CONSTRUCTION AND EVALUATION OF THREE CANDIDATE VACCINES EXPRESSING HIV-1 SUBTYPE C MOSAIC GAG

By

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MSc. (MED) Medical Microbiology

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TO MY FAMILY, WITH LOVE

"It always seems impossible until it is done"

Nelson Mandela 1918-2013

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ABSTRACT

Of the 35 million people living with HIV-1 globally, approximately 71.4% are in the resource-limited sub-Saharan Africa. The immense sequence diversity of HIV-1, even within subtypes, makes it challenging to develop effective vaccines that target a wide range of HIV subtypes. Mosaic immunogens have been computationally designed to specifically overcome this hurdle by maximizing the inclusion of common T cell epitopes. When compared to consensus immunogens, polyvalent mosaic immunogens of HIV-1 group M have shown increased breadth and depth of antigen-specific T-cell responses. More than 90% of HIV positive individuals in sub-Saharan Africa are infected with HIV-1 subtype C (HIV-1C). We therefore designed, constructed, and evaluated candidate vaccines expressing HIV-1C mosaic Gag (Gag^M) in a proof of concept study. Gag was chosen as the most appropriate target for a T cell-based vaccine as there are many studies correlating control of HIV viral load with T cell responses to Gag. The immunogen was designed by Fischer *et al.*, 2007 (1). Three different vaccine platforms were chosen based on their different strengths to be used in prime-boost regimens to determine the immunogenicity of HIV-1C GagM in mice. The first was a pantothenic auxotroph of the tuberculosis vaccine Mycobacterium bovis Bacille Calmette Guérin (BCG). The second was a DNA vaccine vector with enhanced expression of transgenes due to a novel enhancer element from porcine circovirus type 1, which has been demonstrated to increase gene expression. The third vaccine vector selected was the well characterised poxvirus modified vaccinia Ankara (MVA).

BCG, DNA, and MVA vaccines expressing the HIV-1C Gag^M immunogen were successfully constructed (BCG-Gag^M, DNA-Gag^M, and MVA-Gag^M, respectively). The Gag^M immunogen, although computationally generated, was expressed as a correctly sized protein of 55kD, budded from cells infected with MVA-Gag^M, and formed virus-like particles (VLPs). The BCG-Gag^M vaccine retained its integrity in vaccine stocks and 11.5 weeks post vaccination in BALB/c mice.

Immune responses to these candidate vaccines were evaluated in homologous and heterologous prime-boost vaccinations. Heterologous vaccination regimens were more immunogenic than the homologous vaccination regimens with each of the individual vaccines. Although the Gag^M immunogen was the same in all vaccines, the use of

different vectors gave different types of immune responses. The DNA homologous prime boost vaccination elicited predominantly $CD8^+$ T cells while the homologous BCG and MVA vaccination induced predominantly $CD4^+$ T cells. The heterologous prime boost vaccinations where MVA-Gag^M was used as a boost induced a more balanced $CD4^+$ and $CD8^+$ T cell response.

The BCG-Gag^M prime MVA-Gag^M boost regimen (BCG-Gag^M/MVA-Gag^M) elicited T cell responses of high magnitude and quality. Gag-specific IFN-γ ELISPOT responses and frequencies of cytokine-positive $CD8^+$ and $CD4^+$ T cells were at least two-fold higher than those induced by a control BCG prime (without a *gag* insert; BCG^E), MVA-Gag^M boost. The DNA-Gag^M prime, MVA-Gag^M boost vaccination regimen (DNA-Gag^M/MVA-Gag^M) was more immunogenic than the BCG-Gag^M/MVA-Gag^M prime boost regimen. A DNA-Gag^M/MVA-Gag^M prime boost regimen induced a high magnitude of HIV Gag-specific IFN-γ ELISPOT responses that was 7.1-fold higher than those induced by a control DNA prime (without a *gag* insert; DNA^E), MVA-Gag^M boost (2675 ± 292.8 sfu/10^6 and 375 ± 70.74 sfu/10^6splenocytes, respectively). Cytokine-positive $CD8^+$ and $CD4^+$ T cells were 2 and 1.4-fold greater, respectively, for the DNA-Gag^M/MVA-Gag^M prime boost vaccination regimen in comparison to the control regimen (DNA^E/MVA-Gag^M). Both the BCG-Gag^M/MVA-Gag^M and the DNA-Gag^M/MVA-Gag^M prime boost vaccination regimens generated cytokine-positive $CD8^+$ and $CD4^+$ T cells with predominant effector memory phenotype. These vaccination regimens also induced responses with a Th1 bias as determined by the predominant secretion of IFN-γ, TNF-α, and IL-2.

Superior immune responses were elicited by the mosaic (Gag^M) in comparison to those elicited by an HIV-1C natural Gag (Gag^N). This was done by directly comparing the DNA vaccines expressing these antigens. DNA-Gag^N expresses a natural HIV-1C Gag with a sequence that was closest to the consensus sequence of subtype C viruses sampled in South Africa. A DNA-Gag^M homologous vaccination (DNA-Gag^M/DNA-Gag^M) induced cumulative HIV-1 Gag-specific IFN-γ ELISPOT responses that were 6.5-fold higher than those induced by the DNA-Gag^N vaccination (DNA-Gag^N/DNA-Gag^N; 882.3 ± 297.8 sfu/10^6 and 135.7 ± 14.01 sfu/10^6splenocytes, respectively). The frequencies of cytokine-positive $CD8^+$ and $CD4^+$ T cells were 7 and 1.2-fold greater, respectively, for

the DNA-Gag^{M}/DNA-Gag^{M} homologous vaccination regimen in comparison to the DNA-Gag^{N}/DNA-Gag^{N} homologous vaccination regimen.

A low dose of MVA (10^4 pfu) effectively boosted the BCG prime and a low dose prime of the novel DNA vaccine (10μg) expressing the same mosaic immunogen. This could result in dose sparing of MVA and DNA vaccines and is particularly attractive for resource-limited countries. In this study, we have demonstrated novel ways of improving immunogenicity of subtype-specific vaccines even further by using a mosaic antigen and a novel enhancer element in the DNA vaccine. The breadth of the immune response elicited by these vaccines would need to be assessed in non-human primates in a separate study. However, our findings are particularly attractive for a vaccine that will eventually be used in the resource limited countries of sub-Saharan Africa and India where the predominating virus is subtype C. An ideal HIV-1 vaccine should induce both T cell and humoral immune responses. We have made one component that induces T cell responses. Future work will include vaccinating in conjunction with antibody-generating mosaic immunogens or boosting with HIV-1C Env protein.

DECLARATION

The work described in this thesis was done at the Division of Medical Virology, Department of Clinical Laboratory Science and Institute of Infectious Diseases and Molecular Medicine (IDM), University of Cape Town, under the supervision and guidance of Professor Anna-Lise Williamson, Dr Ros Chapman, Dr Nicola Douglas, and Dr Gerald Chege. This is my own work, and where use has been made of others, their contribution has been acknowledged.

