immune response (440, 441). Apart from the vectors immune-stimulatory functions, one must consider stability, safety, large scale manufacturing, capacity for an immunogen and pre-existing anti-vector immunity when choosing a vector (438, 441). Three vectors were employed in this project, a recombinant plasmid DNA vector and two recombinant viral vectors, chimp adenovirus virus serotype 63 (ChAdV63) and modified vaccinia virus Ankara (MVA).

**Recombinant plasmid DNA vaccine vectors**

The concept of plasmid DNA vaccines was first described in the early 1990's, when it was noted that naked DNA can transfect animal cell line *in-vivo* (442, 443). A study by Wolff *et al.* in 1990 demonstrated protein expression from DNA vectors encoding chloramphenicol acetyltransferase, luciferase or β-galactosidase injected into mouse muscle, up to two month post injection (442). This introduced the concept of using non-replicating plasmid DNA encoding protein from foreign pathogen as a mean of immunisation. In the following years several groups used DNA plasmids as vectors for vaccines against HIV-1, influenza, hepatitis B and SIV (444-447). In the case for influenza, a plasmid DNA encoding influenza A nucleoprotein was injected into BALB/c mice, generating CTL and IgG Ab inducing protection following subsequent challenges (445). Similarly with the HIV-1 study, a plasmid DNA encoding gp160 was injected intramuscularly into BALB/c, eliciting both T and B cell immune response (444, 447).

Since these first few publications, plasmid DNA has become a popular vector due to its production simplicity, safety, stability, tranfecting different cell types and ability to elicit both cellular and humoral immune responses. Furthermore and most importantly, plasmid

DNA vaccines can be used repeatedly to boost without induction of anti-vector immunity and immune interference (448). The design of plasmid DNA vectors is simple and has not evolved much. They are normally made of a minimal backbone containing a selectable marker such as a kanamycin resistance gene, an origin of replication site active in *Escherichia coli* (E.coli), a viral promoter most commonly CMV intron A promoter and a transcription termination site or a polyadenelations site e.g. BGH poly-A. Different delivery modalities of plasmid DNA vaccines have an effect on the type and magnitude of response elicited. They can be delivered via injection intradermal or intramuscular, in the latter whe used in large volumes, the DNA moves free in the blood to the spleen whereby they get picked up and processed by antigen presenting cells and initiate an immune response (449). However, although very promising in mice, plasmid DNA vaccines have not been very immunogenic in humans, unable to stimulate a strong vaccine specific immune response on there own.

Recent advances in DNA vaccine delivery using electroporation, gene gun or biojector devices have been shown to enhance immunogenicity (443, 450-452). Several recent studies in macaques monkeys have shown electroporation of HIV-1 DNA vaccines induced long lived cellular and humoral immune responses without subsequent boosting (453-455). One such study in macaque monkeys showed electroporation into the muscle of DNA vaccines with a HIV-1 env transgene followed by a gp120 protein boost elicited strong CTL responses with increased perforin and CD107a expression and a strong ant-gp120 antibody response (453). Other studies using the multigenic ADVAX HIV-1 DNA vaccine have shown when injected intramuscularly with electroporation, the magnitude of T cell responses increased by 70 fold with greater breadth, producing multiple cytokines, eliciting CD4+ and CD8+ T cells and gp120 specific immune responses (451, 456).

Other significant improvements in DNA vaccine technology include the use of cytokine adjuvants, stronger promoters, codon optimization and signal peptide such as

tissue plasminogen activator (tPA) to enhance protein expression (discussed in result chapter 5) (457-461). Challenge studies in macaques have revealed that co-administration of a purified fusion protein IL-2/Ig or a plasmid encoding IL-12 or IL-15 with a DNA vaccine encoding, SIVgag, whole SIVmac239 proteome or SIVgag plus HIV-1 Env, greatly improved the immunogenicity of the vaccine (412, 460, 462, 463). Cell mediated and humoral immune responses were significantly enhanced with the cytokine adjuvant, leading to better viremia control and clinical outcome following the challenge. They noted an improvement in the responses breadth and magnitude, an increase in IFN-γ cytokine and granzyme B production, enhancing proliferation of CD8+ and CD4+ T cells (412, 460, 462, 463). In human clinical trials, IL-12 and IL-15 plasmid cytokine adjuvants administered in conjunction with different HIV-1 vaccines, were found it to be safe and well tolerated with no adverse affects but did not enhance the immune response unless used in conjunction with electroporation (458, 459). Administering an IL-2/Ig plasmid cytokine two days post DNA vaccination (HIV-1 clade A, B, and C Env and a clade B /Gag/Pol/Nef poly protein) significantly enhanced immune response, measured by IFN-γ and IL-2 ELISPOT, in a human phase I trial (464). Agonists of TLR-4 and TLR-5 (toll like receptors), glucopyranosyl lipid A and flagellin respectively, were shown to enhance both T cell and antibody responses when used as adjuvants with a DNA vaccine (465, 466).

To date, there are four DNA vaccines licensed for use in animals against; (i) West Nile virus in horses; (ii) infectious haematopoietic necrosis virus in salmon; (iii) melanoma in dogs; (iv) growth hormone releasing hormone vaccine for foetal loss in swine. Despite their poor immunogenicity on their own in human, DNA vaccines are still widely used in the field in heterologous regimens with other vectored vaccines or proteins, whereby they have shown greater immunogenicity. Despite all the advances discussed above, the best results with HIV-1 DNA vaccines till now have been when used as a prime in a vaccine combination (discussed later in the chapter).

**Adenovirus vaccine vectors**

In 1954, adenoviruses were isolated from adenoid tissue for the first time in an attempt to find the causative agents of acute respiratory illness (467, 468). Since then a total of 52 human adenoviruses (Ad), classified into seven viral species (A-G) and a number of non-human primate adenoviruses (Chimpanzees, Bonobos, Gorillas, Macaques) have been identified (469, 470). Adenoviruses can infect a wide variety of cells via its attachment to the coxsackie and adenovirus receptor (CAR) and CD46, the latter being expressed on most nucleated cells as an inhibitory complement receptor (471-474). Transcription of the adenovirus genome is divided into three phases, early transcription which contains 5 units (E1A, E1B, E3 and E4), intermediate transcription containing two units (IX, Iva2) and one late transcriptional unit (475). Deleting the E1 transcriptional unit renders the virus replication defective, thus facilitating its use as non-replicating recombinant viral vector. Human embryonic kidney-293 (HEK-293) cells, developed in 1977 by Frank Graham, were shown to complement the E1 deletion in adenoviruses, as E1 is integrated into the cells chromosome, thus enabling the virus to propagate (476). This facilitated the development of the first replication defective E1 deleted adenovirus vectors in the late 1980's (477). The capacity of the vector for a transgene is limited by the size of the genome that can be packaged into the viral capsid, thus the final recombinant vector genome size cannot exceed 105% that of the parental genome. To increase the transgene capacity, E3 transcription unit is often deleted, increasing the insert capacity to 8 kbp of dsDNA without affecting viral replication (478, 479).

Since their development, adenoviruses have proven to be potent vectors, eliciting cell mediated and humoral immune responses. They elicit superior CD8+ T cells, up to 5 - 10 fold stronger T cell responses in comparison to DNA or MVA vaccine elicited T cells in HIV-1 and malaria pre-clinical studies (380, 480-484). Replication defective adenoviruses

have been shown to predominantly elicit effector memory CD8+ T cell responses, which are one of the strongest correlates of protection in malaria and HIV-1, due to their rapid effector function and immediate viremia control at sites of infection e.g. genital mucosa (399, 485-488). In contrast to other viral vectors, transgene expression from adenoviral vectors persist for prolonged periods of time, up to one-year post immunisation (489). Post immunisation with adenoviruses, CD8+ T cell responses exhibit a protracted contraction phase and a sustained memory population, which is indicative of a sustained low level antigen (Ag) expression or infection such as with CMV (490). This prolonged low level of immunogen expression is responsible for the long-lived immunity, continuously priming naïve T cells and maintaining an effector memory T cell response active (486-490). Induction of the innate immune response and type I interferon is integral to the adaptive immune response to adenoviruses as noted in knockout mice lacking interferon α and β receptors (IFNαβR-/-) (491). Adenoviruses mediate the induction of innate immunity via TLR2 and TLR9 (toll like receptor) in a MyD88 dependent manner, activating plasmacytoid dendritic cells (pDCc) and inducing high level production of type I interferon (491-493). Due to this, adenoviruses are potent inducers of pro-inflammatory cytokines and chemokines including TNFα, IL-12, IL-6, IFN-γ, MIP-1α, MIP-1β and RANTES (492, 494, 495).

Human adenovirus serotype 5 (Ad5) is a well characterized virus and the most studied recombinant Ad (rAd) vector showed very promising results in preclinical trials as a vector for a HIV-1 vaccine (483). However, the first human clinical trial using a rAd5 vector as a booster, expressing one of gag, pol or nef HIV-1 genes (phase IIb STEP trial) was a failure (428-430). The clinical trial was halted prematurely due to the ineffectiveness of the vaccine and the high rate of HIV-1 infection among vaccinated participant in comparison to the placebo (428-430). Pre-existing immunity to Ad5 is very high in humans, as it is a very common virus and a large percentage of the population have high

titers of Ad5 neutralising antibodies. Therefore, when used as a vector, a strong anti-vector response hinders the delivery of the transgene, rendering the vaccine ineffective (428-430, 496, 497).

This was a huge set back in HIV-1 vaccine development, but it lead to the search and development of vaccines using adenoviruses of rare serotypes including those from chimpanzees to circumvent pre-existing anti-vector immunity (411, 480, 498-503). Human adenoviruses serotype 26 and 35 are two leading candidates as vaccine vectors, due to their rarity in the human population and low neutralizing antibody titers prevalence in the population (411, 501-505). In pre-clinical animal studies, both vectors were highly immunogenic, inducing a strong CTL and antibody response (501, 504, 505). In the first human clinical trial of Ad26 vector expressing HIV-1 env from clade A, data show the induction of cellular and antibody responses with viral inhibitory capacity. The vaccine induced Env specific effector and memory CD8+ and CD4+ T cells, with degranulation, viral inhibition and antibody dependent cell mediated phagocytosis functional activity (506, 507). A second human trial was conducted using Ad35 as a vector expressing Env or GRIN (gag, reverse integrase transcriptase and nef) in healthy volunteers. The vaccine was highly immunogenic, inducing polyfunctional CD8+ and CD4+ T cells (508).

Chimpanzee adenoviruses vectors have recently emerged as an attractive alternative due to their low sero-prevalence and lack of cross reactivity with human adenoviruses (498, 500, 509). They are very immunogenic in humans, eliciting strong T cell and antibody responses, even in humans with pre-excising Ad5 immunity (436, 480, 484, 485, 510, 511). Chimp adenovirus serotype 63 (ChAdV63) has been assessed for the first time in human as a vector for a malaria vaccine (499). The outcome showed that was safe and very immunogenic in humans, eliciting antigen specific CD8+ and CD4+ T cells to unprecedented levels. They were also very efficient inducing antigen specific neutralizing antibodies (499, 512, 513). Recently ChAdV63 vector employed in a HIV-1 clinical trial,

expressing the HIVconsv immunogen, has shown very promising results. The vaccine was safe with no adverse effects in volunteers, highly immunogenic, inducing antigen specific CD8+ T cell at levels higher than that of STEP trail (396, 436, 514). ChAdV63 is one of three vectors employed in my research.

**Recombinant modified vaccinia virus Ankara (MVA) vaccine**

The history of vaccinology started with poxviruses, when Edward Jenner used a cowpox pustule to vaccinate an eight year old boy against smallpox. Two centuries later, the world health organization was successful in eradicating smallpox using an attenuated poxvirus, vaccinia virus, later developed and used as a vector. Modified vaccinia virus Ankara (MVA) was attenuated during the 1960's, via the passaging of a vaccinia virus strain 570 times in chicken embryo fibroblast, losing approximately 15% of its genome, rendering the virus replication defective in mammalian cells (515-518). MVA is a third generation vaccine for smallpox, it was used to vaccinate more than 120,000 people in southern Germany and Turkey between 1966-1980 during the smallpox eradication program (516, 519). Since then, MVA has been developed and used as vaccine vector due to its excellent safety record, ease of large scale production, its large genome thus accommodating large inserts and their adjuvant stimulatory properties (517, 518, 520).

Although MVA have been used as a smallpox vaccine in the 1970's, its first use in humans as a vector expressing a malaria antigen was in 2000 by a group in Oxford University (521). MVA is a potent immunostimulator, infecting monocytes, B cells and dendritic cells, upregulating the expression of co-stimulatory molecules and MHC-II (522). Type I interferon are subsequently released from infected dendritic cells leading to maturation and activation of uninfected dendritic cells which in turn activate cytotoxic T cell response (522-524). This cross presentation activation is the dominant mechanism of MVA's activation of CD8+ T cell (522, 524). MVA can stimulate innate immunity via

TLR 2 – TLR 6 and TLR 9, leading to the expression of type I interferon and various chemokine (CCL2, CCR1, CXC10) for cell homing and migration (522-524). MVA potent immunostimulatory is attributed to the absence of genes involved in immune evasion, normally blocking IFN-α, IFN-β and TNF-α, thus allowing strong induction of innate immunity (517, 522-524). MVA induction of strong T cell responses, specifically CD8+ T cells, has been well established now for intracellular pathogens such as HIV-1 and malaria, especially when used as a booster (396, 411, 436, 485). Most importantly, anti-vector immunity to MVA is very low and does not affect transgene delivery or the quality of antigen specific elicited immune response (525).

In clinical settings, MVA was shown to induce protective cellular and antibody responses to malaria, TB and HIV-1 (396, 436, 512, 513). In a phase I clinical trial of HIVA immunogen, HIV-1 clade A p24/p17 gag linked to a string of CTL epitopes, MVA was used as a booster following a DNA prime, was found to be safe and immunogenic, inducing multifunctional and proliferative CD8+ and CD4+ T cell responses in more than 70% of volunteers (394, 526, 527). Furthermore, preclinical and phase I/II clinical trials of HIVconsv immunogen have shown MVA to induce very strong antigen specific polyfunctional CD8+ T cells when delivered in a prime boost heterologous regimen (395, 396, 436). Preclinical and clinical data strongly support the use of MVA as a vector, especially as a booster as it induces a very strong cytolytic and humoral response. MVA is one of three vectors employed in my research presented here.

**Heterologous prime boost strategies for enhanced HIV vaccine immunogenicity**

The use of a heterologous prime boost strategy in vaccination is widely considered the most efficient mean at inducing superior quality and quantity of vaccine specific immune responses. Unlike DNA vaccine, multiple administration of viral vectors risks the development of host responses to the viral vector itself, thus reducing transgene delivery

and induction of antigen specific immune response, thus rendering the vaccine ineffective (394, 438, 528, 529). Using different vectors in a prime boost regimen limits the development of anti-vector immunity and enhances vaccine specific immune responses. Furthermore, prime boost regimens have been shown to increase the frequency of memory T cells, and the number of number of immunisations affecting the phenotype of vaccine specific memory T-cells. It has been noted, secondary and tertiary immunisations generate effector memory T cells which accumulate in non-lymphoid organs (399, 485, 530). Furthermore, certain vectors have shown efficacy only as a prime or a as booster, e.g. DNA and MVA respectively, thus supporting the concept of using different vectors in heterologous regimen.

Plasmid DNA vaccines by themselves are insufficient at inducing a strong immunogenic response, but have consistently shown better immunogenicity when used as a prime in conjunction with a viral vector or protein boost (394, 396, 432). DNA and protein co-immunisations have shown to induce long lived cellular and humoral immune responses which disseminate to mucosal sites (455). Furthermore, studies in our groups have consistently shown when DNA vaccines are used as a prime followed by an adenovirus or MVA boost, they generate long lasting polyfunctional CD8+ T cell responses (395, 396, 432, 436, 438, 526, 531, 532).

Adenoviruses vectors have been shown to be potent when used as prime or boost in different regimens, inducing strong cellular and humoral immune responses. They have been used in prime boost regimens with DNA, MVA or with an adenovirus of a different serotype (396, 432, 436, 501, 533). MVA superiority as a booster rather than as a prime is now well established as a result of many pre-clinical and human clinical trial, especially following an adenovirus prime (394-396, 436, 526, 527, 531, 532, 534). MVA are effective in inducing a strong antigen specific immune response, in agreement with its high transgene expression, however it is very short lived unlike adenoviruses, whereby low levels of

expression occur for prolonged periods after the initial vaccination. Therefore, adenoviruses are effective at eliciting effector memory CD8+ T cells whereas MVA elicit a strong burst of effector response. However, when used as a booster, MVA expands the pre-existing transgene response. The underlying mechanism for MVA's potency as a booster vector is not well understood, considering its weak priming responses. However, its efficiency to present antigens to dendritic cells and triggering of CXCL9 and CXCL10 expression are critical factors in this vectors ability to expand memory T cells (523, 535).

In a recent SIV challenge study in rhesus macaques, protection was achieved used Ad26/MVA or Ad35/Ad26 vaccine regimen expressing gag/pol/env. They demonstrated induction of neutralizing antibody and cellular immunity achieved protection from acquisition and disease progression (501). More recently, a phaseI/II clinical trial of HIVconsv immunogen delivered in a DNA, ChAdV63 MVA (DDDCM) or ChAdV63 and MVA boost (CM) showed unprecedented magnitude levels of T cell responses and viral inhibition (396). Furthermore, the RV144 trial, the only study to date showing protection used ALVAC (poxvirus vector) to prime antibody and T cell responses followed by a protein boost (433). In this study, three vectors have been used to express the various mosaic antigens, a plasmid DNA, ChAdV63 and MVA to be used in two heterologous prime boost regimens. The first regimen is three DNA, three weeks apart, followed by a ChAdV63 boost and a MVA boost six weeks later (DDDCM). The second regimen is ChAdV63 prime, followed by a MVA boost six weeks later (CM).

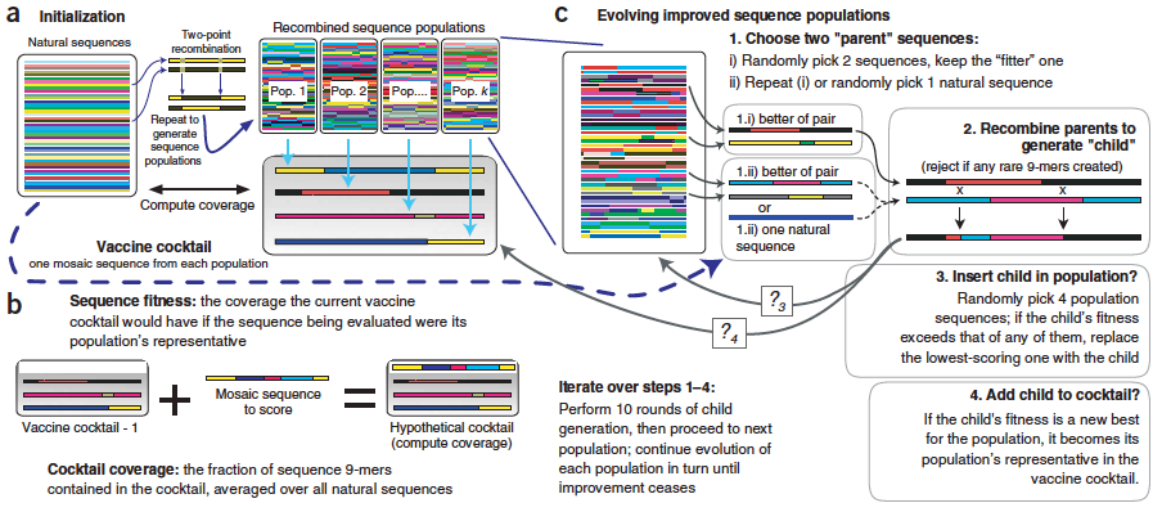



**Figure 3.1. Development of mosaic immunogens**

**(A)** A set of populations is generated by random two-point recombination of natural sequences. One sequence from each population is chosen (initially at random) for the mosaic cocktail, which is subsequently optimized. The cocktail sequences are scored by computing coverage, defined as the mean fraction of natural-sequence 9-mers included in the cocktail, averaged over all natural sequences in the input data set. Any new sequence that covers more epitopes will increase the score of the whole cocktail. (b) The fitness score of any individual sequence is the coverage of a cocktail containing that sequence plus the current representatives from other populations. (c) Optimization occurs in the following steps. **1**. Two pair of recombined sequences 'parents' are chosen. The sequence with the higher-score of the pair is chosen. **2**. Two-point recombination between the two parents is generates a 'child' sequence. If the child contains unnatural or rare 9-mers it is immediately rejected; otherwise it is scored. **3**. If the score is higher than that of any of four randomly selected population members, the child is inserted in the population in place of the weakest of the four. **4**. If its score is a new high score, the new child replaces the current cocktail member from its population. Ten cycles of child generation are repeated for each population in turn, and the process iterates until improvement stalls. (adapted from Fischer *et al.* (439))

## 3.2 Results

### 3.2.1 Mosaic Immunogens

Better Korber using the 14 conserved regions of HIVconsv has designed four mosaic immunogens for us. The first immunogen, HIVconsv mosaic (tHIVcmo) was designed to complement HIVconsv immunogen. Meaning that, only variants of epitopes present in HIVconsv are included in HIVcmo (Fig. 3.2A). The second set of mosaics, conserved 1/2/3 (tConsv1, tConsv2, tConsv3) designed to complement each other and to be used in a trivalent vaccine cocktail, each containing a different epitope variant (Fig. 3.2A). These immunogens were designed to increase epitope coverage in comparison to HIVconsv (Fig. 3.2B). The aim is to determine which of these combinations would induce a response with the greatest breadth and depth. All four mosaic immunogens and HIVconsv were expressed from a plasmid DNA, ChAdV63 and a MVA vector (Fig. 3.2A). All immunogens have a tPA leader sequence indicated by the letter (t) before the immunogen name. Hanke's group provided the tHIVconsv immunogen, I only developed the mosaic vaccines.

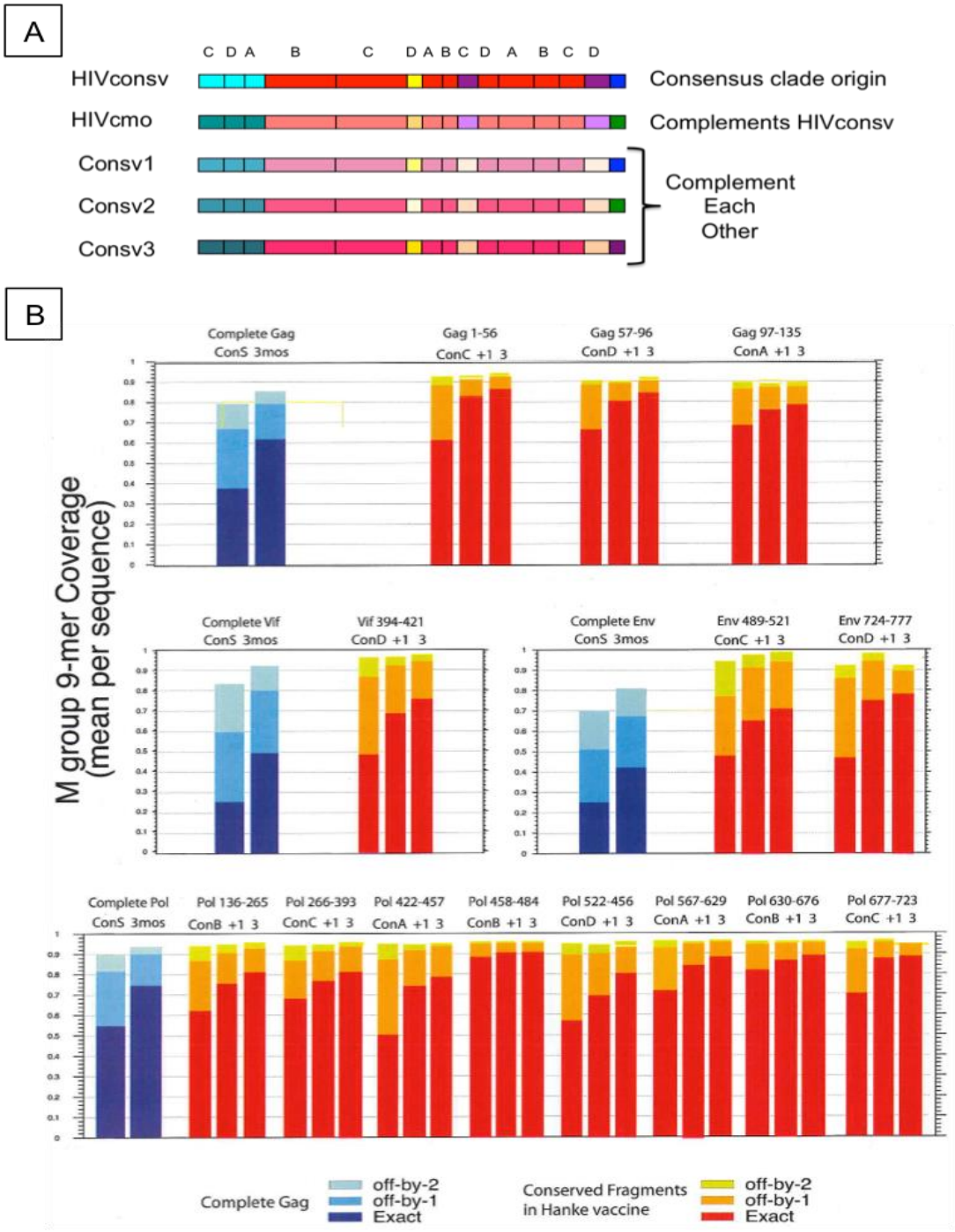



**Figure 3.2. Design and global coverage of four conserved mosaic immunogens**
**(A)** An illustration of the four mosaic immunogens and the 14 segments they are comprised of. HIVcmo is designed to complement and to be used in conjunction with HIVconsv. The Consv1, Consv2 and Consv3 mosaics are complementary to each other, used in a polyvalent cocktail. **(B)** Comparing the coverage of 9-mers (potential T-cell epitopes) of group M HIV-1 in each of the 14 segments. Full size protein (Gag, Vif, Env, Pol) and the tHIVconsv region amino acid positions as in HXB2 are listed above the graphs. For complete proteins, the consensus (ConS) and trivalent mosaic (3mos) are shown in blue. For the conserved 14 fragments, consensus with the clade used (e.g. ConC) for tHIVconsv, consensus plus one mosaic (+1) for tHIVconsv+tHIVcmo and trivalent mosaic (3) for tConsv1+tConsv2+tConsv3 are shown in red/orange/yellow. Dark blue/red – 9/9 aa match; medium blue/orange – 8/9 aa match; and light/yellow – 7/9 aa match.

### 3.2.2 Construction of Plasmid DNA (pSG2) Vaccines

To generate DNA vaccines, the mosaic immunogens of interest (tHIVcmo, tConsv1, tConsv2, tConsv3) were cloned into plasmid DNA (pSG2) vector. Gene Art provided the four immunogen genes in a shuttle plasmid DNA vector. The pSG2 vector plasmid (provided by Prof. Sarah Gilbert) comprises of a Cytomegalovirus immediate early promoter (CMV-IE) plus intron A, a Bovine growth hormone polyadenylation signal (BGH poly-A) and a Kanamycin resistance gene (Kan-R) utilised for colony screening. CMV IE intron A initiates and promotes transcription of an inserted immunogen. BGH poly-A signal is necessary for the translation of the mRNA into proteins as it is involved in the mRNA processing by ribosomes, stabilising and protecting the mRNA from hydrolytic enzymes and serves as a transcription termination sequence.

Both, pSG2 vector and the shuttle plasmids were digested via two unique enzyme restriction sites, BamHI and NotI, to isolate the immunogen genes (2500 bp) and facilitate their ligation into pSG2 (4370 bp) (Fig. 3.3A). Isolated DNA from *E.coli* DH5α screening colonies was digested to confirm ligation (Fig. 3.3B). All four vaccines were sequenced confirming the successful cloning of the immunogens between the CMV IE promoter and the BGH poly-A sequence in pSG2 (Fig. 3.4). The DNA vaccines were made with all procedures conforming to Good Laboratory Practice (GLP).

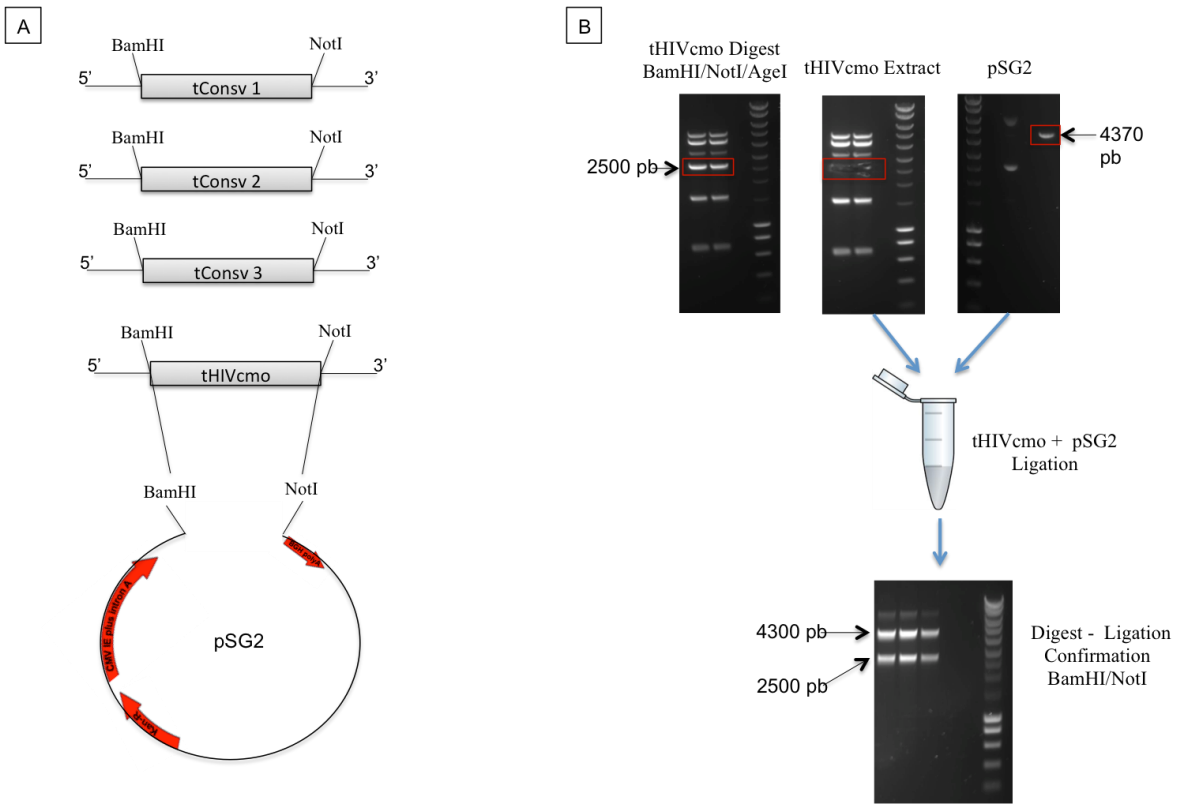



**Figure 3.3. Construction of DNA plasmid vaccines**

**(A)** Schematic representation of the cloning strategy of the four mosaic immunogens and the DNA vector pSG2. The restriction sites used for cloning (BamHI and NotI) are highlighted. **(B)** Top images of the gel electrophoresis highlighting the isolated immunogen (tHIVcmo) and the linearised vector. Bottom gel image of the vaccine digest confirming ligation of the immunogen (2500 bp) into pSG2 (4300 bp) using BamHI and NotI restriction enzymes.

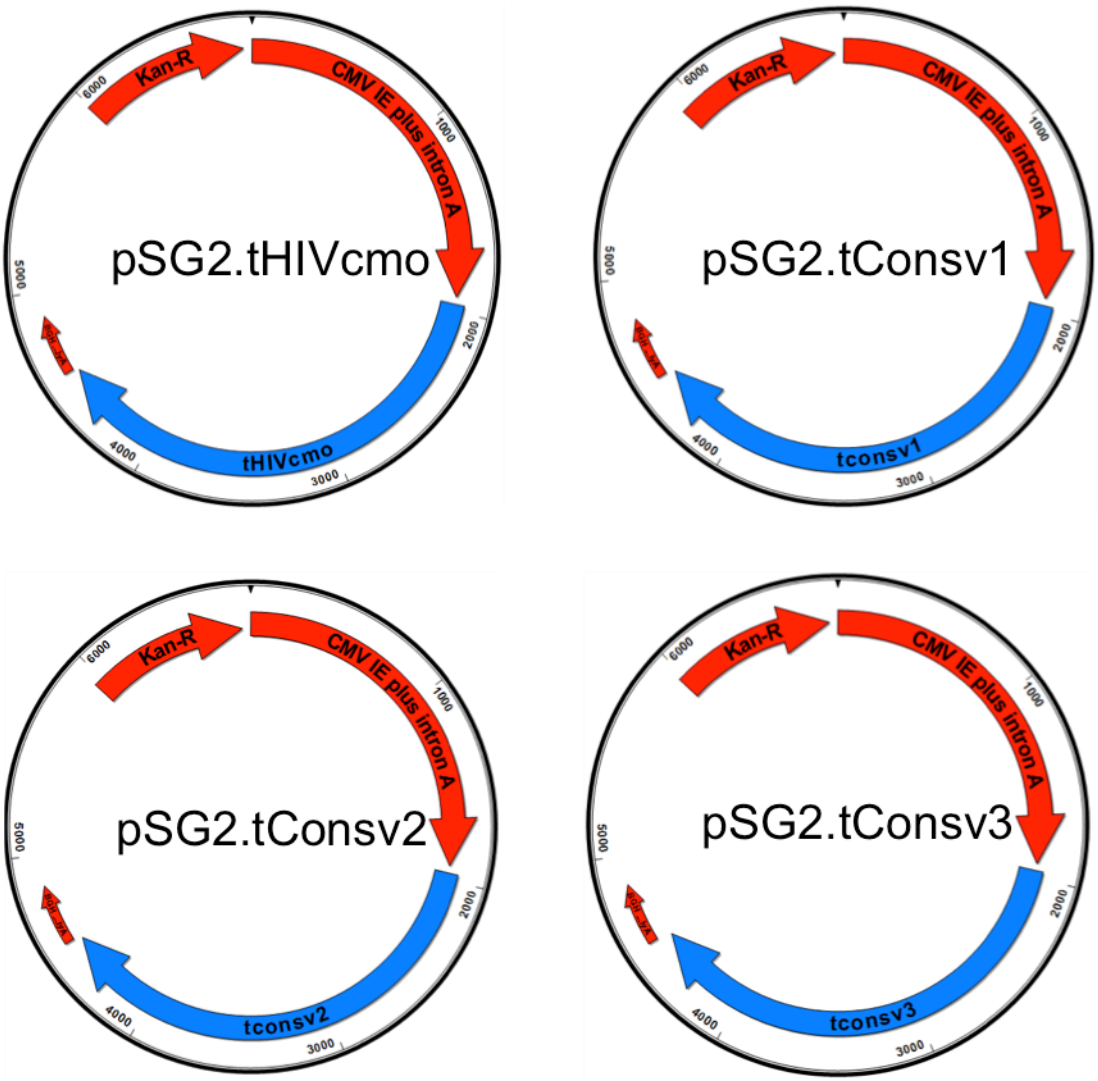



**Figure 3.4. Plasmid maps of the DNA vaccines.**
Plasmid maps of all four DNA vaccines, highlighting the Kanamycin resistance sequence (Kan-R), the Cytomegalovirus immediate early promoter (CMV IE) plus intron A, the mosaic immunogens (blue) and the Bovine growth hormone polyadenylation signal (BGH poly-A).

### 3.2.3 Construction of recombinant MVA shuttle vectors p434 and p856

For the production of the recombinant MVA (rMVA) vaccines, two shuttle plasmid vectors, p434 and p856, were used to introduce the immunogens into the virus non-essential thymidine kinase (TK) locus. The four mosaic immunogens were cloned into the shuttle plasmids between the TK left and right flanking regions (TKL and TKR) to later insert the immunogens into the virus genome via homologous recombination between the TK flanking regions. Selection for the rMVA is achieved via green fluorescent protein (GFP). Both shuttle plasmids contain the GFP gene but at different locations within the plasmid. The p434 shuttle plasmid contains the GFP between the TK flanking regions, thus retaining the GFP gene in the final rMVA vaccine; whereas in the p856 plasmid the GFP gene is located outside the TK flanking regions, therefore is lost in subsequent passages of the rMVA, producing a markerless vaccine, suitable for human clinical trial use.

Key features of both shuttle plasmids are the following: TKL and TKR, a p7.5 promoter, an ampicillin resistance gene and a GFP marker gene. Both plasmids were modified prior to cloning of the immunogens. For p434 plasmid, the p7.5 promoter and a multi cloning site (MCS), totaling 330 bp, were excised via SnaBI and PstI restriction sites from the plasmid followed by two modifications. Firstly, a new MCS (KpnI, SacII, XmaI, SalI, PstI) was inserted into the plasmid to facilitate the cloning of the immunogens, designated p434.1 (Fig. 3.5A.1). Secondly, an mH5 promoter was inserted replacing the p7.5 promoter as it supports higher protein expression and is more immunogenic, designated p434.2 (Fig. 3.5A.2) (536, 537). The mH5 promoter was excised from pEcomH5 plasmid (Gene Art) via XmaI and SalI restriction sites and ligated into the p434.1 plasmid, producing the final modified product p434.2 (Fig. 3.5B). As for p856 plasmid, a single modification was made, replacing the p7.5 promoter with the more efficient mH5 promoter via MfeI and XmaI restriction sites, designated p856.1 (Fig. 3.6).

