Identification of Immunological Targets for HIV-1 Vaccine and Cure Strategies

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Thesis submitted in partial fulfilment of the requirement for the degree of Doctor of Philosophy

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Abstract

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HIV-1 chronically infected individuals represent a large disease burden, making the development of a therapeutic vaccine for use in chronic infection a priority. However, therapeutic vaccination has not been successful to date. Most approaches have employed viral vectored vaccines encoding full length viral proteins, which aimed to boost pre-existing CD8+ T cell responses by mimicking natural HIV-1 infection. Simply boosting these pre-existing CD8+ T cell responses which have previously failed to control the virus may be insufficient. Although HIV-1 has a huge capacity to diversify, certain regions are less tolerant of mutations due to structural and functional constraints. We therefore hypothesised that it would be necessary to redirect the immune response to more vulnerable regions of the proteome, such as conserved regions.

HIVconsv is a rationally designed conserved region vaccine. Vaccination of 19 chronically HIV-1 infected HAART treated patients with MVA.HIVconsv was safe and well tolerated. There was a significant increase in the magnitude of HIVconsv-specific T cell response following vaccination ($p = 0.001$), as measured by IFN-ɣ Elispot assay, but changes observed in vaccinees did not reach statistical significance when compared with placebo recipients ($p = 0.48$).

The capacity of CD8+ T cells to inhibit HIV-1 replication *in vitro* is highly predictive of virus control *in vivo* and is thus a possible surrogate of vaccine efficacy. There was a trend towards increased CD8+ T cell mediated inhibition following vaccination with $2x10^8$pfu MVA.HIVconsv (17% inhibition pre- vs 54% inhibition post-vaccination, $p = 0.06$). However, measurement of the latent HIV-1 reservoir by quantification of total HIV-1 DNA in circulating CD4+ T cells by droplet digital PCR showed no reduction in size upon vaccination, indicating CD8+ T cells induced by vaccination with MVA.HIVconsv were not of sufficient potency to impact the reservoir.

In a second cohort of HIV-1 infected individuals, antiviral inhibitory activity was measured in 36 HIV-1-infected STEP and Phambili trial participants. Sustained potent CD8+ T cell antiviral inhibitory responses were rare but were strongly correlated with IFN-γ responses to so-called 'beneficial' low entropy regions in HIV-1 Gag and Pol, that had been reported previously to be associated with HIV-1 control, ($r = 0.69$, $p = 0.0001$). This correlation was still significant after controlling for protective HLA alleles, whereas responses to conserved elements were only weakly correlated with viral inhibition ($r = 0.41$, $p = 0.04$).

These data indicate that immunogens that are based on the selection of regions within the viral proteome by conservation score alone may not induce the most effective HIV-1-specific T cell responses and they highlight the importance of systematically selecting specific regions associated with HIV-1 control, together with exclusion of immunodominant decoy epitopes.

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List of Abbreviations

Aa	Amino acid
Ab	antibody
Ad5	Adenovirus 5
ADCC	Antibody dependent cellular cytotoxicity
AIDS	Acquired immune deficiency syndrome
ANOVA	Analysis of variance
APC	Antigen presenting cell
APC	Allophycocyanin
APOBEC	Apolipoprotein B mRNA-editing enzyme-catalytic polypeptide like
BFA	Brefeldin A
BNAb	Broadly neutralising enzyme
BSA	Bovine serum albumin
CCR5	Cysteine-cysteine linked chemokine receptor 5
CD	Cluster of differentiation
CDR	Complementarity determining region
CE	Conserved elements
CFSE	Carboxyfluorescein succinimidyl ester
CRF	Circulating recombinant form
CMV	Cytomegalovirus
CPE	Cytopathic effects
CTL	Cytotoxic T lymphocyte
CTLA-4	Cytotoxic T lymphocyte-associated antigen 4
CXCR4	Cysteine-x-cysteine linked chemokines receptor 4
D	Diversity chain
DC	Dendritic cell
DC-SIGN	DC-specific intracellular adhesion molecule-3-grabbing non-integrin
ddPCR	Droplet digital polymerase chain reaction
HDAC	Histone deacetylase inhibitor
DMSO	Dimethyl sulfoxide
DN	Double negative
DNA	Deoxyribonucleic acid
DP	Double positive
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme linked immunoassay
ELISPOT	Enzyme linked immunospot assay
ER	Endoplasmic reticulum
ESCRT	Endosomal sorting complex required for transport
FACS	Fluorescence-activated cell sorting
FEC	Influenza virus, Epstein Bar Virus, Cytomegalovirus optimal epitopes
FCS	Fetal calf serum
FITC	Fluorescein isothiocyanate, green
FoxP3	Forkhead box p3
Gag	Group antigen
GALT	Gut associated lymphoid tissue
Gp	Glycoprotein
HAART	Highly active antiretroviral therapy
HIV-1	Human immunodeficiency syndrome type 1
HIV-2	Human immunodeficiency syndrome type 2

HLA	Human leukocyte antigen
HRP	Horse radish peroxidase
HVTN	HIV Vaccine Trials Network
ICS	Intracellular cytokine staining
IDU	Intravenous drug user
IFN	Interferon
Ig	Immunoglobulin
Li	Invariant chain
IκB	Inhibitor of kappa B
IL	Interleukin
ITAM	Immunoreceptor tyrosine-based activation motif
IUPM	Infectious units per million
J	Joining chain
KIR	Killer cell immunoglobulin like receptor
LANL	Los Alamos National Laboratory
LCMV	Lymphocytic Choriomeningitis Virus
LPS	Lipopolysaccharide
LTNP	Long term non-progressor
LTR	Long terminal repeat
mAb	Monoclonal antibody
MACS	Magnetic-activated cell sorting
MDDC	Monocyte derived dendritic cell
MFI	Mean fluorescence intensity
MHC	Major histocompatibility complex
MIP-1α	Macrophage inflammatory protein-alpha
MIP-1β	Macrophage inflammatory protein-beta
µl	Microlitre
ml	Milliletre
mRNA	Messenger ribonucleic acid
MVA	Modified vaccinia Ankara
MOI	Multiplicity of infection
Nab	Neutralising antibody
Nef	Negative regulatory factor
NF-κB	Nuclear factor kappa B
NHP	Non-human primate
NIAID	National institute for allergy and infectious diseases
NIH	National institutes for health
NK	Natural killer
NKT	Natural killer T cell
NP-40	Nonidet P-40
OLP	Overlapping peptides
PAMP	Pathogen associated molecular pattern
PBMC	Peripheral blood mononuclear cells
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PD-1	Programmed death
PE	Phycoerythrin
PerCP	Peridinin chlorophyll
PFA	Paraformaldehyde

PFU	Plaque forming unit
PHA	Phaseolus vulgaris (red kidney bean) Phytohemagglutinin
PHI	Primary HIV infection
PI	Phosphatidylinositol
PKC	Protein kinase C
pMHC	Peptide-major histocompatibility complex
RANTES	Regulated upon activation of normal T cells, expressed and secreted
RNA	Ribonucleic acid
RT	Reverse transcriptase
RT-PCR	Real-time polymerase chain reaction
SD	Standard deviation
SEB	Staphylococcus enterotoxin B
SFU	Spot forming unit
SIV	Simian immunodeficiency virus
STCL	Short term cell line
STI	Sexually transmitted infection
STI	Structured treatment interruption
TAP	Transported associated with antigen processing
Tat	Transactivating protein
TCID	Tissue culture infectivity dose
TCM	Central memory T cell
TCR	T cell receptor
TEM	Effector memory T cell
TFH	T follicular helper cell
Th	T helper cell
TGF-β	Transforming growth factor beta
TLR	Toll like receptor
TNF-α	Tumour necrosis factor alpha
Treg	Regulatory T cell
TRIM5α	Tripartite motif-containing protein 5
TTM	Transitional memory T cell
URF	Unique recombinant forms
V	Variable Chain
Vif	Virus infectivity factor
VL	Viral load
VOA	Viral outgrowth assay
Vpu	Viral protein U

1. Introduction

Acquired Immune Deficiency Syndrome (AIDS) was first described in a paper by Gottlieb and colleagues in June 1981[1]. They reported cases of previously healthy homosexual men who succumbed to unusual opportunistic infections and rare malignancies. Human Immunodeficiency Virus Type 1 (HIV-1) was described as the causative agent in 1983[2] following which 60 million people have become infected with HIV-1, 25 million of whom have since died[3]. Although the number of new infections has seen a steady decline over recent years, the number of people living with HIV-1 has increased. An estimated 35.3 million people, predominately in sub-Saharan Africa, were living with HIV-1 in 2012 compared to 29 million in 2001, an increase of 17% in 11 years[3]. This is in part due the introduction of Highly Active Antiretroviral therapy (HAART), which has turned HIV-1 from a fatal infection into a chronic manageable condition.

With the increase in HIV-1 patients' life expectancy, challenges with long term treatment emerge. Because of the existence of reservoirs of latently infected CD4+ T cells which are established early after infection[4,5], cessation of therapy results in plasma viraemia rebound to pre-treatment levels. Therefore, to control the infection treatment must be lifelong and maybe associated with long-term toxicity. Cardiovascular[6] (reviewed in[7]) and metabolic diseases (reviewed in [8]) have been associated with antiretroviral therapy. Failure to adhere to strict drug regimens can also result in drug resistance, with ensuing treatment failure, and also the risk of transmission of drug-resistant viruses. Access to therapy is not universal: even though sub-Saharan Africa, for example, has been greatly affected by the epidemic, not everyone needing therapy is

receiving it. This is due not only to the high cost but also the instability of governments, health care systems, transport problems and competing health priorities such as clean water and adequate nutrition[9].

1.1 Origin of HIV-1

There are two types of HIV that cause disease in humans, HIV-1 and HIV-2. HIV-1 is responsible for the majority of the pandemic and this introduction is limited to discussions about HIV-1 unless otherwise stated. HIV-1 is comprised of 4 distinct lineages, M, N, O and the newly discovered P. Each lineage arose from independent cross-species transmissions of simian immunodeficiency virus (SIV) from non-human primates into humans in West and Central Africa, likely through bushmeat hunting[10,11]. Group M is the most prevalent, caused by the lentivirus SIVcpz, it is the oldest HIV-1 lineage in humans with cross-species transmission being estimated to have occurred between 1850-1900[12,13]. It is divided into 9 subtypes (Clades A-D, F-H, J and K), which differ by 25-35% in the sequence of the env gene and 15% in the gag gene[14]. Clade B is the predominant circulating strain in the Americas, Europe and Australia, Clade C is most common in Southern Africa and A/G and B/C recombinant strains are present at high frequency in West Africa and China respectively. The other three lineages account for only a small percentage of infections and have not spread far beyond their country of origin[13,15].

Infection of a single cell with two different viruses can result in a recombinant virus. This can occur between viruses from different lineages (e.g. M and O), but also between

subtypes within a single lineage. Two types of recombinant virus exist, circulating recombinant forms (CRFs) are those that are identified in three or more epidemiologically unlinked individuals and unique recombinant forms (URFs) are recombinants identified in a single individual or in linked individuals[16]. 30 CRFs have been identified to date[14,17] and the number is increasing as a result of new recombinations occurring in 'recombination hotspots' where several subtypes and CRFs co-circulate. This is a major driving force in the generation of diversity in the HIV-1 pandemic.

1.2 Structure of HIV-1

HIV-1 is a lentivirus from the retroviridae family, composed of two identical copies of single-stranded RNA molecules enclosed by a capsid. As is typical of all retroviruses, HIV-1 encodes for three structural genes; gag pol and env, but also encodes a further 6 regulatory/accessory proteins; tat, rev, nef, vif, vpu and vpr unique to HIV-1 which are involved in modulating virus replication.

Gag (group specific antigen)

The gag gene encodes the Gag polyprotein precursor Pr55Gag. The polyprotein can mediate all essential events in virion assembly, however, cleavage of Pr55 by HIV-1 protease induces structural changes which are essential for the generation of infectious virus particles[18]. The cleavage products are;

Matrix (MA or p17)

In the polyprotein, MA targets Gag to the plasma membrane and aids in virus assembly. It has also been implicated in recruitment and incorporation of Env into virions during virus budding. In the maturing virion, MA coats the inner leaflet of the viral membrane and on infection of a new cell it facilitates transport of the viral genome to the nucleus.

Capsid (CA or p24)

CA mediates protein-protein interactions required for virus assembly. In the maturing virion CA creates a conical shell around the viral core[19].

Nucleocapsid (NC or p7)

NC binds to the packaging signal on viral RNA resulting in encapsidation of the RNA genome into virions during assembly.

p6

The p6 polypeptide region contains binding sites for Vif and proteins of the ESCRT (endosomal sorting complex required for transport) pathway allowing for efficient release of budding virions[20].

Two spacer proteins (p1 and p2)

Regulate conformational changes that accompany viral maturation.

Pol (Polymerase)

The Gag-Pol fusion protein is the precursor for the viral enzymes Protease (Pro), Reverse transcriptase (RT) and Integrase (IN)[21]. Protease is required for cleavage of the Gag and Gag-Pol polyprotein precursors, an essential step in virus maturation. Reverse transcriptase catalyses the transcription of the dimer of single stranded genomic RNA in the virion into double stranded DNA. Polymerase contains no proof reading activity and therefore replication is error-prone. Following transcription double stranded DNA is imported into the nucleus where it can insert into the genomic DNA of the host cell through the activity of integrase[22]. As well as existing as an integrated provirus, HIV-1 DNA can also exist in an unintegrated form, as one or two LTR circles or as linear molecules[23,24] (reviewed in [25]).

Env (Envelope)

The env gene encodes the 160kD Env (gp160) precursor protein which is cleaved by a cellular protease, Furin, into gp120 and gp41[26]. Env forms a trimeric 'spike' on the virus surface which facilitates entry into uninfected cells. The spike is a trimer of heterodimers of gp41 and gp120[27–29]. Binding of gp120 to the CD4 receptor on target cells induces changes in its conformation which causes gp41 to become exposed. A hydrophobic region, the fusion peptide, inserts into the cell membrane allowing fusion of the virus membrane with the cell membrane.

Tat (Trans-activator of transcription)

Tat binds to the transactivation response element (TAR) located in the 5' end of HIV-1 RNA. In binding to TAR, Tat alters the properties of the transcription complex, recruits a positive transcription elongation complex (P-TEFb) and hence increases the production of full-length viral RNA[30]. Tat increases production of viral mRNAs 100-fold and consequently is essential for viral replication[31].

Rev (Regulator of expression of virion proteins)

Rev binds to unspliced viral transcripts in the nucleus through a rev response element (RRE) and transports them to the cytoplasm where a full set of proteins can be produced[32]. Rev contains a leucine-rich nuclear export signal (NES) that allows it to shuttle between the nucleus and cytoplasm[33].

Nef (Negative regulatory factor)

Viral expression of Nef induces numerous changes within the infected cell in order to evade host defence and increase viral infectivity. Functions include;

- Decreasing surface expression of CD4 by increasing its rate of degradation[34]. Down-modulation of CD4 could be critical for HIV-1 pathogenesis by preventing superinfection and promoting virus release and infectivity[35].
- Down-regulation of CTLA-4, an inhibitor of T cell activation, by targeting it for degradation. Resting helper T cells are refractory to productive infection[36].
- Inactivation of bad, a proapototic protein, therefore protecting the infected cell from apoptosis[37].

- Down-regulation of MHC class I, thus decreasing presentation of viral peptides and subsequent killing of infected cells by CD8+ T cells[3838,39].

Vif (Viral infectivity factor)

Vif targets APOBEC3G for ubiquitination and degradation, thereby preventing it from entering a virion during budding from a host cell. APOBEC is a cytidine deaminase enzyme that mutates viral nucleic acids[40,41] (Discussed further in section 1.7.1).

Vpu (Viral Protein Unique)

Newly synthesized Env glycoproteins (gp160) are sometimes retained in the endoplasmic reticulum through interactions with newly synthesized CD4 molecules. Vpu promotes degradation of CD4 in these complexes, thus allowing transport of Env to the cell surface for assembly into viral particles[42]. Vif also inhibits the activity of tetherin which functions to tether budding virions to the surface of the infected cell[43].

Vpr (Viral Protein R)

Vpr regulates nuclear import of the pre-integration complex (PIC)[44]. Vpr also induces G2 cell cycle arrest; the transcriptional activity of the HIV-1 long terminal repeat is most active in the G2 phase of the cell cycle[45–47].

1.3 Life cycle of HIV-1

Binding and entry

Entry into the cell is initiated through binding of the gp41/gp120 trimer with the CD4 receptor. This receptor is found on 60% of circulating T cells, on T cell precursors in the bone marrow and thymus, dendritic cells (DC), macrophages, eosinophils and brain microglia. Binding of gp120 to CD4 induces a conformational change in the envelope complex which exposes its chemokine-binding domain, allowing it to interact with the chemokine receptors on the cell surface. HIV-1 commonly uses CCR5 (R5-tropic strains) and CXCR4 (X4-tropic strains). Binding to CD4 and the chemokine receptor allows for a stable two-pronged attachment, following which gp41 can penetrate the cell membrane allowing for fusion of the membranes and entry of the viral capsid into the cell. Homozygosity for a 32 base pair deletion in the gene encoding CCR5 can protect against infection with R5 isolates and a heterozygous deletion is associated with delayed disease progression[48–50].

Uncoating

Once the membranes have fused, the viral core is uncoated and released into the cytoplasm.

Reverse transcription

The viral enzyme reverse transcriptase catalyses the production of a double stranded DNA molecule and the formation of a preintegration complex (PIC).

Provirus integration

This viral DNA is inserted into the host DNA by the enzyme integrase which acts to catalyze two reactions: firstly, the production of sticky ends on the viral DNA and secondly, the covalent ligation of the viral DNA with the host DNA. Integrated DNA may be transcriptionally silent, resulting in a reservoir of latently infected cells.

Virus protein synthesis and assembly

In activated cells proviral DNA is transcribed into mRNA. The regulatory proteins Tat and Rev are the first to be translated, followed by the structural proteins. Env is transported to the plasma membrane. Gag (p55) and Gag-Pol form the nucleus of the maturing virus particle and coat two copies of the viral RNA to form the capsid.

Budding

The immature virion starts to bud from the host cell plasma membrane during which envelope glycoproteins are incorporated. The budded virion is still immature and not infectious until the precursor proteins are cleaved by HIV-1 protease. Cleavage leads to core condensation and the generation of a mature infectious virion.

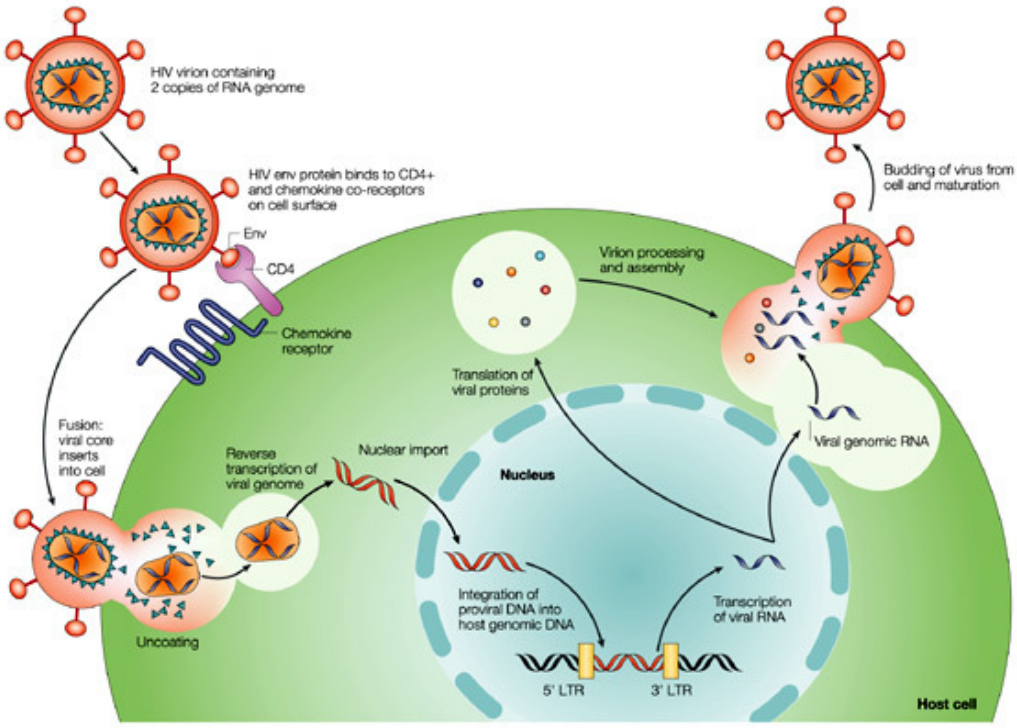


Figure 1.1 HIV life cycle (figure from Nature)

1.4 Viral latency

In order for HIV-1 to replicate, the viral genome has to be reverse transcribed into DNA, transported into the nucleus and integrated into the host genome. Pre-integration latency results from a partial or complete block of the viral life cycle at steps prior to the integration of the virus into the host genome. Mechanisms include; insufficient ATP supply for PIC transport into the nucleus, an inadequate supply of the host cell's cytoplasmic nucleotide pool, and the presence of polynucleotide-cytidine deaminases, such as APOBEC3G[51]. Integration restraints can lead to the accumulation of linear and circular forms of unintegrated HIV-1 DNA. However due to the short half-life of unintegrated DNA it is thought to have a small impact on the generation of a stable pool

of latently infected T cells. From this state, the virus may either surpass the cellular blocks and move to a productive infection or in the majority of cases, become inactivated and be cleared from the cell.

Post-integration latency, which is more relevant for HIV-1 persistence, occurs when a provirus fails to effectively express its genome and is reversibly silenced after integration into the host cell genome. Mechanisms contributing to the establishment of HIV-1 post-integration latency may operate at the post-transcriptional level (inhibition of nuclear RNA export and inhibition of HIV-1 translation by microRNAs). However, in the majority of latently infected cells, HIV-1 infection appears to be blocked at the transcriptional level. Mechanisms causing blocks at the transcriptional level, which may possibly act simultaneously, include 1) transcriptional interference owing to the site and orientation of the provirus in the cell chromosome; 2) the absence of nuclear host transcription factors, such as NF-κB (nuclear factor- κB) or NFAT (Nuclear Factor of Activated T-cells); 3) the presence of cellular transcriptional repressors such as yin and yang 1; 4) the chromatin structure of the HIV-1 promoter and the presence of a repressive nucleosome; 5) epigenetic silencing, by posttranscriptional modifications (e.g. hypoacetylation by histone deacetylases) of the histone tails that form the nucleosome core and modulate the chromatin structure; 6) the sequestration of the cellular positive transcription elongation factor b (P-TEFb), in an inactive form; 7) the sub-optimal concentration of the viral transactivator Tat[52].

This latent state is exceptionally stable and a cell can remain latently infected for its natural life span as the lack of viral proteins protects it from the cytopathic effects of the

viral infection while it remains undetected by the host immune system. Although transcriptionally silent, this reservoir is fully capable of producing infectious virus when the host cell is reactivated by recall antigen or various cytokines or when HAART is discontinued[53].

1.5 Transmission of HIV-1

There are three routes by which HIV-1 can be transmitted;

- Sexual exposure; heterosexual and homosexual
- Blood-born transmission; IV drug users & contaminated blood products
- Mother to child; in-utero, intra-partum and post-partum

Sexual contact is the most common route of transmission and 70% of HIV-1 infections worldwide are due to heterosexual transmission (reviewed in [54]). Transmission probability per coital act is estimated to be 1 in 200 to 1 in 3000 for heterosexual transmission although can be higher for MSM[55], but is affected by compounding risk factors such as sexually transmitted infections (STIs), male circumcision and plasma viral load. Semen can contain free HIV-1 particles, as well as HIV-1-infected cells and it is unclear if transmission is mediated by free virions or cell-associated virus. *In vitro* experiments using human cervical explants suggested that rates of transmission between the two were comparable[56]. Within hours of infection HIV-1 crosses the mucosal epithelial barrier[57] by transcytosis through epithelial cells[58,59], direct penetration or the infection of Langerhans cells[60,61]. A single virion or "founder virus" establishes

infection[62,63] in 80% of heterosexual subjects despite a highly diverse circulating viral quasispecies within the transmitting partner, due to the formidable barrier that intact genital mucosal tissues provide.

1.6 Course of HIV-1 infection

The course of HIV-1 infection can be divided into three stages; an acute phase that lasts 3-12 weeks[64–66] followed by a clinically latent chronic phase lasting 3-10 years which, in the absence of treatment results in Acquired Immunodeficiency Syndrome (AIDS).

Acute infection refers to the period between infection and seroconversion, which is when HIV-1-specific antibodies can first be detected in the blood. Following transmission of HIV-1 across the mucosal barrier, virions enter resting CD4+CCR5+ T cells situated immediately below and within the genital tract epithelial layers. In the SIV model these are the first cells to be infected after exposure[67,68]. Most sexually transmitted viruses utilize CCR5, CXCR4 viruses are rarely transmitted and generally appear only in late infection. This initial replication of virus locally in the genital mucosa constitutes the eclipse phase, a period lasting approximately 10 days during which HIV-1 RNA is not detectable in blood. Once replication reaches a threshold, virus and / or virus-infected cells reach the draining lymph nodes (LNs), where there is a plentiful supply of activated CD4+CCR5+ T cells which allow for massive virus replication and dissemination around the body to other lymphoid tissues. Dendritic cells can enhance infection by binding to gp120 through DC-specific ICAM3-grabbing non-integrin (DC-SIGN) which functions as a unique HIV-1 trans-receptor. HIV-1 is then

internalized into the DC and delivered to CD4+ T cells where the interaction between DC-SIGN and ICAM-3 facilitates infection[69]. There is a high frequency of memory CD4+CCR5+ T cells in the gut-associated lymphoid tissue (GALT)[70,71]. It is estimated that 80% of GALT CD4+ T cells are depleted within the first three weeks of infection due to both direct cytopathy of infected CD4+ T cells and bystander activation-induced apoptosis of uninfected cells[72–74]. Plasma viraemia increases exponentially until it reaches a peak of 10^7-10^8 RNA copies per ml of blood at 21-28 days after HIV-1 infection. CD4+ T cell numbers may be reduced at the time of peak viraemia but later rebound, although rarely to normal levels in the blood[68,71,72]. Around this time of infection patients frequently experience non-specific flu- like symptoms such as headache, muscle aches, sore throat and fever known as 'seroconversion illness' (or acute retroviral syndrome).

Following peak viraemia, virus levels fall by 10-200 fold over a 12-20 week period to a stable level known as the virus set point[73,75,76]. This plateauing of the plasma viral load marks the start of the chronic phase of infection, which may be asymptomatic and lasts for an average 10 years in the absence of HAART. The decline in plasma viraemia is concomitant with the emergence of HIV-1-specific CD8+ T cells[77,78]. Rapid viral replication of up to 10^{12} virions per day[79] continues in lymphoid tissue despite the presence of neutralising antibodies and strong CD8+ T cell responses. However, an equilibrium develops between virus replication and anti-viral immune responses, resulting in a period of clinical latency during which the viral load remains relatively stable. Host genetic factors can influence the period of clinical latency and a lower set point has been shown to correlate with slower disease progression[75,80]. Viral

subpopulations or quasispecies become more heterogeneous due to viral evolution driven largely by immune pressure.

Due to chronic immune activation[81,82] a gradual decline in circulating CD4+ T cells is observed during the chronic phase[83,84] and when the CD4+ T cell count falls below 200cells/µl, viral replication accelerates, CD8+ T cell activity decreases and AIDS defining illnesses can develop. The time from infection to death in the absence of HAART is on average 10 years[85]; however a great deal of heterogeneity exists. 5-8% of HIV-1 infected patients remain clinically stable for decades in the absence of HAART. These viraemic controllers/Long term non-progressors (LTNPs) have plasma RNA levels <2000copies/ml and / or maintain normal CD4 T cell counts with a reduced rate of CD4+ T cell loss (18cells/µl/year) as compared to typical progressors (60 cells/µl/year)[86,87]. On the other hand, 10% of HIV-1 infected individuals known as rapid progressors (RP) show fast CD4+ T cell decline soon after seroconversion and have high levels of viraemia. They develop symptoms of AIDS within five years[88]. Viraemic non-progressors have high virus replication during chronic phase, but remain asymptomatic with high CD4 counts.

1.7 The immune response to HIV-1 infection

1.7.1 Innate immune responses

The first immune responses to HIV-1 are mediated by the components of the innate immune system: these include complement and acute phase proteins (synthesised by the

liver) and cytokines and chemokines produced by cellular components (Monocytes/macrophages, dendritic cells, neutrophils and natural killer cells).

Soluble factors

Complement

The complement system is the first line of defence against many pathogens, including HIV-1. Activation of the complement system can occur by three independent pathways (classical/alternative/lectin) but all ultimately converge with the cleavage and activation of C3[89]. Subsequent cleavage and activation events result in the formation of the membrane attack complex (MAC). This complex forms a lytic pore in the cell membrane, disrupting the integrity of the cell and resulting in the death of the infected cell. HIV-1 can directly activate the complement system via gp120 and gp41: this can occur in the absence of antibodies but is further enhanced when they are present[90,91]. In acute infection there is an inverse correlation between complement lysis activity and plasma viral load[92]. However it is also thought that the complement system may enhance the infectivity of HIV-1 in the host. HIV-1 acquires regulators of complement activation such as CD59 and CD55 during budding from host cells, allowing it to escape complement-mediated lysis. Virions not killed by complement-mediated lysis remain covered with C3 fragments, allowing them to interact with complement receptor-expressing cells such as DCs, macrophages, natural killer (NK) cells, B cells or follicular dendritic cells[90,93,94] and so facilitating the spread of HIV-1.

Cytokines and chemokines

Cytokines are diverse group of small proteins which have an extensive range of effects on many different cell types. The main cytokines involved in an antiviral response are the interferons (IFN-α, IFN-β and IFN-ɣ) and the tumour necrosis family (TNF-α and TNF-β). Type I IFNs (IFN-α/β) are secreted by monocytes and plasmacytoid dendritic cells (pDC) in direct response to virus infection[95]. In addition to their direct antiviral effect, type I IFNs exert multiple immunomodulatory effects, such as enhancement of antibody production by B cells[96], enhancement of T and NK cell cytotoxicity[97] and inhibition of T suppressor/regulatory cells[98–100]. IFN-α in particular has shown potent anti-HIV-1 effects *in vitro* which include inhibiting multiple steps of the virus life cycle, such as reverse transcription and viral expression after integration of the provirus[101,102]. These pleiotropic activities are mediated via the up-regulation of a diverse spectrum of IFN-stimulated genes (ISG). Type II IFNs (IFN-γ) are synthesized in response to the recognition of infected cells by activated T lymphocytes and NK cells. Whereas both type I and II IFNs can enhance the expression of MHC class I, only IFN-γ is capable of inducing the expression of MHC class II, thus promoting CD4+ T cell responses.

Chemokines, a subset of cytokines, are small proteins whose main role is to act as a chemoattractant during homeostatic cell-trafficking or inflammatory responses (reviewed in [103]). The chemokines MIP-1α (CCL3), MIP-1β (CCL4) and RANTES (CCL5) are produced early in HIV-1 infection by activation of macrophages, DCs, T cells and NK cells[104]. These chemokines can block the CCR5 coreceptor and prevent infection by R5-tropic viruses[105]. Stacey et al. showed in a longitudinal study in primary HIV-1 infection that there is an ordered increase in plasma levels of ~30 cytokines and chemokines[106]. IFNα and IL-15 exhibited a rapid but transient elevation, succeeded by a

large and more sustained increase in inducible protein 10 (IP-10) and TNF-α. There is a slow induction of the pro-inflammatory cytokines IL-6, IL-8, IL-12 and IFN-γ, but this occurs before the up-regulation of the anti-inflammatory cytokine IL-10. There was little change in cytokine levels from plasma samples collected during the same phase of Hepatitis B infection, indicating that a systemic cytokine response of this magnitude is not required for viral clearance.

Cellular components

Phagocytic cells

The primary cells of the innate system are phagocytes (monocytes, macrophages, and neutrophils) which engulf pathogens into an intracellular vesicle called a phagosome. Phagocytes either recognise pathogen-associated molecular patterns (PAMPs), opsonising antibodies or complement bound to the pathogen surface[107–111]. Fusion of a lysosome with the phagosome and the release of reactive oxygen species, reactive nitrogen intermediates or antimicrobial peptides results in the death of the pathogen. Macrophages are recognised as the second cellular target of HIV-1[112] and a crucial virus reservoir[113].

Cytotoxic cells

Natural Killer (NK) cells become activated during acute HIV-1 infection[95,114]. They can control HIV-1 replication through direct cytolysis of infected cells and through the production of antiviral cytokines and chemokines such as IFN-ɣ, TNF-α, MIP1-α and β. Activation of NK cells is controlled by the balance of signals received through inhibitory receptors (iNKRs) and activating receptors that recognise molecules up-

regulated on infected cells[115–117]. NK cells express a repertoire of inhibitory receptors including (1) killer cell immunoglobulin-like receptors (KIRs), which recognize HLA-A, -B, and -C molecules; (2) the CD94/NKG2A-B heterodimers, which belong to the C-type lectin superfamily and (3) immunoglobulin-like transcripts (ILTs) / leukocyte Ig-like receptors (LIRs). NK cells do not possess one dominant activating receptor, but instead rely on a vast combinatorial array of receptors to initiate effector functions such as NKG2, CD16, Ly49. Bryceson et al. demonstrated that none of the activating receptors alone, with the exception of CD16, were able to elicit cytolytic activity or cytokine secretion. However, when different pairs or combinations of receptors were simultaneously cross-linked, effector functions were triggered[118].

Under normal conditions the inhibitory signals are dominant and the cell is spared from lysis by the NK cell. However virus infected cells, including HIV-1 infected cells down-regulate HLA class I molecules in order to evade the CD8+ T cell response[119,120], meaning the inhibitory signal is removed. This alone is not sufficient to activate the NK cell, a second activating signal, such as the up-regulation of stress induced ligands is also required. Alternatively, target cells that up-regulate activating NK receptor ligands to levels that outcompete the dominant inhibitory signals delivered through normal MHC recognition can also result in NK cell activation. NK mediated cytolysis of target cells can occur by death receptor dependent killing or granule dependent cell killing. The former involves up-regulation of death receptors on the NK cell which bind their receptors on target cells, initiating an apoptotic cascade. The latter involves cytotoxic granules within the NK cell that contain granzymes and perforin[121]. Activation of the NK cell results in the release of the contents of these granules followed by target cell

death. NK cells also have receptors for the Fc fragment of the antibody molecule, allowing them to kill pathogens coated with opsonising antibodies by a process called antibody-dependant cellular cytotoxicity (ADCC).

Natural Killer T cells are a subset of thymus-derived lymphocytes that express a semi-invariant T cell receptor (TCR) which recognises glycolipids presented by CD1d[122,123] they therefore bridge the gap between the innate and adaptive immune systems. Activation of NKT cells following signalling through the TCR induces immunoregulatory and effector functions via production of T helper type 1 (Th1) and type 2 (Th2) cytokines and via cytolysis. Peripheral blood NKT cell numbers are known to be reduced from early HIV-1 infection onwards which may be due in part to the susceptibility of a subset of NKT cells expressing both CD4 and CCR5 which are highly susceptible to infection with R5-tropic HIV-1[124–126].

Antigen presenting cells

Cells of the innate immune system play a pivotal role in the initiation of the adaptive immune response by presenting antigen to T lymphocytes. Dendritic cells are found in epithelial, mucosal and submucosal tissues where they are primed to capture and present foreign antigens to naïve T cells. Dendritic cells as well as macrophages and monocytes are professional antigen presenting cell (APCs). The expression of co-stimulatory molecules such as CD80 (B7.1) and CD86 (B7.2), which interact with complementary molecules on T cells, such as CD28 is a defining feature of professional APCs[127,128]. This co-stimulatory signal is required for the activation of naïve T cells[127].

Cellular restriction factors

Mammalian cells have evolved a number of proteins, termed restriction factors, whose function is to suppress virus replication. They provide an early line of defence against infection as a component of, or even preceding, innate antiviral responses. Four HIV-1 restriction factors which have been intensively characterised are APOBEC3G (Apolipoprotein B mRNA-editing enzyme, catalytic polypeptide like 3G), TRIM5-α (tripartite motif-containing protein 5-α), Tetherin and SAMHD1 (SAM domain and HD domain-containing protein 1).

APOBEC3G deaminates cytidine residues to uridine which causes guanosine-to-adenosine hypermutation in the opposite strand, thereby introducing an unnatural base and inhibiting virus replication[129]. HIV-1 counteracts APOBEC3G through the activity of viral infectivity factor (Vif), which triggers the ubiquitination and degradation of APOBEC3G via the proteasomal pathway[130]. There is now evidence that APOBEC3G can directly inhibit integration by modifying the linear cDNA substrate, thus rendering it unsuitable for provirus formation[131,132].

TRIM5-α blocks HIV-1 infectivity by recognising motifs within the capsid proteins and interfering with the uncoating process, therefore preventing successful reverse transcription and transport to the nucleus of the viral genome[133,134].

Tetherin is a membrane protein which functions to restrict the release of fully formed progeny virions from infected cells. When the virion buds from the surface of the cell, the N-terminus of tetherin remains attached to the infected cell while the glycosyl-

phosphatidylinositol (gpi) anchor attaches to budding virions[43,135]. It is antagonized by the viral protein Vpu which is thought to work by targeting tetherin for degradation[43,135].

SAMHD1 functions as a dNTP triphosphohydrolase (dNTPase) depleting the pool of nucleotides available to reverse transcriptase for viral cDNA synthesis[136]. It is responsible for blocking replication of HIV-1 in dendritic cells[137], macrophages[138] and monocytes[139].

1.7.2 Adaptive immune responses

Humoral responses

The first antibody response to HIV-1, detectable within eight to ten days after plasma virus detection, fails to neutralise virus[140]. Acute gp41 and gp120 specific antibodies do not select escape mutations indicating they are ineffective against HIV-1[141–143]. Neutralising antibodies (nAb) appear 12 weeks after infection, generally when viral load has already reached set point[144,145]. They are targeted at the surface glycoproteins gp120 and gp41 in order to block binding and entry of HIV-1 to target cells. nAbs fall into 2 categories: those that bind to the variable regions of HIV-1 and that neutralize a relatively narrow group of isolates and those that bind to conserved regions of the virus and that can neutralize a wider range of isolates, including those in different subtypes or from diverse geographical regions, termed broadly neutralising antibodies (bnAbs)[26]. The majority of nAb responses fall into the first category, with just 20% of patients developing bnAbs years after infection[146]. HIV-1 infection in humanized mice and SHIV infection in macaques can be prevented by passive transfer of bnAbs[147–150] and it

is widely believed that a vaccine that elicits such antibodies would be effective in protecting against HIV-1 infection. bnAbs target a small number of regions of the viral envelope, are often polyreactive which increases binding avidity when the low number of envelope spikes in HIV-1 virions prevents simultaneous binding of both arms of the antibody[151] and are extensively hypermutated[151]. Affinity maturation may be delayed or hampered due to dysregulation and loss of CD4+ T cell help necessary for full activation of B cells. Avoidance of neutralisation involves at least three mechanisms: i) point mutations within the epitopes which prevent antibodies binding[152] ii) concealment of epitopes within deep recesses that are inaccessible to antibodies until gp120 binding[153], and iii) epitopes may be hidden behind constantly shifting glycan shields which are largely nonimmunogenic[145]. This means neutralizing antibodies preferentially recognize and inhibit preceding but not the current autologous viral strains, as confirmed by studies showing that antibodies isolated from a host are effective against virus isolates from earlier time points but are unable to neutralise current virus strains[145,154,155]. The majority of envelope-specific antibodies generated fail to interact with functional envelope spikes and therefore lack neutralising activity. These non-neutralising antibodies can promote viral lysis via the complement pathway[92], phagocytosis[156,157] or antibody dependent cellular cytotoxicity (ADCC) by NK cells[158]. Non-neutralising antibodies can therefore eliminate HIV-1-infected cells, thus reducing production of progeny, limiting cell-to-cell spread and potentially also lowering the pool of infected cells that revert to latency. Antibodies which mediate ADCC develop rapidly in the majority of patients with HIV-1 infection and can be detected within a few weeks after the onset of symptoms of acute infection[159,160]. The recent RV144 vaccine trial, used a recombinant canary pox priming vaccination followed by an envelope protein

boosting vaccination and was shown to generate modest protection against the acquisition of HIV-1 infection[161]. Since this vaccine regimen elicited non-neutralizing antibody responses, attention is turning to the possibility that non-neutralizing antibodies may have mediated the protection[162,163].

Cellular responses

T cell development and thymic selection

T cell progenitors arise from bone marrow hematopoietic stem cells. After exiting the bone marrow they travel to the thymus via the bloodstream. The newly arriving immature thymocytes lack both the CD4 and CD8 coreceptors and are therefore described as double negative (DN) cells. As they migrate through the thymic cortex they express a randomly generated TCR (discussed below) and also become double positive (DP) cells by switching on expression of both CD4 and CD8. Thymocytes then undergo a process of thymic education involving two steps; positive selection and negative selection. During positive selection thymocytes are selected for their ability to bind self-peptides displayed on MHC molecules of thymic epithelial cells. DP cells that are able to bind self pMHC with low affinity receive survival signals and are thus protected from apoptosis. If a thymocyte is unable to bind self pMHC or if it binds it with high affinity it will die. Recognition of peptide on MHC class II results in the down-regulation of CD8 synthesis and commitment of these cells to the CD4 lineage, while recognition of peptide on MHC class I has the opposite effect, creating T cells of the CD8 lineage. These single positive thymocytes then undergo negative selection to prevent the release of potentially harmful self-reactive T cells into the circulation. T cells binding too

strongly to self-antigens are eliminated from the population. Following this final selection process, surviving cells undergo terminal differentiation and exit the thymus.

T cell receptor

The TCR is a transmembrane heterodimer composed of either an α and β polypeptide chain or more rarely (1-5% of T-cells) a ɣ and δ polypeptide chain. The β chain is formed by recombination between variable (V), diversity (D), joining (J) and constant (C) segments, whilst α chains are formed from recombined V, J and C segments. There are multiple copies of each segment and geometric recombination of these segments generates a large number of different VDJ or VJ sequences. Diversity can be further increased by the random insertion and deletion of nucleotides and the generation of palindromic sequences arising from the formation of DNA hairpins during the recombination process. Each variable domain has three hypervariable complementarity determining regions (CDRs), CDR1, CDR2 and CDR3; CDR3 is the most variable region and is generated by V(D)J gene recombination, it is this region which is responsible for recognising processed antigen. Random rearrangement of the germline gene segments combined with junctional flexibility generates an enormous TCR repertoire and it has been estimated that the circulating TCR repertoire of an individual comprises approximately $2.5x10^7$ clonotypes[164].

Antigen presentation

Naïve T cells become activated following recognition of antigen peptides presented by Major histocompatibility complex (MHC) class I or II molecules of professional APCs such as dendritic cell, macrophages and B cells. There are two antigen processing

pathways that lead to the generation of peptide for presentation in association with class I or II MHC molecules.

- MHC class I pathway of antigen presentation

The MHC class I pathway processes proteins from the cytosol, including viral proteins and is known as the endogenous pathway. These proteins are first ubiquitinated which targets them to the proteasome where they undergo proteolytic cleavage to form peptides. The peptides are transported into the endoplasmic reticulum (ER) by the transporters associated with antigen processing (TAP) 1 and TAP2. In the ER the peptides are further trimmed at their N-terminus by an ER aminopeptidase (ERAP). The peptide loading complex (PLC) comprising TAP1/2 with calreticulin, tapasin and ERp57 facilitates loading of the trimmed peptides into the groove of the membrane bound class I MHC. The peptide-MHC complex is released from the PLC, translocates to the Golgi complex and subsequently appears on the cell surface ready for presentation to a T-cell receptor. Many viruses are not tropic for dendritic cells and are therefore not present in the cytosol of professional APCs; however, exogenous antigens can be presented to MHC class I molecules via a process called cross-presentation. Phagocytosed / endocytosed antigens from apoptotic or necrotic virus-infected cells can leave the vacuole in which they have been engulfed and gain entry to the cytosol, where they are subsequently degraded by the proteasome, transported to the ER and presented by MHC class I to CD8+ T cells. The half-life of peptide-MHC complexes upon the surface of DC has been estimated to be short (~10hours). Therefore in order to achieve successful presentation there must be sustained and continuous synthesis of HLA class I molecules and loading of antigenic peptide (Reviewed in [165]).

- MHC class II pathway of antigen presentation

Newly synthesised MHC class II molecules in the ER bind to the transmembrane invariant chain (Ii), inactivating a retention signal and allowing transport through the Golgi to the trans-Golgi reticulum. Here the MHC class II-containing vacuole fuses with a late endosome containing peptides generated by degradation of exogenous antigens internalised by phagocytosis / endocytosis by immature DCs and macrophages. Within these late endosomes, the acidic environment results in the degradation of Ii, except for the part sitting in the MHC groove referred to as CLIP (Class II-associated invariant chain peptide). A MHC class II-like structure, HLA-DM catalyses the removal of the CLIP peptide, allowing endosomal peptides to bind. Initial peptide binding is determined by the concentration of peptide and the on-rate, but HLA-DM plays an important role in "editing" the peptide repertoire by assisting in the removal of lower affinity peptides allowing replacement with high affinity peptides. The MHC class II enriched compartment is then transported to the cell surface for presentation to CD4+ T helper cells. Antigen presented upon HLA class II molecules has an extremely long half-life, estimated to be around 100 hours (Reviewed in [165]).

T cell activation

Stimulation of the TCR by MHC-peptide is not sufficient to fully activate naïve T cells. Two signals are required: Ligation of the TCR by MHC-peptide provides signal one; signal two is provided by the costimulatory molecules CD80 and CD86 present on professional APCs such as mature dendritic cells that bind to CD28 on the naïve T cell. Engagement of the TCR by pMHC without the accompanying costimulatory signal results in the T cell becoming anergic. Binding of the MHC-peptide complex by the

TCR and CD4 (for MHC class II molecules) or CD8 (MHC class I molecules) in the presence of costimulatory signals results in the phosphorylation of immunoreceptor tyrosine based activation motifs (ITAMs) present within the CD3 coreceptor complex. These ITAMs are phosphorylated by the protein kinase Lck, which is constitutively associated with CD4 and CD8. This induces a signalling cascade via pathways including the Ras/MAPK pathway and the phosphatidylinositol pathway which result in the activation of transcription factors and subsequent transcription of genes involved in the proliferation of T cells.

CD4+ T cells and their role in HIV-1 infection

CD4+ T cells, referred to as helper T cells, orchestrate responses of other cells of the immune system to antigens including those derived from pathogens. They recognise peptide in the context of MHC class II molecules. Studies of murine T cell clones suggested that following activation, uncommitted 'Th0' CD4+ T cells differentiate into either Th1 or Th2 effector cells as defined by their cytokine output[166,167]. However, the Th1 / Th2 paradigm has been expanded by the identification of other CD4+ T cell subsets. Th1 cells function against intracellular bacterial and viral infections. They secrete IL-2, IFN-ɣ and TNF-α which results in activation of macrophages to enhance killing of phagocytosed pathogens and proliferation of cytotoxic CD8+ T cells[168]. Th2 cells produce the cytokines IL-4, IL-5, IL-6, IL-10 and IL-13 which promote B cell differentiation and proliferation in response to extracellular pathogens[169,170]. Th cell polarization is determined during the primary interaction of the naïve CD4+ T cell with the professional APC. IL-12 and IFN-ɣ produced by dendritic cells in response to activation of TLRs by microbial infections induces a Th1 polarization[171]. Th2

polarization follows the early production of IL-4 during the primary response[172]. The essential transcription factors of Th lineages are T-bet/Stat4 for Th1[173], GATA-3/STAT5 for Th2[174,175], RORγt/STAT3 for Th17[176], and Foxp3/STAT5 for Treg[177,178]. A third subset of CD4+ effector Th cells, named Th17 cells, was identified in 2003[179]. Th17 cells produce IL-17 which promotes neutrophil recruitment, activation and migration to tissues and induces the production of inflammatory cytokines. Their main role is in the clearance of extracellular pathogens[180]. A further subset, follicular helper T (Tfh) cells, that express CXCR5, provide specialised help for B cell responses[181].

In the periphery, regulatory T cells (Tregs) are able to control autoreactive cells that have escaped central tolerance. Natural Tregs originate and achieve their regulatory phenotype in the thymus[182], they are identified by the expression of CD4, CD25 and FoxP3[183]. They suppress effector T cell function by direct cell lysis of effector T cells, inhibition of proliferation via competition for IL-2 (CD25 is the high affinity receptor for IL-2) and the production of immunomodulatory cytokines such as TGF-β and IL-10[184]. A second group of Treg cells, adaptive Tregs, arise from the thymus as conventional effector T cells and acquire regulatory properties in the periphery.

A growing body of evidence highlights the importance of an effective CD4+ T cell response in many viral infections for effective pathogen control and elimination. There are three main roles proposed for CD4+ T cells in HIV-1 infection; B cell help, T cell help and direct killing of infected cells. CD4+ T cell help is essential for the optimal functioning of CD8+ T cells during infection. CD4+ T cell help is required not only during the priming of naïve CD8+ T cells[185,186], but it is also necessary for the

maintenance of effector functions of CD8+ T cells during chronic infection[187]. In the LCMV model, removal of CD4+ T cells by an anti-CD4 antibody resulted in the mice being unable to clear the virus and becoming chronically infected[187]. CD4+ T cells are the primary targets of HIV-1 and are massively depleted during acute infection, particularly from the GALT[188]. In addition, remaining CD4+ T helper cells become dysfunctional, exhibiting a deficiency in IL-2 production and a lack of proliferative capacity[189–191]. The proliferation and maintenance of memory CD8+ T cell responses capable of expansion on secondary exposure to antigen is dependent in the production of IL-2 by CD4+ T cells[192–194]. It is inferred, therefore, that the rapid and profound loss of CD4+ T cells contributes to the failure of HIV-1-specific CD8+ T cells to clear infection. HIV-1 controllers and / or long-term non-progressors show stronger CD4+ T cell proliferative responses and IL-2 secretion *in vitro* than non-controllers or progressors[191,195]. Furthermore, Lichterfeld et al. demonstrated that CD4+ T cell helper function could be restored by the adoptive transfer of autologous CD4+ T cells to chronically infected individuals and this was associated with restoration of HIV-1-specific CD8+ T cell function *in vitro*[196]. A small fraction of HIV-1-specific CD4+ T cells have been shown to have some direct cytolytic activity[197,198]. Degranulatory activity, as demonstrated by surface expression of CD107a, and expression of perforin and granzyme have been detected in CD4+ T cells. Soghoian et al. showed that expression of granzyme A on CD4+ T cells strongly correlated with slower disease progression in HIV-1 infected individuals[199]. Rosenberg et al. showed that initiation of antiretroviral therapy during primary HIV-1 infection was associated with preservation of CD4+ T cell helper functions such as *in vitro* proliferative capacity[200].

CD8+ T cells and their role in HIV-1 infection

On recognition of pMHC Class I on the surface of APCs, HIV-1-specific CD8+ T cells release cytotoxic granules containing perforin and granzymes which induce apoptosis in target cells. Cytotoxic CD8+ T cells can also induce death by surface expression of FasL, which delivers apoptosis-inducing signals to target cells expressing Fas. CD8+ T cells can also secrete a range of cytokines including IFN-ɣ and TNF-α, which can inhibit viral replication, and chemokines such as RANTES and MIP-1α and MIP1-β which act as viral entry inhibitors[105,201,202].

There is a substantial body of evidence demonstrating the importance of CD8+ T-cells in the control of HIV-1 virus replication. In the acute phase, the appearance of HIV-1-specific CD8+ T-cells coincides with a decline in viral load (VL) towards the set point[77,78]. In macaques, depletion of CD8+ T-cells using an anti-CD8 antibody during primary SIV infection resulted in sustained high viraemia, which only decreased when the effect of the infused antibody waned[203]. Subsequent studies in macaques showed that stimulation of CD8+ T cells by vaccination could partially protect against SIV challenge[204–206]. Vaccination of macaques with DNA and Ad5-vectored SIV vaccines in prime-boost regimens resulted in a 10-fold reduction in VL compared to controls; however this was short-lived due to viral escape from vaccine-induced T-cell responses[206–208]. More recently, a CMV-vectored vaccine elicited a persistent and rapid effector T cell response to SIV antigens which resulted in control of infection in 50% of animals, and ultimately cleared the infection, thus demonstrating for the first time that clearance of an AIDS virus infection can be achieved by vaccine-induced CD8+ T cells[209,210]. A second line of evidence supporting the role of CD8+ T cells in the control

of HIV-1 replication is the association between certain MHC class I molecules and disease outcome. Several studies have shown an association between HLA-B57 and HLA-B27 and slow progression to AIDS, whereas alleles such as HLA-B35 are associated with rapid progression to AIDS[211–215]. Fellay et al. conducted a genome wide association study (GWAS) and identified an association between HLA-B57 and viral load set point[214]. These associations are consistent with a contribution of HLA class I restricted CD8+ T cell responses to virus control. Further, the appearance of escape mutations within targeted epitopes from the earliest stages of infection indicates the strong selection pressure HIV-1-specific CD8+ T cells exert on the virus[216–218] . 80% of acute infections are established by a single founder virus; however, the sequence of this founder virus begins to diversify at around the time of peak viraemia[63,219]. Mutations occur via the acquisition of amino acid changes in and around T cell epitopes that impair epitope processing (if in flanking regions), presentation (binding to HLA class I) or T cell receptor recognition. These mutations can lead to a complete replacement of the original sequence of the founder virus within 10 days of infection[216].

There are very few studies which have examined the CD8+ T cell response during the early stages of HIV-1 infection (p24 antigen positive but negative antibody response). A study by Goonetilleke et al. showed that the initial CD8+ T cell response is very narrow, targeting only one to three epitopes[216]. It remains narrowly directed as viral load set point is reached[220,221]. Early responses appear to be targeted to epitopes of higher entropy for example epitopes within Env[222–225]. During chronic infection responses broaden with the average person targeting a median of 14 epitopes[226]. However these CD8+ T cells ultimately fail to control virus replication and prevent progression to

AIDS. Escape can lead to reduced viral fitness, as demonstrated by the reversion of escape mutations to a more wild-type sequence following transmission to an HLA-mismatched recipient who cannot recognize the epitope[227–230]. However, this can be overcome by compensatory mutations[231,232]. In addition to mutational escape, loss and early dysfunction of HIV-1-specific CD4+ T cell function[71] and chronic antigen stimulation results in exhaustion of CD8+ T cells[233]. *In vitro* studies have shown progressive loss of functions: proliferative capacity, secretion of different cytokines and chemokines, cytolytic activity, and finally they enter a stage of full exhaustion followed by physical deletion[234,235]. CD4+ and CD8+ T cell exhaustion has been demonstrated in chronic HIV-1 infection[236,237] and is associated with increased expression of negative regulators of T cell activation such as programmed death (PD-1) and cytotoxic T lymphocyte antigen-4 (CTLA-4). Following binding to its ligands PDL-1 and PDL-2, PD-1 dampens TCR-induced cytokine production. Its expression on CD8+ T cells during chronic HIV-1 infection correlates with viral load and LTNPs exhibit markedly lower expression levels as compared to progressors[236–238]. CTLA-4 suppresses TCR signaling by binding to CD80 and CD86 on APCs with much higher affinity than CD28[239].

Characteristics of an effective CD8+ T cell response against HIV-1

The introduction of tests for viral load enabled the identification of a subset of patients known as HIV-1 controllers who are able to exert long-term control over HIV-1 replication in the absence of treatment. Increasing evidence suggests that the CD8+ T cell response plays a critical role in this control. No studies have demonstrated a correlation between the frequency of IFN-ɣ secreting HIV-1-specific cells and viral

load[226,240], but qualitative differences in the HIV-specific CD8+ T cell response have been observed in HIV-1 controllers as compared with progressors.