Signed by candidate .....

Signature removed

Tsungai Ivai Jongwe

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- God for getting me through this experience. I could not have made it without you Lord!

I have fought the good fight, I have finished the race, I have kept the faith.

2 Timothy 4:7*New International Version (NIV)*

LIST OF ACRONYMS AND ABBREVIATIONS

%	percent
˚C	degrees Celcius
®	registered
ADCC	antobody-dependent cellular cytotoxicity
ADCVI	antibody-dependant cellular-mediated virus inhibition
AIDS	Acquired Immunodeficiency Syndrome
AmpR	ampicilin resistance
APC	antigen presenting cell
APOBEC	apolipoprotein B mRNA editing enzyme, catalytic polypeptide-like 3G
ARP	AIDS reagent program
ARV	anti-retroviral
BALB	Bagg ALBino
BCG	Bacille Calmette Guérin
BFA	brefaldin A
BHK	baby hampster kidney cells
bNAb	broadly neutralising antibody
bp	base pairs
BSD	blasticidin
CA	capsid
CBA	cytometric bead array
CCR5	C-C chemokine receptor 5
CD	cluster of differerentiation
CD	cluster of differerentiation
CEF	chick emryonic fibroblasts
cfu	coloy forming units
cm	centimetres
CmpR	chloramphenicol resistance
CMV	cytomegalovirus
CRF	circulating recombinant form
CXCR4	C-X-C chemokine receptor 4
DC	dendritic cell
DMEM	Dulbecco's Modified Eagle's medium
DMSO	dimethyl sulphoxide
E. coli	*Escherichia coli*
EC	elite controller
EDTA	ethyllendiamine tetra-acetic acid
ELISA	enzyme-linked immunosorbent assay
ELISPOT	enzyme-linked immunospot
Env	envelope
ER	endoplasmic reticulum
ESCRT	endosomal-sorting complex required for transport
FCS	fetal calf serum
Gag	group specific antigen
GFP	green flourescent protein
h	hour
h.p.i.	hour post infection
HEK	human embryonic kidney
HIV	human imunodeficiency virus
HIV-1C	human imunodeficiency virus subtype C
HPLC	high performance liquid chromatography
HTLV-1	human T-cell leukemia virus type 1
ICS	intracellular cytokine staining
IFN-γ	interferon gamma
IL-2	interleukin 2
IN	integrase
IV	immature virus
KanR	kanamycin resitance
kb	kilo base
kDa	kilo dalton
kV	kilo volt
LB	luria bertani
LNTP	long term nn-progressors
LTR	long terminal repeats
M.	Mycobecterium
MA	matrix
mH5	modified H5 promoter
ml	mililitre
mm	millimeter
MOI	multiplicity of infection
mRNA	messenger RNA
MVA	modified vaccinia Ankara
NAb	neutralising antibody
NBT/BCIP	Nitro blue tetrazolium chloride/ 5-bromo-4-chloro-3-indolyl phosphate
NC	nucleocapsid
Nef	negative regulatory factor
NHLS	National Health Laboratory Services

NHP	non-human primates
nm	nano metre
NoNAb	Non-neutralizing antibodies
NYVAC	New York vaccinia
OADC	oleic acid-albumin-dextrose-catalase
OD	optical density
ORF	open reading frame
OriE	E. coli origin of replication
OriM	Mycobactrial origin of replication
pan	pantonthenic acid
PBMC	peripheral blood mononuclear cells
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PCV	porcine circovirus
pfu	plaque forming units
pg	pico gram
Pol	polymerase
PR	protease
r	recombinant
Rev	regulator of virion expression
rpm	rotations per minute
RT	reverse transcriptase
SD	standard deviation
SDS-PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
sfu	spot-forming unit
SHIV	simian-human immunodeficiency virus
SIV	simian immunodeficiency virus
SU	surface protein
SV40	simian virus 40
Tat	trans-activator of transcription
TB	tuberculosis
TC	tissue culture
TCM	T cell central memory
TEM	T cell effector memory
Th1/2	helper cell type 1/type 2
TM	transmembrane
TNF-α	tumor necrosis factor-alpha
™	trademark
UV	ultra violet
V	voltage
v/v	volume per volume
VACV	vaccinia virus
VC	viral crescents
Vif	viral protein regulatory
VIV2	variable regions 1 and 2
VLP	virus-like particle
Vpr	viral protein regulatory
Vpu	viral protein unknown
wt	wild type

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CHAPTER 1: LITERATURE REVIEW

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1.1 THE ORIGIN OF HIV

Human Immunodeficiency Virus (HIV) is a retrovirus that belongs to the *Lentiviridae* family. It was discovered in the early 1980s as the causative agent of Acquired Immunodeficiency Syndrome (AIDS; (2,3)). HIV originates from the zoonotic transmission of Simian Immunodeficiency Viruses (SIV), most likely through hunting, butchering, and handling contaminated uncooked meat (4-6). The earliest evidence of HIV infection in humans was from a plasma sample collected in 1959 from an adult Bantu man living in Congo (6-8). However, HIV is estimated to have been introduced into the human population between 1915 and 1941. This was estimated using in-depth full-length sequence alignment of the *envelope* gene. These were used to estimate the phylogeny and origin of HIV using intensive computational algorithms (9).

1.2 CLASSIFICATION OF HIV

There are two species of HIV, HIV-1 and HIV-2. They differ in terms of geographical distribution, pathogenicity (10), and genomic organization (11). The more pathogenic of the two lineages is HIV-1, which is responsible for the global epidemic. Cases of HIV-2 infection on the other hand, have been reported mostly in West Africa (12,13). HIV-1 consists of four sub-lineages (M-Major; O-Outlier; N-non-M and non-O; and P) which arose from autonomous zoonotic infections (14-19). The HIV global epidemic is a result of group M infections (20,21). This group is further subdivided into nine subtypes (A, B, C, D, F, G, H, J, K, and L), six sub-subtypes (A1-A4; F1-F2), and over 70 inter-subtype circulating recombinant forms (CRF) based on phylogenetic relatedness (20-23). There are also many fully sequenced, but unclassified group M HIVs. These are too diverse to be included in the existing CRF grouping or subtype (24).

1.3 GLOBAL IMPACT OF HIV-1 SUBTYPE C

There are 35 million people living with HIV-1 globally. Approximately 71.4% (25 million) are living in sub-Saharan Africa and the same region makes up 73% of the 1.5 million AIDS-related deaths worldwide (25). In South Africa, HIV-1 prevalence in

women attending government antenatal clinics is 29.5%, and an estimated 6.4 million South Africans are infected with HIV (25,26).