Genes coding for the immunogens tConsv1, tConsv2 and tConsv3 (2500 bp each) were each cloned into the shuttle vector p434.2 (5500 bp) in between the TK flanking regions and adjacent to the mH5 promoter (Fig. 3.7A). Shuttle plasmids containing tConsv1, tConsv2 and tConsv3 immunogen genes (Gene Art) were digested using XmaI, KpnI and AgeI restriction sites to isolate the genes and ligate them into a p434.2 plasmid digested with XmaI and KpnI restriction enzymes (Fig. 3.7B). Ligation was confirmed via digest of the final product DNA product via XmaI and KpnI restriction enzymes yielding two distinct bands of 2500 and 5500 bp corresponding to the immunogen gene and vector plasmid (Fig. 3.7B). The confirmed plasmid was then sequenced confirming the successful cloning of the three immunogen genes (Fig. 3.9A). The tHIVcmo genes (2500 bp) was cloned into p856.1 (5800 bp) shuttle vector in between the TK flanking regions and adjacent to the mH5 promoter using via XmaI and KpnI restriction sites (Fig. 3.8A). Cloning was first confirmed via restriction enzyme digest followed by sequencing as with the other plasmid (Fig. 3.8B and 3.9B).

Following the sequence results confirming the successful cloning of the various immunogen genes, the shuttle vectors were passed on to the Vector Core Facility to generate the rMVA. This is achieved by infecting cells with parental MVA and transfecting with the shuttle vectors, allowing spontaneous homologous recombination via the TK flanking regions of plasmid and virus, thus producing a rMVA containing the immunogen gene, the final vaccine product. Identity, flank and purity PCR checks were performed during the different production stages of the virus to determine contamination, presence of parental virus and the identity of the virus. The rMVA vaccines were made with all procedures conforming to GLP.

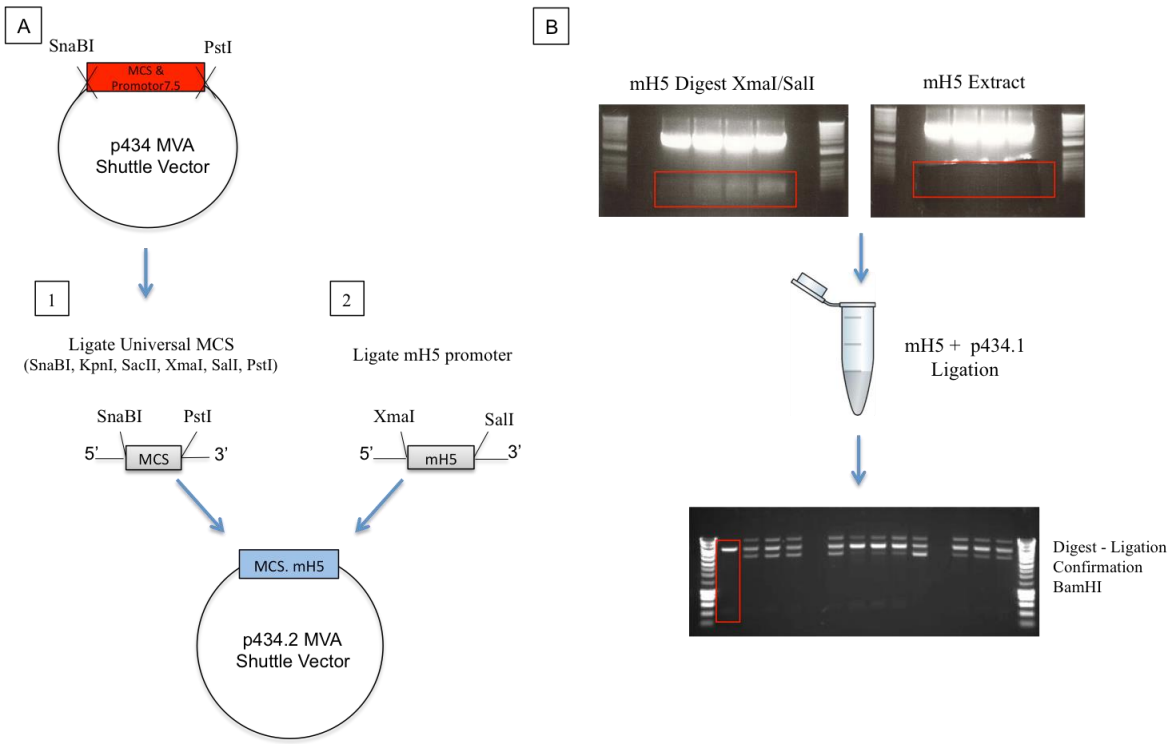



**Figure 3.5. Modification of MVA shuttle vector p434**

**(A)** Schematic representation of p434 shuttle vector modification, whereby a multi cloning site (MCS) and the p7.5 promoter were excised from the plasmid via SnaBI and PstI restriction sites. (1) First modification of the shuttle plasmid, inserting a new MCS generating a new shuttle plasmid (p434.1). (2) Second modification, insertion of mH5 promoter into p434.1, generating the final product p434.2 plasmid. **(B)** Top gel electrophoresis image of the mH5 promoter excised and extracted (red box) from its parental plasmid (pEcomH5) to be ligated into p434.1 shuttle vector. Bottom gel image of the restriction enzyme digest using BamHI to confirm the ligation of mH5 into p434.1.

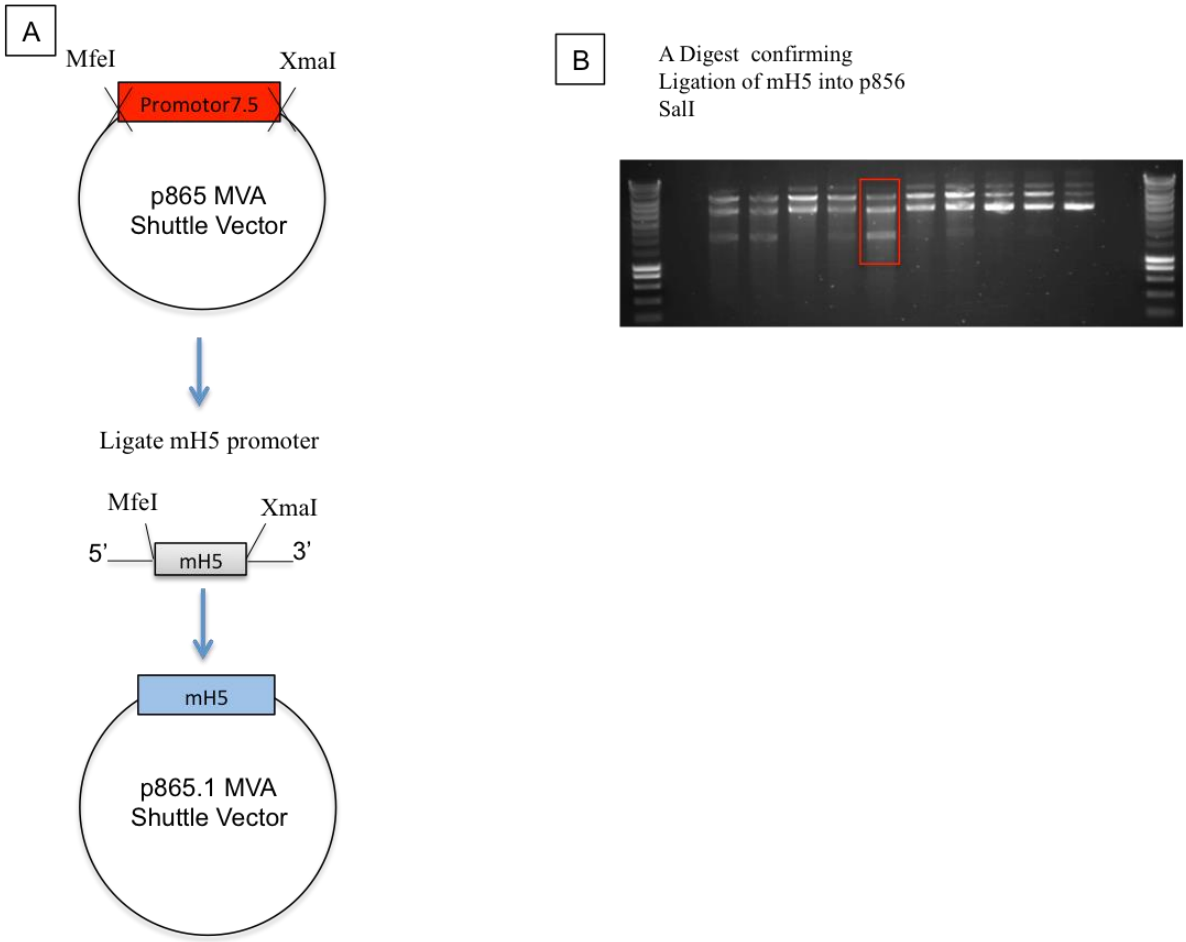



**Figure 3.6. Modification of MVA shuttle vector p856**

**(A)** Schematic representation of p856 shuttle vector modification. The p7.5 promoter was excised from the plasmid via MfeI and XmaI restriction sites and replaced with the mH5 promoter, generating the final product p856.1 plasmid. **(B)** Gel electrophoresis image of the restriction enzyme digest using SalI to confirm the ligation of mH5 into p856.

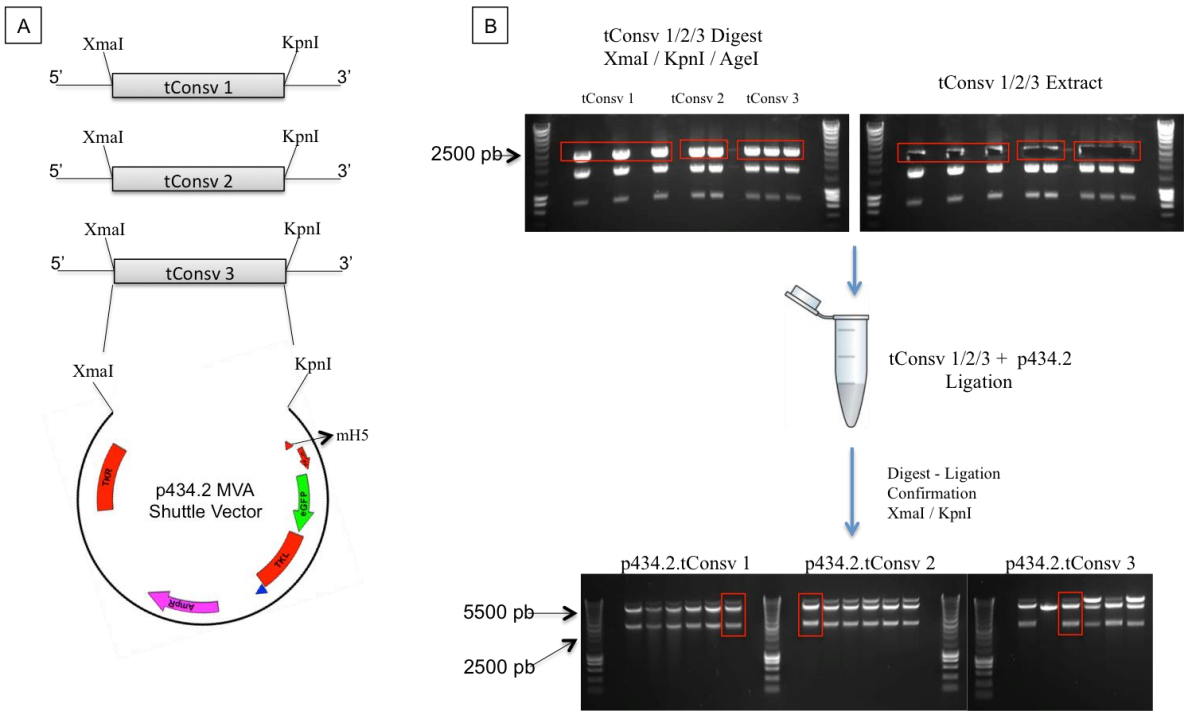



**Figure 3.7. Cloning of tConsv 1/2/3 into the shuttle vector p434.2**

**(A)** Schematic representation of the cloning strategy utilised to insert the immunogens into the MVA shuttle vector p434.2 plasmid via the restriction sites XmaI and KpnI. **(B)** Top gel electrophoresis image of tConsv1, tConsv2 and tConsv3 (2500 bp) excised and extracted (red box) from its parental plasmid via XmaI, KpnI and AgeI restriction site to be ligated into p434.2 shuttle vector. Bottom gel image of the restriction digest using XmaI and KpnI enzymes, resulting in two distinct bands of 2500 bp (immunogen) and 5500 bp (vector), confirm the ligation of the immunogens into the p434.2 plasmid.

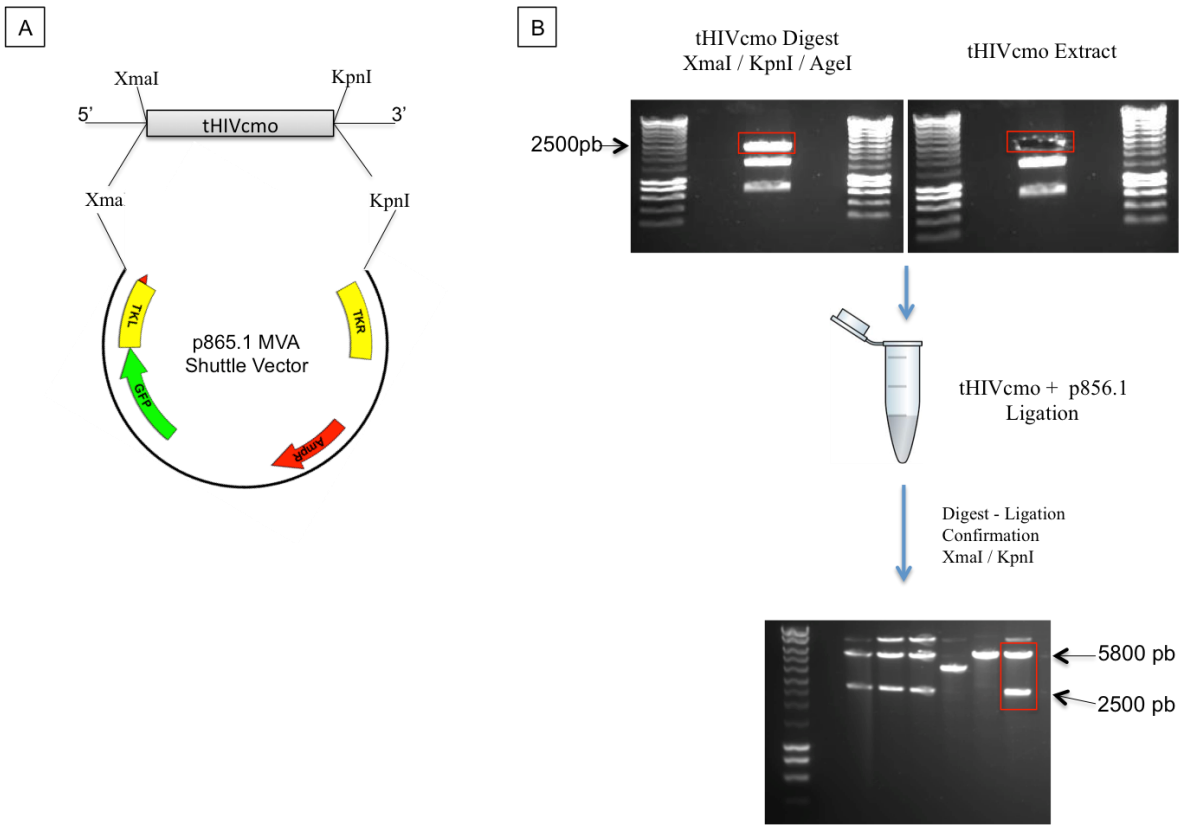



**Figure 3.8. Cloning of tHIVcmo into the shuttle vector p856.1**

**(A)** Schematic representation of the cloning strategy utilised to insert tHIVcmo into the MVA shuttle vector p856.1 plasmid via the restriction sites XmaI and KpnI. **(B)** Top gel electrophoresis image of tHIVcmo (2500 bp) excised and extracted (red box) from its parental plasmid via XmaI, KpnI and AgeI restriction site to be ligated into p856.1 shuttle vector. Bottom gel image of the restriction digest using XmaI and KpnI enzymes, resulting in two distinct bands of 2500 bp (tHIVcmo) and 5800 bp (p856.1) confirming ligation.

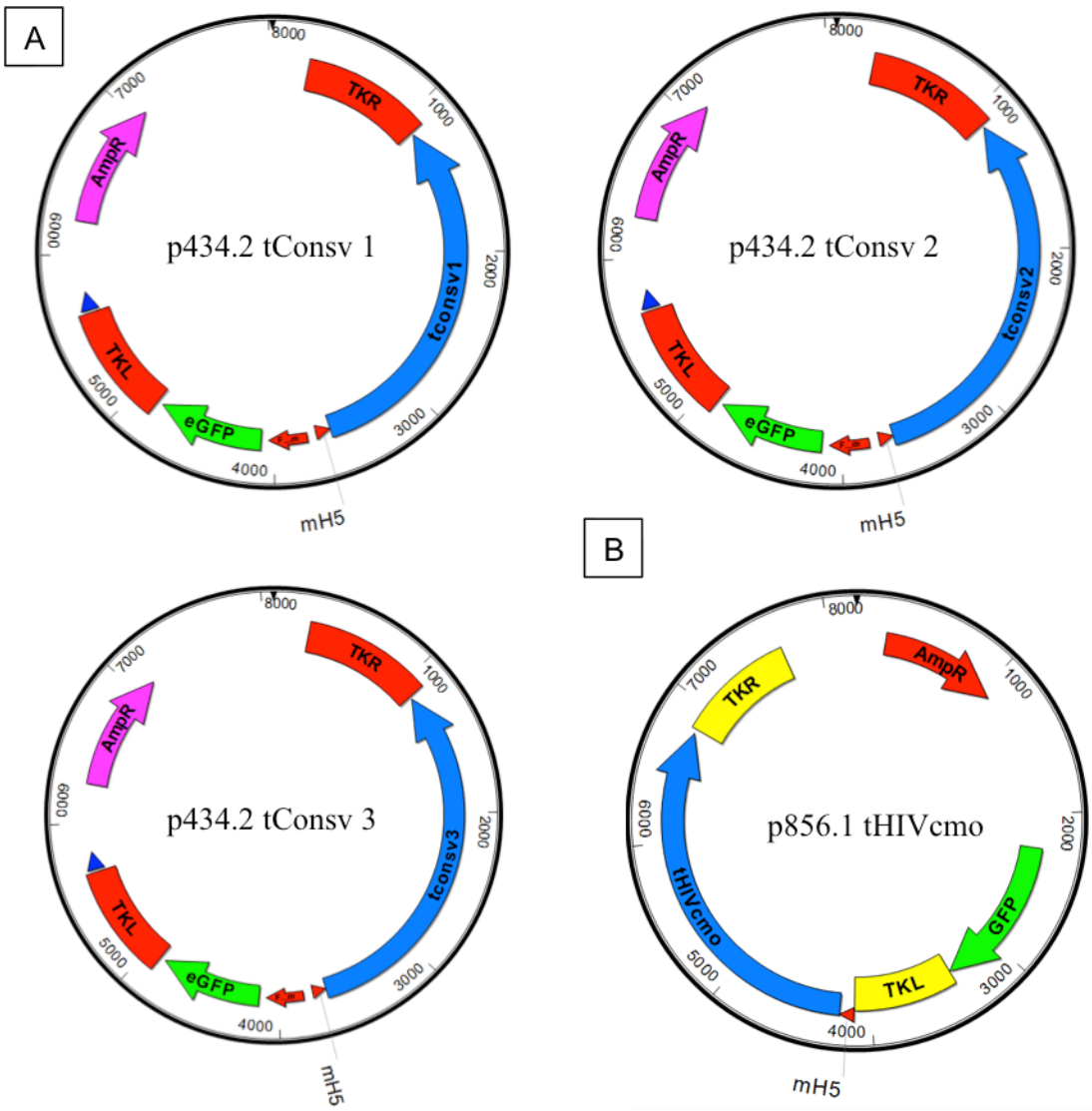



**Figure 3.9. Plasmid maps of MVA shuttle vectors p434.2 and p856.1**
**(A)** Plasmid maps p434.2 shuttle vector with the mosaic immunogen (blue) cloned in between the TK flanking regions, TKR and TKL (red), with the mH5 promoter highlighted next to the immunogen. The GFP marker gene is also located between the TK flanking regions. **(B)** Plasmid map of the p856.1 shuttle vector with tHIVcmo (blue) cloned in between the TK flanking regions, TKR and TKL (yellow), with the mH5 promoter highlighted next to the immunogen and the GFP marker gene located outside the TK flanking regions.

### 3.2.4 Construction of Chimp adenovirus 63 (ChAdV63) vaccines

Recombinant ChAdV63 vaccines were generated using site specific homologous recombination properties of bacteriophage lambda through the use of Gateway technology. Two vector plasmids, an entry vector and a destination vector, are used to assemble the rChAdV63 genome. The key features of the entry vector are an attL flanking regions, a CMV IE promoter and a BGH poly-A sequence in between the flanking regions and kanamycin resistance gene located outside the flanking the regions. The key features of the destination vector, which contains the ChAdV63 genome backbone (35 kb), are an ampicillin resistance gene and a chloramphenicol resistance gene flanked by attR sites. These sites allow the recombination and exchange of the sequence within attL (entry vector) and attR (destination vector) via LR clonase reaction.

The first step was cloning the four mosaic immunogen genes (tHIVcmo, tConsv1, tConsv2, tConsv3) into a ChAdV63 entry vector. The transgene is cloned in between the attL flanking regions. The four transgenes were excised from their parental plasmid (Gene Art) and ligated into the entry vector between the KpnI and NotI sites (Fig. 3.10A). This inserted the transgene between the CMV IE promoter and the BGH poly-A sequence, which lie within the attL flanking regions (Fig. 3.10A). Insertion of the genes was then confirmed by restriction enzyme digest. The transgene was next transferred into the Gateway destination vector via LR clonase reaction. Once recombination is complete, the transgene is inserted into the destination vector replacing the chloramphenicol resistance gene (Fig. 3.10B). The loss of chloramphenicol allows for the selection of the correct destination vector via colony screening. Destination vectors with the correct insert would be ampicillin resistant and chloramphenicol sensitive. Following confirmation, the destination vector is linearised and the ampicillin resistance gene is excised via restriction enzyme digest. Once linearised and ampicillin free, the destination vector was passed on to the vector core facility, whereby they transfected 293 T-cells with the vector to generate

and rescue the virus. The ChAdV63 vaccines were made with all procedures conforming to GLP.

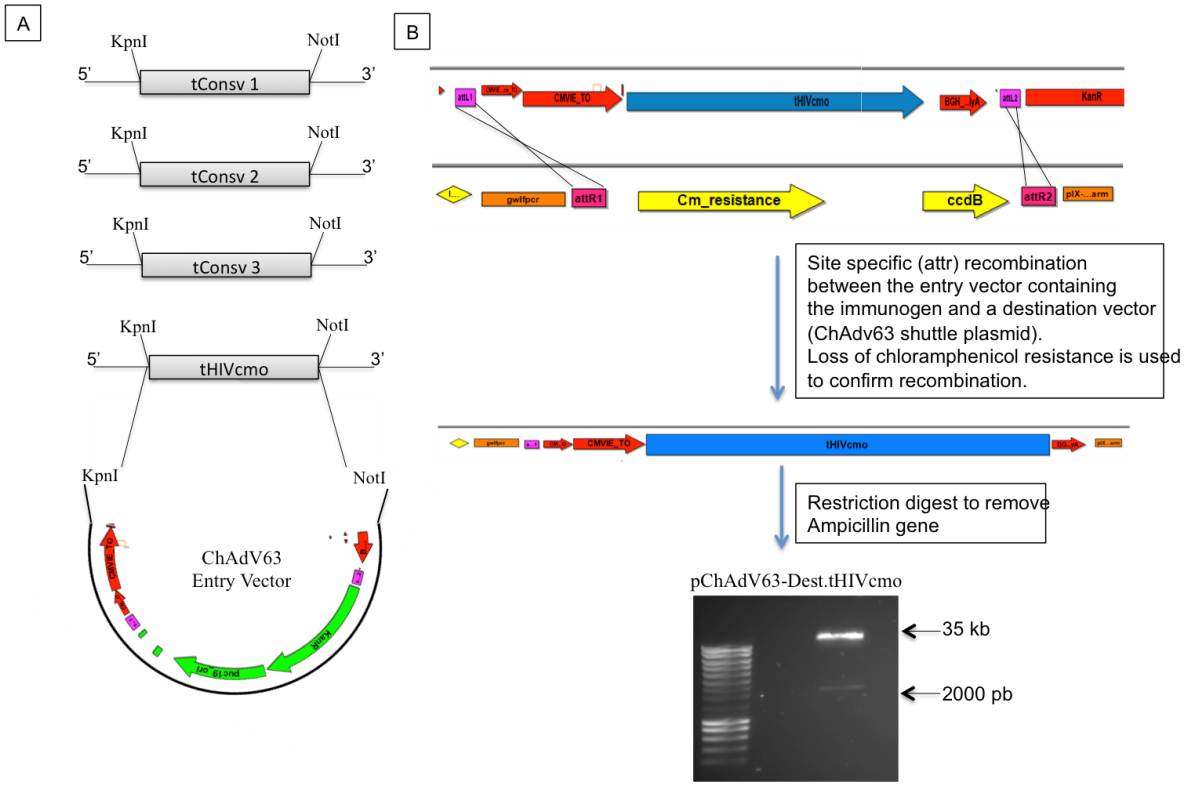



**Figure 3.10. Construction of ChAdV63 vaccines via Gateway cloning**

**(A)** Schematic representation of the cloning strategy utilised to insert the four mosaic immunogens into the ChAdV63 entry vector via the KpnI and NotI sites. The immunogen is cloned within the attL flanking regions (violet) between the CMV IE promoter and the BGF poly-A sequence. **(B)** Gateway LR clonase between the entry and destination vector. Recombination occurs between the attL of the entry vector (violet) and attR destination vector (pink) sites, resulting in the insertion of the immunogen (blue) into the destination vector replacing the chloramphenicol resistance gene (yellow). Gel electrophoresis image of the restriction digest product removing the ampicillin resistance gene and linearising the destination vector.

### 3.2.5 Construction of single epitope DNA plasmid vaccine (mini-genes)

Preliminary analysis of the depth of T-cell responses from HHD mice vaccinated with tHIVconsv and tHIVcmo have identified eleven epitope variants unable to induce a response. The lack of responses indicates that these epitopes are subdominant within the tHIVconsv and tHIVcmo immunogens. To better examine the depth of the CD8+ T cell responses elicited by these two vaccines, these eleven subdominant epitopes were cloned and expressed individually using the pSG2 DNA plasmid vector, designated mini-gene vaccines.

Double strands of DNA oligonucleotide sequences of the eleven epitopes were developed (Eurofins) with an EcoRI overhang and phosphate group in the 5' end to assist in the cloning (Fig. 3.11). Prior to cloning of the mini-genes into the DNA plasmid vector, pSG2 was modified by inserting part of the fragment C of tetanus toxin (FrC). The first domain sequence (DOM) (provided by Dr. Freda Stevenson, University of Southampton) of the tetanus toxin FrC with was cloned into pSG2 to act as an immune booster (Fig. 3.12A). The DOM sequence contains the p30 epitope, a well described universal helper epitope binding to both mouse and human MHCII and inducing CD4+ T helper cells (538-540). The insertion of the DOM sequence in DNA plasmid vaccines was shown to increase the frequency of CD8+ and CD4+ T cell responses (538-540). The DOM sequence was cloned into pSG2 between the CMV IE promoter and BGH poly-A sequence via KpnI and NotI restriction sites (Fig. 3.12B). Each mini-gene oligonucleotide was cloned individually into pSG2.DOM via an EcoRI restriction site in the C-terminus of the DOM sequence (Fig. 3.12C). All 11 vaccines were sequenced to confirm the cloning was successful (Fig. 3.12D).

| | | |
|---|---|---|
| 1) EPFRDYVDRF | (F) | 5'PHO-**aatt**TGAGCCCTTCCGCGACTACGTGGACCGCTTCT-3' |
| | (R) | 5'PHO-**aatt**AGAAGCGGTCCACGTAGTCGCGGAAGGGCTCA-3' |
| 2) FLGKIWPSR | (F) | 5'PHO-**aatt**TTTCCTGGGCAAGATCTGGCCCTCCCGCT-3' |
| | (R) | 5'PHO-**aatt**AGCGGGAGGGCCAGATCTTGCCCAGGAAA-3' |
| 3) VLDVGDAYFSV | (F) | 5'PHO-**aatt**TGTGCTGGACGTGGGCGACGCCTACTTCTCCGTGT-3' |
| | (R) | 5'PHO-**aatt**ACACGGAGAAGTAGGCGTCGCCCACGTCCAGCACA-3' |
| 4) IISLWDQSL | (F) | 5'PHO-**aatt**TATCATCTCCCTGTGGGACCAGTCCCTGT-3' |
| | (R) | 5'PHO-**aatt**ACAGGGACTGGTCCCACAGGGAGATGATA-3' |
| 5) EPFREYVDRF | (F) | 5'PHO-**aatt**TGAGCCCTTCCGCGAGTACGTGGACCGCTTCT-3' |
| | (R) | 5'PHO-**aatt**AGAAGCGGTCCACGTACTCGCGGAAGGGCTCA-3' |
| 6) FLGRIWPSK | (F) | 5'PHO-**aatt**TTTCCTGGGCCGCATCTGGCCCTCCAAGT-3' |
| | (R) | 5'PHO-**aatt**ACTTGGAGGGCCAGATGCGGCCCAGGAAA-3' |
| 7) ILDVGDAYFSV | (F) | 5'PHO-**aatt**TATCCTGGACGTGGGCGACGCCTACTTCTCCGTGT-3' |
| | (R) | 5'PHO-**aatt**ACACGGAGAAGTAGGCGTCGCCCACGTCCAGGATA-3' |
| 8) IISLWDESL | (F) | 5'PHO-**aatt**TATCATCTCCCTGTGGGACGAGTCCCTGT-3' |
| | (R) | 5'PHO-**aatt**ACAGGGACTCGTCCCACAGGGAGATGATA-3' |
| 9) KMIGGIGGFI | (F) | 5'PHO-**aatt**TATGATCGGCGGCATCGGCGGCTTCATCT-3' |
| | (R) | 5'PHO-**aatt**AGATGAAGCCGCCGATGCCGCCGATCATA-3' |
| 10) PLVKLWYQL | (F) | 5'PHO-**aatt**TCCCCTGGTGAAGCTGTGGTATCAGCTGT-3' |
| | (R) | 5'PHO-**aatt**ACAGCTGATACCACAGCTTCACCAGGGGA-3' |
| 11) LLWKGEGA | (F) | 5'PHO-**aatt**TCTGCTGTGGAAGGGAGAGGGCGCCGTGT-3' |
| | (R) | 5'PHO-**aatt**ACACGGCGCCCTCTCCCTTCCACAGCAGA-3' |

**Figure 3.11. Mini-gene sequences**

List of the 11 sub-dominant epitopes and their respective oligonucleotide double strand sequences. The EcoRI overhang is highlighted in bold.

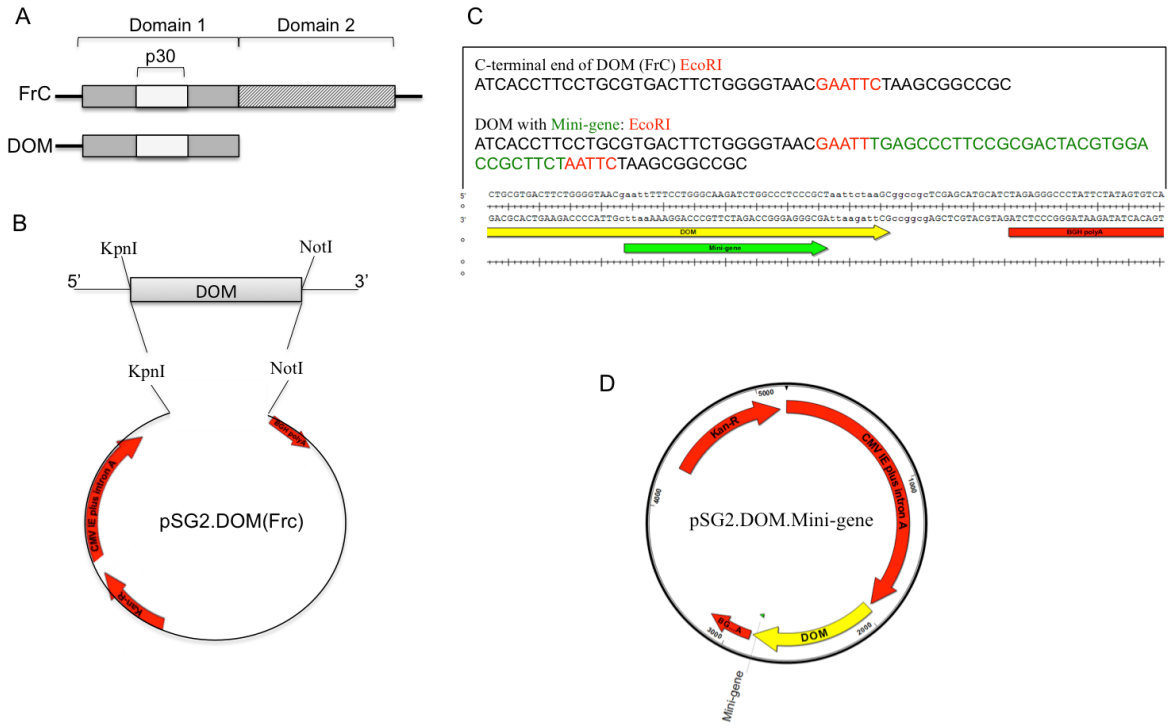



**Figure 3.12. Construction of the mini-gene vaccines**

(A) Schematic representation of tetanus toxin fragment C (FrC) and DOM. (B) Cloning of the DOM sequence into pSG2 between the CMV IE promoter and the BGH poly-A sequence via KpnI and NotI restriction sites. (C) Cloning of the mini-gene (green) sequence into pSG2 via the EcoRI (red) site at the C-terminus end of DOM. (D) Plasmid map of a mini-gene vaccine, highlighting DOM (yellow) and the mini-gene (green).

### 3.2.6 Expression of the mosaic immunogens from the DNA, ChAdV63 and MVA vaccines

Immunofluorescence analysis was employed to confirm the expression of the four immunogens from the vaccines. This assay does not provide quantitative data on the level of expression of the immunogen but rather confirms expression from the vaccines. Each of the four immunogens had a tag sequence at the C terminal end, which was targeted to detect expression. Immunogens tHIVcmo and tConsv1 had a histidine tag (His) (HHHHHH), tConsv2 had a PK tag (IPNPLLGLD) and tConsv3 had a c-myc tag (EQKLISEEDL). Monoclonal antibodies (mAb) specific to each tag were used to detect the expression of each immunogen in transfected or infected HeLa cells.

To confirm expression of the immunogens from the DNA vector, HeLe cells were transfected with 2500 ng of pSG2.tHIVcmo, pSG2.tConsv1, pSG2.tConsv2 or pSG2.tConsv3 vaccine separately for 24 hours. Following transfection cells were stained with a FITC (green) conjugated mAb (anti-His, anti-Pk, anti-c-myc) specific to each immunogen and DAPI stain for the nucleus. All four immunogens were successfully expressed from the DNA vaccines in HeLa cells (Fig. 3.13).

To confirm expression of the immunogens from the ChAdV63 vaccines, HeLe cells were infected with ChAdV63.tHIVcmo, ChAdV63.tConsv1, ChAdV63.tConsv2 or ChAdV63.tConsv3 with a multiplicity of infection (MOI) of 10. As for the MVA vaccines, HeLa cells were infected with MVA.tHIVcmo, MVA.tConsv1, MVA.tConsv2 or MVA.tConsv3 with a MOI of 5. Cells were infected (ChAdV63 or MVA) for 2 hours only and then incubated, in complete media only, for 24 hours prior to staining. FITC (green) conjugated mAb was used to stain all ChAdV63 infected cells and MVA.tHIVcmo. Cells infected with MVA.tConsv1, MVA.tConsv2 or MVA.tConsv3 were stained with a primary tag specific mAb and a secondary Alexa fluora 594 (red) conjugated anti-mouse Ab, due to

the presence of GFP in the MVA. All four immunogens were successfully expressed from the ChAdV63 (Fig. 3.14) and MVA vaccines in HeLa cells (Fig. 3.15).

Three controls were employed to ensure staining was specific to the expression and to the tag. The first control was staining of un-infected cells with DAPI and anti-His, anti-PK or anti-cmyc mAb conjugated to either FITC or Alexa fluora 594. As expected no FITC or Alexa fluora signal was detected, confirming no unspecific binding to HeLa cells (Fig. 3.16A). The second control was staining infected cells (ChAdV63) with a mAb specific for a different tag e.g. HeLa cells infected with ChAdV63.tHIVcmo, containing the His tag, were stained with using an anti-Pk mAb and DAPI. This is to ensure that signals detected are immunogen specific. No signal was detected for all three tags control (Fig. 3.16B). The third control was staining infected cells (ChAdV63) with the Alexa fluora 594 conjugated secondary Ab only. No signal was detected, confirming no unspecific binding to the cell or the immunogen (Fig. 3.16C).