Host genetics

The common feature amongst this group of individuals is the over-representation of a certain group of HLA-B alleles as compared with the broader population. These include HLA-B57, HLA-B58, HLA-B27, HLA-B51, HLA-B81, HLA-B44 and HLA-B14. Large population studies show that protective HLA alleles are expressed in 67% of controllers but are only present at a frequency of 37% in HIV-1 progressors or in HIV-1-negative populations[241]. However the majority of infected individuals expressing protective alleles still develop progressive disease[242] and some controllers with undetectable viral loads do not possess protective HLA class I alleles[215]. The exact mechanism of virus control observed in subjects with protective HLA alleles is not fully understood however these alleles preferentially present epitopes within Gag, particularly within highly conserved regions[243–245]. Wang et al. noted that CD8+ T cell responses in HIV-1 infected patients with protective HLA alleles preferentially targeted highly conserved regions of the virus and this correlated with their ability to control HIV-1[246]. In addition to the restriction of antiviral CD8+ T cell-mediated immune responses, polymorphisms in HLA class I alleles might affect HIV-1 immune control through interactions with other molecules of the immune system, for example specific Killer-cell immunoglobulin-like receptors (KIR). The expression of particular activating KIRs in conjunction with their ligands have a marked effect on HIV-1 disease outcome[247,248], however no enrichment for protective KIR–HLA combinations in HIV-1 controllers has been observed[249].

Viral targets

The ability of CD8+ T cells to target particularly vulnerable regions of HIV-1 may provide controllers with a better outcome. Preyera et al. demonstrated that CD8+ T cells from a cohort of HIV-1 controllers with diverse HLA class I alleles preferentially targeted Gag over other proteins whereas responses were more broadly distributed in persons with progressive infection[241]. In a large study of clade C HIV-1 infected subjects, the broader the Gag-specific response the lower the viral load[250]. The effectiveness of CD8+ T cells targeting Gag and particularly conserved regions within Gag maybe explained by the impact of immune selected escape mutations within these regions on viral fitness. Due to its essential role in viral assembly, maturation, uncoating, and nuclear import, mutations within Gag may have a higher likelihood of inducing a fitness defect as compared with mutations within variable regions such as Env. Indeed mutations within a highly conserved p24 Gag epitope presented by HLA-B5701 reduced replicative capacity *in vitro* in a cohort of individuals expressing this allele[251]. Rihn et al. showed that in a large randomly mutagenized library of gag capsid (CA) sequences, ~70% of random single nucleotide substitutions lead to a >50-fold reduction in replicative fitness[252]. Alternatively, the enhanced antiviral effect of Gag-specific CD8+ T cells may be linked to an early expression (4 hours) of Gag epitopes on the surface of infected cells. Preformed Gag protein is introduced into the cytoplasm during initial virus entry and therefore it can be immediately processed and presented to CD8+ T cells without the requirement for de novo protein synthesis. Whereas epitopes from Env are not presented until 24 hours after infection thus delaying the cytotoxic response[253].

Functional characteristics

CD8+ T cells from controllers synthesise greater amounts of cytotoxic granule components such as perforin and granzyme, which are essential for cell mediated cytotoxicity[242,254–256]. Migueles et al. also showed increased perforin and granzyme in CD8+ T cells of controllers but extended the analysis further by measuring only functional granzyme B that had been delivered to the target cell, not inactive granzyme B stored within cytotoxic granules to demonstrate that these CD8+ T cells had increased cytotoxic capacity[256]. The increased expression of these cytotoxic granule components seems to be partly under the control of the transcription factor T-bet which is able to directly bind to the promoter regions of perforin and granzyme B genes and has been shown to be up-regulated in HIV-1-specific T cells from elite controllers[255].

Studies have also demonstrated that CD8+ T cells from controllers are 'polyfunctional'[234,241] rather than 'monofunctional' as observed in non-controllers. Betts et al. used polychromatic flow cytometry to assess five CD8+T cell functions, including degranulation (CD107a) and cytokine (IFN-ɣ, TNF-α, and IL-2) and chemokine production (MIP-1β)[234]. Responses in controllers had a higher degree of functionality (four or five different functions simultaneously) than in the progressors who were limited to the expression of various combinations of CD107a, IFN-ɣ, and MIP-1β, with little expression of TNF-α or IL-2[234].

Additionally CD8+ T cells of controllers have strong proliferative responses[257,258]. Migueles et al. examined the ability of CD8+ T cells to respond to HIV-1-infected primary autologous CD4+ T cells and showed that although CD8+ T cells from both

controllers and progressors were able to produce IFN-ɣ in response to HIV-1-infected CD4+ T cells, CD8+ T cells from controllers had a greater capacity to proliferate than those from progressors. Proliferation was tightly coupled to increases in perforin expression[257].

HIV-1-specific CD8+ T cells of higher avidity have also been observed in HIV-1 infected individuals controlling virus replication[259,260]. Berger et al. studied the functional avidities of HIV-1-specific CTL responses targeting 20 different, optimally defined CTL epitopes in a cohort of 44 HIV-1 controllers and 68 HIV-1 non-controllers. The functional avidities of Gag-specific and HLA-B-restricted responses were higher in HIV-1 controllers than in non-controllers and were not restored in HIV-1 non-controllers initiating HAART[259]. This was confirmed by work from Mothe et al. which demonstrated responses to p24 of comparable magnitude in controllers and non-controllers, but the responses in controllers were of higher avidity[260].

The limitation with many of these studies is their cross sectional design which makes it difficult to ascertain whether the attributes of HIV-1-specific CD8+ T cells observed in controllers as mentioned above are a cause or consequence of virus control. HIV-1-specific CD8+ T cells from controllers have a greater ability to inhibit HIV-1 replication in *ex vivo* infected autologous CD4+ T cells as compared with CD8+ T cells from non-controllers[261,262]. The ability of HIV-1 controllers to effectively inhibit virus replication is likely a composite of all the above functional characteristics and therefore prospective measurement of antiviral activity can overcome the limitations of cross-sectional studies.

1.8 HIV-1 vaccines

Historically, vaccines have been the most effective weapon for preventing and even eradicating global infectious diseases such as smallpox, polio, measles, and yellow fever. They safely and cost-effectively prevent illness, disability and death. The World Health Organization estimates that each year more than 120 different types of vaccines save 2.5 million lives[263]. A mathematical modelling analysis showed that the implementation of combined strategies such as medical male circumcision, earlier HAART and pre-exposure prophylaxis (PrEP) could lead to dramatic declines in HIV-1 incidence but they will not stop transmission completely[264]. Therefore, a HIV-1 vaccine remains the best hope for controlling the HIV-1/AIDS pandemic.

1.8.1 Vaccine approaches

Attenuated live virus

Propagation of a virus *in vitro* generates mutations which can attenuate the pathogenicity of the virus *in vivo*. These viruses can elicit a robust immune response but cannot disseminate and vaccination with such pathogenically attenuated viruses has been very successful for many viral infections such as measles, polio, and chicken pox. A live attenuated HIV-1 vaccine has severe safety limitations due to the inherent risk of vaccine strains acquiring full virulence. Preliminary studies with the SIV-macaque model demonstrated that six rhesus monkeys infected with a live attenuated virus (SIVmac239 with a deletion in nef) maintained low virus burdens, normal CD4+ T cell counts and remained healthy for more than three years after experimental inoculation. The monkeys were protected from infection following challenge with homologous wild

type virus[265]. However, the vaccine strain was found to have repaired the nef deletion over time. Further work showed that newborn monkeys or adult monkeys infected for a long period of time with attenuated SIV eventually developed AIDS and died[266].

Subunit/Recombinant protein vaccines

Subunit vaccines include only the antigens that are considered critical for induction of protective immunity. Experiments in nonhuman primates indicated that passive transfer of neutralizing antibodies could protect against experimental challenge from primate lentiviruses[267–269]. The initial strategy for HIV-1 vaccine development used recombinant HIV-1 envelope proteins (gp160 or gp120) in an attempt to elicit neutralizing antibodies. However a study by Berman et al. showed that vaccination of chimpanzees with recombinant HIV-1 envelope glycoprotein as an immunogen provided only modest protection, and only against homologous challenge virus[270]. The first HIV-1 vaccine developed and tested in humans, was a subunit vaccine comprising gp160[271].

DNA vaccines

DNA vaccines consist of a plasmid encoding a protein of interest. Injection of plasmid DNA carrying a gene encoding an antigen under the control of an appropriate mammalian transcription promoter leads to the expression of the antigen in situ and triggers CD4+ and CD8+ T cell responses. The potential advantages of DNA vaccines are: completely synthetic, easy to engineer, genetically stable, thermostable, immunogenic in animal models and can be administered repeatedly without generating immunity against the vector. Although vaccination with DNA plasmids induced

substantial cellular responses and demonstrated promise in animal models, early candidate DNA vaccines have been poorly immunogenic in humans for a number of viruses[272,273]. Immunogenicity can be increased by improving delivery methods, such as intramuscular electroporation[274] and by the use of molecular adjuvants[275].

Dendritic cell based vaccines

Dendritic cells are the most potent professional APC with the ability to present antigen via the MHC class I and class II pathway and therefore induce both a CD4+ and CD8+ T cell response[276–279]. Animals vaccinated with DCs loaded with HIV-1 viral lysate, envelope glycoproteins, or inactivated virus were shown to mount a potent immune response against HIV-1[280–283].

Attenuated viral vector vaccines

Serial passage or targeted gene deletions of certain viruses renders them replication-defective and therefore safe to use as vectors for the delivery of foreign genes. Viral vector vaccines have a similar mode of action to DNA vaccines, with the added advantage that the vector may induce innate immune responses (similarly to the parental virus) and thus act as adjuvants. Following administration of a viral vectored vaccine and infection of a target cell, the encoded antigens are produced intracellularly and therefore enter the MHC class I processing pathway and so are particularly effective at eliciting a CD8+ T cell response.

i) Poxviridae:

The best studied vaccine vectors in humans are the pox family of viruses (Reviewed in[284]). They are able to carry multiple or large foreign genes (~25kb) which are stably expressed[285]. Poxviruses are thermostable and therefore may be good candidates for use in settings where it is difficult to maintain a cold chain. The prototype member of this family is vaccinia, the replication-competent virus which was used in the worldwide smallpox eradication campaign; however several studies indicated that vaccinia virus could disseminate and caused complications in immunocompromised people. This led to the development of replication defective vaccinia strains such as Modified vaccinia Ankara (MVA) and NYVAC. Attenuation of MVA was achieved by serial passage in chick embryo fibroblasts (CEF) resulting in loss of 15% of the parental genome. It cannot replicate in mammalian cells but can complete a replication cycle which results in expression of passenger proteins and induction of CD4+ and CD8+ T cell responses. NYVAC, derived from the Copenhagen strain of vaccinia, was rendered replication-defective by deletion of 18 different open reading frames from the original viral genome (Reviewed in[286]).

Other poxvirus vector-based HIV-1 vaccines have been derived from the family of avipoxviruses and include canarypox (ALVAC)[287] and fowlpox viruses. ALVAC does not replicate in human cells and further attenuation was produced through more than 200 passages in chick embryo fibroblasts (Reviewed in[286]). The recent phase III RV-144 trial in Thailand which involved 16402 healthy volunteers evaluated the efficacy of four priming injections of ALVAC-HIV (vCP1521) in combination with two booster immunizations of a recombinant gp120 subunit vaccine[161].

ii) Adenoviridae:

These are medium sized (90-110nm) non-enveloped DNA viruses that typically cause mild respiratory tract infections. They can be made replication-defective by deletion or inactivation of the E1 and E3 genes. This also enables them to carry large transgenes (~8 kb)[288,289] which are expressed under the control of promiscuous promoters such as human cytomegalovirus promoter. Of the 51 currently known serotypes, adenovirus serotype 5 has been most widely tested in humans, initially for gene therapy and more recently as a vaccine vector. However, as Ad5 is widespread, the prevalence of Ad5 neutralising antibodies in the general population is high (up to 90% in several regions of Africa and 60% in Europe / N. America[290–292]). In the recently halted STEP trial, individuals that were seropositive for Ad5 showed increased rates of HIV-1 infection following vaccination with an Ad5-based HIV-1 vaccine. It has been hypothesized that rAd5 vaccination in individuals with pre-existing immunity against Ad5 may have resulted in preferential activation and expansion of HIV-susceptible CD4+ T cells expressing a mucosal homing phenotype, and these cells could have trafficked to mucosal sites and served as increased targets for HIV-1 infection. Benlahrech et al demonstrated that stimulation of T cells from healthy Ad5-seropositive individuals with Ad5 induced preferential expansion of adenovirus memory CD4+ T cells expressing α4β7 integrins and CCR9, indicating a mucosal-homing phenotype[293]. However Hutnik et al found that Ad5-seropositive and Ad5-seronegative individuals vaccinated with the same Ad5-based HIV-1 vaccine used in the STEP study did not demonstrate any significant differences in either the frequency or function (IFN-ɣ, IL-2, MIP-1α, TNF-α and/or perforin) of Ad5-specific CD4+ T cells[294]. A number of alternatives are being evaluated, including rare human serotypes such as Ad26 and Ad35[295] and non-human

adenoviruses from ovine, porcine, bovine, and chimpanzee sources[296–299]. Ad35 has been shown to be immunogenic and not inhibited by pre-existing anti-Ad5 antibodies in mice[300]. However, rare serotypes of human Ad including Ad24, Ad26, Ad34 and Ad35 are less potent as vaccine vectors than Ad5 in mice and NHPs[301], and they showed lower protective efficacy in NHPs[206,302].

Adenoviruses derived from chimpanzees (ChAd) were advanced to the clinic because of their low/no seroprevalence in the human population[299,303,304] and comparable immunological potency to human Ad serotype 5. In NHPs a single ChAd injection elicited high magnitude (1000–2000 IFN-γ SFU per million PBMCs) and similar quality of T cell responses to the clinically validated Ad5 and Ad6[301]. The first replication-defective adenovirus vector of chimpanzee origin tested in humans was derived from the serotype ChAd63. ChAd63 was engineered to encode three relevant malaria antigens, ME-TRAP, AMA1 and MSP1. Phase I clinical trials reported median IFN-ɣ Elispot responses at peak of approximately 1000 SFU/10^6PBMCs for ME-TRAP[305], 900 SFU/10^6PBMCs for AMA1[306] and 2000 SFU/10^6PBMCs for MSP1[307]. In HIV-1 uninfected volunteers, ChAd63 encoding a conserved region immunogen, HIVconsv, induced peak responses of 443 IFN-γ SFU/10^6 PBMCs and 5150 IFN-γ SFU/10^6 PBMCs following boosting with MVA.HIVconsv[308].

ChAd3 was used as vaccine vector for the development of a T cell-based vaccine to HCV. Safety and immunogenicity of ChAd3 encoding the non-structural region (NS) transgene (ChAd3NS) was assessed in a Phase I clinical trial in healthy volunteers[309]. Peak responses were observed between day 14 and 28, with median IFN-ɣ Elispot

responses at peak exceeding 1000 SFU/10^6PBMCs. Similar data were obtained with an Ad6 vector encoding the same antigen (Ad6NS) confirming that the two vectors had comparable immunogenicity[310].

Because attenuated viral vectors only infect a single cell and have limited replicative capacity before the infected cell dies this can result in a short-lived immune response compared to parental virus strains that persist for longer. Replicating and/or persistent vectors can infect many more cells and therefore more closely mimic a natural viral infection by inducing potent innate immune responses, which in turn enhance adaptive cellular and humoral responses. In addition, a lower dose can be given to achieve the same magnitude of immune response. There is renewed interest in the use of replicating viral vectors following a study by Hansen et al. which demonstrated control of SIVmac239 infection in macaques after vaccination with a CMV vectored vaccine[209,210]. CMV persistence results in continuous, low-level immune stimulation which maintains an expanded pool of effector-differentiated T cells. Other replicating viral vectors under investigation include adeno-associated virus (AAV), Venezuelan equine encephalitis virus (VEE), vesicular stomatitis virus (VSV) as well as replication competent poxviruses and adenoviruses. An important advantage of replication-competent adenovirus vectors is their natural ability to infect mucosal surfaces, allowing for oral administration. Adenovirus serotypes 4 (Ad4) and (Ad7) have substantial safety data following vaccination of US military personnel for prevention of respiratory and enteric illness[311]. An Ad4-HIV Env vaccine was shown to induce both Env-specific humoral and cellular immunity in rabbits[312].

Prime-boost vaccines

DNA vaccines are weakly immunogenic in humans when used alone, and anti-vector immune responses limit the efficacy of homologous viral vector regimens. These disadvantages may be overcome by combining the two approaches in heterologous prime-boost vaccination strategies. Such a regimen may generate immune responses that are higher in magnitude and better in quality for example an increased frequency of polyfunctional cells, a higher IFN-ɣ MFI on CD8+ T cells indicating more efficacious effectors and CD8+ T cells of higher avidity[313–318] compared with homologous priming and boosting[206,319,320]. One of the most extensively studied strategies is recombinant DNA prime-poxvirus boost[321,322], which can induce T cell responses of 10 times greater magnitude than either vaccine given separately[323]. HIV-1 immunogens delivered by DNA-MVA, DNA-NYVAC, fowlpox virus-MVA regimens have been tested in many clinical trials[324–326]. While they are very safe and well tolerated, a major limitation is that they induce CD4+ but not CD8+ T cell responses[316]. MVA has been shown to efficiently boost ChAd63-primed T cell responses. O'Hara et al. and Sheehy et al. demonstrated that priming with ChAd63 expressing ME-TRAP, AMA1 or MSP1 can be boosted by MVA encoding the same antigen by a factor of two- to eight-fold with up to 7000 SFU/10^6PBMCs median peak IFN-ɣ Elispot responses[305–307]. Vaccination induced well-balanced CD8+ and Th1 CD4+ T cells with a substantial proportion of polyfunctional cells (secreting a combination of IFN-γ, TNF-α and IL-12). As mentioned earlier a prime boost of ChAd63.HIVconsv / MVA.HIVconsv induced responses of 5150 IFN-γ SFU/10^6PBMCs compared with 443 IFN-γ SFU/10^6PBMCs when ChAd63.HIVconsv was administered alone[308].

1.8.2 Prophylactic HIV-1 vaccines

Complete protection against HIV-1 infection with no generation of a latent reservoir requires sterilising immunity. This will likely only be achieved with a vaccine that can induce broadly neutralising antibodies. However, as this is a major challenge it is not likely that this could be achieved with any current vaccine candidate. Assuming that breakthrough HIV-1 infections will occur, a secondary goal would be to induce immune responses that can control early virus replication in vaccinees who become infected, by reducing peak and set point viral load. As CD8+ T cells play a key role in controlling primary viraemia in SIV and HIV-1 infections, it is hypothesised that a CD8+ T cell-inducing vaccine could reduce HIV-1 infections by decreasing infectiousness. Recently a SIV protein expressing CMV vectored vaccine cleared SIV infection in rhesus macaques[210].

Despite an extensive number of prophylactic HIV-1 vaccine candidates being investigated in phase I trials, only five have entered clinical efficacy studies. The first two efficacy studies, VAX004 and VAX003, evaluated a bivalent gp120 subunit (AIDSVAX) designed to elicit antibodies to the viral envelope (Env). VAX004, based on two different isolates of subtype B viruses, was tested among 5400 people at risk of sexual transmission of HIV-1 in a randomised placebo-controlled trial in the United States, Canada, Puerto Rico and the Netherlands. Although the vaccine did elicit antibodies, it failed to prevent HIV-1 acquisition or reduce viral load in those who became infected[327]. A second trial using VAX003, based on Thai subtype E and subtype B viruses, recruited 2500 injecting drug users in Thailand and also found no evidence of protection[328–330]. Antibodies elicited were not capable of neutralizing primary virus

isolates i.e. genetically diverse circulating HIV-1 strains[331]. The challenges associated with antibody-based vaccine candidates, coupled with studies in the SIV model showing that T cell vaccines could reduce early SIV replication after challenge led to interest in the development of vaccines to generate cellular immune responses.

The first test-of-concept studies for this approach were the STEP and Phambili trials, which evaluated Merck's trivalent Ad5-HIV-1 vaccine, in high-risk MSM and heterosexual men and women in the Americas and Australia (STEP/HVTN 502) and heterosexual men and women in South Africa (Phambili/HVTN 503). The vaccine candidate was a mix of rAd5 vectors expressing HIV-1 clade B Gag, Pol and Nef antigens which were delivered at months 0, 1 and 6 in a homologous regimen[332,333]. Vaccinations were halted in 2007 after results from the first interim analysis showed the vaccine failed to prevent infection or reduce early plasma viral load in vaccinees compared with placebo recipients, despite inducing T cell responses of similar magnitude and breadth to those observed in earlier trials[334]. Furthermore, a post-hoc analysis indicated there was a trend of enhanced risk of infection in uncircumcised men with pre-existing immunity to adenovirus serotype 5[335]. The failure of the STEP/Phambili trials highlighted limitations in the animal models used to assess vaccine efficacy. NHP vaccinated with Ad5-SIV and challenged with SHIV or SIVmac did not predict the lack of effect of Ad5-SIV vaccination on viral load set point post HIV-1 infection. Also, vaccinees who acquired HIV-1 infection showed a similar magnitude of response to vaccination to those who did not become infected indicating methods used to measure the immunogenicity of the Merck Ad5 gag/pol/nef vaccine in phase I/II trials give misleading results. The breadth of responses in vaccinees was

extremely limited, with CD8+ T cell responses targeting a median of one epitope per protein, with a bias towards less conserved epitopes. However, sieve analysis of the earliest breakthrough isolates in HIV-1-infected STEP vaccinees demonstrated that the vaccine induced T-cell–mediated immune pressure on the viruses, particularly in individuals with protective HLA class I alleles[336]. A post-hoc analysis of STEP trial participants who received the Merck Ad5 gag/pol/nef vaccine, showed that individuals who targeted ≥3 epitopes in Gag achieved a lower viral load than subjects without Gag responses[337]. These observations suggest that T cells induced by vaccination can exert pressure on the virus following acquisition, but that more potent and broad CD8+ T cell responses would be needed to contain early viral replication.

The fourth efficacy trial, RV144, tested a heterologous prime-boost vaccine regimen which aimed to induce both cellular and humoral immune responses. It consisted of four injections of a recombinant canarypox vector vaccine expressing gag, pol, and env (ALVAC vCP1521) followed by two injections of a bivalent gp120 subunit vaccine (AIDSVAX) boost over a six month period. The gp120 protein was identical to the immunogen used in VAX003/VAX004[161]. The trial recruited > 16000 individuals at low risk of HIV-1 infection. Follow up lasted three years and vaccination resulted in a 31% reduction in new infections compared to the placebo group. The RV144 vaccine did not elicit measurable CD8+ T-cell responses which is consistent with the lack of effect on viral load in individuals who acquired HIV-1. It also did not elicit BnAbs, however, it induced antibody-dependent cellular cytotoxicity (ADCC) responses highlighting the importance of investigating Fc-related antibody activities in future vaccine strategies[338]. Post hoc analyses demonstrated that the level of plasma IgG antibodies binding to the V1V2 loop region of gp120 was associated with decreased risk

of HIV-1 and also high plasma level of IgA antibodies to Env was associated with increased infection risk[339].

HVTN 505 was a phase IIB, randomized, placebo-controlled, double-blind clinical trial of a multigene, multiclade DNA prime–recombinant adenovirus type 5 vector boost (DNA/rAd5) vaccine. The DNA prime consisted of six closed circular plasmids (in a 1:1:1:1:1:1 ratio) expressing HIV-1 clade B Gag, Pol, and Nef and Env proteins from clades A, B, and C and was administered at week 0, week 4, and week 8. The rAd5 boost consisted of four rAd5 vectors (in a 3:1:1:1 ratio) expressing an HIV-1 clade B Gag-Pol fusion protein and Env glycoproteins from clades A, B, and C. The primary efficacy end points were HIV-1 infections diagnosed after week 28 and viral load set point. Vaccinations were stopped in April 2013 due to initial results showing that the vaccine was ineffective in preventing HIV-1 infections and lowering viral load among those participants who had become infected with HIV-1[340].

1.8.3 Therapeutic HIV-1 vaccines

Scale up of HAART has led to impressive reductions in morbidity and mortality among HIV-1 patients, however it has limitations. The cost of medication, the requirement for lifelong therapy which carries risks of toxicity and drug resistance[6], its failure to restore effective HIV-1-specific immune responses[254,256,258] and inability to deplete the reservoir of latently infected CD4+ T cells established early after infection[5,341–344] remain unresolved problems. In the early HAART era, drug combinations were toxic, poorly tolerated and too costly for many, and therapeutic vaccination was considered as a possible means to limit use of HAART. As drug regimens improved considerably,

with many patients achieving sustained virological suppression on one or two pills a day, and the costs of antiretroviral drugs have declined, interest in developing therapeutic vaccines waned. However, there are still no drugs that are able to reduce latent viral reservoirs and new treatment strategies are required for viral eradication. Only one individual is known to have achieved a 'functional cure'. Timothy Ray Brown received an allogenic hematopoietic stem cell transplant (HSTC), using a homozygous CCR5Δ32 donor, for the treatment of leukaemia. The patient is to date, five years later undetectable for HIV-1 DNA and RNA and no replication-competent virus can be cultured from his PBMCs[345]. HSTC is not a feasible strategy for cure in the majority. Nevertheless, it has sparked interest in other approaches such as combining latency reversing agents with another strategy to eliminate reactivated CD4+ T cells (shock / kill etc.). Therapeutic vaccination is one option for the latter. The main objective of a therapeutic HIV-1 vaccine is to induce HIV-1-specific immune responses that are substantially different in magnitude and / or quality from those induced by HIV-1 itself. The goal of early therapeutic studies was to achieve control of viral replication to an undetectable level off HAART, mimicking the phenotype of HIV-1 controllers. As HAART interruptions in the absence of any other intervention are no longer considered ethical (post-SMART study), the current goal is to combine a therapeutic vaccine with agents to reactivate latently infected cells. If significant reductions in various measures of the latent reservoir could be demonstrated, interruption of HAART may be considered in order to ascertain whether a functional cure has been achieved.

Initial studies in macaques supported the rationale for the development of therapeutic vaccines. Vaccination with DNA or poxvirus-vectored SIV immunogens to SIVmac251 chronically infected rhesus macaques treated with HAART resulted in significant

lowering of viral set point upon treatment cessation[208,346]. Most of the therapeutic vaccines tested were the same candidates as those developed for prevention and they were tested in trials involving an analytical treatment interruption. Unfortunately, therapeutic vaccination in HAART treated HIV-1 infected patients has given disappointing results.

Recombinant gp120 or gp160 were tested in several clinical trials in HAART treated HIV-1 infected patients without benefit in terms of CD4+ T cell count or viral load[347–350]. Other recombinant HIV-1 proteins such as Gag and Tat subunits have been tested in HIV-1 infected individuals[351–355]. Vacc-4x consists of four synthetic peptides corresponding to conserved regions of the HIV-1 protein p24. When administered intradermally with granulocyte-macrophage colony-stimulating factor (GM-CSF) in a prospective Phase II trial to patients on therapy, it induced HIV-1-specific immune responses in 90% of patients[352]. However in a placebo controlled Phase II trial vaccination did not reduce time to HAART resumption or slow CD4+ T cell decline as compared with placebos during a scheduled treatment interruption[356]. Vaccination of HAART treated individuals with Tat-protein based vaccines induced Tat-specific T helper cell responses, reduced cellular and biochemical activation markers and increased regulatory T cells, but no treatment interruption was conducted[353–355].

DNA vectored vaccines have also been extensively studied in therapeutic trials. DNA plasmids encoding nef, rev or tat regulatory genes were able to induce HIV-1-specific cellular responses in HAART treated patients but could not control viral replication or change CD4+ T cell counts[357,358]. A further two DNA vectored candidates, the first

expressing env/rev and gag/pol and the second encoding HIVA (a clade A p24/p17 fused to a string of cytotoxic T cell epitopes) have been tested. Both vaccines were safe but the ability to boost CD4+ and CD8+ T cells was poor[359,360].

Several trials have been conducted in HAART treated patients with HIV-1 vaccine candidates vectored by adenoviruses and poxviruses (canarypox, fowlpox, NYVAC and MVA). The most frequently studied vector has been canarypox (ALVAC). The QUEST study assessed, in a randomized, double-blind, placebo-controlled trial, whether the addition of ALVAC-HIV (vCP1452) or ALVAC-HIV and Remune to HAART initiated during acute infection could reduce plasma viral load upon treatment interruption compared with placebo. Despite increased HIV-1–specific CD4+ and CD8+ T cell responses in vaccinees, following discontinuation of therapy there was no difference in viral rebound dynamics or viral loads when compared with placebos[361]. Similar results have been obtained with vaccination of chronically infected patients with ALVAC-HIV vCP1452[362,363]. Harrer et al. investigated a recombinant HIV-1 nef expressing MVA vaccine in 14 HAART treated patients. Vaccination was associated with recognition of new HIV-1 T cell epitopes and after treatment interruption viraemia remained below the pre-HAART viral load in 9/14 patients and six patients remained off therapy for a median 64 weeks[364]. In another study using MVA as a vector, 16 infected individuals on HAART received MVA-HIVA. Frequencies of CD8+ and CD4+ T cells were significantly increased and enhanced proliferative responses to Gag were observed[365–367]. Three patients received a third dose of MVA-HIVA and underwent a scheduled treatment interruption however viral loads had risen to more than 10^5 copies/ml by day 28[368]. In a large randomised controlled trial, HIV-1 infected subjects receiving a rAd5

gag vaccine demonstrated a 0.5 log_{10} lower plasma viral load after a 16 week analytic treatment interruption compared with placebos[369,370].

Monocyte-derived dendritic cells pulsed with heat inactivated autologous virus administered to HAART treated patients induced a partial and transient control of viral replication following treatment interruption when compared with patients receiving unpulsed dendritic cells. However all patients eventually rebounded[371,372]. Routy et al. vaccinated HAART treated patients with monocyte-derived dendritic cells electroporated with mRNA encoding autologous gag, nef, rev and vpr. Although an uncontrolled study, by week 12 of the treatment interruption 16/24 subjects demonstrated a mean reduction in viral load of -1.2 log compared with pre-HAART viral load values[373].

In summary, the efficacy of therapeutic vaccines tested to date has been modest with no lasting effect on viral load observed following treatment interruption.

1.9 Challenges for the development of a HIV-1 vaccine

The literature reviewed in the preceding sections illustrates that there are many challenges associated with the design of an effective HIV-1 vaccine. In brief, these include;

- The ability of HIV-1 to integrate as a provirus into host DNA, thus forming a long lived latent reservoir that is inaccessible to antiretroviral drugs or the immune system

- The huge antigenic diversity of HIV-1 is a challenge for induction of cross-protective immune responses and enables HIV-1 to escape from humoral and cell-mediated immune responses

- Natural infection does not result in spontaneous clearance therefore there is no correlate of protective immunity

- Conserved regions of the envelope glycoprotein such as the CD4 binding site are cryptic and poorly accessible to the immune system, enabling HIV-1 to avoid neutralisation; broadly neutralising antibodies only develop after a long period of antigen exposure.

One of the largest hurdles is the tremendous genetic diversity of globally circulating strains of HIV-1. This diversity is due to the error prone nature of HIV-1 reverse transcriptase, the large number of replication cycles of the virus, the influence of innate and adaptive immune responses, and the ability for HIV-1 to tolerate this diversity. The main subtypes within the M group, A-D, F-H, and J-K differ by 25-35% in env sequences and approximately 15% in gag[14]. A rational and systematic approach to immunogen design is needed to address the enormous global antigenic diversity of circulating viral strains. Several approaches are currently being explored.

1.9.1 Centralised sequence immunogens

These use consensus/average sequences common to a clade or group ancestor to minimise the sequence difference between the immunogen and circulating strains. There are three different approaches to achieve this; ancestral reconstructions, consensus sequences and antigens designed on the basis of their central position in a phylogenetic

tree (centre-of-tree/COT). All centralised sequence antigens tested to date are well expressed and immunogenic in small animals[374–378]. An M env group consensus/ancestral env vaccine compared with a natural subtype B env vaccine in rhesus macaques was shown to enhance the cross-reactive potential of T cell responses[379].

1.9.2 Polyvalent immunogens

These incorporate natural antigens from multiple clades of HIV-1. Such vaccines have been used successfully for other pathogens like influenza[380]. In rhesus macaques a polyvalent env vaccine elicited T cell and neutralising antibody responses of a greater breadth than a monovalent env vaccine[381]. Phase I clinical trials of polyvalent vaccines report enhanced breadth of response[382–384].

1.9.3 Mosaic immunogens

Mosaic immunogens are polyvalent cocktails of synthetic proteins assembled from sets of natural sequences that are optimized by in silico recombination to provide maximal coverage of potential T cell epitopes for a given clade or group[385]. They are designed to minimise rare epitopes, exclude junctional epitopes and because they encode intact proteins, mimic natural processing and expression[386]. Vaccination of macaques with a rAd26 gag/pol/env mosaic immunogen induced broader and more cross reactive T cell responses than vaccines encoding M consensus or single clade sequences[387]. In addition, the mosaic HIV-1 vaccines demonstrated partial protection against acquisition of

infection following repetitive, intrarectal, heterologous SHIV-SF162P3 challenges as compared with placebos[388].

1.9.4 Conserved region immunogens

These immunogens are designed to include only highly invariant regions of the HIV-1 proteome linked in a chimeric protein. It is thought that immune responses specific for conserved HIV-1 regions will recognize a multitude of different HIV-1 subtypes as the diverse strains all share a common highly conserved epitope target. Further, immune responses to such conserved regions are hypothesised to limit the potential for the virus to escape without a significant fitness cost[246,389–391]. Limitations to this approach include the possibility of such regions being immunologically 'inert' with diminished recognition in natural infection and the introduction of artificial junctional epitopes.

1.10 HIVconsv

HIVconsv is a chimeric protein comprising the 14 most conserved regions of the HIV-1 proteome. Each segment is a consensus sequence from one of the four major HIV-1 clades A, B, C and D, which alternate to ensure equal clade coverage. The HIVconsv gene was inserted into three viral vectors, plasmid DNA, Ad5 and MVA and delivered in various prime-boost vaccination regimens to Balb/c and HLA-A2 transgenic (HHD) mice. Vaccination induced HIVconsv-specific responses with the DNA, Ad5, MVA (DAM) sequence of vaccinations inducing CD8+ T cell responses > 2000 SFU/million PBMC in IFN-ɣ Elispot assays which was superior to other combinations of these vaccines. HIVconsv-specific T cell responses could also be detected by *ex vivo* IFN-ɣ

Elispot in HIV-1-infected people, indicating that natural infection generates T cells specific to the conserved regions contained within HIVconsv[392]. High levels of immunogenicity have also been demonstrated in macaques[393–395]. HIVconsv vectored using prime-boost regimens of DNA, simian adenovirus and modified vaccinia virus Ankara was administered to uninfected UK volunteers. The vaccine induced high levels of effector T cells that recognized virus-infected autologous CD4+ T cells and inhibited HIV-1 replication by up to 5.79 $\log_{10}$. The virus inhibition was mediated by both Gag and Pol specific effector CD8+ T cells targeting epitopes that are typically subdominant in natural infection[308].

1.11 Thesis Aims

This thesis aimed to address whether a vaccine targeting conserved regions of the HIV-1 proteome could induce effective T cell responses in HIV-1-positive HAART-treated patients. Specific questions were:

- Could vaccination with MVA.HIVconsv increase the frequency and/or breadth of T cell responses to HIV-1 conserved regions in HAART treated patients, either through induction of new responses or boosting of existing subdominant responses?

- Does MVA.HIVconsv vaccination improve the antiviral function of HIV-1-specific CD8+ T cells?

- Is therapeutic vaccination with MVA.HIVconsv associated with a decrease in the size of the latent HIV-1 reservoir in CD4+ T cells?

- Is CD8+ T cell mediated virus inhibitory activity a function of targeting of conserved regions of the viral proteome?

2. Materials and Methods

2.1 Study subjects

2.1.1 MVA.HIVconsv trial participants

Twenty three volunteers with chronic HIV-1 infection were recruited from the Genitourinary Medicine and Infectious Diseases Departments, Churchill Hospital, Oxford University Hospitals NHS Trust and the Department of Sexual Health, Royal Berkshire NHS Foundation Trust, Reading. Principal inclusion criteria were: HIV-1 seropositive individuals, aged 18-60 who had been treated continuously with three or more antiretroviral agents for at least 12 months, had a pVL below the limit of detection (< 50 copies/ml) and a CD4+ cell count of >350 cells/µl. This study was approved by the Gene Therapy Advisory Committee (GTAC 165), the Medicines and Health Products Regulatory Agency (Eudract No. 2009-012662-31) and the Oxford University Hospitals NHS Trust. Written informed consent was obtained from all participants.

2.1.2 STEP/Phambili participants

PBMC sampled from HIV-1-positive STEP/Phambili participants (HVTN 502 / Merck 023 and HVTN 503) 12 months after HIV-1 acquisition were made available through the HIV Vaccine Trials Network (HVTN) (STEP Ancillary Study, ref. SAP-034). The cohort included 20 samples from the STEP trial (13 vaccinees, 7 placebos) and 16 samples from the Phambili trial (10 vaccinees, 6 placebos). Individuals were HAART-naïve 12 months after infection, with CD4+ cell counts >350 cells µl.

2.1.3 HIV-1 seronegative donors

Healthy HIV-seronegative donor samples were obtained from the NHS Blood Transfusion Service, Bristol, England, which were received as buffy coat samples and were processed within one day of collection.

2.2 HLA typing

An aliquot of 1-2 million PBMC was centrifuged at 600 x g for one minute. The supernatant was discarded, the tube was vortexed vigorously and the pellet was resuspended in 300µl of cell lysis solution. The tube was vortexed again and if clumps were still visible, it was incubated at 37°C and 5% CO_2 for 5 minutes. The cell suspension was mixed with 3µl of RNase, and the tube was inverted 25 times, after which 100µl of protein precipitation solution was added. The tube was vortexed for 20 seconds and centrifuged at 600 x g for 10 minutes. Supernatant was carefully discarded and the pellet was resuspended in 300µl of 100% isopropanol. The tube was inverted 50 times to enable precipitation of DNA, and then centrifuged at 600 x g for 10 minutes. The pellet was then resuspended in 300µl of 70% ethanol in PBS. The contents of the tube were transferred to a 2ml cryovial and centrifuged at 600 x g for 10 minutes. Using a Pasteur pipette, the ethanol supernatant was removed and replaced with 50µl of DNA hydration solution. HLA typing was performed by Tim Rostron at the DNA Sequencing/HLA Typing Laboratory (WIMM, John Radcliffe Hospital, OX3 9DS) using ARMS-PCR with sequence-specific primers according to standard procedures. HLA typing for STEP/Phambili subjects was conducted by the HVTN.

2.3 The MVA.HIVconsv vaccine

The Immunogen HIVconsv is a chimeric protein comprising the 14 most conserved regions of the HIV-1 proteome and has been described previously[392]. The gene coding for HIVconsv was inserted in the vector, Modified Vaccinia virus Ankara (MVA). The transgene has been inserted into the thymidine kinase locus of the MVA genome under the control of the promoter p7.5.

2.3.1 MVA.HIVconsv vaccination schedule

Volunteers were screened up to 28 days before vaccination and were followed for 6 months after the last vaccination. The total study duration from screening to completion of follow-up was 38 weeks. Ten volunteers in Group A were randomised to receive either MVA.HIVconsv $5x10^7$ pfu or placebo (4:1 ratio) on days 0, 28 and 84 (Visits 2, 4 and 7); 9 volunteers in Group B were randomised to receive either MVA.HIVconsv $2x10^8$ pfu or placebo (4:1 ratio; 10 subjects were planned for this arm but the study was stopped after 9 were enrolled because of slow recruitment) by intramuscular injection. The placebo was the formulation buffer for the vaccine, comprising 10mM Tris-HCl, 140 mM NaCl, pH 7.7. Blood was sampled at the screening visit and on days 0, 14, 28, 42, 56, 84, 112, 182 and 266 (Figure 2.1).

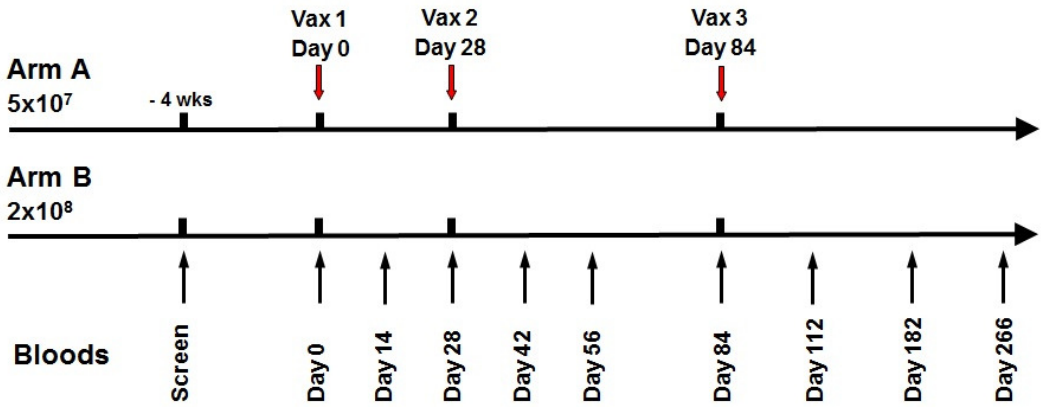


Figure 2.1 MVA.HIVconsv vaccination schedule

2.4 Reagents and buffers

R0: 500ml RPMI 1640 with 5ml Penicillin/Streptomycin and 5ml L-glutamine

R10: 500ml RPMI 1640 with 50ml FCS, 5ml Penicillin/Streptomycin and 5ml L-glutamine

MACS buffer: 500ml PBS with 33ml 7.5% BSA and 2ml 0.5M EDTA

PBS tween: 100ml PBS with 50µl Tween 20

20µg/ml lysolecithin in 4% paraformaldehyde: 40g paraformaldehyde was dissolved in 100ml 1M NaOH and titrated to pH 7.2 using 4M HCL. PBS was added to bring the total volume to a litre and 20mg lysolecithin was dissolved in the solution.

NP-40: 499.5ml PBS with 0.5ml Nonidet P-40

2.5 Peptides

2.5.1 HIVconsv matrix peptides

The matrix pools were designed using “Deconvolute This” (version 1.0 Roederer). This software allows the construction of peptide pools that can be used to identify responses to individual peptides using the least number of cells possible. By using peptides pooled in a matrix format, a two-step process enables deconvolution of the response and identification of individual peptide-specific T-cell responses. In the first step, peptides were pooled into a matrix format, each pool containing a different mixture of peptides. These matrix pools were designed using the software ‘deconvolute this’, which employs several criteria to optimize the matrix configuration. These included the expected number of positive peptides, the desired coverage and pool size (a larger pool size would enable fewer pools to be tested in first round). The coverage indicated the number of pools in which one individual peptide would appear. A coverage of 4 was used, i.e. each peptide was present in 4 different pools. This was expected to reduce the false positive rate compared to a coverage of 2 or 3. The matrix was checked by running simulations to return several different matrix pool configurations. An ambiguity score close to 1 identified a configuration which should require the least number of peptides to be tested in the second round, therefore preserving valuable patient samples. Following testing using the matrix pools, all peptides within negative pools can be discounted; however there is still uncertainty which peptides are true positives and others which may be negative but appear in a pool with other positive peptides. The positive pools are entered into ‘deconvolute this’ and it returns peptides that require

testing in the second round assay. Peptides can then either be confirmed as true positive peptides, or discounted in subsequent IFN-γ Elispot assays.

A total of 199 15-17mer peptides (with a 11 amino acid overlap) cover the HIVconsv immunogen. The sequences of the peptides are shown in the Appendix. The design giving the best ambiguity was 80 pools with 10 peptides in each pool. Each pool was made by diluting 4mg/ml stock peptides in R0 to give a concentration of 4µg/ml. These pools were aliquoted into 96 well plates along with PHA (4µg/ml) as a positive control and R0 (0.1% DMSO) only as a negative control (Figure 2.2). Sufficient peptide plates for the whole of the trial were prepared in advance and stored at -80ºC in order to minimise interassay variation in peptide dispensing. On the day of the elispot assay, the peptides were thawed and added to cell suspensions of equal volume to give a final peptide concentration of 2µg/ml. MVA was added on the day of the assay (MOI of 1) in order to measure vector-specific T cell responses. 'Flu, EBV and CMV (FEC) peptides (Appendix) were pooled into one pool and used as an additional positive control (final concentration of 2µg/ml).

Pool 1	Pool 9	Pool 17	Pool 25	Pool 33	Pool 41	Pool 49	Pool 57	Pool 65	Pool 73	SP 1	FEC
Pool 2	Pool 10	Pool 18	Pool 26	Pool 34	Pool 42	Pool 50	Pool 58	Pool 66	Pool 74	SP 2	FEC
Pool 3	Pool 11	Pool 19	Pool 27	Pool 35	Pool 43	Pool 51	Pool 59	Pool 67	Pool 75	SP 3	Negative
Pool 4	Pool 12	Pool 20	Pool 28	Pool 36	Pool 44	Pool 52	Pool 60	Pool 68	Pool 76	SP 4	Negative
Pool 5	Pool 13	Pool 21	Pool 29	Pool 37	Pool 45	Pool 53	Pool 61	Pool 69	Pool 77	SP 5	Positive
Pool 6	Pool 14	Pool 22	Pool 30	Pool 38	Pool 46	Pool 54	Pool 62	Pool 70	Pool 78	SP 6	Positive
Pool 7	Pool 15	Pool 23	Pool 31	Pool 39	Pool 47	Pool 55	Pool 63	Pool 71	Pool 79	MVA	Positive
Pool 8	Pool 16	Pool 24	Pool 32	Pool 40	Pool 48	Pool 56	Pool 64	Pool 72	Pool 80	MVA	Positive

Figure 2.2 Layout of HIVconsv matrix peptide pools (Pools 1-80) and single pools (SP1-6) with negative and positive control wells. HIVconsv and single pool peptides were at a concentration of 4µg/ml.

2.5.2 HIVconsv single pool peptides

The 199 15-17mer peptides that span the HIVconsv immunogen were divided sequentially into 6 peptide pools;

- SP1 (Gag Clade C, D and A)
- SP2 (Pol Clade B)
- SP3 (Pol Clade C)
- SP4 (Vif Clade D, Pol Clade A, Env Clade C)
- SP5 (Pol Clade D, B and A)
- SP6 (Env Clade D, Pol Clade C)

The concentration of stock peptide pools was 15µg/ml and working solutions were made at 4µg/ml. On the day of the assay, peptide pools were added to PBMCs to give a final concentration of 2µg/ml.

2.5.3 Clade B consensus peptides

HIV-1 subtype B peptides were obtained through the AIDS Research and Reference Reagent Program Division of AIDS, NIAID, NIH, USA. All peptides were 15-mers overlapping by 11 amino acids. Peptides were dissolved in 20% DMSO/PBS to give a stock solution of 10mg/ml. From these stock solutions a working stock solution of 40µg/ml was generated. Peptides were then pooled at a concentration of 4µg/ml as follows; Gag pool, Pol pool 1, Pol pool 2, Env gp41 pool, Env gp120 pool, Nef pool, Rev pool, Tat pool, Vif pool, Vpr pool and Vpu pool. On the day of the assay, peptide pools were added to PBMCs to give a final concentration of 2µg/ml.

2.5.4 FEC peptides

FEC peptides restricted by common HLA class I alleles were synthesized in house, dissolved in 20% DMSO/PBS and pooled to create a 40µg/ml working stock. Final concentration in assays was 2µg/ml. The sequences of the peptides are shown in the Appendix.

2.5.5 Beneficial peptides for STEP/Phambili study

Beneficial overlapping 15-18mer peptides (OLPs) were identified by Mothe et al.[396]. 26 beneficial OLPs (10 in Gag, 12 in Pol, 3 in Vif and 1 in Nef) were identified in a Clade B population and 22 (18 in Gag, 3 in Pol and 1 in Vif) in a Clade C population. Individual Clade B and Clade C specific beneficial peptide sets were made using clade B and C consensus peptides to cover the sequences of the beneficial OLPs. Beneficial OLPs were divided into pools based on their protein source and protective ratio. OLP sequences and pools are shown in the Appendix. Working solutions were made at 4µg/ml and on the day of the assay, peptide pools were added to purified CD8+ T cells to give a final concentration of 2µg/ml.

2.5.6 Conserved elements peptides for STEP/Phambili study

Seven conserved elements, CE 1-7, were identified by Rolland et al.[390]. These were split into 2 pools based on data from Mothe et al. (personal communication and [260]) showing that CEs 4, 5 and 6 were targeted more frequently in HIV-1 controllers compared with non-controllers. Clade B and C consensus peptides were used to span the sequences of the conserved elements peptides. Peptide sequences and pools are shown in the

Appendix. Working solutions were 4µg/ml and on the day of the assay, peptide pools were added to purified CD8+ T cells to give a final concentration of 2µg/ml.

2.6 PBMC separation

Fresh blood was separated by Ficoll-Hypaque density gradient centrifugation. The lymphocyte layer was removed and washed twice with R0. Cells were resuspended in R10 and counted. Cells were then centrifuged at 1500rpm for 5 minutes and resuspended in cold fetal calf serum at a concentration of $5x10^6$/250µl. Aliquots of 250µl/vial were made and 250µl of cold 20%DMSO/FCS added. Vials were transferred to an iso-propanol-containing cooling box and stored overnight at -80ºC. The following day the cells were transferred to vapour phase liquid nitrogen, where they were stored until required.

2.7 Thawing of cells

Frozen cells were thawed in a water bath until a small ice pellet remained and then transferred to a 15ml round bottom tube. R10 benzonase was added dropwise and then the cell suspension was washed twice at 1500rpm for 5 minutes, following which the cells were resuspended in R10 and counted. Cells were rested at 37ºC for a minimum of 2 hours before being used in assays. Viability was assessed using a 1:1 dilution of cells:trypan blue.

2.8 IFN-γ Elispot assay

Cells were separated as described, resuspended to give a final concentration of $1x10^6$/ml PBMC and dispensed into 96-well nitrocellulose-backed plates pre-coated with 1-DIK Mab (15μg/ml). Cells were stimulated with peptide pools at a final concentration of 2μg/ml or with a positive control PHA (4μg/ml) or negative control (R10) for 16-18 hours. The following day, cells were discarded, plates washed 6 times with PBS/0.05% Tween 20 and a secondary biotinylated antibody, anti-IFNγ-Mab (7-B6-1) added at a concentration of 1μg/ml for 3 hours at room temperature. Plates were washed another 6 times before the addition of streptavidin-conjugated alkaline phosphatase (1:1000) for 2 hours at room temperature. Plates were developed with an alkaline phosphatase substrate until the PHA control wells appeared saturated by eye. Blue spots representing spot-forming cells were counted with an automated plate reader (AID Systems, Germany). The frequencies of HIV-1-specific IFN-γ-releasing cells were expressed as IFN-γ spot-forming units (SFU)/million PBMC, after the subtraction of SFU values in negative control wells.

2.9 Short term cell lines (STCLs)

In selected assays PBMCs were removed from the elispot plate after the 16-18 hour incubation. These cells were washed three times with PBS and resuspended at a concentration of $2x10^6$/ml in R10+IL-7 (25ng/ml) medium. 1ml of cell suspension was aliquoted per well of a 12 well plate. On day 3, 10μl of IL-2 was added to each well to give a final concentration of $1.8x10^3$units/ml. Plates were swirled gently and returned to incubator. On day 7, medium was replaced with 1ml of fresh R10/IL-2. On day 10, cells

were starved of IL-2 by washing with PBS 4 times and resting for 30 hours ($1x10^6$ cells/ml). Following this cells were used in an IFN-ɣ Elispot assay.

2.10 CD8+ T cell separation

After being thawed, cells were incubated with 20µl CD8 microbeads at 4°C for 20 minutes. Cells were then washed by adding 1-2ml of MACS buffer per 10^7 cells and centrifuging at 1500rpm for 10 minutes. Supernatant was removed and the cells resuspended in 500µl of buffer. A MACS separation column was placed in the magnetic field of a MACS separator and prepared by rinsing with 500µl of MACS buffer. Once the column had emptied completely, the cell suspension was applied to the column. The column was washed 3 times with 500µl MACS buffer and the total effluent collected (unlabelled fraction). The column was then removed from the separator and placed over a 15ml falcon; 1ml of MACS buffer was added to the column, which was then immediately flushed out by applying the plunger. This was the labelled as the CD8+ positive fraction.

2.11 Intracellular cytokine staining for CD107ab and IFN-γ

PBMC were thawed and then rested for 2 hours. Following resting, cells were counted, resuspended in R10 and aliquoted at $1x10^5$ cells/well into a 96 well plate. Cells were stimulated with either; SEB 1µg/ml (positive control), R0 (negative control) or HIVconsv single peptide pools 1-6 2ug/ml. A volume of 5µl of each FITC-conjugated CD107 a and b antibodies were added at the same time and cultures were incubated for 30 minutes at 37°C. Monensin and BFA were then added and the cells incubated for a

further 5.5 hours at 37°C, following which the plates were placed at 4°C overnight. The next day cells were washed 2 times with FACS wash buffer (PBS 5% FCS) and then incubated with Aqua Live/Dead Fixable stain for 20 minutes at room temperature. Cells were then washed again and stained with surface antibodies (CD3-APCCy7, CD4-PerCP, and CD8-APC) for 20 minutes, room temperature in the dark. Following another wash, cells were permeabilised by incubating with 100ul of Fix/perm solution for 20mins, room temperature in the dark. The cells were then washed 2 times with FACS perm/wash buffer and stained with IFNγ-PE for 20 minutes at 4°C. Following a final wash cells were fixed in 200µl 1% PFA and acquired on a CyAn flow cytometer.

2.12 HLA-B27 Gag Dextramer staining

PBMC were thawed and then rested for 2 hours. Following resting, cells were counted and resuspended in R10 at a density of $1x10^6$ cells/ml. 0.5-$1x10^6$ cells were transferred to a 12 x 75 mm polystyrene test tube and then washed with 5ml of PBS containing 5% FCS. The cell suspension was centrifuged at 1500rpm for 5 minutes, the supernatant removed and the cells resuspended in 50µl of PBS containing 5% FCS. 2µl of 0.32nM KK10 Dextramer was added, the suspension vortexed gently and incubated in the dark at room temperature for 10 minutes. Cells were then washed again with 2ml PBS containing 5% FCS and then incubated with Aqua Live/Dead Fixable stain, CD3-APCCy7 and CD8-APC for 20 minutes, room temperature in the dark. Following a final wash, 15µl of cytofix was added 15 minutes prior to acquiring the cells on a CyAn flow cytometer. The dextramer consists of HLA-B27 bound to the HIV-1 Gag

KRWIILGLNK (KK10) peptide on a dextran polymer backbone conjugated to PE. It was obtained from Immudex and titrated prior to use.

2.13 Viral inhibition assay

2.13.1 Propagation of HIV-1 isolates

HIV-1 isolates were obtained from the Centre for AIDS Reagents, National Institute for Biological Standards and Control (NIBSC), Health Protection Agency, UK. The laboratory-adapted CCR5-tropic clade B isolate BaL (ARP118) was used in all virus inhibition assays. Other laboratory-adapted and primary isolates of various clades were used in selected experiments. These were HIV-1_{IIIB} (clade B, CXCR4-tropic), HIV-$1_{92UG029}$ (clade A, CCR5/CXCR4 dual-tropic), HIV-$1_{RW93024}$ (clade A, CXCR4-tropic) and HIV-$1_{ES\ X\text{-}1936}$ (clade C, CCR5-tropic).

High titre stocks of these virus isolates were generated as follows. PBMCs from HIV-seronegative donors were resuspended in R10 medium at a density of $2x10^6$ cells/ml with PHA at a final concentration of 5µg/ml and incubated for 3 days. On day 3 of incubation, PHA-stimulated PBMCs were harvested, washed twice and infected with one of the five HIV-1 isolates by spinoculation at 2000rpm for two hours at 25ºC. Following spinoculation, 80% of the supernatant was removed and cells were resuspended at a density of $1x10^6$ cells/ml in R10 supplemented with IL-2 (20 IU/ml), transferred to a 25ml tissue culture flask, and incubated at 5% CO2 and 37°C for 5-7 days. Aliquots of infected cells were taken at days 2, 3, 5 and 7 post-infection for analysis of the percentage of infected cells by flow cytometry (below). On days three

and five, the supernatant, which contained the virus particles, was harvested from 25ml tissue culture flask for storage at -80°C in 2 ml freezer vials and the culture medium (R10/IL-2) was replenished as necessary.