HIV-1 subtype C (HIV-1C) is the most prevalent, accounting for over 50% of all the global infections (27,28). This subtype was initially described in Karonga District, Malawi from sequenced blood samples that had been collected between 1982 and 1984 (29,30). HIV-1C is also commonly associated with heterosexual transmission and has since spread to east, central, and southern Africa (31-34). In southern Africa, HIV-1C accounts for more than 95% of all HIV-1 infections (35-38). HIV-1C infections among heterosexual couples who attend sexually transmitted infections clinics in Wales and England have been on the increase. These findings are notably among young attendees, suggesting recent transmission and spread of this subtype (39). Brazil has recently reported an exponential increase on the spread of HIV-1 subtype B and C isolates. HIV-1C is spreading at a rate that is almost double that of the B subtype and seems to be spreading throughout South America (40,41). India accounts for 6% of the HIV-1 global prevalence (26), the second after South Africa, and subtype C is the dominant subtype (38,42-44).

The prevalence, transmissibility, and pathogenesis of HIV-1C, particularly in heterosexual couples, are not fully understood. However, some research groups have made some interesting findings. Ball and colleagues (2003; (45)) compared the viral fitness of HIV-1 subtype A, B and C using a growth competition assay. They co-infected macrophages, $CD4^+$ T cells, peripheral blood mononuclear cells (PBMCs), and Langerhans cells with a combination of two viruses at a time. HIV-1C was outcompeted in all cell lines except in Langerhans cells. This was an interesting finding as Langerhans cells line the genital tract mucosa and are therefore the first immune cells to encounter HIV-1 following infection (46). In a different study, Rodriguez *et al*., (2009) found HIV-1C isolates to be more fit than HIV-1A isolates in a growth competition assay on infected PBMCs. PBMCs were infected with an HIV-1 A/C recombinant that had the 5'end from HIV-1A, and the 3' end from HIV-1C. Interestingly, the recombinant was equally fit as the HIV-1A isolate. The viruses were also used to infect some *ex vivo* cervical tissue. HIV-1C had greater transmissibility than the subtype A and recombinant A/C viruses. It is therefore likely that HIV-1C 3' terminus region has features that make it highly transmissible and fit compared to other subtypes (47).

1.4 MOTIVATION FOR MAKING HIV-1 VACCINES

The number of deaths associated with HIV-1 infection and HIV-1-related illnesses have been reduced over the years (25). Various strategies have contributed to this, including expansion of educational programs, use of condoms, male circumcision, as well as the use of anti-retroviral (ARV) drugs. ARVs are used as pre-exposure prophylaxis, in microbicides, against mother-to-child transmission, and for HIV-1 treatment in infected individuals. The biggest challenge with some of the interventions is that they require lifetime adherence. Non-compliance of these strategies is therefore a major drawback regarding HIV-1 eradication. ARV usage on the other hand is prone to viral resistance (48-50) and patients often have undesirable side effects (51,52).

Vaccination, however, has demonstrated to be one of the most successful medical measures to reduce mortality and morbidity. It has liberated the human population from some threatening infectious diseases like measles (53), smallpox (54), and polio (55,56) by complete or near eradication. A prophylactic HIV-1 vaccine is the best long-term means of preventing the spread of this epidemic. There is therefore need for increased efforts toward the development for an HIV-1 vaccine to ensure its eradication.

1.5 THE STRUCTURE AND MORPHOGENESIS OF HIV-1

The HI virion is roughly spherical in structure and has a lipid bilayer envelope with a diameter of 80-120nm. Within the virion are two copies of the genome, viral enzymes, and nucleocapsid proteins enclosed by a conical capsid (Figure 1.1A). The HIV genome is a positive sense, single-stranded RNA of 9.8 kilo base (kb). It is made up of 9 genes that code for 15 proteins (Figure 1.1B). The group specific antigen (Gag), polymerase (Pol) and envelope (Env) are structural genes; regulator of virion expression (Rev) and trans-activator of transcription (Tat) are regulatory genes; the rest, viral protein unknown (Vpu), viral protein regulatory (Vpr), viral infectivity factor (Vif), and negative regulatory factor (Nef) are accessory proteins. Two non-coding regions (long terminal repeats; LTR) are located on either terminal of the viral genome. The 5' LTR is involved in the regulation of viral gene expression. It has a transcriptional initiation site as well as several transcriptional factor binding sites (57,58). A single messenger (m) RNA is transcribed, from which 9 open reading frames (ORFs) are translated. An HIV-1 Gag