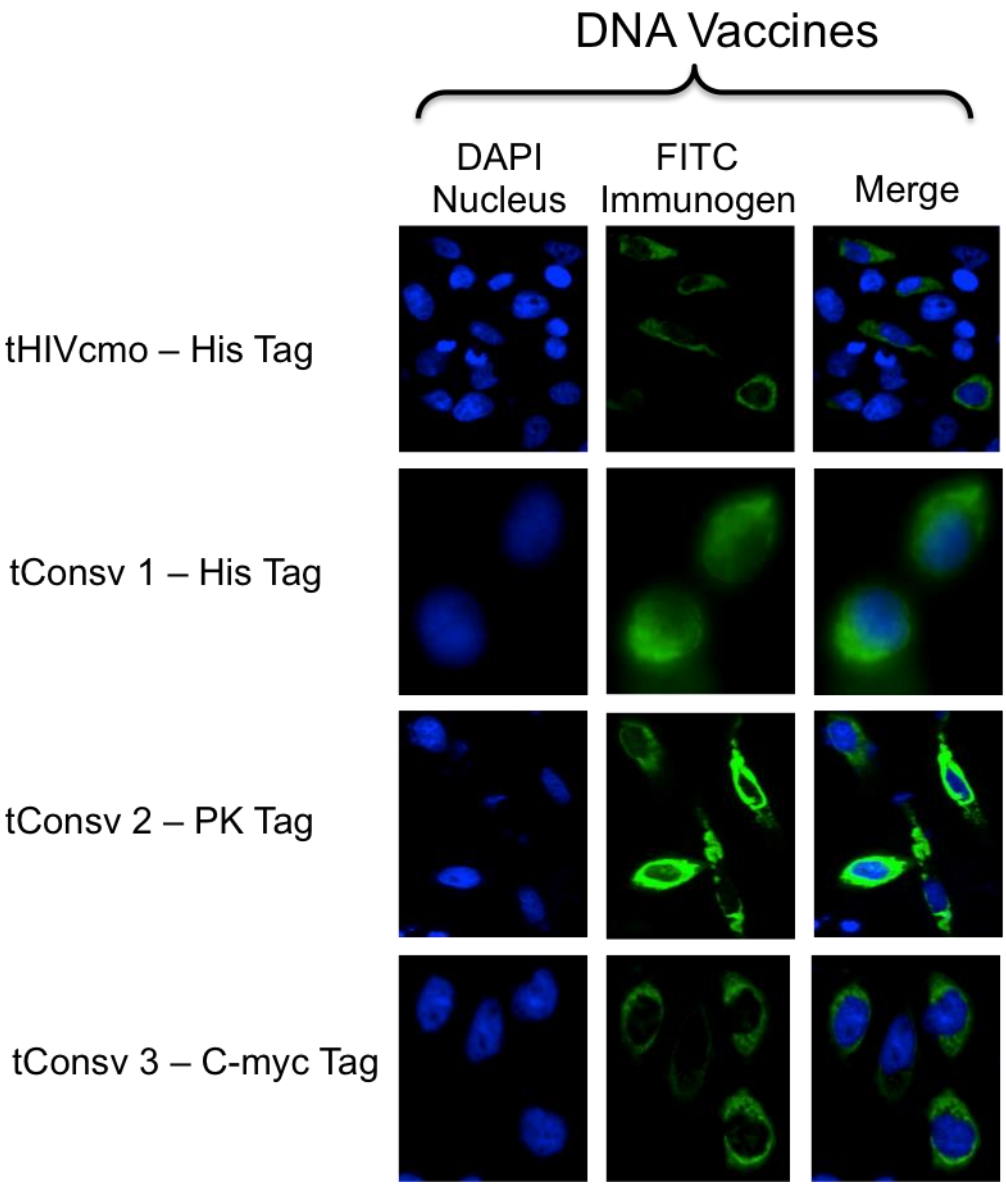



**Figure 3.13. Expression of DNA vectored mosaic immunogens**
Immunofluorescence images of HeLa cells transfected with 2500 ng of pSG2.tHIVcmo (His tag), pSG2.tConsv1 (His tag), pSG2.tConsv2 (Pk tag) or pSG2.tConsv3 (C-myc tag). Cells were stained with a FITC conjugated mAb antibody (anti-His, anti-Pk or anti-c-myc) targeting the C-terminal tag in the immunogen. Blue represents DAPI stain for the nucleus, while green represents the tag staining of the immunogens.

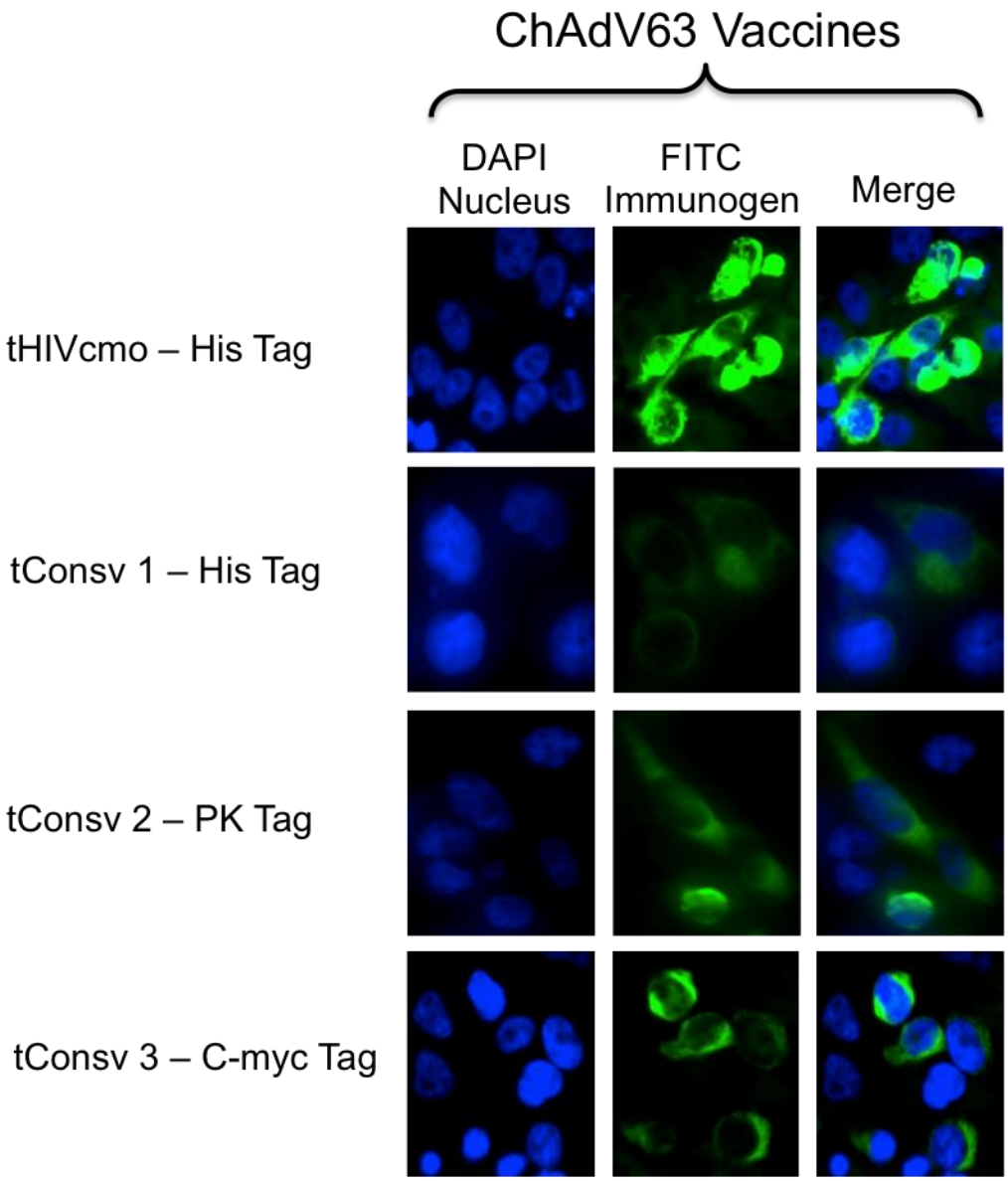



**Figure 3.14. Expression of ChAdV63 vectored mosaic immunogens**
Immunofluorescence images of HeLa cells infected with ChAdV63.tHIVcmo (His tag), ChAdV63.tConsv1 (His tag), ChAdV63.tConsv2 (Pk tag) or ChAdV63.tConsv3 (c-myc tag). HeLa cells were infected with ChAdV63 at a MOI of 10. Cells were stained with a FITC conjugated mAb antibody (anti-His, anti-Pk or anti-c-myc) targeting the C-terminal tag in the immunogens. Blue represents DAPI stain for the nucleus, while green represents the tag staining of the immunogens.

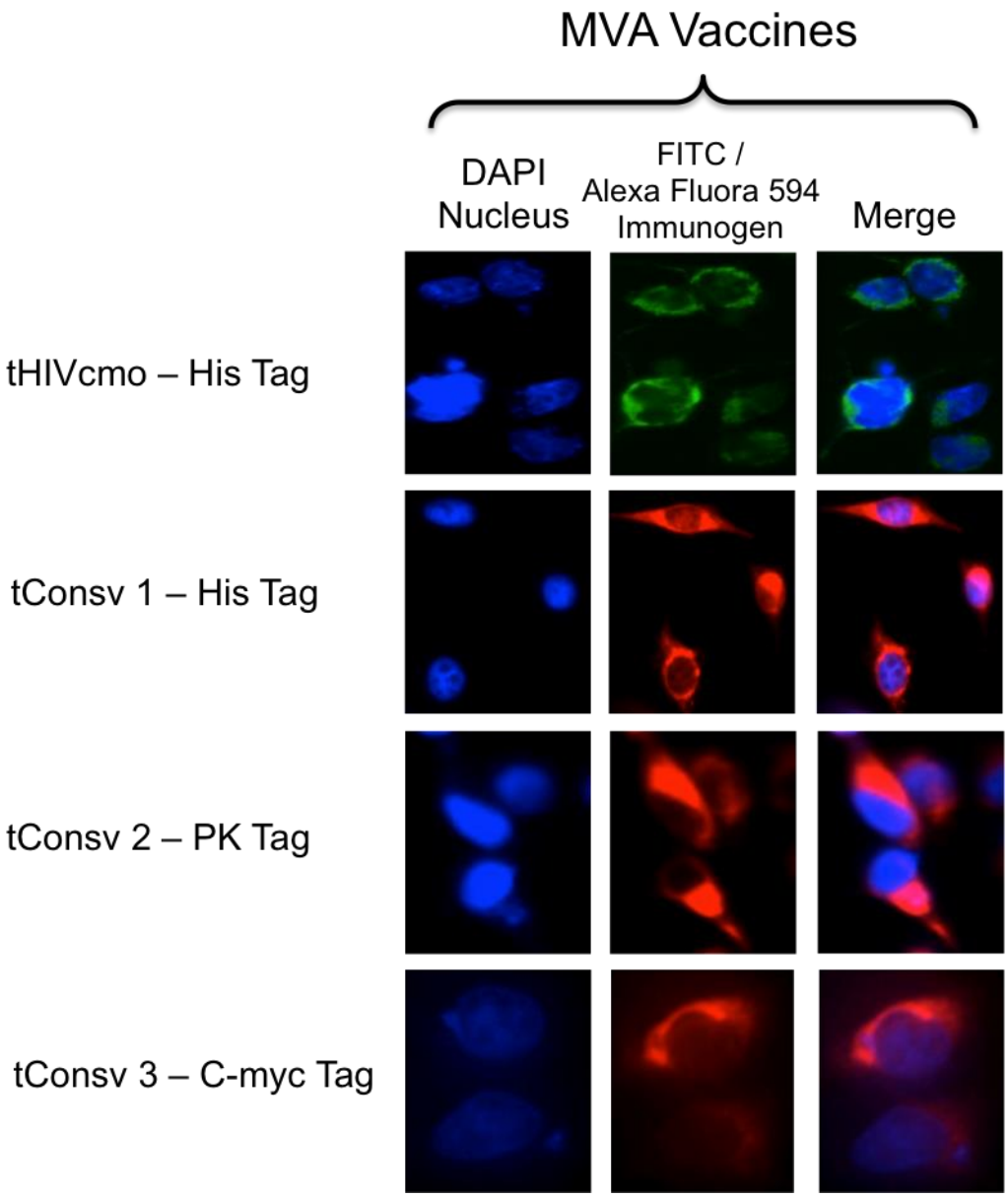



**Figure 3.15. Expression of rMVA vectored mosaic immunogens**
Immunofluorescence images of HeLa cells infected with MVA vectored vaccines at a MOI of 5. MVA.tHIVcmo infected cells were stained with a FITC conjugated anti-His antibody (anti-His, anti-Pk or anti-c-myc) targeting the C-terminal His tag in the immunogen. HeLa cells infected with MVA.tConsv1 (His tag), MVA.tConsv2 (Pk tag) or MVA.tConsv3 (c-myc tag) were stained with an Alexa fluora 594 conjugated mAb (anti-His, anti-Pk or anti-cmyc) targeting the C-terminal tag in the immunogens. Blue represents DAPI stain for the nucleus, while green and red represents the tag staining of the immunogens.

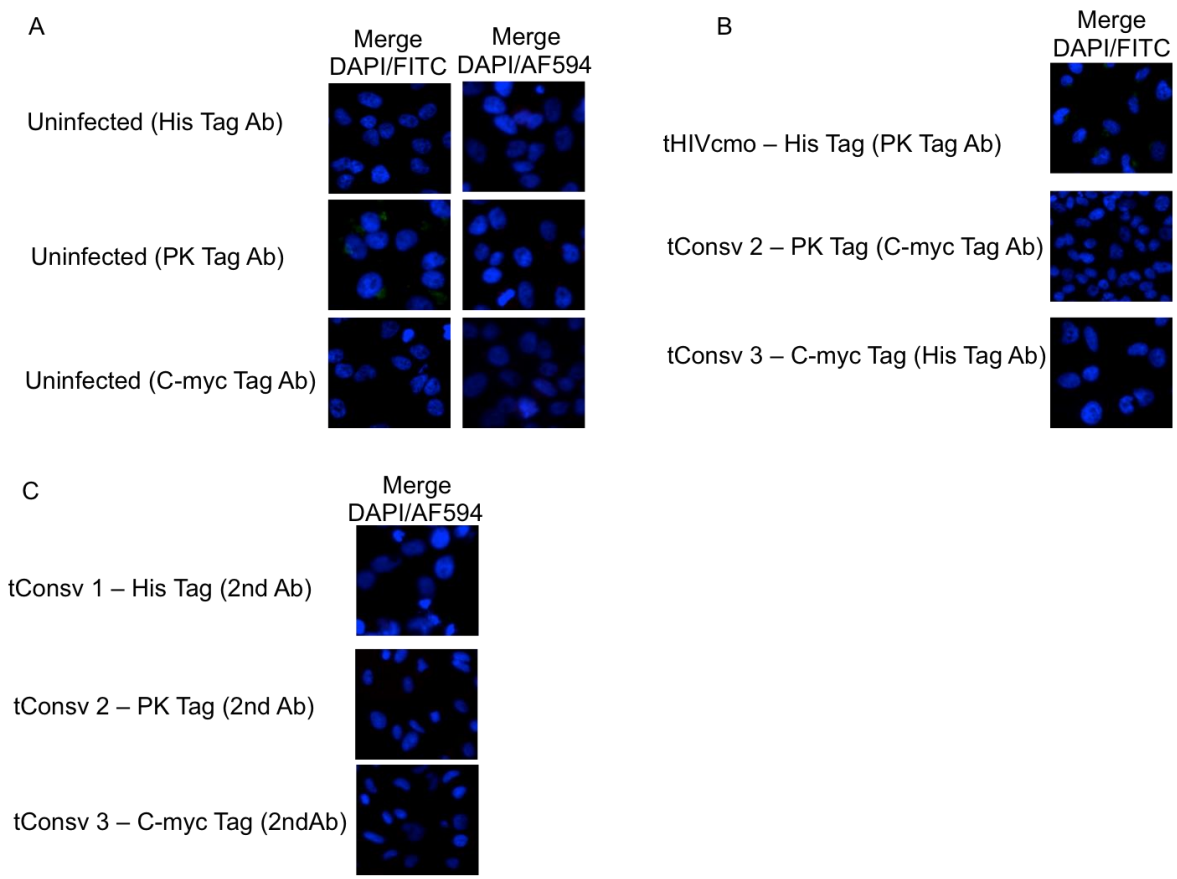



**Figure 3.16. Immunofluorescence negative controls**

**(A)** Uninfected HeLa cells stained with FITC or Alexa fluora 594 conjugated mAb antibody (anti-His, anti-Pk or anti-c-myc) and DAPI nucleus stain. **(B)** ChAdV63 infected HeLa cells stained with FITC conjugated mAb antibody (anti-His, anti-Pk or anti-c-myc) not matching the immunogens Tag and DAPI nucleus stain. Cells infected with ChAdV63.tHIVcmo (His tag) were stained with an anti-Pk antibody. Cells infected with ChAdV63.tConsv2 (Pk tag) were stained with an anti-c-myc antibody. Cells infected with ChAdV63.tConsv3 (C-myc tag) were stained with an anti-His antibody. **(C)** HeLa cells infected with ChAdV63.tConsv1, ChAdV63.tConsv2 or ChAdV63.tConsv3 were stained with DAPI nucleus stain and Alexa fluora 594 conjugated secondary antibody. Blue represents DAPI stain for the nucleus, while green and red represents the tag staining of the immunogens.

## 3.3 Discussion

The four mosaic immunogens (tHIVcom, tConsv 1, tConsv 2, tConsv 3) were all successfully cloned and expressed from the three designated vectors (pSG2 DNA, ChAdV63, MVA). The mini-gene vaccines were also all cloned successfully into the plasmid DNA vector. This work was completed in the first 18 month of my project. The vaccines were then tested in mice using multiple heterologous prime boost regimens to determine their immunogenicity, breadth and depth of the vaccine elicited immune responses.

# Chapter 4:

## *Immunogenicity of the Conserved Mosaic Vaccines in HHD Mice*

# 4. Comparing the Immunogenicity of the Conserved Mosaic Vaccines to HIVconsv in HHD Mice

## 4.1 Introduction

Developing an effective HIV-1 vaccine to protect people from infection or developing AIDS remains a major focus of HIV research. Ideally a vaccine against HIV-1 may have to induce both humoral and cellular immune responses. Eliciting any single arm of these, if effective, may impact the AIDS pandemic (167, 541). The vaccines I developed aim to maximize the induction of protective T cell responses. Although CD8 T cells cannot prevent infection, increasing evidence points to their central role in the control of HIV-1 (97, 542-544). Cytotoxic T lymphocytes (CTL) directly kill infected, virus producing cells thereby reducing the production of more virions. They produce cytokines such as IFN-$\gamma$ and TNF-$\alpha$ with antiviral activities and β-chemokines RANTES, MIP-1α and MIP-1β, which compete for the CCR5 receptor, thus inhibiting infection by blocking the attachment of HIV-1 and entry into the cell (284, 399, 542, 545). Furthermore, CTL responses are a driving force in the evolution of HIV-1 at an individual and population level, selecting virus mutation escaping T-cell recognition (288, 296, 435, 546). This is driven by the human leukocyte antigen (HLA) class I molecules, which present virus-derived peptides (epitopes) on the surface of infected cells to CD8 T cells. Some HLA alleles e.g. B*27, B*57 and B*58 are associated with T-cell immunity and strong control of HIV-1 infection. These alleles present epitopes from functionally conserved regions of the HIV-1 proteome, in which escape would incur a price on the virus fitness (98, 101, 102, 547). Furthermore, increases in viral loads were noted in rhesus macaques post of $CD8^+$ T-cell depletion. This model was also used to demonstrate the effectiveness of T-cell based vaccines against simian immunodeficiency virus (SIV) challenge (399, 548). These and other findings

support the development of an HIV-1 vaccine stimulating strong cellular responses (details in main introduction).

The biggest challenge developing a HIV vaccine has been the variability of HIV-1 caused by the high error rate of reverse trascriptase (371, 549). Due to this high rate of mutagenesis, HIV-1 is a very diverse virus, classed into three universal groups (M, O and N). Group M, the most common globally, is further sub-divided into 15 subtypes, with four major subtypes (clades A, B, C and D) corresponding to the amino-acid diversity found in different isolates from different geographical regions (392, 550). Variations between clades can be up to 20% in relatively conserved regions and up to 35% in the more variable envelope region (392, 551). This huge diversity must be taken into consideration when designing a HIV-1 vaccine.

**Polyvalent Mosaic Antigens**

To further tackle HIV-1 diversity and minimize amino-acid mismatches between circulating viruses and vaccine immunogens, Bette Korber *et al.* have developed an algorithm to computationally design polyvalent antigens (mosaic) *in-silico* maximizing the sequence coverage by T cell based vaccines (344, 439). Mosaic immunogens are developed via random two-point recombination of natural HIV-1 sequences, resulting in recombinant proteins that are rated and scored based on the number of epitopes (natural 9-mers) and their frequency (344, 439). They are optimised to cover the maximum number of common natural 9-mer's and exclude rare or unnatural 9-mer CD8 epitopes that would not confer any protection (344, 439, 505). To avoid artificial junctional epitopes, boundary regions spanning recombination breakpoints are embedded in short regions that recur in natural sequences, thus ensuring they resemble and align natural proteins. This is of great importance, to ensure the processing and presentation of mosaic antigens mimic that of natural proteins, so epitopes eliciting a response from the vaccine will be the same as ones

found in natural infection (344, 439). Ndhlovu et al. confirmed this, demonstrating known T-cell epitopes expressed from mosaic immunogens in human cells in a physiologically relevant form and were able to stimulate and direct CD8+ T cells to multiple protective epitopes. They also noted that mosaic immunogens were able to elicit strong immune responses to subdominant epitopes (505).

The aim with the development of these mosaic antigens is to increase the breadth and depth of T-cell response, thus enhancing T-cell cross reactivity and recognition of different co-circulating strains worldwide. The breadth is defined as the number of different epitopes recognised. The depth is defined as the number of variants of each epitope recognised. Therefore, the algorithm generates a number of mosaic antigens, as specified by the user, as a final product to be used together in a polyvalent vaccine. Depending on the number of mosaics generated during optimization, if bivalent mosaic immunogens were generated, they would be two full-length proteins incorporating the two most common variants of each epitope. If trivalent were generated then a third variant would be included and so on (552). By increasing the number of mosaic's generated, one increases the number of variants included thus increasing coverage. However, this risks incorporating rare epitopes into the mosaic cocktail as the number of variants increase and potentially diluting each variant in the cocktail (552). Therefore, specifying the number of mosaic immunogens included into the polyvalent vaccine should be balanced between increasing coverage and reducing epitope representation, as well as considering the complexity associated with the number of multiple variants in a vaccine cocktail (344, 439, 552).

To examine epitope coverage by a polyvalent mosaic vaccine, mosaics used in a 2-valent, 3-valent, 4-valent and 6-valent vaccines were compared to vaccines using either a consensus or a natural strain sequence of Gag and Nef (344, 439, 552). Comparison of coverage was done within the clade, between clades and globally, optimized on group M. Coverage was assessed by the number of 9-mer epitopes that are exact match or have 1 or 2

amino acid mismatch compared to a natural sequences. Coverage of a single mosaic was comparable to that of a consensus or the natural strain vaccine within and between clades but not globally. When using multiple mosaics 3 or more, the number of perfectly matched 9-mers increased drastically in comparison to the other two vaccines. Most importantly, when examining coverage of the global vaccines (optimized on group M), coverage with consensus and the natural strain was low, whereas using three or more mosaics had a perfect match of approximately 75-80% (439). This was noted in another study comparing a 2-valent and 3-valent mosaic vaccines to a consensus and natural strain sequence of the Env protein. Again, coverage of perfect match epitopes by the natural sequence and consensus was 17% and 26% respectively, whereas the 2-valent and 3-valent mosaic vaccine coverage was 38% and 44% respectively (552). Breadth and depth of the T-cells response was shown to increase in both studies using a polyvalent mosaic vaccine when compared to either a consensus or natural strain vaccine (439, 552). The optimal number of mosaic immunogens in a vaccine cocktail is three.

Theoretical predictions of mosaic vaccines have been confirmed in rhesus monkeys, whereby mosaic vaccines increased the breadth and depth of the T-cell response. The first group compared a bivalent mosaic vaccine consisting of HIV-1 Gag, Pol and Env proteins in a recombinant adenovirus serotype 26 (rAd26) vector to a consensus and a natural sequence vaccines. Cellular immune responses in monkeys immunised with the mosaic vaccines had greater breadth and depth in comparison to the other vaccines (504). Furthermore, the mosaic vaccines induced a more superior response to clade C Gag, number of epitopes recognised, than the optimal natural clade C vaccine. The greater breadth and depth was also noted in responses between different clades. Moreover, magnitudes of the responses induced by the different vaccines were comparable, demonstrating that mosaic vaccines expanded the breadth and depth of the response without compromising the magnitude (504). This was noted in a second monkey study, employing

the Gag and Nef proteins for the immunogens. Similarly their results demonstrated the superiority of the mosaic vaccines in comparison to the consensus vaccine. Mosaic vaccinated monkeys T-cell responses had greater cross reactivity, recognising more Gag and Nef epitopes and variants than T-cells induced by the consensus immunogen. They also noted a larger number of CD8+ T-cells recognising multiple epitopes elicited by the mosaic immunogen (553). Both studies have noted that mosaic immunogens have a greater effect on CD8+ T-cells than CD4+ T-cells, which is of great significance, as CD8+ cytotoxic T lymphocytes are responsible for the killing of infected cells and controlling HIV-1 infection (504, 552, 553). A challenge study in rhesus monkeys using a difficult to neutralise strain of SHIV-1 was conducted to test the efficacy of an Env/Gag/Pol bi-valent mosaic vaccine (501). The vaccines were expressed using an Ad26 and MVA vectors to be used in heterologous prime-boost regimens, Ad26 prime - MVA boost or Ad26 prime and boost. Although most vaccinated monkeys were infected by the end of the challenge, there was an 87-90% reduction in the pre-exposure relative risk of acquisition of infection. It took several (4-6) high doses of intrarectal challenges for the vaccinated monkeys to get infected, whereas 83% of the unvaccinated control group were infected after the first challenge and 100% after the third challenge. The partial protection noted in this study was attributed to Env specific antibodies as no correlation was noted between T-cells and protection (501).

Mosaic vaccines have been utilized recently for other diseases with high variability such as hepatitis C virus (HCV) and avian influenza (554-556). For avian flu, who have recently developed a mosaic vaccine using the conserved hemagglutinin sequence of H5N1 expressed in an MVA vector. Studies in mice have shown broad protective responses to several H5N1 viruses and a prolonged protection against challenge with a highly pathogenic avian flu virus. It induced broad breadth of CD4 and CD8 responses and broad neutralizing antibodies against multiple variants of the virus. Most importantly it provided

sterilizing immunity of the lung against several H5N1 viruses (554). A mosaic hepatitis C virus genotype 1 vaccine was developed to tackle the diversity of HCV. The mosaic vaccine induced stronger responses and was more immunogenic than a natural strain vaccine. It improved epitope coverage significantly for all HCV genotype 1 proteins compared to a consensus and natural strain based vaccines and inducing both CD4 and CD8 effector responses (555, 556). All these findings from the various vaccines targeting HIV-1, HCV and avian flu strongly support the use of mosaic vaccines to tackle diversity of infectious disease.

The focus of our group is using conserved regions of HIV-1 as vaccine immunogens. Since HIVconsv has shown promising results in pre-clinical and clinical trials, we have decided to employ this technique to design conserved mosaic immunogens. Bette Korber using the 14 conserved regions of HIVconsv designed four conserved mosaic immunogens that will be the focus of my work. The first immunogen, HIVconsv mosaic (tHIVcmo), was designed to complement HIVconsv. The three other immunogens, conserved1/2/3 (tConsv1, tConsv2, tConsv3), were designed to complement each other in a 3-valent mosaic cocktail vaccine. Previous studies have all used whole proteins of HIV-1 e.g. Gag, Pol, Nef etc, while the immunogens employed in these sets of experiments are based only on the conserved regions across the HIV-1 sequence, thus making this the first conserved mosaic immunogen tested to date. To ensure the relevance of these results to humans, experiments were done using HHD transgenic mice expressing the human HLA-A2. They express a chimeric HLA-A*0201 MHC I, with a human α-1 & α-2 domain, for peptide binding and presentation to T cell receptors and a mouse α-3 domain. The α-3 heavy chain is linked covalently to the human $\beta_2$m light chain spanning the plasma membrane and interacting with T-cell co-receptors (414, 415, 557, 558). HLA-A2 is the most prevalent HLA-A allele family in the world and has been studied extensively in relation to several diseases including HIV-1 (559-562). With regards to vaccines and HIV-

1, HLA-A02 has been implicated to have a role in the partial efficacy observed in the RV144 clinical trial (563). Furthermore, HLA-A2 has been shown to bind a number of optimal CTL epitopes known to control HIV-1 (307). Using this model ensures the data are relevant to a well defined human HLA associated with HIV-1 control across different geographical regions. The aim of this chapter is to determine the breadth and depth of the response elicited by conserved mosaic vaccines and whether they are an improvement to the consensus HIVconsv vaccine.

## 4.2 Results

### 4.2.1 Assessing the depth of T-cell responses to conserved HIV-1 epitopes in a ChAdV63-prime MVA-boost (CM) regimen

The aim of this experiment is to investigate whether the addition of a mosaic vaccine to HIVconsv in a CM regimen would increase the depth of the response relative to immunisation with the HIVconsv vaccine alone. HIV conserved mosaic, designated tHIVcmo, was the mosaic immunogen designed to complement tHIVconsv and used in this experiment. To determine the breadth and depth of the response with relevance to humans, HHD transgenic mice expressing a chimeric HLA-A*0201 were used.

First, using the Los Alamos national laboratory HIV database, all known HIV-1 HLA-A*0201 epitopes were identified and crosschecked with the immunogens tHIVconsv and tHIVcmo, identifying 21 epitopes. Next, I searched the database for all known variants across the four major HIV-1 clades in group M (A, B, C and D) and identified the 76 most common variants i.e. present more than 1% in the population (Table 4.1 and 4.5). These epitopes (peptides) were identified and acquired at different times, thus experiments at different times used different number of peptides (Table. 4.1). Furthermore, preliminary experiments identified several sub-dominant epitopes that were excluded from experiments looking at depth and were used in a separate set of experiments (Table 4.5) (discussed later). For these experiment 37 peptides were used (Table 4.1).

To compare and assess the depth of the response, HHD mice (n = 5) were either immunised with tHIVconsv or tHIVcmo alone or both vaccines combined. Mice were immunised intra-muscularly (i.m.) in both legs, using a heterologous regimen of ChAdv63 prime and an MVA boost, at a final concentration of $10^8$ infectious unit (IU) and $10^6$ plaque forming units (pfu) respectively. There were two groups of mice receiving both tHIVconsv and tHIVcmo vaccines, each in a separate leg. The first group received a half dose of each vaccine i.e. $5x10^7$ IU of ChAdv63.tHIVconsv and ChAdv63.tHIVcmo, and $5 x10^5$ pfu of

MVA.tHIVconsv and MVA.tHIVcmo, thus receiving the same final dosage as mice vaccinated with a single vaccine. The second group received a full dose of each vaccine i.e. $10^8$ IU and $10^6$ pfu of ChAdV63 and MVA respectively of tHIVconsv and tHIVcmo, thus receiving double the dosage in comparison to mice vaccinated with a single vaccine. The prime and boost were 6 weeks apart, mice were sacrificed and spleens were collected one week after the MVA boost (Fig. 4.1A).

IFN-γ responses from the four groups of HHD mice were assessed individually in an IFN-γ ELISPOT assay against the 37 defined peptides to determine the magnitude, breadth and depth of the response. The average of the negative controls in the assay, splenocytes without peptide stimulation, has been subtracted from all the results presented. The positive threshold cut off point was determined as the mean of the negative controls plus three standard deviations, thus yielding a 99.7% positive accuracy. A positive response was defined by the median of the group response being above the positive threshold for each individual peptide. Non-parametric one-way ANOVA (Kruskal Wallis test) was applied for statistical analysis.

Mice immunised with either vaccine alone or combined elicited a positive response to the same four epitopes and their variants (VLVGPTPVNI, LVGPTPVNI, YQYMDDLYV and IYQYMDDLYV) (Fig. 4.1). Furthermore, responses to YQYMDDLYV and IYQYMDDLYV epitopes and their variants yielded the strongest responses in all four groups of mice (Fig. 4.1). Responses to the other epitopes and their variants were negative, except for three notable responses, whereby the median response of the group was above the positive threshold (Fig 4.2). The first was against epitope ILKDPVHGV, where by a positive response was noted for mice immunised with tHIVconsv alone and tHIVconsv + tHIVcmo at half dose (Fig. 4.2). The second was against IIYQYMDDL epitope, inducing a positive response in the tHIVconsv + tHIVcmo at half dose group (Fig. 4.2B). The third was against SLVKHHLTEE epitope, inducing a response

in the tHIVconsv group (Fig. 4.2B). There was no significant difference in the magnitude of the response between the four groups of mice in response to all the individual peptides (Fig. 4.1 & 4.2). However, the results do illustrate that combining the mosaic vaccine tHIVcmo with tHIVconsv does increase the breadth and depth of the response (Fig. 4.5). Mice immunised with tHIVconsv alone had a positive response to 15 epitopes and variants, whereas mice immunised with the combined vaccine at half dose induced a positive response to 19 epitopes and variants (Fig. 4.5).

**Table 4.1:** List of HIV-1 HLA-A*02:01 epitopes (blue) and variants (red). Epitope correspondences to immunogen have been identified.
tHIVconsv: consv  tHIVcmo: cmo  tConsv 1: con1  tConsv 2: con2  tConsv 3: con3

| Immunogen | HLA-A*02:01 Epitopes | Variants | Immunogen | HLA-A*02:01 Epitopes | Variants |
|---|---|---|---|---|---|
| consv, cmo, con3 | IYQYMDDLYV | | cmo, con3 | RTLNAWVKV | |
| con1 | | IYQYVDDLYV | - | | TLNAWVKVV |
| con2 | | IYQYMDDLYI | - | | TLNAWVKVI |
| - | | ICQYMDDLYV | - | | TLNAWVAVV |
| - | | IYSYMDDLYV | consv, con2 | TAFTIPSI | |
| consv, cmo, con3 | YQYMDDLYV | | cmo, con3 | | TAFTIPST |
| con1 | | YQYVDDLYV | con1 | | TAFTIPSV |
| con2 | | YQYMDDLYI | - | | TAFTIPSL |
| - | | CQYMDDLYV | - | | TAFTIPSR |
| consv, cmo, con2&3 | VIYQYMDDL | | - | SLVKHHMYV | |
| con1 | | VIYQYVDDL | consv, con1&3 | | SLVKHHLTE |
| - | | IIYQYMDDL | cmo, con2 | | SLVKHHLTK |
| - | | EICQYMDDL | - | | SLVKHHMYI |
| consv, con3 | VLVGPTPVNI | | - | | SLVKHHMHI |
| cmo, con2 | | VLIGPTPVNII | consv, con2&3 | KRWIILGLNK | |
| con1 | | VLVGPTPINI | - | | KRWIIMGLNK |
| - | | VLIGPTPVNI | - | | KRWIVLGLNK |
| - | | VLVGPTPANI | - | | KKWIIMGLNK |
| consv, con3 | LVGPTPVNI | | - | | RRWIILGLNK |
| cmo, con2 | | LIGPTPVNI | consv, con2&3 | ILGLNKIV | |
| con1 | | LVGPTPINI | cmo, con1 | | IMGLNKIV |
| - | | LVGPTPANI | - | | VLGLNKIV |
| consv, cmo, con1&3 | QMHEDIISL | | - | | IIGLNKIV |
| con2 | | QMHEDVISL | consv, con1&3 | KAFSPEVIPMF | |
| - | | QMQEDIISL | con2 | | KGFNPEVIPMF |
| - | | QMHTDIISL | - | | KAFSPEIIPMF |
| - | | QMHEDIISI | - | | KAFNPEVIPMF |
| - | | QMQTDIISL | - | | RAFSPEVIPMF |
| - | | QMHADIISL | cmo, con2 | TSTLQEQIGW | |
| - | EILKEPVGHV | | - | | TSTLQEQIAW |
| consv | | EILKDPVHGV | - | | TSNLQEQIAW |
| cmo, con1 | | EILKEPVHGV | - | | TSNLQEQIGW |
| cmo, con1 | ILKEPVHGV | | - | | TSTPQEQIGW |
| consv | | ILKDPVHGV | | | |
| con2 | | ILREPVHGV | | | |
| con3 | | ILKEPVHGA | | | |
| - | | ILKTPVHGV | | | |
| - | | ILKNPVHGV | | | |

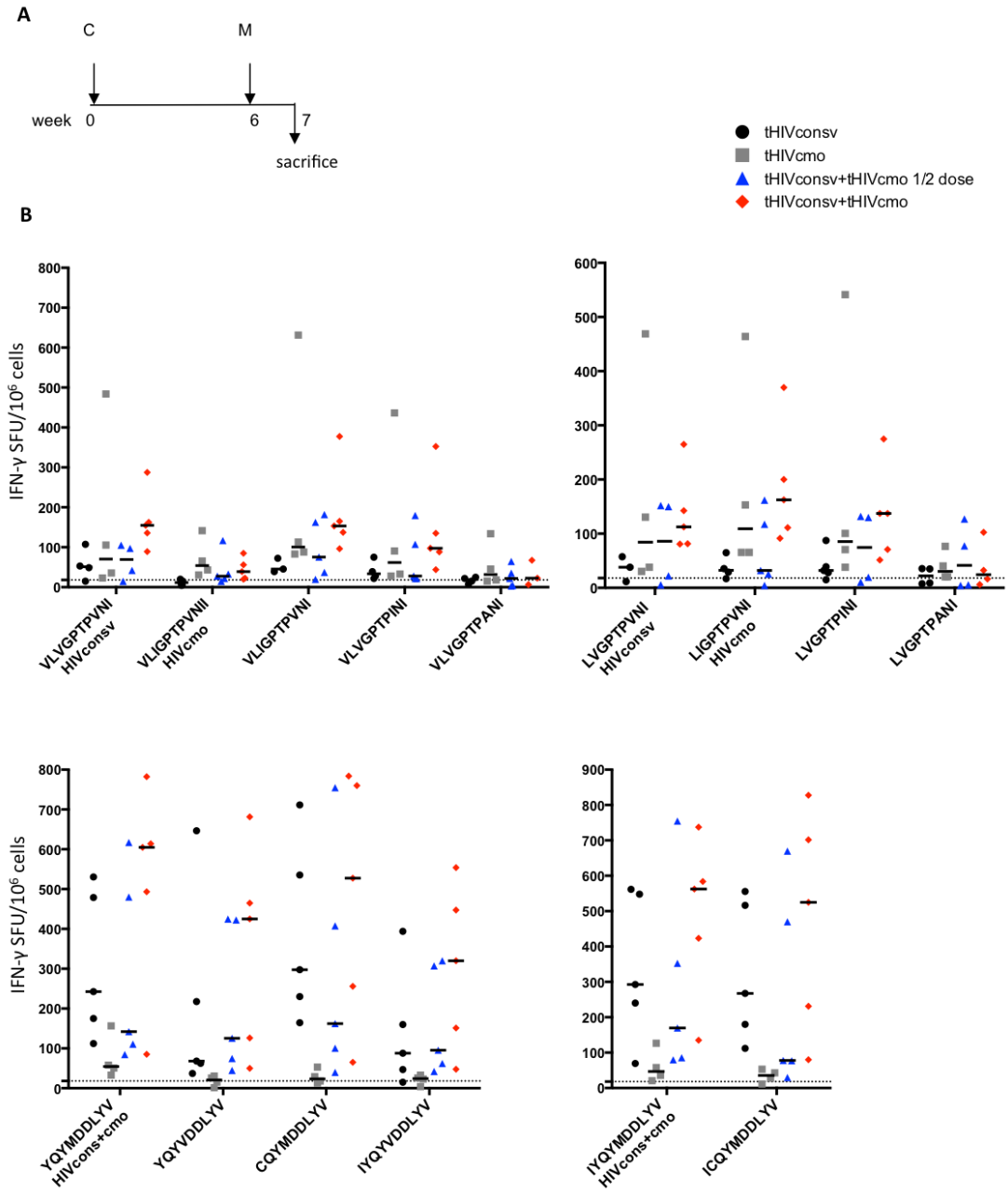



**Figure 4.1. Depth of responses induced by tHIVconsv and tHIVcmo in a CM regimen**
**(A)** Immunization schedule for groups of HHD mice immunised intramuscularly (n = 5). C represents ChAdV63 and M represents MVA vectored vaccines. Mice were vaccinated either with a full dose receiving $10^8$ IU of C and $10^6$ pfu of M of each vaccine; or a half dose receiving $5x10^7$ IU of C and $5x10^5$ pfu of M of each vaccine **(B)** Mice were immunised with tHIVconsv (•), tHIVcmo (■), tHIVconsv + tHIVcmo at 1/2 dose (Δ), tHIVconsv + tHIVcmo (◆). Frequencies of responding splenocytes were tested by IFN-γ ELISPOT assay against individual peptides (X-axis). Under each peptide is the immunogen corresponding to it; undefined peptides are variants. Data are shown as individual (shape) and median (horizontal line) of spots forming units (SFU) per million cells (Y-axis). Means of the negative controls have been subtracted from all responses. Positive threshold cut off point (dotted line) was determined as the mean of the negative controls plus three standard deviations.