2.13.2 Titration of HIV-1 isolates

Titres of stock viruses were determined by infection of PBMC with ten-fold serial dilutions of supernatants of each virus stock in R10/IL-2, using four replicate wells per dilution. Culture plates were incubated at 37°C with 5% CO_2 for 6 days. After 6 days, the percentage of infected cells was determined by staining for intracellular p24 antigen. $TCID_{50}$ was calculated using the Reed-Muench accumulative method

2.13.3 Isolation and infection of CD4+ T cells (target cells)

On day 0 of the virus inhibition assay, cryopreserved PBMC were thawed, washed and resuspended in R10. PBMCs were depleted of CD8+ T cells by magnetic bead selection following the CD8 separation protocol (described in 2.10 CD8+ T cell separation) and collecting the unlabelled fraction. These CD8- CD4+ cells were resuspended in R10 at a density of $1x10^6$ cells/ml and stimulated with PHA at a concentration of 5µg/ml for 3 days. On day 3, the cells were washed 3 times with fresh R10, counted and then infected with HIV-1 isolates at multiplicities of infection (MOI) ranging from 0.001 to 0.01 by spinoculation for 2 hours at 25 °C. MOIs for each virus isolate are shown in the Appendix. 80% of the supernatant was removed from the infected cells which were then resuspended at a density of $1x10^6$ cells/ml in R10/IL-2.

2.13.4 Isolation of CD8+ T cells (effectors) and virus inhibition assay set-up

Simultaneously on day 3 of the virus inhibition assay, a second vial of PBMCs was thawed and the CD8+ cells isolated by magnetic bead selection (see 2.10 CD8+ T cell separation). HIV-(super)infected CD4+ T cells were dispensed at $5x10^4$/well and cultured in duplicate (in R10 supplemented with 20IU/ml IL-2) either alone or with the freshly isolated autologous unstimulated CD8+ T cells at various effector:target (E:T) ratios according to cell numbers available (1:1 as a minimum). Cells were harvested either on day 3 (HIV-1_{A2}) or day 6 (HIV-1_{BaL}, HIV-1_{A1}, HIV-$_C$ and HIV-1_{IIIB}) and stained for intracellular p24 antigen.

2.13.5 Measurement of inhibition by CD8+ T cells by quantification of p24 antigen positive cells

Cell cultures described above were harvested, pelleted and washed in PBS. Cells were stained with Aqua Live/Dead Fixable stain for 20 minutes at room temperature, washed and then fixed with 20mg/ml lysolecithin in 4% paraformaldehyde for 2 minutes at room temperature. Cells were then permeabilised with cold 50% methanol for 15 minutes at 4ºC followed by further permeabilisation with 0.1% Nonidet P-40 for 5 minutes at 4ºC[397]. Cells were then stained with p24 antibody (KC-57 FITC) and cell surface antibodies (CD3-APCCy7, CD4-PerCP and CD8-APC) for 15 minutes at room temperature. Samples were acquired on a CyAn flow cytometer and data analysed using FlowJo software (version 9.2). CD8+ T cell antiviral inhibitory activity was expressed as percentage inhibition and determined as follows: [(fraction of p24+ cells in CD4+ T-

cells cultured alone) – (fraction of p24+ in CD4+ T-cells cultured with CD8+ cells)] / (fraction of p24+ cells in CD4+ T-cells cultured alone) x 100.

2.14 Quantification of integrated HIV-1 DNA in CD4+ T cells

2.14.1 Isolation of CD4+ T cells

PBMCs were thawed as described (section 2.7) and CD4+ T cells isolated using the CD4 Miltenyi isolation kit. Briefly, samples were centrifuged for 5 minutes at 1450rpm, the supernatant discarded using a pipette and the cells resuspended in 40µl of wash buffer. 10µl of the CD4+ T cell Biotin-Antibody cocktail was added. Cells were incubated for 5 minutes at 4°C. 30µl of wash buffer and 20µl of CD4+ T cell MicroBeads Cocktail were then added to the suspension which was incubated for a further 10 minutes at 4°C. Cells were resuspended in 1ml of wash buffer and separated using an autoMACS separator. The positive fraction (CD4+) was collected and kept on ice. 50µl of the positive fraction was placed in a cytometer tube with 50µl of PerfectCount Microbeads and 100µl of formaldehyde. The cells were counted using a FACS Kalibur.

2.14.2 DNA extraction

The samples were transferred into 1.5ml thread tubes and centrifuged using a minicentrifuge for 5 minutes at 2500rpm. The supernatant was discarded using first a p1000 pipette and then the residual volume removed using a p200 pipette. The appropriate quantity of lysis buffer (10 mM Tris HCl pH9, 0.1% Triton x-100,

400ug/ml Proteinasa K and deionized water) was added to each sample. Samples were then heated using a thermoblock at 55ºC for 10 hours and then 95ºC for 5 minutes. Samples were vortexed and kept at -20ºC until required.

2.14.3 Primers and probes

Each reaction consisted of a 20µl solution containing 10µl of mastermix, 1µl of primer-probe mix, 7µl of H_20 and 2µl of template DNA. An RPP30 primer/probe set was used for host genomic DNA quantification. The reaction mixture was loaded into a Bio-Rad droplet generator plate along with 70µl of oil. The plate was placed in a Bio-Rad QX-100 device and droplets generated following the manufacturer's instructions. The droplets were transferred to a 96-well PCR plate and sealed. DNA was amplified using an Applied Biosystems GeneAmp 9700 thermal cycler with the following cycling conditions: 10 minutes at 95°C, 40 cycles each consisting of a 30 second denaturation at 94°C followed by a 58°C extension for 60 seconds, and a final 10 minutes at 98°C. After cycling droplets were analysed immediately using a QX100 droplet reader, which analyses each droplet individually using a two-colour detection system (set to detect FAM and HEX). Analysis of copies/µl of target DNA was done using the manufacturer's software (Bio-Rad QuantaSoft v.1.2). QuantaSoft software measures the numbers of droplets that are positive (above a manually set threshold) and negative for each fluorophore in a sample. The fraction of positive droplets was then fitted to a Poisson distribution to determine the absolute initial copy number of the target DNA molecule in the input reaction mixture in units of copies/µl.

2.15 Entropy calculations

A Shannon entropy score was calculated for all beneficial/conserved elements peptides[398]. For each peptide, the LANL sequence database was used to identify all known sequences that belong to HIV-1 clade B (for Clade B specific beneficial peptide set and conserved elements set) or HIV-1 clade C (for Clade C specific beneficial peptide set). All gaps within sequences were removed and sequences were aligned and gaps included at either terminus to maximize similarity between sequences. The Shannon entropy of each amino acid position in the epitope was then calculated with the software Simon's Protein Entropy Widget (Simon Brackenridge) using the following formula (–∑ni=1pilogepi), where pi stands for the frequency of a given amino acid at position i. A peptide's entropy score was calculated as a mean of entropy scores of amino acid positions included in the peptide. The higher the Shannon entropy score the more variable the peptide.

2.16 Statistics

Statistical analysis and graphical presentation were performed using GraphPad Prism version 5.0 software. p values < 0.05 were considered significant. Linear mixed model analyses were conducted in collaboration with Dr. Eleni. Giannoulatou. Briefly, linear mixed effects models were fitted to the data following a described model formulation[399] and computational framework[400]. Linear mixed effects models describe a relationship between a response variable (in this case the SFU per million PBMCs) and covariates that have been measured or observed along with the response. They incorporate both fixed-effects parameters and random effects: here, the fixed categorical variables (i.e.

days and group [that is placebo or vaccinated]) are modelled by fixed effects, while random effects are used to capture inter-individual variability. The Wald test was used to test the null hypothesis. Models to explore predictors of inter-subject variation in viral inhibition by CD8+ T cells for STEP/Phambili participants were tested using multiple linear regression. Analyses were performed using SPSS version 2.2.

3. HIV-1-specific T cell responses following MVA.HIVconsv vaccination

3.1 Introduction

Highly active antiretroviral therapy (HAART) has had a huge impact on the life expectancy of HIV-1 positive individuals; because of its success HIV-1 has evolved into a chronic disease. However, patients require lifelong treatment with three or more antiretroviral agents to prevent disease progression. Treatment carries the risk of long-term toxicity and drug resistance[401], has no effect on the reservoir of latently infected CD4+ T cells established early in infection[5,341–344] and fails to restore effective HIV-1-specific immune responses[254,256,258]. Additionally, limited availability and the cost of antiretroviral drugs means approximately two-thirds of the 35 million people currently living with HIV-1 infection who are in immediate need of treatment are not receiving it. There is a need to develop new therapeutic approaches, with one possible approach being therapeutic vaccination.

The number of new HIV- 1 infections has fallen from 3.1 million in 2001 to 2.3 million in 2013; however the number of people living with HIV-1 has increased; 35.3 million in 2012 compared to 34 million at the end of 2010[3]. HIV-1 chronically infected individuals therefore represent the largest burden of the disease, thus making the development of a therapeutic vaccine for use in chronic infection a priority. The aim of therapeutic vaccination in the chronic stage of infection is to augment virus-specific host immune responses, both CD4+ T cell helper and CD8+ T cell responses, using a planned exposure to HIV-1 antigens whilst the virus is still suppressed by HAART. It assumes

that the natural response to HIV-1, which in most cases fails to prevent disease progression, can be modified by inducing entirely different responses from those primed by HIV-1. The ultimate goal is to completely clear infection, either through vaccination alone or in combination with latency reversing agents, in order to purge the reservoirs of latently infected cells. A more attainable objective might be to control viral replication to undetectable levels, as observed in HIV-1 controllers, to allow individuals to cease using HAART or to have extended periods without therapy ('remission') (Reviewed in[402]). However evolving treatment guidelines make HAART interruption difficult to justify and it will be necessary to demonstrate an impact on the latent HIV-1 reservoir before HAART interruption is considered.

In the early HAART era, several animal studies supported the rationale for therapeutic vaccination by showing partial control of viraemia after therapeutic vaccination during established infection with SIV. A study by Tryniszewska et al. demonstrated that vaccination of HAART-treated rhesus macaques with chronic SIVmac251 infection resulted in a ten-fold reduction in the viral set point upon treatment interruption as compared with controls[346]. Von Gegerfelt et al. used DNA or poxvirus-vectored SIV immunogens to vaccinate SIVmac251 infected rhesus macaques treated with antiretrovirals. Vaccination increased cellular immune responses and following cessation of therapy viraemia in 12 vaccinated animals was significantly lower compared to the 11 animals treated only with antiretroviral drugs[208].

In HIV-1 infected patients treated with HAART, therapeutic vaccination has been shown to boost cell mediated immune responses but had no significant impact on virus

rebound after cessation of therapy[363,365,403,404]. Dorrell et al. tested a MVA-vectored vaccine encoding HIV-1 clade A Gag p24/p17 sequences and multiple CD8+ T cell epitopes in 16 HAART treated HIV-1 infected individuals. Although the vaccine was immunogenic as demonstrated by augmentation of HIV-1-specific IFN-ɣ-secreting CD8+ and CD4+ T cells, three patients who underwent a supervised short-term HAART interruption following a third booster vaccination experienced rapid viral rebound[365,368]. An additional two studies have also shown MVA-vectored therapeutic vaccines to be immunogenic in HAART treated HIV-1 infected individuals[364,405]. These studies all employed viral vectored vaccines encoding full length proteins and were therefore likely to have boosted pre-existing CD8+ T cell responses by mimicking natural HIV-1 infection.

The first HIV-1-specific CD8+ T cell responses in primary infection are dominated by responses to variable regions of the virus[222]. Although highly immunogenic, these regions mutate easily under selective pressure from the immune system allowing the virus to rapidly escape from these immunodominant T cell responses. This is highlighted by work from Liu et al. which demonstrated that immunodominance and epitope entropy, a measure of conservation, could explain 49% of the variation in the time to escape[218]. These variable regions serve as decoys, diverting the immune response away from potentially beneficial regions of the proteome. Although HIV-1 has a huge capacity to diversify, certain regions are less tolerant of mutations due to their need to maintain an exact structure or carry out a precise function. Targeting these conserved regions by vaccination has several potential advantages. Firstly, it should redirect the natural response which has failed to eliminate infection towards less

variable, protective regions. Studies have shown a correlation between breadth of response to conserved regions and viral load[222,396,406] and a study by Li et al. demonstrated that the Ad5 gag/pol/nef vaccine used in the STEP trial induced responses biased towards less-conserved regions, which could partially explain the failure of the vaccine to control early viral replication[407]. Secondly, targeting conserved regions should limit the potential for the virus to escape and if escape were to occur it would be predicted to result in a significant fitness cost to the virus[244,408]. Wang et al. noted that CD8+ T cell responses in HIV-1 infected patients with protective HLA alleles preferentially targeted highly conserved regions of the virus; suggesting that CD8+ T cell responses capable of selecting for escape mutations within such regions may be critical to the control of virus[246]. Finally, because these regions are shared across clades a single immunogen could potentially be used universally.

HIVconsv is a chimeric protein comprising the 14 most conserved regions of the HIV-1 proteome[392]. The HIVconsv gene was inserted into three vectors, plasmid DNA, Adenovirus type 5 (Ad5) and Modified vaccinia Ankara (MVA) and delivered in various prime-boost vaccine regimens and it was shown to induce HIVconsv-specific responses in inbred mice. HIVconsv-specific T cell responses could also be detected by *ex vivo* IFN-γ Elispot in HIV-1-infected people, indicating that natural infection generates T cells specific to the conserved regions contained within HIVconsv[392].

This chapter investigates the effect of vaccination with MVA.HIVconsv, on the magnitude of the T cell response in HIV-1 infected HAART treated patients.

3.2 Results

3.2.1 Study subjects

Twenty three volunteers with chronic HIV-1 infection were recruited from the Genitourinary Medicine and Infectious Diseases Departments, Churchill Hospital, Oxford University Hospitals NHS Trust and the Department of Sexual Health, Royal Berkshire NHS Foundation Trust, Reading. Principal inclusion criteria were: HIV-1 seropositive individuals who had been treated continuously with three or more antiretroviral agents for at least 12 months, had a plasma viral load (pVL) below the limit of detection (< 50 copies/ml) and a CD4+ T cell count of >350 cells/µl. Ten volunteers were randomised to receive either MVA.HIVconsv ($5x10^7$ pfu) or placebo in a 4:1 ratio. It was intended to recruit and randomise a further 10 volunteers to receive either a higher dose of MVA.HIVconsv ($2x10^8$ pfu) or placebo in a 4:1 ratio. However, because of slow recruitment, only 9 subjects were enrolled in the higher dose group. This was a double-blind study. Table 3.1 summarises the clinical characteristics of the 19 volunteers who were vaccinated. Median CD4+ T cell count at enrolment was 630 cells/µl (range 360-2010), HAART had been administered for a median of 37 months (range 12-144) and the predominant virus subtype, where this was known, was B. Trial participants continued their normal HAART regimen throughout the study period and plasma HIV-1 RNA remained undetectable (<50 copies/ml).

Subject	Sex	Age	HAART duration (Months)	Viral subtype	CD4+ count (Cells/µ)l	CD4+ nadir pre-HAART (Cells/µl)
201	M	46	25	B	390	160
202	M	55	144	B	2010	70
203	M	50	64	NK	560	90
204	M	45	48	C*	650	170
205	F	43	60	C*	680	150
206	F	37	48	NK	440	40
207	M	39	37	B*	450	230
209	M	48	24	B	630	230
210	M	43	24	NK	930	NK
213	M	60	12	C*	360	159
214	M	55	14	B*	640	310
215	F	27	16	D	1270	NK
216	M	54	12	B	580	270
217	M	55	28	C	540	280
218	M	44	56	B	490	229
218	F	44	108	C*	990	NK
221	F	46	25	C*	430	NK
222	M	40	144	B	1060	NK
223	M	46	41	B*	730	NK
Median	-	**46**	**37**	**-**	**630**	**170**

Table 3.1 Clinical characteristics of trial participants. *not confirmed but predicted from patient history. NK; not known.

3.2.2 Vaccination schedule

On days 0, 28 and 84 volunteers in the low dose group (group A) received either MVA.HIVconsv $5x10^7$ pfu or placebo; volunteers in the high dose group (group B) received either MVA.HIVconsv $2x10^8$ pfu or placebo as described in Materials and Methods (Section 2.3.1). Figure 3.1 illustrates volunteer recruitment and retention throughout the study.

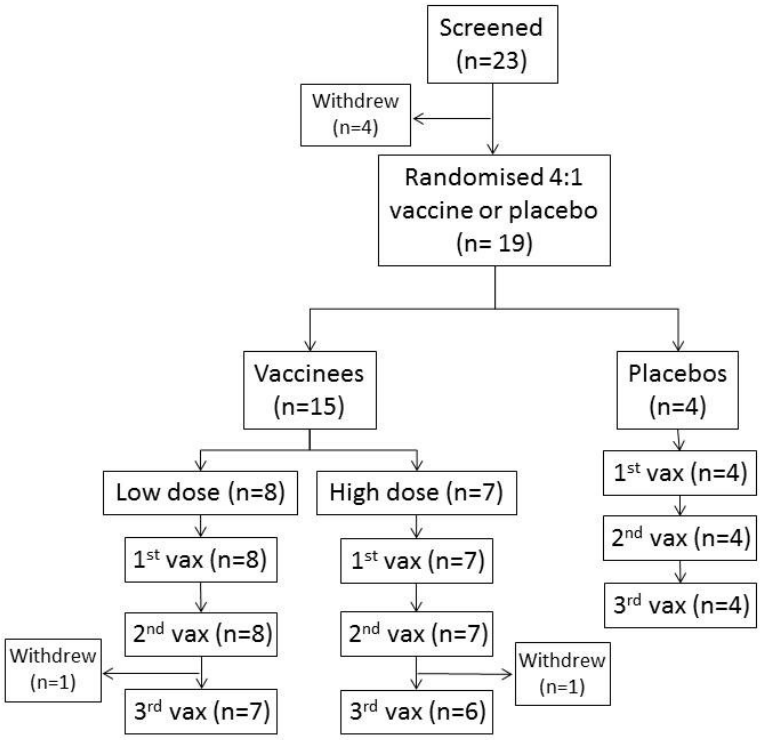
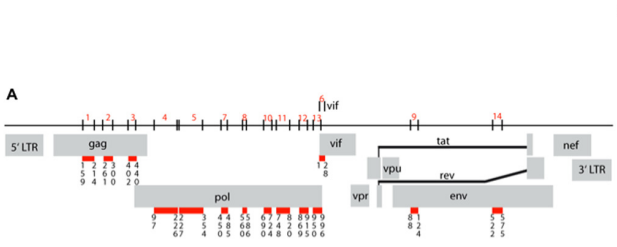


Figure 3.1 Volunteer recruitment and retention throughout the MVA.HIVconsv study

3.2.3 MVA.HIVconsv

The HIVconsv gene encodes the 14 most conserved regions of HIV-1 (Figure 3.2) and was inserted into the vector Modified Vaccinia Ankara (MVA).



Fragm	Protein	Clade	Identical consensus	HIV_{CONSV} (aa)
1	Gag	C	A2, CRF08	1-56
2	Gag	D	M, A2, F, G, K, CRF01	57-96
3	Gag	A	M, C, G, CRF01	97-135
4	Pol	B		136-265
5	Pol	C		266-393
6	Vif	D	B	394-421
7	Pol	A		422-457
8	Pol	B	M, A, C, D, F, CRF01, CRF02, CRF08	458-484
9	Env	C		485-521
10	Pol	D		522-556
11	Pol	A		557-629
12	Pol	B	M, A2, C, D, F, G	630-676
13	Pol	C	CRF08	677-723
14	Env	D	B	724-777
15	Added macaque, mouse and mAb epitopes			778-806

Figure 3.2 The HIVconsv immunogen. (A) Location of the HIVconsv segments across the HIV-1 proteome. (B) Summary of the fragments in HIVconsv showing the protein in which the fragment is located and the clade of the sequence. Figure from Létourneau et al, Plos One, 2007.

3.2.4 Assessment of non-specific (background) IFN-γ production in the Elispot assay

HIVconsv-specific T cells were enumerated using the standard interferon (IFN)-γ enzyme linked immunoabsorbent spot (ELISPOT) assay (IFN-γ Elispot) as described in Materials and Methods (Section 2.8). A total of 178 IFN-γ Elispot assays using freshly isolated peripheral blood mononuclear cells (PBMCs) and 98 IFN-γ Elispot assays using frozen PBMCs were performed. The mean of the negative control wells in the IFN-γ Elispot assays using fresh PBMCs was 33 spot forming units/million PBMCs (SFU/million PBMCs) (Figure 3.3), standard deviation was 30 SFU/million PBMCs and the mean plus 2*SD was 93 SFU/million PBMCs. In the 98 IFN-γ Elispots using frozen PBMCs the mean was 38 SFU/million PBMCs (figure 3.3), standard deviation was 28 SFU/million PBMCs and mean plus 2*SD was 94 SFU/million PBMCs. Assays were accepted if the PHA (positive control) wells were saturated. For the purposes of analysing magnitude and breadth a response was considered positive if it was > mean + 2*SD of the negative wells.

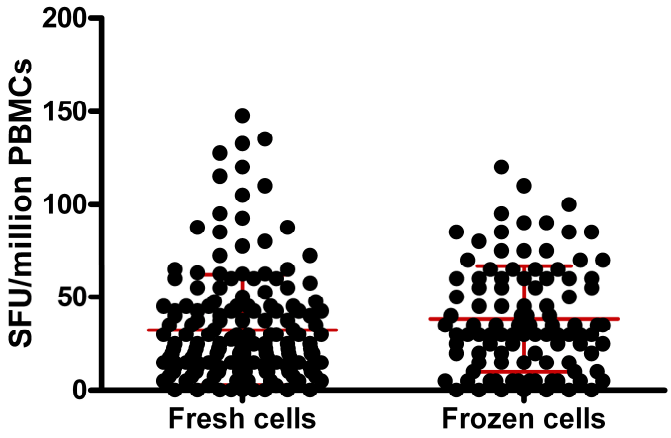


Figure 3.3 SFU/million PBMCs in the negative control wells for 178 IFN-γ Elispot assays using freshly isolated PBMCs and 98 IFN-γ Elispot assays using frozen PBMCs.$1x10^5$ PBMCs/well. Horizontal red lines indicate mean and standard deviation.

3.2.5 Rationale for and preliminary testing of the HIVconsv peptide matrix

To investigate changes in the magnitude of the T cell response to vaccination with MVA.HIVconsv a peptide matrix was used. This not only provides information on the magnitude of the response but can also enable efficient identification of the epitopic regions within the immunogen that are targeted. Determination of which epitopes are targeted by T cells after vaccination would entail testing each of the 199 HIVconsv peptides individually in an IFN-γ Elispot assay. This would require $10x10^6$ cells to test each peptide just once. By using peptides pooled in a matrix format, identification of individual peptide-specific T cell responses can be achieved with fewer cells. A 4D peptide matrix was designed using the software 'deconvolute this'[409], as described in Materials and Methods (Section 2.5.1).

Cells from an HIV-negative donor buffy coat were used to test the HIVconsv peptide matrix for non-specific responses. 64/80 pools gave a response of 0 SFU/million PBMCs and the remaining 20 pools all gave a response of 20 SFU/million PBMCs, which is below the mean plus 2*SD of negative control wells from fresh IFN-γ Elispot assays (100 SFU/million PBMCs) (Figure 3.4A).

When the peptide matrix was used in two separate IFN-γ Elispot assays carried out on different days but using PBMCs from the same HIV infected donor, positive pools were the same between the two assays and the list of peptides returned by 'deconvolute this' for retesting were identical (Figure 3.4B)

In order to confirm that the HIVconsv peptide matrix would accurately detect HIV-specific T cells, it was tested in parallel with a set of overlapping Clade B whole proteome consensus peptides using PBMCs from a HIV positive donor. (Figure 3.4C and D). Positive peptides identified for retesting by deconvolution of the matrix peptide were located within proteins for which the Clade B peptide pools also showed positivity (Table 3.2).

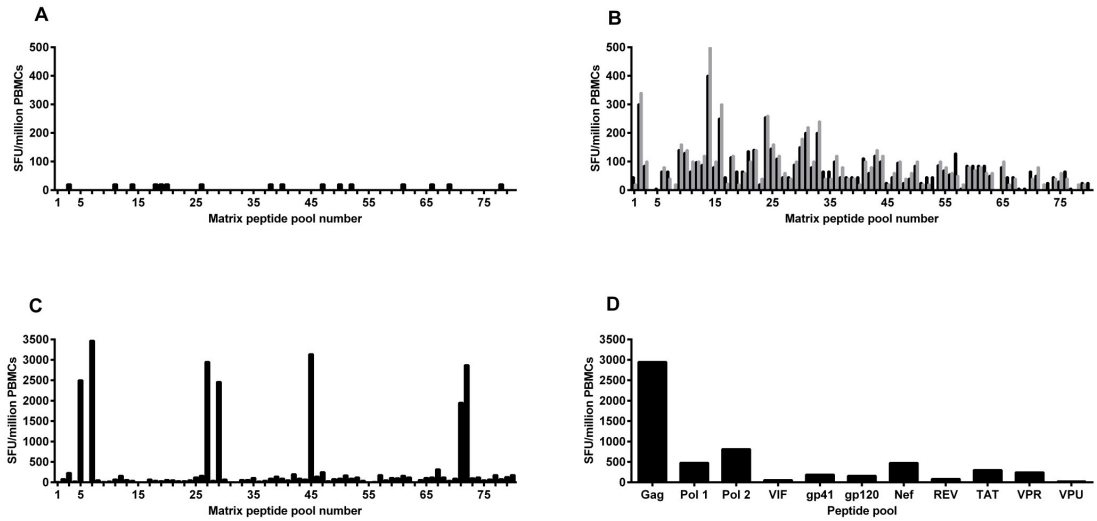


Figure 3.4 Preliminary testing of the HIVconsv peptide matrix. (A) PBMCs from a HIV-1 negative donor were used in an IFN-γ Elispot assay to test the HIVconsv peptide matrix for non-specific responses. (B) Responses to HIVconsv matrix peptides from two separate IFN-γ Elispot assays using PBMCs from a HIV-1 infected donor. Bars in grey indicate the second assay. Comparison of the IFN-γ response in a HIV-1 infected donor using (C) HIVconsv matrix peptides and (D) clade B whole proteome consensus peptides. Each pool in the HIVconsv peptide matrix contained 10 15mer HIVconsv peptides. Clade B whole proteome peptides were pooled according to protein source in a sequential manner. $1x10^5$ PBMCs/well and a final peptide concentration of 2µg/ml were used for all assays.

HIVconsv peptide returned for retesting	Protein origin of peptide	Protein positive in Clade B peptide set IFN-γ Elispot?[a]
5	Gag	Yes
6	Gag	Yes
9	Gag	Yes
49	Pol	Yes
68	Pol	Yes
76	Pol	Yes

Table 3.2 Peptides returned for retesting from first round HIVconsv peptide matrix IFN-γ Elispot assay in a HIV-1 infected donor. [a] positive if above mean plus 2*SD of negative control wells from fresh IFN-γ Elispot assay (100 SFU/million PBMCs). $1x10^5$ PBMCs/well and a final peptide concentration of 2μg/ml were used in all assays.

3.2.6 Quantification of HIVconsv-specific T cells pre and post-vaccination

IFN-γ Elispot assays with the 4D HIVconsv peptide matrix were performed using fresh PBMCs in all lower dose group subjects at all time-points in the trial schedule. In the higher dose group, IFN-γ Elispot assays with the 4D HIVconsv peptide matrix were performed on fresh PBMCs at screen/day 0, day 14, day 42 and day 266 time-points because it was observed that carrying out the assay at every time point did not return a higher number of peptides for retesting. All deconvoluted matrix data up to day 56 were collated and individual peptide sets for re-testing were devised for each subject based on these data. Responses to these peptides were then tested using frozen PBMC from each time-point in a single IFN-γ Elispot assay. The day 56 visit was chosen because it was anticipated that the peak response would be attained after two vaccinations as demonstrated in other therapeutic vaccine trials [364,365,368]; although one study reported further boosting with a third vaccination[410]. The total HIVconsv specific response was defined as the sum of the magnitude of all HIVconsv peptides retested in the second round assay that gave a response above the mean plus 2*SD of negative control wells in frozen IFN-γ Elispot assays (100

SFU/million PBMCs). Pre-vaccination responses were assessed at screening or day 0 and were detected in 17/19 volunteers, with a median frequency of 736 SFU/million PBMCs (range 123-6807). Figure 3.5, Figure 3.6 and Figure 3.7show the response to all retested peptides in low dose vaccinees, high dose vaccinees and placebos respectively at pre-vaccination and post-vaccination time points.

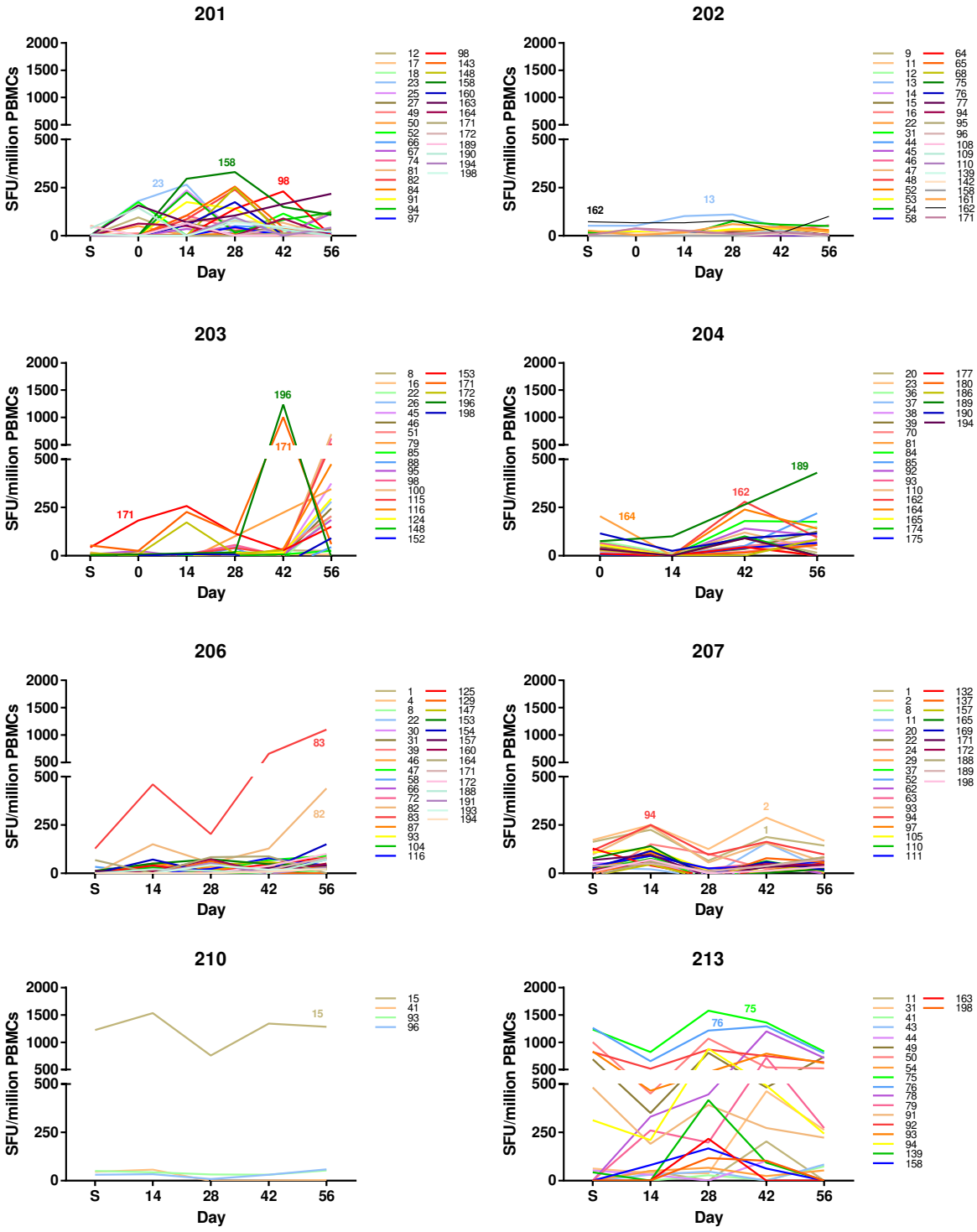


Figure 3.5 Response to MVA.HIVconsv vaccination in the lower dose vaccinee group. Frozen PBMCs were stimulated with individual HIVconsv peptides, identified for second round testing from deconvolution of the peptide matrix, in an IFN-γ Elispot assay. $1x10^5$ PBMC/well were used in the assay with a final peptide concentration of 2µg/ml. Graphs show all peptides retested in the second round assays irrespective of positivity above the negative cut-off. Results expressed as SFU/million PBMCs following background subtraction. Volunteers were vaccinated on days 0, 28 and 84 with $5x10^7$ pfu MVA.HIVconsv. Note 202 terminated the trial early and only received two vaccinations. Coloured numbers correspond to peptides with highest magnitude response.

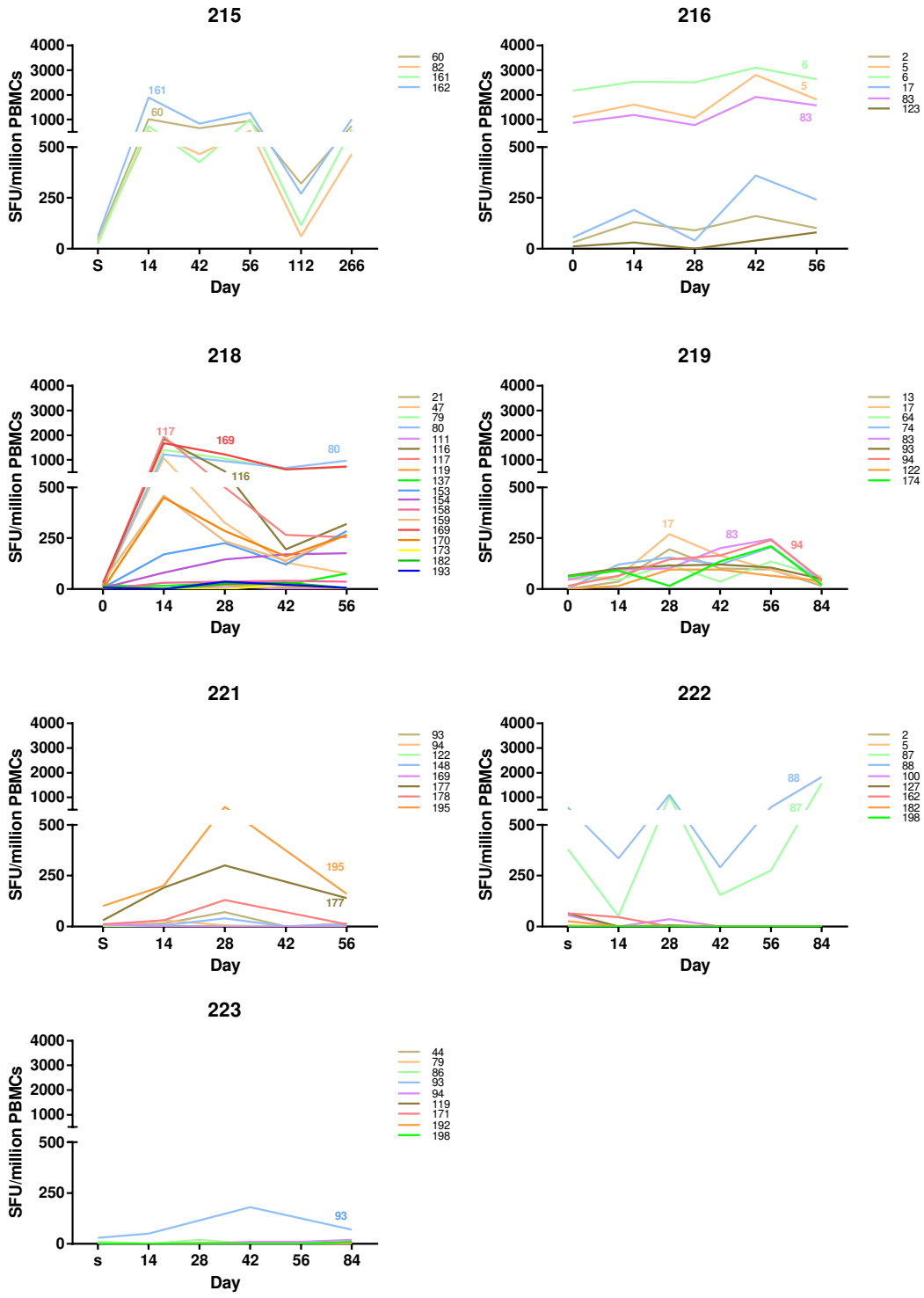


Figure 3.6 Response to MVA.HIVconsv vaccination in the higher dose vaccinee group. Frozen PBMCs were stimulated with individual HIVconsv peptides, identified for second round testing from deconvolution of the peptide matrix, in an IFN-γ Elispot assay. $1x10^5$ PBMC/well were used in the assay with a final peptide concentration of 2µg/ml. Graphs show all peptides retested in the second round assays. Results expressed as SFU/million PBMCs following background subtraction. Volunteers were vaccinated on days 0, 28 and 84 with $2x10^8$ pfu MVA.HIVconsv. Note 221 terminated the trial early and only received two vaccinations. . Coloured numbers correspond to peptides with highest magnitude response

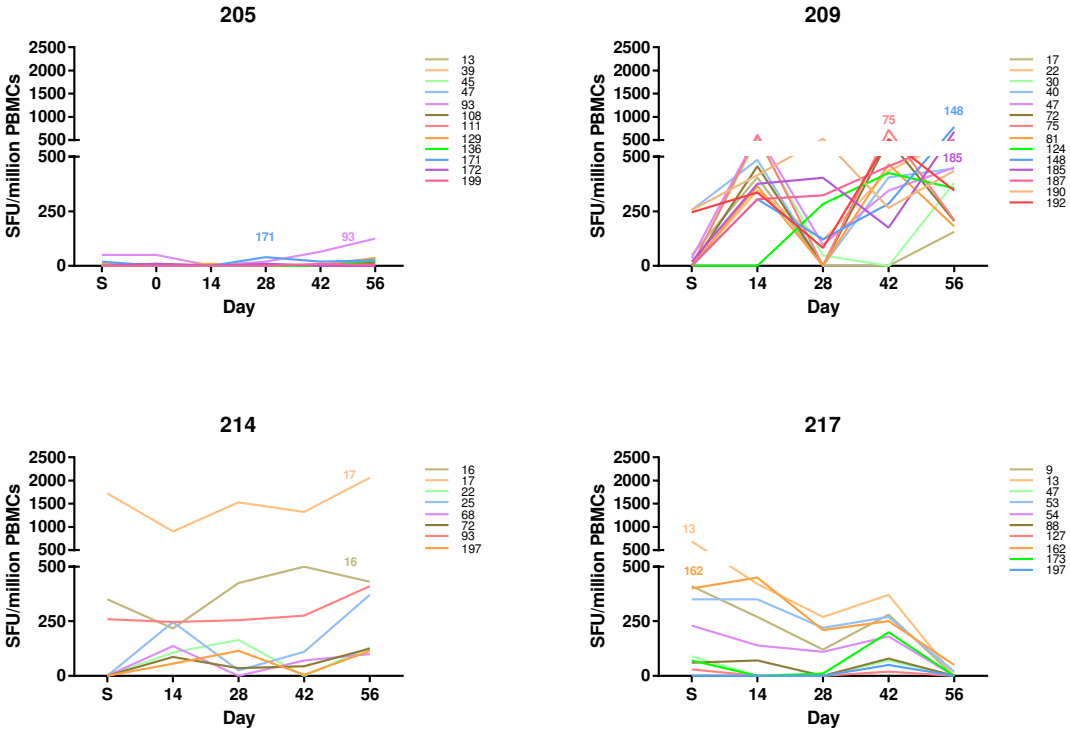


Figure 3.7 Response to MVA.HIVconsv vaccination in the placebo group. Frozen PBMCs were stimulated with individual HIVconsv peptides, identified for second round testing from deconvolution of the peptide matrix, in an IFN-γ Elispot assay. $1x10^5$ PBMC/well were used in the assay with a final peptide concentration of 2µg/ml. Graphs show all peptides retested in the second round assays. Results expressed as SFU/million PBMCs following background subtraction. Volunteers were vaccinated on days 0, 28 and 84 with placebo. Coloured numbers correspond to peptides with highest magnitude response

Following vaccination the peak response was not synchronised among the volunteers, as had been observed in previous studies of therapeutic HIV-1 vaccines based on full-length gene sequences[316,364,367], and occurred between days 14 and 56. All 19 volunteers had HIVconsv-specific responses above 100 SFU/million PBMCs at the peak of response and the median frequency was 1790 SFU/million PBMCs (range 125-10300 SFU/million PBMCs). There was a significant correlation between magnitude of response at baseline and magnitude at the peak of response in the vaccinees ($r = 0.66$, $p = 0.01$) (Figure 3.8) suggesting that MVA.HIVconsv vaccinations had boosted pre-existing responses. MVA has been shown to be effective at boosting T cell responses[306,314,411].

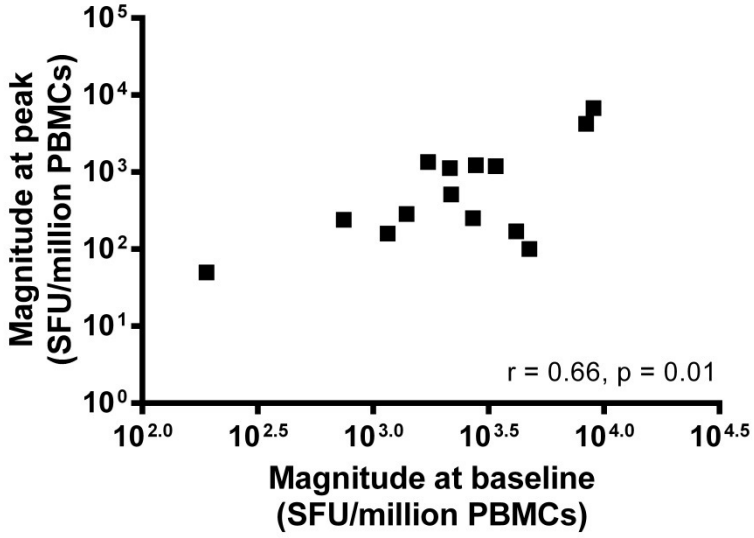


Figure 3.8 Correlation between magnitude of response to HIVconsv at baseline and at peak of response in vaccinees. Magnitude of response to HIVconsv is defined as the sum of all peptides retested in second round assays with a response above 100 SFU/million PBMCs. Statistical analysis performed using the Pearson correlation test.

In view of the inter-subject variation in baseline responses to HIVconsv, together with lack of synchronicity in responses to vaccination, linear mixed models were used to determine whether any changes in the magnitude of response over time, quantified as the sum of all peptides retested in second round assays with a response above 100 SFU/million PBMCs, were due to vaccination with MVA.HIVconsv. Although there was a significant difference in the magnitude of response over time ($p = 0.001$), there was no significant difference between vaccinees and placebos ($p = 0.48$) (Table 3.3). The change in magnitude of response observed in placebos over the course of the study highlighted the natural variation in the HIV-1-specific T cell response during the course of long-term HAART treatment. Participants' IFN-γ Elispot responses in negative control wells and to a pool of FEC peptides (data shown in appendix) did not vary over time, confirming that variation in assay conditions e.g. cell input numbers, was not the reason for the change in magnitude of response to HIVconsv observed in placebo

Variable	dF	F	p
Intercept	644	17.2	0.00004
Days	644	3.3	0.00100
Group	17	0.5	0.48300

Table 3.3 Linear mixed model investigating the effect of MVA.HIVconsv vaccination on the magnitude of response over time (Variable; days) and between vaccinees and placebos (Variable; group).

subjects.

Figure 3.9 shows the location of HIVconsv peptides that had a response greater than 100 SFU/million PBMCs in second round testing across the HIVconsv immunogen. Individual positive peptides confirmed for each patient and possible epitopes within these peptides (according to the Immunology database, Los Alamos National Laboratories) are given in the Appendix. Pre-vaccination responses to HIVconsv were

distributed evenly across the immunogen, as were new responses post-vaccination, indicating all regions of the immunogen were similarly immunogenic. The median breadth of response at baseline was one peptide (range 0-9), this increased to a median of five peptides (range 1-20) at the peak of response. However, linear mixed models indicated that there was no significant difference between vaccinees and placebos in the breadth of response following vaccination ($p = 0.45$) (Table 3.4)

Variable	dF	F	p
Intercept	70	28.1	0.000001
Days	70	2.9	0.007900
Group	16	0.59	0.450000

Table 3.4 Linear mixed model investigating the effect of MVA.HIVconsv vaccination on the breadth of response over time (Variable; days) and between vaccinees and placebos (Variable; group).

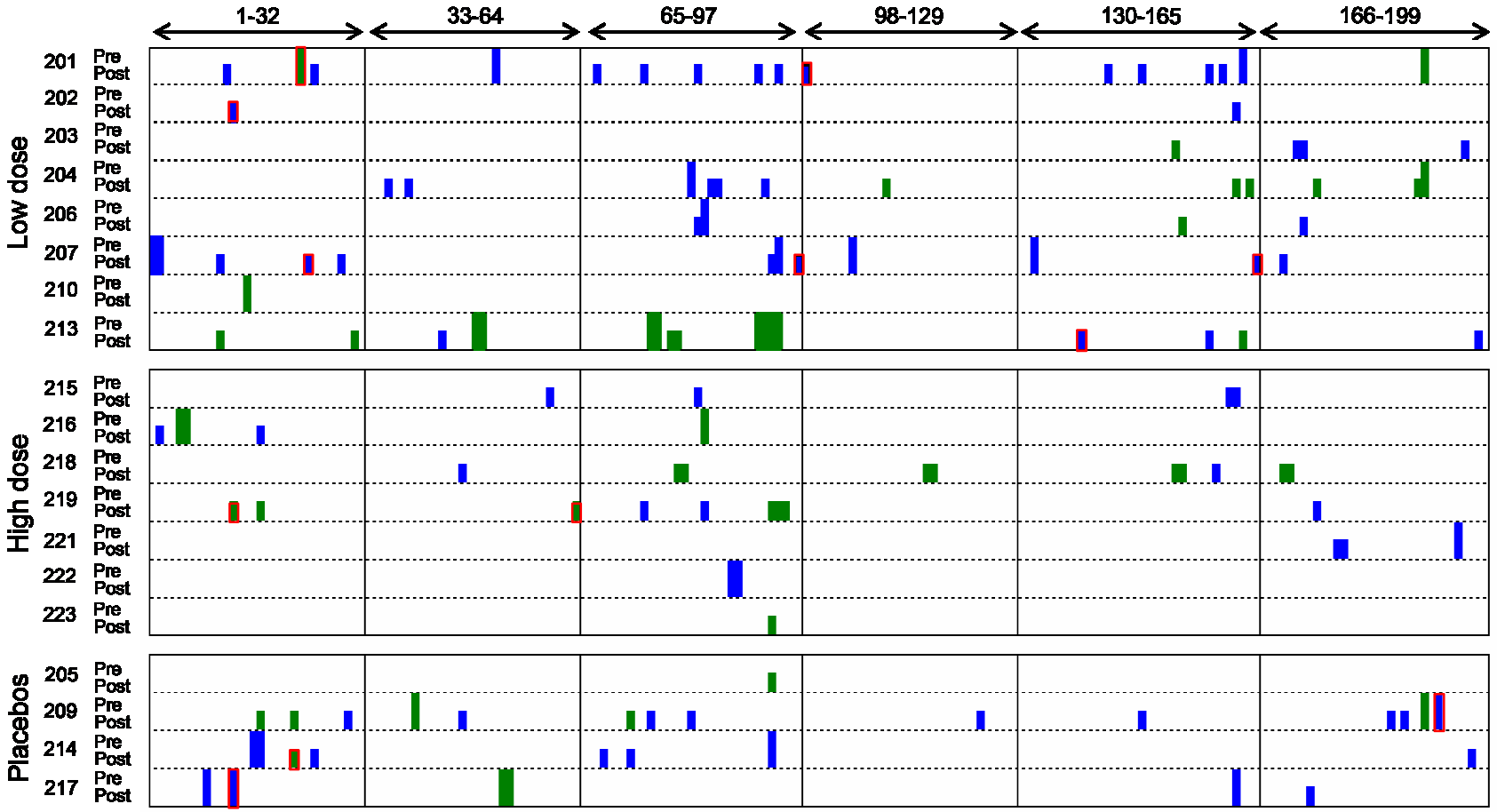


Figure 3.9 Location of confirmed positive peptides (above 100 SFU/million PBMC) across the HIVconsv immunogen pre and post-vaccination (included if peptide was above 100 SFU/million PBMCs at any time point after screening/day 0). Green boxes indicate epitopes which had been confirmed for the HLA of that volunteer in the LANL database, blue boxes show epitopes that had not been mapped for the HLA of that particular volunteer. Boxes with a red outline indicate junctional peptides which span two proteins.

Confirmatory IFN-γ Elispot assays were carried out at all trial visit time points using peptide pools in which the 199 HIVconsv peptides were divided sequentially into 6 'conventional' pools (described in Materials and Methods section 2.5.2) (Figure 3.10). There was a good correlation between the magnitude of response measured using the matrix peptide pools and the sum of the responses to the single peptide pools (r = 0.86, p = <0.0001) (Figure 3.11).

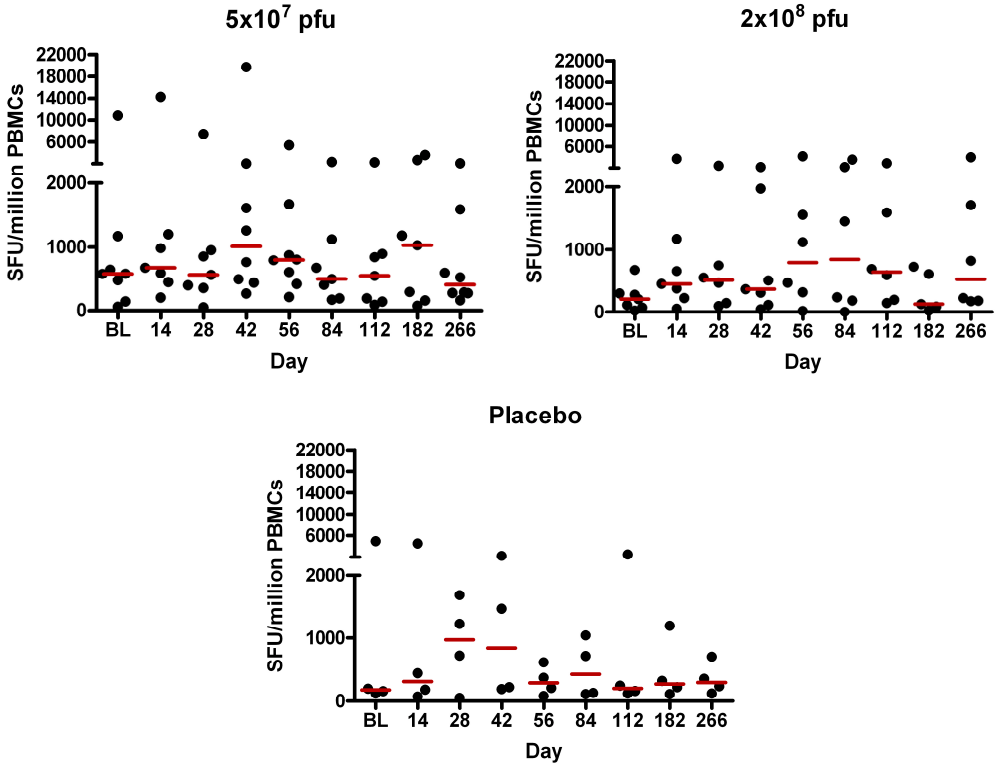


Figure 3.10 Magnitude of response to MVA.HIVconsv vaccination in the lower ($5x10^7$ pfu) and higher dose ($2x10^8$ pfu) groups and placebos quantified using single peptide pools. Freshly isolated PBMCs from each time point were stimulated with 6 pools of peptides in an IFN-γ Elispot assay. Peptides were at a final concentration of 2µg/ml. Data shown is the sum of responses to pools 1-6. Horizontal red line shows median response at each time point. Baseline is the mean of Screen and day 0.

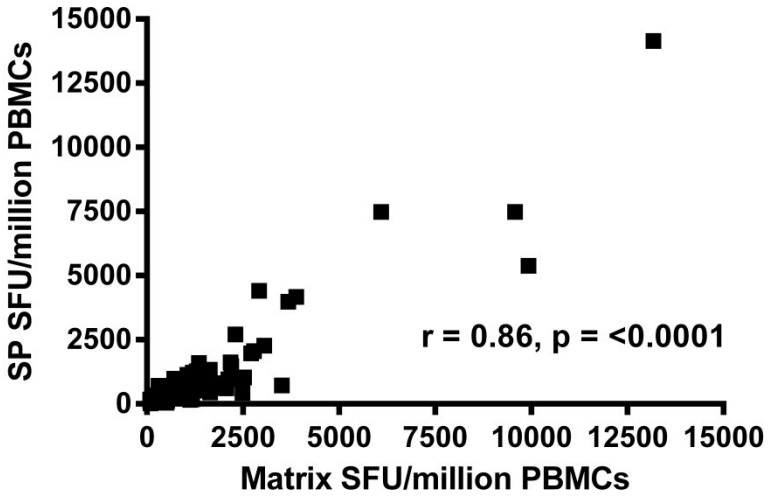


Figure 3.11 Correlation between the magnitude of response to HIVconsv matrix peptide pools and single pools measured by IFN-γ Elispot assay. Statistical analysis conducted using the Spearman rank test. The correlation persists on removal of the three outliers (r = 0.84, p = 0.0001).

Linear mixed models were again used to assess changes in magnitude of response to single pools 1 to 6; this analysis confirmed that there was no difference in the magnitude of response following vaccination with MVA.HIVconsv between vaccinees and placebos to any of the individual pools. This was also true when the vaccinees were split into low and high dose groups (results shown in the Appendix). This indicates that there was not a significant increase in the magnitude of response to one particular area of the immunogen that was being masked by analysing responses to the immunogen as a whole.

3.2.7 MVA.HIVconsv vaccination induces T cell responses to junctional regions in a minority of participants

Due to the chimaeric nature of HIVconsv, a potential problem is the induction of T cell responses to unnatural, irrelevant epitopes at the boundaries of fragments[308]. There are 14 such junctional regions within HIVconsv (Figure 3.12A). The HIVconsv peptides are

15mers overlapping by 11 amino acids; this means there are 3 separate HIVconsv peptides that span each junctional region (Figure 3.12B).