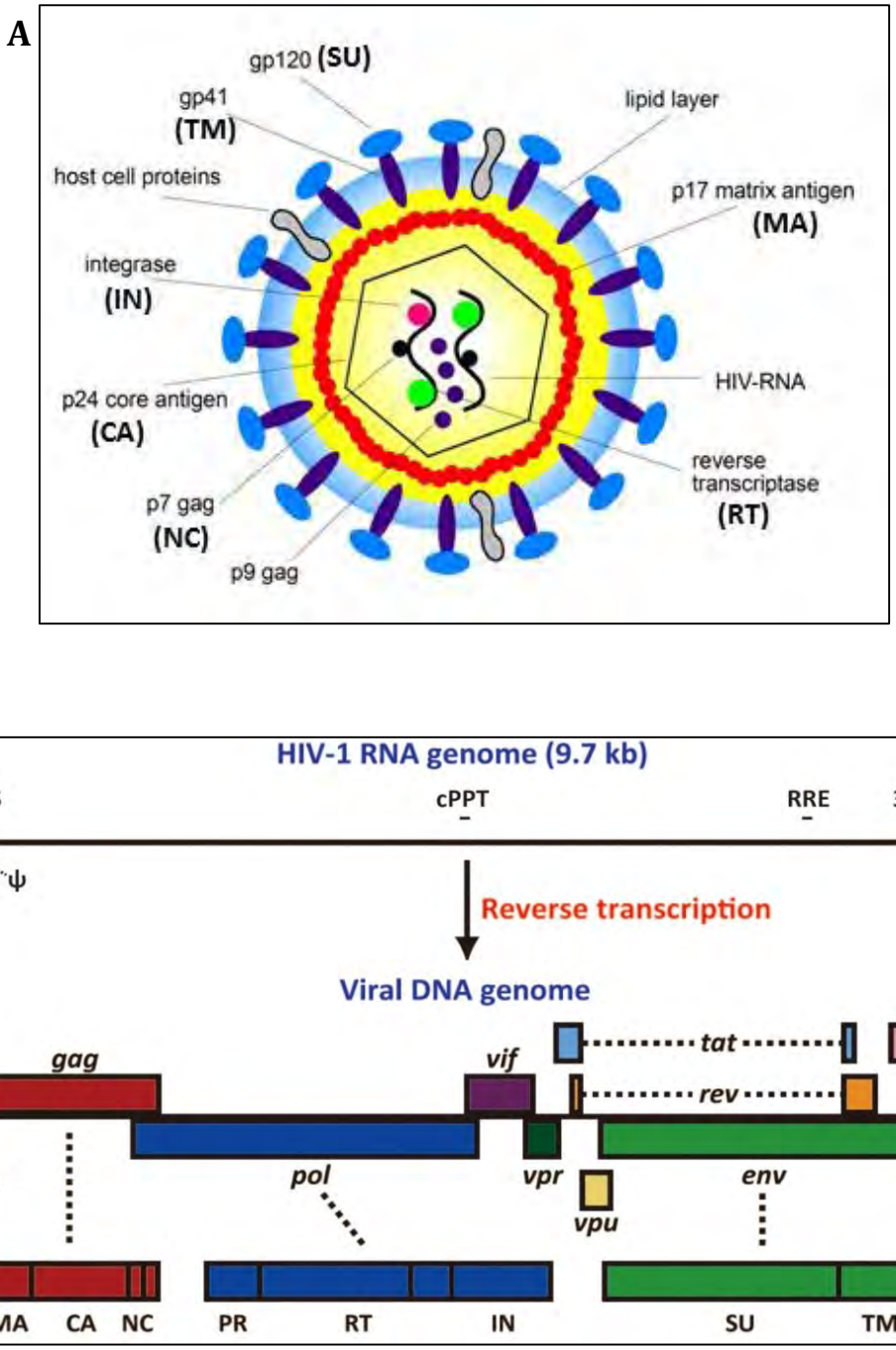


Figure 1.1: Structure and genomic organization of HIV-1. (**A**) **Structure of the HIV-1 virion.** Envelope glycoproteins gp120 and gp41 are protruded from a lipid bilayer originated from the host cell plasma membrane. The viral core consists of two copies of positive single stranded RNA enclosed by the viral capsid protein p24 together with the reverse transcriptase and integrase enzymes required for the formation of new HIV particles (Adapted from Rubbert *et al*., 2011; (59)). (**B**) **The genetic organisation of HIV genome.** The proviral genome is composed of nine genes that are flanked by long terminal repeats (LTRs; 5' and 3'). The nine open reading frames code for at least 15 proteins. The *gag* gene is translated into the structural Gag precursor that is cleaved into matrix (MA), capsid (CA), nucleocapsid (NC). The *pol* gene is translated by a -1 ribosomal frameshift as a Gag-Pol precursor polypeptide that yields the four enzymes Reverse Transcriptase (RT), Integrase (IN), Protease (PR) and Ribonuclease H (RNaseH) upon cleavage. The *env* gene encodes the anchor structural precursor Env, gp160, which is cleaved into the surface (SU; gp120) and trans-membrane (TM; gp41) glycoproteins. The Gag-Pol mRNAs are spliced to encode the regulatory (Rev, Tat) and accessory (Nef, Vpr, Vpu and Vif) proteins. Taken from Yasutsugo *et al*.,(60).

immunogen was used for the vaccines made in this study, HIV-1 Gag is therefore reviewed in Section 1.5.1. The functions of the other proteins are listed in Table 1.1. The morphogenesis of HIV-1 has several distinct stages (Figure 1.2) and is regulated by both viral and host cell proteins.

Table 1.1: Functions of HIV-1 proteins

	Precursor polyprotein	**protein**	**Function and properties**
Structural proteins	**Gag**	p55	Structural protein. p55 is post translationally cleaved into MA, CA, NC, p6, and spacer regions 1 and 2 (sp1 and sp2) Reviewed in Section 1.3.1
	Pol	p10 (PR)	Post-translational processing of viral proteins; Gag-Pol cleavage and maturation
		p66, p51 (RT)	Reverse transcription of viral RNA genome; has DNA polymerase activity
		p15 (Rnase H)	Rnase H activity; cleaves RNA from DNA-RNA hybrids
		p32 (IN)	DNA provirus integration into host genome
	Env	gp120 (SU)	Envelope surface protein; interacts with CD4 and co receptors
		gp41 (TM)	Envelope transmembrane protein
Regulatory proteins	**Tat**	p14	Transcriptional activation; not virion associated
	Rev	p19	Regulation of viral mRNA expression; not virion associated
Accessory proteins	**Vpr**	p15	Helps with virus replication and transactivation
	Vpu	p16	Assists with virus release; disrupts gp160-CD4 complexes; inhibits expression of HLA I molecules; not virion associated
	Nef	p27	Can increase or decrease virus replication; down regulation of surface HLA I and II molecules; inhibits expression of CD4; CCR5 and CXCR4 down regulation to avoid superinfection; prevents apoptosis of the infected cell
	Vif	p23	Increases virus infectivity and cell-to-cell transmission; helps with proviral DNA synthesis and/or virion assembly; down regulation of the antiviral restriction factor APOBEC3G

APOBEC3G - apolipoprotein B mRNA editing enzyme, catalytic polypeptide-like 3G. Adapted from Chakraborty *et al*., 2014 (61)