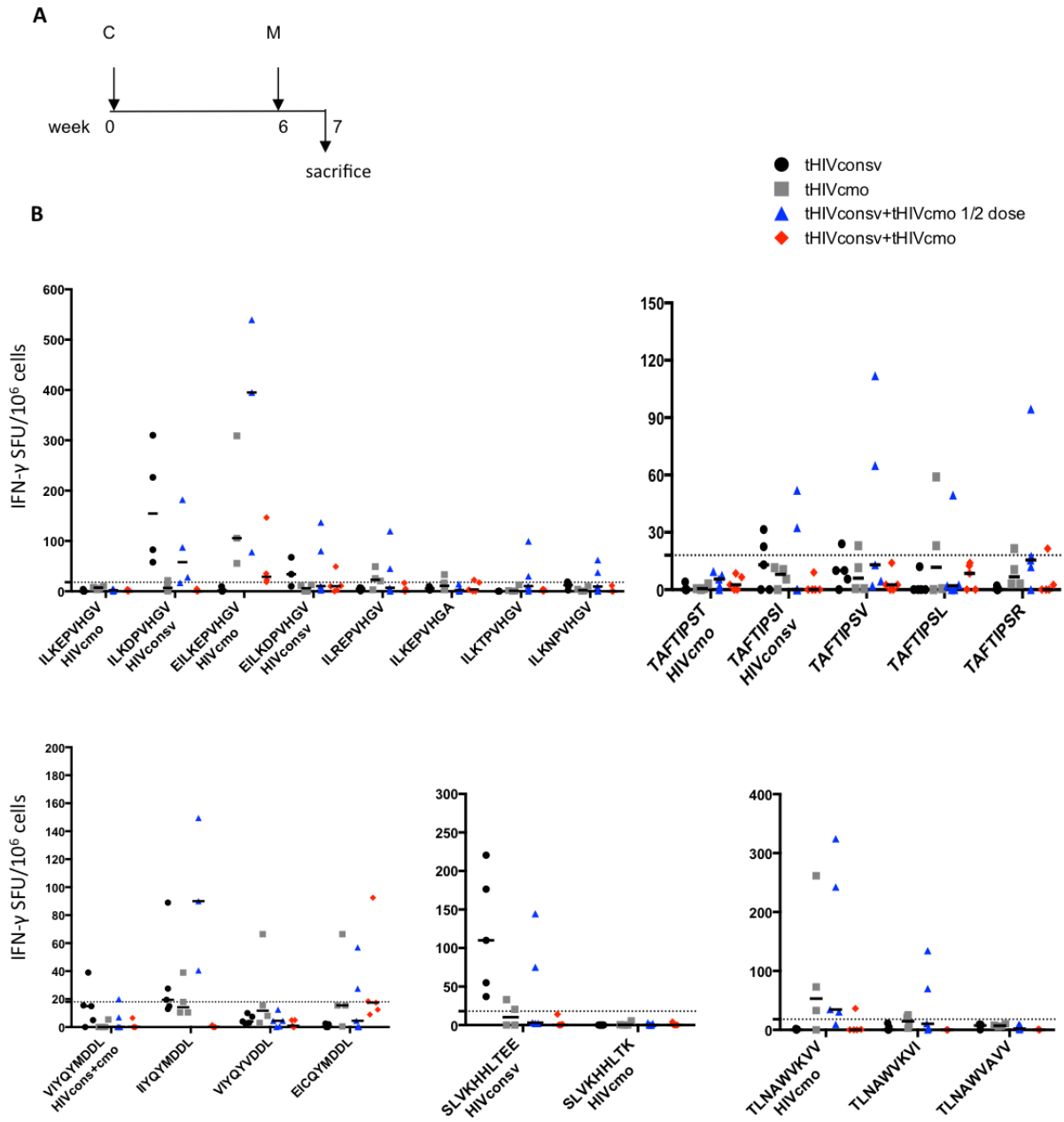



**Figure 4.2: Depth of responses induced by tHIVconsv and tHIVcmo in a CM regimen**
**(A)** Immunization schedule for groups of HHD mice immunised intramuscularly (n = 5). C represents ChAdV63 and M represents MVA vectored vaccines. Mice were vaccinated either with a full dose receiving $10^8$ IU of C and $10^6$ pfu of M of each vaccine; or a half dose receiving $5x10^7$ IU of C and $5x10^5$ pfu of M of each vaccine **(B)** Mice were immunised with tHIVconsv (•), tHIVcmo (■), tHIVconsv + tHIVcmo at 1/2 dose (Δ), tHIVconsv + tHIVcmo (♦). Frequencies of responding splenocytes were tested by IFN-γ ELISPOT assay against individual peptides (X-axis). Under each peptide is the immunogen corresponding to it; undefined peptides are variants. Data are shown as individual (shape) and median (horizontal line) of spots forming units (SFU) per million cells (Y-axis). Means of the negative controls have been subtracted from all responses. Positive threshold cut off point (dotted line) was determined as the mean of the negative controls plus three standard deviations.

### 4.2.2 Evaluating the effects of a DNA prime followed by a viral vector boost on the depth of T-cell responses to conserved HIV-1 epitopes in a DDDCM regimen

Priming with DNA vaccines followed by a viral vector boost has been shown to be an effective regimen in inducing a strong immune response. In this experiment DNA vaccines were used as a prime to determine whether it has an affect on epitope recognition by enhancing the depth of the response. HHD mice were immunised with 3 DNA vaccines and a ChAdV63 boost, 3 weeks apart, followed by a MVA final boost six weeks later (DDDCM) before being sacrificed a week post last immunisation (Fig. 4.3A). Again there were four groups of mice (n = 4), two groups received either tHIVconsv or tHIVcmo alone and two groups received the two vaccines simultaneously in separate legs, with one group receiving half the dosage of each vaccine. DNA was given to mice at a final concentration of 100 μg, except for the group immunised with a half dose receiving 50 μg. The ChAdV63 and MVA vaccines were administered at the same dosages described in the previous experiment. IFN-γ responses from the four groups of HHD mice were assessed individually in an IFN-γ ELISPOT assay against 57 defined peptides to determine the magnitude, breadth and depth of the response. The number of peptides used in this experiment is greater than the previous experiment as new variants and epitopes were identified and acquired (Table 1). IFN-γ responses and statistical analysis were determined as described previously.

The four groups of vaccinated mice induced a positive response to the same four groups of epitopes (VLVGPTPVNI, LVGPTPVNI, YQYMDDLYV and IYQYMDDLYV) (Fig. 4.3B). New variants were added to the YQYMDDLYV and IYQYMDDLYV set of epitopes and positive responses were induced successfully. The magnitude of the responses did not differ significantly between the different groups and did not differ from the previous CM regimen. Furthermore, responses were not induced to any other epitope, unlike the

previous experiment, including the new sets of epitopes except for tHIVconsv positive response to ILKDPVHGV (Fig. 4.4B). These result demonstrate a greater breadth with tHIVconsv alone, where by 5 different epitopes induced a positive response in comparison to tHIVconsv + tHIVcmo which only induced responses to 4 epitopes (Fig. 4.5). However for the depth of the response, the addition of the mosaic vaccine tHIVcmo to tHIVconsv was positive, albeit small, as the combined vaccines induced positive T-cell responses to 16 epitope variants compared to tHIVconsv alone 15 (Fig. 4.5).

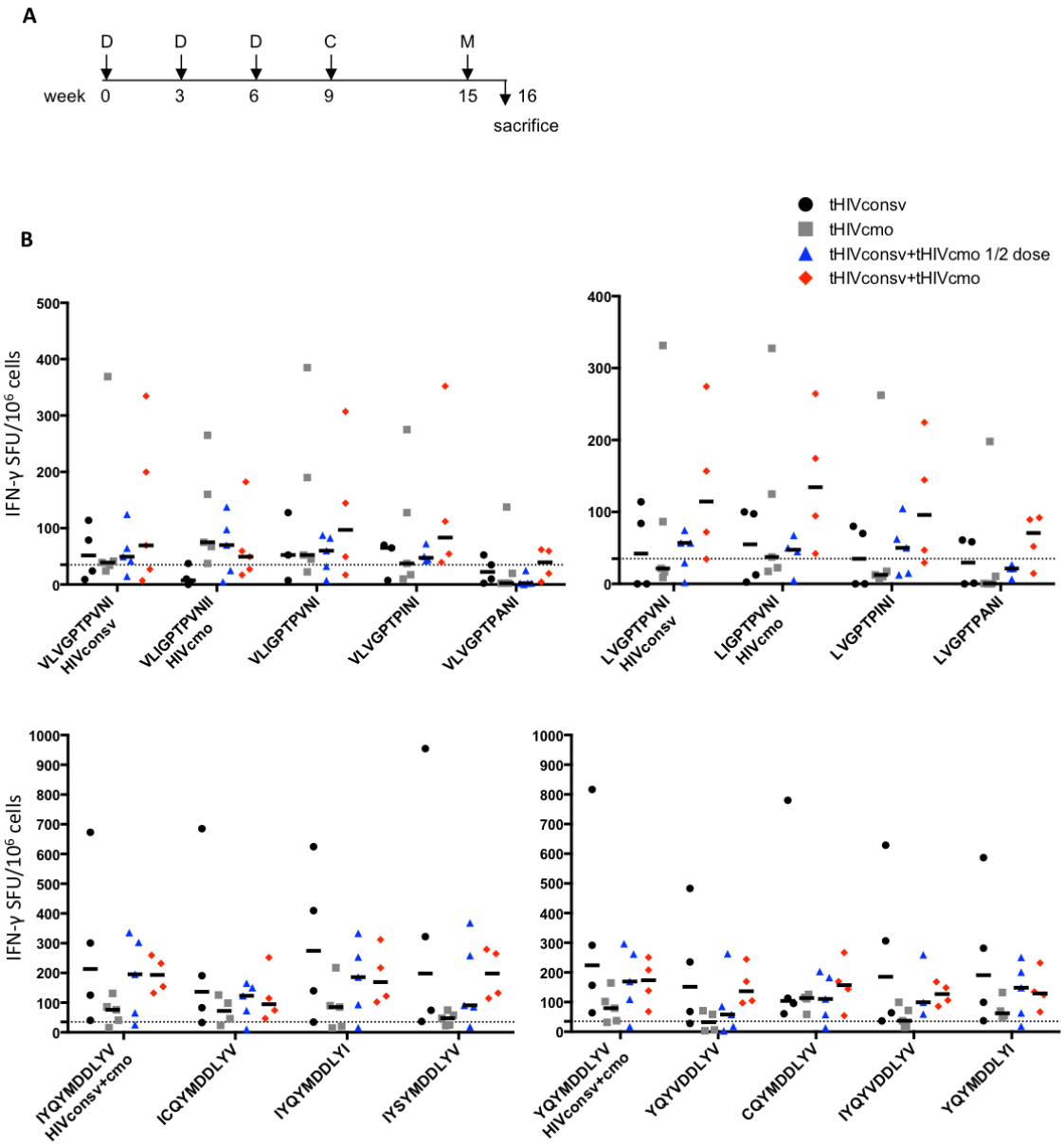



**Figure 4.3. Depth of responses induced by tHIVconsv and tHIVcmo in a DDDCM regimen**

**(A)** Immunization schedule for groups of HHD mice immunised intramuscularly (n = 4). D represents plasmid DNA, C represents ChAdV63 and M represents MVA vectored vaccine. Mice were vaccinated either with a full dose receiving 100 µg of D, $10^8$ IU of C and $10^6$ pfu of M of each vaccine; or a half dose receiving 50 µg of D, $5x10^7$ IU of C and $5x10^5$ pfu of M of each vaccine. **(B)** Mice were immunised with tHIVconsv (•), tHIVcmo (■), tHIVconsv + tHIVcmo at 1/2 dose (Δ), tHIVconsv + tHIVcmo (♦). Frequencies of responding splenocytes were tested by IFN-γ ELISPOT assay against individual peptides (X-axis). Under each peptide is the immunogen corresponding to it; undefined peptides are variants. Data are shown as individual (shape) and median (horizontal line) of spots forming units (SFU) per million cells (Y-axis). Means of the negative controls have been subtracted from all responses. Positive threshold cut off point (dotted line) was determined as the mean of the negative controls plus three standard deviations. This figure illustrates results of peptides inducing positive responses.

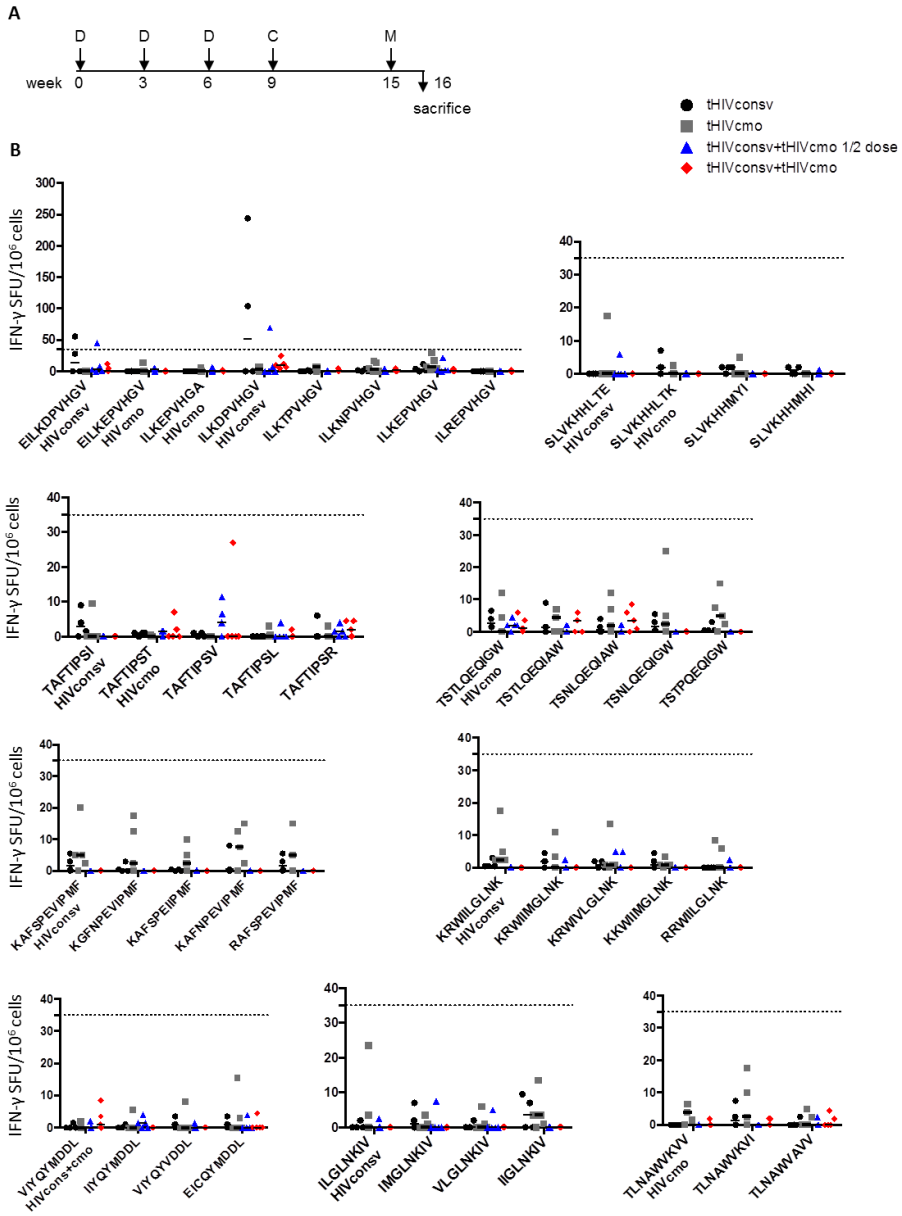



**Figure 4.4. Depth of responses induced by tHIVconsv and tHIVcmo in a DDDCM regimen**

**(A)** Immunization schedule for groups of HHD mice immunised intramuscularly (n = 4). D represents plasmid DNA, C represents ChAdV63 and M represents MVA vectored vaccine. Mice were vaccinated either with a full dose receiving 100 µg of D, $10^8$ IU of C and $10^6$ pfu of M of each vaccine; or a half dose receiving 50 µg of D, $5x10^7$ IU of C and $5x10^5$ pfu of M of each vaccine. **(B)** Mice were immunised with tHIVconsv (•), tHIVcmo (■), tHIVconsv + tHIVcmo at 1/2 dose (Δ), tHIVconsv + tHIVcmo (♦). Frequencies of responding splenocytes were tested by IFN-γ ELISPOT assay against individual peptides (X-axis). Under each peptide is the immunogen corresponding to it; undefined peptides are variants. Data are shown as individual (shape) and median (horizontal line) of spots forming units (SFU) per million cells (Y-axis). Means of the negative controls have been subtracted from all responses. Positive threshold cut off point (dotted line) was determined as the mean of the negative controls plus three standard deviations.

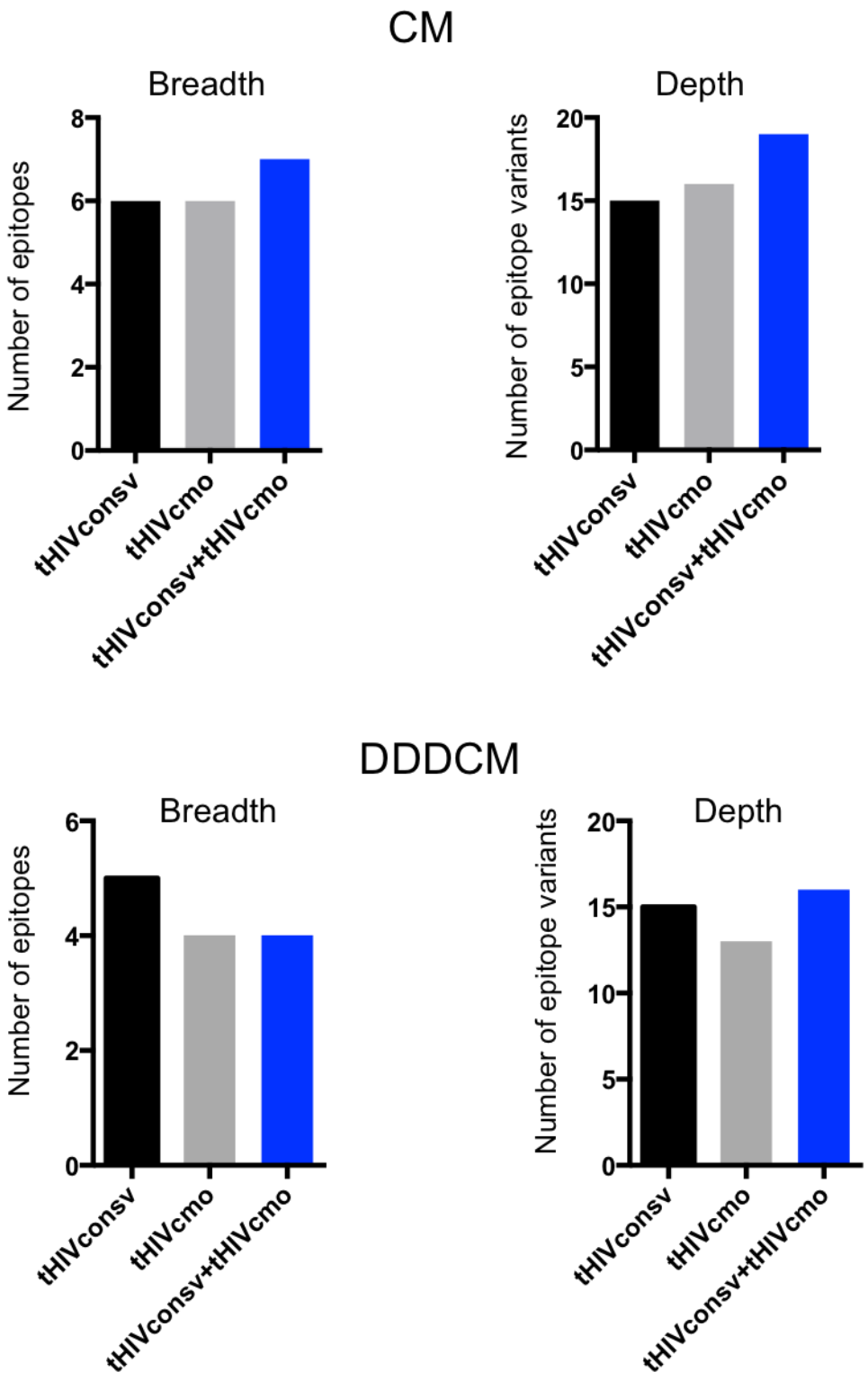



**Figure 4.5. Summary of the responses breadth and depth in a CM and DDDCM regimen**

A summary illustration of the breadth (number of epitopes) and depth (number of variants) of the positive responses induced in an IFN-γ ELISPOT assay. Comparison of responses from three groups of HHD mice vaccinated with tHIVconsv (black), tHIVcmo (gray) and tHIVconsv + tHIVcmo at half dose (blue) using a CM and DDDCM.

### 4.2.3 Determining the avidity of the response induced by tHIVconsv and tHIVcmo in a DDDCM regimen

The responses illustrated in the previous experiments were of great interest as it demonstrated that these vaccines were able to induce T-cell responses recognizing multiple epitopes and variants presented by human HLA. To ensure these responses were biologically relevant and not the result of over stimulation, peptides were titrated at different concentration to determine the avidity of the response. This will also ensure a closer representation of antigen presentation at physiological levels. Four groups of HHD mice (n = 5) were immunised using the DDDCM regimen same as the previous experiment (Fig. 4.6A). IFN-γ responses from the four groups of HHD mice were assessed individually in an IFN-γ ELISPOT assay against 19 peptides at concentrations of 2 μg, 0.2 μg and 0.02 μg to determine the avidity of the T-cell responses. The four sets of epitopes (VLVGPTPVNI, LVGPTPVNI, YQYMDDLYV and IYQYMDDLYV) that induced a positive response in the previous experiment were selected for this experiment with the addition of one new epitope QMHEDIISL. Responses to peptides VLVGPTPVNI, LVGPTPVNI and their variants are not presented due to problems with the ELISPOT plates.

The results illustrate that tHIVconsv alone and tHIVconsv + tHIVcmo were able to elicit positive response to the various epitopes and variants at different concentrations (Fig. 4.6B). Magnitudes of responses decreased with the serial dilution, but positive response were noted for all at 2 μg and 0.2 μg concentration. At the lowest concentration of 0.02 μg, few individual responses were not detected e.g. tHIVconsv alone response was below the positive threshold for peptide YQYVDDLYV at 0.02 μg, nonetheless the pattern from these results indicate responses are present at this lowest dilution (Fig. 4.6B). Responses to QMHEDIISL were very strong across all four groups, thus increasing the breadth of the response. QMHEDIISL was added to subsequent experiments and its variants.

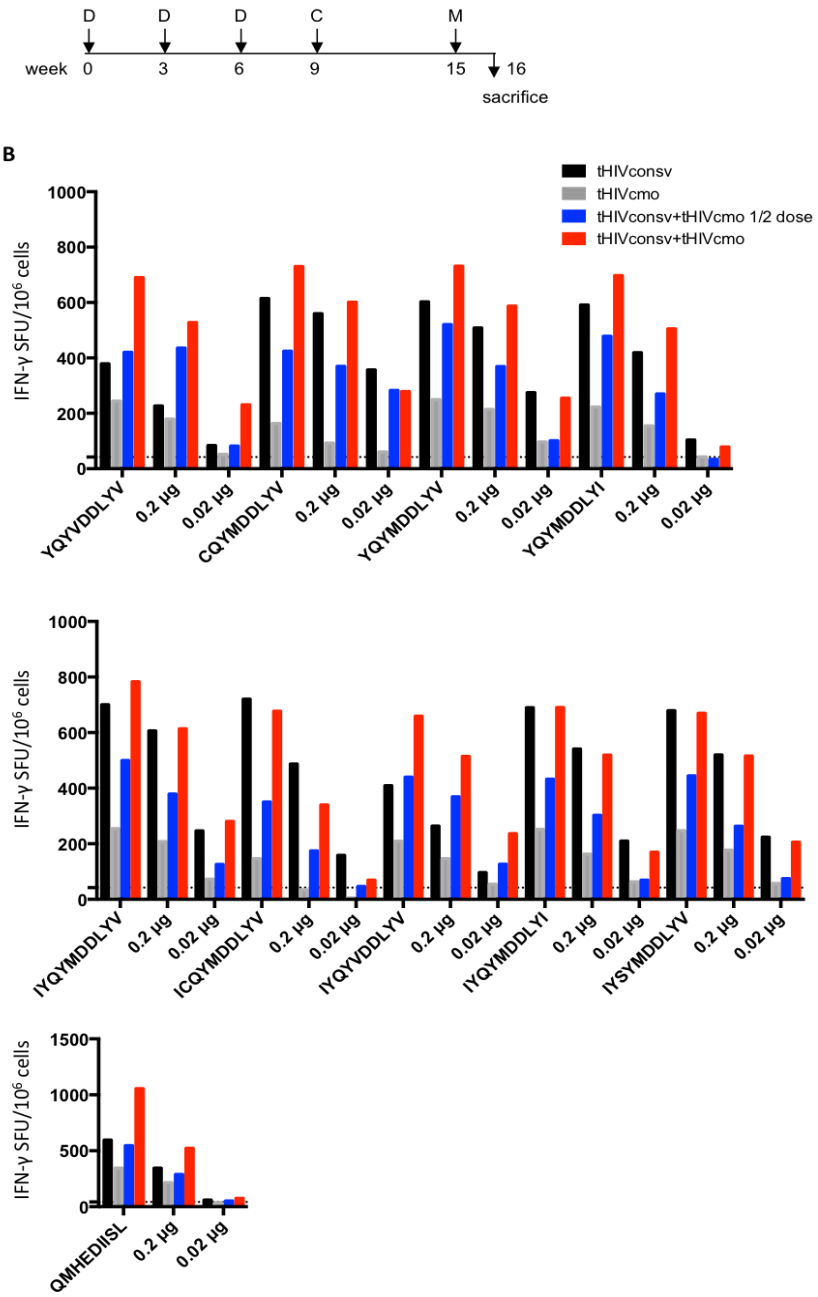



**Figure 4.6. Avidity of vaccine induced T-cell responses**

**(A)** Immunization schedule of HHD mice immunised intramuscularly (n = 5). D represents plasmid DNA, C represents ChAdV63 and M represents MVA vectored vaccine. Mice were vaccinated either with a full dose receiving 100 µg of D, $10^8$ IU of C and $10^6$ pfu of M of each vaccine; or a half dose receiving 50 µg of D, $5x10^7$ IU of C and $5x10^5$ pfu of M of each vaccine. **(B)** Mice were immunised with tHIVconsv (■), tHIVcmo (■), tHIVconsv + tHIVcmo at 1/2 dose (■), tHIVconsv + tHIVcmo (■). Frequencies of responding splenocytes were tested by IFN-γ ELISPOT assay against 10 individual peptides in a serial dilution at concentrations of 2 µg, 0.2 µg and 0.02 µg (X-axis). Data are shown as median of spots forming units (SFU) per million cells (Y-axis). Means of the negative controls have been subtracted from all responses. Positive threshold cut off point (dotted line) was determined as the mean of the negative controls plus three standard deviations.

### 4.2.4 Killing efficacy of tHIVconsv and tHIVconsv + tHIVcmo induced T-cells

The effect of adding a mosaic to tHIVconsv on the depth of the response has been determined and analysed using IFN-γ ELISPOT assay. This indicated an effector cytokine was produced in response to the desired epitopes. An *ex-vivo* killing assay was employed to determine the killing efficacy of vaccine elicited cytotoxic T-cells and confirm if the depth of the response translates into killing. Splenocytes from naïve HHD mice were isolated and labeled with CFSE at two different concentrations, a low and high concentration of 32 nM and 800 nM respectively, whereon cells with the higher CFSE label are pulsed with peptides (individually) rendering them targets for killing and cells with the lower CFSE label are unpulsed. Four groups of HHD mice (n = 3) were immunised using the DDDCM regimen (Fig. 4.8A) at the same concentrations described in the earlier experiments. Splenocytes from the four vaccinated groups were isolated and co-cultured with peptide pulsed target cells and unpulsed cells at an effector to target ratio of 10:1. The numbers of targets and unpulsed cells added to the mix were equal. The assay readout was done using flow cytometry FACS analysis, whereby the percentage of killing is calculated as the difference between the pulsed and unpulsed cell count.

Cells were pulsed with 17 peptides individually selected in accordance to the results from previous experiments. The first two sets of epitopes and variants selected were VLIGPTPVNI and IYQYMDDLYV as they induced strong positive responses in the IFN-γ ELISPOT readout in the previous experiment. The third epitope selected was EILKDPVHGV and its variants. Although it did not induce a consistent positive response in the previous experiment in the different groups of vaccinated mice, some sporadic responses were noted, thus it was of interest to observe how this translated in a killing efficacy assay.

Figure 4.7 outlines the FACS gating strategy and how the two populations of target and unpulsed cells were gated on to determine the percentage of specific killing. Firstly lymphocytes were gated followed by the singlets and the live cells. Within the live population, unstained and CFSE labeled cells were determined, whereby the labeled cells were gated on thus allowing for the high and low labeled cells to be measured (Fig. 4.7). For epitopes group VLIGPTPVNI and EILKDPVHGV, the percentage of specific killing was between 10-20% across the four groups of mice (Fig. 4.8B). There was no significant difference between tHIVconsv and tHIVcons + tHIVcmo, however tHIVconsv alone induced a higher killing percentage (40%) against cells pulsed with (VLVGPTVNI, ILKDPVHGV and ILKNPVHGV) although non-significant (Fig. 4.8B). The IYGYMDDLYV group of epitopes had the highest percentage of killing (30% - 50%) amongst all four groups of mice. Again there was no significant difference in the killing percentage between the different vaccines, although a trend did show better killing with tHIVconsv alone (Fig. 4.8B).

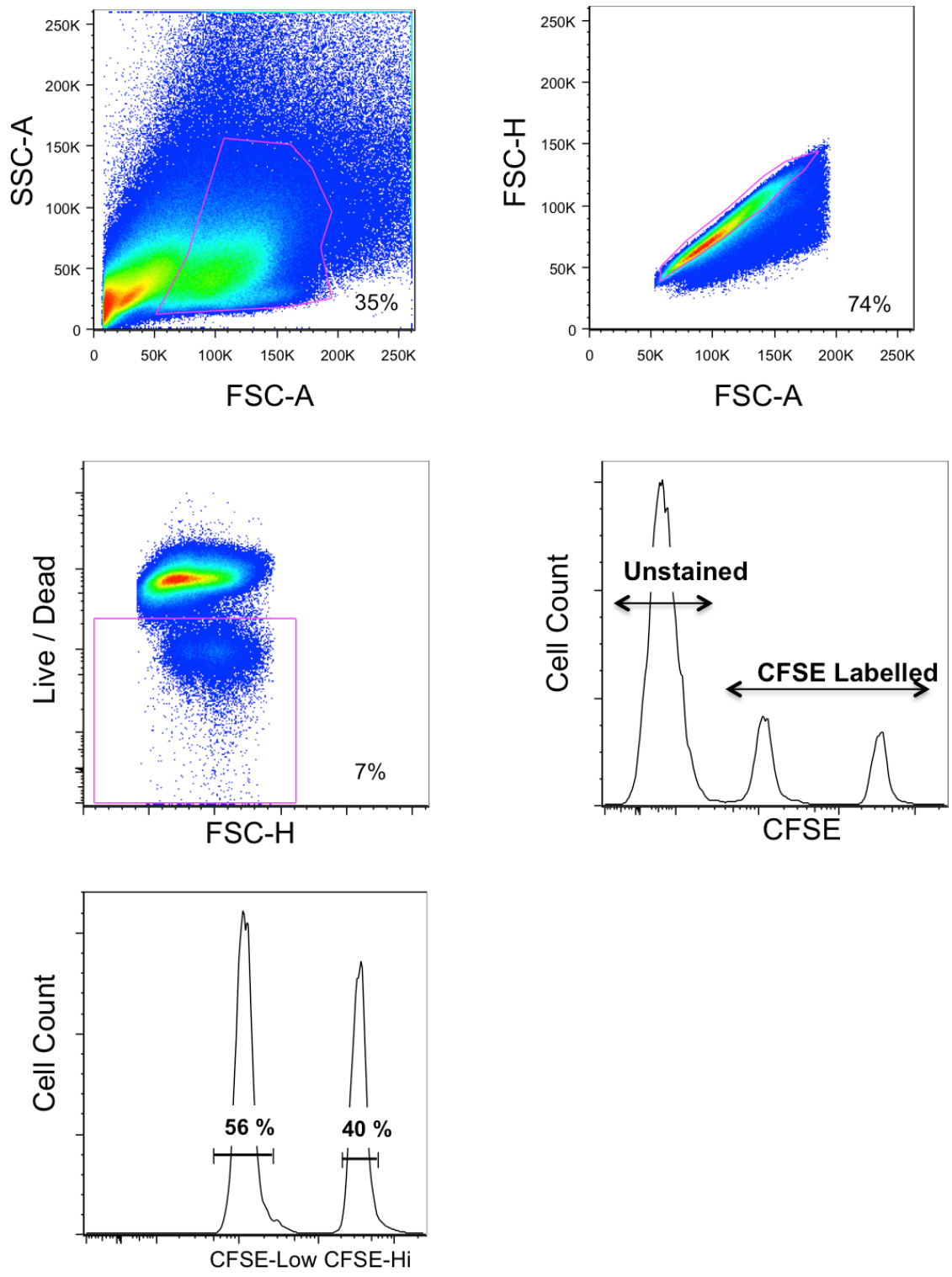



**Figure 4.7. Representative FACS plots of *ex-vivo* killing**

Representative FACS plots showing gating strategy for *ex-vivo* killing. Gating strategy (from top left panel going left) 1) Lymphocytes 2) Singlets 3) Live cells 4) CFSE labeled 5) CFSE low and CFSE high labeled populations. Percentage of each population is indicated on the plot.