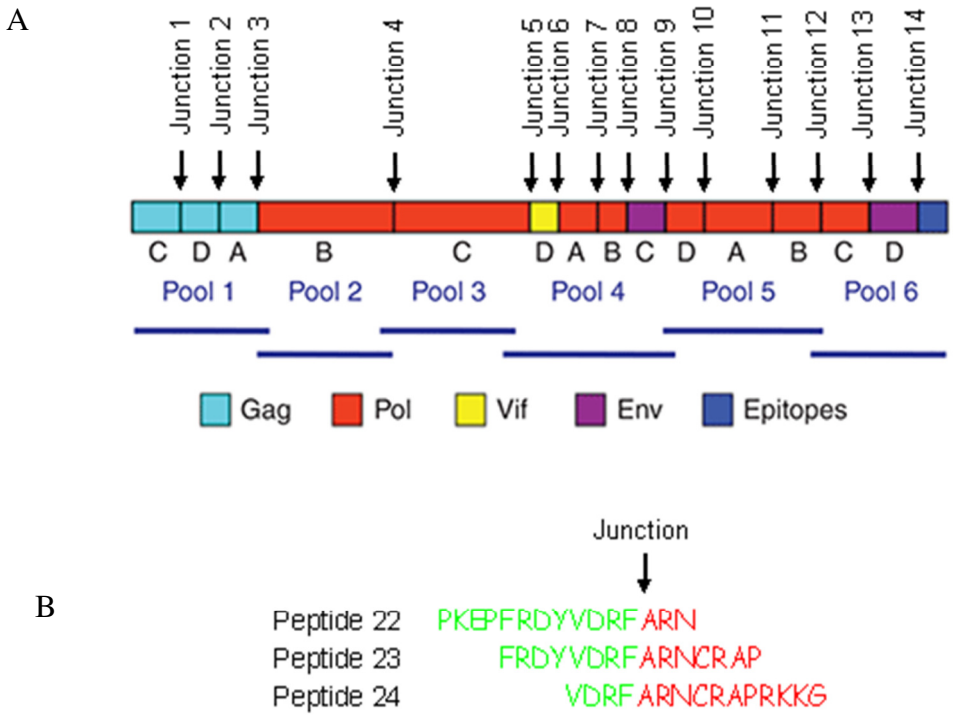


Figure 3.12 (A) Junctional regions across the HIVconsv immunogen formed by the joining of fragments from different proteins or similar proteins from different clades. Figure adapted from Borthwick et al., Molecular Therapy, 2014. (B) Representation of one junctional region showing that three individual HIVconsv peptides span each junction.

Defining a response to a junctional region as a response to any one of the three peptides that span the junction, 3/19 trial participants had a response greater than 100 SFU/million PBMCs at baseline. This increased to 9/19 participants at the peak of response, with four participants targeting more than one junction. However a response to either of the two outer peptides (peptides 22 and 24 in figure 3.9B) is likely to be to an 8-11-mer epitope located to the left or right of the junction respectively rather than to an unnatural epitope spanning the junction. Therefore if defining a true junctional response as only a response to the central peptide, 1/19 trial participants had a response

above 100 SFU/million PBMCs pre-vaccination; this increased to 4/19 volunteers post-vaccination (Table 3.5).

Junction	Region	Patient	Magnitude of response pre-vax	Percent of total HIVconsv response pre-vax	Magnitude of response post-vax	Percent of total HIVconsv response post-vax
1	Gag C → Gag D	202	53	42	110	60
		217	690	32	420	26
		219	0	0	195	17
2	Gag D → Gag A	201	0	0	265	16
		207	0	0	150	8
		209	0	0	520	9
		214	0	0	165	6
3	Gag A → Pol B	None				
4	Pol B → Pol C	219	55	19	135	10
5	Pol C → Vif D	201	0	0	230	26
6	Vif D → Pol A	207	108	11	125	7
7	Pol A → Pol B	None				
8	Pol B → Env C	None				
9	Env C → Pol D	None				
10	Pol D → Pol A	213	43	1	417	5
11	Pol A → Pol B	None				
12	Pol B → Pol C	207	28	3	115	6
		218	30	21	1675	16
13	Pol C → Env D	None				
14	Env D → M&M	209	245	28	525	10

Table 3.5 Magnitude of response to junctional peptides quantified from HIVconsv individual peptide second round retest assays. Post-vaccination response is the time point for which the junctional peptide gave the highest magnitude response. Percent of total HIVconsv response calculated using the sum of all peptides above 100 SFU/million PBMCs from second round assays at the time point concerned. Table shows responses to all 3 peptides spanning a junction, responses to the central peptide of a junction are highlighted in red.

3.2.8 Responses to HIVconsv are subdominant

Conserved epitopes are recognised with a probability similar to that for variable epitopes; however conserved epitopes generally elicit subdominant responses during both primary and chronic infection[406]. In a subset of vaccinees I was able to measure the magnitude of response to whole Gag and Pol using a set of clade B consensus Gag and Pol peptides and compare this to responses to HIVconsv single peptide pools at pre and

post-vaccination time points. The HIVconsv immunogen only covers 25% of the total HIV-1 proteome and comprises mainly sequences from Gag and Pol, therefore, responses to whole Gag and Pol only were used for comparison. At baseline, responses to HIVconsv were of a lower magnitude than responses to whole Gag and Pol in 3/4 volunteers; in 4/4 volunteers HIVconsv responses were lower than whole Gag and Pol responses post-vaccination (Figure 3.13). In volunteer 206, despite no change in the magnitude of response to HIVconsv peptides between baseline and day 42 there was an increase in the magnitude of response to the clade B whole Gag and Pol peptides. This again highlighted the natural variation in HIV-1-specific T cell responses in patients on long term HAART.

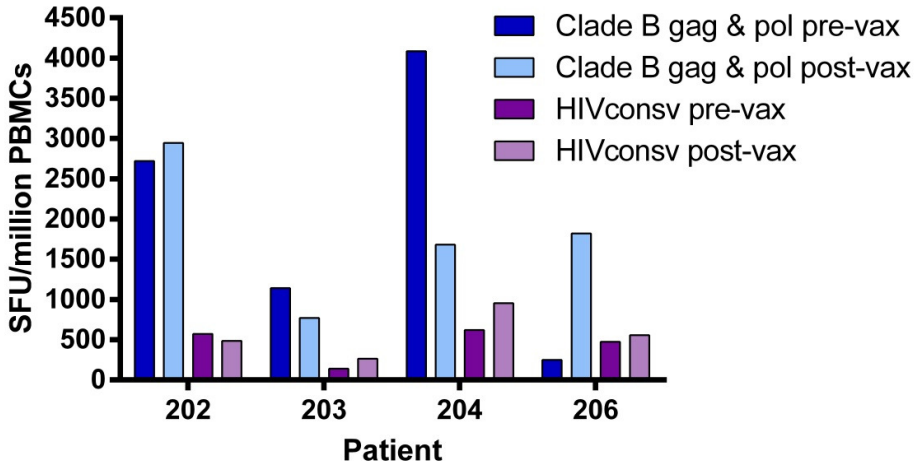


Figure 3.13 Magnitude of response to the whole proteome measured using a clade B consensus peptide set and to HIVconsv peptides, quantified as the sum of responses to single pools 1-6, measured at baseline and post-vaccination by IFN-γ Elispot assay. Post-vaccination time point used was either day 28 or 42. $1x10^5$ PBMCs/well, final peptide concentration was 2μg/ml.

3.2.9 Responses to MVA.HIVconsv are mediated predominantly by CD8+ T cells

To investigate if the response to vaccination with MVA.HIVconsv was mediated by CD8+ T cells, IFN-γ Elispot assays using whole PBMC, positively selected CD8+ T-cells and CD8+ T cell-depleted PBMC were carried out for all subjects in the low dose group. Responses were calculated as spot forming units per million PBMC, per million CD8+ T-cells or per million CD8+ T-cell depleted PBMC. In 7/10 volunteers the response to HIVconsv was predominantly (>50%) CD8+ T cell mediated (Figure 3.14). Interpretation is limited because the CD8+ T-cell fraction was enriched for cells that could secrete IFN-ɣ upon stimulation as compared with the whole PBMC fraction and CD8+ depleted fraction.

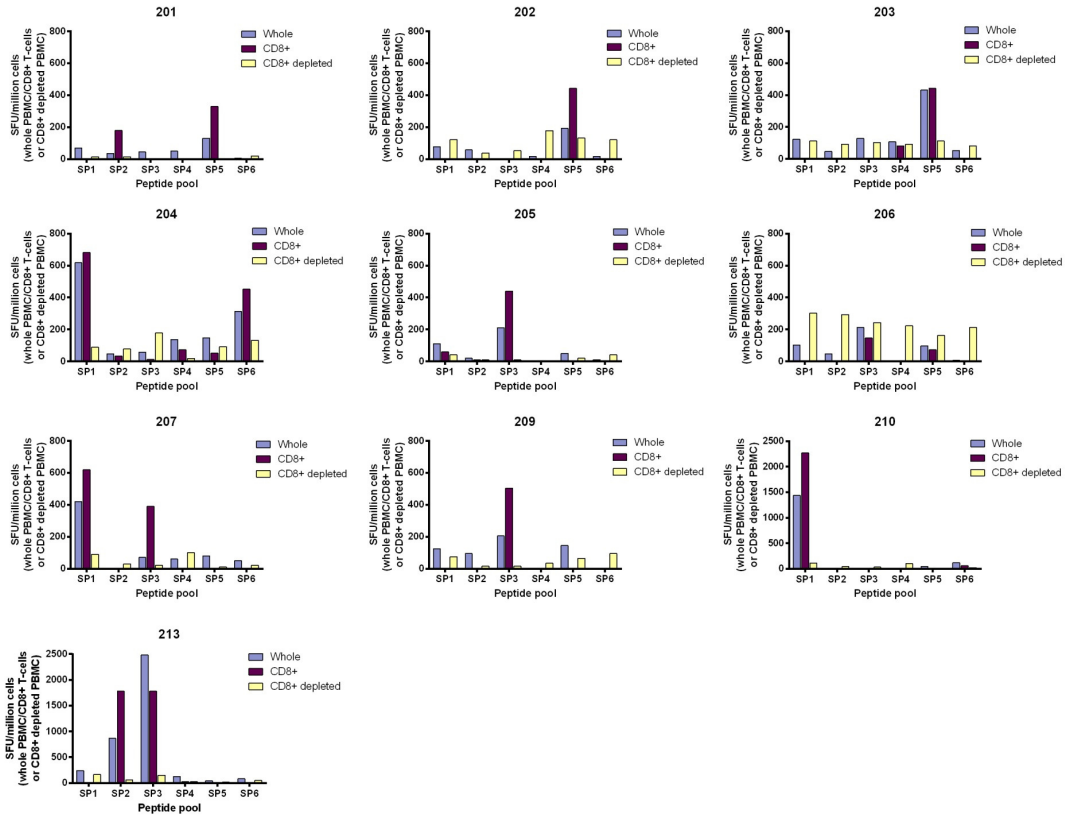


Figure 3.14 IFN-γ Elispot responses to HIVconsv single pools 1-6 using whole PBMCs, a CD8+ T cell positively selected fraction and a CD8+ T cell depleted fraction in the 10 lower dose volunteers. Note 205 and 209 are placebos. $1x10^5$ cells/well, final peptide concentration 2µg/ml.

3.2.10 Vector specific responses are of low magnitude

Volunteers received three vaccinations of MVA.HIVconsv. Since repeated delivery of MVA expressing a cancer antigen was shown to lead to the induction of high magnitude vector-specific T-cells that dampened the response to the immunogen through competition by vector-specific T cells, responses to the MVA vector were measured in the trial participants[412].

T cell responses to the vector were measured by IFN-γ Elispot assay using PBMCs cultured with MVA at an MOI of 1. Responses to MVA were of low magnitude at baseline (Median 30 SFU/million PBMCs) and post-vaccination (33 SFU/million PBMCs) and did not out-grow responses to the immunogen. There was no correlation in the vaccinees between the magnitude of response to MVA and the magnitude of response to HIVconsv at either baseline (r = 0.18, p = 0.51) or the peak of response (r = 0.03, p = 0.87). (Figure 3.15 A&B).

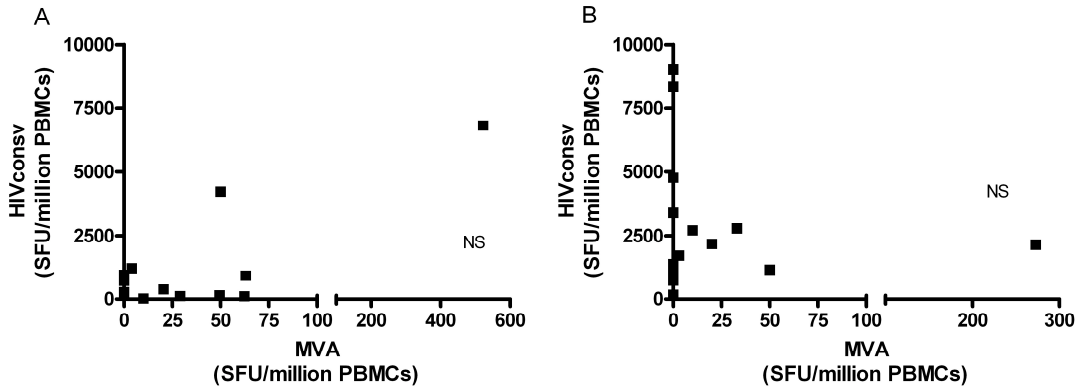


Figure 3.15 No correlation between response to HIVconsv and MVA at (A) baseline and (B) peak of response in 15 vaccinees measured by IFN-γ Elispot assay. Spearman rank test used to assess correlation.

3.3 Discussion

Experiments in this chapter sought to investigate the T cell immunogenicity of a rationally designed, conserved region immunogen tested for the first time in HIV-1 infected individuals on HAART.

CD8+ T cell responses in the majority of HIV-1 infected patients ultimately fail to control virus replication and prevent progression to AIDS. Simply boosting these pre-existing CD8+ T cell responses with a therapeutic vaccine is unlikely to result in better immune control. Studies employing structured treatment interruption (STI) as a means of boosting pre-existing HIV-1-specific responses have failed to show enhancement of viral control[413]. We therefore hypothesised that it would be necessary to redirect the immune response to more vulnerable regions of the proteome, such as conserved regions[222,396,406]. Two other groups to date are developing conserved region immunogens. Rolland et al. identified regions within Gag of at least 12 amino acids in length where each amino acid is found in more than 98% of all available independent group M sequences[414]. Seven such regions, named conserved elements (CEs), were identified within Gag and a prototype DNA vaccine expressing these seven CEs as a single protein (p24CE) was engineered and shown to be immunogenic in rhesus macaques[415]. Mothe et al. used functional data from a cohort of 950 untreated B HIV-1 infected individuals to identify overlapping peptides targeted by individuals with reduced viral loads[396]. Gene segments encoding these regions were assembled and cloned into a DNA plasmid and a MVA vector and shown to be immunogenic in rhesus macaques (Mothe et al., personal communication). Both of these conserved region immunogens await testing in human clinical trials.

Vaccination with MVA.HIVconsv was safe and well tolerated. Responses to HIVconsv were predominantly CD8+ T cell mediated and were subdominant. Encouragingly, these responses were located across the whole of the HIVconsv immunogen indicating that all regions of HIVconsv are immunogenic.

The level of variation in pre-existing responses to HIVconsv in placebo recipients over the course of the study was unanticipated. As a consequence, the observed increases in T cell responses following vaccination with MVA.HIVconsv in 15/15 vaccinees did not reach statistical significance, nor could we confirm induction of significant de novo responses to epitopes within HIVconsv. This was confirmed by using two different approaches – single peptide pools and matrix peptide pools. This not only highlights the importance of the inclusion of placebos in vaccine trials but also demonstrates the variation in T cell response in patients on long-term treatment. Only one study has investigated changes in T cell responses over time during long-term HAART and it showed concurrent expansions (10-1570 SFU/million PBMCs) and contractions (-10 to -1850 SFU/million PBMCs) of T cell responses[416]. Importantly, negative control wells and responses to a set of FEC peptides were consistent throughout the study period in all MVA.HIVconsv trial volunteers and plasma viral loads remained undetectable. The extent of this natural variation in the CD8+ T cell response during HAART needs to be precisely defined in order to understand what level of vaccine effect needs to be observed in order to detect a significant effect of vaccination. This highlights the need for adequately powered studies to detect an effect of vaccination in HAART treated patients above that of placebos.

Reasons for the lack of efficacy of MVA.HIVconsv could include the vector/vaccination regimen used and the trial population studied. These will be discussed in turn below.

The magnitude, quality and durability of response to a vaccine are dependent not only on the design of the immunogen, but also on the delivery system. The delivery system used in this trial was the viral vector, MVA, which was administered in a homologous regimen on days 0, 28 and 84. It is possible that repeated vaccination with the same vector can lead to a damping of immunogen specific responses and a previous trial demonstrated that poxvirus vector-specific T cells were of a higher magnitude than the tumour antigen insert specific T cells[412]. However work from both Goepfert et al. and Harrop et al. showed that repeated administration of an MVA-vectored vaccine was able to re-boost immunogen specific responses to levels observed after the first vaccination[417,418]. In our study there was no reduction in the magnitude of response to HIVconsv due to anti-vector responses. We have also demonstrated this in a previous study[419]. In the four trial participants tested, pre-vaccination and post-vaccination T cell responses to HIVconsv were subdominant, which is consistent with an observational study by Liu et al. in HIV-1-infected individuals[406]. In a therapeutic vaccination setting, vaccine-induced responses to subdominant epitopes, such as those within some conserved regions, may have to compete with pre-existing immunodominant responses; therefore a more immunogenic vector/regimen may be required. Numerous studies have indicated that a heterologous viral vector prime boost regimen such as DNA prime/MVA boost, delivering the same insert generates not only a higher frequency, but also a better ‘quality’ of T cell, for example an increased frequency of polyfunctional

cells, higher IFN-ɣ production on a per cell basis, or, CD8+ T cells of higher avidity[313–315,317,318,365]. Due to the limited immunogenicity of DNA plasmids, the immune response is focused on the encoded vaccine antigens. This is not the case with viral vectors such as MVA, which induce an immune response not only to the transgene product but also to vector antigens. Repeated vaccination with the same MVA vaccine activates both the antigen-specific and vector-specific T and B lymphocyte populations, potentially resulting in competition between these populations for APCs as well as growth and survival factors. By contrast, boosting with a poxvirus vector following a DNA prime, transgene-specific memory T cells induced during the prime have a competitive advantage over naïve T cells that are primed to vector antigens present in the boosting vaccine. Additionally, the presence of short dinucleotide sequences (CpG motifs) in the bacterial plasmid strongly stimulates IL-12 production. IL-12 has been shown to provide a third signal during T cell activation, which is required for the generation of CD8+ T cells with optimal effector function[420]. In the HIV-CORE 002 trial, prime-boost vaccinations in HIV-1 uninfected individuals with the HIVconsv immunogen delivered by a DNA and / or replication-defective simian adenovirus, ChAdV63, followed by MVA, induced median peak T cell responses of 5000 SFU/million PBMCs[308]. Investigation of this regimen in HIV-1 infected patients is underway (ChAd-MVA.HIVconsv-BCN01, Clinicaltrials.gov). The ChAd/MVA vector combination has also been shown to elicit very high frequencies of T cells specific for P. falciparum malaria[306,307,421,422]. MVA is a highly attenuated, non-persisting vector, therefore, the duration of transgene expression is short[423–425]. Estcourt et al. vaccinated C57BL/10 mice with MVA.HIVA-NP and showed that MVA-stimulated T cells first started to proliferate within 3–5 days after vaccination, however after this period no more cells

entered cycling[426]. Whereas mice vaccinated with pTH.HIVA-NP exhibited proliferation of MVA-stimulated T cells for up to 14 days post-vaccination. This indicates that MVA is a non-persisting vaccine and the MVA-infected cells are rapidly cleared from the body. It is possible that a persisting vector will be required to maintain immunogen-specific CD8+ T cell responses at sufficient levels to a) prevent establishment of persistent infection in the case of a preventive vaccine and b) eliminate virus-infected CD4+ T cells and prevent escape in the case of a therapeutic vaccine.

Hansen et al. demonstrated that vaccination of macaques with SIV vaccines vectored by RhCMV, a persistent virus, established effector memory T cell responses which controlled SIVmac239 infection early after mucosal challenge in 13/24 animals. Long term control was observed (<1 year) in 12/13 animals[427]. More recently, Hansen et al. showed that the protected rhesus macaques lost signs of SIV infection over time. Tissues sampled from these animals 69-172 weeks after challenge had no detectable SIV RNA or DNA above background levels by ultrasensitive quantitative PCR analysis. This is the first time clearance of a pathogenic lentiviral infection by a T cell vaccine has been demonstrated[210].

CD4+ T cells are the primary target of HIV-1 infection and during the acute phase of infection there is a massive loss of CD4+ T cells, particularly from the GALT[188]. CD4+ T cell help is essential for the optimal functioning of CD8+ T cells during infection, not only in the priming of new responses[185,186] but also for the maintenance of effector functions during chronic infection[187]. The patients studied in this trial had all initiated antiretroviral therapy during chronic infection and had a low pre-treatment CD4+ cell

nadir. Although they all had CD4+ cell counts >350 cells/µl at enrolment, it is possible that the function of these cells was not fully restored by HAART, resulting in inadequate CD4+ T cell help for CD8+ T cell responses following vaccination. This may have resulted not only in inefficient priming of new CD8+ T cell responses but also reduced function of memory CD8+ T cells upon recall by vaccination, leading to an ineffective antiviral CD8+ T cell response. Initiation of antiretroviral treatment in acute HIV-1 infection has been shown to preserve immune functions such as helper T cell responses[200,221]. This could provide a better environment in which to give a therapeutic vaccination. We are currently conducting a clinical trial with collaborators at the Irsicaixa AIDS Research Institute, Barcelona to investigate therapeutic vaccination with ChAdV63.HIVconsv / MVA.HIVconsv in patients who are diagnosed and treated with HAART during acute infection (ChAd-MVA.HIVconsv-BCN01, Clinicaltrials.gov).

Therapeutic vaccination in early infection could also limit the size of the HIV-1 latent reservoir. HIV-1 controllers have a reduced number of latently infected cells compared to progressors[428–430]. Blankson et al. demonstrated that CD8+ T cells from HIV-1 elite controllers were able to eliminate non-productively infected resting and activated CD4+ T cells more efficiently than CD8+ T cells from progressors. These were cells that were infected *in vitro* with a GFP-tagged HIV-1 isolate but were eliminated in the window period before synthesis of new virions, i.e. prior to expression of GFP[429]. Graf et al. used an *in vitro* model of latency to show that resting CD4+ T cells can produce HIV-1 Gag without producing infectious virus[431,432]. Such cells may have been infected as they were transitioning from an activated to a resting state and could be precursors of latently infected cells. The effective elimination of these cells through recognition of Gag

proteins may partially explain why HIV-1 controllers have a much lower frequency of latently infected cells compared to progressors. A vaccine that elicits potent CD8+ T cell responses early in infection that can eliminate CD4+ T cells through recognition of Gag proteins on incoming virions and before any viral genes are expressed may have the potential to reduce the seeding of the latent reservoir[433].

61% of individual HIVconsv peptides identified as giving a positive response in second round testing did not contain known epitopes according to the Los Alamos National Laboratory HIV Immunology Database and the HLA type of the patients. This suggests that there are many as yet undefined epitopes or possibly that MVA.HIVconsv had primed novel CD8+T cell responses to epitopes that are not usually recognised in natural infection. This implies that an unnatural immunogen can induce a response distinct from that induced by natural HIV-1 infection. To confirm these novel epitopes, further mapping with truncated peptides would need to be performed, followed by fragility analysis to assess their sensitivity to immune selective pressure.

Because HIVconsv is a chimaeric protein, regions overlapping adjoining fragments may elicit T cell responses to new but irrelevant epitopes. In the HIV-CORE 002 clinical trial testing various prime boost regimens of HIVconsv in healthy volunteers, ~20% of all epitope responses were to junctional epitopes[308]. Vaccination of HIV-1 infected individuals with MVA.HIVconsv induced responses to junctional epitopes that accounted for only 4% of all epitope responses. It is possible that responses to these junctional regions were primed in healthy volunteers by ChAdV63.HIVconsv, whereas MVA.HIVconsv in infected individuals preferentially boosted HIV-1-specific T cell

responses primed by natural HIV-1 infection. Responses to junctional regions were subdominant and did not out-compete true anti-HIV-1 responses.

In summary, vaccination of HAART treated patients with MVA.HIVconsv increased the magnitude of HIVconsv-specific IFN-γ-secreting T cells in 15/15 vaccinees but the changes observed did not reach statistical significance when compared with placebo recipients. The IFN-ɣ Elispot assay is a rapid, standardised, cost-effective, high throughput assay which makes it attractive for use in vaccine trials. However, production of IFN-γ by HIV-1-specific CD8+ T cells is an imprecise measure of their antiviral function; furthermore, their capacity to secrete IFN-γ in response to exogenous peptide stimulation, is maintained throughout the pathway to T cell exhaustion[434,435], and therefore may not accurately reflect their functional activity *in vivo*. It may therefore be necessary to define immunogenicity to vaccination in two stages, using the magnitude of induced responses by IFN-γ Elispot assay first, followed by assays that provide a better representation of antiviral functions *in vivo*. The next chapter therefore examines the quality of T cell responses to vaccination with MVA.HIVconsv.

4. Effect of MVA.HIVconsv vaccination on the function of CD8+ T cells

4.1 Introduction

The IFN-ɣ Elispot assay has been widely used to study antigen-specific T cell responses in HIV-1 infected subjects and to assess vaccine immunogenicity. The advantages of the IFN-ɣ Elispot assay are that it is simple, rapid, amenable to high throughput analyses, and has been validated for HIV-1 vaccine trials[436,437]. However, in many trials, the antigenic stimulus in the assay is provided by exogenously added peptides, frequently at non-physiological concentrations, in order to identify cells that can secrete IFN-ɣ as a measure of viral antigen recognition. The Merck vaccine MrkAd5 gag/pol/nef was designed to specifically induce cell-mediated immune responses[332]. The immunogenicity of this vaccine in phase I trials, as measured by the IFN-ɣ Elispot assay, was considered to be good enough to support further testing in efficacy studies[438,439], but failed to prevent HIV-1 infection or reduce viral load upon acquisition in the phase IIb trials[332,333]. This indicated that IFN-γ production by T cells is a poor correlate of protection against HIV-1 and highlighted the need for assessment of other immunological parameters.

These results are perhaps not surprising given that in cross sectional studies, the magnitude or breadth of HIV-1-specific CD8+ T cells measured *in vitro* by IFN-ɣ Elispot assay or intracellular cytokine staining did not correlate with viral load *in vivo*[226,240]. Furthermore, the STEP trial highlighted the limitations of the macaque SIV model in predicting vaccine efficacy in humans[440,441] and the need to understand better

the mechanisms by which HIV-1-specific T cells influence HIV-1 control in humans. HIV-1 controllers are a subset of all HIV-1 infected individuals (<5%) who are able to control virus to low or undetectable levels. CD8+ T cells from HIV-1 controllers typically show differences from progressors in T cell cytokine secretion profiles, perforin expression, lytic capacity and proliferative capacity *in vitro*[234,256,257]. This indicates that control of virus replication is not simply a matter of the quantity of HIV-1-specific CD8+ T cells, but that the quality of the CD8+ T cells may influence the course of infection.

Inhibition of virus replication by CD8+ T cells *in vitro* may provide a more accurate measure of immune control *in vivo* as it relies on recognition of endogenously generated peptides within HIV-1-infected CD4+ T cells. CD4+ T cells are super-infected with an exogenous HIV-1 strain following which viral replication is allowed to proceed for several days. Autologous CD8+ T cells are added to the culture and any reduction in infection represents the inhibitory capacity of the CD8+ T cells. Thus it assesses both direct target cell lysis[202,256] and non-cytolytic mechanisms of virus inhibition including cytokine and chemokine secretion[105,442]. Several studies have demonstrated that viral inhibition measured *in vitro* correlates with control of HIV/SIV *in vivo*. In NHP studies Yamamoto et al. showed that animals with a greater than one log inhibition of virus replication mediated by the effector memory CD8+ T cell compartment showed lower plasma VLs than those with less than one log of inhibition[443]. Other NHP studies demonstrated that CD8+ T cell inhibitory activity induced by vaccination with viral-vectored SIV antigens correlated with control of viraemia[444,445]. In human studies, Yang et al. demonstrated in a cohort of chronically infected individuals a significant

interaction between CD8+ T cell mediated inhibition and the CD4+ T cell gradient, with more efficient inhibition associated with slower CD4+ T cell loss. In addition, in a prospective cohort of recently infected individuals, we found antiviral activity to be predictive of subsequent CD4+ T cell decline. Furthermore CD8+ T cell mediated inhibition was inversely correlated with viral load set point[446], a predictor of disease progression[80,447]. Saez-Cirion et al. reported potent CD8+ T cell inhibitory activity in a cross-sectional study of HIV-1 controllers with HLA-B57 or –B27 alleles, with undetectable virus replication *in vitro* in 9/10 subjects. In contrast, CD8+ T cells from viraemic patients showed poor control[262]. Both Yang and Saez-Cirion showed that inhibition of virus replication was MHC class I restricted and was lost upon titration of the CD8+ T cells from viraemic subjects, whereas in HIV-1 controllers inhibition could still be observed at effector:target ratios as low as 1:10 CD8+/CD4+ T cells[262,446]. Inhibition of virus replication was shown to be stable over a three year period[261]. A study by Lecuroux el al. reported that CD8+ T cell mediated inhibition was generally poor in HIV-1 infected subjects with primary infection, indicating poor inhibition in chronic infection is not a consequence of uncontrolled viral replication. This indicates the superior suppressive capacity of CD8+ T cells from HIV-1 controllers is not a general characteristic of the HIV-1-specific CD8+ T cell response in primary infection[448].

Spentzou et al. and Freel et al. used the virus inhibition assay to study CD8+ T cell mediated inhibition following vaccination of HIV-1 uninfected individuals. Spentzou et al. showed in a blinded analysis of seven participants vaccinated with a DNA prime / rAd5 boost encoding HIV-1 gag, pol, and nef from clade B and env from clades A, B,

and C that no inhibitory activity against HIV-1 IIIB was detectable at baseline but was observed at 6 and 12 weeks after the rAd5 boost in all seven vaccinees. Responses were undetectable in the placebo recipient[449]. Freel et al. also analysed HIV-1 uninfected subjects who had received a similar vaccine regimen. The 40 vaccinees exhibited significantly greater virus inhibition than unvaccinated controls. However, this was still significantly lower than levels of inhibition observed in 16 viraemic controllers. Antiviral activity in vaccinees was strongly associated with increased numbers of CD8+ T cells expressing CD107a or MIP-1β[450].

The studies highlighted above provide evidence to suggest that the quality of CD8+ T cells influences the rate of HIV-1 progression and that the virus inhibition assay can provide more relevant information regarding the quality or antiviral function of CD8+ T cells than cytokine-based assays. The aim of work described in this chapter was to investigate whether therapeutic vaccination with MVA.HIVconsv improved the function of HIV-1-specific CD8+ T cells.

4.2 Results

4.2.1 Assay optimisation

PBMCs from three HIV-1 uninfected donors were depleted of CD8+ T cells by magnetic bead separation using CD8 MicroBeads. A typical depletion removed 97% of CD8+ T cells (Figure 4.1). The CD8+ T cell depleted fraction, termed 'CD4+ positive cells' was activated with PHA for three days and then infected with HIV-1_{BaL} at an MOI of 0.01 (based on previous work by Yang et al.[397]). These cells were cultured for nine days in the absence of CD8+ T cells in order to determine the kinetics of HIV-1_{BaL} replication. Infected CD4+ T cells were identified by flow cytometry as described in Materials and Methods (Section 2.13.5) and a consistent gating strategy used to quantify the percentage of p24 positive CD4+ T cells (Figure 4.2 A-E). Figure 4.2 F is gated on CD3+ CD8- cells to prevent exclusion of CD4+ T cells that have downregulated surface expression of CD4 following infection[451–454]. Replication of HIV-1_{BaL} initially increased exponentially, peaking at day six after which it declined rapidly (Figure 4.3). The kinetics of HIV-1_{BaL} replication, reported here were in agreement with work from Yang et al.[397,446]. Analysis of HIV-1_{BaL} inhibition was therefore performed on day six post infection.

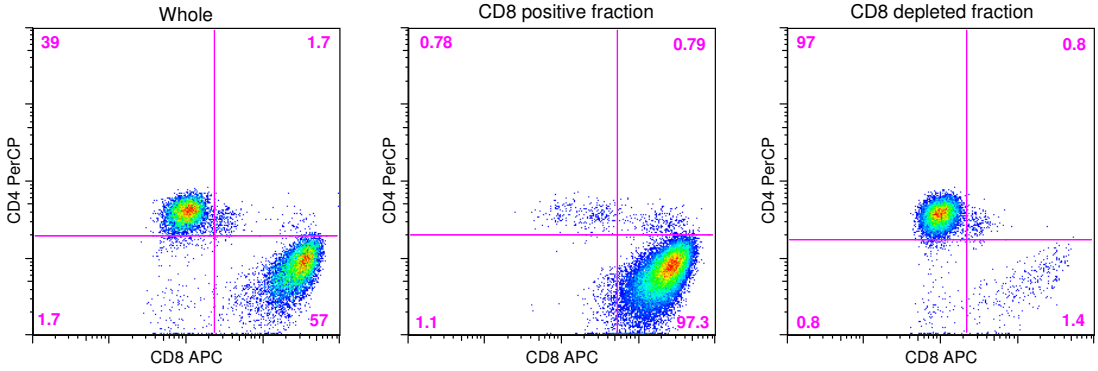


Figure 4.1 Representative FACS plot from a CD8 separation in a HIV-1 uninfected donor showing (A) unseparated PBMC fraction, (B) negatively selected CD4+ fraction and (C) positively selected CD8+ fraction. The cell separation was performed by incubating PBMCs with CD8 antibody-coated magnetic beads for 20 minutes. The cell suspension was run through a magnetic column. Unlabeled cells that passed through the column were collected; this was the 'CD4 positive' fraction. The labelled CD8+ cells that stuck to the column were collected separately; this was the 'CD8 positive' fraction.

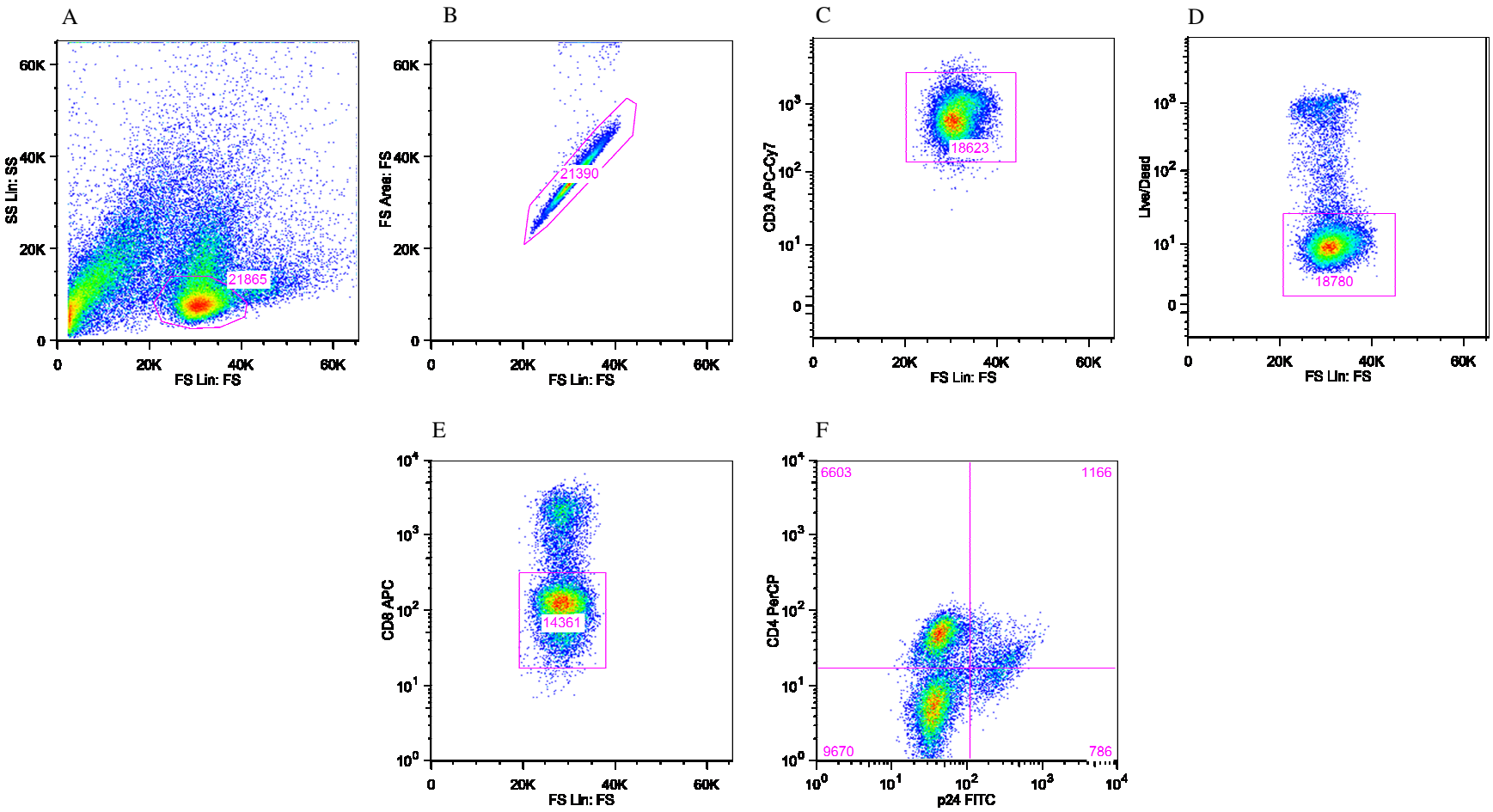


Figure 4.2 Gating strategy for virus inhibition assay. Lymphocytes were identified by (A) forward scatter/side scatter,(B) singlet events by forward scatter linear/forward scatter area, (C) CD3+ cells by forward scatter/CD3 APCCy7+ and (D) live cells by negativity for Aqua live/dead cell stain.(E) CD4+ infected cells are gated on CD8 negative cells to prevent exclusion of CD4+ T cells that have downregulated surface expression of CD4 following infection. (F) HIV-$_{BaL}$ infected CD4+ T-cells cultured alone.

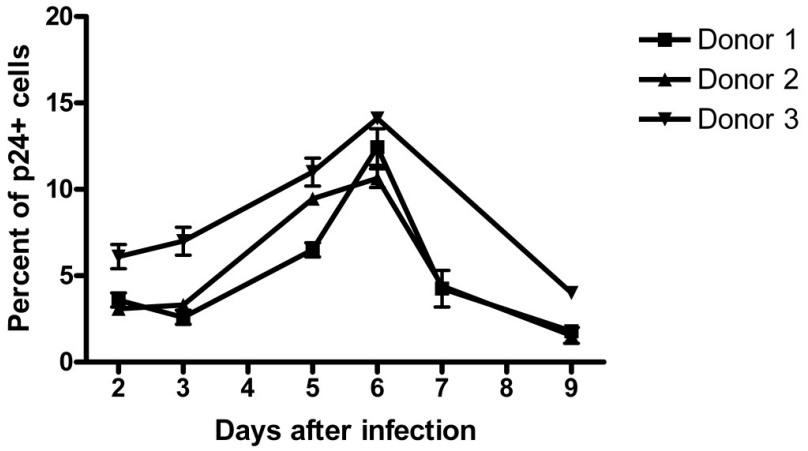


Figure 4.3 Time course of HIV-1_{BaL} superinfection. CD4+ T cells from three HIV-1 uninfected donors were superinfected with HIV-1_{BaL} at an MOI of 0.01 and cultured in duplicate for nine days. p24 was quantified by flow cytometric analysis of intracellular p24 antigen on day 2, 3, 5, 6, 7 and 9 following superinfection. Each data point represents the mean of duplicate wells with standard deviation shown by error bars.

Uninfected activated CD4+ T cells from five HIV-1 negative donors were cultured alone for six days in order to determine the background level of p24 staining. The mean frequency of p24-positive cells measured on day six of culture in these five donors was 0.43%, with minimum and maximum values of 0.1 to 1%. Using the mean plus two standard deviations as a cut-off, values of p24-positive CD4+ T cells above 1% were considered to be a true infection of CD4+ T cells with HIV-1_{BaL}.

To determine a cut-off for CD8+ T cell mediated virus inhibition, CD4+ T cells infected with HIV-1_{BaL} were co-cultured with autologous unstimulated CD8+ T cells from five HIV-1 uninfected donors at a CD8+/CD4+ T cell ratio of 1:1. p24 Ag+ cells were quantified on day six of infection and percent inhibition was calculated using the equation;

$$\% \text{ inhibition} = \frac{(\% \text{ p24}^{+}\text{in CD4 culture} - \% \text{ p24}^{+}\text{ in CD4/CD8 co-culture})}{\% \text{ p24}^{+}\text{in CD4 culture}}$$

The mean virus inhibitory activity of CD8+ T cells from HIV-1 uninfected donors measured on day six of co-culture was 4%. The cut off for assay positivity was defined as the mean plus two standard deviations, which was 19%. CD8+ T cell mediated inhibition measured above this value was therefore defined as HIV-1-specific.

4.2.2 MVA.HIVconsv trial participants demonstrate moderate CD8+ T cell mediated virus inhibition at baseline

CD8+ T cell antiviral activity was measured in 19 MVA.HIVconsv trial participants at both pre-vaccination (Screen or day 0) and post-vaccination time points. CD4+ T cells to be superinfected were always isolated from a pre-vaccination time point in order to minimize inter-assay variation. Antiviral activity was always determined on day six of co-culture using a 1:1 CD8+/CD4+ T cell ratio and a 2:1 and/or 1:10 when cell number permitted. Pre-vaccination median inhibition measured at a 1:1 CD4+/CD8+ T cell ratio was 20% (range 0-85%) and was significantly higher than in uninfected individuals ($p = 0.03$) (Figure 4.4). This value is consistent with previous data from our lab in which the median inhibition observed in a cohort of 22 chronically infected patients on HAART was 38% (range 0-94%) (Yang H et al., unpublished data). There was no correlation between percent inhibition and the frequency of infected CD4+ T cells ($r = -0.07$, $p = 0.79$) (Figure 4.5), indicating that higher antiviral activity was not simply a consequence of lower infection of CD4+ T cells.

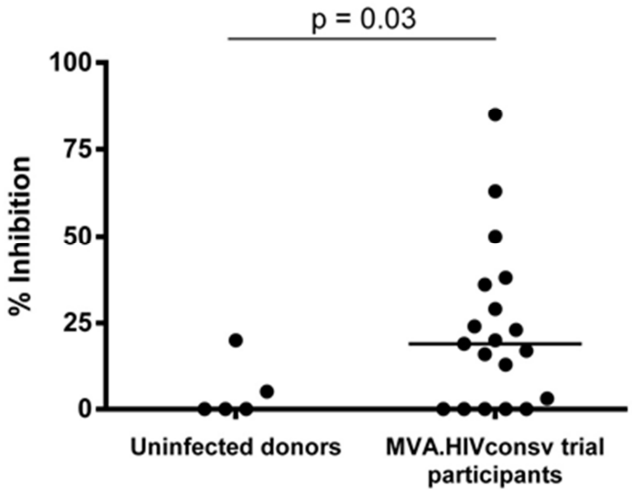


Figure 4.4 HIV-1 infected HIVconsv trial participants demonstrate higher CD8+ T cell mediated inhibition than HIV-1 negative donors. Activated CD4+ T cells were superinfected with HIV-1_{BaL} at an MOI of 0.01 and co-cultured with autologous CD8+ T cells at a CD8+/CD4+ T cell ratio of 1:1. HIV-1 infected CD4+ T cells were quantified by flow cytometric analysis of intracellular p24 antigen on day 6 and percent inhibition calculated as described in Materials & Methods. Horizontal black lines represent median values. Statistical analysis conducted using the Mann Whitney test.

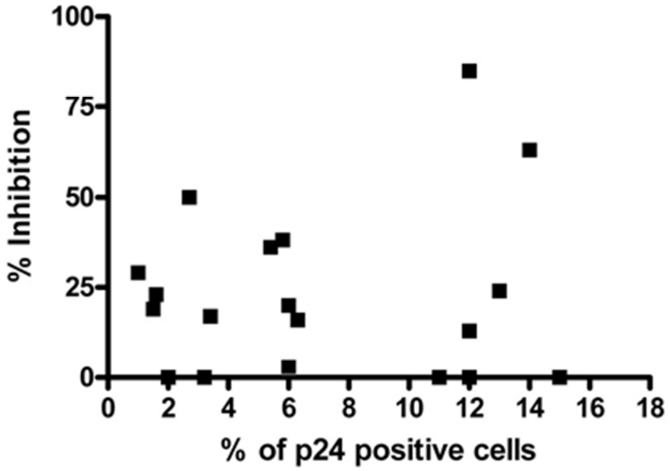


Figure 4.5 CD4+ T cell infection levels do not correlate with percent inhibition. Activated CD4+ T cells from 19 HIVconsv trial participants were superinfected with HIV-1_{BaL} at an MOI of 0.01 and cultured in duplicate. HIV-1 infected CD4+ T cells were quantified by flow cytometric analysis of intracellular p24 antigen on day 6 and percent inhibition calculated as described in Materials & Methods. Analysis of correlation performed using the Spearman rank correlation test.

4.2.3 CD8+ T cell mediated antiviral activity is increased following vaccination with MVA.HIVconsv at a dose of $2x10^8$ pfu

To determine if vaccination with MVA.HIVconsv could enhance the capacity of CD8+ T cells to inhibit virus replication, CD8+ T cell mediated inhibition at a post-vaccination time point was compared with inhibition pre-vaccination (screen or day 0). The post-vaccination time point selected was that at which the peak of response was observed for each subject as confirmed from second round IFN-ɣ Elispot assays (Section 3.2.6). Median inhibition pre-vaccination measured at a 1:1 CD8+/CD4+ T cell ratio was 0%, 30.5% and 17% in placebos, low dose vaccinees and high dose vaccinees respectively. Trial participants were randomly assigned to their vaccine group and it is unfortunate that the placebos selected had significantly lower pre-vaccination inhibition values than vaccinees ($p = 0.0003$). There was no significant difference in pre-vaccination CD8+ T cell mediated inhibition between low and high dose vaccinees ($p = 0.12$), allowing for comparison between these two groups. At the peak of response post-vaccination median inhibition was 4.6%, 12.5% and 54% in placebos, low dose vaccinees and high dose vaccinees respectively when measured at a 1:1 CD8+/CD4+ T cell ratio. In the high dose vaccinees there was a trend towards increased inhibition following vaccination with MVA.HIVconsv ($p = 0.06$) (Figure 4.6 C), but no such trend was observed in the placebos or low dose vaccinees ($p = 0.49$ and 0.31 respectively) (Figure 4.6 A&B).

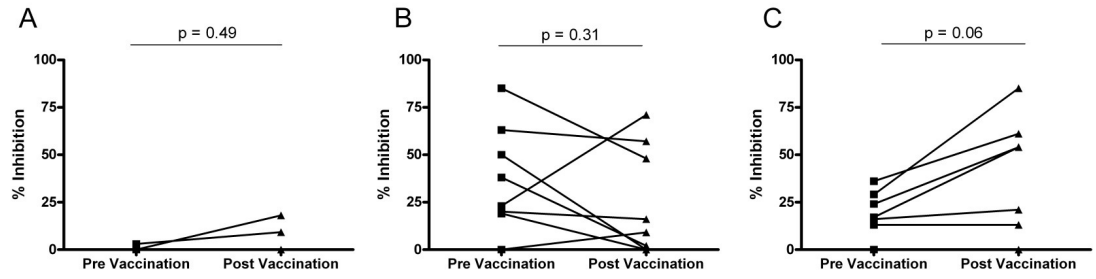


Figure 4.6 CD8+ T cell mediated inhibition is increased following vaccination with $2x10^8$ pfu of MVA.HIVconsv. Activated CD4+ T cells from (A) placebos, (B) lower dose vaccinees and (C) higher dose vaccinees were superinfected with HIV-1_{BaL} at an MOI of 0.01 and co-cultured with autologous CD8+ T cells from a pre and post-vaccination time point at a CD8+/CD4+ T cell ratio of 1:1. HIV-1 infected CD4+ T cells were quantified by flow cytometric analysis of intracellular p24 antigen on day 6 and percent inhibition calculated as described in Materials & Methods. Statistical analysis was performed using the Wilcoxon signed rank test.

CD8+ T cell mediated inhibition was measured at a lower CD8+/CD4+ T cell ratio of 1:10 pre and post-vaccination, unfortunately cell number did not permit this in the placebo group. As expected, inhibition measured at a 1:10 CD8+/CD4+ T cell ratio was lower than that measured at a 1:1 CD8+/CD4+ T cell ratio, consistent with work from Saez-cirion et al. which showed that the antiviral activity of CD8+ T cells from non-controllers was lost following titration of CD8+ T cells[262]. In both vaccine groups inhibition measured at a 1:10 CD8+/CD4+ T cell ratio was not significantly increased following vaccination (Figure 4.7).

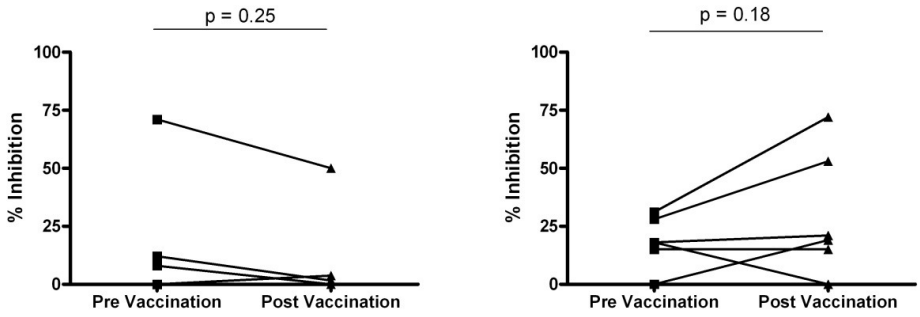


Figure 4.7 Activated CD4+ T cells from (A) low dose vaccinees and (B) high dose vaccinees were superinfected with HIV-1_{BaL} at an MOI of 0.01 and co-cultured with autologous CD8+ T cells from a pre and post-vaccination time point. CD4+ T cells were co-cultured with CD8+ T cells at a CD8+/CD4+ T cell ratio of 1:10. HIV-1 infected CD4+ T cells were quantified by flow cytometric analysis of intracellular p24 antigen on day 6 and percent inhibition calculated as described in Materials & Methods. Statistical analysis performed using the Wilcoxon signed rank test

In order to increase the power of the analysis of inhibition pre and post-vaccination, data for inhibition measured at a 1:1 and 1:10 CD8+/CD4+ T cell ratio were analysed by calculating the area under curve (AUC). Figure 4.8 shows inhibition values at a 1:1 and 1:10 CD8+/CD4+ T cell ratio pre and post-vaccination in subject 221 which were used to calculate AUC. Comparison of AUC values at pre and post-vaccination time points confirmed a trend towards increased inhibition in the higher dose vaccine group following vaccination with MVA.HIVconsv) (Figure 4.9).

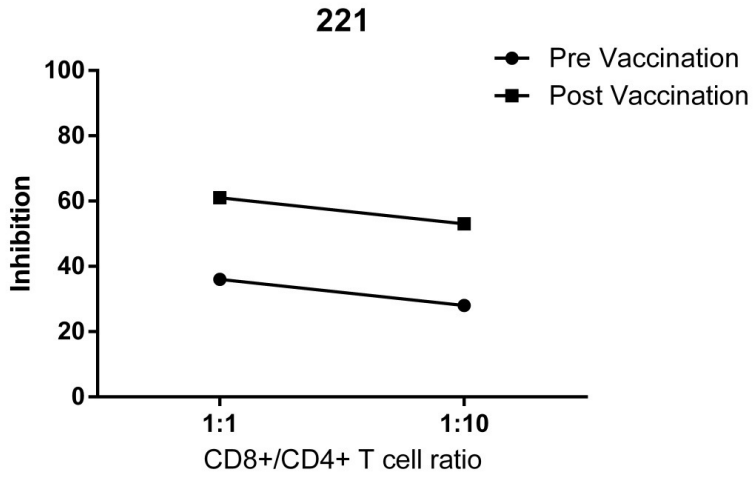


Figure 4.8 CD8+ T cell mediated inhibition measured at a 1:1 and 1:10 CD8+/CD4+ T cell ratio pre and post-vaccination in trial participant 221.

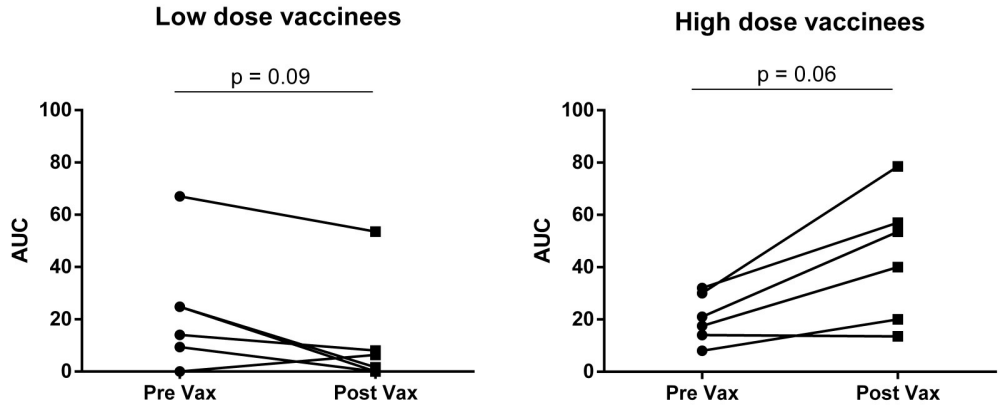


Figure 4.9 AUC was calculated for each trial participant using inhibition measured at a 1:1 and 1:10 CD8+/CD4+ T cell ratio at pre and post-vaccination time points. Statistical analysis performed using the

4.2.4 CD107a/CD107b expression is not increased following MVA.HIVconsv vaccination

Lysosomal associated membrane glycoproteins (LAMPs), including CD107a (LAMP-1) and CD107b (LAMP-2) are transiently expressed on a cell membrane when the cell is about to degranulate[455,456]. Measurement of CD107a and CD107b on the cell surface of responding antigen-specific T cells provides a positive marker of degranulation[456].

CD107a and CD107b are encoded on different chromosomes and are therefore likely to be differentially regulated and expressed[457]. A study by Betts et al. thus recommended that in order to ensure greatest sensitivity both CD107a and b be measured[456].

PBMCs from trial participants were stained simultaneously for CD107a and CD107b as well as IFN-ɣ following stimulation with; R0 (negative control), SEB 1µg/ml (positive control) or HIVconsv single peptide pools 1-6 2µg/ml. This was to obtain information as to whether IFN-ɣ-positive cells identified by Elispot had lytic potential or if two different populations of HIVconsv-specific T cells existed which do not overlap in their function to express IFN-ɣ or degranulate. Stimulation of PBMCs from HIV-1 uninfected donors with HIVconsv single peptide pools did not result in the upregulation of CD107a/b on either CD8+ or CD4+ T cells (Figure 4.10F&C), however, CD107a/b could be detected when cells were stimulated with SEB (Figure 4.10B&E). CD107a/b expression was then measured at pre-vaccination and post-vaccination time points in all trial participants. Day 42 was selected for the post-vaccination time point as it was hypothesized from other therapeutic vaccine studies that the peak response would have occurred by this time and the assays were conducted before trial participants had completed the study schedule. Stimulation of PBMCs from trial participants with R0 and SEB resulted in similar CD107a/b expression levels on CD8+ and CD4+ T cells to those observed in HIV-1 uninfected donors (Figure 4.11). Following stimulation of PBMCs with HIVconsv single pool peptides, there was no significant difference in the frequency of cells expressing CD107a/b in either the CD8+ or CD4+ T-cell populations post-vaccination as compared with pre-vaccination in placebos, low dose vaccinees or high dose vaccinees (Figure 4.12 A & B).