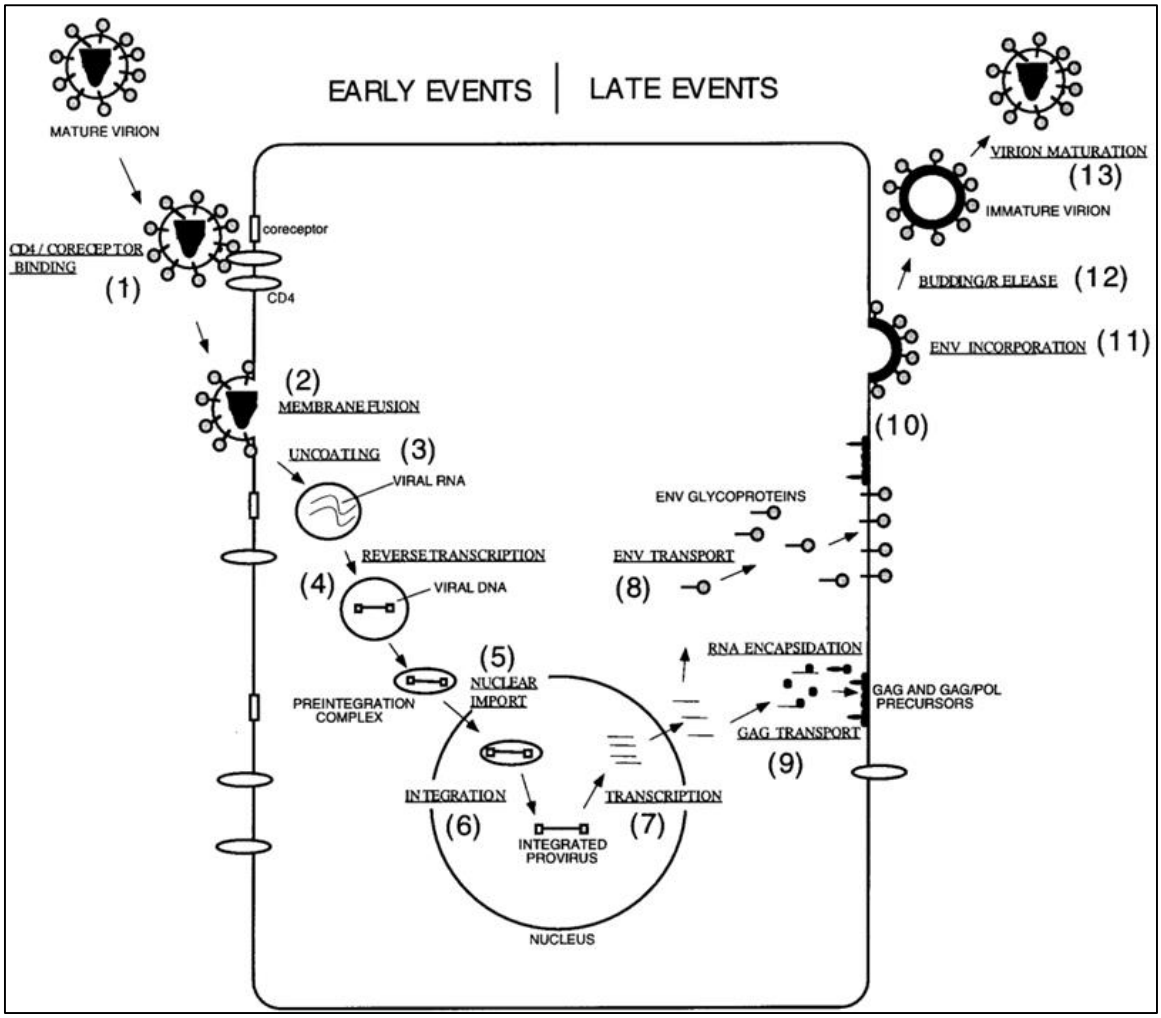


Figure 1.2: The HIV-1 life cycle. (1) The infection process begins when gp120 binds CD4 and interacts with a coreceptor. (2) A membrane fusion reaction, induced by gp41, occurs between the lipid bilayer of the virion and the host cell plasma membrane, releasing the viral core into the cytoplasm. (3) A collective series of steps, referred to as uncoating, takes place. During this process, CA is lost, while at least some MA, as well as NC, the pol-encoded enzymes IN and RT, and Vpr are thought to be retained as part of a high-molecular weight complex. (4) During uncoating, reverse transcription of the viral RNA to generate a double-stranded DNA copy is largely completed. (5) The high-molecular-weight complex, now referred to as the pre-integration complex is transported across the nuclear membrane. (6) In the nucleus, integration of the viral DNA into the host cell chromosome is catalyzed by IN. (7) The integrated viral DNA, known as the provirus, serves as the template for the synthesis of viral RNAs, which are transported to the cytoplasm. (8) The Env glycoproteins are synthesized in the endoplasmic reticulum (ER) and are transported to the plasma membrane via the secretory pathway. (9) The Gag and Gag-Pol polyprotein precursors are synthesized and transported to the plasma membrane. During or after transport, the Gag precursor recruits two copies of the single-stranded viral RNA genome, interacts with the Gag-Pol precursor, and assembles into structures visible by electron microscopy as dense patches lining the inner face of the plasma membrane. (10) The assembled Gag protein complex induces membrane curvature, leading to the formation of a bud. (11) During budding, the viral Env glycoproteins are incorporated into the nascent particles. (12) Budding is completed as the immature virus particle pinches off from the plasma membrane. (13) During or immediately after budding, the viral PR cleaves the Gag and Gag-Pol polyprotein precursors to the mature Gag and Pol proteins. PR cleavage leads to core condensation and the generation of a mature, infectious virion which is now capable of initiating a new round of infection. Major steps in the cycle are underlined. The life cycle is subdivided into early and late stages. Taken from Freed 1998 (62).

1.5.1 Functional properties of HIV-1 Gag

The HIV-1 Gag precursor protein, $Pr55^{Gag}$, is the main viral structural protein. $Pr55^{Gag}$, through its various domains, is responsible for complexing with genomic RNA (63-66) and host proteins (67-69). These are then incorporated into the virion particle (70). $Pr55^{Gag}$ is then responsible for directing itself towards the plasma membrane for oligomerization, and for exploiting the host's cellular endosomal-sorting complex required for transport (ESCRT) to facilitate budding. Several thousand $Pr55^{Gag}$ poly-proteins assemble at the membrane of virus-producing cells in regions that are fatty-acid rich (71-74). Within these regions, the $Pr55^{Gag}$ molecules are organised into a lattice that eventually start to bud as immature virus-like particles (VLPs). HIV-1 Env is also incorporated at this stage at the plasma membrane (Figure 1.2 step 11). In the presence of viral PR, the $Pr55^{Gag}$ polyprotein is sequentially cleaved at five different sites into different proteins and peptides; the matrix (MA; $p17^{MA}$), capsid (CA; $p24^{CA}$), nucleocapsid (NC; $p7^{NC}$), and p6 (75). Two small "spacer" peptides (sp1 and sp2) flank the NC (Figures 1.3A and B). Protease cleavage of viral precursor proteins leads to the formation of mature virions (Figure 1.4). $Pr55^{Gag}$ can also form immature VLPs in the absence of any other HIV-1 proteins (76-78). The immature virions are also slightly larger (120-140nm) and more circular than mature virions (100-110nm) (79,80).

1.5.1.1 Function of the matrix (MA) protein (p17)

The MA protein forms the N-terminal region of $Pr55^{Gag}$ and is made up of 128 amino acids (81). The MA is a part of $Pr55^{Gag}$ and p41 (MA, CA, and p2) before it is finally cleaved into the mature p17 protein (reviewed by Bell and Lever 2013; (82)). It is involved in both the early and late stages of the HIV-1 replication cycle (reviewed by Bukrinskaya 2007; (83)). The MA is involved in the transport of the viral genome to the nucleus. It has a conserved nuclear localisation signal (GKKXYKLKH; Figure 1.3B) similar to that of the simian virus 40 (SV40) large T antigen (84). Various studies have detected the MA protein in the pre-integration complex (85,86). Heterologous proteins coupled to the HIV-1 MA protein have been targeted to the nucleus in non-replicating cells (87).

The MA N-terminal domain has properties that enhance translation of $Pr55^{Gag}$ (88). The signal recognised by $Pr55^{Gag}$ to initiate genome packaging is in the 5' LTR of the HIV-1 RNA (89). This is also the region were the translation initiation complex forms (57,58).