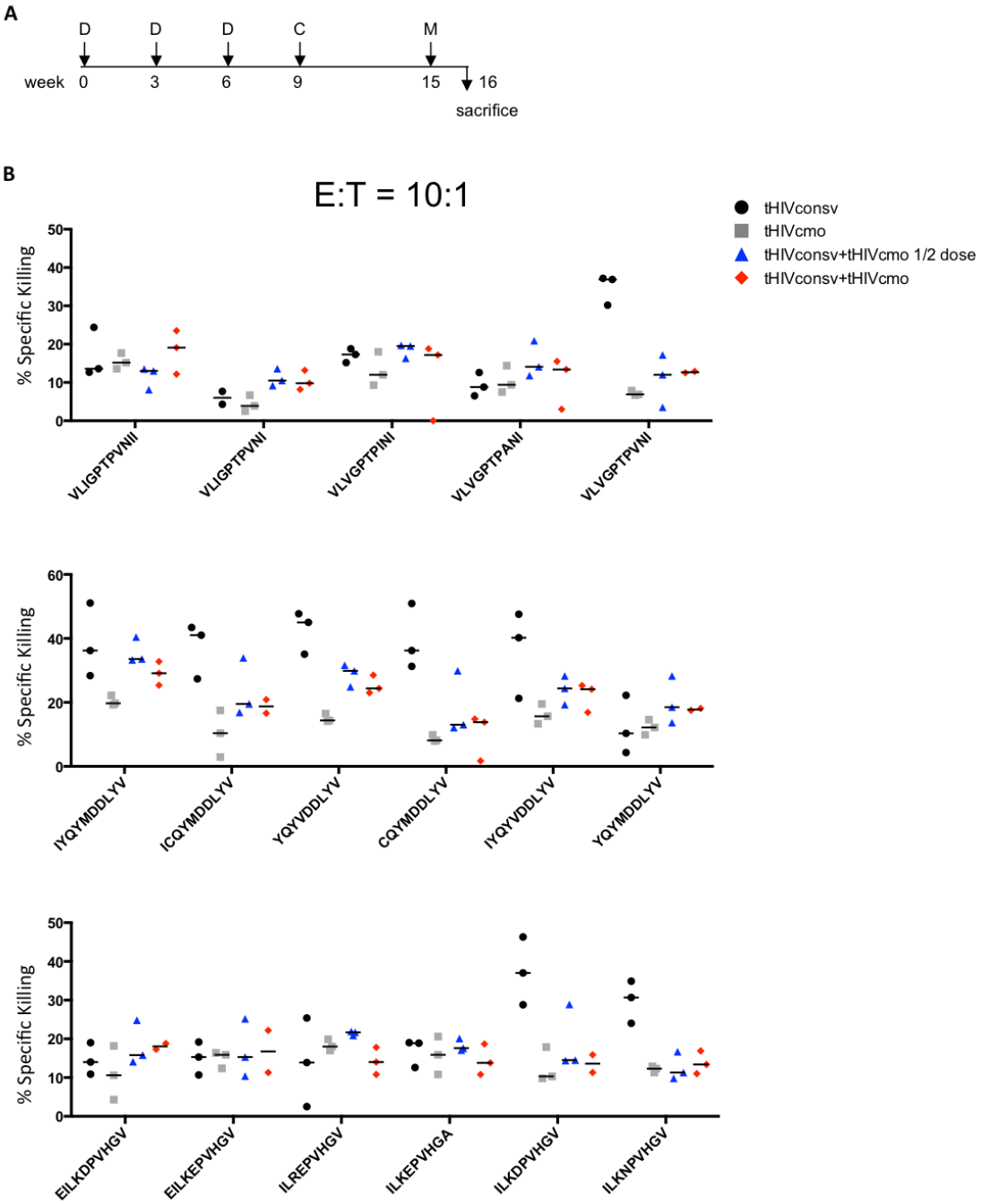



**Figure 4.8. Killing efficacy of vaccine induced T-cells in a DDDCM regimen**

**(A)** Immunization schedule of HHD mice immunised intramuscularly (n = 3). D represents plasmid DNA, C represents ChAdV63 and M represents MVA vectored vaccine. Mice were vaccinated either with a full dose receiving 100 µg of D, $10^8$ IU of C and $10^6$ pfu of M of each vaccine; or a half dose receiving 50 µg of D, $5x10^7$ IU of C and $5x10^5$ pfu of M of each vaccine. **(B)** Mice were immunised with tHIVconsv (•), tHIVcmo (■), tHIVconsv + tHIVcmo at 1/2 dose (Δ), tHIVconsv + tHIVcmo (♦). Frequency of splenocytes specific killing was determined using an *Ex-vivo* killing assay against target splenocytes cells pulsed with 17 peptides individually (X-axis). Effector to target ratio was 10:1. Data are shown as percentage of specific killing with a horizontal line depicting the median (Y-axis).

### 4.2.5 Assessing the depth of T-cell responses induced by three mosaic vaccines

Next I set to assess the breadth and depth of T-cell responses induced in HHD mice vaccinated with the three complementary mosaic vaccines, tConsv1, tConsv2 and tConsv3. The three immunogens were each expressed in a DNA plasmid vector and viral vectors ChAdV63 and MVA, giving a total of nine vaccines. HHD mice (n = 5) were immunised with a mix of all three vaccines at full dose and a third of the dose using the DDDCM regimen (Fig. 4.9A). The group receiving the full dose received 100 μg of each DNA vaccine, $10^8$ IU of each ChAdV63 vaccine and $10^6$ pfu of each MVA vaccine. Mice receiving the lesser dose received 33.3 μg of each DNA vaccine, $3.3x10^7$ IU of each ChAdV63 and $3.3x10^5$ pfu of each MVA vaccine, thus when combined each would equal one full dose. The breadth and depth of the responses from the two groups of mice were assessed using an IFN-γ ELISPOT assay against 64 individual peptides. New epitopes and variants were identified, thus adding to the list of peptides tested (Table 1). Positive IFN-γ responses were determined and analysed in the same manner as the previous experiment.

As with the previous experiments, positive responses were detected to the same five established groups of epitopes and their variants, VLVGPTPVNI, LVGPTPVNI, IYQYMDDLYV, YQYMDDLYV and QMHEDIISL with the addition of ALTAICEEM (Fig. 4.9B). The median of the responses magnitude was higher for these two groups of mice when compared to the previous experiments, however the breadth and depth was the same. The median of the responses magnitude to peptides IYQYMDDLYV, YQYMDDLYV and their variants was 600 SFU/million for the trivalent mosaic vaccines. The median of the responses magnitude to peptides IYQYMDDLYV, YQYMDDLYV from the previous experiment was 200 SFU/million. Positive responses were detected for 18 epitopes and their variants from the group receiving third of the dose (Fig. 4.9B). IYQYMDDLYV, YQYMDDLYV and QMHEDIISL epitopes and variants induced the

strongest responses, in terms of magnitude (Fig. 4.9B). Responses against the other 9 groups of epitopes and their 44 variants were negative; these included the negative epitopes used in the previous experiments and new ones (Fig. 4.10B).

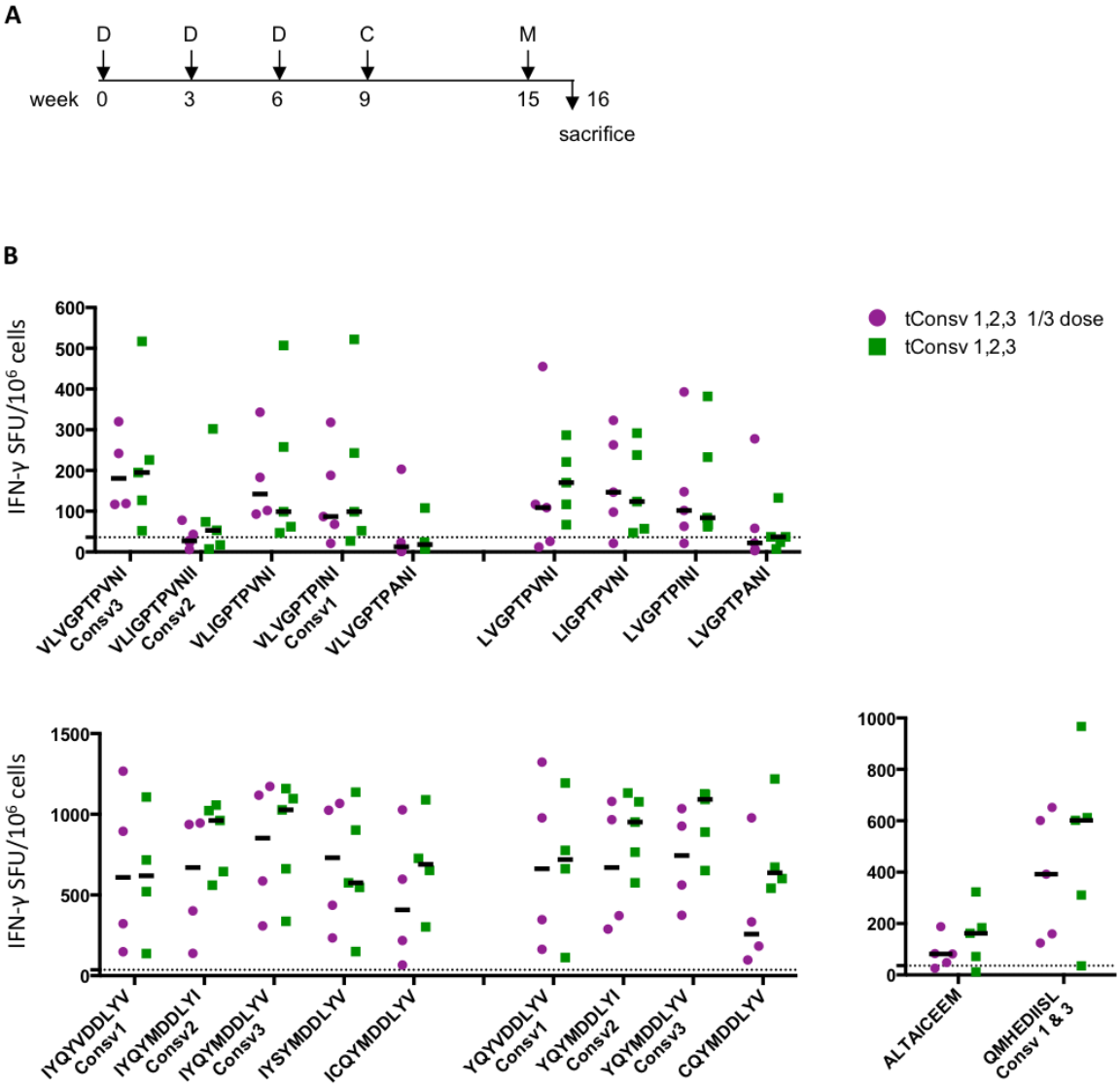



**Figure 4.9. The breadth and depth of polyvalent mosaic vaccine induced T-cells**
**(A)** Immunization schedule of HHD mice immunised intramuscularly (n = 5). D represents plasmid DNA, C represents ChAdV63 and M represents MVA vectored vaccine. Mice were vaccinated either with a trivalent vaccine at a full dose receiving 100 µg of D, $10^8$ IU of C and $10^6$ pfu of M of each vaccine; or a third dose receiving 33.3 µg of D, $3.3x10^7$ IU of C and $3.3x10^5$ pfu of M of each vaccine. **(B)** Mice were immunised with tConsv1+2+3 at 1/3 dose of each (•) or tConsv1+2+3 at full dose (■). Frequencies of responding splenocytes were tested by IFN-γ ELISPOT assay against individual peptides (X-axis). Under each peptide is the immunogen corresponding to it; undefined peptides are variants. Data are shown as individual (shape) and median (horizontal line) of spots forming units (SFU) per million cells (Y-axis). Means of the negative controls have been subtracted from all responses. Positive threshold cut off point (dotted line) was determined as the mean of the negative controls plus three standard deviations. This figure illustrates results of peptides inducing positive responses.

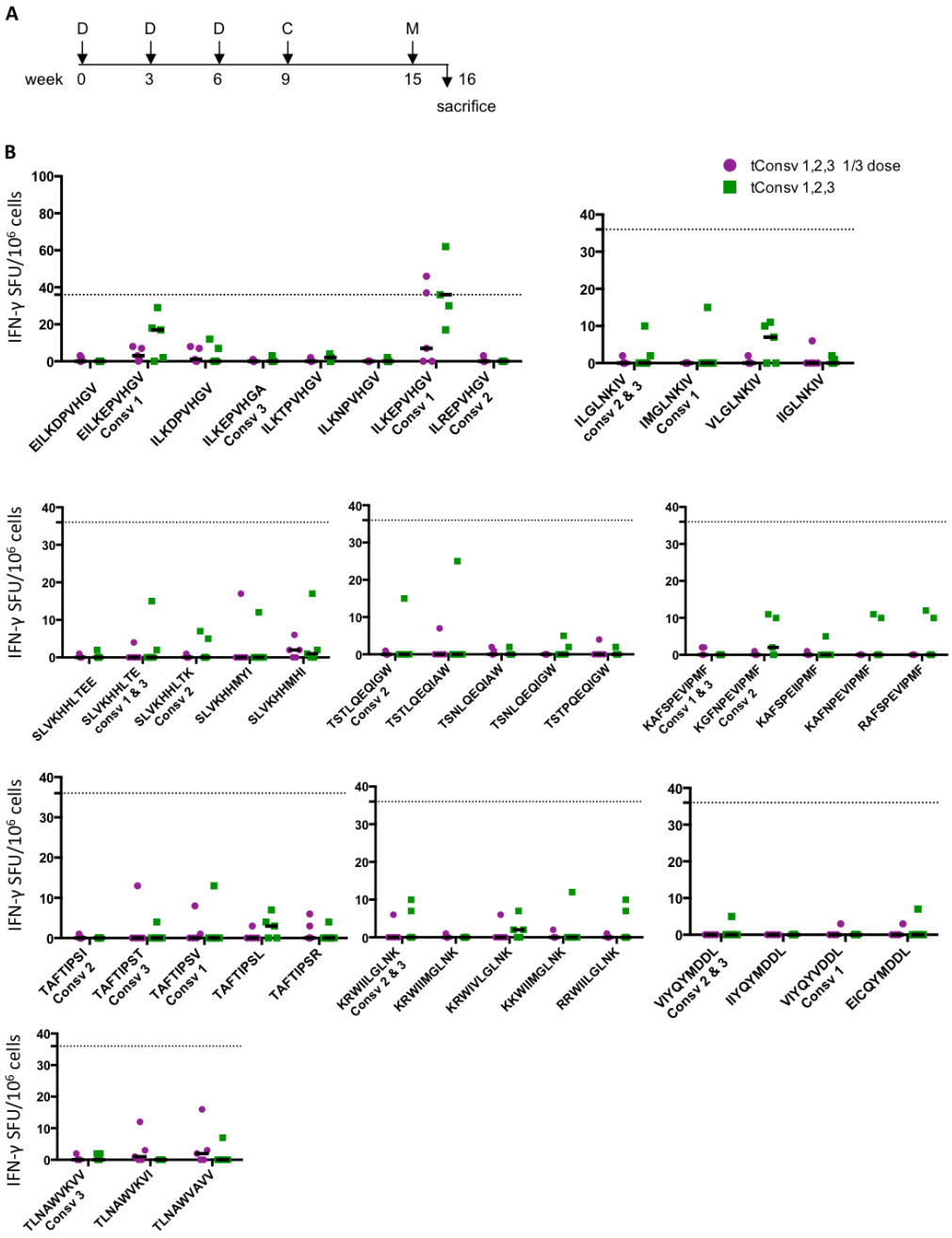



**Figure 4.10. The breadth and depth of polyvalent mosaic vaccine induced T-cells**
**(A)** Immunization schedule of HHD mice immunised intramuscularly (n = 5). D represents plasmid DNA, C represents ChAdV63 and M represents MVA vectored vaccine. Mice were vaccinated either with a trivalent vaccine at a full dose receiving 100 µg of D, $10^8$ IU of C and $10^6$ pfu of M of each vaccine; or a third dose receiving 33.3 µg of D, $3.3x10^7$ IU of C and $3.3x10^5$ pfu of M of each vaccine. **(B)** Mice were immunised with tConsv1+2+3 at 1/3 dose of each (•) or tConsv1+2+3 at full dose (■). Frequencies of responding splenocytes were tested by IFN-γ ELISPOT assay against individual peptides (X-axis). Under each peptide is the immunogen corresponding to it; undefined peptides are variants. Data are shown as individual (shape) and median (horizontal line) of spots forming units (SFU) per million cells (Y-axis). Means of the negative controls have been subtracted from all responses. Positive threshold cut off point (dotted line) was determined as the mean of the negative controls plus three standard deviations.

### 4.2.6 Avidity of functional T cells induced by the trivalent mosaic vaccine

In consistence with our previous vaccine functional analysis, I sought to determine the avidity of the positive response reported for tConsv1, tConsv2 and tConsv3. One group of HHD mice (n = 5) were vaccinated using the DDDCM regimen with a third of the dose as described in the prevoius experiment (Fig. 4.11A). The avidity of the response was assessed using an IFN-γ ELISPOT assay against 21 individual peptides. Peptides that induced a positive response in the previous experiment were selected for this study. Peptides were titrated and used at the following dilutions: 2 μg, 0.2 μg, 0.02 μg and 0.002 μg. In the previous avidity experiment the lowest dilution used was 0.02 μg. A further serial dilution was included in this study as the responses magnitudes were higher from mice vaccinated with the trivalent mosaic vaccine.

Positive responses were noted for all peptides at 2 μg and 0.2 μg except for VLVGPTPANI, LVGPTPANI, ALVEICTEM and ALTAICEEM. Furthermore, responses to 12 epitopes were noted at 0.02 μg and one was noted at 0.002 μg for peptide LIGPTPVNI (Fig. 4.11B). The magnitude of the responses declined in relation to the dilutions, confirming genuine responses and not a result of over stimulation (Fig. 4.11B).

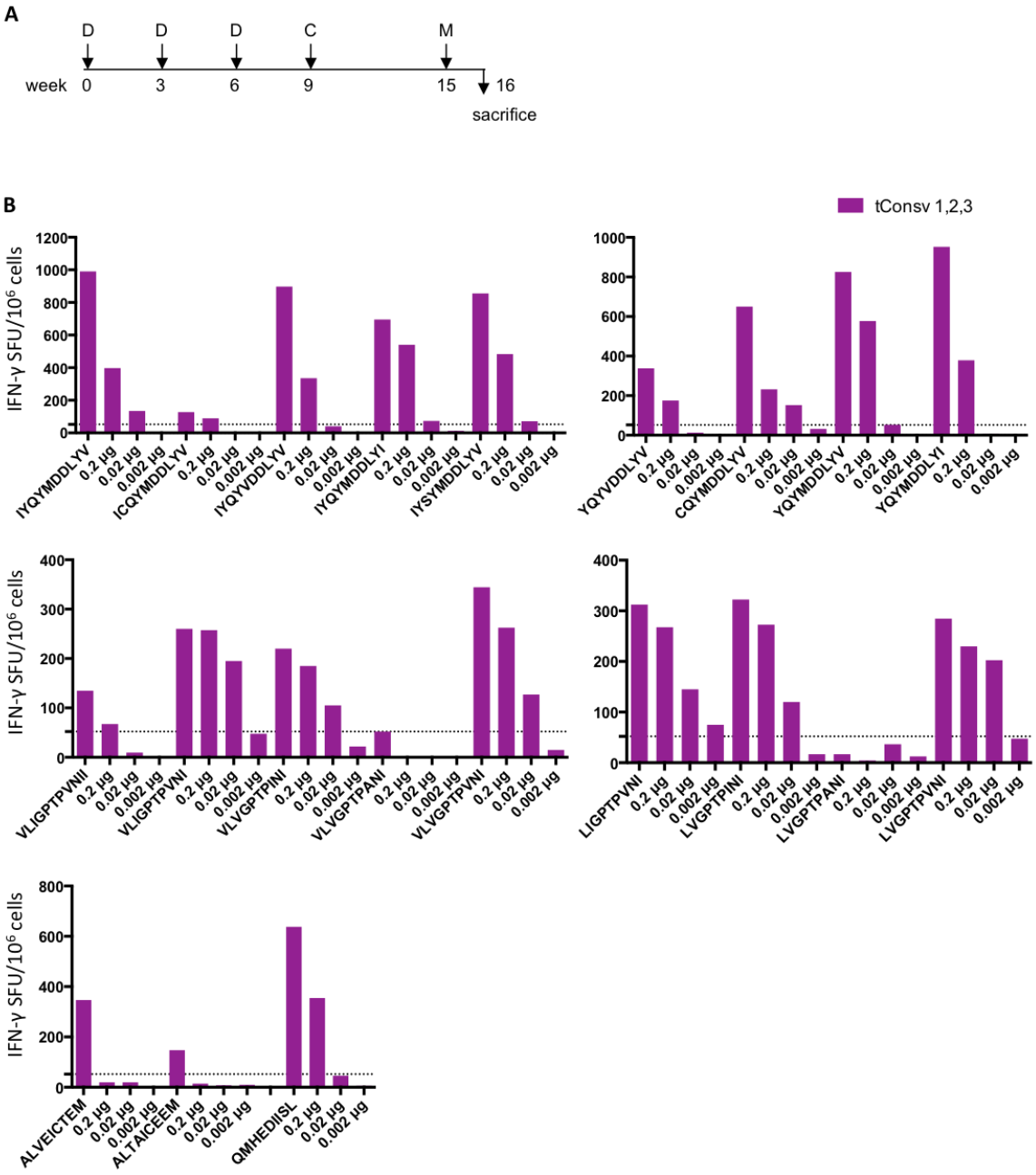



**Figure 4.11. Avidity of vaccine induced T-cell responses**

**(A)** Immunization schedule of HHD mice immunised intramuscularly (n = 5). D represents plasmid DNA, C represents ChAdV63 and M represents MVA vectored vaccine. Mice were vaccinated with a trivalent vaccine at a third of the dose receiving 33.3 µg of D, $3.3x10^7$ IU of C and $3.3x10^5$ pfu of M of each vaccine. **(B)** Mice immunised with tConsv1+2+3 at 1/3 dose of each (■). Frequencies of responding splenocytes were tested by IFN-γ ELISPOT assay against 21 individual peptides in a serial dilution at concentrations of 2 µg, 0.2 µg, 0.02 µg and 0.002 µg (X-axis). Data are shown as median of spots forming units (SFU) per million cells (Y-axis). Means of the negative controls have been subtracted from all responses. Positive threshold cut off point (dotted line) was determined as the mean of the negative controls plus three standard deviations.

### 4.2.7 Killing potential of conserved mosaic induced T-cells

An *ex-vivo* killing assay was employed to determine the potential of cytolytic T-cells elicited by tConsv1, tConsv2 and tConsv3 trivalent vaccine. Two groups of HHD mice (n = 5) were vaccinated with either the full dose or a third of dose of vaccines in a DDDCM regimen. The assay was implemented as described in the previous killing assay, with the gating strategy (Fig. 4.7) and analysis also performed in the same manner. Target cells were stimulated with five peptides in total (IYQYMDDLYV, ICQYMDDLYV, IYSYMDDLYV, YQYVDDLYV, YQYMDDLYI). These peptides induced strong responses in the IFN-γ ELISPOT assay. This experiment was not intended to examine the depth as with the previous killing assay, hence the low number of peptides used. Killing was induced against all target cells, with a percentage range of 45% - 55% specific killing recorded (Fig. 4.12B). There was no significant difference between the two groups of mice, both inducing efficient killing of target cells.

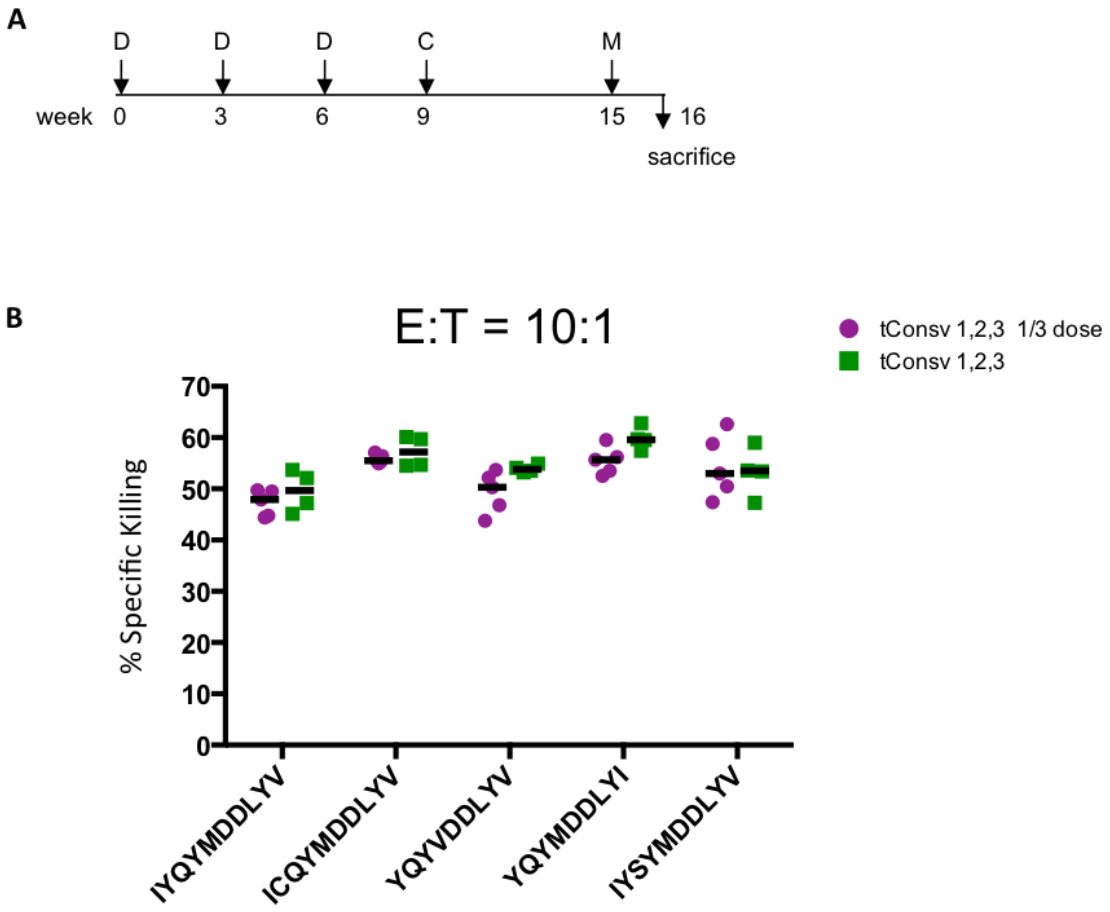



**Figure 4.12. Killing efficacy of vaccine induced T-cells in a DDDCM regimen**
**(A)** Immunization schedule of HHD mice immunised intramuscularly (n = 5). D represents plasmid DNA, C represents ChAdV63 and M represents MVA vectored vaccine. Mice were vaccinated either with a trivalent vaccine at a full dose receiving 100 µg of D, $10^8$ IU of C and $10^6$ pfu of M of each vaccine; or a third dose receiving 33.3 µg of D, $3.3x10^7$ IU of C and $3.3x10^5$ pfu of M of each vaccine. **(B)** Mice immunised with tConsv1+2+3 at 1/3 dose of each (•) or tConsv1+2+3 at full dose (■). Frequency of splenocytes specific killing was determined using an *Ex-vivo* killing assay against target splenocytes cells pulsed with 5 peptides individually (X-axis). Effector to target ratio was 10:1. Data are shown as percentage of specific killing with a horizontal line depicting the median (Y-axis).

### 4.2.8 Comparing the depth of T cell responses induced by the three sets of vaccines in a CM regimen

The previous experiments have examined the depth of the response induced by the different vaccines separately. Although comparison can be made between the three mosaics and tHIVconsv with and without the addition of a mosaic, experimental variations can alter findings and conclusions. To overcome this, in this experiment I compared tHIVconsv versus tHIVconsv + tHIVcmo versus tConsv1 + tConsv2 + tConsv3 to get a better understanding and conclusive results on their effects on epitope recognition. Furthermore, previous experiments have demonstrated when using a vaccine combination, lower doses were sufficient to induce the desired responses, therefore in this experiment the half and third doses were used in groups receiving multiple vaccines. This would also ensure a fair comparison between the groups and any difference seen in the response can be attributed to the immunogen rather than the vector concentration. Furthermore, two regimens (CM and DDDCM) have been used in the previous experiments and there were no major differences between them, both inducing strong responses and similar depth. Therefore for this experiment the CM regimen was employed, as it only takes 7 weeks compared to 16 weeks it would have taken using the DDDCM regimen.

Three groups of HHD mice (n = 6) were vaccinated with tHIVconsv, tHIVconsv + tHIVcmo (1/2 dose each) or tConsv1 + tConsv2 + tConsv3 (1/3 dose each). Concentration for each vaccine is described above. IFN-γ responses were measured to determine the depth of the T cell response in an IFN-γ ELISPOT assay against 60 individual peptides. Assay readout and analysis were done as described previously. Positive responses from all three groups of mice were noted for 5 epitopes and their variants as seen in the previous experiments (Fig. 4.13B). Epitopes that did not induce a positive response are not shown in this section. The depth of the response was greatest in mice immunised with the trivalent mosaic vaccine (tConsv1,2,3), whereby they induced a positive response to 21 epitopes

compared to 14 epitopes for tHIVconsv alone and 19 epitopes for tHIVconsv + tHIVcmo (Fig. 4.13C). Mice vaccinated with the trivalent mosaic vaccine elicited responses with significantly higher magnitude in response to several epitopes in comparison to the two other groups (Fig. 4.13B). Statistically higher responses were noted against 13 epitopes (Table 4.2).

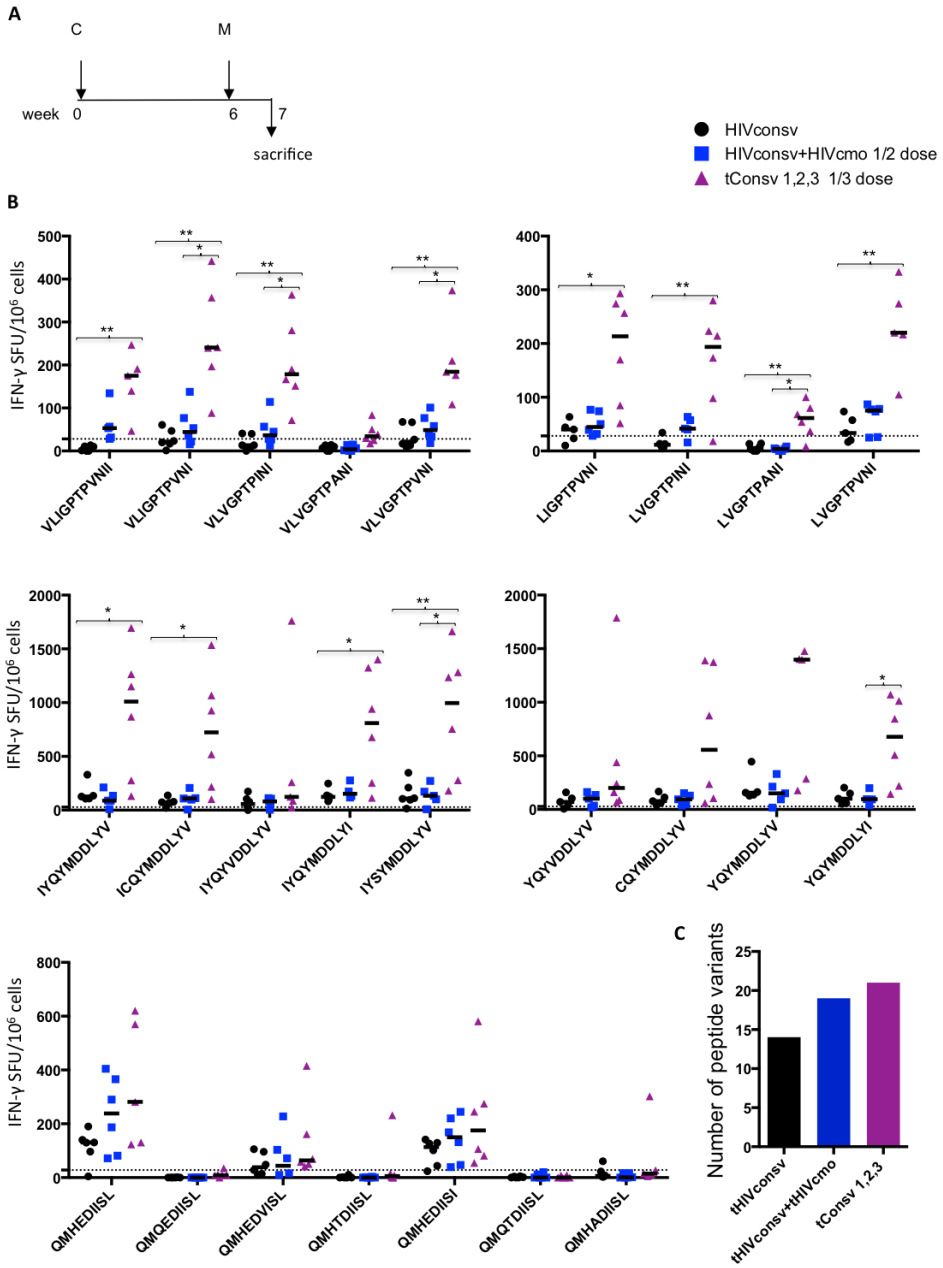



**Figure 4.13. Depth of T cell responses induced by HIVconsv and mosaic vaccines**
**(A)** Immunization schedule of HHD mice immunised intramuscularly (n = 6). C represents ChAdV63 and M represents MVA vectored vaccine. Mice were vaccinated either with a full dose receiving $10^8$ IU of C and $10^6$ pfu of M of each vaccine; or a half dose $5x10^7$ IU of C and $5x10^5$ pfu of M of each vaccine, or a third dose receiving $3.3x10^7$ IU of C and $3.3x10^5$ pfu of M of each vaccine. **(B)** Mice were immunised with tHIVconsv (•), tHIVconsv + tHIVcmo at 1/2 dose (■), tConsv1+2+3 at 1/3 dose of each (Δ). Frequencies of responding splenocytes were tested by IFN-γ ELISPOT assay against 25 individual peptides (X-axis). Data are shown as individual (shape) and median (horizontal line) of spots forming units (SFU) per million cells (Y-axis). Means of the negative controls have been subtracted from all responses. Positive threshold cut off point (dotted line) was determined as the mean of the negative controls plus three standard deviations. ANOVA test was used to determine statistical significant differences between the groups ($p<0.05$), indicated with (*). **(C)** Summary of the number of epitopes and variants recognised by vaccine elicited immune responses.

**Table 4.2.** A summary of the ANOVA statistical analysis results from the IFN-γ ELISPOT assay results. Analysis between three groups of vaccinated HHD mice 1) tHIVconsv 2) tHIVconsv + tHIVcmo (half dose) 3) tConsv1+2+3 (third dose). The table summarises the statistical significant differences (p value) in IFN-γ responses induced by the different vaccines (blue) against specific peptide (red). **Bold** indicates the vaccine with the greater significance.

| Epitope | Vaccine comparison | P value |
|---|---|---|
| VLIGPTPVNII | **tConsv 1,2,3** vs tHIVconsv | 0.0018 |
| VLIGPTPVNI | **tConsv 1,2,3** vs tHIVconsv | 0.0085 |
| | **tConsv 1,2,3** vs tHIVconsv+tHIVcmo | 0.03 |
| VLVGPTPINI | **tConsv 1,2,3** vs tHIVconsv | 0.0044 |
| | **tConsv 1,2,3** vs tHIVconsv+tHIVcmo | 0.049 |
| VLVGPTPVNI | **tConsv 1,2,3** vs tHIVconsv | 0.0068 |
| | **tConsv 1,2,3** vs tHIVconsv+tHIVcmo | 0.0456 |
| LIGPTPVNI | **tConsv 1,2,3** vs tHIVconsv | 0.012 |
| LVGPTPINI | **tConsv 1,2,3** vs tHIVconsv | 0.0022 |
| LVGPTPANI | **tConsv 1,2,3** vs tHIVconsv | 0.037 |
| | **tConsv 1,2,3** vs tHIVconsv+tHIVcmo | 0.0091 |
| LVGPTPVNI | **tConsv 1,2,3** vs tHIVconsv | 0.0038 |
| IYQYMDDLYV | **tConsv 1,2,3** vs tHIVconsv+tHIVcmo | 0.024 |
| ICQYMDDLYV | **tConsv 1,2,3** vs tHIVconsv | 0.016 |
| IYQYMDDLYI | **tConsv 1,2,3** vs tHIVconsv | 0.046 |
| IYSYMDDLYV | **tConsv 1,2,3** vs tHIVconsv | 0.036 |
| | **tConsv 1,2,3** vs tHIVconsv+tHIVcmo | 0.038 |
| YQYMDDLYI | **tConsv 1,2,3** vs tHIVconsv | 0.036 |

### 4.2.9 Comparisons of vaccine induced CD8+ cytolytic function

Potential of the cytolytic function of vaccine induced CD8+ T cells was compared using an *ex-vivo* killing assay. Three groups of HHD mice (n = 8) were vaccinated with either tHIVconsv, tHIVconsv + tHIVcmo (1/2 dose each) or tConsv1 + tConsv2 + tConsv3 (1/3 dose each) using the CM regimen (Fig 4.14A). Concentration for each vaccine is described above. The *ex-vivo* killing assay was performed as described in the previous killing assay, with the gating strategy (Fig. 4.7) and analysis also performed in the same manner. Target cells were stimulated with the strongest 10 epitopes, selected based on the results of the previous IFN-γ ELISPOT assay. The epitopes divide into three groups 1) IYQYMDDLYV, 2) VLIGPTPVNI, 3) QMHEDIISL with variants for each group completing the 10 epitopes.

Splenocytes from the three groups of vaccinated mice were successful in killing all target cells by different degrees. tHIVconsv induced an average of 30% - 40% specific killing across all epitopes (Fig. 4.14B). When coupled with tHIVcmo the average of specific killing was between 50% and 60% across all epitopes (Fig. 4.14B). The trivalent mosaic vaccine induced the highest percentage of specific killing, with an average between 70% and 75% (Fig. 4.14B). The difference in the percentage of specific killing induced by the trivalent vaccine and tHIVconsv across all the targets was statistically significant, whereas when compared to tHIVconsv + tHIVcmo a significant increase was noted for five of the target cells (Fig. 4.14B). Results of the statistical analysis are summarised in table 4.3.