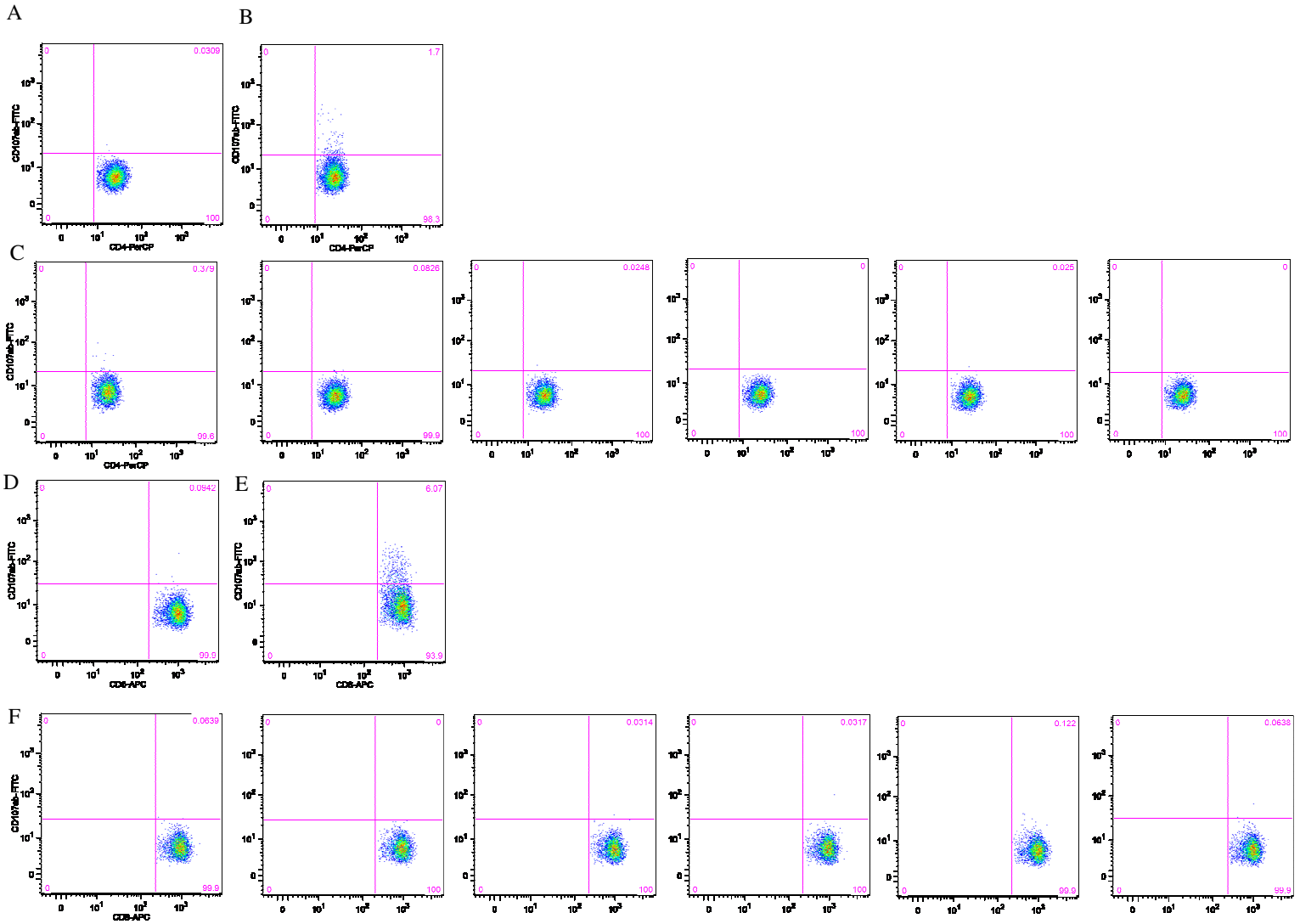


Figure 4.10 CD107ab expression on CD4+ T cells quantified by flow cytometry following stimulation of PBMCs from a HIV-1 uninfected donor with (A) R0 (negative control), (B) SEB 1µg/ml (positive control) or (C) left to right HIVconsv single peptide pools 1-6 2µg/ml. CD107ab expression on CD8+ T cells quantified by flow cytometry following stimulation of PBMCs from a HIV-1 uninfected donor with (D) R0 (negative control), (E) SEB 1µg/ml (positive control) or (F) left to right HIVconsv single peptide pools 1-6 2µg/ml.

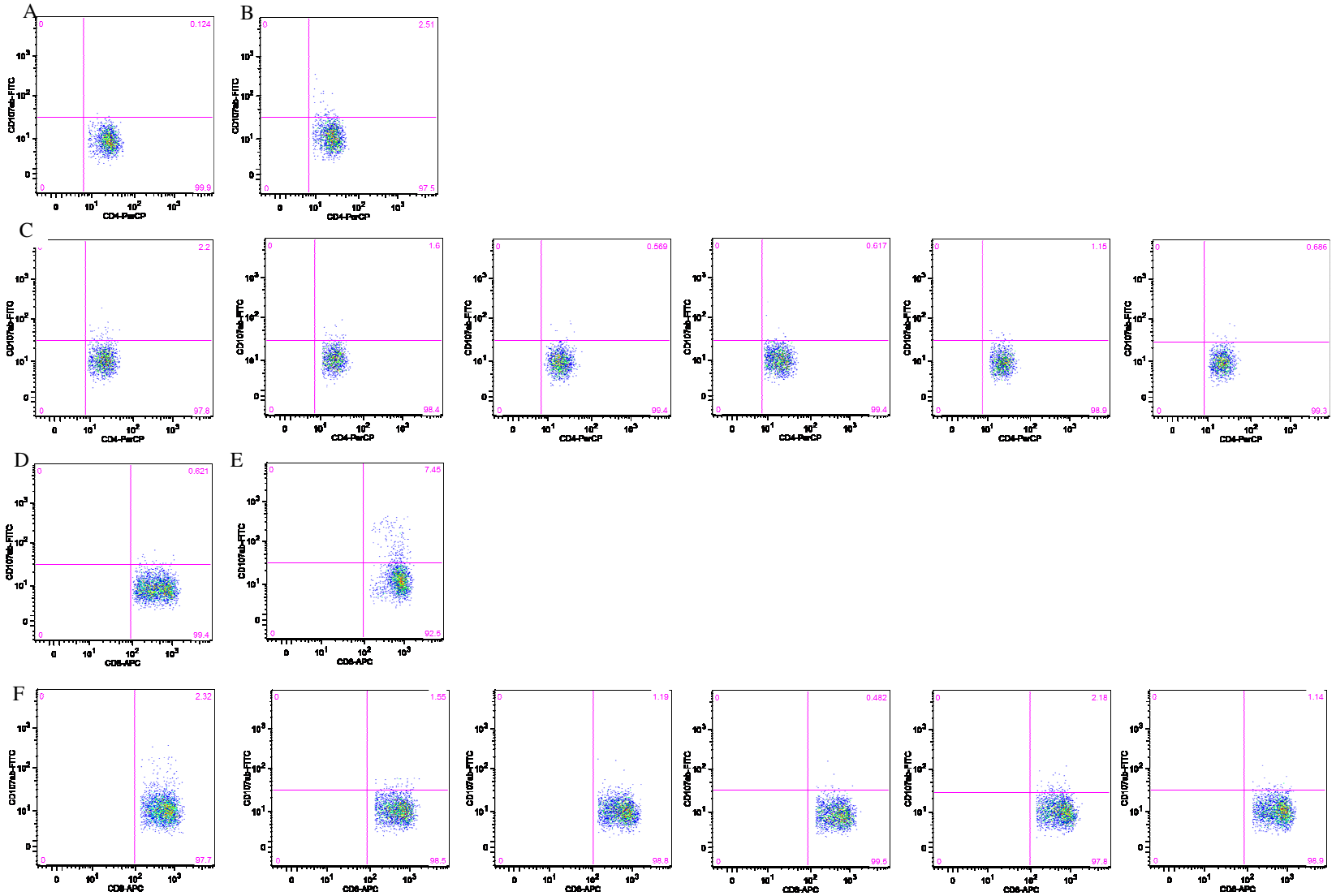


Figure 4.11 CD107ab expression on CD4+ T cells quantified by flow cytometry following stimulation of PBMCs from a HIVconsv trial participant with (A) R0 (negative control), (B) SEB 1µg/ml (positive control) or (C) left to right HIVconsv single peptide pools 1-6 2µg/ml. CD107ab expression on CD8+ T cells quantified by flow cytometry following stimulation of PBMCs from a HIV-1 uninfected donor with (D) R0 (negative control), (E) SEB 1µg/ml (positive control) or (F) left to right HIVconsv single peptide pools 1-6 2µg/ml.

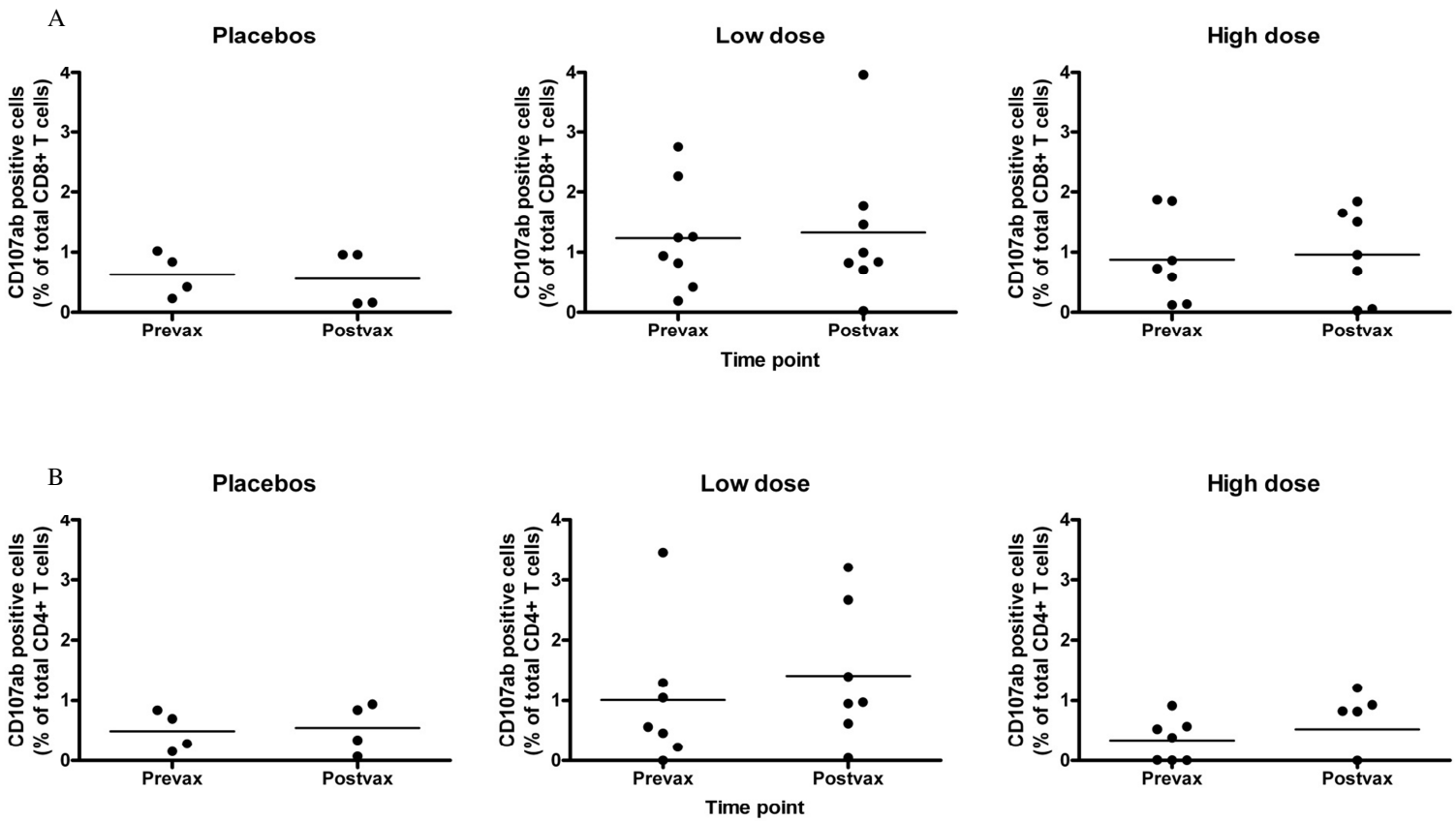


Figure 4.12 CD107ab expression quantified by flow cytometry on (A) CD8+ T cells and (B) CD4+ T cells in (left to right) placebos, low dose vaccinees and high dose vaccinees after stimulation of PBMCs from pre- and post-vaccination time points with 2µg/ml HIVconsv single peptide pools 1-6. CD107ab positive cells expressed as a percentage of total CD4+ or CD8+ T cells following subtraction of CD107ab staining from negative control wells. Statistical analysis performed using the Wilcoxon signed rank test.

CD8+ T cell mediated inhibition and degranulation by CD107ab staining are both considered to be a proxy for the cytolytic capacity of CD8+ T cells[262,446,449,456]. There was, however, no correlation between CD107a/b expression on CD8+ T cells and CD8+ T cell mediated virus inhibition measured at a 1:1 CD8+/CD4+ T cell ratio for either pre-vaccination ($r = 0.19$, $p = 0.41$) (Figure 4.13) or post-vaccination time points ($r = -0.35$, $p = 0.14$) (Figure 4.14).

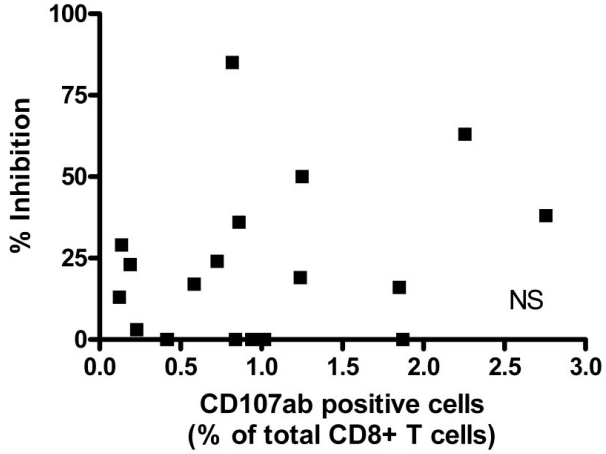


Figure 4.13 No correlation between CD8+ T cell mediated inhibition at a 1:1 CD8+/CD4+ T cell ratio and CD107ab positive CD8+ T cells following stimulation of PBMCs with HIVconsv single peptide pools, both measured pre-vaccination in MVA.HIVconsv trial participants. Statistical analysis conducted using the Spearman rank correlation.

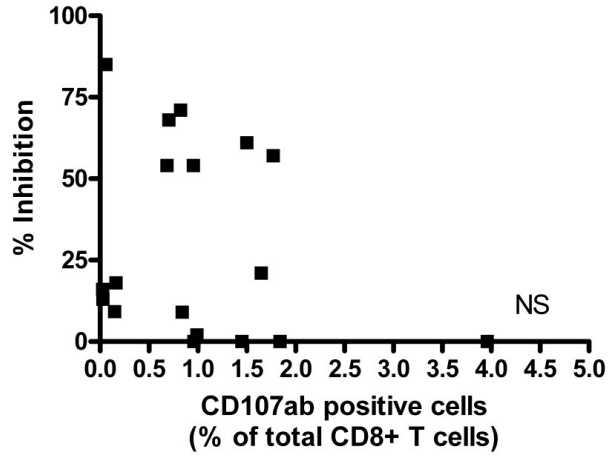


Figure 4.14No correlation between CD8+ T cell mediated inhibition at a 1:1 CD8+/CD4+ T cell ratio and CD107ab positive CD8+ T cells following stimulation of PBMCs with HIVconsv single peptide pools, both measured post-vaccination in MVA.HIVconsv trial participants. Statistical analysis conducted using the Spearman rank correlation.

Figure 4.15 shows CD107ab/IFN-ɣ double positive CD8+ and CD4+ T cells in a HIVconsv trial participant. There was no increase in CD107ab/IFN-ɣ double positive CD8+ or CD4+ T cells following vaccination with MVA.HIVconsv (Figure 4.16). There was also no correlation between CD107ab/IFNγ double positive CD8+ T cells and CD8+ T cell mediated inhibition measured at either pre-vaccination ($r = 0.22$, $p = 0.36$) (Figure 4.17 A) or post-vaccination ($r = 0.14$, $p = 0.17$) (Figure 4.17 B) time points.

Figure 4.15 CD107ab/IFN-ɣ expression on CD4+ T cells quantified by flow cytometry following stimulation of PBMCs from a HIVconsv trial participant with (A) R0 (negative control), (B) SEB 1µg/ml (positive control) or (C) left to right HIVconsv single peptide pools 1-6 2µg/ml. CD107ab expression on CD8+ T cells quantified by flow cytometry following stimulation of PBMCs from a HIV-1 uninfected donor with (D) R0 (negative control), (E) SEB 1µg/ml (positive control) or (F) left to right HIVconsv single peptide pools 1-6 2µg/ml.

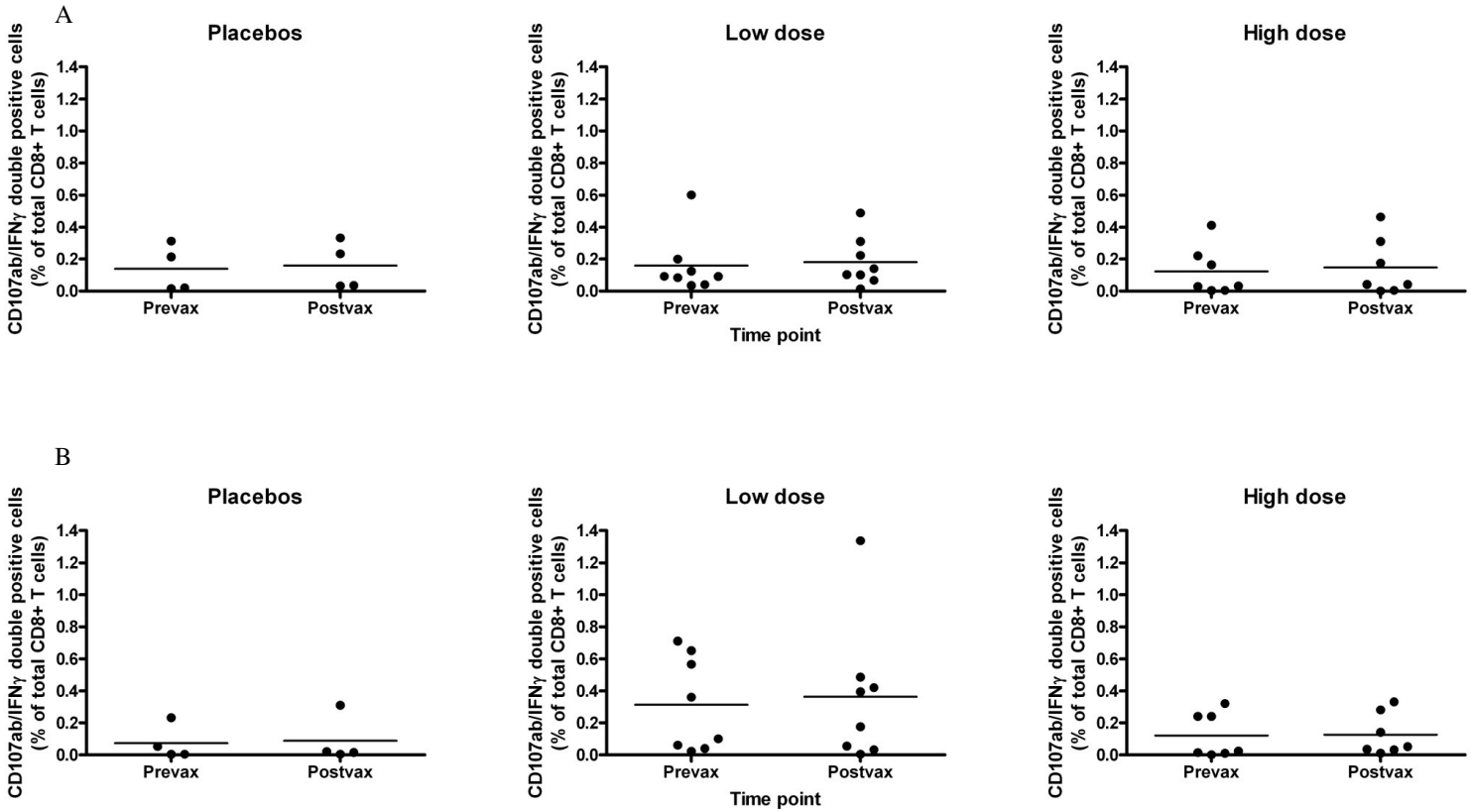


Figure 4.16 CD107ab/ IFN-ɣ expression quantified by flow cytometry on (A) CD8+ T cells and (B) CD4+ T cells in (left to right) placebos, low dose vaccinees and high dose vaccinees after stimulation of PBMCs from pre- and post-vaccination time points with 2µg/ml HIVconsv single peptide pools 1-6. CD107ab IFN-ɣ double positive cells expressed as a percentage of total CD4+ or CD8+ T cells following subtraction of CD107ab/IFN-ɣ staining from negative control wells. Statistical analysis performed using the Wilcoxon signed rank test.

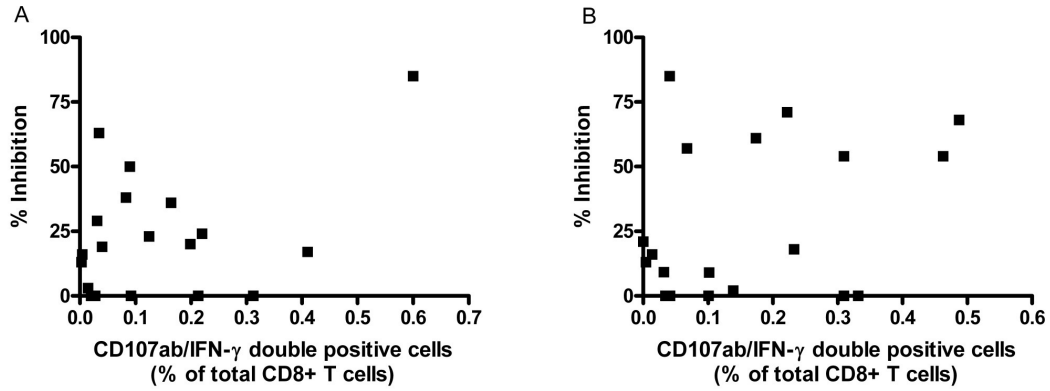


Figure 4.17 No correlation between CD8+ T cell mediated inhibition at a 1:1 CD8+/CD4+ T cell ratio and CD107ab/IFN-ɣ double positive CD8+ T cells following stimulation of PBMCs with HIVconsv single peptide pools, measured at (A) pre-vaccination and (B) post-vaccination time points in MVA.HIVconsv trial participants. Statistical analysis conducted using the Spearman rank correlation.

4.2.5 CD8+ T cells restricted by known protective HLA class I alleles can potently inhibit virus replication

CD8+ T cell mediated inhibition in trial participant 210 measured at a 1:1 CD8+/CD4+ T cell ratio increased from 23% pre-vaccination to 71% post-vaccination. This individual was HLA-B2702-positive. Deconvolution of the peptide matrix IFN-γ Elispot assay from this patient revealed a single peptide, IYKRWIILGLNKIVR, which contains the HLA-B27-restricted epitope KK10. When tested in a confirmatory assay this peptide induced a response of 1537 SFU/million PBMCs (day 14). The HLA-B27 KK10 response has been correlated with slow progression and late escape[231,458–460] and is immunodominant in HLA-B27 positive HIV-1-infected individuals[365,460,461]. Using a KK10 dextramer, KK10-specific CD8+ T cells could be detected in PBMCs from patient 210, at a frequency of 0.7% CD8+ T cells, but not in PBMCs from an HLA-B27-positive HIV-1 uninfected donor (Figure 4.18). PBMCs from patient 210 at screening, day 14, 42, 84 and 186 were stained using the KK10 dextramer in order to track KK10-specific CD8+ T cells (Figure 4.19). The kinetics were similar to those seen

in the IFN-γ Elispot, confirming that an expansion of KK10-specific CD8+ T cells had occurred, particularly after the third MVA.HIVconsv vaccination.

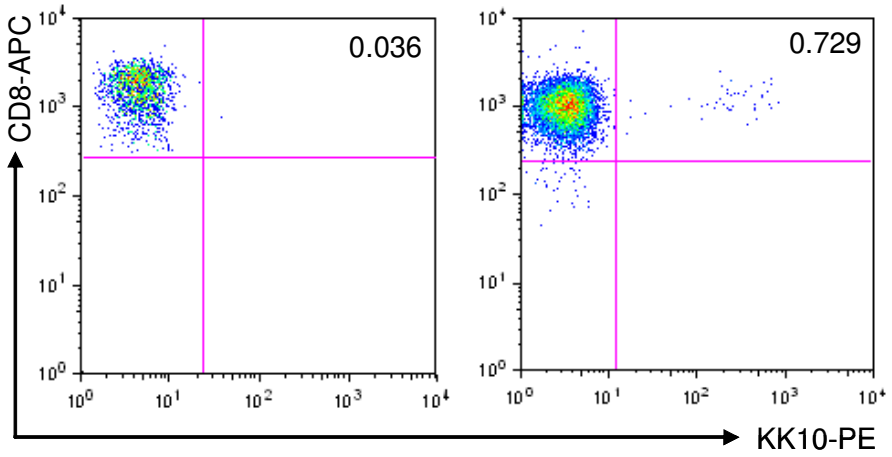


Figure 4.18 KK10 dextramer positive cells can be detected in a B27-positive HIV-1 infected donor. PBMCs from a (A) B27-positive HIV-1 uninfected donor and (B) B27-postive HIV-1 infected HIVconsv trial participant, 210, were stained with a KK10 dextramer, followed by Aqua Live/Dead Fixable stain, CD3-APCCy7 and CD8-APC. Cells were analysed by FACS and were gated on lymphocytes, singlets, live cells, CD3-APCCy7 positive and CD8-APC positive. KK10 dextramer positive cells are expressed as a percentage of total CD8+ T cells.

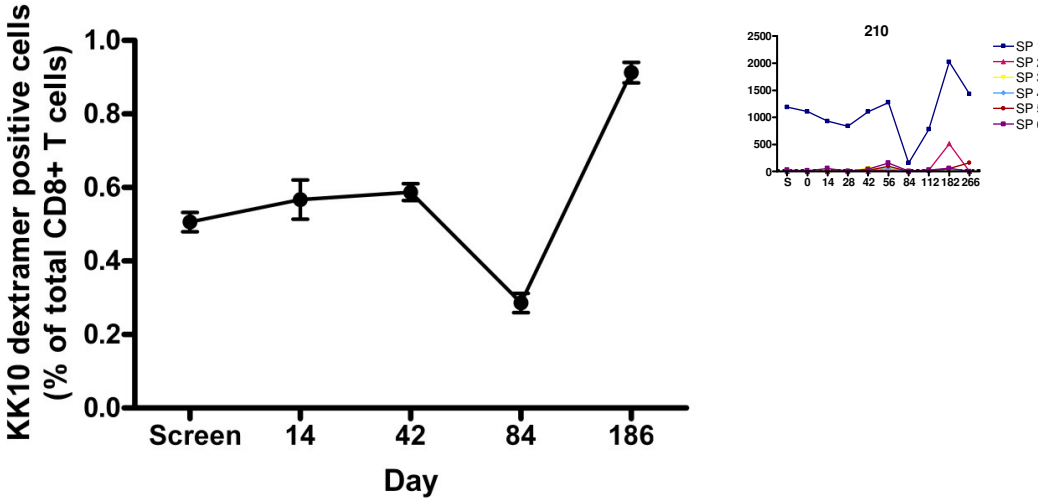


Figure 4.19 KK10 dextramer positive cells increase following vaccination with MVA.HIVconvs. PBMCs from patient 210 were stained with a KK10 dextramer at screen, day 14, 42, 84 and 186. KK10 dextramer positive cells expressed as a percentage of total CD8+ T cells. Insert shows IFN-ɣ Elispot responses to HIVconsv single pools 1-6 in patient 210.

Using anti-PE magnetic beads, KK10-positive CD8+ T cells were isolated from day 186 PBMCs in patient 210. These CD8+ T cells were then used in a virus inhibition assay to determine if KK10-specific CD8+ T cells could inhibit virus replication. Due to the low percentage of KK10 positive cells in bulk PBMCs the virus inhibition assay could only be done at a 1:10 CD8-KK10+/CD4+ T cell ratio. However, even at this low CD8+/CD4+ T cell ratio, KK10 specific cells inhibited virus replication to a greater degree than KK10-negative cells used at the same CD8+/CD4+ T cell ratio (36% vs 18% inhibition) (Figure 4.20).

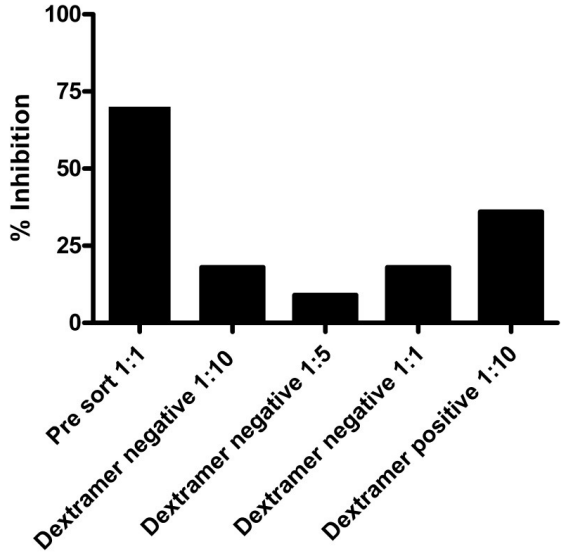


Figure 4.20 KK10 positive cells efficiently inhibit virus replication. Activated CD4+ T cells from patient 210 were superinfected with HIV-1_{BaL} at an MOI of 0.01 and co-cultured with either autologous whole CD8+ T cells, KK10 negative CD8+ T cells or KK10 positive CD8+ T cells at the specified CD8+/CD4+ T cell ratios. HIV-1 infected CD4+ T cells were quantified by flow cytometric analysis of intracellular p24 antigen on day 6 and percent inhibition calculated as described in Materials & Methods.

4.3 Discussion

The role of CD8+ T cells in viral containment during acute and chronic HIV-1 infection has been discussed earlier. Absence of a correlation between magnitude of T cell response and viral load suggests that control of virus replication is not simply a reflection of the frequency of HIV-1-specific CD8+ T cells[226,240]. The efficient inhibition of viral replication by CD8+ T cells from HIV-1 controllers provides compelling evidence that the quality of CD8+ T cells influences HIV-1 control[262,459,462,463] and therefore we hypothesized that inducing potent antiviral inhibitory responses by therapeutic vaccination, or increasing weak responses, is an important goal of HIV-1 eradication strategies.

The capacity of CD8+ T cells from 19 MVA.HIVconsv trial participants to inhibit HIV-1 infection of autologous CD4+ T cells was assessed pre and post-vaccination using a viral inhibition assay. Antiviral inhibition capacity ranged from 0% to 85% at baseline when measured at a 1:1 CD8+/CD4+ T cell ratio. There was a trend towards increased inhibition of virus replication by CD8+ T cells following vaccination of HIV-1 infected HAART treated subjects with the higher dose of MVA.HIVconsv ($2x10^8$ pfu).

A higher dose of the vaccine could increase immunogenicity by two non-mutually exclusive mechanisms. Firstly it may result in increased activation of the innate immune system and therefore subsequently an increased adaptive immune response. An *ex vivo* study by Zak et al. identified innate immune signatures that could predict subsequent HIV-1-specific adaptive immune responses following vaccination of HIV-1 uninfected

individuals with Mrk Ad5 gag/pol/nef. They found that the serum concentrations of two chemokines (MCP-1 and MCP-2) measured 24hr after vaccination could distinguish individuals with high/moderate frequencies of Gag-specific CD8+ T cells from those with no Gag-specific CD8+ T cells detected at 28 days after vaccination[464]. Higher chemokine induction predicted increased likelihood of positive CD8+ T-cell responses, indicating an increased innate immune response may result in an increased T cell response. Secondly, an increased vaccine dose may result in a greater number of cells presenting HIVconsv epitopes and therefore more T cells becoming activated and undergoing proliferation. Estcourt et al. demonstrated in a mouse model a direct correlation between increasing recombinant MVA (rMVA) vaccine doses (MVA.HIVA-NP $5x10^3$, $5x10^5$ or $5x10^7$ PFU) and the frequency of T cells entering the cell cycle[426]. However, an increase in poxvirus doses much beyond $2x10^8$ pfu is likely to, require larger volumes of injection or multiple injections, result in amplified reactogenicity and would pose challenges for manufacturing capacity.

The trend towards increased inhibition following vaccination with a higher dose of MVA.HIVconsv is a signal that it may be possible to increase the number of functional CD8+ T cells in HIV-1 infected HAART treated patients by therapeutic vaccination. However, it is not clear whether the level of CD8+ T mediated inhibition achieved by this vaccination strategy would translate into a beneficial effect in terms of eliminating HIV-1-infected CD4+ T cells. Vaccination of HIV-1 uninfected subjects with a DNA prime and rAd5 boost encoding HIV-1 Env and Gag-Pol in a study by Freel et al. induced substantial CD8+ T cell virus inhibitory responses as compared to unvaccinated HIV-1 uninfected subjects. However levels of inhibition observed were 0.7-log less than

levels in HIV-1 controllers[450]. Yang el al demonstrated using linear mixed models that inhibition in HAART-naïve viraemic subjects of ≥ 90% was associated with very slow CD4+ cell decline (~20 cells/μL blood per year) and that some viraemic controllers showed inhibition of close to 90%[446]. No MVA.HIVconsv trial participants exhibited inhibition of virus replication of this level.

CD8+ T cells specific for epitopes within HIVconsv may be irrelevant for control of virus replication and this could account for the poor potency of the antiviral response following vaccination. Several vaccine studies have demonstrated that the use of full length proteins as vaccine immunogens does not induce more effective CTL responses[332,333] than natural infection. Immunodominant responses to variable regions of the HIV-1 proteome may act as 'decoys' which absorb much of the host adaptive immune response[390]. Responses to conserved regions of the HIV-1 proteome have been associated with control of virus replication[222,396,406]. However, the HIVconsv immunogen was designed by selecting only the most conserved regions of the HIV-1 proteome using HIV-1 sequences from the Los Alamos National Laboratory database, using the centralised sequence method. The design did not take into consideration any functional data from natural HIV-1 infection. Work from two separate groups has shown that despite Gag being a highly conserved protein due to its critical functions such as its role in virus assembly and virus maturation among others (Reviewed in[18]), some particular regions within Gag have a higher genetic fragility than other regions, that is, mutations within these regions result in a greater fitness cost to the virus. Rihn et al. demonstrated that these highly genetically fragile regions were not uniformly distributed across the Gag capsid protein (CA)[252]; in agreement with this, work from

Rolland et al. found no correlation between sequence conservation and fitness cost within the Gag protein[390]. This indicates that even within conserved regions of a protein there are particular sites that would be more advantageous to include in an immunogen. Mothe et al. used functional data from a cohort of 950 untreated HIV-1 clade B and C infected individuals to identify potential immunogen sequence candidates. By studying responses to overlapping peptides (OLPs) spanning the whole proteome, they were able to identify certain 'beneficial' OLPs: these were peptides which were associated with a lower viral load in responders compared with non-responders. These data were used to calculate a protective ratio (PR), which was a ratio of the median viral loads (VL) between OLP non-responders and responders[396]. The 14 conserved segments of HIVconsv only overlap by 57% with the 'beneficial' regions identified by Mothe, so although the segments in HIVconsv are conserved, some of them may have limited functional importance. CD8+ T cells specific for these regions would therefore be predicted to exert only partial control of virus replication. This is explored further in Chapter 6.

The latent HIV-1 reservoir is a major barrier to a cure; long-lived CD4+ T cells harbouring transcriptionally silent but replication-competent viruses are not affected by current antiretroviral drugs or host CD8+ T cell responses, therefore, lifelong HAART is required to prevent viral replication and re-seeding of the reservoir. Shan et al. demonstrated using the Bcl-2 transduced resting CD4+ T cell latency model that latently infected cells reactivated with a HDAC inhibitor did not die due to cytopathic effects and CD8+ T cells from HAART treated patients were unable to kill these reactivated cells. CD8+ T cells from these patients exhibited improved lytic function

when stimulated with Gag peptides *in vitro* and eliminated the HIV-1-infected reactivated cells[465]. They therefore proposed that a therapeutic vaccine could be used in a two pronged approach, involving latency reversal with agents such as HDAC inhibitors and a therapeutic vaccine to stimulate CD8+ T cells to kill reactivated cells ('shock and kill'). The data in this chapter demonstrated that vaccination with MVA.HIVconsv did not stimulate CD8+ T cells with an enhanced potential to kill autologous CD4+ T cells from patients after super-infection with a heterologous virus, which suggests that the 'kill' component of the 'shock and kill' strategy is unlikely to be achieved with current vaccine candidates and needs to be optimized. Furthermore, a recent study from Bullen et al. showed that several latency reversing agents, including HDAC inhibitors, did not reactivate HIV-1 in CD4+ T cells from HAART-treated patients and concluded that the Bcl-2 latency model did not adequately reflect latency reversal in CD4+ T cells from HAART-treated patients[466].

The median level of CD107a/b single positive CD8+ T cells in trial participants pre-vaccination was 0.84% of total CD8+ T cells. This is in agreement with a study by Freel et al.[450]. Expression of CD107a/b was also detected in CD4+ T cells in MVA.HIVconsv trial participants and this has been observed in other studies of HIV-1-infected individuals[467] and HCMV-seropositive individuals[468]. CD4+ T cells can acquire cytotoxic activity and have been identified in PBMCs from HIV-1 infected individuals[197,198,469]. In the MVA.HIVconsv trial participants there was no increase in the frequency of CD107a/b positive CD8+ or CD4+ T cells following vaccination, which contrasted with the increase in CD8+ T cell mediated inhibition observed after vaccination with the higher dose of MVA.HIVconsv. Additionally, CD107a/b

expression was not correlated with CD8+ T cell mediated inhibition in MVA.HIVconsv trial participants at either pre- or post-vaccination time points. Yamamato et al. observed in rhesus macaques that those with strong virus inhibition had significantly higher frequencies of CD8+ T_{EM} cells expressing CD107a than macaques with low virus inhibition[443]; however, CD107a positive cells were expressed as a percentage of effector memory CD8+ T cells. In my experiments CD107a/b positive cells were expressed as a percentage of total CD8+ T cells and it could be that staining for and gating on specific CD8+ T cell subsets would reveal differences in CD107 levels pre- and post-vaccination. The discordance between the viral inhibition assay and CD107 staining could also be due to technical differences between the two assays. Firstly, CD107 a and b expression are measured over a six hour stimulation period whereas the inhibition assay is conducted over a six day period and therefore measures cumulative inhibition of virus replication. Secondly, CD8+ T cells can kill target cells via two major pathways, a granule-dependent (perforin/granzyme) and independent (ligand–ligand induced cell death, e.g. fas-fasL) mechanism (reviewed in[470]). CD107 staining measures only killing by the granule-dependent pathway whereas the virus inhibition assay measures killing by both pathways. The frequency of CD107ab/IFN-ɣ double positive CD4+ and CD8+ T cells detected in MVA.HIVconsv trial participants was very low. Casazza et al. also observed a lower frequency of CD107ab/IFN-ɣ double positive cells compared with the number of either CD107a or IFN-ɣ single positive cells[471]. This could be because different subsets of cells are responsible for IFN-ɣ secretion and cytolysis. Varadarjan et al. evaluated the ability of thousands of individual CD8+ T cells from HIV-1-infected patients to mediate lysis and to produce cytokines. They

demonstrated that the majority of *in vivo* primed, circulating HIV-1-specific CD8+ T cells were discordant for cytolysis and IFN-γ secretion[472].

In the case of patient 210, vaccination with MVA.HIVconsv resulted in an increase in CD8+ T cell mediated virus inhibition despite this patient receiving a low dose of the vaccine. Sorted KK10-specific CD8+ T cells from this patient were more efficient at inhibiting virus replication than KK10-negative CD8+ T cells, suggesting that a single HIV-1-specific T cell population may be responsible for much of the overall antiviral response. KK10-specific CD8+ T-cells have been shown to have a high functional avidity[459] which could explain this and are associated with reduced VL and slow disease progression[459,460]. Several other patients, 215, 221, 222, and 223, also showed increases in viral inhibition after vaccination. The increase in inhibition observed in 215 was of a similar magnitude to that observed in patient 210. It was however not possible to conduct similar experiments because of the limited availability of tetramers/dextramers to match their HLA haplotypes and HIV-1-specific T cell responses.

A limitation of this study is the use of a laboratory-adapted viral isolate in the virus inhibition assay. Laboratory-adapted viruses do not represent circulating viruses in patients. Because this study involved chronically infected patients, it is likely that their virus quasispecies was extremely diverse prior to initiating HAART. The patients' circulating HIV-1 specific CD8+ T cells may therefore be specific for epitopes in archived viruses that were not shared by HIV-1_{BaL}, which was used for superinfection, possibly resulting in an underestimation of the inhibitory capacity of CD8+ T cells. However the goal of this work was to measure virus inhibition by CD8+ T cells

targeting conserved epitopes. Comparison of the sequence of the laboratory strain(s) used in the assay with the corresponding regions of HIVconsv could reveal differences that would account for possible underestimation of the response.

A further limitation is that the virus inhibition assay does not distinguish between inhibition of autologous virus and HIV-1_{BaL} virus. Autologous virus could be detected by intracellular p24 staining in five MVA.HIVconsv trial participants after PHA activation and culture of their CD4+ T cells. A study by Saez-Cirion et al. demonstrated that the level of inhibition against autologous virus was similar to that measured against superinfection, indicating that measurement of both gives comparable results[261]. However this work was carried out using CD8+ T cells from HIV-1 controllers and it may be this observation is not generalizable to all HIV-1 infected individuals. Differences in the capacity of CD8+ T cells from chronically infected patients to inhibit autologous and heterologous viruses may reflect their specificity, avidity or lytic capacity. Work in our group has shown that CD8+ T cells from chronically infected patients were better able to inhibit autologous than heterologous viruses (Yang H., unpublished data). A laboratory adapted strain with mutations that confer resistance to antiretroviral agents, that are then added to the culture to prevent outgrowth of endogenous virus could allow for more accurate measurement of inhibition of superinfection only[430].

In summary, vaccination of HIV-1 infected HAART treated patients with a higher dose of MVA.HIVconsv resulted in a trend towards increased CD8+ T cell mediated inhibition as measured using the viral inhibition assay. However, the level of inhibition

following vaccination was not as potent as that observed in viraemic controllers who could suppress virus replication by 80-90% in this assay. The absence of potent antiviral responses in trial participants may have been due to any or all of: i) incomplete immune reconstitution due to initiation of HAART in chronic infection, ii) the homologous vector regimen, iii) poor potency of the vector or iv) targeting of regions of the HIV-1 proteome by HIVconsv that are not genetically fragile despite being highly conserved and therefore not associated with control of virus replication *in vivo*. This will be explored further in chapter 6. Work in the next chapter investigated whether therapeutic vaccination could affect the size of the latent HIV-1 reservoir.

5. Analysis of the effect of therapeutic vaccination with MVA.HIVconsv on the HIV-1 latent reservoir in chronically infected patients

5.1 Introduction

Treatment of HIV-1 infection with highly active antiretroviral therapy (HAART) results in the rapid control of HIV-1 replication, reducing plasma HIV-1 RNA levels below the limit of clinical detection and partially restoring immune function. Despite this a stable reservoir of infected cells which are replication competent but transcriptionally silent persist and cause virus rebound upon treatment interruption[473,474].

The major reservoir is formed from a small pool of latently infected resting memory CD4+ T cells (<0.05% of the total resting CD4+ T cell population)[475]. Experiments by Chomont et al. demonstrated that 52% of these latently infected resting CD4+ T cells are T_{CM}, 34% are T_{TM} and 14% are T_{EM}[476]. Memory T cells are an ideal cell type for harbouring latent infection due to their long lifespan, however, latency may also occur in other cell subsets such as naïve CD4+ T cells, monocytes and macrophages, astrocytes and microglial cells. Recently, CD4+ T cells with stem cell-like properties (T_{SCM}) have been shown to contain high per cell levels of HIV-1 DNA and due to their ability to self-renew, avoid apoptosis and survive for extended periods of time may allow for long-term viral persistence[477]. HIV-1 persistence is thought to occur due to the long-lived nature and homeostatic proliferation of latently infected cells, however, residual viral replication may also contribute to the replenishment of the latent

reservoir[477–481]. This may occur particularly in privileged anatomical sites such as the gut mucosa, genital tract, lymph nodes and CNS where there is suboptimal penetration of antiretroviral drugs.

Cells may either become latently infected by direct infection of resting CD4+ T cells (pre-activation model) or by infection of activated cells which avoid cell death and then revert to a resting state (post-activation model)[431,482]. The convergence of multiple molecular mechanisms is likely responsible for the silencing of HIV-1 DNA following integration into the host genome. These can either be transcriptional or post transcriptional blocks (Table 5.1).

Transcriptional blocks	Insufficient levels of transcription factors
	Transcriptional interference
	Limited elongation factors
	Chromatin modifications
	Insufficient Tat activity
Post transcriptional blocks	Inefficient mRNA splicing
	Inefficient nuclear export
	miRNA regulation of viral protein expression

Table 5.1 Mechanisms of latency establishment

The latent reservoir cannot be cleared by current therapeutic options, making it a major barrier to HIV-1 cure. There is renewed interest in the development of affordable and safe curative strategies for HIV-1. Cure could be defined as functional (host mediated control in the absence of HAART, similar to the control of virus replication seen in HIV-1 elite controllers) or sterilising (complete elimination of virus from the host). Evidence that a cure is possible has come from several sources, firstly, an HIV-1

infected patient known famously as the 'Berlin patient', received an allogenic hematopoietic stem cell transplant, using a homozygous CCR5Δ32 donor, for the treatment of leukaemia. The patient is to date, five years later undetectable for HIV-1 DNA and RNA and no replication-competent virus can be cultured from his PBMCs[483]. Two further patients, who received allogeneic transplants for the treatment of lymphoma, but from CCR5 wild-type donors, had undetectable HIV-1 DNA and no detectable HIV-1-specific T cell responses by IFNγ-Elispot screenings of total PBMCs for three and five years whilst remaining on therapy. However both rebounded at 12 and 32 weeks respectively after HAART interruption[484,485]. An infant born to an HIV-1 infected mother, who started treatment within 30 hours of being born, remained undetectable for HIV-1 RNA, proviral DNA in PBMCs and HIV-1 antibodies until 47 months of age, despite cessation of HAART at 18 months of age[486]. The mechanisms behind these possible cures are not fully understood and the interventions used are not feasible for use in the general HIV-1 population.

The reservoir is established in primary infection[4] and early treatment can reduce the size of the reservoir but a pool of stably infected cells persists[487–489]. A study by Chun et al. demonstrated that administration of HAART as early as 10 days after the onset of clinical symptoms did not prevent establishment of the latent reservoir[4]. Eradication of latent virus will therefore require a targeted approach to induce provirus gene expression. Attempts to activate transcription of HIV-1 include the use of HDAC inhibitors, compounds targeting the PKC and NF-κB pathways, Disulfiram, PD-1 inhibition and therapeutic vaccination (Reviewed in [490]). Two separate clinical studies investigated the effect of the HDAC inhibitor vorinostat in HIV-1 infected individuals

on treatment. In a study by Archin et al. eight subjects were given a single 400mg dose of vorinostat and an increase in HIV-1 RNA expression in resting CD4+ T cells was observed in all patients[491]. Elliot et al. administered 400 mg of vorinostat daily for 14 days and also observed an increase in HIV-1 RNA but no change in HIV-1 DNA[492]. A key question therefore is the fate of these cells following reactivation of latent virus. An *in vitro* study by Shan et al. showed that latently infected cells reactivated with Vorinostat were not killed by cytopathic effects (CPEs) or the host immune response and that CD8+ T cells from patients on treatment could not kill these cells. Only CD8+ T cells from elite controllers or CD8+ T cells prestimulated with Gag peptides could kill reactivated CD4+ T cells[465]. Elimination of the reservoir may therefore require a two-pronged approach involving viral reactivation together with induction / boosting of effective virus-specific CD8+ T cell responses to eliminate CD4+ T cells harbouring reactivated virus.

It will be necessary to be able to accurately measure the size of the HIV-1 reservoir in order to assess the effectiveness of strategies targeting latently infected cells. The viral outgrowth assay (VOA) is the principal method for measuring HIV-1 persistence during HAART. Highly purified resting CD4+ T cells isolated by fluorescence activated cell sorting are stimulated with PHA or anti-CD3/anti-CD28 to induce T cell activation, virus released from these cells is then expanded in CD4+ T cells from HIV-1 negative donors for 2-3 weeks and a p24 ELISA is used to measure virus in the culture supernatant. The main advantage of this assay is that it measures the frequency of latently infected cells carrying replication competent virus. The pitfalls of this assay are that it is labour intensive, expensive and requires 120-180 ml of blood. An adaptation of

this method reported by Laird et al. uses bead and column-based purification of resting CD4+ T cells, the MOLT-4/CCR5 cell line for viral expansion and a quantitative RT-PCR rather than p24 ELISA for virus detection, making the technique more labour and cost-effective and therefore suitable for use in clinical trials of HIV-1 eradication strategies[493]. A recent paper by Ho et al. raised concerns that not all proviruses are induced during the T cell activation step and therefore the VOA may underestimate the size of the latent reservoir[494]. Other approaches for measuring the latent reservoir involve PCR-based assays. These assays can be used to measure HIV-1 DNA in unfractionated PBMCs or purified resting CD4+ T cells and are able to distinguish between integrated and unintegrated HIV-1 DNA. A new PCR assay, ddPCR has greater sensitivity than standard PCR and therefore is particularly effective for measuring low template numbers such as those in latently infected cells[495]. It will be preferential to specifically target replication competent virus rather than reducing the number of HIV-1 DNA positive cells; PCR-based assays cannot differentiate between defective and replication competent viruses. Eriksson et al. compared 11 different methods for measuring latent HIV-1 in patients on HAART. HIV-1 DNA cell containing frequencies were approximately two logs higher when measured using PCR based assays compared with the viral outgrowth assay, this is possibly due to the presence of a large amount of defective virus which is detected using PCR-based assays[496].

The aim of the work described in this chapter was to investigate the susceptibility of the latent reservoir to CD8+ T cells that have been primed and / or boosted by a rationally

designed vaccine, which targets the most conserved regions of the HIV-1 proteome, in long term HAART treated patients.

5.2 Results

5.2.1 ddPCR optimisation

The efficiency of a PCR reaction is dependent on the binding of the primer to the target DNA sequence. To optimise the detection of HIV-1 DNA in CD4+ T cells by ddPCR 2 primers, Gag and LTR, were used to test baseline samples (Screen or Day 0) from all 19 MVA.HIVconsv trial subjects. The primer giving the highest number of positive droplets (those containing HIV-1 DNA) in each patient was then used to test subsequent time points. Table 5.2 shows the sequence of the primers used and Figure 5.1 shows the location of these primers in the HIV-1 proteome.

Primer	Region	Direction	Position HXB2	Sequence 5'-3'
HIV_F (SCA)	GAG	F	1299	CATGTTTTCAGCATTATC AGAAGGA
HIV_R (SCA)	GAG	R	1359	TGCTTGATGTCCCCCCA CT
HIV PROBE (SCA)	GAG		1325	CCACCCCACAAGATTTA AACACCATGCTAA
LTR U5	LTR U5	R	628	GTT CGG GCG CCA CTG CTA G
LTR	LTR	F	521	TTA AGC CTC AAT AAA GCT TGC C
PROBE NEW -2	LTR		561	CCA GAG TCA CAC AAC AGA CGG GCA

Table 5.2 Sequence of the Gag and LTR primers used in the ddPCR assay.

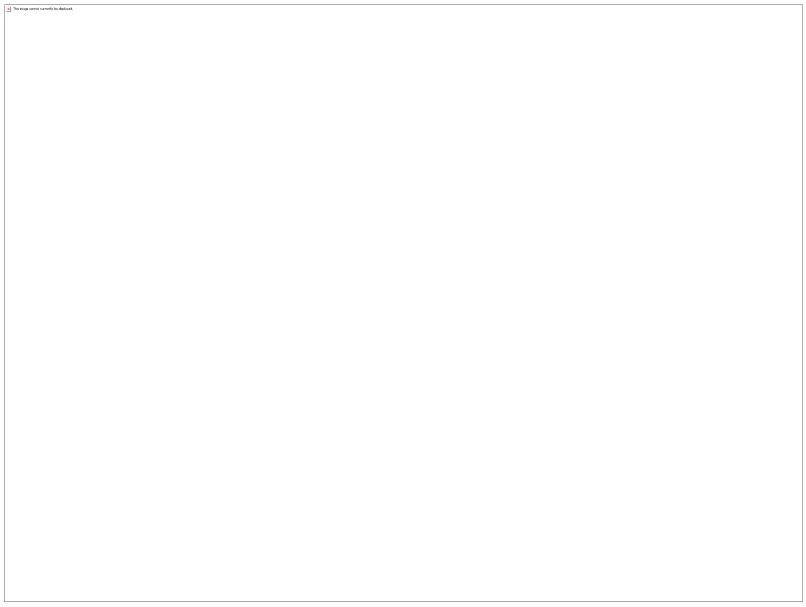


Figure 5.1 Location of the Gag and LTR primers in the HIV-1 proteome

There were only two patients in whom the Gag primer gave a higher concentration of HIV-1 DNA than the LTR primer and was therefore used for analysis of the remaining time points in these two patients. The LTR primer was used for all subsequent time points in the remaining patients.

5.2.2 HIV-1 DNA can be detected in CD4+ T cells from all MVA.HIVconsv trial subjects

The cutoff for detecting HIV-1 DNA by ddPCR is reported to be between 1-14 copies per million cells[492,493]. Using the upper end of 14 copies per million cells as a cutoff, HIV-1 DNA was detected in 19/19 MVA.HIVconsv patient samples at all time points tested. Values varied over a ~2 log range with a median value of 1083 copies HIV-1 DNA/10^6 CD4+ T cells (IQR 592-2128 copies HIV-1 DNA/10^6 CD4+ T cell) for the entire cohort. Figure 5.2 shows positive (blue) and negative (grey) droplets in a baseline

and week 38 sample from a HIV-1 infected patient as well as positive droplets in a HIV-1 negative donor control sample.

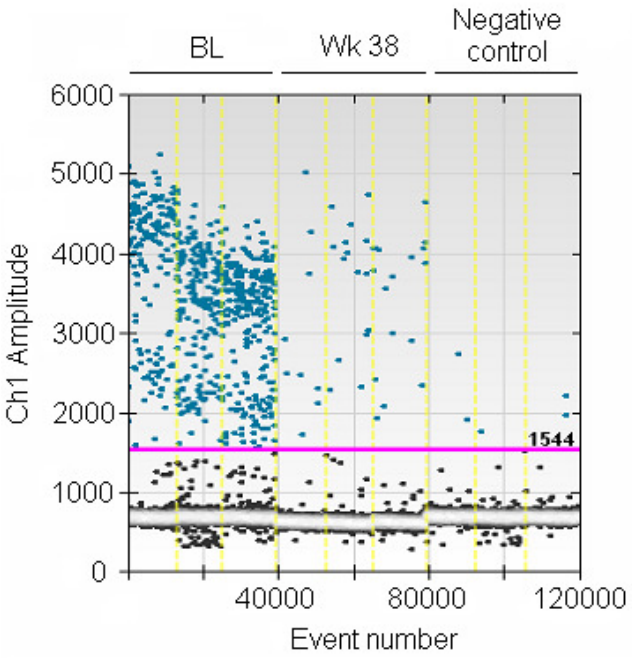


Figure 5.2 Positive droplets containing target DNA (Blue) have a higher fluorescence than negative droplets (grey) which do not contain DNA of interest. Data shown for a baseline and week 38 sample and a HIV-1 negative donor control. Horizontal pink line shows the manually set threshold for droplet positivity. Y-axis is fluorescence intensity and x-axis is total number of events.

5.2.3 Patient-specific characteristics do not predict the size of the HIV-1 reservoir as determined by quantification of CD4+ T cells harbouring HIV-1 DNA

Because establishment of the HIV-1 reservoir occurs in primary infection, clinical factors such as CD4+ T cell nadir, CD4+ T cell count and the time to initiation of treatment could all influence the size of the latent reservoir. There was no correlation between CD4+ T cell nadir ($r = 0.42$, $p = 0.15$), CD4+ T count ($r = -0.23$, $p = 0.33$) or the interval between diagnosis and initiation of treatment ($r = 0.17$, $p = 0.54$) with the

frequency of CD4+ T cells harbouring HIV-1 DNA at baseline in MVA.HIVconsv trial participants (Figure 5.3).