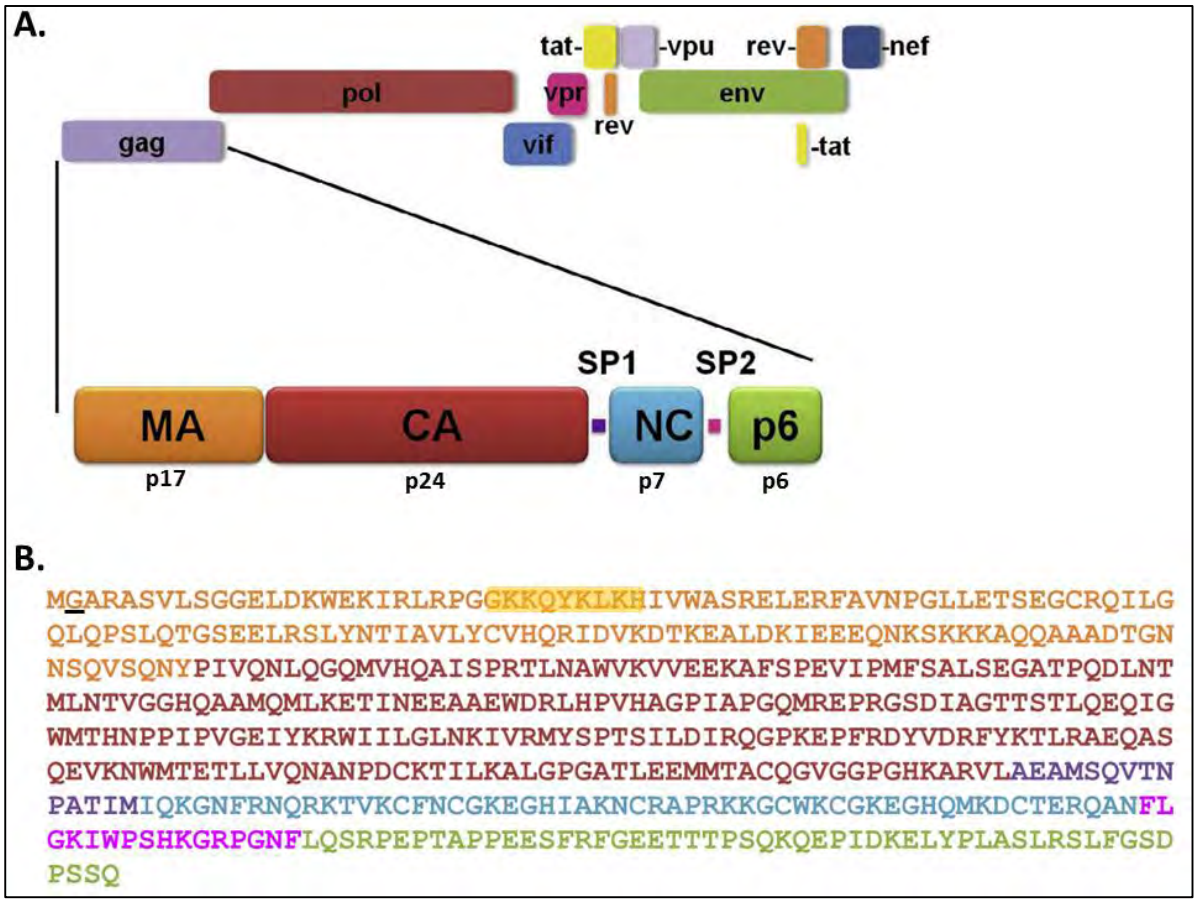


Figure 1.3: HIV-1 $Pr55^{Gag}$. (**A**) Schematic representation of the HIV-1 genome organization, detailing the domain organization of Gag. (**B**) Full length amino acid sequence of HIV-1 $Pr55^{Gag}$ derived from the pNL4.3 HIV molecular clone. The domains are color coded accordingly; matrix – orange, capsid – red, nucleocapsid – blue, and p6 – green, SP1 – purple, SP2 – pink. The second glycine residue (underlined) is post translationally modified by myristylation. The nuclear localisation signal is highlighted in orange. Adapted from McKinstry *et al*., 2014 (90).

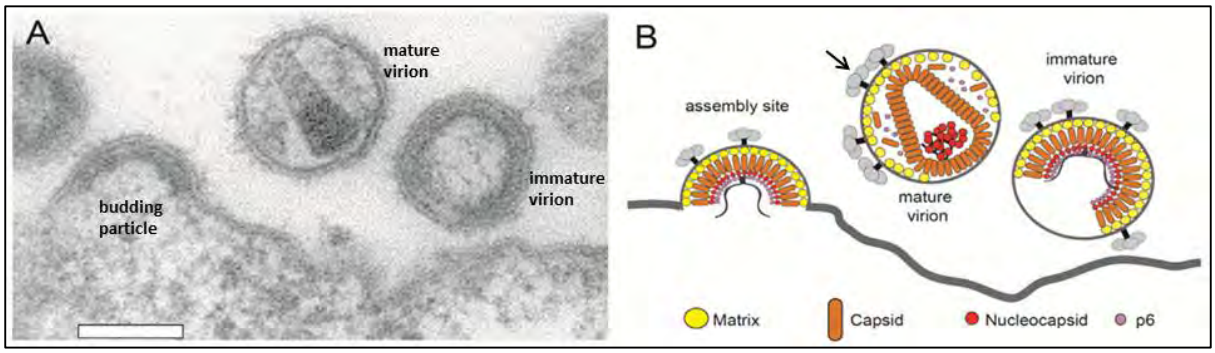


Figure 1.4: Architecture of the HIV-1 budding site and of released particles. (**A**) Electron micrograph of a HIV-1 budding site showing immature and mature virions at the plasma membrane of a virus producing T-cell. Scale bar 100 nm. (**B**) Schematic representation of the structures shown in (**A**). The arrow indicates Env. Adapted from Baumgärtel *et al*., 2014 (91).

Using *in vitro* and *in vivo* models, Anderson and colleagues (2006) reported that translation was initiated at low $Pr55^{Gag}$ levels but inhibited at high levels in order to regulate the translation and packaging processes. They mapped the inhibition process to NC which bound the packaging signal. Using gel mobility shift assays, the MA protein was identified as the domain responsible for the stimulatory activity. A $Pr55^{Gag}$ with the MA deleted was unable to stimulate translation (88).

The MA domain is responsible for targeting Gag to the host plasma membrane (92-94). $Pr55^{Gag}$ is post-translationally modified by the addition of myristic acid at the glycine amino acid residue at position 2 in the N-terminal domain by the enzyme N-myristyltransferase (Figure 1.3B). This modification is crucial for $Pr55^{Gag}$ host cell membrane targeting and virus assembly. The myristic acid is masked within a hydrophobic region in the MA domain and binds to cell membranes by interacting with phosphatidylinositol-4,5-bisphosphate (PIP2; (94-96)). Mutating the glycine residue results in blocking of the virus particle budding and intracellular accumulation of Gag (97,98). Following virus budding, MA molecules remain attached to the virion lipid bilayer to stabilize the virus particle (99). To prevent its non-specific binding to other lipid membranes as it traffics through the host cell, $Pr55^{Gag}$, through the MA domain, binds RNA in the cytoplasm (stage 9 of Figure 1.2; (100)).

1.5.1.2 Function of the capsid (CA) protein (p24)

CA is a component of $Pr55^{Gag}$, p41 (MA, CA, and p2) and p25 (CA and p2) before the mature p24 is generated by further proteolytic cleavage (reviewed by Bell and Lever 2013; (82)). The commonly used p24 ELISA diagnosis assay quantitatively detects the presence of virion-free and/or virion-bound CA by anti-p24 antibodies bound to a microtitration well (101,102).