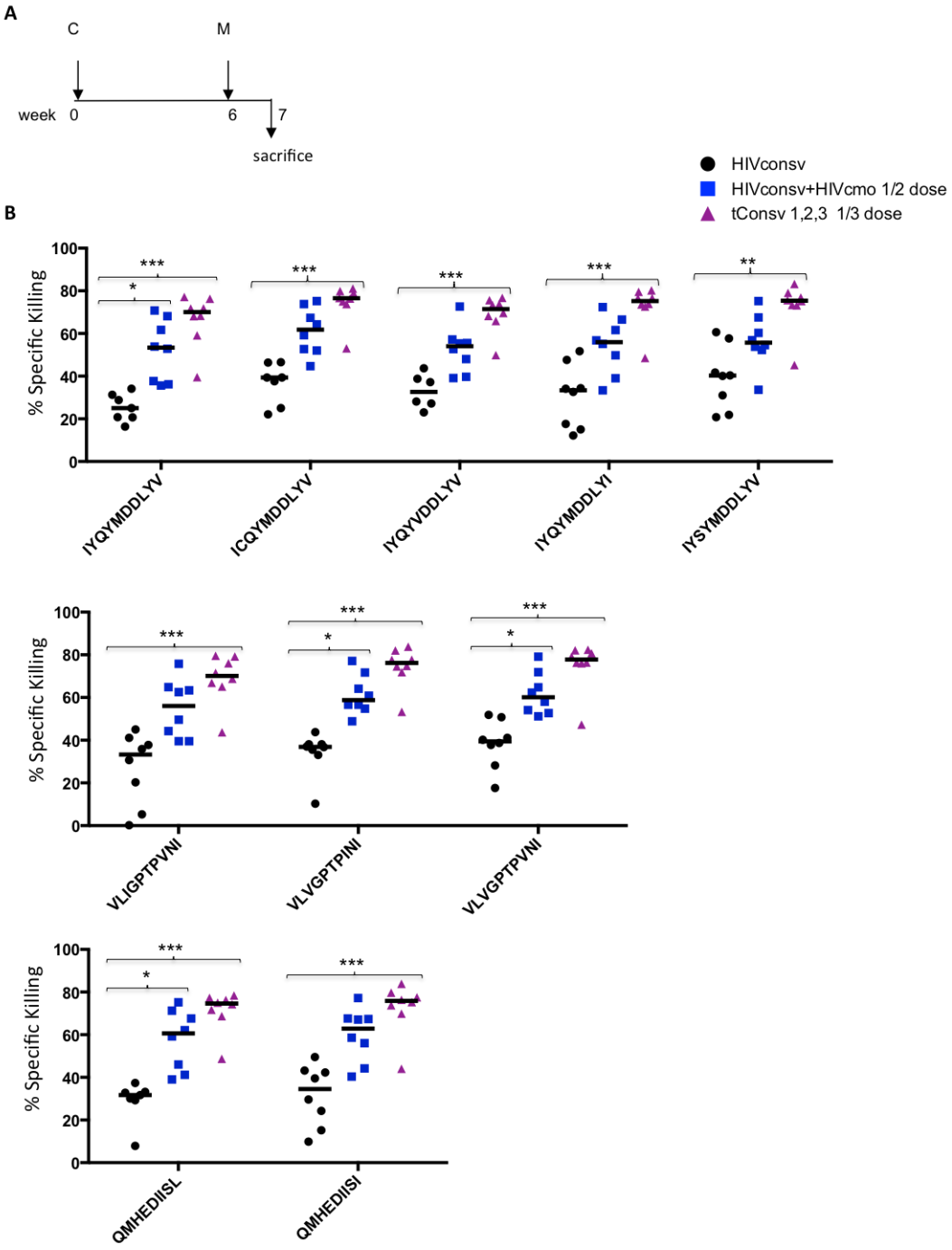



**Figure 4.14. Killing efficacy of vaccine induced T-cells in a CM regimen**

**(A)** Immunization schedule of HHD mice immunised intramuscularly (n = 8). C represents ChAdV63 and M represents MVA vectored vaccine. Mice were vaccinated either with a full dose receiving $10^8$ IU of C and $10^6$ pfu of M of each vaccine; or a half dose $5x10^7$ IU of C and $5x10^5$ pfu of M of each vaccine, or a third dose receiving $3.3x10^7$ IU of C and $3.3x10^5$ pfu of M of each vaccine. **(B)** Mice were immunised with tHIVconsv (•), tHIVconsv + tHIVcmo at 1/2 dose (■), tConsv1+2+3 at 1/3 dose of each (Δ). Frequency of splenocytes specific killing was determined using an *Ex-vivo* killing assay against target splenocytes cells pulsed with 10 peptides individually (X-axis). Effector to target ratio was 10:1. Data are shown as percentage of specific killing with a horizontal line depicting the median (Y-axis). ANOVA test was used to determine statistical significant differences between the groups ($p<0.05$), indicated with (*).

**Table 4.3.** A summary of the ANOVA statistical analysis results from the *ex*-vivo killing assay results. Analysis between three groups of vaccinated HHD mice 1) tHIVconsv 2) tHIVconsv + tHIVcmo (half dose) 3) tConsv1+2+3 (third dose). The table summarises the statistical significant differences (p value) in specific killing induced by the different vaccines (blue) against specific peptide (red). **Bold** indicates the vaccine with the significantly greater killing efficacy.

| Epitope | Vaccine comparison | P value |
|---|---|---|
| IYQYMDDLYV | **tConsv 1,2,3** vs tHIVconsv | 0.0002 |
| | **tHIVconsv+tHIVcmo** vs tHIVconsv | 0.036 |
| ICQYMDDLYV | **tConsv 1,2,3** vs tHIVconsv | 0.0001 |
| IYQYVDDLYV | **tConsv 1,2,3** vs tHIVconsv | 0.0003 |
| IYQYMDDLYI | **tConsv 1,2,3** vs tHIVconsv | 0.0002 |
| IYSYMDDLYV | **tConsv 1,2,3** vs tHIVconsv | 0.0011 |
| VLIGPTPVNI | **tConsv 1,2,3** vs tHIVconsv | 0.0003 |
| VLVGPTPINI | **tConsv 1,2,3** vs tHIVconsv | < 0.0001 |
| | **tHIVconsv+tHIVcmo** vs tHIVconsv | 0.027 |
| VLVGPTPVNI | **tConsv 1,2,3** vs tHIVconsv | 0.0002 |
| | **tHIVconsv+tHIVcmo** vs tHIVconsv | 0.035 |
| QMHEDIISL | **tConsv 1,2,3** vs tHIVconsv | 0.0001 |
| | **tHIVconsv+tHIVcmo** vs tHIVconsv | 0.038 |
| QMHEDIISI | **tConsv 1,2,3** vs tHIVconsv | 0.0003 |

### 4.2.10 Comparison of multi-functional profiles of vaccine induced T-cells in a CM regimen

Next I set to further characterize the effects of mosaic vaccines on the frequency and functional spectrum of vaccine-elicited T cells. To do so, frequencies of T cells expressing CD107a, IFN-γ, TNF-α and IL-2 were examined. This was determined from vaccinated mouse splenocytes stimulated *ex-vivo* with five individual peptides (YQYVDDLYV, CQYMDDLYV, VIYQYMDDL, YQYMDDLYV and YQYMDDLYI) (Fig. 4.16). Three groups of HHD mice (n = 4) were vaccinated with tHIVconsv, tHIVconsv + tHIVcmo (1/2 dose) and tConsv 1, 2 & 3 mix (1/3 dose) in a CM regimen (Fig. 4.16A). Intracellular cytokine staining (ICS) was used to analyse the percentage of CD8+ cells producing CD107a, IFN-γ and TNF-α via flow cytometry.

Analysis and gating strategy of the flow cytometry are illustrated in figure 4.15. CD8+ T-cells were gated on from the live cells within the lymphocyte population. Once identified, the four cytokines were detected within the CD8+ population (Fig. 4.15A). The gates were set in accordance to the negative control (Fig. 4.15B). A two-way ANOVA test was employed for statistical analysis (Table 4.4). The predominant phenotype of T-cells from the three vaccinee groups was CD8+ T cells expressing CD107a and IFN-γ. tConsv 1, 2 and 3 vaccine mix induced significantly higher expression of CD107a, IFN-γ and TNF-α in response to several peptides (Table 4). The average of IFN-γ expression from mice immunised with tHIVconsv and tHIVconsv + tHIVcmo was 3% for all 5 peptides, compared to the 10-15% expression levels seen in tConsv 1, 2, 3 group (Fig. 4.16B). Expression of TNF-α was the lowest, with an average of 1-2% for both groups with tHIVconsv and 2-7% for the tConsv 1, 2, 3 group. As for CD107a, expression was slightly higher with average expression level of 7% for the two groups with tHIVconsv and 15-20% for the tConsv 1, 2, 3 group (Fig. 4.16B). Due to the low magnitude of the responses induced by HHD mice (as shown by ELISPOT) and the sensitivity of this assay, the

readouts from this assay were very low and not conclusive, however the pattern shown is in agreement with the previous results.

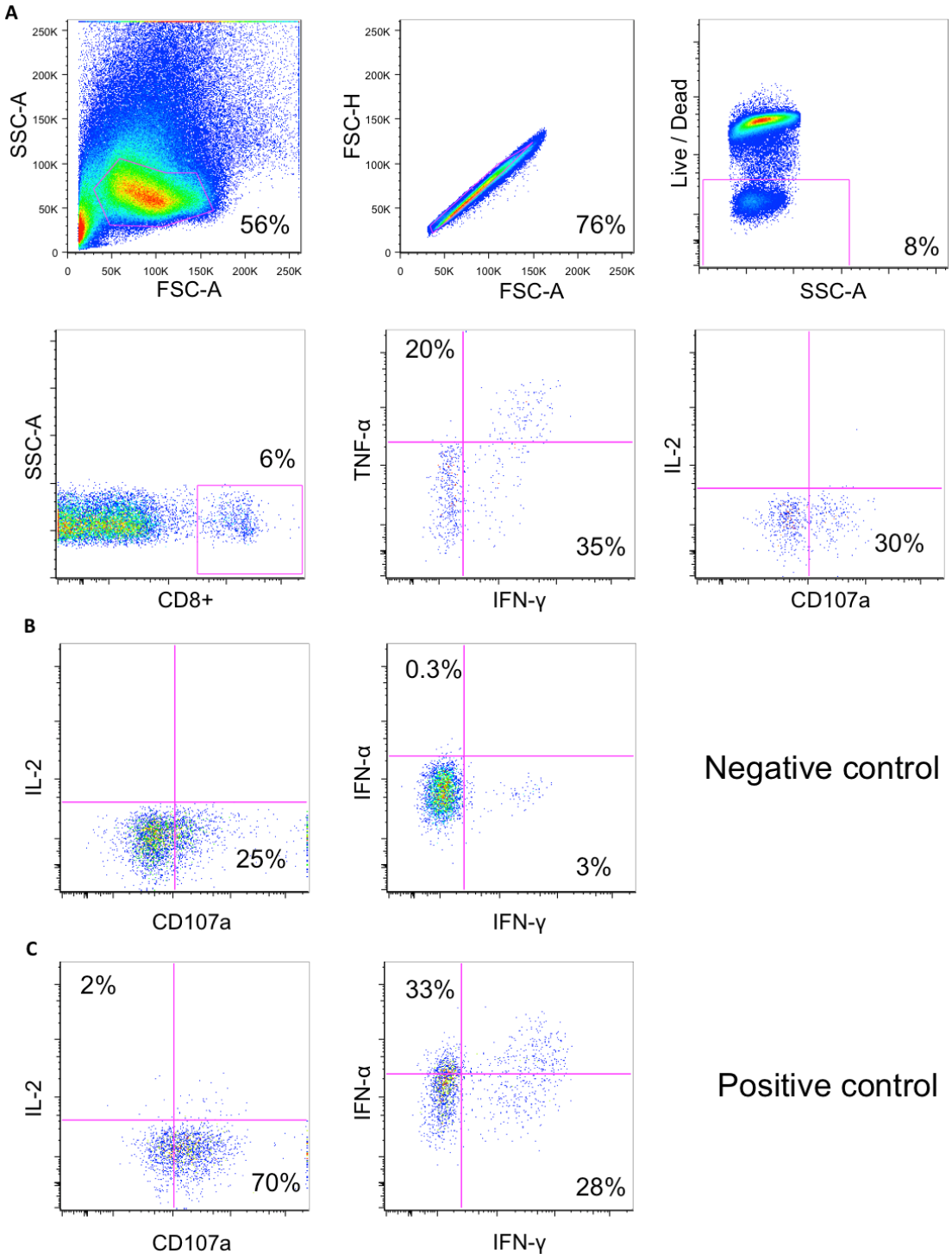



**Figure 4.15. Representative FACS plots of a intracellular cytokine staining (ICS) assay**

**(A)** Representative FACS plots showing gating strategy for an ICS assay of peptide stimulated cells. Gating strategy (from top left panel going left) 1) Lymphocytes 2) Singlets 3) Live cells 4) CD8+ cells 5) CD8+ expressing TNF-α and IFN-γ 6) CD8+ expressing IL-2 and CD107. **(B and C)** FACS plots of negative and positive controls. Gates were set based on the negative controls. Percentage of each population is indicated on the blot.

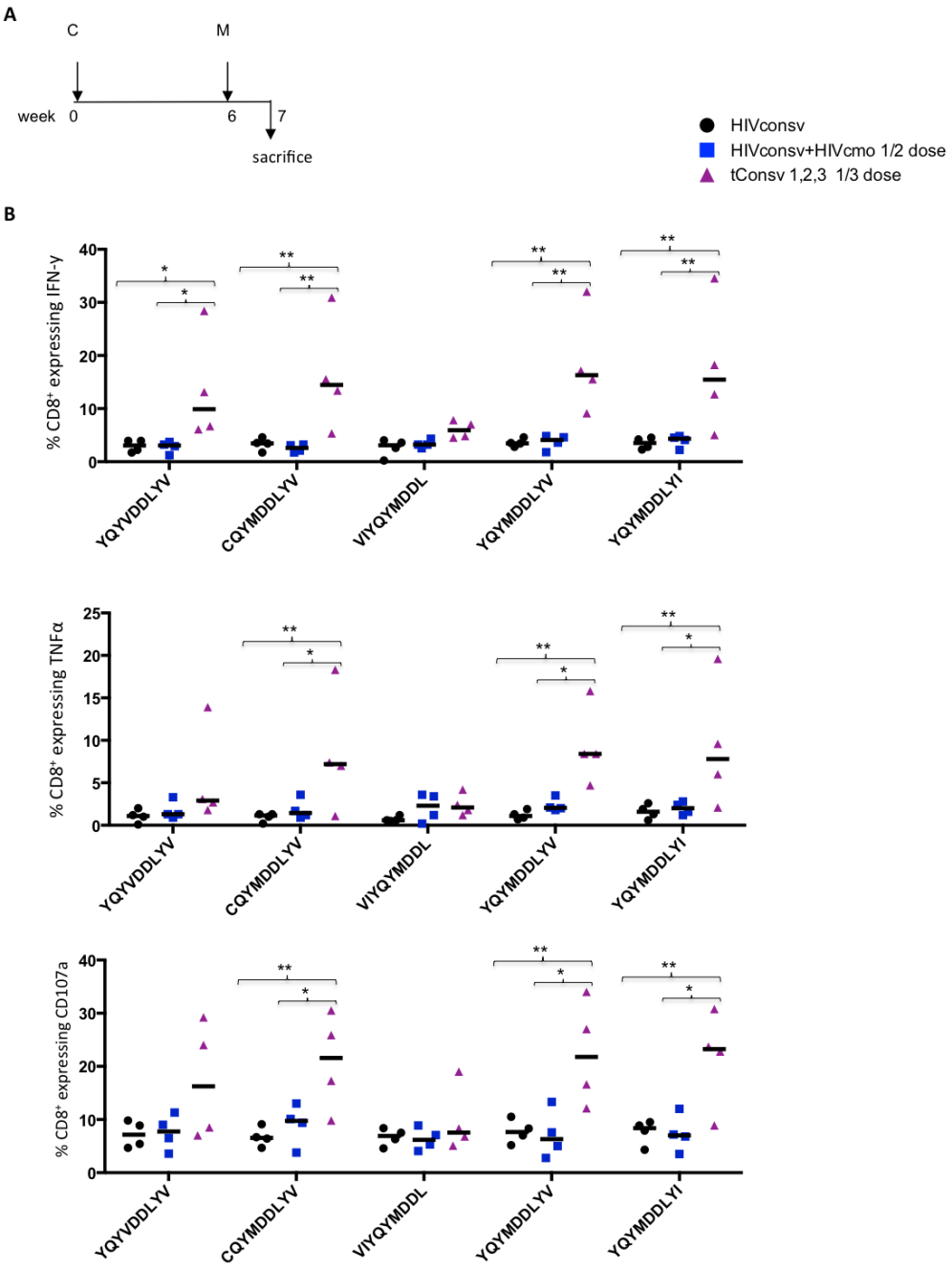



**Figure 4.16. Effects of the different vaccines on cytokine secretion and degranulation** **(A)** Immunization schedule of HHD mice immunised intramuscularly (n = 4). C represents ChAdV63 and M represents MVA vectored vaccine. Mice were vaccinated either with a full dose receiving $10^8$ IU of C and $10^6$ pfu of M of each vaccine; or a half dose $5x10^7$ IU of C and $5x10^5$ pfu of M of each vaccine, or a third dose receiving $3.3x10^7$ IU of C and $3.3x10^5$ pfu of M of each vaccine. **(B)** Mice were immunised with tHIVconsv (•), tHIVconsv + tHIVcmo at 1/2 dose (■), tConsv1+2+3 at 1/3 dose of each (Δ). Intracellular cytokine staining assay was used to determine the percentage of CD8+ T cells expressing CD107a, IFN-γ or TNF-α (Y-axis) via FACS analysis, the horizontal line depicting the median. 5 peptides were used to stimulate splenocytes responses (X-axis). ANOVA test was used to determine statistical significant differences between the groups ($p<0.05$), indicated with (*).

**Table 4.4.** A summary of the ANOVA statistical analysis results from the intracellular cytokine staining assay results. Analysis between three groups of vaccinated HHD mice 1) tHIVconsv 2) tHIVconsv + tHIVcmo (half dose) 3) tConsv1+2+3 (third dose). The table summarises the statistical significant differences (p value) in cytokine production (green) between vaccine (blue) induced T-cell against a specific peptide (red). **Bold** indicates the vaccine with the significantly greater cytokine production.

| Cytokine | Epitope | Vaccine comparison | P value |
|---|---|---|---|
| IFN-γ | YQYVDDLYV | **tConsv 1,2,3** vs tHIVconsv | 0.0299 |
| | | **tConsv 1,2,3** vs tHIVconsv+tHIVcmo | 0.0268 |
| | CQYMDDLYV | **tConsv 1,2,3** vs tHIVconsv | 0.0066 |
| | | **tConsv 1,2,3** vs tHIVconsv+tHIVcmo | 0.0039 |
| | YQYMDDLYV | **tConsv 1,2,3** vs tHIVconsv | 0.0018 |
| | | **tConsv 1,2,3** vs tHIVconsv+tHIVcmo | 0.0019 |
| | YQYMDDLYI | **tConsv 1,2,3** vs tHIVconsv | 0.0029 |
| | | **tConsv 1,2,3** vs tHIVconsv+tHIVcmo | 0.004 |
| | | | |
| TNF-α | CQYMDDLYV | **tConsv 1,2,3** vs tHIVconsv | 0.0084 |
| | | **tConsv 1,2,3** vs tHIVconsv+tHIVcmo | 0.0226 |
| | YQYMDDLYV | **tConsv 1,2,3** vs tHIVconsv | 0.0041 |
| | | **tConsv 1,2,3** vs tHIVconsv+tHIVcmo | 0.0151 |
| | YQYMDDLYI | **tConsv 1,2,3** vs tHIVconsv | 0.0065 |
| | | **tConsv 1,2,3** vs tHIVconsv+tHIVcmo | 0.0103 |
| | | | |
| CD107a | CQYMDDLYV | **tConsv 1,2,3** vs tHIVconsv | 0.0038 |
| | | **tConsv 1,2,3** vs tHIVconsv+tHIVcmo | 0.0178 |
| | YQYMDDLYV | **tConsv 1,2,3** vs tHIVconsv | 0.0027 |
| | | **tConsv 1,2,3** vs tHIVconsv+tHIVcmo | 0.0018 |
| | YQYMDDLYI | **tConsv 1,2,3** vs tHIVconsv | 0.0045 |
| | | **tConsv 1,2,3** vs tHIVconsv+tHIVcmo | 0.0038 |

### 4.2.11 Priming the immune response with single epitope vaccine

Preliminary studies of tHIVconsv and tHIVcmo (data not shown) have identified 12 subdominant epitopes that did not induce a response in an IFN-γ ELISPOT (Table 4.5). These epitopes are likely sub-dominant within the hierarchy of epitopes expressed in these immunogen. However, I was still interested in examining whether a response can be induced to these epitopes, to study the variant recognition.

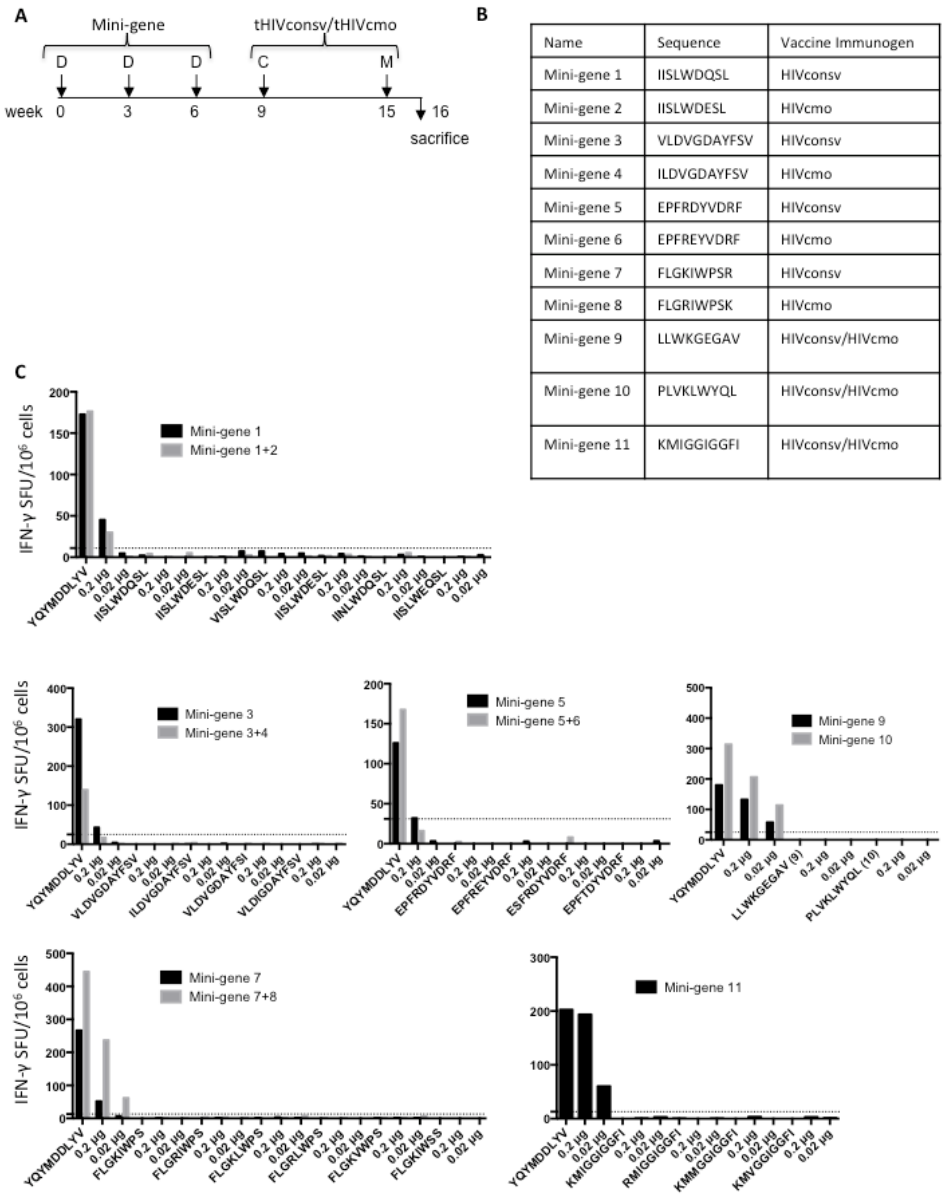
Plasmid DNA vaccines, modified with DOM FrC tetanus, expressing each of these epitopes individually were constructed (mini-gene vaccines), to test whether priming with these singular epitopes can alter the immune-dominance and elicit a response. The twelve epitopes are divided into 7 groups, some epitopes have a variant in each of the two vaccines, tHIVconsv and tHIVcmo, others are found in both vaccines (Table 4.5). HHD mice (n = 4) were vaccinated using the DDDCM regimen, they received a DNA prime of one mini-gene or a pair. To clarify, mini-gene 1 and mini-gene 2 vaccines encode different variants of the same epitope, each found in either tHIVconsv or tHIVcmo; these were tested in two groups of mice, one receiving mini-gene 1 alone the other group receiving mini-gene 1 and 2 simultaneously. Following the three DNA primes, mice were boosted with recombinant ChAdV63 and MVA encoding either tHIVconsv or tHIVconsv + tHIVcmo. The group of mice primed with one mini-gene vaccine were boosted with tHIVconsv, the group primed with the pair of mini-genes vaccine were boosted with tHIVconsv + tHIVcmo (Fig. 4.17A). IFN-γ responses were measured using an IFN-γ ELISPOT against the mini-gene epitopes and their variants. Epitope YQYMDDLYV, shown to elicit strong responses to tHIVconsv and tHIVcmo was used as an internal control. Peptides were used in a serial dilution (2 μg, 0.2 μg, 0.02 μg) to determine functional avidity.

No positive responses were noted for any of the eleven epitopes or their variants (Fig. 4.17B). Positive responses were only noted to epitope YQYMDDLYV as expected.

The priming with one or two mini-genes did not induce any positive response to any of the desired epitopes.

**Table 4.5.** A list of HLA-A*02:01 epitopes (blue) sub-dominant within tHIVconsv and tHIVcmo immunogens and their variants (red).

| Immunogen | Mini-gene epitope | Variants | | | |
|---|---|---|---|---|---|
| HIVconsv | EPFRDYVDRF | ESFRDYVDRF | EPFTDYVDRF | | |
| HIVcmo | EPFREYVDRF | | | | |
| HIVconsv | FLGKIWPS | FLGKLWPS | FLGRLWPS | FLGKVWPS | FLGKIWSS |
| HIVcmo | FLGRIWPS | | | | |
| HIVconsv/HIVcmo | KMIGGIGGFI | RMIGGIGGFI | KMMGGIGGFI | KMVGGIGGFI | |
| HIVconsv | VLDVGDAYFSV | VLDVGDAYFSI | VLDIGDAYFSV | | |
| HIVcmo | ILDVGDAYFSV | | | | |
| HIVconsv/HIVcmo | PLVKLWYQL | | | | |
| HIVconsv/HIVcmo | LLWKGEGAV | | | | |
| HIVconsv | IISLWDQSL | VISLWDQSL | IISLWDESL | IINLWDQSL | IISLWEQSL |
| HIVcmo | IISLWDESL | | | | |




| Name | Sequence | Vaccine Immunogen |
|---|---|---|
| Mini-gene 1 | IISLWDQSL | HIVconsv |
| Mini-gene 2 | IISLWDESL | HIVcmo |
| Mini-gene 3 | VLDVGDAYFSV | HIVconsv |
| Mini-gene 4 | ILDVGDAYFSV | HIVcmo |
| Mini-gene 5 | EPFRDYVDRF | HIVconsv |
| Mini-gene 6 | EPFREYVDRF | HIVcmo |
| Mini-gene 7 | FLGKIWPSR | HIVconsv |
| Mini-gene 8 | FLGRIWPSK | HIVcmo |
| Mini-gene 9 | LLWKGEGAV | HIVconsv/HIVcmo |
| Mini-gene 10 | PLVKLWYQL | HIVconsv/HIVcmo |
| Mini-gene 11 | KMIGGIGGFI | HIVconsv/HIVcmo |

**Figure 4.17. Assessing mini-gene vaccines**

**(A)** Immunization schedule of HHD mice immunised intramuscularly (n = 4). D represents plasmid DNA, C represents ChAdV63 and M represents MVA vectored vaccine. Mice were either vaccinated with a full dose receiving 100 μg of D, $10^8$ IU of C and $10^6$ pfu of M of each vaccine; or a half dose receiving 50 μg of D, $5x10^7$ IU of C and $5x10^5$ pfu of M of each vaccine. **(B)** List of the mini-genes sequences and the corresponding immunogen they originate from. **(C)** Mice were primed with 1 mini-gene and boosted with tHIVconsv (■), two mini-genes and boosted with tHIVconsv + tHIVcmo at ½ dose (■). Frequencies of responding splenocytes were tested by IFN-γ ELISPOT assay against individual peptides in a serial dilution at concentrations of 2 μg, 0.2 μg and 0.02 μg (X-axis). Peptides correspond to the mini-genes and their variants; and YQYMDDLYV peptide was used as control. Data are shown as median of spots forming units (SFU) per million cells (Y-axis). Means of the negative controls have been subtracted from all responses. Positive threshold cut off point (dotted line) was determined as the mean of the negative controls plus three standard deviations.

## 4.3 Discussion

Mosaic immunogens are designed to maximize the coverage of potential T cell epitope variants (439, 504, 505, 553, 564). They are derived from in *silico* recombination of natural sequences, producing immunogens resembling and aligning natural sequences but include only common and exclude rare or unnatural T cell epitopes, avoiding the induction of non-protective, vaccine specific responses (344, 439, 504, 505, 553, 564). Bette Korber, using 14 pre-defined conserved regions of HIV-1, developed four mosaic immunogens for us. The first immunogen tHIVcmo, was designed to complement tHIVconsv. The three other immunogens, tConsv 1/2/3, designed to complement each other in a polyvalent vaccine cocktail. The work done in this chapter is to answer two questions (i) would the addition of a mosaic vaccine tHIVcmo to a consensus vaccine tHIVconsv have an added benefit by enhancing the breadth or depth of the elicited response (ii) would trivalent mosaic vaccines induce a T cell response with greater breadth or depth. This was assessed using HHD transgenic mice expressing a chimeric human HLA-A*0201, to translaye the study to humans. The breadth and depth of the response were characterized and determined against known HIV-1, CD8+ HLA-A02, epitopes and variants across all major clades (A, B, C, D) (Table 1). The immunogens were expressed and delivered via three vectors, a plasmid DNA, ChAdV63 and MVA in two heterologous prime boost regimens, DDDCM and CM (395, 396, 531). Both regimens induced prolific responses with no notable differences between them, thus my discussion and analysis is based on both results, not differentiating between them. The comparisons are based on the groups receiving the lesser dose of combined vaccines, to ensure comparison with the individual vaccine is comparable and to eliminate the vector concentration factor.

During infection, immune responses are induced against variable proteins of HIV-1, allowing escape from immune recognition due to mutations and epitope mismatch. However, on a sub-protein level, some parts of protein are conserved and remain constant

to maintain viral structural and biological function (99, 565-568). Therefore, conserved parts of proteins are potentially effective vaccines targets, as a way of addressing viral diversity and immune evasion. HIVconsv, is a universal HIV-1 vaccine targeting the conserved region of HIV-1 currently in phase IIa human clinical trials. It is a T cell based vaccine, assembled from the 14 most highly conserved regions of HIV-1 irrespective of known CD8+ T-cell epitopes. Each region uses the consensus amino acid sequence from each of the four major clades A, B, C and D (395, 396, 436). Pre-clinical studies in BALB/c mice, non-human primate studies and clinical studies from a phase I/IIa have reported on the high immunogenicity of HIVconsv, inducing very strong and broadly-directed, polyfunctional T cell responses (396, 436, 531, 532). The human clinical trials showed unprecedented high levels of T cell responses to HIV-1 peptides. Vaccine elicited T cells were broadly specific, produced multiple intercellular signaling molecules and showed efficacy *in vitro* inhibiting HIV-1 replication (396). Nonetheless, to further improve coverage, we employed mosaic technology in an effort to enhance the depth of vaccine elicited specific T cells.

Responses induced in an IFN-γ ELISPOT from mice immunised with either the consensus vaccine tHIVconsv or a single mosaic, tHIVcmo, were comparable in magnitude and epitope coverage. Both the breadth and depth of the response was higher for tHIVconsv when using the DDDCM regimen whereas the breadth was the same with the CM regimen while the depth was higher by one variant for tHIVcmo. The increase noted in the DDDCM regimen is due to a response seen to a single epitope (ILKDPVHGV), although it was only noted in 50% of the mice in the group. These results agree with findings from other studies, whereby the performance of a single mosaic was comparable to that of a consensus vaccine (439). This is to be expected as both vaccines both have very similar sequences and only one variant of each epitope present, thus a similar response is expected from both. However, when combining both in a bivalent vaccine, an increase in the depth of the

response was noted, with positive responses to 3 and 2 extra variants in the CM and DDDCM regimens respectively. Responses from all groups were limited to five groups of epitopes and their variants (VLVGPTPVNI, LVGPTVNI, YQYMDDLYV, IYQYMDDLYV, QMHEDIISL).

Next I compared the three sets of vaccines (tHIVconsv, tHIVconsv + tHIVcmo, tConsv1/2/3) in an independent experiment with relevance to the clinical development in a larger group of mice. T-cell responses elicited by the trivalent mosaic vaccines had a significantly higher magnitude and greater depth in the response in comparison to tHIVconsv alone and tHIVconsv + tHIVcmo. Examining the depth, tHIVconsv induced a positive response to 14 epitopes, tHIVconsv + tHIVcmo 18 epitopes and the trivalent mosaic vaccine 21 epitopes. Increase in depth is of great significance, as it ensures protection against infection by genetically diverse viruses and blocks the emergence of common variant escape viruses and is correlated with disease control (373, 569). Furthermore, the magnitude of vaccine elicited specific immune responses from mice vaccinated with the trivalent mosaic was significantly higher to most epitopes, even when the overall dose was the same. Higher magnitudes are correlated with strong CTL responses and strong viral control (504, 533, 553, 564). Although the overall magnitude of responses detected was lower than ones detected in BALB/c mice, this is due to these transgenic mice having a lower CD8+ T cell population (558, 570). These results are in agreement with our hypothesis and other studies in mice and non human primates, illustrating polyvalent mosaic vaccines induced responses with greater depth compared to consensus based vaccines (344, 439, 501, 533).

Conserved epitopes are typically subdominant in natural infection and variable epitopes are dominant. Nonetheless, conserved epitopes induce responses in natural infections and have been associated with lowering viremia and slowing disease progression (395, 398, 571, 572). Employing vaccines targeted at conserved regions may alter the

natural immuno-dominance, focusing the response on highly conserved epitopes as seen in long term non-progressors (395, 397, 573). Mutations in highly conserved regions to escape CTL responses are thought to incur a cost on viral fitness. Therefore, targeting these regions either induces strong viral control or forces a mutation incurring a fitness cost, reducing transmission and replication which is also favourable (396, 574-576). Responses induced in vaccinated mice in all three groups are attributed to three groups of five epitopes, all highly conserved. Epitopes VLVGPTVNI and LVGPTPVNI are 10-mer and 9-mer epitopes from the protease enzyme region in the Pol. Epitopes IYQYMDDLYV and YQYMDDLYV are 10-mer and 9-mer epitopes from the reverse transcriptase region of Pol. Epitope QMHEDIISL is a 9-mer epitope from the gp120 region of Env. No positive responses were detected against any of the Gag epitopes, which is a known conserved region associated with good viral control (100, 397, 567, 575). The majority of responses discussed here are targeted towards Pol, which is not associated with protection unlike Gag. The lack of association between Pol and CD8+ specific responses during infection has been attributed to the low expression of Pol epitopes on virus infected cells (292). Nonetheless, Pol region is highly conserved and could be a good target for vaccination as supported by the recent results from the human clinical trial of HIVconsv, whereby Pol-specific CD8+ T-cell responses correlated better than Gag specific responses with viral inhibition (396).

An *ex vivo* killing assay was utilized to determine the killing potential of vaccine elicited CTL. Trivalent mosaic elicited T cells had the highest percentage of specific killing. In comparison to tHIVconsv, tConsv1/2/3 induction of CTL killing was significantly greater (2 fold). Furthermore, combining a single mosaic to tHIVconsv also induced higher percentage of killing although statistically not significant. This confirms that although mosaics are designed to increase epitope recognition and cross reactivity of T-cells, this does not hinder its induction of cytotoxicity (501, 505, 553, 564). To further characterise the mosaic induced CD8+ T-cells, an ICS assay was employed to determine

polyfunctionalty of these cells. However, due to the low CD8+ count in HHD mice an accurate readout was not possible from this assay. Nonetheless, the results do indicate that vaccine induced CD8+ T cells produced IFN-γ, TNF-α and CD107a, all factors indicative of cytolytic function.

Functional avidity is a biological measure *in vitro* of the strength of T cell responses to given peptide concentration; it describes how well a T cell responds to antigens. T cells with high functional avidity respond *in vitro* to low antigen stimulation whereas T cells with low functional avidity require high concentrations of antigen. Thus functional avidity is inversely correlated with antigen dosage. It is a method of determining T cell function with physiological relevance and is determined by quantifying biological function e.g. IFN-γ production, cytotoxic activity *ex vivo* (577). Several factors have an impact on functional avidity (i) affinity of the T cell receptor (TCR) and MHC molecule (ii) levels of expression of the TCR and the co-receptors CD8 or CD4; reduced levels of expression correlate with reduced functional avidity (iii) distribution and composition of signaling molecule that attenuate TCR signaling and T cell function e.g. co-stimulatory or inhibitory molecules (578-581). Higher functional avidity correlates with higher efficacy in eliminating cancer cells and clearing acute viral infections e.g. high functional avidity Tax specific CD8+ T cells eliminate HTLV-1 cells with higher efficacy than low functional avidity cells (582-584). With regards to HIV-1, protective HIV specific CD8 T cell in non-progressors were of high functional avidity and mediated greater cross-reactive T cells. Furthermore, HLA-B restricted, Gag specific CD8 T cells in elite controllers have been shown to be of higher functional avidity (303, 583, 585). To determine the functional avidity of vaccine elicited T cells, responses were measured against peptides in a serial dilution (2 μg, 0.2 μg, 0.02 μg) in a DDDCM regimen. Responses to all peptides were detected at 2 μg, 0.2 μg and some even at 0.02 μg, proving that the vaccine elicited high functional avidity T cells. This indicates the conserved epitopes in the vaccines were expressed correctly and bind with

high affinity to the MHC and TCR, inducing effective CD8 responses. However, some studies reported that high functioning avidity T cells are detrimental with regards to HIV-1, as they drive mutation and viral escape. HLA's conferring higher functional avidity induce a higher level of sequence mutation and clonal turnover, leading to an increase in viral escape (547, 586). This might be true for T cells targeting variable regions of HIV-1, whereby a strong T cell response will induce mutations and viral escape. But when targeting conserved regions, inducing high functional avidity T cells is always beneficial if T cells are wanted, as these epitopes have a low clonal turnover thus inducing a strong effective response would have an impact on viral control. However, if this does drive mutation in the conserved regions for escape, it would incur a cost on viral fitness which is also harmful to the virus and good for the patient.