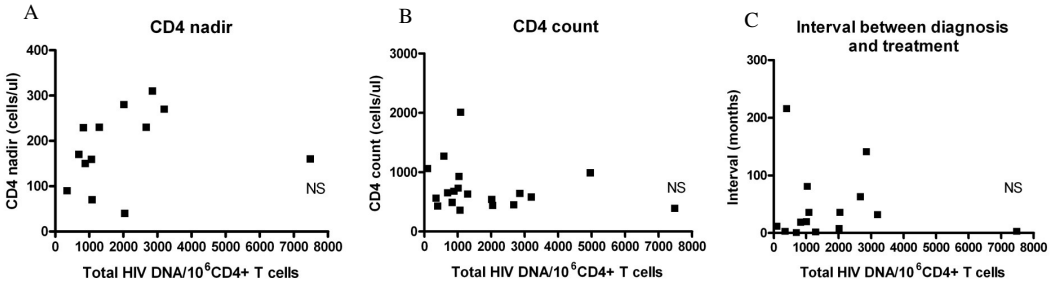


Figure 5.3 No correlation between (A) CD4+ T cell nadir (B) CD4+ T cell count and (C) interval between diagnosis and initiation of treatment with total copies of HIV-1 DNA/10^6 CD4+ T cells from baseline samples, measured using ddPCR. Statistical analysis performed using the Spearman rank correlation test.

The mean time on treatment of the MVA.HIVconsv trial participants at baseline was 49 months (range 12-144 months). There was no correlation between time on therapy and total HIV-1 DNA/10^6CD4+ T cells at baseline (Figure 5.4). Because the frequency of CD4+ T cells harbouring HIV-1 DNA was not measured when treatment was initiated, a possible correlation between the change in the frequency of latently infected cells and time on treatment cannot be calculated.

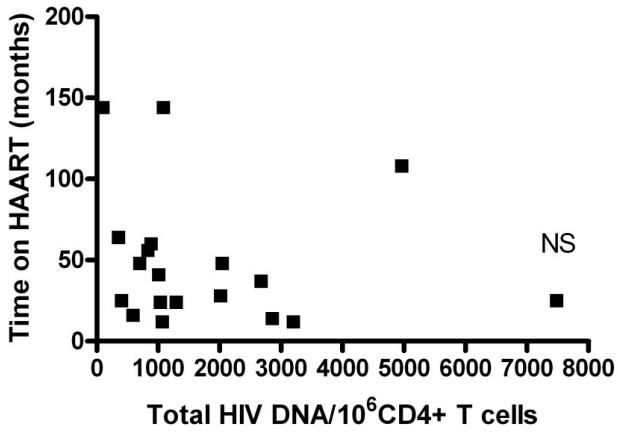


Figure 5.4 No correlation between time on HAART of trial participants and total copies of HIV-1 DNA/10^6 CD4+ T cells at baseline as measured by ddPCR. Spearman rank correlation used to assess correlation.

5.2.4 The role of HIV-1-specific CD8+ T cells in controlling the level of HIV-1 DNA in CD4+ T cells

Potent CD8+ T cell responses could limit the size of the latent reservoir by reducing HIV-1 infection events and eliminating all actively infected CD4+ T cells[463,497]. It was therefore important to examine if CD8+ T cell responses in trial participants correlated with total HIV-1 DNA in CD4+ T cells at baseline. There was a weak correlation, although not significant between CD8+T cell mediated inhibition (as measured in Chapter 4) and the size of the latent reservoir at baseline in MVA.HIVconsv trial partipants ($r = -0.42$, $p = 0.09$) (Figure 5.5).

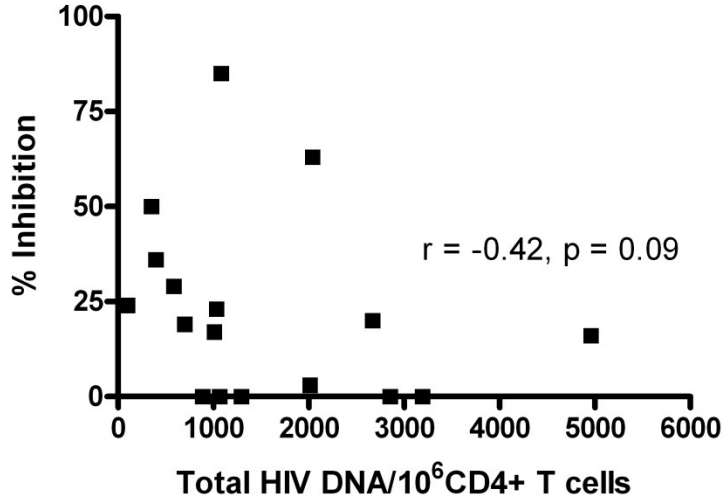


Figure 5.5 Correlation between percent inhibition against HIV-$_{BaL}$ at a 1:1 CD8+/CD4+ T cell ratio measured on day 6 of co-culture from baseline sample and total HIV-1 DNA/10^6 CD4+ T cells at baseline measured by ddPCR. Statistical analysis perfomed using the Spearman rank correlation test.

CD8+ T cell responses to the 14 most conserved regions of the HIV-1 proteome were mapped in the HIVconsv trial participants (Chapter 3); T cell responses to conserved regions have been associated with control of virus replication[222,396,406]. There was however no correlation between the total magnitude of response to the conserved regions in HIVconsv (as measured in Chapter 3) and the size of the reservoir at baseline (Figure 5.6A). An *in vivo* study by Descours et al. demonstrated that HLA-B27 and HLA-B57 restricted CD8+ T cell responses against Gag limited the size of the latent reservoir in LTNPs[497]. In spite of this, there was no correlation between IFN-γ Elispot responses to SP1, a region of HIVconsv that is exclusively Gag, and the size of the reservoir at baseline in the HIVconsv trial participants (Figure 5.6B).

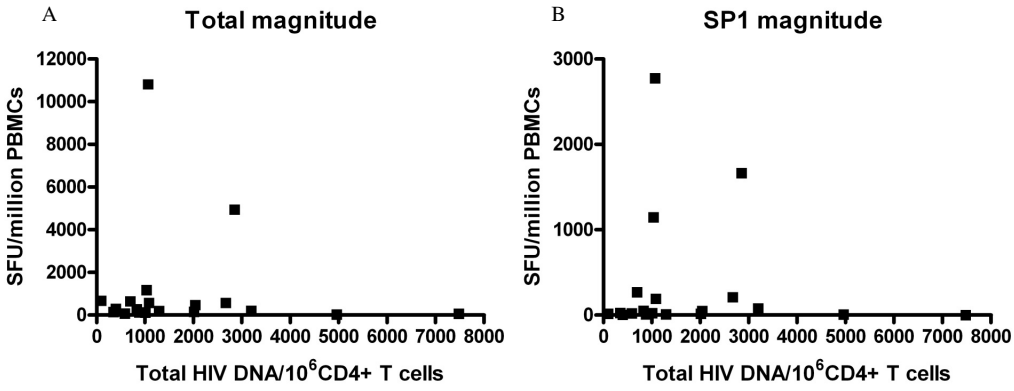


Figure 5.6 No correlation between magnitude of response to (A) all HIVconsv peptides and (B) SP1 peptides measured by IFN-γ Elispot assay at baseline (screen or day 0) with total HIV-1 DNA/10^6 CD4+ T cells quantified by ddPCR at baseline in MVA.HIVconsv trial participants. Statistical analysis perfomed using the Spearman rank test.

5.2.5 Vaccination with MVA.HIVconsv is not associated with a reduction in the level of total HIV-1 DNA in CD4+ T cells

At baseline (Screen or Day 0) there was no significant difference in the size of the HIV-1 reservoir between MVA.HIVconsv vaccinees and placebos measured by dPCR (1032 vs. 1652 copies HIV-1 DNA/10^6 CD4+ T cells, p = 0.45) (Figure 5.7).

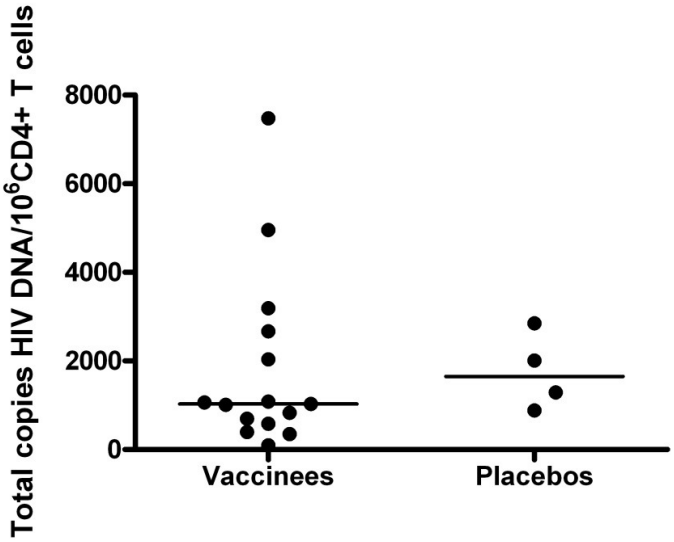


Figure 5.7 Copies of HIV DNA/10^6 CD4+ T cells in 15 vaccinees and 4 placebos at baseline (Screen or Day 0) as measured by ddPCR. Horizontal bars show median values. Statistical analysis carried out using Mann Whitney test.

HIV-1 DNA was measured at two post-vaccination time points: week 6, in order to detect any rapid changes in the size of the latent reservoir occurring after the second vaccination and week 38 to look for slower long term changes. There was no change in the size of the latent reservoir measured at week 6 or week 38 compared to baseline in either the vaccinees (944 vs. 960 vs. 1032 median copies HIV-1 DNA/10^6 CD4+ T cells, p = 0.77; Friedman 1 way ANOVA test) or placebos (2058 vs. 1351 vs. 1652 median copies HIV-1 DNA/10^6 CD4+ T cells, p = 0.65; Friedman 1 way ANOVA test) (Figure 5.8). There was also no change in the size of the reservoir at week 6 or week 38 compared to baseline when separating the vaccinees into the low (1260 vs. 1272 vs. 1074 median copies HIV-1 DNA/10^6 CD4+ T cells, p = 0.65; Friedman 1 way ANOVA test) and high dose groups (721 vs. 577 vs. 827 median copies HIV-1 DNA/10^6 CD4+ T cells, p = 0.23; Friedman 1 way ANOVA test) (Figure 5.9).

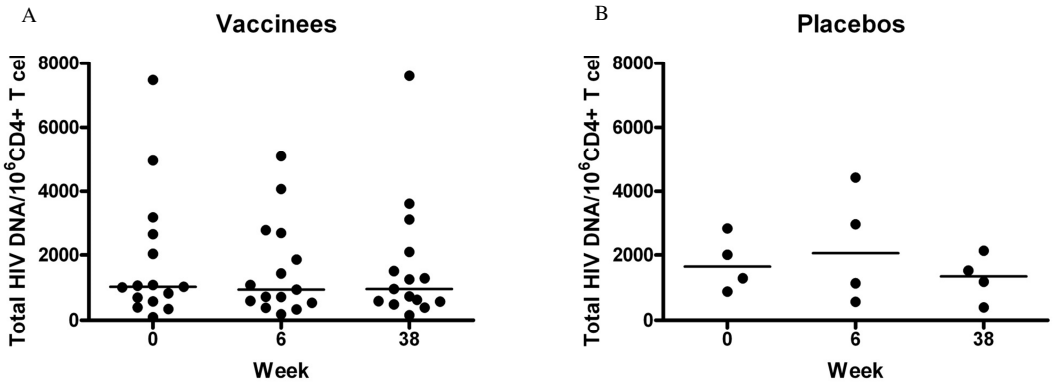


Figure 5.8 Copies of HIV DNA/10^6 CD4+ T cells in (A) 15 vaccinees and (B) 4 placebos at baseline (Screen or Day 0), week 6 and week 38 measured by ddPCR. Horizontal lines show medians. Statistical analysis performed using Friedman 1 way ANOVA test.

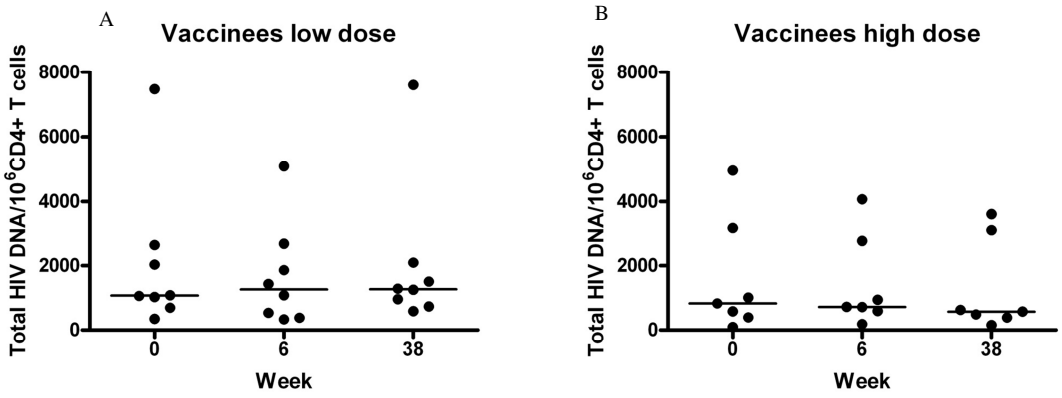


Figure 5.9 Copies of HIV-1 DNA/10^6 CD4+ T cells in (A) eight 'low dose' vaccinees who received $5x10^7$ pfu MVA.HIVConsv and (B) seven 'high dose' vaccinees who received $2x10^8$ pfu MVA.HIVconsv, measured at baseline (Screen or Day 0), week 6 and week 38 by ddPCR. Horizontal lines show medians. Statistical analysis performed using Friedman 1 way ANOVA test.

To allow for differences in the size of the reservoir between patients at baseline, copies of HIV-1 DNA/10^6 CD4+ T cells at week 6 and 38 were expressed relative to the size at baseline. This allows for any increases or decreases in the size of the reservoir with respect to the baseline to be observed (Figure 5.10). This confirmed that there was no significant change in the size of the reservoir during the course of the study.

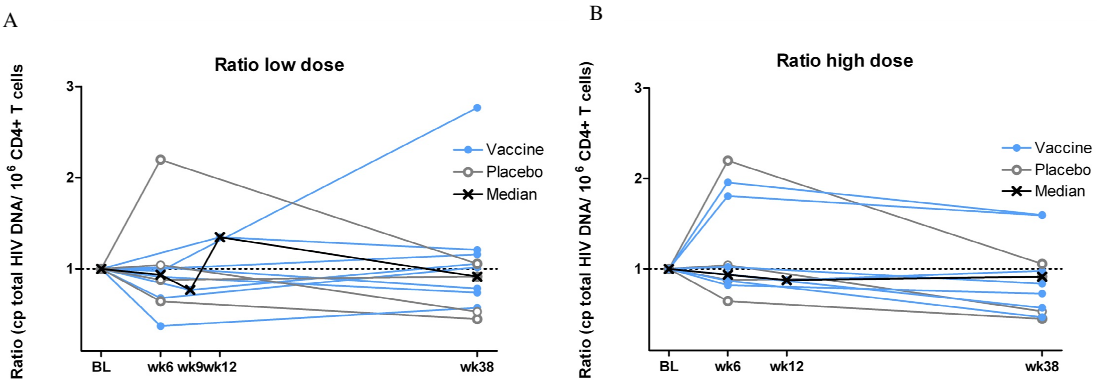


Figure 5.10 Ratio of total HIV-1 DNA/10^6 CD4+ T cells at post-vaccination timepoints as compared with baseline total HIV-1 DNA/106 CD4+ T cells in (A) low and (B) high dose vaccinees.

5.3 Discussion

The experiments in this chapter sought to investigate the effect of therapeutic vaccination on the size of the latent reservoir. I used ddPCR to quantify total HIV-1 DNA in CD4+ T cells prior to and post vaccination of 19 HAART-treated patients who were vaccinated with MVA.HIVconsv or placebo. ddPCR is a third generation PCR-based assay which involves generating thousands of droplets within a sample in order to separate the DNA template molecules. PCR amplification is then carried out within each droplet enabling the measurement of thousands of independent amplification events within a single sample. With conventional PCR the number of amplification cycles is proportional to the starting copy number; ddPCR is an absolute measure of target DNA concentration so eliminates the reliance on uncertain exponential data to quantify the sample. ddPCR provides an increased precision of over four fold to over 20-fold verses real time PCR[495]. The disadvantage of ddPCR is that it does not distinguish between integrated or unintegrated HIV-1 DNA. HIV-1 proviral DNA may not always integrate into the host genome on entry into the cell nucleus. This results in circularized molecules containing one or two copies of the long terminal repeat region. In spite of this, work by Eriksson et al. demonstrated that integrated HIV-1 DNA in resting CD4+ T cells measured by Alu PCR correlated well with total HIV-1 DNA in resting CD4+ T cells measured by ddPCR in HAART-treated patients[496]. This is consistent with data demonstrating that after one year of therapy most HIV-1 DNA is integrated in the host genome, with unintegrated forms making only a small contribution to the size of the latent reservoir[498]. One of the inclusion criteria for this therapeutic vaccination study was treatment with an effective HAART regimen for a

minimum of one year, therefore, it is likely the results obtained here by ddPCR would accurately reflect of the size of the latent reservoir. A further limitation of DNA / RNA quantification is that it does not specifically measure replication-competent virus. A large proportion of infected resting CD4+ T cells contain defective, hypermutated or silenced proviruses[499,500]. Agents to induce HIV-1 replication may not have any effect on defective virus and therefore any successful clearance of latently infected cells could be masked by the large number of cells harbouring defective virus. An additional consideration is the design of the primers used in the ddPCR assay: they were based on conserved regions of the HIV-1 proteome to maximise amplification of diverse viral sequences, however, there is still the risk that some sequences may not be amplified due to inefficient binding to mismatched viral sequences in some patients. This could be avoided by using patient specific primers; however this would be at a greater cost and increased time. In addition, my analysis was limited to CD4+ T cells in the peripheral blood. It is possible that the frequency of latently-infected cells in gut associated lymphoid tissue (GALT), a major site of HIV-1 replication, may differ. Several studies have shown increased levels of latently infected CD4+ T cells in the GALT as compared with the peripheral blood[345,483,496,501]. Eriksson et al. measured HIV-1 DNA in rectal biopsy samples in 19 HAART-treated subjects and using paired comparisons found infected CD4+ T cell frequencies to be significantly higher than infected cell frequencies in the peripheral blood[496]. It would therefore be valuable to measure HIV-1 DNA in CD4+ T cells from the GALT in parallel.

Median copies of HIV-1 DNA/10^6 CD4+ T cells in the 19 MVA.HIVconsv trial subjects were slightly higher than levels reported in a study by Chun et al. examining

the frequency of CD4+ T cells carrying HIV-1 DNA in HAART treated patients. However Chun et al. used real-time PCR to quantify HIV-1 DNA in CD4+ T cells which is less accurate that ddPCR. Patients had also been on treatment for a median 6.5 years, which is longer than patients in the MVA.HIVconsv trial and therefore it is likely that the reservoir of latently infected cells would be smaller[478]. The size of the latent reservoir at baseline did not correlate with CD4+ T cell nadir in the MVA.HIVconsv study subjects; this contrasts with data from Boulassel et al. who observed an inverse correlation between total HIV-1 DNA in CD4+ T cells with CD4+ T cell nadir[502]. The data from Boulassel et al. indicates that higher viral replication in the acute phase of infection resulting in more CD4+ T cell destruction will create a larger latent reservoir. The lack of correlation in MVA.HIVconsv trial participants could be due to the small sample size or the different method used to measure the latent reservoir. There was also no correlation between CD4+ T cell count, time between diagnosis and treatment and time on treatment with total HIV-1 DNA in CD4+ T cells, which is in agreement with data from two other studies[496,503]. The lack of correlation between time on treatment and total HIV-1 DNA in CD4+ T cells reflects the long half-life of the latent reservoir. The reservoir is thought to decrease in a biphasic manner, after an initial rapid decrease reflecting the death of cells in the preintegration phase of latency, the second phase of decay represents long lived cells and has an estimated half-life of 44 months[23,488,504,505]. A study by Sarmati el al measured decay of the reservoir, quantified as HIV-1 DNA in PBMCs by real-time PCR, after a short duration of therapy and found an inverse correlation between treatment duration and HIV-1 DNA[506]. This was likely observed because the study addressed the first phase of decay.

There was a weak correlation between CD8+ T cell mediated inhibition and the frequency of latently infected CD4+ T cells at baseline in MVA.HIVconsv trial participants. Data from several recent studies has demonstrated the importance of an effective cellular immune response in controlling the size of the HIV-1 latent reservoir. An *ex vivo* study by Shan et al. showed that resting CD4+ T cells in which virus had been reactivated with vorinostat could be killed by HIV-1 Gag-specific CD8+ T cells from elite controllers but not HAART treated patients[465]. In HLA-B27 and HLA-B57 positive LTNPs functional antiviral CD8+ T cells are associated with a reduced size of the central memory CD4+ T cell reservoir[497]. Additionally CD8+ T cells from elite controllers can effectively eliminate non-productively HIV-1 infected resting CD4+ T cells. These non-productively infected cells may represent precursors to latently infected cells and therefore the ability of CD8+ T cells from elite controllers to eliminate them may explain why elite controllers have a lower frequency of latently infected cells[507]. Graf et al. demonstrated *in vitro* that resting CD4+ T cells that express Gag without producing infectious virus can be cleared by CD8+ T cells from elite controllers more effectively than CD8+ T cells from non-controllers. The extent of CD8+ T cell clearance *in vitro* correlated with *in vivo* reservoir size[508]. HIV-1 controllers therefore represent a good model for a functional cure. Although a heterogeneous population, HIV-1 controllers can have effective HIV-1-specific CD8+ T cell responses that preferentially target more conserved regions of the HIV-1 virus[241,245,246], there was however no correlation between CD8+ T cell responses to conserved regions and the size of the latent reservoir in MVA.HIVconsv trial participants at baseline. Due to PBMC availability it was not possible to measure

responses to the whole proteome, but responses to conserved regions of the proteome have been associated with control of virus replication[222,396,406].

Therapeutic vaccination could impact on the size of the latent reservoir through direct and indirect but non-mutually exclusive mechanisms. Firstly it may induce reactivation of latently infected cells, for example through immune activation by the viral vector, rendering these activated cells susceptible to HIV-1-specific CD8+ T cell killing or antiretroviral drugs. Secondly it could boost the frequency of circulating HIV-1-specific CD8+ T cells, which are known to decrease in HAART treated patients, to facilitate clearance of infected resting CD4+ T cells. Consequently there is also a risk that therapeutic vaccination may increase the size of the latent reservoir by inducing CD4+ T cell proliferation, thereby increasing target cell availability. Two studies to date have investigated the effect of therapeutic vaccination on the size of the latent reservoir, however, both used vaccines comprising full-length HIV-1 antigens in a heterologous vaccination regimen. Casazza et al. administered to chronic HAART-treated patients three intramuscular injections of a DNA vaccine encoding Clade B Gag, Pol and Nef and clade A, B and C Env followed by a boost with a replication-deficient adenovirus type 5 vaccine which was homologous for the Gag, Pol and Env antigens. Using the viral outgrowth assay they detected no significant reduction in the size of the latent reservoir one month after the rAd5 boost in either the 12 vaccinees or 5 placebo volunteers despite observing an increase in magnitude, polyfunctionality and breadth of the CD8+ T cell response following vaccination[471]. Persaud et al. also used the viral outgrowth assay to measure changes in the reservoir following heterologous vaccinations with MVA and fowlpox encoding env, gag, tat, rev, nef and RT in an

uncontrolled study of HAART-treated adolescents. Changes in the frequency of resting CD4+ T cells containing replication competent HIV-1 in $\log_{10}$ infectious units per million (IUPM) relative to baseline were reported. They observed a significant decrease relative to baseline at 40 weeks after the first MVA vaccination however the confidence interval was very large (-0.60 to -0.03 $\log_{10}$ IUPM) and by week 72 the frequency of CD4+ cells carrying replication-competent virus had almost returned to baseline values (-0.06 $\log_{10}$ IUPM)[509].

In MVA.HIVconsv trial participants there was no significant change in the size of the latent reservoir at week 6 or week 38 post-vaccination in either the vaccine or placebo group. This study used a homologous vaccination regime with an attenuated MVA vector. During serial passage in chick embryo fibroblasts, MVA lost approximately 15% of its parent genome, including genes responsible for the evasion of the host immune response[424]. Upon infection of mammalian cells with MVA, products of both early and late genes are produced but replication is blocked during virion assembly[425]. This restricts the number of cells that will become infected, thereby limiting the resultant immune response to immunization with a MVA-vectored vaccine. It is likely MVA, particularly when administered in a homologous regimen, is too attenuated to induce the immune activation required to reactivate latently infected cells. Prime-boost vaccination regimens have been shown to be more immunogenic than homologous regimens[313–317] and a study of the heterologous regimen of ChAd.HIVconsv prime and MVA.HIVconsv boost in healthy volunteers showed a significant increase in the CD8+ T cell response following vaccination[308]. A phase I clinical trial is currently investigating the effect of vaccination with ChAd-MVA.HIVconsv on the size of the

latent reservoir in early infected individuals 6 months after the initiation of treatment (ChAd-MVA.HIVconsv-BCN01, Clinicaltrials.gov).

MVA.HIVconsv would also be unable to augment clearance of reactivated cells due to enhancement of HIV-1-specific immune responses because as the results in Chapter 3 demonstrate there was no significant increase in the magnitude of the CD8+ T cell response following vaccination. The attributes of an effective CD8+ T cell response, such as that seen in HIV-1 controllers which limits the size of the latent reservoir, are likely to be the same attributes required to eliminate latently infected cells in order to achieve a functional cure. Further work is required to define the particular characteristics such as phenotype and epitope specificity of these CD8+ T cells. Hansen et al. immunised rhesus macaques with rhesus cytomegalovirus (RhCMV) vectored SIV vaccines and upon challenge with SIVmac239 13/24 macaques rapidly controlled virus to undetectable levels. There were occasional transient bursts of viraemia, but after week 52 viral blips rarely occurred and the vast majority of PBMCs from these rhesus macaques were negative for cell-associated SIV RNA and DNA[427]. This study indicates that the T_{EM} cells induced by vaccination were able to prevent the formation of a latent reservoir.

In conclusion, vaccination of HAART treated patients with MVA.HIVconsv did not lead to a reduction in the size of the latent reservoir as determined by quantification of CD4+ T cells harbouring HIV-1 DNA by ddPCR. Only one therapeutic vaccination study to date has shown a reduction in the size of the latent reservoir[509]. It is therefore

likely that therapeutic vaccination will form part of a multi-targeted approach to reduce the HIV-1 latent reservoir.

6. Targets of a beneficial T cell response

6.1 Introduction

Although responses to conserved regions of the HIV-1 proteome have been associated with control of virus replication[222,396,406], data from the previous three chapters demonstrated that vaccination of HAART-treated patients with a rationally designed vaccine targeting such regions failed to: i) boost significantly or induce de novo T cell responses as measured by IFN-γ Elispot assay, ii) increase CD8+ T cell-mediated inhibitory activity to levels observed in HIV-1 controllers, or iii) reduce the size of the latent HIV-1 reservoir. The limited immunogenicity of MVA.HIVconsv could be due to a plethora of reasons, including the immunogenicity and dose of the MVA vector used, the study population and the design of the immunogen. In particular, it brings into question which regions of the HIV-1 proteome should be targeted by vaccination. The definition of a minimal length immunogen which can be used in a broad HLA context is essential.

We and others have shown that the capacity of CD8+ T cells to inhibit HIV-1 virus replication *in vitro* is highly predictive of virus control *in vivo*[262,446,462]; however the essential components of an immunogen that could elicit effective CD8+ T cell responses, as defined in a viral inhibition assay, are undefined. In both clade B and C infection, T cell responses to Gag proteins have most consistently been associated with reduced viral load[250,259,510,511], with generally no such correlations found for other proteins. This association was confirmed by a post-hoc analysis of STEP trial participants who received the MrkAd5 gag/pol/nef vaccine, showing that individuals

who targeted ≥3 epitopes in Gag achieved a lower viral load than subjects without Gag-specific responses[337]. However, both the MrkAd5 HIV-1 vaccine and the ALVAC/gp120 vaccine regimen tested in RV144 failed to impact on HIV-1 acquisition[161,332,333] even though they each included full length Gag proteins. Furthermore, Yang et al. found no correlation between CD8+ T cell mediated virus inhibition and the frequency of IFN-γ secreting T cells specific for Gag proteins as a whole[446]. Additionally it has been shown that not all Gag epitopes are equal in terms of genetic fragility[252].

In a cohort of 950 untreated HIV-1 clade B and C infected individuals Mothe et al. explored responses to the entire proteome using a set of 410 overlapping peptides (OLPs) of 18 amino acids in length. By comparing median viral loads between OLP responders and non-responders a protective ratio was generated for each OLP. An OLP with a PR > 1 indicated that it was preferentially targeted by individuals with reduced viral loads ('beneficial OLP'). OLPs spanning Gag proteins had the highest PR's; however Gag also contained OLPs with a PR<1, indicating that even proteins that are thought to be favorable in terms of eliciting 'effective' T cell responses may contain 'non-beneficial' regions. For the clade B cohort 26 OLP were identified for which the median viral load significantly differed between OLP responders and non-responders ('beneficial' OLP). These beneficial OLP were skewed towards Gag and Pol, with Env containing only OLP with a PR<1 ('non-beneficial' OLP). Only one beneficial OLP was located in Nef despite this protein being frequently targeted in primary infection[216]. In clade C, 22 OLPs with a PR>1 where identified, with distribution patterns similar to

the clade B beneficial OLPs. Importantly, beneficial regions were identified outside of the Gag protein in both clade B and clade C proteomes[396].

The work described in this chapter investigated whether targeting of the regions identified by Mothe et al. as being 'beneficial'[396] was associated with potent CD8+ T cell mediated virus inhibition. Samples from HIV-1-infected individuals from the STEP (HVTN502) and Phambili (HVTN503) vaccine trials were available to me to address this hypothesis. These phase IIb studies evaluated the impact of a replication-defective adenovirus type 5 (Ad5) Merck gag/pol/nef HIV-1 vaccine on HIV-1 infection and on early control of viral replication upon HIV-1 acquisition in cohorts at high risk of HIV-1 exposure. The STEP study enrolled men and women in the Americas, Caribbean and Australia[332]; the Phambili trial enrolled men and women in South Africa[333]. Subjects were randomized to receive three intramuscular injections of MrkAd5 gag/pol/nef or placebo at study enrolment, week 4 and week 26. An interim analysis in the STEP study showed no vaccine efficacy and vaccinations were halted in both the STEP and Phambili studies[332,333]. As I performed a blinded analysis with these samples I was also able to investigate whether vaccination with MrkAd5 gag/pol/nef was associated with greater post-infection CD8+ T cell antiviral activity than that observed in placebo recipients.

6.2 Results

6.2.1 Study subjects

PBMCs from 36 STEP (HVTN 502) and Phambili (HVTN 503) trial participants were used for this study. The STEP samples were obtained from 13 vaccinees and 7 placebos and the Phambili samples were from 10 vaccinees and 6 placebos. All samples were from HIV-1 seropositive individuals who were HAART-naïve 12 months after infection, with CD4+ cell counts >350 cells µl. Clinical characteristics of both groups are shown in Table 6.1.

	HVTN 502 (n=20)	HVTN 503 (n=16)	p value
Sex, male (%)	100	31	-
Age, years	30 (23-35)	23 (22-28)	0.07
Known duration of infection (Days)	364 (363-365)	370 (364-375)	0.10
CD4+ T cell count cells/µl (at time of sample)	633 (487-671)	559 (463-651)	0.46
Plasma viral load at 3 months log10 (copies/ml)	4.19 (3.31-4.70)	4.36 (3.59-5.18)	0.31
Subjects with known protective alleles[a]	4	3	-
Clade			
B	19/20	0/16	
C	0/20	7/16	
Other	1/20	2/16	
Not defined	0/20	7/16	

Table 6.1 Characteristics of STEP (HVTN 502) and Phambili (HVTN 503) trial participants. Data shown are median values (interquartile range) unless otherwise indicated. [a] HLA-B5701/03, B27, B51, B5801, and B8101.

6.2.2 Virus panel development

To investigate the breadth of CD8+ T cell mediated inhibition I developed a panel of five laboratory-adapted and primary HIV-1 isolates representing clades A, B and C for use in the virus inhibition assay. In order to determine the optimum time to assess CD8+ T cell mediated inhibition for each of the viral isolates, PHA-blasted CD4+ T cells from HIV-1 uninfected donors were infected with each viral isolate at multiplicities of infection indicated in the appendix and cultured for 10 days. Infected CD4+ T cells were quantified by flow cytometry as described in Materials and Methods (section 2.13.5) between days 1 and 10. The clade B isolates BaL and IIIB, clade C and clade A(1) isolates showed a plateau in replication between days 6-7 of culture, while the clade A(2) isolate peaked at day 3 (Figure 6.1). Quantification of p24 Ag+ cells to assess virus inhibition was therefore carried out on day 3 for A(2) and day 6 for Bal, A(1), C and IIIB.

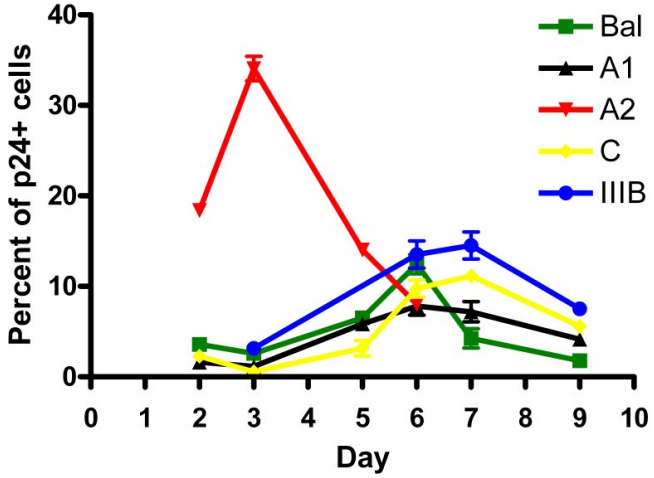


Figure 6.1 Kinetics of five viral isolates. CD4+ T cells from three HIV-1 uninfected donors were superinfected with two clade B viruses (BaL and IIIB), a clade C, and two clade A viruses (A1 and A2) at MOIs indicated in the appendix and cultured in duplicate for 10 days. p24+ cells were quantified by flow cytometric analysis of intracellular p24 antigen between days 2 and 10 post super-infection. Each data point represents the mean of duplicate wells with standard deviation shown by error bars.

6.2.3 HIV-1 positive STEP/Phambili participants show low potency and breadth of CD8+ T cell mediated inhibition

CD8+ T cell mediated antiviral activity was measured in 20 STEP and 16 Phambili trial participants sampled 12 months post infection using a panel of laboratory adapted and primary HIV-1 isolates representing clades A, B and C. Due to limited cell availability, inhibition of one clade B and one clade C virus were tested as a minimum, with the other viruses included if cell number permitted. The potency and breadth of CD8+ T cell-mediated inhibitory responses in these subjects was compared with a 'reference' cohort of viraemic controllers from Duke University (Dr. Georgia Tomaras) which had been characterised previously by other group members Dr. Hongbing Yang and Ms Lisette Yorke (University of Oxford). The data from these two cohorts are shown as a heatmap in Figure 6.2.

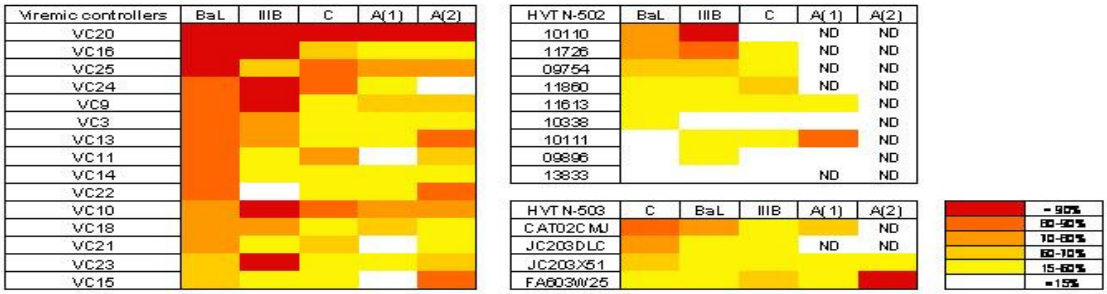


Figure 6.2 Potency and breadth of CD8+ T cell mediated inhibition against a panel of viruses determined by quantification of p24+ cells by flow cytometry on day 3 (A2) or day 6 (Bal, IIIB, C and A1) of co-culture at a 2:1 CD8+/CD4+ T cell ratio in viraemic controllers and STEP (HVTN502)/Phambili (HVTN503) participants. Data only shown for STEP/Phambili participants in which three or more viruses were tested.

Inhibition values observed when using a clade-matched virus in assays with CD8+ T cells from STEP and Phambili participants were consistent with data reported in the literature for early infected individuals[448], but were, however significantly lower than inhibition in the viraemic controller cohort (median inhibition at a 2:1 CD8+/CD4+ T cell ratio 42% vs 85%, p = 0.0002) (Figure 6.3A). This difference was still significant at a 1:10 CD8+/CD4+ T cell ratio, which confirmed the ability of HIV-1 controllers to inhibit virus replication at low effector numbers (median inhibition 18% vs 61%, p<0.0001) (Figure 6.3B) (Hancock et al.; in preparation, [262,446,449,450]).

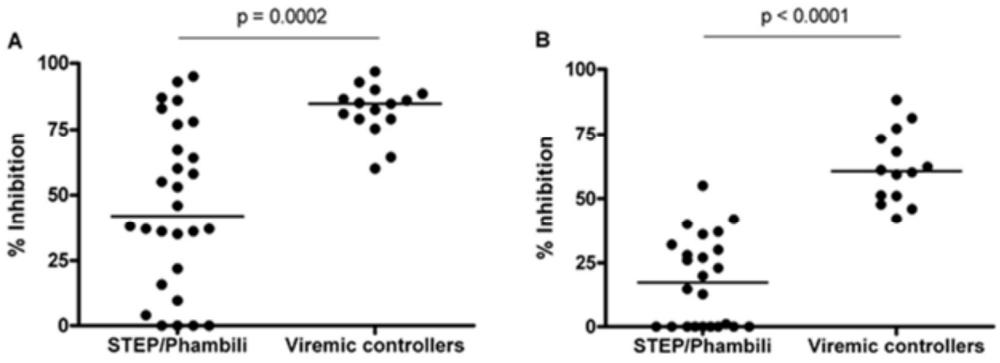


Figure 6.3 Inhibition of a clade matched virus in STEP/Phambili participants and viraemic controllers, determined by quantification of p24+ cells by flow cytometry on day 6 of co-culture at a (A) 2:1 and (B) 1:10 CD8+/CD4+ T cell ratio. Horizontal lines indicate median values. Statistical analysis performed using the Mann Whitney test. For STEP trial participants (clade B) the clade B virus, either Bal or IIIB, giving the highest inhibition value was used in the analysis.

The capacity to inhibit a clade-mismatched virus was lower than for a clade-matched virus in most participants, whether they were viraemic controllers or non-controllers. Nevertheless, cross-clade inhibition was still more potent in viraemic controllers than STEP/Phambili participants at both a 2:1 and 1:10 CD8+/CD4+ T cell ratio (60% vs 29%, p = 0.004 at 2:1 and 43% vs 13%, p = 0.002 at 1:10 CD4+/CD8+ T cell ratio) (Figure 6.4).

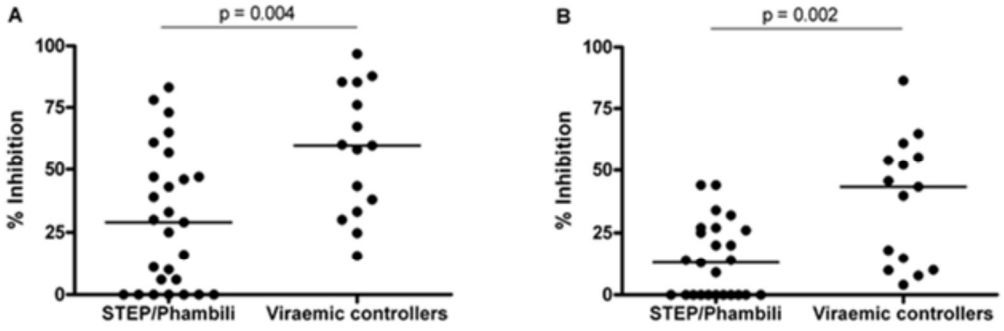


Figure 6.4 Inhibition of a clade mismatched virus in STEP/Phambili participants and viraemic controllers, determined by quantification of p24+ cells by flow cytometry on day 6 of co-culture at a (A) 2:1 and (B) 1:10 CD8+/CD4+ T cell ratio. Horizontal lines indicate medians. Statistical analysis performed using the Mann Whitney test. Inhibition values against the clade C virus were used for STEP trial participants and viraemic controllers and inhibition against either Bal or IIIB, whichever gave the highest value were used for Phambili trial participants.

6.2.4 CD8+ T cell viral inhibitory capacity is inversely related to viral load set point

Yang et al. previously reported a significant inverse correlation between CD8+ T cell mediated inhibition measured at 6 months post-infection and viral load set point, a known predictor of the rate of progression to AIDS[446,512]. To investigate whether a similar relationship existed in this cohort, I calculated viral load set point for MVA.HIVconsv trial participants using the method of Fellay et al. (Viral load data given in the Appendix). The data obtained here also demonstrated a significant inverse correlation between viral-load set point and inhibition in STEP and Phambili trial participants, even though the later was measured 12 months post infection ($r = -0.48$, $p = 0.009$) (Figure 6.5A). This relationship was maintained when viral load values from the viraemic controllers were included in the analysis (viral load value from sample

time point at which inhibition was measured, used in the absence of viral load set point data) (r = -0.41, p = 0.006) (Figure 6.5B).

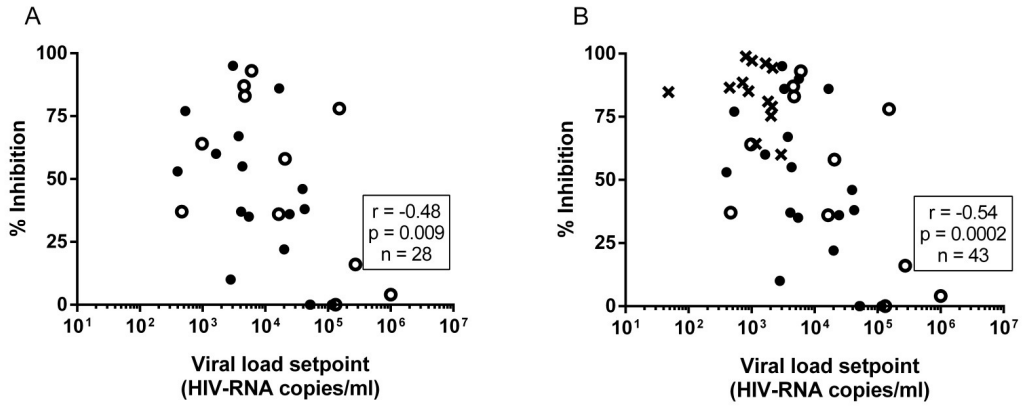


Figure 6.5 Correlation between CD8+ T cell mediated inhibition of a clade matched virus determined by quantification of p24+ cells by flow cytometry on day 6 of co-culture at a 2:1 CD8+/CD4+ T cell ratio and viral load set point (determined using the method of Fellay et al.) in (A) 28 STEP and Phambili participants and (B) 28 STEP/Phambili participants and 15 viraemic controllers. Phambili participants shown with open symbols, viraemic controllers shown as crosses. Average viral load values used for viraemic controllers. Statistical analysis performed using the Spearman rank test.

6.2.5 Vaccination with MrkAd5 gag/pol/nef is not associated with increased CD8+ T cell mediated inhibition one year after infection

The MrkAd5 gag/pol/nef vaccine did not prevent HIV-1 infection and there was no reduction in viraemia in those who subsequently became infected[332,333]. However, post-infection sieve analysis indicated that the vaccine exerted some T cell pressure on breakthrough virus[336]. I hypothesised that this vaccine-induced effect would also be apparent in increased CD8+ T cell mediated inhibition, in STEP and Phambili vaccinees when compared with placebos 12 months post-infection. There was no significant difference in inhibition of a clade-matched virus at a 2:1 CD8+/CD4+ T cell ratio between vaccinees and placebos (median 42% vs 36%, p = 0.59) (Figure 6.6).

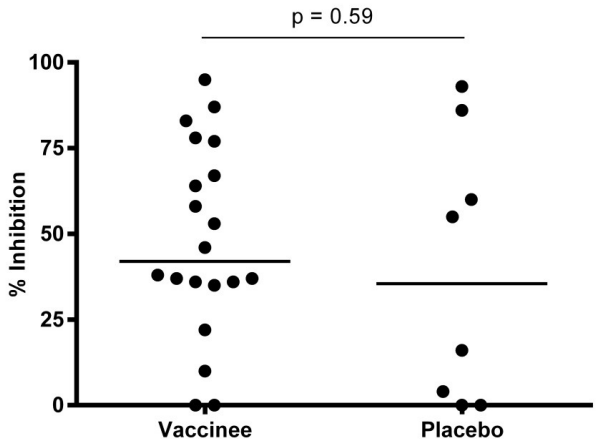


Figure 6.6 Inhibition in STEP/Phambili vaccinees and placebos against a clade matched virus determined by quantification of p24+ cells by flow cytometry on day 6 of co-culture at a 2:1 CD8+/CD4+ T cell ratio. Medians are represented by black horizontal lines and p values reported were generated using the Mann Whitney test.

6.2.6 CD8+ T cell mediated inhibition is strongly associated with targeting of 'beneficial' peptides

To investigate further the mechanisms underlying the limited potency of CD8+ T cell mediated inhibition observed in HIV-1 positive STEP and Phambili trial participants, CD8+ T cell responses to a set of 'beneficial' peptides identified by Mothe et al.[396] and to a set of 'conserved elements' peptides identified by Rolland et al.[414] were measured by IFN-γ Elispot assays using CD8+ T cells sampled from the same time point as those used in the virus inhibition assay. Conserved elements were defined by Rolland et al. as regions within Gag of at least 12 amino acids in length where each amino acid is found in more than 98% of all available independent group M sequences[414]. Gag p24 contains seven such CE segments ranging from 12 to 24 amino acids in length. Different 'beneficial' peptides had been identified previously in clade B and clade C infected populations, therefore, STEP trial participants (Clade B infected) were tested using a

clade B 'beneficial' peptide set and Phambili trial participants (Clade C infected) were tested using a clade C 'beneficial' peptide set (Shown in Appendix). The same set of conserved elements peptides was used for both STEP and Phambili trial participants.

The total magnitude of the CD8+ T cell response targeting either beneficial peptides or conserved elements peptides measured 12 months post-infection was low overall (median 190 and 262 SFU/million CD8+ T cells for the clade B (STEP) and clade C (Phambili) beneficial peptides respectively; median 32.5 SFU/million CD8+ T cells for conserved elements peptides) (Figure 6.7).

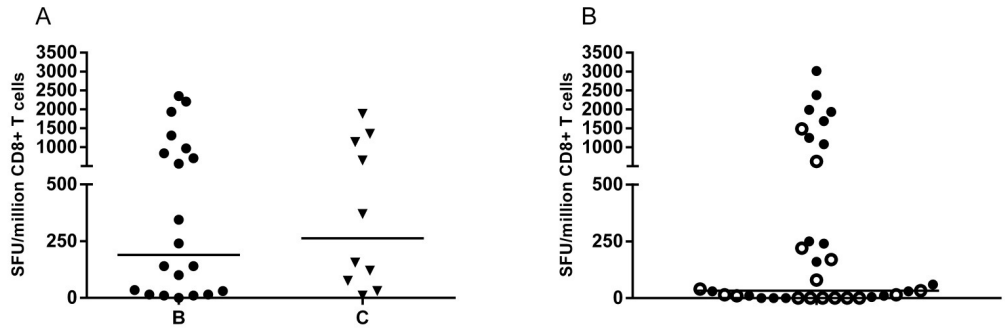


Figure 6.7 CD8+ T cell responses to (A) clade specific 'beneficial' peptides and (B) conserved elements peptides in STEP and Phambili participants, measured by IFN-γ Elispot assay. In graph B. Phambili subjects shown with open symbols. $1x10^5$ CD8+ T cells/well, final peptide concentration of 2μg/ml. Horizontal lines show median values

There was no significant difference between vaccinees and placebos for either peptide sets (240 vs 120 SFU/million PBMCs, p = 0.62 for beneficial peptides and 31 vs 40 SFU/million PBMCs, p = 0.56 for conserved elements peptides) (Figure 6.8).

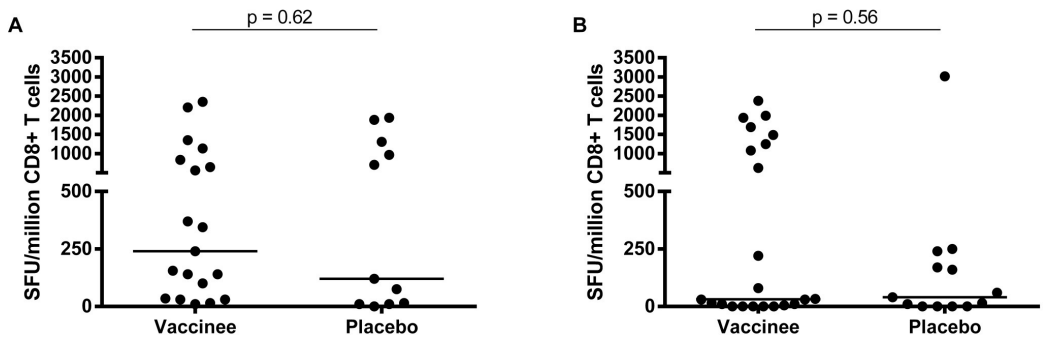


Figure 6.8 CD8+ T cell responses to (A) beneficial peptides and (B) conserved elements peptides in STEP and Phambili vaccinees and placebos measured by IFN-γ Elispot assay. Horizontal black lines indicate medians. $1x10^5$ CD8+ T cells/well, final peptide concentration of 2μg/ml. Statistical analysis performed using the Mann Whitney test.

Due to limited PBMC availability, T cell responses to the whole proteome could not be measured simultaneously. However, quantification of responses to the total proteome by intracellular cytokine staining had been performed independently by Dr. Nicole Frahm (HIV Vaccine Trials Network), which demonstrated that responses to the whole proteome in this cohort, measured four weeks after the second vaccination, were not generally weak overall. The median total proteome response was 1.81% CD8+ T cells, with no significant difference between STEP/Phambili vaccinees and placebos (median 2.1% and 1.5% of CD8+ T cells, p = 0.23). This indicated that the majority of CD8+ T cells were targeting non-beneficial or variable epitopes and responses to beneficial peptides in STEP/Phambili trial participants were subdominant. Despite responses to beneficial peptides being weak, there was a strong correlation between the magnitude of response to beneficial peptides and CD8+ T cell mediated inhibition of a clade matched virus at a 2:1 CD8+/CD4+ T cell ratio (r = 0.69, p = 0.0001) (Figure 6.9A). This correlation was still observed when a 1:1 CD8+/CD4+ T cell ratio was used (r = 0.64, p = 0.0003). The relationship between the magnitude of response to beneficial peptides

and CD8+ T cell mediated inhibition was maintained even after the removal of subjects with protective HLA class I alleles from the analysis (r = 0.71, p = 0.0005), suggesting that the specificity of CD8+ T cells was more critical to their antiviral potency than their HLA class I restriction (Figure 6.9B).

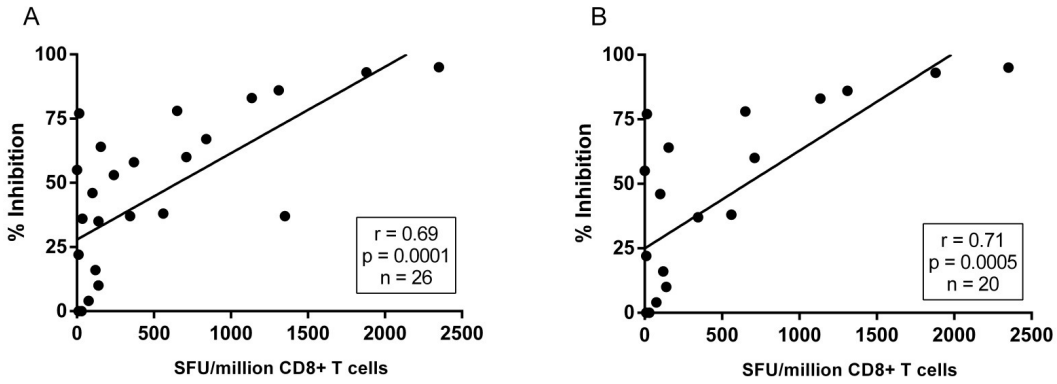


Figure 6.9 (A) Correlation between magnitude of CD8+ T cell IFN-γ Elispot response to beneficial peptides and inhibition of a clade matched virus determined by quantification of p24+ cells by flow cytometry on day 6 of co-culture at a 2:1 CD8+/CD4+ T cell ratio in STEP and Phambili trial participants and (B) after removal of trial participants with protective HLA class I alleles (B5801, B8101, B27, B57 and B51) from the analysis. Statistical analysis conducted using the Spearman rank test.

CD8+ T cells removed from the IFN-γ Elispot assay following a 16 hour incubation were used to grow short term cell lines according to the protocol in Materials and Methods (section 2.9). Short term cell lines were grown successfully in 15 STEP/Phambili participants and individual peptides from the beneficial peptide pool giving a positive response in the first elispot were then retested in order to identify individual positive beneficial peptides (individual positive beneficial peptides shown in the appendix). The magnitude of the response to the individual positive peptide correlated with CD8+ T cell mediated inhibition of a clade matched virus (r = 0.61, p = 0.01) (Figure 6.10).

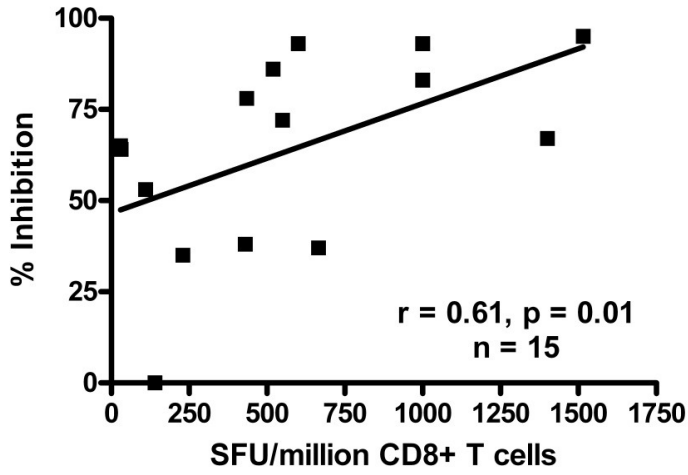


Figure 6.10 Correlation between magnitude of CD8+ T cell IFN-γ Elispot response to individual beneficial peptides and inhibition of a clade matched virus determined by quantification of p24+ cells by flow cytometry on day 6 of co-culture at a 2:1 CD8+/CD4+ T cell ratio. In 15 STEP/Phambili participants short term cell lines were grown from CD8+ T cells removed from the initial IFN-γ Elispot to test beneficial peptide pools. These cells were tested in a second IFN-γ Elispot assay using individual peptides from the positive beneficial peptide pool identified in the first IFN-γ Elispot assay. $1x10^5$ CD8+ T cells/well, final peptide concentration of 2μg/ml. Statistical analysis conducted using the Spearman rank correlation.