The CA domain in $Pr55^{Gag}$ is divided into the N- and C-terminal domains (NTD and CTD respectively) connected by a flexible hinge which is essential for correct assembly of the core, viral replication and viral infectivity (103). The CTD is responsible for the formation of CA-CA dimers (104-106) and for viral core formation. In the mature virion, the CA forms a conical shell around the core-associated proteins and the viral genome (Figures 1.1A and 1.4). The NTD on the other hand forms two different morphological polymers which are necessary for forming this conical shape (107,108). The immature

virion has about 5000 copies of $Pr55^{Gag}$. However, about 1000 - 1500 CA proteins form the core in the mature virion (107,109-112).

1.5.1.3 Function of the nucleocapsid (NC) protein (p7)

NC is a low molecular weight protein with mostly basic amino acid residues, and is conserved in retroviruses that are replication-competent (113,114). During the virion maturation process, NC is a component of the proteins $Pr55^{Gag}$, p15 (NC, p1, and p6) and p9 (NC and p1). The p1 is eventually cleaved from p9 forming a mature NC (reviewed by Bell and Lever 2013; (82)).

Although it is one of the smallest components of Gag, the NC has been extensively researched and actually has many different functions in HIV-1 morphogenesis. NC protects and packages viral RNA within this nucleoprotein core (109,111,115-118). Darlix and colleagues (119) have demonstrated that NC assists with reverse transcription of the viral genome, by destabilizing the nucleic acid double stranded helix and allowing cDNA elongation (120-122). NC has a structural role in forming immature viral particles through Gag-Gag interactions (111,123) and it is also implicated in virus particle budding via the ESCRT-associated protein, ALIX, which binds NC and p6 to facilitate the budding process (115).

1.5.1.4 Function of the p6 protein

The p6 protein is the most variable region of Gag (124). Recently, p6 was shown to bind cyclophilin A and may be implicated in further protection of the HIV-1 virion against TRIM5α (125). HIV-1 Gag takes advantage of the ESCRT for budding out of the host cell (70,126,127) and the p6 C-terminus domain is vital for recruiting this host machinery, particularly the proteins ALIX and TSG101 (64,128,129). p6 is also responsible for recruiting the viral accessory protein, Vpr, into the HIV-1 virion (128). Mutations in the p6 domain of $Pr55^{Gag}$ block incorporation of the Env glycoprotein into the HI virus particle (130) , suggesting that p6 is responsible for this process.

Deletions or mutations in p6 result in a rather dramatic phenotype where virus particles fail to complete budding. Incompletely processed particles can be seen on the host membrane surface bound to the budding stalk (64,131-133), indicating that portions of the p6 domain are involved in releasing the budding virus from the plasma

membrane. When the p6 mutation was reversed, virus particles were released from the host cell (66,134). However, some groups have deleted the p6 domain to stabilise the expression and purification of HIV-1 Gag (64).

The size of some retroviral particles is determined by the CA protein (135). While some authors argue that this is the case for HIV-1 (136), there is compelling evidence suggesting otherwise. Garnier and colleagues 1998 (137) analysed over 30 HIV-1 Gag deletion mutants. The mutations spanned different regions of Gag and the constructs encoded inactivated PR. Using rate zonal gradient assays, constructs with mutations in the MA, CA, and NC regions did not alter virion particle size. However, mutations within p6 resulted in larger virus particles. Rous sarcoma virus (RSV) uses the CA domain to regulate its particle size. RSV CA mutants produce particles of different sizes. Insertion of the HIV-1 p6 domain downstream of an RSV CA mutant resulted in the production of normal-sized particles.

1.5.1.5 Function of the spacer regions, sp1 and sp2

The NC lies between two spacer proteins, sp1 and sp2 in $Pr55^{Gag}$. The p2 protein influences CA assembly as determined by the appearance of clusters of ribonucleoprotein at the host cell plasma membrane following deletion of p2. The p2 domain is part of the p41 (MA, CA, and p2) and p25 (CA and p2) precursors. It has been shown that p2 helices also stabilize the HIV-1 Gag hexamer complexes within the immature particle (138). This implies that p2 cleavage from NC is necessary for CA core condensation and formation of the ribonucleoprotein with genomic RNA. The cleavage of p2 from CA, which occurs later in HIV-1 viral morphogenesis, probably modulates CA-CA interactions as shown by Gross and colleagues (139). Their electron microscopic studies indicated that the CA-p2 integrity was essential for forming the immature CA sphere and cleavage of p25 facilitates viral core maturation and condensation. The p1 peptide of Gag on the other hand is associated with the incorporation of Gag and Pol into the virus during assembly (140).

1.6 IMMUNOLOGICAL RESPONSES TO HIV-1 INFECTION

HIV-1 infects cells vital to the immune system, particularly T helper (h) cells,

macrophages, dendritic cells (DCs), as well as, to a certain extent, microglial cells. These cells all express the primary HIV-1 receptor CD4 (reviewed by Grossman *et al*., 2006 (141), and Peterlin and Trono 2003 (142)). HIV-1 further requires the co-receptors CC-chemokine receptor 5 (CCR5) and CX-chemokine receptor 4 (CXCR4) for host cell infection (143-145). HIV-1 infection most commonly occurs through sexual intercourse, sharing of contaminated needles by intravenous drug users, and from a mother to their baby during birth or breastfeeding (reviewed by Girad *et al*., 2011; (146)). The genital mucosae and gastrointestinal tracts are the main sites of HIV-1 infection (147). Once the target cells are infected, the virus replicates and spreads to lymphoid organs and the bloodstream. Eventually the virus reaches the gut-associated lymphoid tissues where there is the largest amount of $CD4^+$ T cells which eventually causes illness and the onset of AIDS (reviewed by Murphy 2012; (148)). The immune responses to HIV-1 infection are detailed in Figure 1.5.

Neutralising antibodies (NAb) to HIV-1 appear within 3 weeks in almost all infected individuals (Figure 1.5). However, these antibodies are often strain-specific [150] because they target variable regions of HIV-1 Env. Furthermore HIV-1 Env differs markedly between viruses (Section 1.8.1; [151,152]). The immune pressure causes the virus to escape by compensatory mutations, to which new antibodies develop [153,154]. The HIV-1 continues to mutate forming more variants within an infected individual and this causes the production of non-protective cross-reactive NAbs [155]. Broadly NAbs (bNAb), that recognise structurally conserved Env epitopes capable of blocking multiple variants of HIV-1 (80-90%), can protect target cells from being infected. However, these antibodies only develop about 3 years after HIV-1 infection, by which time the virus would have established latent reservoirs and it will be too late to control the infection and restore the immune system ([156]; reviewed by Overbaugh and Morris 2012; [157]). Another hurdle is that only a few infected individuals (15-30%) develop these bNAbs [158-160]. Non-neutralizing antibodies (NoNAbs) to HIV-1 develop after natural infection, but only in about 1% of infected individuals [161]. These cells potently neutralises serum-associated virus using mechanisms mediated by the fragment crystallisable (Fc) region of the antibody. The Fc region recruits antigen-presenting cells (APCs) that promote antibody-dependent cellular cytotoxicity (ADCC) and (ADCVI) involving the secretion of antiviral cytokines and proteases (reviewed by

Lema et al., 2014; [162]).