In preliminary analysis of tHIVconsv and tHIVcmo, eleven epitopes were identified as sub-dominant within the immunogen dominance hierarchy. To further analyse the breadth and depth of the response, I sought to determine whether a response could be induced against these individual epitopes. Thus I constructed eleven DNA plasmid vaccines, each containing one sub-dominant epitope (mini-gene vaccines). They were employed as a prime vaccination in a DDDCM regimen and the viral vectors contained either tHIVconsv or tHIVcmo depending on the epitope. The idea was, priming with a mini-gene vaccine, might alter the immune-dominance hierarchy of the tHIVconsv or tHIVcmo epitopes and the CD8 specific response induced. This was unsuccessful for all eleven mini-gene vaccines, as no responses were detected to any of the sub-dominant epitopes or their variants. The lack of response may indicate a failure altering the epitope immune-dominance. A second explanation could be due to poor expression of the epitopes. These epitopes were cloned individually into the plasmid, thus it is plausible they were not expressed and/or processed correctly.

The work described in this chapter is unique in two ways. First, this was the first fully conserved polyvalent mosaic vaccine developed and tested. Other studies have used mosaic vaccines of the whole Gag, Nef or Pol as conserved proteins, but none have used specifically conserved regions only within these proteins (439, 504, 553). A recent study compared a full length mosaic immunogen versus a conserved mosaic (Gag, Pol, Env). They reported on full length mosaics inducing responses with greater magnitude against conserved HIV-1 epitopes and comparable breadth of specific responses to conserved epitopes. Furthermore, the full length mosaic had a greater breadth of response to HIV-1 (533). This is to be expected as the total number of epitopes in the full length immunogen is greater than that of the conserved immunogen, thus responses would be elicited to a greater number of epitopes. Also the increase in magnitude could be attributed to the fact that processing epitopes from full length proteins is more efficient and presentation is optimal, thus inducing a greater magnitude (533). The greater magnitude could also be attributed to the larger number of epitopes in the full length, which would have induced a response with greater breadth, thus activating a stronger immune response and increasing the expression of cytokines and cytolytic factors. However, this does not ascertain that full-length mosaics are more efficient than conserved mosaics as they have suggested (533). The increase in breadth noted is to non-conserved variable regions, where mutations will most likely occur. Furthermore, although response to the conserved epitopes were comparable when tested in an assay, it is likely that epitopes from the variable regions are more dominant and responses during an infection will more likely be elicited to these epitopes. The aim of using conserved regions only, it is to alter the immune-dominance epitope hierarchy and their T cells responses.

Second, this is the first time the breadth and depth of a mosaic vaccine elicited responses have been described in a human HLA (HLA-A*02). This allowed us to examine responses to known human CD8 epitopes (9-mer and 10-mer) with the caveat of mouse

TCR repertoire and mouse processing machinery. Although this data translates more directly to humans, it is limited to one HLA type, therefore it limits the scope of the breadth and depth examined. Previous studies of mosaic vaccines have been conducted in mice and monkeys, which have multiple MHC I molecules, allowing the analysis of responses against a larger number of potential T cell epitopes (501, 533, 552, 564). However, although these studies show greater breadth and depth, this does not translate directly to humans, due to the different HLA's. Using other transgenic mice expressing different human HLA's (HLA-A*0101, HLA-A*2402, HLA-B*2705, HLA-B*3501) or humanised BLT mice, would be a useful way of analysing the effects of mosaic vaccines on epitope recognition. This would give us a better understanding of the potential of these vaccines and more accurate data relevant to humans. The next step is to test a mosaic vaccine in humans, to determine whether this enhancement in breadth in depth is translated in humans and what limitations it might have. We have currently designed a second generation of conserved bivalent mosaic vaccines, using more refined conserved regions of HIV-1. These are in pre-clinical trials at the moment with the aim of going into human clinical trials in the near future.

# Chapter 5:

## *The Effects of tPA leader sequence on T cell HIV-1 vaccines*

# 5. The Effects of tPA leader Sequence on a HIVconsv

## 5.1 Introduction

One of the main challenges and priorities of vaccine development is enhancing immunogenicity and efficacy to eradicate communicable and non-communicable diseases. To do so scientist have been investigating vectors for better immune induction; adjuvants to boost and alter immune responses in a specific manner; immunogen design to improve response specificity, antigen presentation, magnitude of response, killing efficacy and antibody production. Additionally, high-level expression of pathogen-derived immunogens and intracellular localization of antigens have been a focus to improve vaccine immunogenicity (587-589). Post-transcriptional and post-translational modifications were employed to direct intracellular antigen trafficking to various compartments to enhance antigen processing and presentation to the immune system. Tissue plasminogen activator (tPA) is a human enzyme involved in dissolving fibrin in blood clots by converting plasminogen to plasmin (590). tPA is produced by different cell types and tissues and is comprised of a 530 amino-acid single-chain glycoprotein and a 32 amino-acid leader sequence (590, 591). The tPA leader sequence is a known efficient secretory signal used for many proteins. It is usually incorporated into the 5' coding region of the transgene to increase intracellular transport and expression efficiency by directing proteins to the secretory pathway (457, 592-594).

The tPA leader sequence enhances protein expression by specifically targeting the antigen to the endoplasmic reticulum (ER) and promoting transport to the golgi apparatus (GA) thus facilitating its secretion from the cell (588, 593). Antigens are presented on the MHC complex through the transporter associated with antigen pathway (TAP), which includes processing by proteasomes in the cytoplasm and chaperones in the ER e.g.

calnexin and binding immunoglobulin protein (BiP). By targeting the antigen to the ER, tPA obviates the need to process and translocate antigens to the cytoplasm and ER and also prevents antigen retention in the ER, thus enhancing antigen bioavailability (461, 588, 593, 595, 596).

Accordingly, the inclusion of the tPA leader sequence has been shown to significantly increase the expression and secretion of proteins in vaccine constructs. This has been demonstrated for vaccines encoding immunogens for HIV, TB, influenza A (Flu A), anthrax, hepatitis C (hep C), dengue, malaria, Japanese encephalitis virus (JEV), bovine viral diarrhea virus (BVDV) and more (461, 593, 595-601). tPA has also been effective in secreting non-secretory proteins such as Flu A internal nucleoprotein (NP) and HIV-1 p24 (461, 594). Nucleoprotein is a core-conserved antigen of Flu A and has emerged as a prime target for Flu A vaccines due to the rarity of antigenic variation. DNA vaccine studies have shown NP to localize predominantly in the nucleus, thus reducing the immunogenicity of such vaccines. However, coupling tPA to the NP protein in a DNA vaccine localized the protein to the cytoplasm and yielded high protein expression in the cell lysate and supernatant when compared to the wild type NP vaccine (461). This was also true for p24, which is a non-secretory HIV-1 protein, but when fused to tPA, was directed to the secretory pathway and high levels of expression were detected in both the supernatant and cell lysate (594).

Due to the high level of antigen secretion, tPA efficiently evokes high antibody responses, as much as a 10 fold increase (598, 602-604). When comparing four different TB immunogens in DNA vaccines with and without tPA, it was noted that constructs with tPA were significantly more protective in mice aerogenically challenged with low doses of TB. This was due to the higher level of expression which in turn increased antigen uptake by antigen-presenting cells (APC) and led to an increased induction of responses, inducing higher titers of IgG antibodies (595). Furthermore, a comparison of a malaria DNA vaccine

with circumsporozoite protein (CSP) as an immunogen has also demonstrated that fusing tPA elicited 5 to 10 times more antibodies and reduced the rate of infection more effectively (600).

Although the use of tPA has primarily been focused on enhancing humoral immunity, several studies have shown it to be likewise effective in boosting cellular immunity (461, 593, 596). A flu DNA vaccine encoding the NP has illustrated fusing tPA enhances cytotoxic immunity by inducing about 2 fold increase in the frequency of CD8+ IFN-γ secreting cells (461). This was also noted for a BVDV and JEV DNA vaccines, whereby tPA enhanced cytolytic and helper T cells activity (593, 596). However, this has not been consistent and in some cases tPA has either had a negative effect or no immune enhancement at all (605, 606). One group comparing different dengue-2 envelop DNA vaccines found that inclusion of tPA did increase antigen expression, but that did not translate into better immunogenicity, as no notable difference was observed (607). Similarly the fusion of tPA to Env gp160 in a DNA vaccine failed to enhance the magnitude, breadth or protective efficacy of the response (608). Also a comparative study of a DNA malaria vaccine failed to show any enhancement on the humoral immune response when tPA was included (609). Therefore, whether or not tPA is beneficial for vaccine expression and immunogenicity remains controversial and as such the immune-enhancing effects of tPA should be tested for every antigen and the type of immune responses being targeted.

In this study, the effect of attaching tPA to HIVconsv on induction of T cells was assessed in BALB/c mice in a series of immunisation protocols employing the plasmid DNA, ChAdV63 and MVA vectors. The power of immunogenicity analysis was increased by a detailed mapping of the CD8+ T-cell epitopes across the HIVconsv immunogen, comprehensive functional analysis by intracellular cytokine staining and by measuring *ex-vivo* cytotoxicity to peptide-pulsed targets. The addition of the tPA leader sequence did not

augment the magnitude, functional diversity or cytotoxic potential of HIVconsv-specific T cells in DDD, DDDCM or CM immunization regimens. These results are important for informing future development of this vaccine strategy. This work was done in collaboration with Dr. Beatrice Ondondo.

## 5.2 Results

### 5.2.1 Characterisation of tPA leader signal sequence-fused HIVconsv vaccine (tHIVconsv)

The leader signal sequence of tPA (MDAMKRGLCCVLLLCGAVFVSAR) was coupled to the N-terminus of HIVconsv to generate tHIVconsv (Fig. 5.1A). Both HIVconsv and tHIVconsv immunogens were expressed from three vectors: a plasmid DNA (pSG2) (DNA.HIVconsv and DNA.tHIVconsv), ChAdV63 (ChAdV63.HIVconsv and ChAdV63.tHIVconsv) and MVA (MVA.HIVconsv and MVA.tHIVconsv). Since tPA was shown to enhance antigen expression and secretion, I first sought to determine whether coupling tPA to HIVconsv resulted in increased antigen expression levels in comparison to the unmodified HIVconsv vaccines. *In vitro* antigen expression levels of HIVconsv and tHIVconsv were evaluated using immunofluorescence by targeting the Pk tag located at the C-terminus of the HIVconsv proteins (Fig. 5.1B). HeLa cells were DNA-transfected and infected with ChAdV63 and MVA at MOI 10 and 5 respectively. Expression was evaluated 24 hours post transfection or infection using an anti-PK antibody FITC conjugated. Immunofluorescence staining revealed a similar cellular distribution pattern for tHIVconsv and HIVconsv proteins within the cytoplasm and no notable difference in expression level (Fig. 5.1B).

In flow cytometry analysis, the frequencies of DNA transfected HeLa cells (percentage of cells expressing Pk) were relatively higher for tHIVconsv compared to HIVconsv with a median of 5.7% and 2.3% respectively (Fig. 5.1D). This increase was also seen with ChAdV63 infection with a median of 56% and 18% for tHIVconsv and HIVconsv respectively, although both increases did not reach statistical significance (Mann-Whitney test) (Fig. 5.1D). As for the MVA vaccine, coupling tPA to HIVconsv did not affect antigen expression (Fig. 5.1D). Furthermore, measurements of median fluorescence intensities (MFI) were used to compare the level of antigen expression

between HIVconsv and tHIVconsv (Fig. 5.1D). Results indicate there were no significant differences between the levels of antigen expression of the two immunogens. Nonetheless, a slight trend for increased expression was observed for tHIVconsv in the DNA and ChAdV63 vaccines over HIVconsv. The later producing a notable increase in tHIVconsv expression compared to HIVconsv with a median of 1375 MFI and 921 MFI respectively (Fig. 5.1D). Taken together, these data suggest that coupling of the tPA leader sequence did not significantly alter the expression properties of HIVconsv delivered by DNA, ChAdV63 or MVA vectors.

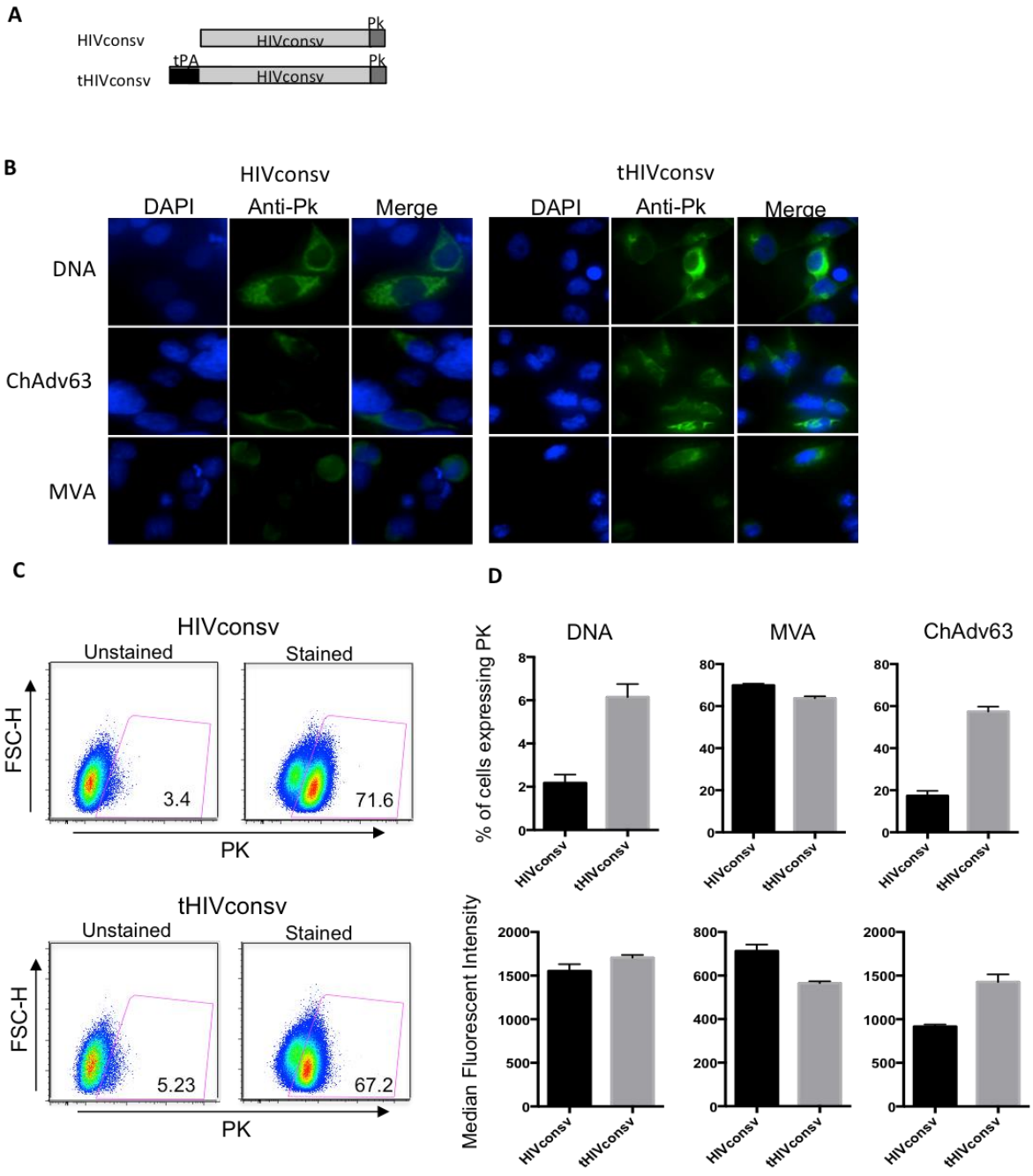



**Figure 5.1: Characterization of tHIVconsv expression from DNA, ChAdV63 and MVA vectors**

**(A)** Schematic diagrams of HIVconsv and tPA leader signal sequence-fused HIVconsv immunogens. **(B)** Immunofluorescence of HeLa cells either transfected with DNA plasmid pSG2, or infected with ChAdV63 or MVA expressing HIVconsv or tHIVconsv. Cells were stained with a FITC conjugated anti-Pk antibody targeting the C-terminal Pk-tag in the immunogens. Blue represents DAPI stain for the nucleus, while green represents the PK tag staining for HIVconsv. **(C and D)** Transfected or infected HeLa cells were stained and analysed by flow cytometry using the anti-Pk-tag antibody. **(C)** Representative FACS plots showing HIVconsv expression from MVA and gating strategy. **(D)** Percentages of cells expressing HIVconsv and tHIVconsv are shown as Pk+ cells (Top graphs) and the median fluorescence intensity (MFI) of the Pk expression is shown in the bottom graphs. Data are represented as mean + SEM (n = 3).

### 5.2.2 Effects of tPA leader sequence on induction of T cells by DNA immunization (DDD) regimen

I sought to determine whether the addition of the tPA leader sequence could influence induction of T cells and alter the magnitude of T-cell responses specific to the HIVconsv immunogen using the DNA.tHIVconsv vaccine alone. Two groups of BALB/c mice (n = 6) were immunised intra-muscularly (i.m.) with 3 doses of recombinant DNA (DDD regimen) 7 weeks apart with a 100 μg of DNA.HIVconsv or DNA.tHIVconsv and sacrificed two weeks later (Fig. 5.2A). Immune responses were then tested in an IFN-γ ELISPOT assay, stimulating splenocytes with six HIVconsv peptide pools (P1-P6) spanning the entire HIVconsv immunogen and the immunodominant peptide H (RGPGRAFVTI). 199 15-mer peptides overlapping by 11 amino acids (15/11) that were combined into 6 pools, each containing 32 to 35 individual peptides. There was no statistical significant difference between the total frequencies of the DNA.HIVconsv and DNA.tHIVconsv vaccine induced cells (Fig. 5.2B). Both immunogens induced a high frequency of IFN-γ producing cells in response to pool 6 (P6) containing peptide H, and lower frequencies of IFN-γ producing cells in responses to the rest of the peptide pools, indicating a lack of tPA enhancing effect (Fig. 5.2B). Moreover, no differences in IFN-γ responses were observed when the optimum 9-mer immunodominant peptide H epitope was used in the ELISPOT assay (Fig. 5.2C). In a separate independent experiment, two groups of BALB/c mice (n = 3) received a similar DDD immunisation schedule, their IFN-γ responses were tested against eight previously identified HIVconsv peptides, most notably immune-dominant peptides 42 and 112 (534). Analysis of the results showed no significant differences between tHIVconsv and HIVconsv, although there was a slight higher trend in favor of tHIVconsv with peptide 42, 112, 151 and peptide H (Fig. 5.2D).

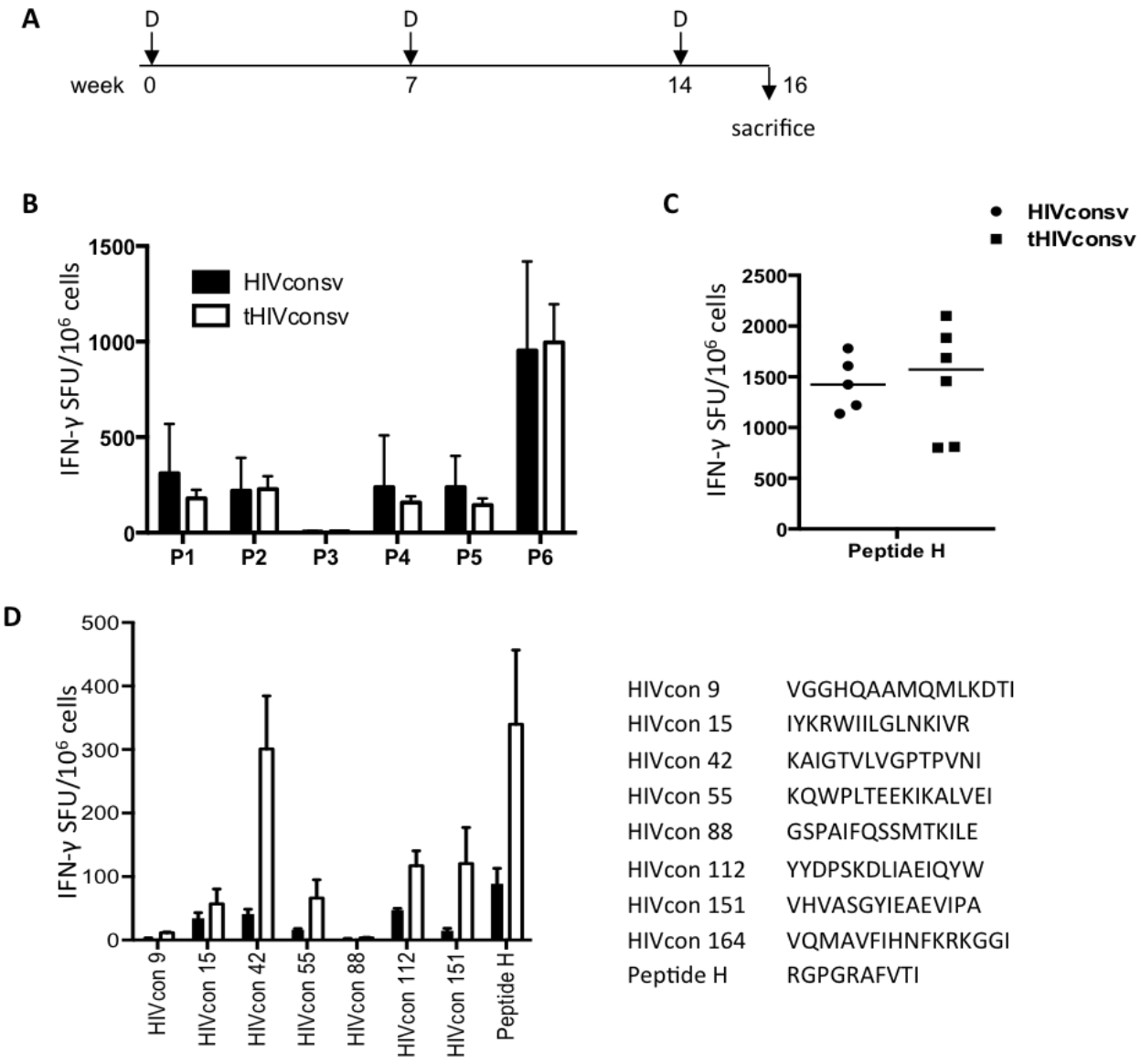



**Figure 5.2. Effect of tPA leader sequence in a DNA alone regimen**

**(A)** Immunization schedule for groups of BALB/c mice immunized intramuscularly with 100 µg of either DNA.HIVconsv or DNA.tHIVconsv and sacrificed 2 weeks after the last immunization (week 16). **(B and C)** Frequencies of responding splenocytes were tested by IFN-γ ELISPOT assay. **(B)** T cell responses to six peptide pools P1–P6 of HIVconsv. Results are shown as the mean + SEM of spots forming units (SFU) per million cells (n = 6). **(C)** Responses of the individual mice to the immune-dominant peptide H. Horizontal line depicts the median SFU per million. **(D)** Responses to 8 selected HIVconsv peptides were tested by IFN-γ ELISPOT. Data are shown as mean + SEM (n = 3). Amino acid sequences of the 8 peptides are as indicated.

### 5.2.3 Effect of tPA leader sequence on T-cell induction in a DNA-prime ChAdV63 and MVA viral vector-boost (DDDCM) regimen

In these sets of experiments, I determined the effects of tPA in a regimen using DNA as a prime of the immune response followed by two boost with the live viral vectors: ChAdV63 and MVA (i.e. DDDCM regimen; Fig. 5.3A) expressing HIVconsv or tHIVconsv. The DNA prime vaccines were given three weeks apart, followed by a ChAdv63 vaccine boost three weeks later and an MVA vaccine boost six weeks later before mice were sacrificed a week after (Fig. 5.3A). IFN-γ responses from two groups of BALB/c mice (n = 4) vaccinated with HIVconsv or tHIVconsv were assessed in an IFN-γ ELISPOT assay against the six HIVconsv peptide pools (Fig. 5.3B) and the nine individual peptides including peptide H (Fig. 5.3C). Similarly, no significant difference was observed in the IFN-γ responses to the six peptide pools, with a strong magnitude response against pools 2, 4 and 6, which contain the immunodominant peptides 42, 112, and H respectively (Fig. 5.3B). However, there was a trend of immunenhancement with tHIVconsv against pools 2, 4 and 6 but not significant (Fig. 5.3B). The results for the individual peptides showed a similar pattern, no significant difference between the two immunogens with a trend of enhanced immune response with tHIVconsv against peptide 112 and H (Fig. 5.3C).

Next I sought to evaluate the effects of tPA on the effector functions of HIVconsv-specific T cells employing an *ex-vivo* killing assay, whereby P185 target cells were pulsed with peptide to assess the killing efficacy of CD8+ T cells from mice vaccinated with HIVconsv and tHIVconsv. Target cells were pulsed with peptides 42, 112 and H separately and incubated with splenocytes from both groups of vaccinated mice at an effector to target ratio of 10:1 and 5:1. tHIVconsv induced a significantly higher specific killing of peptide H (RGPGRAFVTI) pulsed target cells ($p = 0.02$) (Fig. 5.3D). For peptides 42 (LVGPTPVNI) and 112 (YYDPSKDLI) pulsed target cells, no significant differences were observed in killing efficacy (Fig. 5.3D).

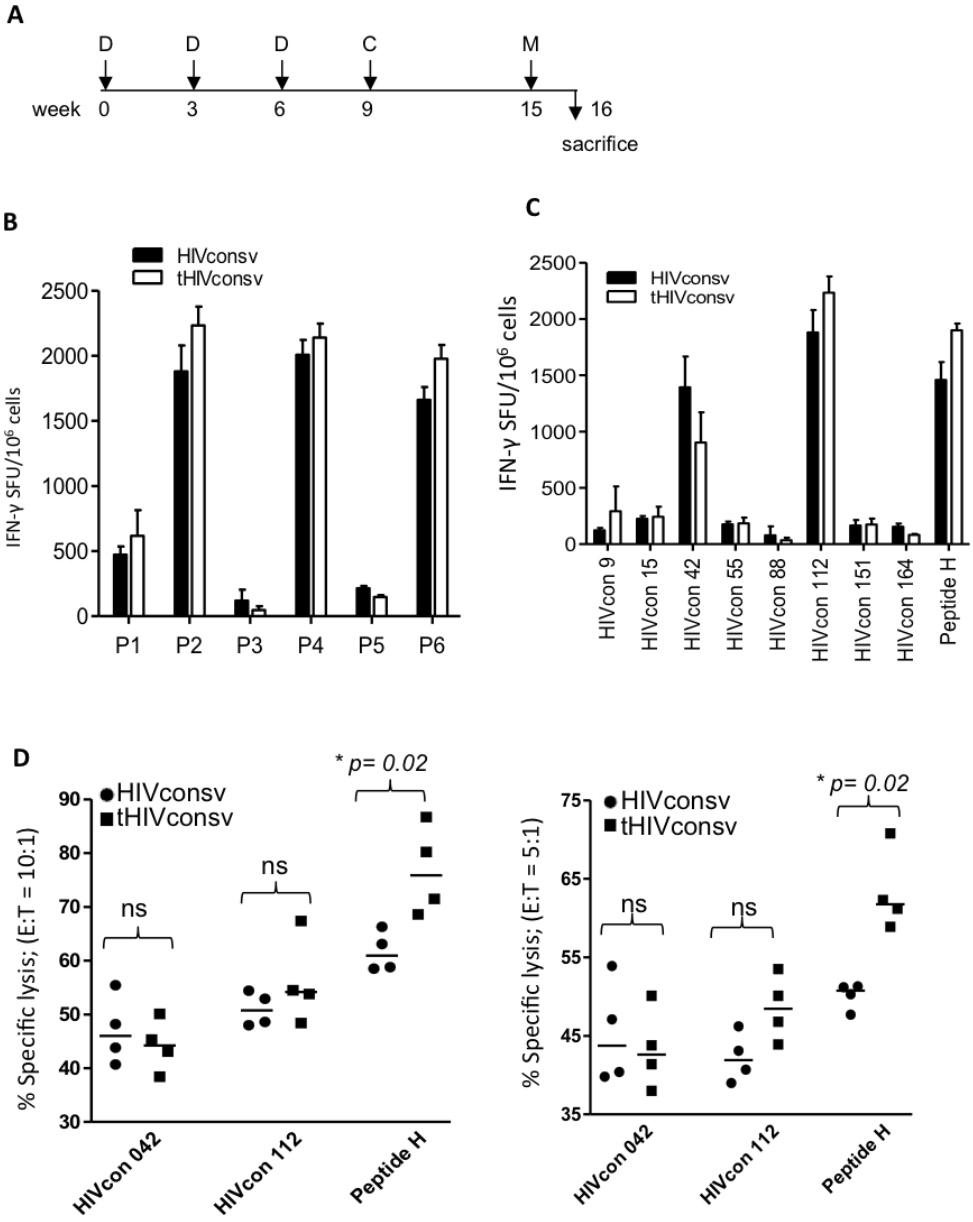



**Figure 5.3. Effect of tPA leader sequence in the DDDCM regimen**

**(A)** Immunization schedule of BALB/c mice. D represents 100 µg of either DNA.HIVconsv or DNA.tHIVconsv; C represents $10^8$ IU of ChAdV63.HIVconsv or ChAdV63.tHIVconsv; M represents $10^6$ pfu of MVA.HIVconsv or MVA.tHIVconsv. **(B and C)** Frequencies of responding splenocytes were tested by IFN-γ ELISPOT assay. Data is represented as mean + SEM of spots forming units (SFU) per million cells (n = 4). **(B)** T-cell responses against six HIVconsv peptide pools P1-P6 or **(C)** eight selected individual peptides, including two dominant peptides HIVCON 42, 112 and the immuno-dominant peptide H. **(D)** *Ex-vivo* killing of peptide-pulsed P815 target cells at an effector to target ratio of 10:1 (Left panel) or effector to target ratio of 5:1 (Right panel). Cells were pulsed with 3 peptides 42, 112 and H. Data are represented as percentage of specific killing with a horizontal line depicting the mean (n = 4). Statistically significant differences are marked with the asterisk (*) and the p values are as indicated.

### 5.2.4 Effect of tPA leader sequence on T-cell induction in a ChAdv63-prime MVA-boost (CM) regimen

Next I set to further assess the effects of tPA but using a different immunisation regimen, whereby ChAdV63 vaccine is used as prime followed by an MVA vaccine boost (i.e. CM regimen) 6 weeks later (Fig. 5.4A). To evaluate the effect of the tPA leader sequence on T cell responses, IFN-γ responses from two groups of BALB/c mice (n = 10) vaccinated with HIVconsv or tHIVconsv were assessed in an IFN-γ ELISPOT assay against the six HIVconsv peptide pool (Fig. 5.4B); and the eight individual peptides including peptide H (Fig. 5.4C). A larger number of animals (n = 10) were used to increase the power of detecting statistically relevant differences. A higher magnitude of HIVconsv-specific T-cell responses were induced against peptide pools 2, 4 and 6, exceeding a mean of 3000 spots forming unit (SFU) per million cells for peptide 6 (Fig. 5.4B). These responses are in agreement with the results achieved against the individual peptides whereby similarly high magnitude responses where recorded for peptides 42, 112 and H (Fig. 5.4C). There was no significant difference in the responses between the two immunogens, however a trend indicates tPA to decrease the magnitude of the response as HIVconsv immunogen induced the strongest of responses in both assays. An *ex-vivo* killing assay of peptide-pulsed P185 target cells revealed similar results to the ELISPOT (Fig. 5.4D). Cytotoxicity data indicate that killing efficacy decreased with tPA, whereby the killing of peptide 42 (LVGPTPVNI) and 112 (YYDPSKDLI) pulsed targets by splenocytes from tHIVconsv-immunized mice decreased: $p = 0.045$ for peptide 42 and $p = 0.02$ for peptide 112 (Fig. 5.4D).

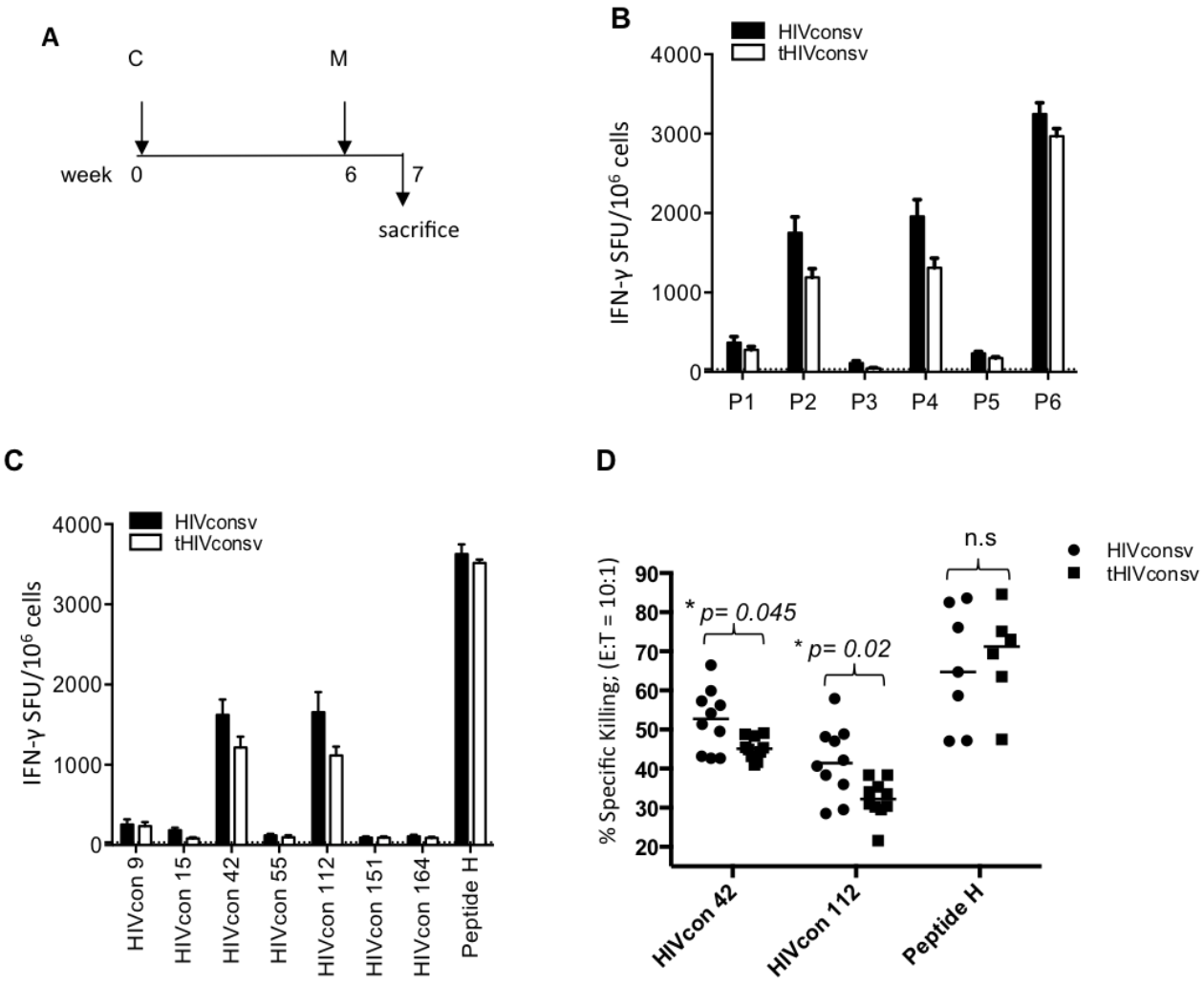



**Figure 5.4. tPA leader sequence effect in the CM regimen**

**(A)** Immunization schedule of BALB/c mice whereby, C represents $10^8$ IU of ChAdV63.HIVconsv or ChAdV63.tHIVconsv and M represents $10^6$ pfu MVA.HIVconsv or MVA.tHIVconsv. **(B and C)** Frequencies of responding splenocytes were tested by IFN-γ ELISPOT assay. Data is represented as mean + SEM of spots forming units (SFU) per million cells (n = 10). **(B)** T-cell responses against six HIVconsv peptide pools P1-P6 or **(C)** eight selected individual peptides, including two dominant peptides HIVCON 42, 112 and the immuno-dominant peptide H. **(D)** *Ex-vivo* killing of peptide-pulsed P815 target cells at an effector to target ratio of 10:1. Cells were pulsed with peptides 42, 112 and H. Data are represented as percentage of specific killing with a horizontal line depicting the mean (n = 10). Statistically significant differences are marked with the asterisk (*) and the p values are as indicated.