Unexpectedly, a weaker relationship between responses to the conserved elements peptides and CD8+ T cell mediated inhibition was observed (r = 0.41, p = 0.04) (Figure 6.11A). This relationship was largely driven by responses to peptides within conserved elements pool B (r = 0.41, p = 0.04), which contains CE 4, 5 and 6, rather than responses to pool A (r = 0.17, p = 0.41) (Figure 6.11B&C). Mothe et al. reported that conserved elements 4, 5 and 6 were targeted more frequently by patients with a reduced viral load[260].

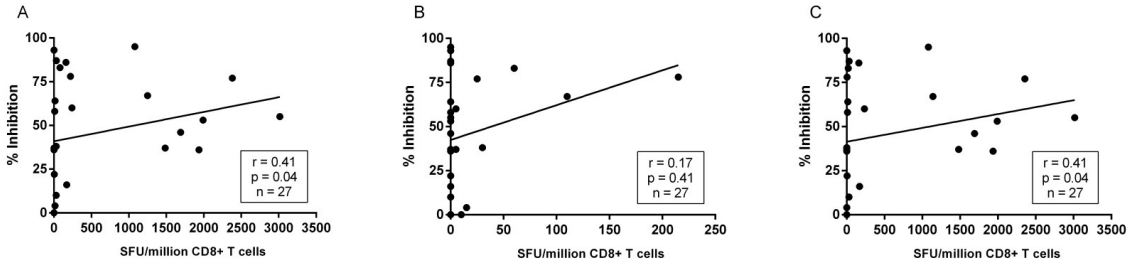


Figure 6.11 Correlation between CD8+ T cell IFN-γ Elispot responses to (A) all conserved elements peptides, (B) conserved elements Pool A peptides and (B) conserved elements pool B peptides and inhibition of a clade matched virus determined by quantification of p24+ cells by flow cytometry on day 6 of co-culture at a 2:1 CD8+/CD4+ T cell ratio. Statistical analysis conducted using the Spearman rank correlation.

Importantly there was no significant correlation between magnitude of response to the whole proteome (as measured by Dr. Nicole Frahm, HVTN using ICS) and virus inhibition (r = 0.11, p = 0.64) (Figure 6.12).

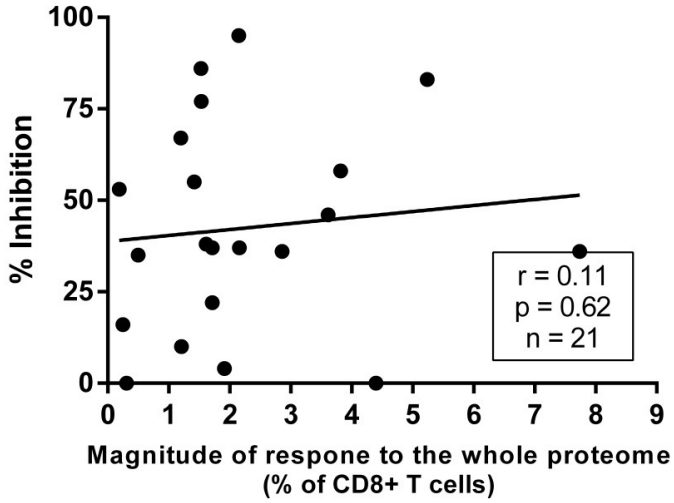


Figure 6.12 No correlation between magnitude of IFN-γ CD8+ T cell response to the whole proteome measured by intracellular cytokine staining and inhibition of a clade matched virus determined by quantification of p24+ cells by flow cytometry on day 6 of co-culture at a 2:1 CD8+/CD4+ T cell ratio. Statistical analysis conducted using the Spearman rank correlation.

As the responses to beneficial peptides were measured using purified CD8+ T cells it was possible to calculate the ratio of the beneficial CD8+ T cell response to the total proteome CD8+ T cell response; this ratio was shown to correlate significantly with CD8+ T cell mediated virus inhibition (r = 0.49, p = 0.02) (Figure 6.13). This suggested that not only the absolute magnitude but also the dominance of the response to beneficial peptides was a determinant of antiviral efficacy.

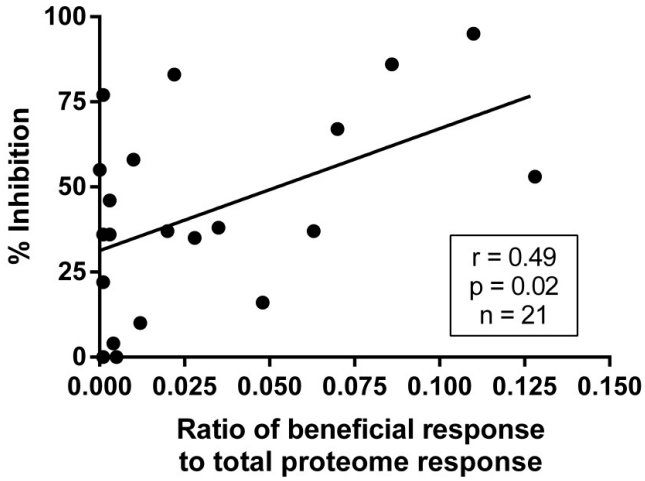


Figure 6.13 Correlation between ratio of beneficial response to total proteome response measured by IFN-γ Elispot assay/intracellular cytokine staining and inhibition of a clade matched virus determined by quantification of p24+ cells by flow cytometry on day 6 of co-culture at a 2:1 CD8+/CD4+ T cell ratio. Statistical analysis conducted using the Spearman rank correlation.

6.2.7 Magnitude of response to beneficial peptides alone explains nearly 40% of the variation in CD8+ T cell antiviral efficacy

In view of the unexpected correlations between viral inhibition and targeting of beneficial versus conserved elements peptides, hierarchical and standard multiple regression analyses were used to determine if CD8+ T cell antiviral efficacy could be best explained by either i) the magnitude of CD8+ T cell response to beneficial peptides

('absolute magnitude') ii) Shannon entropy for each beneficial peptide pool iii) the ratio of magnitude of responses to beneficial peptides to the total proteome response (relative magnitude or immunodominance) or iv) the presence of protective ('good') or non-protective ('bad') HLA class I alleles. In the hierarchical models, (Model 1) CD8+ T cell inhibition was the dependent variable and magnitude of response to beneficial peptides was the independent variable ('absolute magnitude'). Absolute magnitude explained 38% of the variation in CD8+ T cell mediated inhibition; this fit was improved to 47% in model 2 by including entropy as a second independent variable. However after controlling for magnitude, the addition of entropy was not significant (Table 6.2). In three standard multiple regression models comprising magnitude of response to beneficial peptides together with (i) relative magnitude, (ii) entropy and 'good' HLA class I alleles and (iii) entropy and 'bad' HLA class I alleles, the model as a whole explained 39-49% of the variation in CD8+ T cell mediated inhibition, with magnitude of response to beneficial peptides making the strongest unique contribution (Table 6.3).

	Model 1	**Model 2**
R^2	0.38	0.471
Adjusted R^2	0.351	0.42
Standard error of estimate	25.037	23.675
F change	13.465	3.604
Signif F change	0.001	0.071

Table 6.2 Hierarchical regression model: % inhibition as dependent variable. Model 1: magnitude (response to beneficial peptides). Model 2: magnitude plus entropy

	β	p
Predictor variable		
Model 1: r^2 = 38.8, p = 0.015		
Magnitude	0.531	0.059
Relative magnitude	0.123	0.645
Model 2: r^2 = 0.472, p = 0.006		
Magnitude	0.578	0.003
Entropy	0.315	0.091
Good HLA allele*	-0.038	0.833
Model 3: r^2 = 0.489, p = 0.005		
Magnitude	0.585	0.002
Entropy	0.294	0.092
Bad HLA allele§	-0.135	0.424

Table 6.3 Standard multiple regression model: % inhibition as dependent variable. * HLA-B*27, B*51, B*5701, B*5801, B*81. § HLA-B*35 (Py), HLA-B*53

6.2.8 Vaccines encoding full length proteins skew responses to non-beneficial regions of the HIV-1 proteome

The specificity of T cell responses to the MrkAd5 gag/pol/nef immunogen were mapped by Dr. Nicole Frahm, HVTN, in a subset of STEP participants four weeks after the second vaccination and prior to HIV-1 acquisition. This allowed me to determine if a vaccine expressing full length proteins could prime responses to regions defined previously as beneficial or conserved. I compared the magnitude of vaccine-induced responses to the clade B set of beneficial peptides and to conserved elements peptides to the magnitude of responses to the entire immunogen in 13 STEP vaccinees. In view of the small number of subjects, responses to these two peptide sets were combined. Vaccination primed responses to beneficial/conserved elements peptides in 8/13 STEP vaccinees, which accounted for a median 6.2% of the response to the entire immunogen (Figure 6.14).

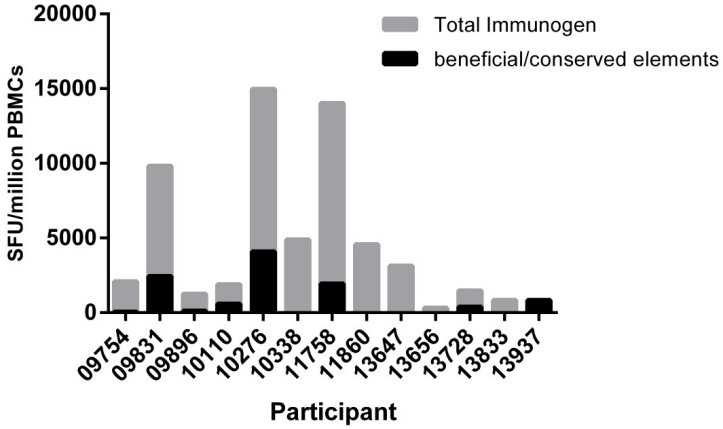


Figure 6.14 Magnitude of response to the MrkAd5 gag/pol/neg immunogen (grey bars) and beneficial/conserved elements peptides (black bars) measured four weeks after the second vaccination and prior to HIV-1 infection by IFN-γ Elispot assay.

6.3 Discussion

Until recently, little attention was devoted to defining which regions of the HIV-1 genome should be included in a vaccine and most immunogens incorporated full-length gene sequences for many viral proteins. In the absence of a reliable correlate of protective immunity, efforts to develop a T cell-based vaccine have been founded on the principle of recapitulating the immunological phenotype of individuals who maintain control of viraemia in the absence of HAART. Many studies in HIV-1 controllers have limitations such as cross-sectional design and enrichment for subjects with protective HLA alleles that are known to present conserved epitopes within Gag[459,460]. It is difficult to disentangle the contribution of these factors to the observed outcome. Work in this chapter sought to investigate whether potent CD8+ T cell-mediated virus inhibition is dependent on targeting of regions of the HIV-1 proteome that had been shown in an independent study of two large cohorts to associate with control of viraemia[396].

CD8+ T cell-mediated inhibition measured in STEP and Phambili trial participants was weak and of limited breadth compared to that in viraemic controllers, consistent with data from other studies[262,446,448]. There was no increase in inhibition in STEP/Phambili vaccinees compared with placebos which is in line with the results of both the phase II trials which showed no reduction in viral load upon HIV-1 acquisition[332,333]. Data in this chapter confirmed an earlier observation made by Yang et al. that CD8+ T cell virus inhibition is inversely correlated with viral load set point[446]. These observations add further weight to the clinical/biological relevance of CD8+ T cell inhibitory activity and

support the use of the viral inhibition assay as a measure of the overall efficacy of CD8+ T cell responses against HIV-1. CD8+ T cell responses to beneficial peptides and conserved elements peptides were of low magnitude overall, in both absolute and relative terms. This indicates that CD8+ T cell responses to these beneficial/conserved elements peptides are subdominant. However, the most important finding from this study was that CD8+ T cell responses to these beneficial peptides were strongly correlated with CD8+ T cell mediated inhibition and this association persisted after removal of subjects with protective HLA class I alleles. Furthermore, the association with responses to beneficial peptides was much stronger than for conserved elements peptides. Indeed, the weak correlation with conserved elements peptides was mediated predominantly by responses to CE pool B, which contains CE segments 4, 5 and 6. Mothe et al. showed than CE segments 4, 5 and 6 were recognised by 50% more controllers than non-controllers and that the magnitude of response to CE segments 4, 5 and 6 was significantly inversely correlated with viral load in the cohort of 50 HIV-1 controllers and non-controllers, indicating that stronger responses to these regions may mediate better viral control. No such association was observed for CE segments 1, 2, 3 and 7. These observations were confirmed in the regression analyses, which showed that magnitude of the response to beneficial peptides explained variation in inhibitory activity better than all the other variables that were considered, which included entropy. This data suggests that conservation of an epitope alone does not mediate an effective T cell response and this is consistent with other studies demonstrating that conservation does not predict viral fitness[252,406]. Figure 6.15 is from work by Rihn et al. showing the fitness, as a percentage of the wild-type virus fitness, for a library of capsid mutants, overlaid with the Shannon entropy values for every residue in capsid. There are

discrepancies between Shannon entropy and fitness scores across the capsid. This may also be true in other proteins of HIV-1.

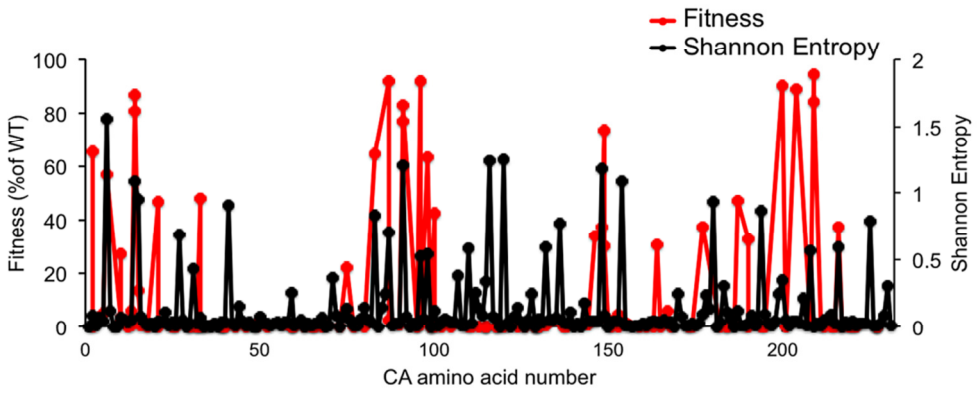


Figure 6.15 Fitness (red), as a percentage of the wild-type virus fitness, for a library of capsid mutants. Shannon entropy values (black) for every residue in the Gag capsid protein. Figure adapted from Rihn et al, Plos Pathogens, 2013.

The observation that the correlation between responses to conserved regions and CD8+ T cell mediated inhibition is weaker than the correlation between responses to beneficial regions and inhibition supports data in previous chapters showing that an immunogen selected on conserved regions alone is not effective There is only a 57% overlap between HIVconsv regions and beneficial regions (of the 14 regions of HIVconsv, 8 overlap at least partially with the beneficial regions) and 35% of conserved elements peptides are covered by clade B specific beneficial peptides. Therefore HIVconsv couldn't be expected to induce enough responses to critical regions identified by Mothe et al.[396].

CD8+ T cell epitopes are not evenly distributed over the entire viral genome. Rather, there are regions where many epitopes overlap, epitope clusters/hotspots as well as regions deficient of any epitope clusters, epitope poor regions. This phenomenon has

been described as early as 1993 and a recent paper by Chakraborty et al.[513] which studied the protein location of 269 experimentally validated optimal epitopes identified two such epitope clusters in gp160, two clusters in Nef, two in p17, four in p24 and only one in RT. Several factors could influence the observed CD8+ T cell epitope clustering, and Yusim et al. used the complete set of optimal HIV-1-derived CTL epitopes included in the Los Alamos HIV Immunology Database to show that regions of HIV-1 proteins that were epitope rich were i) conserved, ii) had a lower frequency of amino acids that do not serve as C-terminal anchor residues compared to regions of proteins that had a lack of CD8+ T cell epitopes and iii) had a high tendency to be cleaved by the immunoproteasome[514]. Frahm et al. identified that there are still some relatively conserved peptides that do not seem to induce a detectable CD8+ T cell response in natural HIV-1 infection[515] and it could be that these peptides may lie within stretches of viral proteins that are relatively resistant to proteasomal digestions or may have a high frequency of forbidden anchor residues. Not only are the beneficial regions identified by Mothe et al. conserved[396], because they have been identified using functional data they are likely to be efficiently processed and presented and therefore according to the criteria defined by Yusim et[514] al are likely to be rich in epitopes. Regions of HIVconsv however, although conserved may be poorly processed and presented.

In HIV-uninfected volunteers DNA, MVA and ChAd63 vectored HIVconsv administered in various prime-boost regimens was shown to inhibit virus replication by up to 5.79 $\log_{10}$. Overall, 7/7, 3/8, and 5/8 vaccine recipients in the respective CM, DDDCM, and DDDMC groups inhibited both clade B (IIIB) and A (U455) viruses above background inhibition. Placebos showed no detectable inhibition[308]. However the

protocol used by Borthwick et al. to determine CD8+ T cell mediated inhibition differed from that used in this thesis. CD8+ T cells were expanded *in vitro* for seven days in the presence of a bi-specific anti CD3-CD4 antibody prior to co-culture with autologous superinfected CD4+ T cells. Additionally infection was measured on day 13, as opposed to day six and the level of p24 was determined by p24 ELISA. This protocol hasn't been shown to correlate with clinical outcome e.g. viral load set point as has the inhibition assay used in this thesis[446].

Analysis of post-vaccination pre-infection responses in a subset of STEP vaccinees showed that CD8+ T cell responses to beneficial/conserved elements peptides were primed by vaccination in a minority of vaccinees and accounted for only 6% of the response to the whole immunogen. This shows that vaccination by MrkAd5 gag/pol/nef, which expresses full length Gag, Pol and Nef, preferentially elicited responses to non-beneficial/variable regions. This is confirmed by Hertz et al. who demonstrated that MrkAd5 gag/pol/nef vaccine induced CD8+ T cell responses clustered in immunodominant 'hotspots' that were located in variable regions of the proteome[407,516]. The MrkAd5 gag/pol/nef immunogen contained the majority of the beneficial regions identified by Mothe et al.[396], it is therefore concerning that vaccination preferentially induced responses to variable regions. These observations highlight the need to develop a HIV-1 vaccine to overcome natural immunodominance hierarchies in humans by the inclusion of specific critical regions of the viral proteome, coupled with the exclusion of decoy regions.

Mothe et al. have designed a HIV-1 immunogen using the 26 beneficial regions they identified in clade B infected subjects. These regions were aligned and if in close proximity they were fused using the clade B consensus sequence. Further modifications included inclusion of specific epitopes that were considered important, shortening of segments to exclude immunodominant epitopes for which no published evidence supports their beneficial effects on viral control, extensions to cover flanking regions known to affect epitope processing, introduction of single amino acid changes to cover common drug-resistance and inclusion of two additional regions in Gag to complete the coverage of all previously described hot-spots of T-helper cell activity[517]. The resulting immunogen was a subunit construct of 16 segments ranging from 11-78 amino acids in length representing Gag (45%), Pol (44%), Vif (8%) and Nef (3%) sequences (Mothe et al., personal communication).

This approach could be further refined by the inclusion of sequences that pre-empt predictable escape mutations. Valkenburg et al. demonstrated using an influenza mouse model that priming against predicted escape mutants induced secondary CD8+ T cells responses to these escape mutants upon challenge with influenza[518]. If this is also true for other RNA viruses like HIV-1, priming against predicted mutants could function to prevent the emergence of escape variants in infected hosts.

The experiments conducted in this chapter have limitations. Firstly, the study by Mothe et al. that described beneficial regions within the HIV-1 proteome was conducted in chronically infected individuals[396]. It will be important to investigate whether the targets of effective CD8+ T cell responses are the same in acute infection as in chronic

infection and if early dominant responses to these targets during acute infection predicts long-term control of viral replication in chronic infection. Secondly, the PBMC samples were obtained one year post-infection from HAART-naïve individuals; it is assumed that they had either declined or were not considered eligible at that time. It is possible that this cohort was therefore enriched for slow progressors who as such may have efficacious CD8+ T cell responses, although this is not supported by the CD4+ cell count and viral load data. Furthermore, post-vaccination pre-infection responses to the MrkAd5 gag/pol/nef vaccine could only be assessed in 13 STEP vaccinees. It is uncertain therefore how representative these individuals were of the whole vaccinated population with regard to priming of responses to beneficial regions by the Ad5 HIV-1 vaccine. Lastly, I was unable to sort CD8+ T cells specific for the beneficial peptides. It would have been interesting to isolate these cells and use them in a virus inhibition assay in order to confirm that they were mediating most of the inhibitory activity observed when bulk CD8+ T cells were used in the assay. It would have also allowed me to further define these cells in terms of avidity, proliferation and polyfunctionality in order to understand why CD8+ T cells targeting these beneficial regions are efficient at inhibiting virus replication.

The data presented in this chapter supports the inclusion of specific regions associated with control of viraemia in a HIV-1 vaccine immunogen. Selection of regions based on conservation alone is not effective and this is likely due to the fact such a method doesn't take into account epitope density, processing preferences of certain regions and genetic fragility across proteins. Definitive evidence that an immunogen containing

these beneficial regions can control HIV-1 infection will only be gained though clinical testing of the HIVACAT immunogen.

7. Final Discussion

The work presented in this thesis aimed to evaluate the immunogenicity of a rationally designed conserved region vaccine, MVA.HIVconsv, in HIV-1 seropositive HAART treated patients. One of the biggest challenges in the development of an effective vaccine against HIV-1, whether for prevention or therapy, is virus diversity and escape from immune responses. In natural infection, the very first CD8+ T cell responses are directed to variable regions of the virus[216,222] which although highly immunogenic, mutate easily under immune pressure allowing the virus to escape. Targeting the most conserved regions of the virus should redirect the immune response to more vulnerable regions of the proteome where escape results in a significant fitness cost to the virus[244,408]. Studies have shown a correlation between breadth of response to conserved regions and viral load[222,396,406].

Linear mixed models were used to determine whether any changes in the magnitude of response over time, quantified as the sum of all confirmed peptides eliciting a response above 100 SFU/million PBMCs, were due to vaccination with MVA.HIVconsv. Although there was a significant difference in the magnitude of response over time ($p = 0.001$), there was no significant difference between vaccinees and placebos ($p= 0.48$). The change in magnitude of response observed in placebos over the course of the study highlighted the natural variation in the HIV-1-specific T cell response during the course of long-term HAART treatment, which has not been fully appreciated before[416]. Analysis of the breadth of response by linear mixed models also demonstrated that there was no significant induction of de novo responses to epitopes within HIVconsv in

vaccinees compared with placebo subjects ($p = 0.45$). There was, however, a trend towards increased CD8+ T cell mediated inhibition following vaccination with a higher dose of MVA.HIVconsv, although this did not reach statistical significance. Measurement of inhibition at additional time points is planned in order to increase the power of the analysis. In a second cohort of HIV-1 infected subjects antiviral activity was shown to correlate strongly with targeting of regions of the HIV-1 proteome that had been identified as being 'beneficial' by Mothe et al. due to an association with lower viral loads in subjects targeting these regions[396]. Although these 'beneficial' regions are also highly conserved, the poor results obtained for HIVconsv and the weaker correlation between responses to conserved elements and CD8+ T cell mediated inhibition, suggests that the favorable nature of these 'beneficial' regions is not solely due to their conservation.

The hypothesis underpinning the design of immunogens that exclusively target such regions is that these regions are conserved due to functional / structural constraints and that immune pressure against these regions that drives the virus to escape results in selection of less fit viruses. Liu et al. investigated the fitness impact of mutations of highly conserved amino acid sites (HCS) within the Gag-p24 and Env-gp120 proteins. Amino acid sites over 98% conserved in HIV-1 group M in the Los Alamos HIV Sequence Database were defined as HCS and others as variable sites (VS). Virus sequenced from an antiretroviral therapy naïve subtype B subject contained ten mutations at highly conserved amino acid sites in gag (all in p24) and nine in env (all in gp120) over the first four years of infection, all involving single nucleotide changes. Seven of the ten gag mutations were lethal, with the remaining three reducing viral

fitness. In env all nine mutations were non-lethal, but five did result in reduced viral fitness. When these mutations were introduced into founder viruses from two other clade B patients similar results were obtained[519]. This work demonstrates that although the majority of mutations within highly conserved regions in p24 gag were lethal, there are some mutations that can be tolerated by the virus, indicating that there are viable mutational pathways for some highly conserved HIV-1 segments. Vaccination with HIVconsv may induce T cells which target parts of the HIV-1 proteome that, although highly conserved, can tolerate mutations thereby negating any effect of the immune response on viral fitness. Future vaccine immunogens should consider information on fitness constrains as well as the conservation level of a region. This is further supported by work from Rolland et al. and Rihn et al. Rolland et al. evaluated the *in vitro* fitness cost of 23 mutations engineered in the HIV-1 subtype B Gag-p24 Centre-of-Tree (COT) protein through fitness competition assays. They found that there was no overall strong relationship between sequence conservation and replicative capacity[390]. Rihn et al. generated a library of single amino acid substitution mutants, encompassing almost half the residues in the Gag capsid protein (CA) and observed significant discrepancies between the Shannon entropy and the fitness of CA mutant viruses. For example, in helices 5, 6, and 7 Shannon entropy values were quite high, but fitness measurements revealed this region to be highly genetically fragile[252]. Much work has investigated the fitness constraints within the Gag protein; it will be important to elucidate the effect of mutations within other HIV-1 proteins in order to identify areas outside of Gag that have a high genetic fragility and would therefore be desirable to include in future immunogens.

Selection of regions for inclusion in a vaccine immunogen based on conservation alone is further compounded by the fact that conservation as defined by the Shannon entropy is restricted to single amino acid residues and therefore largely ignores mutational couplings that are known to be important determinants of viral replicative fitness[232,520,521]. Co-coordinately linked combinations of mutations are important, due to the structural proximity of groups of residues within 3D protein structure or participation of the group in a particular viral function involving multiple proteins. Immune targeting of multiple sites in such a multidimensionally conserved region might render the virus particularly vulnerable, because viable escape pathways would be greatly restricted. Dahirel et al. analysed available HIV-1 sequences using a method from physics to reveal distinct groups of amino acids whose mutations are collectively coordinated, they termed these "HIV sectors". Many sites do not co-evolve with any other sites; however in Gag there were five strongly coupled groups of sites (sectors 1-5). A negative correlation between sites implies the multiple mutant is observed less frequently than if individual mutations were to arise independently and therefore the multiple mutant is likely to be less fit compared with the single mutant. Gag sector 3 contains a large number of negative correlations, suggesting that it is the most immunologically vulnerable multidimensionally constrained region in Gag. 52/57 sites in sector 3 are in p24: although the sites were not linear on the protein sequence, when superimposed on the structure of the p24 hexamer these sites locate to the interfaces between p24 proteins in a hexamer or at interfaces between the hexamers that form the capsid[521]. Manocheewa et al. demonstrated that mutations at the hexamer interface were more likely to be lethal and had a greater negative impact on viral replication fitness than mutations at non-interface sites[522]. So although one mutation within a hexamer

may still allow it to function in a normal manner, multiple mutations would be highly detrimental. HIV-1 elite controllers were shown to target multiple sites in sector 3[521]. The beneficial regions identified by Mothe et al.[396] may contain these multidimensionally constrained regions / sectors. Groups of residues containing mutational couplings which render the virus particularly vulnerable could be promising new targets for HIV-1 vaccines, both prophylactic and therapeutic.

Immunogen design is also being aided by the application of reverse vaccinology. Reverse vaccinology involves the use of bioinformatics to screen sequences of an entire pathogen's genome to detect epitopes recognised by CD4+ and CD8+ T cells in order to identify new vaccine antigens. The incorporation of computational methods to identify immunogenic epitopes can further enhance the process. Three major steps contribute to the pathway of MHC class I antigen processing and shape the repertoire of presented peptides; processing by the proteasome, binding to transporters associated with antigen presentation (TAP) and binding of peptides to nascent MHC molecules. Each of these antigen processing steps can be modelled using specialised programmes and incorporated in epitope prediction tools in order to improve the reliability of epitope prediction[523–526]. For example, EpiMatrix models the interactions between peptides and MHC molecules. ClustiMer scans EpiMatrix results for T cell epitope clusters. EpiVax Inc. has used such an approach for the design of cross-subtype HIV-1 immunogens. They began with ~18000 GenBank HIV-1 env sequences available from the Los Alamos National Laboratory HIV database. Next, they used an in-house algorithm, Conservatrix, to identify protein or DNA segments that were highly conserved between the input sequences. Identified peptides were then scanned for interactions with HLA

molecules, after which, peptides with significant sequence homology to human or common viral and bacterial genomes were removed. The remaining peptides were shown to be immunogenic *in vitro*, as evaluated by Elispot assay using PBMCs from HIV-1-infected patients[524]. The HIVconsv immunogen was designed by sequence alignments of conserved regions and did not take into account processing and presentation preferences. Tools such as those described above could therefore be used to improve the HIVconsv immunogen.

Responses to conserved / beneficial regions are likely to be subdominant to responses to variable regions. This has been demonstrated in this thesis; conserved region responses were subdominant to whole proteome clade B consensus responses in a subset of MVA.HIVconsv trial participants tested and beneficial / conserved elements responses were subdominant to whole proteome responses in STEP/Phambili trial participants. Immune responses to conserved regions will have to compete for recognition with rapidly changing immunodominant epitopes. There is therefore a need to identify vaccine vectors with greater immunogenicity, either by improving existing vectors or by using novel vectors (e.g. simian adenoviruses).

Although MVA is currently being tested as a viral vector for vaccines against many infectious diseases such as HIV-1[316,365], TB[317,527,528] and malaria[529–531] as well as cancers[532–534], responses have tended to be of low magnitude. There is a need to improve its immunogenicity in order to enhance the magnitude, breadth, polyfunctionality and durability of the immune responses to HIV-1 antigens. The immunogenicity of MVA could be improved by either the addition of costimulatory

molecules and various cytokines or the removal of genes encoding immunomodulatory proteins that attenuate adaptive immune responses but are not essential for virus replication *in vitro*. MVA was obtained by extensive passage of VACV Ankara in chicken embryo fibroblasts (CEFs)[535] which resulted in the loss of approximately 15% of its parent genome, including many genes regulating virus–host interactions[536,537]. However the process of attenuation was non-selective and genes lost through continuous passage might not be the most advantageous. Indeed, some viral genes with immunomodulatory function that block components of the host response to infection are maintained in the MVA genome[538–540]. MVA recombinants with deletions of two MVA immunomodulatory genes, B16R and A41L have been generated and showed some immunological benefit. A41L encodes a secreted glycoprotein which binds CC-chemokines CCL21, CCL25, CCL226 and CCL28[541,542]. These chemokines play a central role in the development of the T cell response; for example, CCL21 is pivotal for priming T cell responses, providing co-stimulatory signals for the expansion of naïve CD4+ and CD8+ T cells and inducing Th1 polarization[543]. Inoculation of mice with MVA in which A41L had been deleted resulted in enhanced immunogenicity against the vector and better protection against subsequent challenge with the pathogenic strain western reserve (WR)[541,542,544]. B16R encodes a secreted glycoprotein which functions as a viral soluble receptor for IL-1β (vIL-1βR) that blocks inflammatory and febrile host responses to infection[545,546]. Deletion of B16R in the MVA genome resulted in a virus with enhanced CD8+ T cell memory responses to the virus and provided greater protection following challenge with WR in a mouse model[545].

Garcia-arriaza et al. compared the wild type MVA vector expressing HIV-1 Env, Gag, Pol and Nef subtype B antigens (MVA-B) with a double deletion MVA mutant expressing the same antigens in which both A41L and B16R genes were deleted (MVA-B ΔA41L/ΔB16R). Administered in a DNA prime/MVA boost to BALB/c mice, the double mutant significantly improved the magnitude of the HIV-1-specific CD4+ and CD8+ T cell response by 2.8 and 3.9 fold respectively as compared with MVA-B[548]. In an NHP model Garber et al. demonstrated that MVA with simultaneous deletion of four genes (an interleukin-18 (IL-18) binding protein, a soluble IL-1β receptor, a dominant negative Toll/IL-1 signaling adapter, and CC-chemokine binding protein) encoding HIV-1 consensus subtype C Env and Gag antigens elicited frequencies of HIV-1-specific CD8+ and CD4+ T cells that were of higher magnitudes than those elicited by a parental control vaccine[549]. These studies indicate that the rational deletion of genes from MVA can improve its immunogenicity.

Approaches to improve the immunogenicity of MVA by the co-expression of various cytokines including IL-12, IL-15 and granulocyte-macrophage colony-stimulating factor (GM-CSF) as well as co-stimulatory molecules, such as CD80 and 4-1BB ligand are also being investigated in mouse models and have been reported to achieve a two- to four-fold increase in peak IFN-γ T cell responses[550–553].

Immunogenicity of vaccines is also being improved by the use of novel vectors. Live attenuated viruses have been the most effective experimental vaccines for preventing AIDS (Reviewed in[554]) when tested in NHP models. However, applying this strategy to HIV-1 has been ruled out because of safety risks such as reversion to wild type HIV-1

with subsequent disease induction[266,555]. A more practical approach is to utilize replication-competent vectors: low level replication ensures ongoing antigen expression and presentation to the immune system, resulting in a sustained effector memory T cell response. This may be more effective at preventing or controlling HIV-1 infection than anamnestic responses induced by replication-defective vectors[556].

Cytomegaloviruses (CMV) are members of the herpesvirus family which persist indefinitely in their host. Persistent antigen exposure results in continuous low level immune stimulation and the generation of an expanded pool of effector T cells. RhCMV/SIV vectors were shown to establish and maintain high-frequency SIV-specific, T_{EM}-biased, CD4+ and CD8+ T cell responses indefinitely in diverse tissue sites of RhCMV+ rhesus macaques[427]. Vaccination of macaques with CMV vectors expressing SIV genes resulted in 50% of macaques suppressing virus replication to undetectable levels following repeated rectal challenge with SIV and 70 weeks after challenge, vaccinated animals had no remnants of SIV DNA or RNA, or replication-competent SIV, in blood or tissues[210,427].

Due to the tight host restriction, human CMV vectors must be developed and validated. One concern with the use of CMV as a vector is that although CMV infection is not pathogenic in the majority of humans, it can pose a risk of pathogenesis in pregnant women and immunocompromised individuals. However, it has been demonstrated in NHP and mouse models that immunogenicity of CMV is not dependent on full replication competence. Genetically modified CMV constructs with a reduced ability to

replicate and spread after the initial round of infection have been shown to elicit high frequencies of T_{EM} responses that appear similar to wild type responses[557].

Adenoviruses that are deleted only in the E3 region are replication-competent, in contrast to E1 and E3-deleted adenoviruses, which are replication-defective. It is deletion of the E1 region which attenuates replication *in vivo*; adenoviruses without deletion of E1 can infect mucosal surfaces and subsequently replicate. However, foreign insert capacity is limited to three to four kb when E1 is retained. To date, preclinical studies using replicating Ad-HIV and SIV recombinants have been shown to elicit potent cellular immunity[312,558–560]. Xiao et al. showed in a NHP model that replication-competent Ad5-SIV vectors expressing Gag, Env, and Rev were immunogenic and partial control over SIVmac251 infection was observed when used in combination with an SIV Env gp120 boost[559]. Replicating and non-replicating Ad-HIV recombinants encoding identical gene products compared head to head in chimpanzees demonstrated that vaccination with the replication competent vector induced a greater frequency of HIV-1-specific IFN-ɣ secreting T cells[556]. Replicating Ad-HIV candidate vaccines have not yet progressed to clinical trials.

An alternative approach to vectored vaccines for HIV-1 immunotherapy / eradication is the use of engineered T cells. This approach has several advantages: it provides an immediate effector response whereas adaptive immune responses take time to develop; the precise specificity of T cell receptors can be better controlled, and the affinity of the TCR for its target epitope can be enhanced by several orders of magnitude. Many studies have investigated ex vivo genetic modification of T cells before reinfusion back

into the patient in a process termed adoptive cell transfer (ACT). This approach has recently been used successfully to augment tumour-specific immunity in melanoma patients. CD8+ T cells that were genetically engineered to recognise MART-1, a tumour-associated antigen, were infused into patients and displayed durable engraftment which was associated with the regression of some tumours[561]. *In vitro* studies by Varela-Rohena et al. showed that T cells expressing an affinity enhanced TCR specific for the HLA-A*0201-restricted Gag p17 epitope, SL9, had enhanced effector functions, including the capacity to eliminate HIV-1-infected cells expressing wild-type and escape variants of SL9, compared to T cells transduced with the wild type TCR[562]. A Phase I clinical study testing the *in vivo* efficacy of these high-affinity Gag-specific T cells in HAART-treated patients is currently underway. Joseph et al. transduced autologous peripheral CD8+ T lymphocytes with lentiviral vectors encoding the HIV-1-specific TCR-α and TCR-β chains for an HIV-1 Gag epitope, SL9 in order to redirect their antigen specificity. Transduction with this lentiviral vector efficiently converted primary human CD8+ lymphocytes into HIV-1-specific CD8+ T cells with potent antiviral effector function *in vitro* and in a humanised mouse model[563]. The potential of this approach is limited by the requirement for *ex vivo* manipulation of CD8+ T cells, which is expensive, labour intensive and not feasible in resource-poor settings. An alternative approach is to redirect the antigen specificity of T cells using bi-specific soluble reagents, termed immune mobilizing monoclonal T-cell receptors against cancer (ImmTAC) or viruses (ImmTAVs), which comprise a soluble affinity enhanced monoclonal TCR (mTCR) fused to a humanised CD3-specific single chain variable fragment (scFv). High affinity TCRs are generated by targeted mutations in the CDRs through phage display[564]. The TCR moiety binds to its target cell and a wide range of

effector T cells are recruited via interaction with the CD3-specific sFv leading to their activation, followed by killing of the target cell. This technology has shown promise in animal models and clinical trials for melanoma[565–567]. Yang et al. demonstrated that a SL9-specific ImmTAV, could redirect autologous CD8+ cells to suppress replication of endogenous virus in activated and resting CD4+T cells from HIV-1-infected HAART treated patients (Yang 2014, submitted). ImmTAVs specific for epitopes within the beneficial regions identified by Mothe et al. could be developed. This approach has potential for HIV-1 eradication as it could be used in combination with agents to reactivate latent virus, e.g. HDAC inhibitors; the high affinity of ImmTAVs should enable killing of cells with reactivated virus that may only be expressing low levels of cognate epitope, thereby helping to clear the latent reservoir.

Another approach bypassing the use of vectors is vaccination with peptides as they bind directly to HLA class I molecules, thereby bypassing the need for intracellular processing and presentation. One such peptide vaccine, Vacc-4x consists of a mixture of four peptides of 20–27 amino acids in length, which were based on consensus sequences within the HIV-1 core protein p24. The regions of p24 covered by Vacc 4x are largely synonymous with the conserved immunologically vulnerable sector 3 regions discussed earlier[521]. The peptides have been modified by amino acid substitution to improve antigen processing and presentation on diverse HLAs. In a phase II randomized, placebo-controlled study of Vacc 4x, 134 HIV-1-infected people from Europe and the US received four primary vaccinations intradermally after administration of adjuvant. Booster vaccinations were given at weeks 16 and 18. At week 28, HAART was interrupted for up to 24 weeks. There was no difference in the co-primary endpoints

HAART resumption and changes in CD4+ counts during treatment interruption between vaccinees and placebos. However a significant difference in viral load, although small, was noted for the Vacc-4× group both at week 48 and week 52[356].

In a NHP study by De Rose et al. autologous PBMCs were pulsed with overlapping SIV peptides for one hour before reinfusion into macaques. An initial set of four vaccinations followed by a booster set of three vaccinations six months later was administered to HAART treated macaques. High-level, SIV-specific CD4+ and CD8+ T cell responses were induced following vaccination, and following HAART interruption virus levels were durably ~10-fold lower for one year in vaccinated animals compared to controls[568]. However a similar approach using autologous white blood cells pulsed with 120 peptides spanning clade C Gag tested in a phase I clinical trial was found to be only weakly immunogenic[569] and caused serious adverse reactions[570].

T cell vaccine trials to date have assessed vaccine efficacy by measurement of T cell function in vaccinees compared with placebo recipients. Techniques have included measurement of epitope recognition by Elispot assay, intracellular cytokine production and cellular phenotype using flow cytometry and characterisation of antigen-specific cells using HLA class I and class II / peptide tetramers. However, these methods assess vaccine efficacy at the single cell level rather than measuring the total immune response, which involves a complex interplay between different cell types and soluble factors. Not only is it important to assess T and B cells, it has become increasingly clear that the early innate immune response has a large impact on shaping the adaptive response, making analysis of early responses to vaccines important (Reviewed in[571]).

Systems biology can be used to elucidate clusters of signatures, based on gene expression, protein or metabolite levels, or even miRNA expression, that correlate with vaccine immunogenicity. This could facilitate not only the systematic evaluation of vaccine candidates but also the formulation of new hypotheses on how vaccines mediate long-term protective immune responses and therefore aid in the subsequent design of new vaccines.

Systems biology has been used successfully to study the immune response induced by the live-attenuated yellow fever virus vaccine YF-17D. Transcriptional profiling coupled with multiplex analysis of plasma cytokines and chemokines gave a global picture of the immune response to vaccination. Computational methods were employed to use the information gathered to identify early gene expression signatures that predicted T cell and antibody responses and these signatures were validated in other vaccine recipient cohorts. These signatures could predict, with up to 90% accuracy, the strength of the immune response to the yellow fever vaccine[572,573]. These results underline a strong correlation between the early innate immune response and protective vaccine response and provided the first demonstration that systems biology approaches can be used to predict the immunogenicity of vaccines.

In the HIV-1 field, systems biology analysis of differences between the HIV-1-specific innate and adaptive immune responses of HIV-1 controllers and those of HIV-1 non-controllers may provide information on the particular immune responses that might confer protection or control of HIV-1. A recent study by Rotger et al. analysed transcriptional profiles in purified CD4+ T cells from HIV-1 progressors and elite

controllers. They observed an upregulation of the interferon pathways, cell cycle, and ubiquitin-proteasome degradation complex in progressors with high viral loads as compared with elite controllers[574]. Loke et al. analysed the expression of several immune parameters in peripheral blood and rectosigmoid biopsy samples from HIV-1 controllers, including elite controllers and LTNPs, and non-controllers. HIV-1 controllers showed a decrease in the IFN-related inflammatory response in the gut compared with progressors and results indicated that immune activation and inflammation are associated with HIV-1 progression[575]. Although HIV-1 controllers exhibit natural control of HIV-1, the problem is to know what is cause and effect. Does a potent CD8+ T cell response help to control viraemia or is it a consequence of low viraemia allowing for maintenance of an effective immune response? A gene signature based on the immune response of HIV-1 controllers may not be able to answer this question unless studied longitudinally from primary infection. Highly exposed seronegative individuals remain uninfected despite repeatedly engaging in activities that are high risk for HIV-1 acquisition, such as unprotected sex and needle-sharing with HIV-1-infected partners. They may represent the ideal study cohort for identification of a gene signature associated with protection from infection.

In the RV144 trial, systems biology could be used to identify a gene signature for study subjects who were protected from HIV-1 infection. Validation of this signature would then allow for its use to assess efficacy in other vaccine trials. Additionally, revealing the mechanism of action of protection in RV144 as well as identifying attributes of highly exposed seronegative individuals or controllers compared with non-controllers, systems biology will help generate novel hypotheses that will aid with the rational

design of new vaccines, both immunogens and vectors. Work in this thesis demonstrated a trend towards increased inhibition following vaccination with MVA.HIVconsv. However, the level of inhibition did not reach that of viraemic controllers. Transcriptional profiling of CD8+ T cells from HIV-1 controllers and non-controllers after culture with autologous CD4+ T cells could enable the identification of a gene signature associated with potent inhibition and is planned. If a signature is identified, there is the potential to validate it in other patient cohorts and to assess the immunogenicity, and ultimately, the efficacy of new vaccines.

In conclusion, I have demonstrated that targeting of 'beneficial' regions of the HIV-1 proteome is correlated with CD8+ T cell mediated inhibition, a highly predictive measure of virus control *in vivo*. A weaker correlation between inhibition and targeting of conserved elements and the disappointing results from the MVA.HIVconsv trial indicate that targeting of conserved regions alone is not sufficient to control virus replication. Although further investigations are needed to confirm the reasons why T cells specific for these 'beneficial' regions are effective at inhibiting virus replication, the data presented here provides support for testing of these regions in a HIV-1 vaccine.

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Appendix

HIVconsv peptide sequences

Name	Sequence	Pool
HIVCON 1	MEEKAFSPEVIPMFT	Pool 1
HIVCON 2	AFSPEVIPMFTALSE	Pool 1
HIVCON 3	EVIPMFTALSEGATP	Pool 1
HIVCON 4	MFTALSEGATPQDLN	Pool 1
HIVCON 5	LSEGATPQDLNTMLN	Pool 1
HIVCON 6	ATPQDLNTMLNTVGG	Pool 1
HIVCON 7R	DLNTMLNTVGGHQAAKK	Pool 1
HIVCON 8	MLNTVGGHQAAMQML	Pool 1
HIVCON 9	VGGHQAAMQMLKDTI	Pool 1
HIVCON 10	KQAAMQMLKDTINEEA	Pool 1
HIVCON 11	KQMLKDTINEEAAEWD	Pool 1
HIVCON 12	DTINEEAAEWDRIYK	Pool 1
HIVCON 13	EEAAEWDRIYKRWII	Pool 1
HIVCON 14	EWDRIYKRWIILGLN	Pool 1
HIVCON 15	IYKRWIILGLNKIVR	Pool 1
HIVCON 16	WIILGLNKIVRMYSP	Pool 1
HIVCON 17	GLNKIVRMYSPVSIL	Pool 1
HIVCON 18	IVRMYSPVSILDIRQ	Pool 1
HIVCON 19	YSPVSILDIRQGPKE	Pool 1
HIVCON 20	SILDIRQGPKEPFRD	Pool 1
HIVCON 21	IRQGPKEPFRDYVDR	Pool 1
HIVCON 22	PKEPFRDYVDRFARN	Pool 1
HIVCON 23	FRDYVDRFARNCRAP	Pool 1
HIVCON 24	VDRFARNCRAPRKKG	Pool 1
HIVCON 25	ARNCRAPRKKGCWKC	Pool 1
HIVCON 26	RAPRKKGCWKCGKEG	Pool 1
HIVCON 27	KKGCWKCGKEGHQMK	Pool 1
HIVCON 28	WKCGKEGHQMKDCTE	Pool 1
HIVCON 29	KEGHQMKDCTERQAN	Pool 1
HIVCON 30	KQMKDCTERQANFLGK	Pool 1
HIVCON 31	CTERQANFLGKIWPS	Pool 1
HIVCON 32	KQANFLGKIWPSRWKP	Pool 1
HIVCON 33	LGKIWPSRWKPKMIG	Pool 2
HIVCON 34	WPSRWKPKMIGGIGG	Pool 2
HIVCON 35	WKPKMIGGIGGFIKV	Pool 2
HIVCON 36	MIGGIGGFIKVRQYD	Pool 2
HIVCON 37	IGGFIKVRQYDQILI	Pool 2
HIVCON 38R	IKVRQYDQILIEICGKK	Pool 2
HIVCON 39	KQYDQILIEICGHKAI	Pool 2
HIVCON 40	ILIEICGHKAIGTVL	Pool 2
HIVCON 41	ICGHKAIGTVLVGPT	Pool 2
HIVCON 42	KAIGTVLVGPTPVNI	Pool 2
HIVCON 43	TVLVGPTPVNIIGRN	Pool 2
HIVCON 44	GPTPVNIIGRNLLTQ	Pool 2
HIVCON 45R	VNIIGRNLLTQIGCTKK	Pool 2
HIVCON 46R	GRNLLTQIGCTLNFPKK	Pool 2
HIVCON 47	LTQIGCTLNFPISPI	Pool 2
HIVCON 48	GCTLNFPISPIETVP	Pool 2
HIVCON 49	KNFPISPIETVPVKLK	Pool 2
HIVCON 50	SPIETVPVKLKPGMD	Pool 2
HIVCON 51	TVPVKLKPGMDGPKV	Pool 2
HIVCON 52	KLKPGMDGPKVKQWP	Pool 2
HIVCON 53	GMDGPKVKQWPLTEE	Pool 2
HIVCON 54	PKVKQWPLTEEKIKA	Pool 2
HIVCON 55	KQWPLTEEKIKALVEI	Pool 2
HIVCON 56	TEEKIKALVEICTEM	Pool 2
HIVCON 57	IKALVEICTEMEKEG	Pool 2
HIVCON 58	VEICTEMEKEGKISK	Pool 2
HIVCON 59	TEMEKEGKISKIGPE	Pool 2
HIVCON 60	KEGKISKIGPENPYN	Pool 2
HIVCON 61	ISKIGPENPYNTPVF	Pool 2
HIVCON 62	GPENPYNTPVFAIKK	Pool 2
HIVCON 63	PYNTPVFAIKKKDST	Pool 2
HIVCON 64	PVFAIKKKDSTKWRK	Pool 2
HIVCON 65	IKKKDSTKWRKLVDF	Pool 3
HIVCON 66	DSTKWRKLVDFRELN	Pool 3
HIVCON 67	WRKLVDFRELNKRTQ	Pool 3
HIVCON 68	VDFRELNKRTQDFWE	Pool 3
HIVCON 69	ELNKRTQDFWEVQLG	Pool 3
HIVCON 70	RTQDFWEVQLGIPHP	Pool 3
HIVCON 71	FWEVQLGIPHPAGLK	Pool 3
HIVCON 72	KQLGIPHPAGLKKKKS	Pool 3
HIVCON 73	PHPAGLKKKKSVTVL	Pool 3
HIVCON 74	GLKKKKSVTVLDVGD	Pool 3
HIVCON 75	KKSVTVLDVGDAYFS	Pool 3
HIVCON 76	TVLDVGDAYFSVPLD	Pool 3
HIVCON 77	VGDAYFSVPLDEGFR	Pool 3
HIVCON 78	YFSVPLDEGFRKYTA	Pool 3
HIVCON 79	PLDEGFRKYTAFTIP	Pool 3
HIVCON 80	GFRKYTAFTIPSINN	Pool 3
HIVCON 81	YTAFTIPSINNETPG	Pool 3
HIVCON 82	TIPSINNETPGIRYQ	Pool 3
HIVCON 83	INNETPGIRYQYNVL	Pool 3
HIVCON 84	TPGIRYQYNVLPQGW	Pool 3
HIVCON 85	RYQYNVLPQGWKGSP	Pool 3
HIVCON 86	KNVLPQGWKGSPAIFQ	Pool 3
HIVCON 87	KQGWKGSPAIFQSSMT	Pool 3
HIVCON 88	GSPAIFQSSMTKILE	Pool 3
HIVCON 89	IFQSSMTKILEPFRA	Pool 3
HIVCON 90	SMTKILEPFRAQNPE	Pool 3
HIVCON 91	ILEPFRAQNPEIVIY	Pool 3
HIVCON 92R	FRAQNPEIVIYQYMDKK	Pool 3
HIVCON 93	KNPEIVIYQYMDDLYV	Pool 3
HIVCON 94	VIYQYMDDLYVGSDL	Pool 3
HIVCON 95	YMDDLYVGSDLEIGQ	Pool 3
HIVCON 96	LYVGSDLEIGQHRME	Pool 3
HIVCON 97	SDLEIGQHRMENRWQ	Pool 3
HIVCON 98	IGQHRMENRWQVMIV	Pool 4
HIVCON 99	RMENRWQVMIVWQVD	Pool 4
HIVCON 100	RWQVMIVWQVDRMRI	Pool 4
HIVCON 101	MIVWQVDRMRIRTWK	Pool 4
HIVCON 102	KQVDRMRIRTWKSLVK	Pool 4
HIVCON 103	MRIRTWKSLVKHHLT	Pool 4
HIVCON 104	TWKSLVKHHLTEEAE	Pool 4
HIVCON 105	LVKHHLTEEAELELA	Pool 4
HIVCON 106	HLTEEAELELAENRE	Pool 4
HIVCON 107	EAELELAENREILKD	Pool 4
HIVCON 108	ELAENREILKDPVHG	Pool 4
HIVCON 109	KNREILKDPVHGVYYD	Pool 4
HIVCON 110	LKDPVHGVYYDPSKD	Pool 4
HIVCON 111	VHGVYYDPSKDLIAE	Pool 4
HIVCON 112	YYDPSKDLIAEIQYW	Pool 4
HIVCON 113	SKDLIAEIQYWQATW	Pool 4
HIVCON 114	IAEIQYWQATWIPEW	Pool 4
HIVCON 115	KQYWQATWIPEWEFVN	Pool 4
HIVCON 116	ATWIPEWEFVNTPPL	Pool 4
HIVCON 117	PEWEFVNTPPLVKLW	Pool 4
HIVCON 118	FVNTPPLVKLWYQLE	Pool 4
HIVCON 119	PPLVKLWYQLEKNVT	Pool 4
HIVCON 120	KLWYQLEKNVTENFN	Pool 4
HIVCON 121	KQLEKNVTENFNMWKN	Pool 4
HIVCON 122	KNVTENFNMWKNDMVD	Pool 4
HIVCON 123	KNFNMWKNDMVDQMHE	Pool 4
HIVCON 124	WKNDMVDQMHEDIIS	Pool 4
HIVCON 125	MVDQMHEDIISLWDQ	Pool 4
HIVCON 126	MHEDIISLWDQSLKP	Pool 4
HIVCON 127	IISLWDQSLKPCVKL	Pool 4
HIVCON 128	WDQSLKPCVKLTPWV	Pool 4
HIVCON 129	LKPCVKLTPWVPAHK	Pool 4
HIVCON 130	VKLTPWVPAHKGIGG	Pool 5
HIVCON 131	PWVPAHKGIGGNEQV	Pool 5
HIVCON 132	AHKGIGGNEQVDKLV	Pool 5
HIVCON 133	IGGNEQVDKLVSQGI	Pool 5
HIVCON 134	EQVDKLVSQGIRKVL	Pool 5
HIVCON 135	KLVSQGIRKVLFLDG	Pool 5
HIVCON 136	SQGIRKVLFLDGIDKA	Pool 5
HIVCON 137	KVLFLDGIDKAQAKE	Pool 5
HIVCON 138	LDGIDKAQAKEIVAS	Pool 5
HIVCON 139	DKAQAKEIVASCDKC	Pool 5
HIVCON 140	AKEIVASCDKCQLKG	Pool 5
HIVCON 141	VASCDKCQLKGEAMH	Pool 5
HIVCON 142	DKCQLKGEAMHGQVD	Pool 5
HIVCON 143	LKGEAMHGQVDCSPG	Pool 5
HIVCON 144	AMHGQVDCSPGIWQL	Pool 5
HIVCON 145	GQVDCSPGIWQLDCTH	Pool 5
HIVCON 146	SPGIWQLDCTHLEGK	Pool 5
HIVCON 147	WQLDCTHLEGKVILV	Pool 5
HIVCON 148	CTHLEGKVILVAVHV	Pool 5
HIVCON 149	EGKVILVAVHVASGY	Pool 5
HIVCON 150	ILVAVHVASGYIEAE	Pool 5
HIVCON 151	VHVASGYIEAEVIPA	Pool 5
HIVCON 152	SGYIEAEVIPAETGQ	Pool 5
HIVCON 153	EAEVIPAETGQETAY	Pool 5
HIVCON 154	IPAETGQETAYFLLK	Pool 5
HIVCON 155R	TGQETAYFLLKLAMNKK	Pool 5
HIVCON 156	TAYFLLKLAMNKELK	Pool 5
HIVCON 157	LLKLAMNKELKKIIG	Pool 5
HIVCON 158	AMNKELKKIIGQVRD	Pool 5
HIVCON 159	ELKKIIGQVRDQAEH	Pool 5
HIVCON 160	IIGQVRDQAEHLKTA	Pool 5
HIVCON 161	VRDQAEHLKTAVQMA	Pool 5
HIVCON 162	AEHLKTAVQMAVFIH	Pool 5
HIVCON 163	KTAVQMAVFIHNFKR	Pool 5
HIVCON 164	VQMAVFIHNFKRKGGI	Pool 5
HIVCON 165	FIHNFKRKGGIGGYS	Pool 5
HIVCON 166	FKRKGGIGGYSAGER	Pool 6
HIVCON 167	GGIGGYSAGERIWKG	Pool 6
HIVCON 168	GYSAGERIWKGPAKL	Pool 6
HIVCON 169	GERIWKGPAKLLWKG	Pool 6
HIVCON 170	WKGPAKLLWKGEGAV	Pool 6
HIVCON 171	AKLLWKGEGAVVIQD	Pool 6
HIVCON 172	WKGEGAVVIQDNSDI	Pool 6
HIVCON 173	GAVVIQDNSDIKVVP	Pool 6
HIVCON 174	IQDNSDIKVVPRRKA	Pool 6
HIVCON 175	SDIKVVPRRKAKIIR	Pool 6
HIVCON 176	VVPRRKAKIIRDYGK	Pool 6
HIVCON 177	RKAKIIRDYGKQMAG	Pool 6
HIVCON 178	IIRDYGKQMAGADCV	Pool 6
HIVCON 179	YGKQMAGADCVFLGA	Pool 6
HIVCON 180R	MAGADCVFLGAAGSTKK	Pool 6
HIVCON 181R	DCVFLGAAGSTMGAAKK	Pool 6
HIVCON 182R	LGAAGSTMGAASMTLKK	Pool 6
HIVCON 183R	GSTMGAASMTLTVQAKK	Pool 6
HIVCON 184R	GAASMTLTVQARQLLKK	Pool 6
HIVCON 185R	MTLTVQARQLLSGIVKK	Pool 6
HIVCON 186	VQARQLLSGIVQQQN	Pool 6
HIVCON 187	RQLLSGIVQQQNNLLR	Pool 6
HIVCON 188	GIVQQQNNLLRAIEA	Pool 6
HIVCON 189	VQQQNNLLRAIEAQQHL	Pool 6
HIVCON 190	LLRAIEAQQHLLQLT	Pool 6
HIVCON 191	IEAQQHLLQLTVWGI	Pool 6
HIVCON 192	QHLLQLTVWGIKQAC	Pool 6
HIVCON 193	LQLTVWGIKQACTPYD	Pool 6
HIVCON 194	WGIKQACTPYDINQM	Pool 6
HIVCON 195	KQACTPYDINQMLRGP	Pool 6
HIVCON 196	PYDINQMLRGPGRAF	Pool 6
HIVCON 197	INQMLRGPGRAFVTIP	Pool 6
HIVCON 198	RGPGRAFVTIPNPLL	Pool 6