### 5.2.5 Effect of tPA leader sequence on breadth and epitope hierarchy of HIVconsv specific T cells

To determine whether tPA affected the breadth of vaccine-induced T cell responses or altered the hierarchy of epitope recognition, IFN-γ responses against the 199 individual 15-mer peptides, spanning the entire HIVconsv proteome, were assessed in an IFN-γ ELISPOT assay. Splenocytes from the two groups of BALB/c mice (n = 3) immunised with HIVconsv and tHIVconsv following the CM regimen (Fig. 5.4A) were pooled together for this screening (Fig. 5.5). Both immunogen induced T cells with broad specificities; however, there was no tPA signal sequence enhancement effect on the magnitude, breadth or epitope recognition patterns in this prime boost regimen (Fig. 5.5). Both immunogens induced strong T cell responses to the same epitopes, with the highest IFN-γ frequencies produced in responses to the previously defined immunodominant epitopes. There was no significant difference in the responses between the two immunogens, with a trend of higher responses with HIVconsv without tPA. However, tHIVconsv induced some low IFN-γ frequency responses, statistically not significant, to several peptides in pool 3 that were not observed with HIVconsv (Fig. 5.5). In a second independent experiment, splenocytes from BALB/c mice (n = 3) immunised with HIVconsv and tHIVconsv following the DDDCM regimen (Fig. 5.3A) were pooled together for screening against the 199 peptides (Fig. 5.6). Results observed in this experiment were very similar to that of the previous one, both immunogen inducing broad specific T cells, with the highest frequency responses noted against the 9 immunodominant peptides with no added benefit with tPA (Fig. 5.6). There was no significant difference between the responses, but a trend shows HIVconsv to induce higher frequency responses as seen in the CM regimen, however, responses in pool 1 were higher with tHIVconsv (Fig. 5.6).

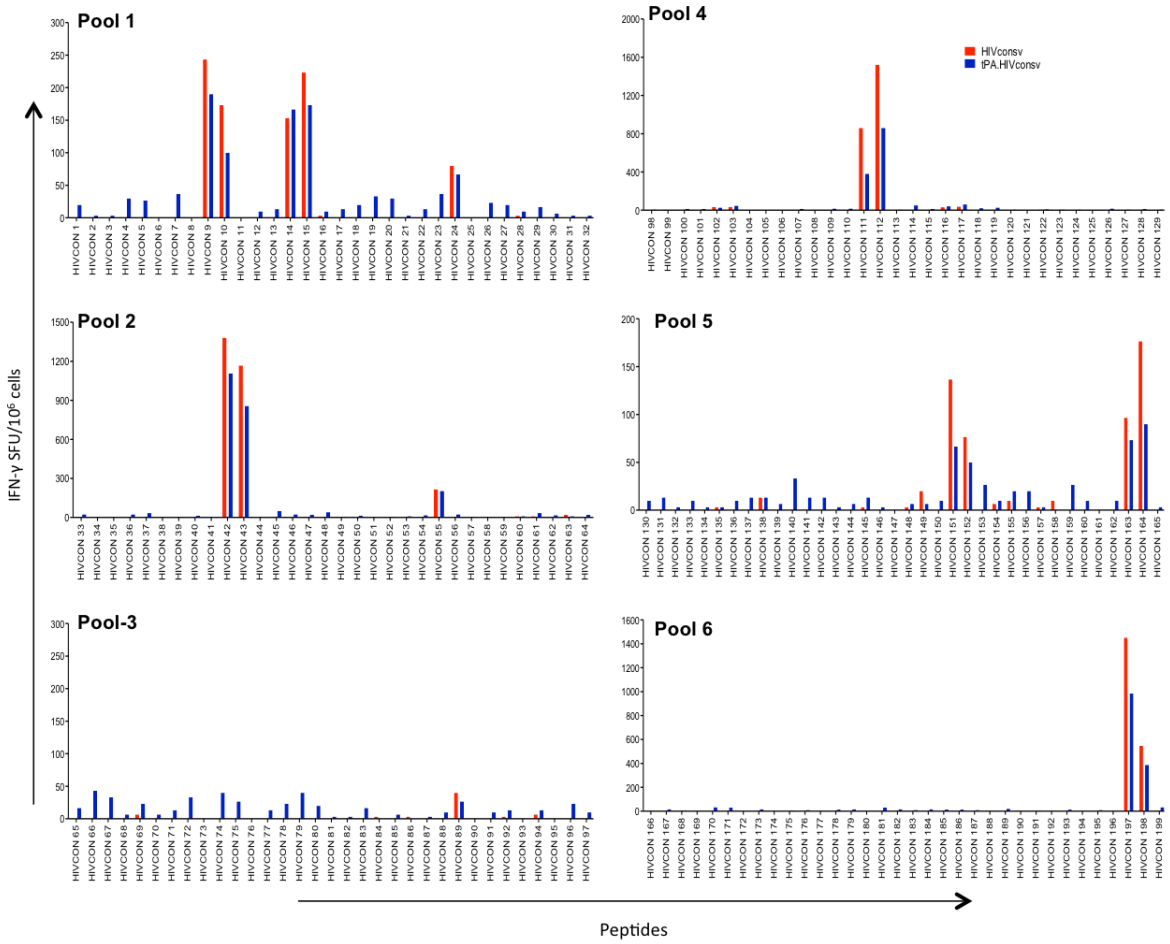



**Figure 5.5. Effect of tPA leader sequence on epitope recognition and breadth of T-cell responses using the CM regimen**

Mice (n = 3) were immunized with HIVconsv or tHIVconsv in the standard CM regimen where C represents $10^8$ IU of ChAdV63.HIVconsv or ChAdV63.tHIVconsv; followed 6 weeks later by M, representing $10^6$ pfu of MVA.HIVconsv or MVA.tHIVconsv. One week after the MVA immunization, T-cell responses from pooled splenocytes were tested in an IFN-γ ELISPOT assay against 199 15-mer peptides overlapping by 11 amino acids covering all HIVconsv peptides. Results are divided into six graphs (pools) of 32-35 peptides, with responses to each peptide indicated as spots forming unit (SFU) per million cells. Red bars represents HIVconsv; Blue bars represents tHIVconsv.

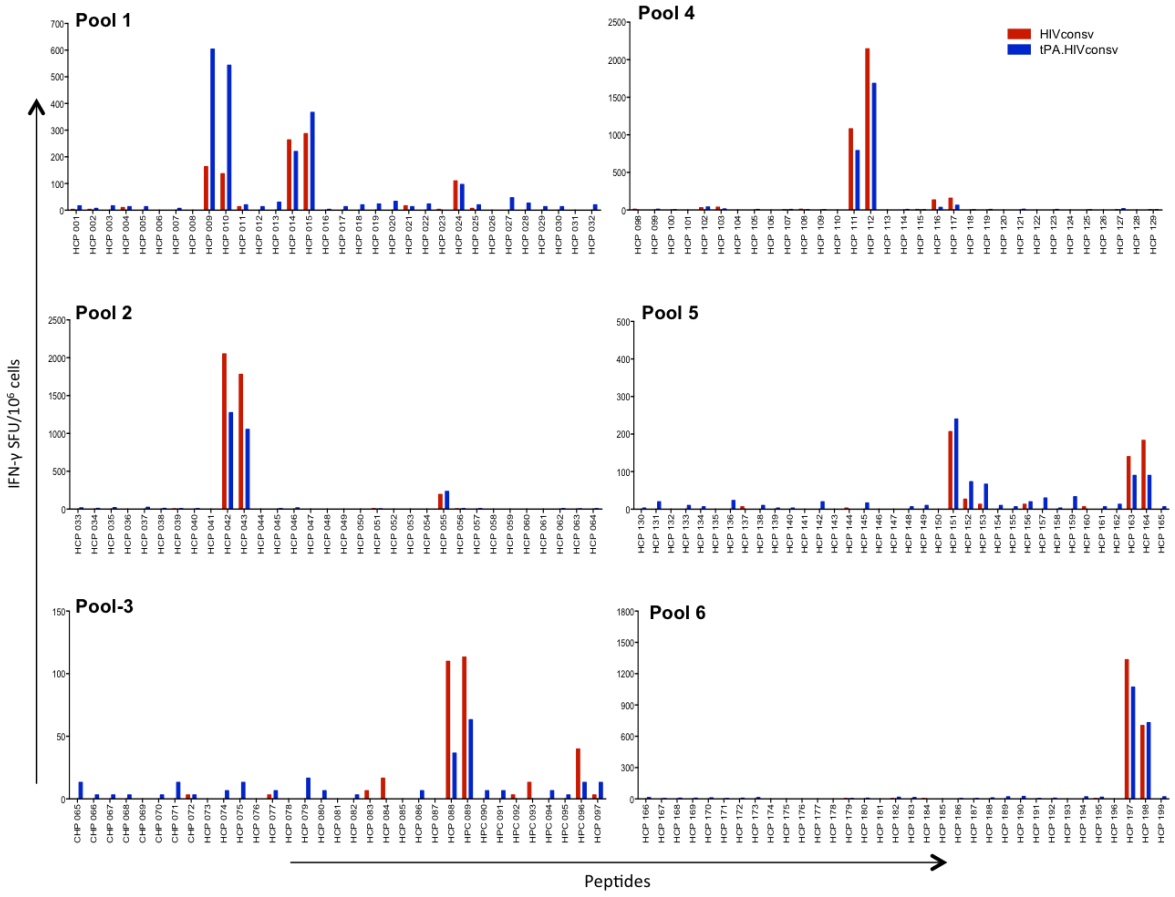



**Figure 5.6. Effect of tPA leader sequence on epitope recognition and breadth of T-cell responses using the DDDCM regimen**

Mice (n = 3) were immunized with HIVconsv or tHIVconsv in the standard DDDCM regimen where D represent 100 μg of DNA.HIVconsv or DNA.tHIVconsv, C represents $10^8$ IU of ChAdV63.HIVconsv or ChAdV63.tHIVconsv; representing $10^6$ pfu of MVA.HIVconsv or MVA.tHIVconsv. One week after the MVA immunization, T-cell responses from pooled splenocytes were tested in an IFN-γ ELISPOT assay against 199 15-mer peptides overlapping by 11 amino acids covering all HIVconsv peptides. Results are divided into six graphs (pools) of 32-35 peptides, with responses to each peptide indicated as spots forming unit (SFU) per million cells. Red bars represents HIVconsv; Blue bars represents tHIVconsv.

### 5.2.6 Effects of tPA leader sequence on multi-functional profiles of T-cells induced by a CM regimen

To further characterize the effects of tPA on the frequency and functional spectrum of vaccine-elicited T cells, frequencies of T cells expressing CD107a, IFN-γ, TNF-α and IL-2 were compared from splenocytes re-stimulated *ex-vivo* with peptides 42, 112 and peptide H (Fig. 5.7). Analysis was done using intracellular cytokine staining on two groups of BALB/c mice (n = 10) immunised with HIVconsv or tHIVconsv using the CM regimen (Fig. 5.7A and 5.7B). The predominant phenotype of T-cells from both HIVconsv and tHIVconsv vaccines are CD8+ T cells expressing CD107a and IFN-γ, with over 25% expression following peptide H stimulation and between 5-10% following peptides 42 and 112 stimulation (Fig. 5.7C). Both vaccines also induced a high frequency of CD8+ T cells secreting TNF-α, 12-15% for peptide H and 3-12% for peptides 42 and 112. However both failed to induce IL-2 secreting CD8+ T cells, 0.5 – 3% following peptide stimulation (Fig. 5.7C).

Finally, a multi-functional profile comparison of HIVconsv and tHIVconsv induced CD8+ T cells was produced using peptides 42, 112 and H (Fig. 5.8). The analysis revealed a higher percentage of peptide-H-specific CD8+ T cells co-expressed 2 or 3 functions (pie chart colors blue and purple, respectively), predominantly co-expressing CD107a, IFN-γ and TNF-α or CD107a, IFN-γ together (Fig. 5.8B). As for peptides 42 and 112 specific CD8+ T cells were mainly mono-functional (green in pie chart) mainly CD107a, or co-expressed three functions (blue in pie chart) CD107a, IFN-γ and TNF-α (Fig. 5.8B). tPA had no effect on the frequency of CD8+ T cells expressing 2 or 4 functions for all three peptides, however it did have an affect on cells expressing 1 or 3 functions. tPA was associated with significantly higher percentage of mono-functional CD8+ T cells expressing CD107a, especially for peptides 42 ($p = 0.02$) and 112 ($p = 0.002$). Likewise, a

significantly higher proportion of TNF-α+ mono-functional CD8+ T cells ($p = 0.027$) were observed for tHIVconsv following stimulation with peptide 112 (Fig. 5.8B).

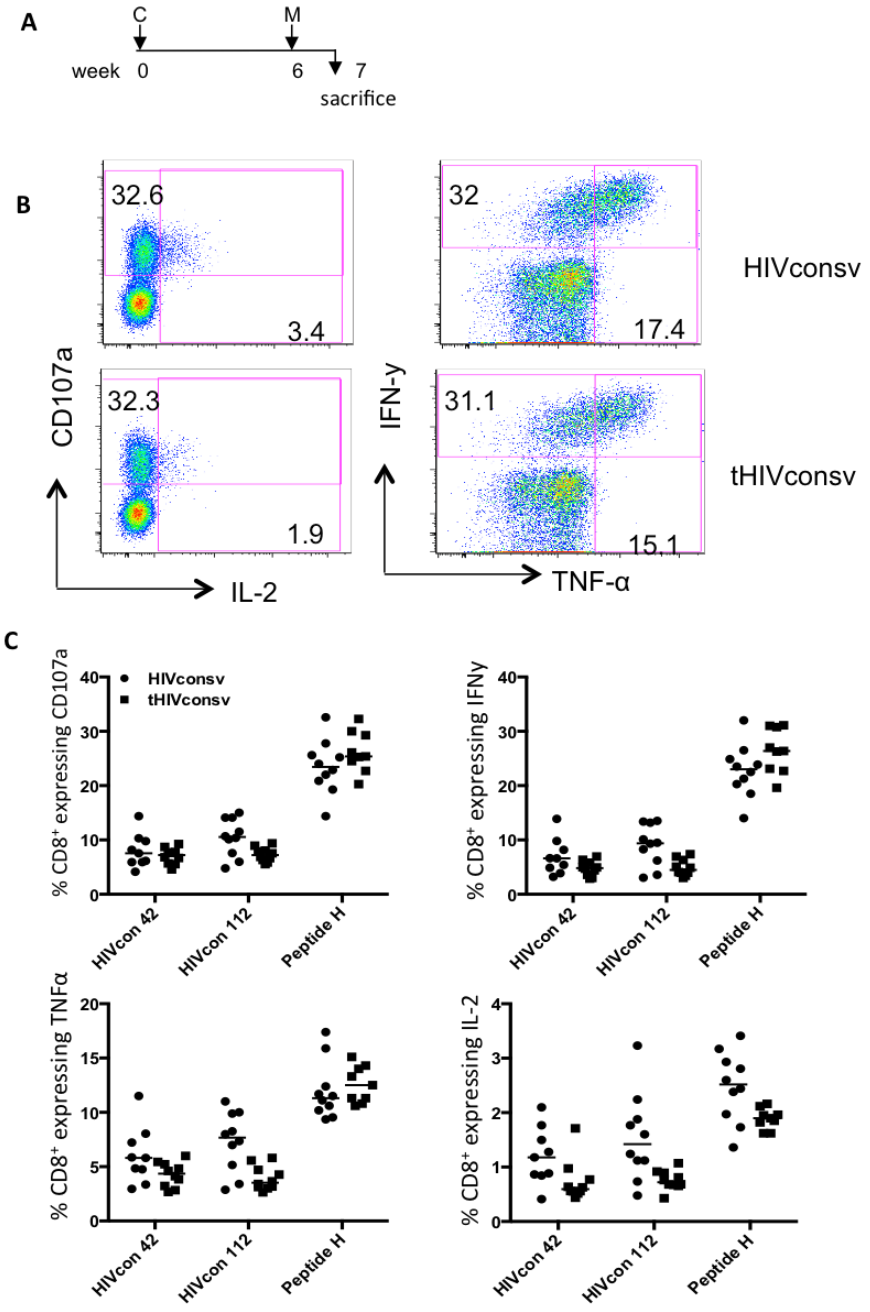



**Figure 5.7. Effect of tPA on cytokine secretion and degranulation in a CM delivery regimen**

**(A)** Immunization schedule: C is $10^8$ IU of ChAdV63.HIVconsv or ChAdV63.tHIVconsv; M is $10^6$ pfu of MVA.HIVconsv or MVA.tHIVconsv. **(B and C)** One week after MVA immunization, splenocytes (n = 10) were stimulated with peptides 42, 112 and peptide H and T-cell responses were measured via intracellular cytokine staining assay analysing the expression of CD107a, IFN-γ, TNF-α and IL-2. **(B)** Representative FACS plots: HIVconsv (Top plots); tHIVconsv (Bottom plots). Numbers in the FACS plots represent the frequencies of total CD8+ T cells expressing the specified cytokines or markers. Cells are gated on live CD8+ lymphocytes. **(C)**. Frequencies of CD8+ T cells expressing CD107a, IFN-γ, TNF-α or IL-2 amongst animals immunized with HIVconsv (circles) or tHIVconsv (squares). The horizontal line depicts the median.

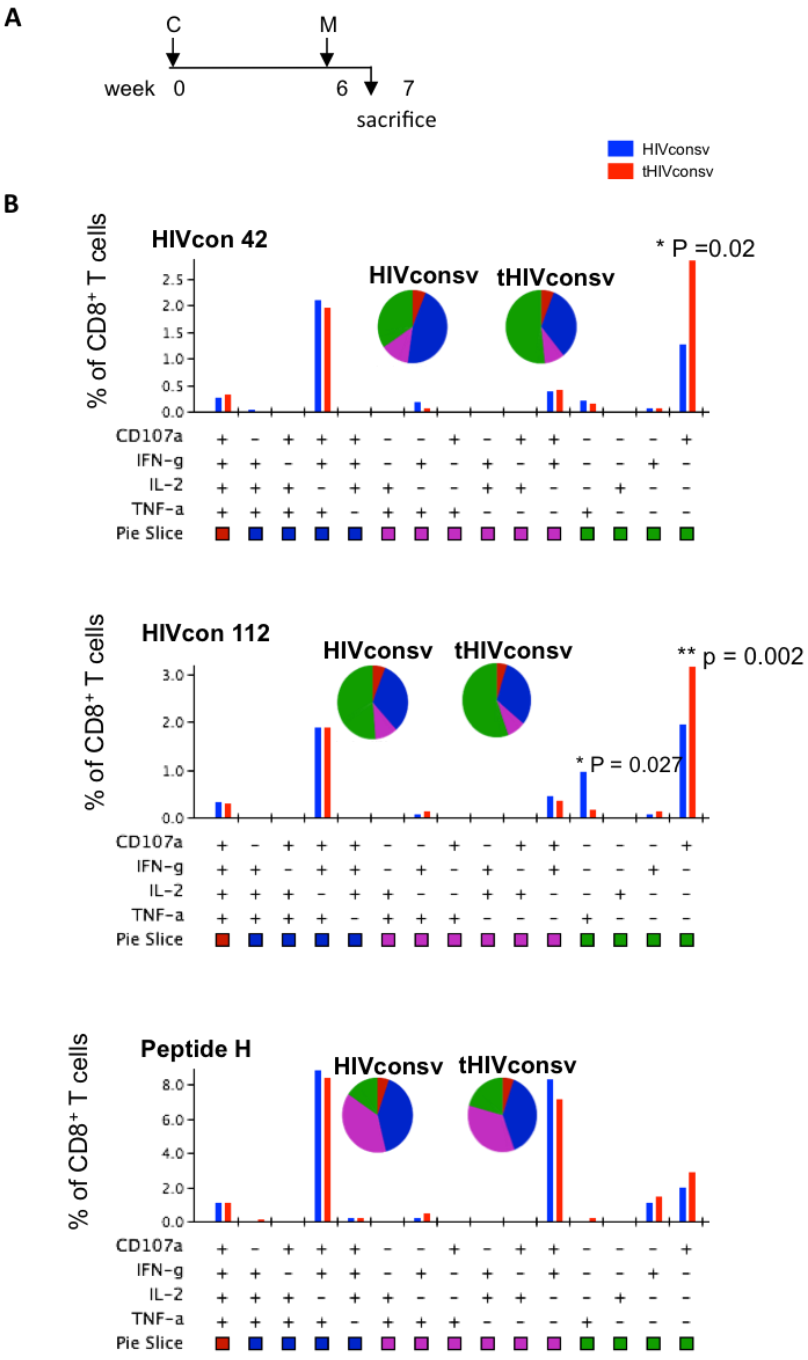



**Figure 5.8. Effect of tPA on the multi-functional profiles of CD8+ T cells in CM regimen**

**(A)** Immunization schedule: C is $10^8$ IU of ChAdV63.HIVconsv or ChAdV63.tHIVconsv; M is $10^6$ pfu of MVA.HIVconsv or MVA.tHIVconsv. One week post-MVA.HIVconsv, an intracellular cytokine staining assay was used to analyse expression of CD107a, IFN-γ, TNF-α and IL-2 following stimulation with peptides 42, 112 and peptide H**. (B)** Analysis of multi-functional profiles. Bar charts represent the frequencies of cells expressing various combinations of the specified functions. Blue represents HIVconsv while Red represents tHIVconsv. Data are represented as medians (n = 10). The pie charts depict the overall number of functions expressed: Green (mono-functional); Purple (any 2 functions); Blue (any 3 functions); Red (all the four functions). Statistically significant differences are marked with the asterisk (*) and the p values are as indicated.

## 5.3 Discussion

HIVconsv is a universal HIV-1 immunogen targeting the conserved region of HIV-1 currently in phase II human clinical trials. It is a T cell based immunogen delivered by three vectors, a plasmid DNA, ChAdV63 and MVA (395, 534). Recent studies reported on the high immunogenicity of HIVconsv, inducing very strong and broadly-directed, polyfunctional T cell responses in pre-clinical studies in BALB/c mice, non-human primate studies and in phase I/II human clinical trials (discussed above) (395, 396, 436, 531, 532, 534, 610). Nonetheless, efforts continue exploring other potential strategies to further improve the magnitude, breadth and functional quality of HIVconsv-induced T cells in order to maximise vaccine efficacy. One strategy of doing so is the inclusion of tPA into the vaccine. An increasing body of literature supports such strategy, as research into vaccines have shown tPA being prolific in enhancing immunogenicity and protective efficacy of antigens fused to tPA (593, 595). Majority of studies have shown an enhancement of immune response with the addition of tPA, mainly the humoral arm of the immune response, enhancing antibody titres and protective efficacy, however, few have also shown an improvement in cellular immunity (596, 598, 603, 611, 612). Therefore, tPA was a leading candidate to enhancing HIVconsv immunogenicity. The primary aim of this study was to explore whether tPA could enhance the immunogenicity and efficacy of HIVconsv vaccine. Thus, the effects of tPA on the magnitude, breadth and functional profiles of HIVconsv-specific T cells were tested for the immunogen delivered using recombinant DNA and/or non-replicating viral vectors (ChAdV63 and MVA).

Enhanced immunogenicity and protective efficacy induced by tPA was attributed to tPA's ability to increase the level of expression and secretion of the antigen fused to it (592, 594, 599, 613). Expression level analysis by flow cytometry revealed an increase in the expression of tHIVconsv in the plasmid DNA and ChAdV63 vectors (3 fold) but not in MVA. This is in accordance with others findings, specifically for the DNA plasmid (457,

592, 601, 602) as there are no studies presenting data on tPA expression from viral vectors. These results are the first to show a comparison of antigen expression from live viral vectors with and without tPA. Furthermore, intracellular localization of HIVconsv and tHIVconsv was examined, since tPA enhances expression and secretion by targeting the antigen to the ER obviating the need for antigen processing and translocation (461, 588, 593). Looking at expression of the antigens in HeLa cells via immunofluorescence microscopy, no notable differences were observed in the localisation of the antigen when fused to the tPA leader sequence.

In this study, experiments have been performed using different prime boost regimens with three vectors to examine the effects of tPA on the magnitude of the T cell response. IFN-γ ELISPOT and *ex-vivo* killing assays were the readouts used to determine the magnitude and efficacy of the responses elicited by HIVconsv and tHIVconsv in BALB/c mice. Responses were targeted against 6 pools of peptides covering the entire HIVconsv proteome, 8 individual peptides identified, with peptide 42 and 112 identified as immunodominant and peptide H, a known BALB/c immunodominant peptide (534).

In the regimens whereby the plasmid DNA vector was used, either alone (DDD) or as prime to the viral vectors (DDDCM) a non-significant increase in IFN-γ frequency against several peptides was observed from mice vaccinated with tHIVconsv. This was also noted in the killing of target cells pulsed with peptide H, whereby tPA enhanced the killing significantly. Since this enhancement in killing was not seen for targets pulsed with peptide 42 and 112, the strong response may be a result of strong stimulation from peptide H rather than tPA. The data from the CM regimen show a slight non-significant decrease in IFN-γ responses with tPA and a significant decrease in killing efficacy of targets pulsed with peptide 42 and 112, although the statistical power is low. Also when examining the effects of tPA on the breadth of the response, looking at responses induced against the 199 peptides spanning the entire HIVconsv proteome, no differences were noted with and

without tPA. Furthermore, looking at the cytokine profile to assess the functional quality of the vaccine induced T-cells revealed that tPA had no effect on the production levels of CD107a, IFN-γ, TNFα and IL-2, although it did alter the poly-functionality profile of the cells. These results suggest that fusing tPA to HIVconsv has no beneficial effect on the vaccine induced T cell responses.

Although these findings are contradictory to the studies cited above and in particular those which have shown enhancement of T cell responses in the BALB/c model, it is not quite as a few other studies also failed to see an advantage of tPA (605-608, 614). One such study, done by Wallace et al., 2013 compared four DNA vaccines with the Gag protein as the immunogen targeting T cell induction. They compared vaccines with and without tPA fused to the wild type Gag gene or a mutated Gag (mutation in the zinc finger) (605). As with my results, they have shown fusion of tPA resulted in an increased expression of the immunogen and secretion into the supernatant and cell lysate. This was accompanied by higher titres of Gag-specific IgG antibodies in mice immunised with the tPA-Gag DNA vaccine (605). However, when measuring Gag-specific T cell responses the opposite was true. They measured CD8+ T cell responses by IFN-γ ELISPOT, ICS and CTL assays targeting AMQMLKETI, a known Gag p24 epitope. Their results illustrate a significant decrease in IFN-γ production and cytolytic responses (605).

This is consistent with the HIVconsv results, whereby the increase in expression by tPA did not translate into cellular immune enhancement. It is noteworthy to mention that the magnitude of the responses induced by the native HIVconsv and Gag DNA vaccines were very high. Previous studies that show tPA to enhance cellular immunity had lower magnitude responses (612). Therefore its is possible that tPA is most effective in less immunogenic vaccines, whereby increasing antigen expression in turn enhances the immune response. Whereas in highly immunogenic vaccines, antigens are processed effectively thus a post-translational modification may have a negative or no effect on the

response, thus rendering tPA useless in such vaccines (605). Another study with an HIV-1 env gp160 DNA vaccine fused to tPA also failed to demonstrate any enhancement in the induction of env-specific cellular responses (608). This might suggest that in the context of HIV antigens, tPA may be more suited for augmentation of antibody rather than T cell responses.

Looking at the effects of tPA on the humoral immune response, many studies reported enhancement in antibody production conferring protection (592, 600, 603, 613), however a few other studies show tPA to be ineffective with regards to antibody production (606, 609). A study looking at neutralizing antibody responses to a DNA vaccine expressing dengue virus envelope antigens VecD2 and VRD2 reported that fusion of tPA failed to improve immunogenicity and antibody responses (607). The failure of tPA to enhance immunogenicity in these vaccines suggest that the effects of tPA on humoral or cellular immune induction are varied. It is likely that the effects of tPA depend on the type and characteristics of the antigen, the type of immune response and probably other uncharacterised confounding factors.

Vaccine immunogenicity and efficacy is to some extent governed by the quantity of expressed antigen, in addition to the quality of antigen presentation. The observation that there was increased expression of HIVconsv by the tPA constructs (DNA.tHIVconsv and ChAdV63.tHIVconsv), but no enhancement of immunogenicity may suggest inefficient antigen presentation despite abundant antigen availability. However, our observations are limited to measurement of *in vitro* antigen expression in HeLa cells, which might be totally distinct from the *in vivo* expression profiles following immunisation. Furthermore, the expression profiles of natural whole protein antigens may be distinct from those of chimeric gene fragments such as HIVconsv, as this is likely to depend on various properties of the antigen and in particular the native conformational structures. It is therefore possible that

what seems like increased antigen expression *in vitro* may not necessarily be equivalent to antigen bioavailability within the *in vivo* immune microenvironment.

In conclusion, this study provides definitive evidence that tPA-induced redirected trafficking of the HIVconsv vaccine is unlikely to enhance induction of cellular immunity or vaccine efficacy in both plasmid DNA and viral vector delivery regimens in BALB/c mice. Studies in human HLA transgenic (e.g. HLA-A02 (HHD), HLA-B27, BLA-B57) or humanized BLT mice will be instrumental in ascertaining any beneficial aspects of tPA to the HIVconsv vaccine in humans. Such studies will significantly inform the clinical development of HIVconsv vaccines expressing the tPA signal peptide.

# Chapter 6:

## *Discussion*

# 6. Discussion

In the present work, I describe the design, construction and pre-clinical testing of novel candidate T-cell vaccines against HIV-1. The vaccines described here target T cells to conserved regions of HIV-1, ensuring broader universal coverage and potent control of infection (572, 573). The mosaic concept was employed in the immunogen design, using 14 conserved regions of HIV-1 used in the HIVconsv vaccine, to increase the breadth and depth of T cell responses (439, 501, 505, 552). As set out earlier, the first aim was to asses whether using conserved mosaic vaccines would induce responses with greater breadth, depth and magnitude in comparison to the HIVconsv vaccine (395, 436). This was achieved by comparing vaccine induced responses from tHIVconsv, tHIVconsv plus tHIVcmo and a tri-valent mosaic cocktail of tConsv 1/2/3. The vaccines were vectored using a plasmid DNA, ChAdV63 and MVA to be used in different prime-boost regimens. Pre-clinical studies were performed in HHD transgenic mice, to safeguard the data on the breadth and depth were relevant to humans. As expected, the tri-valent mosaic vaccine cocktail induced potent $CD8^+$ T cells with greater depth in comparison to the other two vaccines. Furthermore, the magnitude of responses and killing efficacy were significantly higher from mice vaccinated with the tri-valent mosaic cocktail. The second part of my project was to asses the effects of the leader sequence tPA, whether or not adding it to the immunogens would be beneficial. A thorough comparison of immunogen expression, magnitude, breadth, depth and poly-functionality of $CD8^+$ T cell was done on responses induced by vaccines with and without tPA. Results show that tPA had neither a positive nor a negative effect on the vaccine specific induced responses.

The failures of the STEP/Phambili and HVTN 505 trials had cast some doubt on HIV-1 T cell based vaccines. However, their failures are due to flaws in the vaccine designs rather than the concept itself of targeting T cell for a HIV-1 vaccine. Follow up analysis on

cohorts from the STEP and Phambili trials have confirmed that vaccination induced enhancement of HIV-1 acquisition in male cohorts (428-430, 615, 616). Most infections occurred in uncircumcised / Ad5 seropositive vaccinees compared to the placebo, more so in the first 18 month of follow up, as risk of infection did decrease with time (428, 430, 615, 616). According to later follow ups and analysis, there was no statistically significant increase in infection among vaccinees. Nonetheless, early data suggested a link between pre-existing Ad5 immunity and increased susceptibility to infection, although was not proven. It was hypothesized the increase in the rate of infection amongst the Ad5 seropositive participants was due to the expansion and higher levels of Ad5 specific $CD4^+$ T cell, targets for HIV-1 (428, 430, 615, 616). However, two human clinical trials have rebutted this hypothesis, illustrating that Ad5 specific $CD4^+$ T-cells were unlikely to have caused the increase in susceptibility to infection (617, 618). They revealed the presence of Ad5 nAb was not predictive of Ad5 specific $CD4^+$ T cell responses as there was no correlation between the two. Also, Ad5 specific $CD4^+$ T cells expanded post immunisation in both, Ad5 seropositive and seronegative individuals, the later becoming seropositive after a single immunisation (617, 618). Furthermore, the HVTN 505 trial, targeting the induction of T cells and antibodies, also used Ad5 as a vector for the boost vaccine and was also terminated prematurely (432). However, the HVTN 505 trial was terminated due to lack of efficacy rather than vaccine induced enhancement of infection, and was conducted on circumcised / Ad5 seronegative individuals (432). This further casts doubt to Ad5 preexisting immunity being the cause of failure in the STEP and Phambili trials, as the lack of efficacy in HVTN 505 was in Ad5 seronegative participants. Nevertheless, pre-existing immunity to Ad5 would decrease the efficacy of the vaccine, as responses would target the vector itself and limit uptake of the insert and consequently reducing the induction of HIV-1 specific T cell responses.

The second flaw in the MRKAd-5 vaccine design is the immunogen, most likely cause of the trials efficacy failure. The immunogens were clade B Gag, Pol and Nef natural isolates. Immunological analysis of vaccinated individuals revealed HIV-1 specific responses were induced against two epitopes on average and unable to recognise the transmitting viral strain due to the inadequate breadth of HIV-1 specific $CD8^+$ T cell responses (430). Although the vaccine was immunogenic, inducing HIV-1 specific $CD8^+$ T cells that were capable of producing multiple cytokines, the specificity of the response was more towards variable epitopes (619). A comprehensive analysis of T cell epitope responses revealed a bias towards variable epitopes, as the vaccine elicited responses tend to target variable epitopes in Gag, Pol and Nef and very few highly or even less conserved epitopes (619). Furthermore, vaccine induced responses tended to cluster into immunodominant epitope hotspots, a region containing several epitopes presented by different HLA that are immunodominant and are targeted more frequently (620). Epitope mapping data revealed that hotspots targeted by the vaccine were different than ones targeted in chronic infection. Also analysis of the HLA targeting efficiency score, defined by Spearman correlation between scores of binding and amino acid conservation in a protein, revealed the relationship between the targeted hotspots and evolutionary conservations. A positive efficiency score was recorded for natural infection epitopes of Gag and Nef, indicating a binding preference to conserved sites; while the vaccine epitope maps produced a negative efficiency score, indicating a preference to binding variable site (620). The vaccine targeted immunodominant hotspots in Gag and Nef contained very highly variable sites (620). Targeting of variable sites, increases the chance of viral escape and decreases the breadth and depth of the response, and the recognition and targeting of the founder transmitted virus to induce potent viral control (272).

These problems and design flaws have been addressed in the design of our conserved mosaic vaccines, ensuring the vaccine specific responses induced are of high

frequency, magnitude, breadth and depth. To circumvent the problems observed with the Ad5 vector, a chimp adenovirus was used as a vector, guaranteeing non or little anti-vector immunity existing that might hinder the vaccine efficacy. Furthermore, the conserved mosaic vaccines are delivered in a heterologous prime boost regimen, utilizing three vectors (plasmid DNA, ChAdV and MVA), so as not to use the same vector repeatedly and induce anti-vector immunity and therefore safeguarding sufficient antigen uptake. For further development, it would be interesting to develop a conserved vaccine using CMV as a vector, which has produced some very interesting results in pre-clinical testing in non human primates. As for the immunogen, by using conserved regions only, we minimize the induction of responses to immunodominant variable epitopes but rather induce beneficial responses known to control HIV-1 and exert mutational pressure with a fitness cost. Moreover, utilizing the mosaic technology ensures broad T cell responses with greater breadth and depth in the response, increasing the likelihood of recognising the founder transmitted virus and escape mutants thus better control of HIV-1 replication.

The way forward from this point is to test conserved mosaic vaccines in humans. Based on the findings presented here, we have developed a new set of 2nd generation conserved mosaic vaccines to be tested in human clinical trials in the near future. With new advancements made in the field of HIV-1 in the past four years, a new set of mosaic immunogens have been designed, however using slightly different conserved sites. Whereas the first generation was based on 14 conserved regions across Gag, Pol, Vif and Env, the 2nd generation mosaics consist of 6 conserved regions across Gag and Pol only. Although Env protein did contain conserved areas, recent findings suggest that it contained no beneficial epitopes and induced dominant T-cell responses that were not associated with viral control (437, 621). Furthermore, beneficial $CD8^+$ T cell epitopes have been selected for in the immunogens design, based on data from more than 1,000 HIV-1 clade B and C infected individuals (621). The most commonly targeted areas of the HIV-1 proteome in

individuals with low and high viral loads were identified. Gag and Pol were identified as the proteins most associated with beneficial responses. Also, sequences with lower entropy i.e. conserved, were associated with protective responses, even within Gag or Pol (621). These beneficial epitopes were used in the design of HIVACAT T-cell immunogen (HTI), which was shown to induce a broad and balanced $CD8^+$ T cell response (622). Our final product were two mosaic immunogen sequences (bivalent cocktail) expressed in a plasmid DNA, chimp adenovirus Y25 (ChAdOx1) and MVA vectors (tHIVconsvX) (509). The six regions of the two mosaics were arranged differently, so no single junction in present more than once in the vaccine cocktail, minimising responses to unnatural juntional epitopes.

The coverage of perfectly matched or singly mismatched amino acid epitopes has significantly improved in tHIVconsvX immunogens. Furthermore, 33 protective epitopes (associated with low viral load) and only 3 non-protective epitopes are incorporated in the new mosaics regions in comparison to 15 and 25 protective and non-protective epitopes respectively in the older vaccines (621). These protective epitopes are based on ones identified in the study discussed above, from individuals in Spain, Peru and South Africa. Additionally, 10 epitopes that were recently identified as correlates of strong viral control in HIV-1 infected Japanese individuals are also found in tHIVconsvX (573). Murakoshi *et al.* have examined $CD8^+$ T cell responses in 400 Japanese individuals infected with HIV-1 clade B, and have identified 13 protective epitopes, 9 of which have never been reported previously, inducing high specific $CD8^+$ T cell responses controlling HIV-1 replication. More importantly, these epitopes were not restricted to a single HLA, they were recognised by 5 different HLA haplotypes (573). Pre-clinical testing in mice showed that the 2nd generation vaccines induced responses with similar frequencies to those responses induced by the 1st generation vaccines. However, these new vaccines benefit from the advantages of the new mosaic design, and therefore, have the potential to induce broad and more specific, potent $CD8^+$ T cell responses against conserved regions found globally.

The second generation conserved mosaic vaccines are very promising and deserve timely clinical evaluation. Only human data will provide the ultimate information on the protective potential of this HIV-1 vaccine approach.

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