FEC peptide sequences

Peptide Code	Sequence	HLA type	Protein
FEC 1 (27)	GILGFVFTL	A2	Flu M
FEC 2 (28)	FMYSDFHFI	A2	Flu
FEC 3 (29)	SIIPSGPLK	A3, A11, A6805	Flu
FEC 4 (30)	RVLSFIKGTK	A3	Flu NP
FEC 5 (31)	KTGGPIYKR	A68	Flu NP
FEC 6 (32)	LPFDKTTVM	B7	Flu NP
FEC 7 (33)	ELRSRYWAI	B8	Flu NP
FEC 8 (34)	SRYWAIRTE	B27	Flu NP
FEC 9 (35)	ASCMGLIY	B27	Flu M
FEC 10 (36)	CLGGLLTMV	A2	EBV LMP2A
FEC 11 (37)	GLCTLVAML	A2	EBV BMLFE
FEC 12 (38)	AVFDRKSDAK	A11	EBV EBNA4NP
FEC 13			
FEC 14 (39)	DYCNVLNKEF	A24	EBV RTA
FEC 15 (40)	RAKFKQLL	B8	EBV B2LF-1
FEC 16 (41)	FLRGRAYGL	B8	EBV EBNA3A
FEC 17 (42)	QAKWRLQTL	B8	EBV EBNA3
FEC 18 (43)	RRIYDLIEL	B27	EBV EBNA3C
FEC 19 (44)	YPLHEQHGL	B35	EBV EBNA3A
FEC 20 (45)	NLVPMVATV	A0201	CMV pp65
FEC 21 (46)	TPRVTGGGAM	B0702	CMV
FEC 22 (47)	SDEEEAIVAYTL	B18	CMV
FEC 23 (48)	IPSINVHHY	B35	CMV pp65

Clade B specific beneficial peptides

	OPL no.	PR	Entropy	Protective peptide sequence
Gag pool 1 (PR1.2+)	31	1.281	0.12	IAPGQMREPRGSDIA
				GP----------
				----------GT
	34	1.207	0.20	STLQEQIGWMTNNPPIPV

				----------G
Gag pool 2 (PR 1.1+)	6	1.083	0.11	ASRELERFAVNPGLL

	48	1.107	0.10	ACQGVGGPGHKARVLAEA

				----------M
Gag pool 3 (PR1.0+)	3	1.053	0.24	EKIRLRPGGKKKYKLKHI

				----------V
	7	1.04	0.23	ERFAVNPGLLETSEGCR

				----------Q
	10	1.085	0.29	QLQPSLQTGSEELRSLY
				LG----------

	12	1.044	0.37	SLYNTVATLYCVHQRIEV

				----------K
	23	1.048	0.10	AFSPEVIPMFSALSEGA
				EK----------

	60	1.083	0.36	GKIWPSHKGRPGNFLQSR
				FL----------

				----------PEP
Pol pool 1 (PR 1.2+)	160	1.277	0.34	FIKVRQYDQILIEICGHK

				----------A
	171	1.391	0.16	LVEICTEMEKEGKISKI

				----------GP
	195	1.219	0.30	LRWGFTTPDKKHQKEPPF

				----------L
Pol Pool 2 (PR 1.1+)	161	1.149	0.35	QILIEICGHKAIGTVLV

				----------GP
	196	1.136	0.09	DKKHQKEPPFLWMGYELH

				----------P
	269	1.192	0.14	TKELQKQITKIQNFRVYY

				----------R
	276	1.192	0.06	KIIRDYGKQMAGDDCVA

				----------SR
Pol Pool 3 (PR 1.0+)	159	1.094	0.19	KMIGGIGGFIKVRQYDQI

				----------L
	163	1.055	0.16	LVGPTPVNIIGRNLLTQI
				GTV----------

				----------GC
	210	1.068	0.24	EIQKQGQGQWTYQIY
				SKDLIA---------

	270	1.073	0.13	TKIQNFRVYYRDSRDPLW

				----------K
	271	1.032	0.09	YYRDSRDPLWKGPAKLLW

				----------K
Vif pool 1	405	1.084	0.53	VKHHMYISGKAKGWFYRH

	406	1.084	0.54	GKAKGWFYRHHYESTHPR
				MYIS----------

				----------I
	424	1.137	0.25	TKLTEDRWNKPQKTKGHR
				SV----------

				----------GSH
Nef pool 1	75	1.108	0.40	WLEAQEEEEVGFPVRPQV

				----------P

Clade C specific beneficial peptides

	OPL no.	PR	Entropy	Protective peptide sequence
Gag pool 1 (PR 1.23 - 1.34)	7	1.25	0.23	ERFALNPGLLETSEGCK
				L----------
	26	1.23	0.08	NTMLNTVGGHQAAMQMLK

				----------E
	39	1.34	0.15	SILDIKQGPKEPFRDYV
				MYSPV----------
				----------DRFF
Gag pool 2 (PR 1.21 - 1.22)	6	1.22	0.24	ASRELERFALNPGLL
				HLVW----------
				----------ETSEGCK
	60	1.21	0.36	GKIWPSHKGRPGNFLQSR
				FL----------

				----------PEP
	63	1.21	0.84	TAPPAESFRFEETTPAPK
				PEP----------
				----------QEPKD
Gag pool 3 (PR 1.18 - 1.20)	22	1.18	0.14	WVKVIEEKAFSPEVIPMF
				PRTLNA----------
				----------T
	31	1.2	0.15	IAPGQMREPRGSDIA
				GP----------
				----------GT
	55	1.19	0.14	HIARNCRAPRKKGCWK
				KEG----------
				----------CGKEGHQ
Gag pool 4 (PR 1.16 - 1.17)	27	1.16	0.08	GGHQAAMQMLKDTINEEA
				LNTV----------
				----------AEWD
	37	1.17	0.08	WIILGLNKIVRMYSPVSI
				DIYKR----------
				----------LDI
	59	1.16	0.18	RQANFLGKIWPSHKGR

Gag pool 5 (PR 1.09 - 1.13)	3	1.09	0.31	EKIRLRPGGKKHYMLKHL

	42	1.09	0.19	LRAEQATQDVKNWMTDTL
				FFKT----------
				----------LVQN
	33	1.13	0.17	SDIAGTTSTLQEQIAWM
				MREPRG----------
				----------TSN
Gag pool 6 (PR 1.06 - 1.07)	29	1.07	0.23	AAEWDRLHPVHAGPIA

				----------PGQ
	41	1.07	0.17	YVDRFFKTLRAEQATQDV
				PFRD----------
				----------KNWM
	25	1.06	0.12	GATPQDLNTMLNTVGGH
				E----------
				----------Q
Pol pool 1	181	1.19	0.14	LDVGDAYFSVPLDEDFRK
				KKKSVTV----------
				----------Y
	199	1.2	0.16	TVQPIQLPEKDSWTVNDI

	216	1.09	0.24	QKIAMESIVIWGKTPKFR
				KQLTEAV----------
				----------L
Vif pool 1	417	1.5	0.26	CFADSAIRKAILGHIV
				D----------I

Conserved elements peptides

	Conserved element	Entropy	Sequence
Pool A	CE1	0.21	ISPRTLNAWVKV
			QA----------V
	CE2	0.13	VIPMFSALSEGATPQDLN
			SPE----------

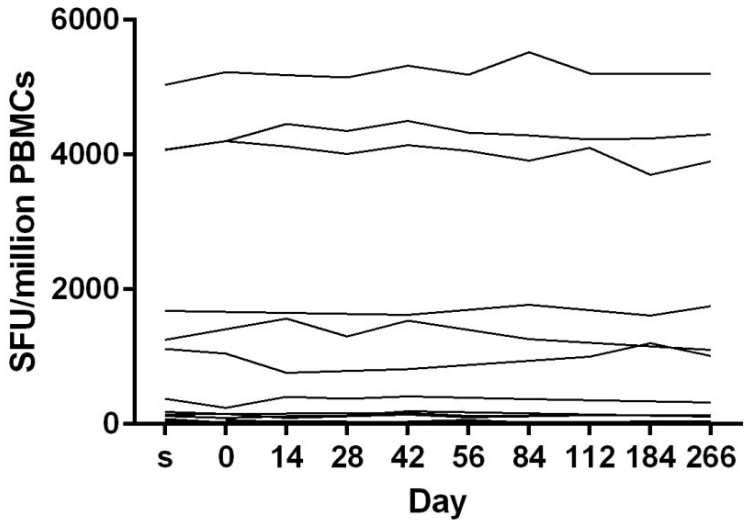
			----------TM
	CE3	0.11	VGGHQAAMQMLKDTINEEAAEWDR
			NT----------

			----------L
	CE7	0.09	LEEMMTACQGVGGPSHK
			AT----------
			----------G--
Pool B	CE4	0.11	PRGSDIAGTTSTLQEQIGW
			GPIAPGQMRE-----
			PGQMRE---------
			RE----------

			----------MT
	CE5	0.09	KRWIILGLNKIVRMYSPVSI
			IY----------

			----------T--L
	CE6	0.15	YVDRFFKTLRAEQA
			FRD-----Y------
			----Y--------SQ

FEC responses measured by IFNγ-Elispot in all 19 MVA.HIVconsv trial participants



Response to FEC peptides in MVA.HIVconsv trial participants. Freshly isolated PBMCs from each time point were stimulated with a pool of FEC peptides in an IFN-γ Elispot assay. $1x10^5$ PBMC/well were used in the assay with a final peptide concentration of 2µg/ml. Results expressed as SFU/million PBMCs following background subtraction.

Virus isolates

Virus subtype	Primary Isolate	Co-receptor Tropism	MOI
A1	Yes	R5 or X4	0.001
A2	Yes	X4	0.003
IIIB	No	X4	0.009
BaL	No	R5	0.01
C	Yes	R5	0.002

Possible epitopes within confirmed positive peptides from second round testing

Patient	Peptide no.	Sequence	Peak Magnitude	Protein	Epitopes (Name)	Restriction	Patients HLA
201 CD8	12	DTINEEAAEWDRIYK	130 SFU (d56)	Gag clade C	**LK**DTINEEAAEWDRLHPV	A*6801	A*2601, A*3201 B*440301, B*4001 Bw4, Bw6 Cw*0304, Cw*1601 DRB1*0401, DRB1*0701 DRB4 DQB1*02, DQB1*0302
					KDTINEEAAEWDRL	A	
					DTINEEAAEW (Gag-DW10)	B53	
	23	FRDYVDRFARNCRAP	265 SFU (d14)	Gag C/D junc	**EP**FRDYVDRF	A*0201	
					PFRDYVDRF		
					LARNCRAP**RK**	A3	
					IAKNCRAP**RK** (IAKK10)	A3	
					CRAP**RKKGC** (CC9)	B14	
	25	ARNCRAPRKKGCWKC	235 SFU (d14)	Gag clade D	TARNCRAPRK	A3, A*3101	
	52	KLKPGMDGPKVKQWP	175 SFU (d0)	Pol Clade B	**IETVPV**KLKPGMDGPKVKQWP**LTEE**	B8	
					PGMDGPKVKQ (1276)	A11	
	67	WRKLVDFRELNKRTQ	115 SFU (d56)	Pol Clade C	KLVDFRELNK (A3-KK10)	A*0301	
	74	GLKKKKSVTVLDVGD	105 SFU (d14)	Pol Clade C	AGLKKKKSVTVLDVGD	Cw4	
					KKKSVTVLDVGD**AYF**		
					TVLDVGD**AY** (Pol-TY9)	B35	
	82	TIPSINNETPGIRYQ	240 SFU (d28)	Pol Clade C	FTIPSINNETPGIRY	A*25	
					NNETPGIRY	B*1801	
					NETPGIRYQ**Y**	B*18	
	91	ILEPFRAQNPEIVIY	175 SFU (d14)	Pol Clade C	AQNPEIVIY	A*3002	
	94	VIYQYMDDLYVGSDL	225 SFU (d14)	Pol Clade C	**NPDI**VIYQYMDDLYVGSDL**EIGQHR**	A11	
					VIYQYMDDL	A2	
					IYQYMDDLYV	A2	
	98	IGQHRMENRWQVMIV	230 SFU (d42)	pol/vif junction	NRWQVMIV**W** (NW9)	B27	
	143	LKGEAMHGQVDCSPG	255 SFU (d28)	Pol Clade A	QVDCSPG**I**	B	
	148	CTHLEGKVILVAVHV	250 SFU (d28)	Pol Clade A	**QLD**CTHLEGK (1335)	A3	
					THLEGKVIL	B15	
	158	AMNKELKKIIGQVRD	330 SFU (d28)	Pol Clade B	ELKKIIGQVR	A33	
	160	IIGQVRDQAEHLKTA	175 SFU (d28)	Pol Clade B	QVRDQAEHL	A02	
					VRDQAEHL	Cw18	
	163	KTAVQMAVFIHNFKR	217 SFU (d28)	Pol Clade B	**HL**KTAVQMAV (1247)	A2	
					KTAVQMAVF (KF9)	B*5801, B57	
					QMAVFIHNFK (Pol929)	A3, A*0301	
					MAVFIHNFK	A3	
					AVFIHNFKR	A*0301	

Patient	Peptide no.	Sequence	Peak Magnitude	Protein	Epitopes (Name)	Restriction	Patients HLA
202 CD8	13	EEAAEWDRIYKRWII	110 SFU (d28)	Gag Clade C	EEAAEWDRL (EL9-B40)	B40	A*01, A*01 B*08, B*08 Bw6 Cw*0701 DRB1*0101 DRB1*0301 DRB3 DQB1*02 DQB1*05
	162	AEHLKTAVQMAVFIH	100 SFU (d56)	Pol Clade B	QMAVFIH**NFK**	A0301	
					MAVFIHNFK	A3 supertype	
					AVFIHNFKR	A3 supertype, A11, A68	
					AVFIHNFKRK	A3 supertype, A11, A68	

Patient	Peptide no.	Sequence	Peak Magnitude	Protein	Epitopes (Name)	Restriction	Patients HLA
203 CD8	153	EAEVIPAETGQETAY	257 SFU (d14)	Pol Clade A	**GYI**EAEVI (Pol797-8)	A*2402	A*0101, A*2601 B*3801, B*5701 Bw4 Cw*0602, Cw*1203 DRB1*0602, DRB1*0701 DRB4 DQB1*05, DQB1*0303
					YIEAEVIPA		
	171	AKLLWKGEGAVVIQD	1000 SFU (d42)	Pol Clade C	LLWKGEGAV (LR28/L9V)	A0201	
	172	WKGEGAVVIQDNSDI	172 SFU (d14)	Pol Clade C	**LL**WKGEGAV	A0201	
	196	PYDINQMLRGPGRAF	1238 SFU (d42)	M&M	cross reactive with HIV-1 variants TPQDLNTML and TPQDLNMML)	B53, A*0101, A*2402, B*07, B*5801	

Patient	Peptide no.	Sequence	Peak Magnitude	Protein	epitopes (Name)	Restriction	Patients HLA
204 CD8	36	MIGGIGGFIKVRQYD	150 SFU (d56)	Pol Clade B	**K**MIGGIGGF (KF9)	B62, A*02	A*3301, A*6802 B*1510, B*1517 Cw*0304, Cw*0501
					KMIGGIGGFI (KI10)	A2, B62	
					KVRQYD**QILI**	A2	
	39	KQYDQILIEICGHKAI	125 SFU (d56)	Pol Clade B	RQYDQILIEI	B13	
					QYDQILIEI	Cw0401	
					ILIEICGHK	A3	
					IEICGHKAI**G** (IG10)	B18, B44, B40	
	81	YTAFTIPSINNETPG	205 SFU (d0)	Pol Clade C	**K**YTAFTIPSI	A2	
					YTAFTIPSI (YI9)	A*0205	
					TAFTIPSI (TI8)	A*0201, B51	
	84	TPGIRYQYNVLPQGW	175 SFU (d56)	Pol Clade C	**NE**TPGIRYQY (NY10)	B18	
					TPGIRYQYNV (Pol1157)	B7	
					IRYQYNVL (IL9)	B14	
	85	RYQYNVLPQGWKGSP	220 SFU (d56)	Pol Clade C	**I**RYQYNVL (IL9)	B14	
					LPQGWKGSP**A** (LA10)	B*5401	
					QGWKGSPA**I**	B5101	
	92	FRAQNPEIVIYQYMDKK	140 SFU (d42)	Pol Clade C	KQNPDIVIY (KY9)	A30	
					NPDIVIYQ	B51, B35, B18	
					NPDIVIYQY (NY9)	A35	
					NPDIVIYQYM	B51	
	110	LKDPVHGVYYDPSKD	120 SFU (d42)	Pol Clade A	**I**LKDPVHGV	A2	
					ILKDPVHGVY	A2, B15, B62	
					VYYDPSKDL	A24	
	162	AEHLKTAVQMAVFIH	280 SFU (d42)	Pol Clade B	QMAVFIH**NFK**	A0301	
					MAVFIHNFK	A3 supertype	
					AVFIHNFKR	A3 supertype, A11, A68	
					AVFIHNFKRK	A3 supertype, A11, A68	
	164	VQMAVFIHNFKRKGGI	240 SFU (d42)	Pol Clade B	QMAVFIHNFK (Pol 929)	A03	
					MAVFIHNFK	A3	
					AVFIHNFKR	A3	
					AVFIHNFKRK	A3, A11, A68	
					FKRKGGIGGY	B15	
	174	IQDNSDIKVVPRRKA	100 SFU (d42)	Pol Clade C	KVVPRRKAK		
					KVVPRRKAKIIR**DYGKQM**		
					RKAKIIR**DY** (B15-RY9)	B15	
	189	VQQQNNLLRAIEAQQHL	430 SFU (d56)	Env Clade D	IVQQQSNLLRAIEAQQHL (Env 75)		
					RAIEAQQHL (E3/RL9)	B15, B51, Cw08	
					RAIEAQQHL**L**	B15, B51, B57, B58, B63	
					LLRAIEAQQHL	A11	
	190	LLRAIEAQQHLLQLT	115 (d56)	Env Clade D	**IVQQQSN**LLRAIEAQQHL (Env 75)		
					LLRAIEAQQHL	A11	

Patient	Peptide no.	Sequence	Peak Magnitude	Protein	Epitopes (Name)	Restriction	Patients HLA
205 CD8	93	KNPEIVIYQYMDDLYV	125 SFU (d56)	Pol Clade C	**K**QNPDIVIY	A30, B15	A*0201, A*0201 B*3501, B*4501 Bw6, Bw6 Cw*0401, Cw*1601 DRB1*1503, DRB1*0801
					KQNPDIVIYQY	A30	
					NPDIVIYQY	B18, B35	
					NPDIVIYQYM	B51	
					VIYQYMDDL	A2	
					IYQYMDDLYV	A2	
					YQYMDDLYV	A2	

Patient	Peptide no.	Sequence	Peak Magnitude	Protein	Epitopes (Name)	Restriction	Patients HLA
206 CD8	82	TIPSINNETPGIRYQ	440 SFU (d56)	Pol Clade C	FTIPSINNETPGIRY	A25	A*3002, A*4301 B*1402, B*4403
					NNETPGIRY	B*1801	
					NETPGIRYQY (NY10)	B18	
					TPGIRYQYNV (Pol1157)	B7	
	83	INNETPGIRYQYNVL	1100 SFU d56)	Pol Clade C	IRYQYNVL (IL9)	B*1401	
	154	IPAETGQETAYFLLK	150 SFU (d56)	Pol Clade A	IPAETGQETA (1294)	B56, B7	
					AETGQETAYF	B4403	
					ETGQETAY	A2601	
					ETAYFLLK**L**	A6802	
	172	WKGEGAVVIQDNSDI	100 SFU (d56)	Pol Clade C	**LL**WKGEGAV	A0201	

Patient	Peptide no.	Sequence	Peak Magnitude	Protein	Epitopes (Name)	Restriction	Patients HLA
207 CD8	93	KNPEIVIYQYMDDLYV	245 SFU (d14)	Pol Clade C	**K**QNPDIVIY	A30, B15	B4402, B5102 Bw4, Bw4
					KQNPDIVIYQY	A30	
					NPDIVIYQY	B18, B35	
					NPDIVIYQYM	B51	
					VIYQYMDDL	A2	
					IYQYMDDLYV	A2	
					YQYMDDLYV	A2	
	94	VIYQYMDDLYVGSDL	250 SFU (d14)	Pol Clade C	**NPDI**VIYQYMDDLYVGSDL**EIGQHR**	A11	
					VIYQYMDDL	A2	
					IYQYMDDLYV	A2	
	97	SDLEIGQHRMENRWQ	105 SFU (d14)	Pol Clade C	DLEIGQHRTK	A3	
	105	LVKHHLTEEAELELA	125 SFU (d14)	Vif Clade D/Pol Clade A	**S**LVKHH**MYV** (Vif 23)	A2	
					IPLTEEAEL	B0702, B1501, B3501, B51	
	132	AHKGIGGNEQVDKLV	127 SFU (d0)	Pol Clade D	Nothing		
	165	FIHNFKRKGGIGGYS	140 SFU (d14)	Pol Clade B	FKRKGGIGGY	B15	
					KRKGGIGGY	B2705	
	169	GERIWKGPAKLLWKG	115 SFU (d14)	Pol Clade B/ Pol Clade C	**DPI**WKGPAKL (Pol 1143)	C	

Patient	Peptide no.	Sequence	Peak Magnitude	Protein	Epitopes (Name)	Restriction	Patients HLA
209 CD8	17	GLNKIVRMYSPVSIL	405 SFU (d14)	Gag Clade D	**KWIL**GLNKIVRMY	B27	A*1101, A*2601 B*1801, B*5501 Bw6, Bw6 Cw*0303, Cw*0701 DRB1*1401, DRB1*1104
					ILGLNKIVRMY	B7	
					IILGLNKIVR (IR10)	A*03, A*11	
					IILGLNKIV (IV9)	A*02	
					ILGLNKIV	A2	
					NKIVRMYSPVSIL**DI**		
					RMYSPVSIL		
	22	PKEPFRDYVDRFARN	520 SFU (d56)	Gag Clade D	**LDIRQG**PKEPFRDYVDRFYK		
					GPKEPFRDY (Gag1164)	B7	
					GPKEPFRDYVDRFYKTLR (Gag40)		
					EPFRDYVDRF	A*0201, A2	
					FRDYVDRFF	Cw*18	
	30	KQMKDCTERQANFLGK	525 SFU (d14)	Gag Clade A	**KEG**HQMKDCTERQANF	A2	
					DCTERQANFLG		
					CTERQANFL	B61	
					TERQANFL (TL8)	B40	
					ERQANFLGK**IW**		
					RQANFLGKI (R19)	B13	
	40	ILIEICGHKAIGTVL	485 SFU (d14)	Pol Clade B	ILIEICGHK (IK9)	A3	
					IEICGHKAIG (IG10)	B18, B40, B44	
					GKKAIGTVL (GL9)	B15	
					HKAIGTVL**VGPTPVN**		
					KAIGTVLV (KV8)	B57	
	47	LTQIGCTLNFPISPI	625 SFU (d14)	Pol Clade B	TQIGCTLNF	B15, B62	
					CTLNFPISPI	A2	
					TLNFPISPI	A2	
	72	KQLGIPHPAGLKKKKS	505 SFU (d42)	Pol Clade C	**FWE**VQLGIPHPAGLKKKK	A*6801	
					GIPHPAGLKK (P248-257)		
					GIPHPAGLK (A3-GK9 Pol)	A*0301	
					AGLKKKKS**VTVLDVGD**	Cw4	
	75	KKSVTVLDVGDAYFS	725 SFU (d42)	Pol Clade C	**K**KKSVTVLDVGDAYFS**V**	Cw*0401	
					KKSVTVLDVGDAYFS	Cw*0401	
					TVLDVGDAY (TY9)	B35	
					VLDVGDAYFS**V** (V11V)	A*02	
					DAYFS**VPL**	B51	
	81	YTAFTIPSINNETPG	465 SFU (d42)	Pol Clade C	**K**YTAFTIPSI	A2	
					YTAFTIPSI (YI9)	A*0205	
					TAFTIPSI (TI8)	A*0201, B51	
	124	WKNDMVDQMHEDIIS	425 SFU (d42)	Env Clade C	QMHEDIIS**L**	A*0201	
					MHEDIIS**LW**	B38	
					HEDIIS**LWDQSLK**	A2	
	148	CTHLEGKVILVAVHV	785 SFU (d56)	Pol Clade A	**QLD**CTHLEGK (1335)	A3	
					THLEGKVIL	B15	
	185	MTLTVQARQLLSGIVKK	685 SFU (d56)	Env Clade D	Nothing		
	187	RQLLSGIVQQQNNLLR	515 SFU (d56)	Env Clade D	IVQQQSNLLRAIEAQQHL (Env 75)		
	190	LLRAIEAQQHLLQLT	532 SFU (d28)	Env Clade D	**IVQQQSN**LLRAIEAQQHL (Env 75)		
					LLRAIEAQQHL	A11	
	192	QHLLQLTVWGIKQAC	525 SFU (d42)	Env Clade D	LLQLTVWGI (Env 565)	A2	

Patient	Peptide no.	Sequence	Peak Magnitude	Protein	Epitopes (Name)	Restriction	Patients HLA
210 CD8	15	IYKRWIILGLNKIVR	1537 SFU (d14)	Gag Clade D	**GE**IYKRWIIL (GL10)	A*24, B*08	A*0301, A*11 B*0801, B*2702 Bw4, Bw6 Cw*0202, Cw*0701 DRB1*1501, DRB1*0301 DRB5, DRB3 DBQ1*02, DBQ1*0602
					EIYKRWIIL (EL9)	A2	
					IYKRWIILGLNKIVR	A24, B27	
					IYKRWIILGL (IL10)	A*24, B*27	
					IYKRWIIL (IL8)	A*24, B*08	
					KRWIILGLNK (KK10)	B27	

Patient	Peptide no.	Sequence	Peak Magnitude	Protein	Epitopes (Name)	Restriction	Patients HLA
213 CD8	11	KQMLKDTINEEAAEWD	203 SFU (d42)	Gag Clade C	**A**MQMLK**E**TINEEAAE	A2, A24, B15, B40	A*0301, A*3001 B*3501, B*4001 Bw6 Cw*0304, Cw*0401 DRB1*0401, DRB1*1302 DRB3, DRB4 DQB1*0301, DQB1*0605
					LKDTINEEAAEWD**RLHPV**	A6801	
					LK**E**TINEEAAEWD**RV**	A25, A3, B18, B27	
					KDTINEEAAEWD**RL**		
					K**E**TINEEAA (KA9)	B40	
					ETINEEAAEW (EW10)	A25	
	31	CTERQANFLGKIWPS	463 SFU (d42)	Gag Clade D	CTERQANFL	B61	
					TERQANFL (TL8)	B40	
					RQANFLGKIWPS**YKG**	A2	
					RQANFLGKI (RI9)	B13	
					FLGKIWPS**HK** (FK10)	A*02	
	44	GPTPVNIIGRNLLTQ	103 SFU (d42)	Pol Clade B	PTPVNIIGRN**L**	B63	
					TPVNIIGRNL (POL1151)	B7	
	49	KNFPISPIETVPVKLK	807 SFU (d28)	Pol Clade B	PISPIETVPVKL**KPGM**	B3910	
					SPIETVPVKL (SL10/1307)	A2, B61, B7, B8	
					IETVPVKL (IL8)	B40	
	50	SPIETVPVKLKPGMD	1067 SFU (d28)	Pol Clade B	**PI**SPIETVPVKLKPGM	B3910	
					SPIETVPVKL (SL10/1307)	A2, B61, B7, B8	
					IETVPVKL (IL8)	B40	
	75	KKSVTVLDVGDAYFS	1577 SFU (d28)	Pol Clade C	**K**KKSVTVLDVGDAYFS**V**	Cw*0401	
					KKSVTVLDVGDAYFS	Cw4	
					TVLDVGDAY (Pol-TY9)	B35	
	76	TVLDVGDAYFSVPLD	1293 SFU (d42)	Pol Clade C	VLDVGDAYFSVPLDE	Cw4	
						A24, B*5101	
					FSVPLD**EDF**	B35, B*5702, B*5703	
	78	YFSVPLDEGFRKYTA	1203 SFU (d42)	Pol Clade C	FSVPLDEDFRKYTA**FTIPSI**		
					FSVPLDEDF	B35, B*5702, B*5704	
					VPLDEDFRKY (Pol-VY10)	B35	
	79	PLDEGFRKYTAFTIP	723 SFU (d42)	Pol Clade C	FSVPLDEDFRKYTAFTIP**SI**		
					VPLDEDFRKY (Pol-VY10)	B35	
					KYTAFTIP**SI**	A2	
					YTAFTIP**SI (A2-IY9)**	A*0205	
					TAFTIPSI (TI8)	A*0217, B*5101	
	91	ILEPFRAQNPEIVIY	483 SFU (S)	Pol Clade C	AQNPEIVIY	A*3002	
	92	FRAQNPEIVIYQYMDKK	867 SFU (28)	Pol Clade C	KQNPDIVIY (KY9)	A30	
					NPDIVIYQ	B51, B35, B18	
					NPDIVIYQY (NY9)	A35	
					NPDIVIYQYM	B51	
	93	KNPEIVIYQYMDDLYV	833 SFU (S)	Pol Clade C	**K**QNPDIVIY	A30, B15	
					KQNPDIVIYQY	A30	
					NPDIVIYQY	B18, B35	
					NPDIVIYQYM	B51	
					VIYQYMDDL	A2	
					IYQYMDDLYV	A2	
					YQYMDDLYV	A2	
	94	VIYQYMDDLYVGSDL	882 SFU (d28)	Pol Clade C	**NPDI**VIYQYMDDLYVGSDL**EIGQHR**	A11	
					VIYQYMDDL	A2	
					IYQYMDDLYV	A2	
	139	DKAQAKEIVASCDKC	417 SFU (d28)	Junc Pol	**PPIV**AKEIVASCDKC**QLK**	B*8101	
					EIVASCDKC**QL**	B*4201	
					VASCDKC**QL (VL9)**	B*8101	
	158	AMNKELKKIIGQVRD	167 SFU (d28)	Pol Clade B	ELKKIIGQVR	A33	
	163	KTAVQMAVFIHNFKR	217 SFU (d28)	Pol Clade B	**HL**KTAVQMAV (1247)	A2	
					KTAVQMAVF (KF9)	B*5801, B57	
					QMAVFIHNFK (Pol929)	A3, A*0301	
					MAVFIHNFK	A3	
					AVFIHNFKR	A*0301	
	198	RGPGRAFVTIPNPLL	117 SFU (d28)	M&M	RGPGRAFVTI (LR25)	A*0201	

Patient	Peptide no.	Sequence	Peak Magnitude	Protein	epitopes (Name)	Restriction	Patients HLA
221 CD8	195	KQACTPYDINQMLRGP	610 (d28)	M&M	e cross reactive with HIV-1 variants TPQ	B53, A*0101, A*2402,	A301, A3002 B1403, B1403 Cw0602, Cw1701 DRB0101, DRB1301 DRB3 DQB05, DQB0602
	177	RKAKIIRDYGKQMAG	300 (d28)	Pol Clade C	KVVPRRKAKIIRDYGKQM		
					RKAKIIRDY (B15-RY9)	B15	
					KIIRDYGK		
					KIIRDYGKQM	B42	
	178	IIRDYGKQMAGADCV	130 (d28)		KIIRDYGK		
					KIIRDYGKQM	B42	

Patient	Peptide no.	Sequence	Peak Magnitude	Protein	epitopes (Name)	Restriction	Patients HLA
221 CD8	88	GSPAIFQSSMTKILE	1820 (d84)	Pol Clade C	QGWKGSPAIFQSSMTKIL		A0224, A6802 B1510, B2705 Cw0702, Cw0802 DRB0102, DRB0401 DRB4 DQB05, DQB0301
					WKGSPAIFQSSMT	B7	
					WKGSPAIFQSSMTKI		
					SPAIFQSSM (SM9)	B*07, B*3501, B*4202	
					SPAIFQSSMT	B7	
	87	KQGWKGSPAIFQSSMT	1575 (d84)	Pol Clade C	LPQGWKGSPA (LA10)	B*5401, B*3910	
					QGWKGSPAIFQSSMTKIL		
					QGWKGSPAI	B*5101	
					WKGSPAIFQSSMT	B7	
					WKGSPAIFQSSMTKI		

Patient	Peptide no.	Sequence	Peak Magnitude	Protein	epitopes (Name)	Restriction	Patients HLA
223 CD8	93	KNPEIVIYQYMDDLYV	180 (d42)	Pol Clade C	KQNPDIVIY	A30, B15	A0201, A0301 B1801, B8101 Cw0303, Cw0501 DRB0101, DRB0401 DRB4 DQB05, DQB0301
					KQNPDIVIYQY	A30	
					NPDIVIYQY	B18, B35	
					NPDIVIYQYM	B51	
					VIYQYMDDL	A2	
					IYQYMDDLYV	A2	
					YQYMDDLYV	A2	

Linear mixed analysis for low dose vaccinees using results from IFN-ɣ Elispot assays with single pool peptides

SP1

	numDF	denDF	F-value	p-value
(Intercept)	1	63	6.00235426	0.01707746
Days	9	63	1.21302601	0.30322313
Group	1	8	1.14542467	0.31573326
Days:Group	9	63	0.32385348	0.96410516

SP2

	numDF	denDF	F-value	p-value
(Intercept)	1	63	1.28455772	0.26134805
Days	9	63	0.8026696	0.61523633
Group	1	8	0.26013207	0.62379959
Days:Group	9	63	0.21778457	0.99089091

SP3

	numDF	denDF	F-value	p-value
(Intercept)	1	63	2.17731835	0.14503898
Days	9	63	1.30908107	0.25031328
Group	1	8	0.26460676	0.62087801
Days:Group	9	63	0.32233437	0.9646507

SP4

	numDF	denDF	F-value	p-value
(Intercept)	1	63	1.77682262	0.18734055
Days	9	63	0.84034762	0.58210466
Group	1	8	0.39236731	0.54850994
Days:Group	9	63	0.21044848	0.9919619

SP5

	numDF	denDF	F-value	p-value
(Intercept)	1	63	3.98939422	0.05011061
Days	9	63	1.13100663	0.35485516
Group	1	8	0.67285762	0.43581437
Days:Group	9	63	0.2929507	0.97424372

SP6

	numDF	denDF	F-value	p-value
(Intercept)	1	63	2.06690558	0.15547539
Days	9	63	1.04849873	0.41279196
Group	1	8	0.37343809	0.55809592
Days:Group	9	63	0.26228448	0.98235215

Linear mixed analysis for high dose vaccinees using results from IFN-ɣ Elispot assays with single pool peptides

SP1

	numDF	denDF	F-value	p-value
(Intercept)	1	54	5.46694134	0.02311336
Days	9	54	0.48951381	0.87527234
Group	1	7	2.26692064	0.17588204
Days:Group	9	54	1.65350111	0.12358099

SP2

	numDF	denDF	F-value	p-value
(Intercept)	1	54	3.38653204	0.07122915
Days	9	54	0.87552652	0.55248623
Group	1	7	0.55693558	0.47980494
Days:Group	9	54	2.72881092	0.01063187

SP3

	numDF	denDF	F-value	p-value
(Intercept)	1	54	8.09496069	0.00625942
Days	9	54	0.86656088	0.56012303
Group	1	7	0.68823009	0.43414243
Days:Group	9	54	0.75766951	0.65512005

SP4

	numDF	denDF	F-value	p-value
(Intercept)	1	54	5.52611294	0.02241625
Days	9	54	0.95711666	0.48506614
Group	1	7	0.07588313	0.79091031
Days:Group	9	54	1.19561549	0.31690742

SP5

	numDF	denDF	F-value	p-value
(Intercept)	1	54	2.77211373	0.10171177
Days	9	54	0.94971422	0.49100945
Group	1	7	0.12393634	0.73516402
Days:Group	9	54	0.27610208	0.97854421

SP6

	numDF	denDF	F-value	p-value
(Intercept)	1	54	2.18166167	0.14547222
Days	9	54	0.59610297	0.79453486
Group	1	7	0.0004375	0.98389606
Days:Group	9	54	1.99591386	0.05765065

Viral load data for STEP participants

Patient	VT_DT	CD4	VL	VISIT	Diagnosis date	VL setpoint
9754	09-Aug-05	492		2	17th Aug 06	3750
	21-Mar-06		400	8		
	17-Aug-06		2500	9		
	18-Sep-06	500	1600	100		
	18-Oct-06	646	800	101		
	06-Nov-06	463	1100	102		
	30-Nov-06	433	4000	103		
	07-Mar-07	340	3100	104		
	04-Sep-07	371		105		
	02-Oct-07		3600	105.1		
	15-Apr-08	335	400	106		
9831	14-Sep-05	1303		2	13th Sep 06	24500
	18-Apr-06		400	8		
	13-Sep-06		100000	9		
	20-Sep-06	499	46000	100		
	28-Sep-06	750	29000	101		
	09-Nov-06	810	21000	102		
	15-May-07	614	28000	104		
	19-Sep-07	659	27000	105		
	18-Mar-08	482	34000	106		
	18-Sep-08	542	8000	107		
9896	27-Feb-06	795		2	27th Sept 06	4133
	31-May-06		400	6		
	29-Aug-06		27000	7		
	27-Sep-06		20000	8		
	16-Oct-06	580	4800	100		
	23-Oct-06	598	2400	101		
	11-Dec-06	672	2300	102		
	08-Jan-07	737	7700	103		
	16-Apr-07	611	9500	104		
	10-Oct-07	500	4500	105		
	22-Apr-08	471	30000	106		
10110	09-May-06	994		2	23rd May 07	3050
	01-Aug-06		400	6		
	17-Nov-06		400	7		
	15-Dec-06		400	8		
	23-May-07		670000	9		
	07-Jun-07	442	330000	100		
	13-Jun-07	737	150000	101		
	18-Jul-07	1047	14000	102		
	15-Aug-07	764	3400	103		
	20-Nov-07	973	2700	104		
	14-May-08	824	400	105		
	30-Jan-09	712	2800	106		
10111	27-Apr-06		400	1	7th aug 06??	1645
	17-May-06	914	400	2		
	14-Jun-06		400	4		
	12-Jul-06		120000	5		
	07-Aug-06		7000	6		
	21-Aug-06	855	1800	100		
	28-Aug-06	944	3400	101		
	02-Oct-06	1045	980	102		
	30-Oct-06	756	400	103		
	29-Jan-07	729	27000	104		
	09-Aug-07	623	49000	105		
	02-Nov-07	551	140000	105.1		
	08-Feb-08	519	65000	106		
	03-Dec-08	476	25000	107		
	04-Feb-09	463	18000	108		

Patient	VT_DT	CD4	VL	VISIT	Diagnosis date	VL setpoint
10276	30-Aug-06	1037		2	29th Nov 06	52667
	27-Sep-06		400	4		
	01-Nov-06		420000	5		
	29-Nov-06		130000	6		
	08-Dec-06	800	84000	100		
	21-Dec-06	690	39000	101		
	30-Jan-07	856	52000	102		
	27-Feb-07	734	67000	103		
	05-Jun-07	586	53000	104		
	13-Nov-07	659	81000	105		
	03-Jun-08	553	50000	106		
	09-Dec-08	529	35000	107		
10338	25-Sep-06	915		2	18th Dec 07	5475
	18-Dec-07		3100	9.2		
	02-Jan-08	765	4600	100		
	07-Jan-08	660	4300	101		
	12-Feb-08	1059	9700	102		
	04-Mar-08	1032	3300	103		
	11-Jun-08	625	43000	104		
	17-Dec-08	705	42000	105		
11613	07-Mar-05		400	1	1st dec 05	4300
	06-Apr-05	609	400	2		
	05-May-05		400	4		
	28-Jun-05		400	6		
	03-Oct-05		700	6.2		
	20-Oct-05		400	7.1		
	10-Nov-05		1200	8		
	01-Dec-05	502	450	100		
	15-Dec-05		610	101		
	31-Jan-06	712	3800	102		
	16-Feb-06	683	4300	103		
	31-May-06	534	49000	104		
	14-Dec-06	522	120000	105		
	23-May-07	252	140000	106		
	06-Dec-07	340	140000	107		
	06-May-08	425	400	108		
	01-Dec-08	684	400	109		
11645	30-Jun-05	523		2	28th Jun 07	
	14-Dec-06		400	10		
	28-Jun-07		44000	11		
	17-Jul-07	462	40000	100		
	24-Jul-07	461	21000	101		
	21-Aug-07	468	13000	102		
	18-Sep-07	547	28000	103		
	18-Dec-07	590	240000	104		
	02-Jul-08	415	16000	105		
	22-Jan-09	566	7100	106		
11726	09-Nov-05	864		2	8th May 07	16667
	15-Nov-06		400	9		
	08-May-07		28000	10		
	29-May-07	415	29000	100		
	11-Jun-07	499		101		
	21-Jun-07		15000	101.2		
	12-Jul-07	579	14000	102		
	13-Aug-07	407	21000	103		
	15-Nov-07	779	8000	104		
	15-May-08	649	2500	105		
	12-Nov-08	640	910	106		

Patient	VT_DT	CD4	VL	VISIT	Diagnosis date	VL setpoint
11758	04-Jan-06	670		2	10th Jan 07	10433
	02-Aug-06		400	8		
	10-Jan-07		50000	9		
	26-Jan-07	513	21000	100		
	06-Feb-07	671	5000	101		
	19-Mar-07	530	9300	102		
	17-Apr-07	575	17000	103		
	24-Jul-07	527	23000	104		
	09-Jan-08	642	7200	105		
	11-Jul-08	505	11000	106		
11860	08-Aug-06	593		2	22nd Feb 07	39333
	31-Oct-06		400	6		
	25-Jan-07		88000	7		
	22-Feb-07		18000	8		
	12-Mar-07	481	26000	100		
	04-Apr-07	448	27000	101		
	07-May-07	482	65000	102		
	05-Jun-07	341	110000	103		
	10-Sep-07	391	110000	104		
	10-Mar-08	384	85000	105		
	15-Sep-08	466	120000	106		
13640	12-Oct-05	735		2	11th Apr 07	115000
	11-Oct-06		400	9		
	11-Apr-07		190000	10		
	20-Apr-07	759	200000	100		
	26-Apr-07	550	120000	101		
	12-Jun-07	676	120000	102		
	09-Jul-07	624	110000	103		
	10-Oct-07	613	85000	104		
	09-Apr-08	754	38000	105		
	08-Oct-08	777	55000	106		
	16-Apr-09	653	41000	107		
13647	05-Jan-06	1333		3	23rd Jun 06	42500
	15-Mar-06		400	6		
	09-Jun-06		540000	7		
	23-Jun-06		77000	7.1		
	12-Jul-06	505	140000	100		
	18-Jul-06	480	300000	101		
	29-Aug-06	684	40000	102		
	12-Sep-06	687	45000	103		
	21-Dec-06	516	64000	104		
	26-Jun-07	603	74000	105		
	27-Dec-07	707	34000	106		
	04-Nov-05		400	1	20th feb 06	20000
	22-Nov-05	1366	400	2		
	19-Dec-05		400	4		
	13-Jan-06		400	5		
	20-Feb-06		390000	6		
	03-Mar-06	1143	78000	100		
	21-Mar-06	1215	31000	101		
	13-Apr-06	1267	30000	102		
	12-May-06	1014	14000	103		
	06-Sep-06	939	19000	104		
	01-Mar-07	865	31000	105		
	20-Aug-07	1030	72000	106		
	29-Apr-08	616	48000	107		
13728	25-Apr-06	606		2	23rd Apr 07	530
	09-Nov-06		400	6.1		
	13-Dec-06		400	8		
	05-Feb-07		400	8.1		
	23-Apr-07		400	9		
	09-May-07	477	400	100		
	21-May-07	374	400	101		
	18-Jun-07	515	400	102		
	25-Jul-07	578	530	103		
	19-Nov-07	608	400	104		
	29-Apr-08	645	2700	105		
	20-Oct-08	386	4100	106		

Patient	VT_DT	CD4	VL	VISIT	Diagnosis date	VL setpoint
13777	06-Feb-06	600		2	18th Jan 07	
	23-Aug-06		400	8		
	18-Jan-07		2300000	9		
	05-Feb-07	354	610000	100		
	12-Feb-07	386	560000	101		
	02-Apr-07	299	1100000	102		
	03-May-07	306	630000	103		
	02-Aug-07	295	510000	104		
	05-Feb-08	354	280000	105		
	11-Aug-08	329	250000	106		
13833	23-Mar-06	949		2	22nd Mar 07	2800
	19-Oct-06		400	8		
	22-Mar-07		700	9		
	11-Apr-07	754	8100	100		
	18-Apr-07	789	4400	101		
	10-Jul-07	724	1200	103		
	04-Oct-07	938	3600	104		
	20-Mar-08	566	22000	105		
	17-Sep-08	420	5800	106		
13915	14-Aug-06	1556		2	12th Oct 07	
	17-Apr-07		400	8		
	16-Aug-07		400	9		
	12-Oct-07		500000	9.2		
	02-Nov-07	643	620000	100		
	26-Nov-07	831	600000	101		
	10-Dec-07	816	450000	102		
	11-Jan-08	703	400000	103		
	11-Apr-08	620	610000	104		
	17-Oct-08	901	160000	105		
13937	26-May-06		400	1	25th sep 06	400
	29-Jun-06	315	100000	2		
	31-Jul-06		400	4		
	28-Aug-06		400	5		
	25-Sep-06		400	6		
	10-Oct-06	523	400	100		
	25-Oct-06	424	400	101		
	04-Dec-06	540	400	102		
	18-Dec-06	522	400	103		
	26-Mar-07	446	400	104		
	24-Sep-07	533	400	105		
	07-Apr-08	616	400	106		
	22-Sep-08	488	400	107		

Viral load data for Phambili participants

Patient	visit	drawdt	CD4	viral_load	Diagnosis date	VL Setpoint
BC2024J4	201	14-Jun-07	927		12th June 08	
	911	01-Jul-08	786	235		
	5101	30-Jul-08	790	716		
	5301	12-Sep-08	675	1710		
	5401	03-Oct-08	715	1160		
	5501	30-Oct-08	682	68100		
	5601	10-Dec-08	802	5750		
	5701	10-Jun-09	650	2050		
	5801	09-Dec-09	759	1740		
JC203DLC	201	18-May-07	1275		13th Feb 09	151500
	1211	06-Mar-09	600	148000		
	5101	01-Apr-09	498	2600		
	5201	21-Apr-09	513	449000		
	5301	08-May-09	474	150000		
	5401	05-Jun-09	613	153000		
	5501	03-Jul-09	660	520000		
	5601	14-Aug-09	550	64300		
	5701	12-Feb-10		131000		
	5701	19-Feb-10	444			
	5801	13-Aug-10	399	161000		
DC202KKR	201	07-Sep-07	983		5th Sep 08	6070
	911	08-Oct-08	690	17600		
	5101	20-Oct-08	859	16900		
	5201	06-Nov-08	771	9600		
	5301	27-Nov-08	696	3350		
	5401	18-Dec-08	445	6580		
	5501	22-Jan-09	878	11400		
	5601	06-Mar-09	916	5130		
	5701	03-Sep-09	766	10200		
	5801	05-Mar-10	523	3750		
KC2010BQ	201	17-Aug-07	1122		9th Nov 07	
	621	22-Feb-08	885	265		
	5311	19-Mar-08	974	104		
	5321	08-Apr-08	942	400		
	5401	08-May-08	1098	174		
	5701	13-Nov-08	699	11700		
	5801	08-May-09	655	1930		
CC200ZOL	201	11-Sep-07	880		6th Nov 07	4735
	601	04-Dec-07		84300		
	5301	24-Jan-08	550	5940		
	5401	10-Apr-08	584	3530		
	5701	04-Nov-08	570	45400		
	5801	06-May-09	506	471		
JC2031JX	201	29-May-07	1262		25th Nov 08	20600
	1111	10-Dec-08	572	71100		
	5201	29-Jan-09	745	145000		
	5301	26-Feb-09	728	12100		
	5401	17-Mar-09	461	29100		
	5601	27-May-09	775	146000		
	5701	24-Nov-09	625	24500		
	5801	25-May-10	496	6630		
JC200YRJ	201	13-Aug-07	1170		5th Nov 07	
	611	29-Nov-07		248000		
	5101	13-Dec-07	692	207000		
	5201	09-Jan-08	633	694000		
	5301	05-Feb-08	501	288000		
	5401	09-Apr-08	615	164000		
	5701	03-Nov-08	492	72000		
	5801	18-May-09	633	17700		

Patient	visit	drawdt	CD4	viral_load	Diagnosis date	VL Setpoint
JC203X51	201	24-Aug-07	1082	338250	22nd May 09	978
	1211	12-Jun-09	599	2740		
	5101	30-Jun-09	660	1340		
	5201	17-Jul-09	432	1380		
	5301	14-Aug-09	511	323		
	5401	11-Sep-09	452	1810		
	5501	09-Oct-09	393	400		
	5601	20-Nov-09	404	76200		
	5701	21-May-10	499	88500		
	5801	09-Nov-10	403	3520		
FA603W25	201	08-Mar-07	975		11th Oct 07	465
	811	10-Dec-07	667	215		
	5311	12-Feb-08	549	716		
	5401	23-Apr-08	604	376		
	5701	09-Oct-08	607	268		
	5801	26-Mar-09	616	1450		
GA604NJH	201	03-Jul-07	993		1st July 08	
	911	14-Jul-08	675	17700		
	5101	30-Jul-08	544	24800		
	5201	19-Aug-08	691	22700		
	5301	23-Sep-08	540	65300		
	5401	21-Oct-08	611	30800		
	5501	12-Nov-08	432	7490		
	5601	06-Jan-09	469	19900		
	5701	06-Jul-09	563	10600		
	5801	09-Dec-09	513	3890		
KA5060GL	201	07-Sep-07	1194		6th Oct 08	51364
	921	17-Oct-08	697	12600		
	5201	09-Dec-08	851	46500		
	5311	13-Jan-09	810	29900		
	5501	23-Feb-09	794	77700		
	5601	07-Apr-09	642	233000		
	5701	27-Oct-09	640	61100		
	5801	07-Apr-10	607	42400		
CAT02CMJ	201	18-Apr-07	612		10th Oct 08	4595
	1011	30-Oct-08	636	6060		
	5101	20-Nov-08	669	5130		
	5201	18-Dec-08	641	4090		
	5701	17-Aug-09	428	41100		
	5801	02-Jul-10	769	23300		
F7Q04CBP	201	28-Aug-07	1337		25th feb 09	274333
	1111	11-Mar-09		319000		
	5101	25-Mar-09	941	150000		
	5201	20-Apr-09	956	193000		
	5301	19-May-09	983	480000		
	5401	17-Jun-09	788	77900		
	5501	15-Jul-09	659	69000		
	5601	17-Aug-09	696	93800		
	5701	24-Feb-10	871	106000		
	5801	06-Sep-10	861	15700		
D7Q03GZG	201	20-Aug-07	1006		4th Aug 08	16350
	911	20-Aug-08	423	25400		
	5101	01-Sep-08	455	26800		
	5201	22-Sep-08	401	16700		
	5301	22-Oct-08	374	16000		
	5601	20-Apr-09	322	6090		
	5701	03-Aug-09	350	12500		
	5801	01-Feb-10	146	581		
K7Q04G4G	201	30-Jul-07	933		28th Jan 09	1000000
	1111	11-Feb-09	344	1000000		
	5111	05-Mar-09	339	1000000		
	5201	08-Apr-09	361	674000		
	5301	23-Apr-09	303	1000000		
	5401	20-May-09	374	867000		
	5501	17-Jun-09	277	1000000		
	5601	05-Aug-09	353	1000000		
	5701	27-Jan-10	361	15600		
	5801	27-Jul-10	282	353000		
DCG00GH2	201	06-Sep-07	1402		21st Jan 09	132500
	1021	17-Feb-09	547	334000		
	5101	10-Mar-09	593	371000		
	5201	01-Apr-09	593	106000		
	5401	21-May-09	628	159000		
	5601	02-Jul-09	620	237000		
	5701	21-Jan-10	548	41400		
	5801	17-Sep-10	5	9950		

Individual positive beneficial peptides

Patient ID	% Inhibition	1st elispot		Retest with STCL			
		Pool	Magnitude	Pool - peptide no.	Magnitude	PR	Entropy
JC203DIC	**78**	V1	415	V1	435	1.5	0.26
DC202KKR	**93**	P1	1535	P1-1/P1-3	600/1000	1.19/1.09	0.14/0.24
CC200ZOL	**83**	G6	430	G6-3	1000	1.06	0.17
JC203X51	64	G5	75	G5-1	32	1.09	0.31

Patient ID	% Inhibition	1st elispot		Retest with STCL			
		Pool	Magnitude	Pool	Magnitude	PR	Entropy
09896	37	G2	290	G2-1 (7881)	665	1.1	0.11
10338	35	G3	130	G3-3 & G3-4	230	1.085/1.044	0.29/0.37
13833	10	G2	100	G2-1 (7881)	1025	1.083	0.11
10110	95	G3	2350	G3-1	1515	1.053	0.24
11768	53	G3	2160	G3-1 & G3-6	1505/1850	1.053/1.083	0.24/0.36
9754	67	G3	780	G3-5	1400	1.048	0.1
13647	38	G3	380	G3-1	430	1.053	0.24
13937	53	G3	240	G3-3	110	1.085	0.29
11726	86	G2	735	G2-2	520	1.04	0.10
13640	0	G2	5	G2-1 (7881)	140	1.083	0.11
11645	72	G3	950	G3-5	550	1.048	0.10
11860	65	G3	60	G3-6	30	1.083	0.36