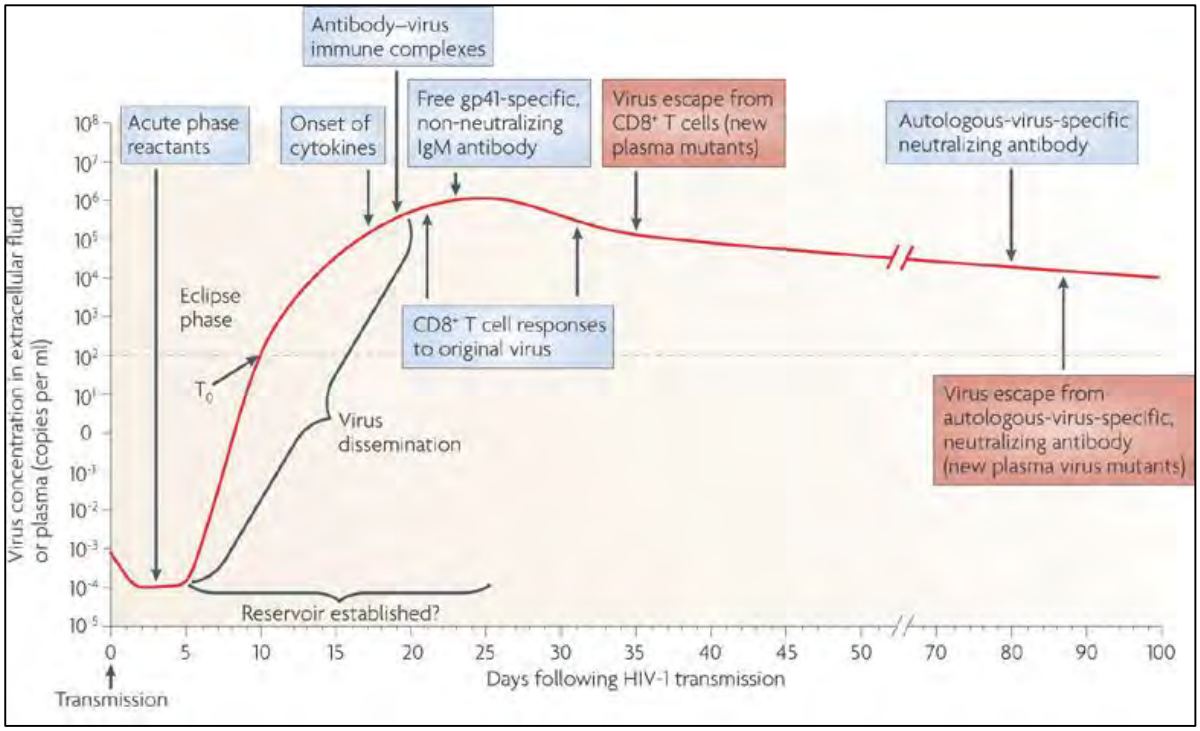


Figure 1.5: The earliest innate and adaptive immune responses detected after HIV-1 transmission. The first systemically detectable immune responses to HIV-1 infection are the increases in levels of acute-phase proteins in the plasma, which are observed when virus replication is still largely restricted to the mucosal tissues and draining lymph nodes (eclipse phase). When virus is first detected in the plasma (T_0), broad and dynamic increases in plasma cytokine levels are also observed. Within days, as plasma viraemia is still increasing exponentially, the first antibody–virus immune complexes are detected. Expansion of the earliest HIV-1-specific $CD8^+$ T cell responses also commences prior to peak viraemia, followed by detection of the first free glycoprotein (gp)41-specific but non-neutralizing IgM antibodies. Complete virus escape from the first $CD8^+$ T cell responses can occur rapidly, within 10 days of T cell expansion. By this time, viral reservoirs exist, possibly becoming established within days of infection. The earliest autologous-virus-neutralizing antibodies are detected around day 80 following infection, as viral loads are still declining prior to the onset of the viral set point. Antibody escape virus mutants emerge in the plasma within the following week. Taken from McMichael *et al*., 2010 (149).

T cell responses on the other hand develop much quicker compared to antibody responses in natural infection (Figure 1.5). HIV-1-specific $CD8^+$ T cells become detectable 2-3 weeks post exposure (reviewed by Walker *et al*., 1990; (150,151)). These cells can inhibit HIV-1 replication *in vitro* (152). The $CD8^+$ T cell frequency continues to increase until they reach a peak point when HIV-1 virus levels decline (149). About 30 days post-infection and at the time of peak $CD8^+$ T cell responses, HIV-1 begins to mutate in an effort to escape the $CD8^+$ T cell pressure (153-155). Meanwhile, the $CD8^+$ T cells corresponding to the initial viral epitopes begin to rapidly decrease and new $CD8^+$ T cells that recognise the mutated HIV-1 sequences may arise. This causes new selection pressure on the virus, which in turn fuels new compensatory mutations (156,157). The number of HIV-1 epitopes targeted broadens and HIV-1-specific $CD8^+$ T cells persist at

high frequencies during chronic infection (158,159). Throughout the asymptomatic phase of infection, this race between the $CD8^+$ T cell responses and HIV-1 compensatory mutations takes place. However, eventually, the virus may restore its infectious ability with the acquired mutations (160-164). The $CD8^+$ T cells eventually become progressively exhausted and have limited efficacy in controlling HIV-1 replication (159).

HIV-1-specific $CD8^+$ T cells function by producing cytokines (interferon-gamma (IFN-γ) and tumour necrosis factor alpha (TNF-α)) that induce antiviral activity in infected cells (165); secreting chemokines (CCL3 and CCL4) that block co-receptor molecules, thus preventing the entry of HIV-1 into target cells (166-168); and other unidentified viral factors which directly suppress viral replication (reviewed by McMichael *et al*., 2001 (169)). $CD8^+$ T cells also secrete cytotoxic granules like granzyme B and perforin, which directly lyse viral-infected cells or trigger a programmed cell death process (170-172).

$CD8^+$ T cells require help from $CD4^+$ T cells to function maximally (173,174). $CD4^+$ T cells are vastly depleted from the lymphoid system during acute HIV-1 infection. As demonstrated in mouse models that have depleted $CD4^+$ T cells, their $CD8^+$ T cell population becomes greatly impaired (175-177), and rapidly reaches a state of exhaustion (178). Although $CD4^+$ T cells are greatly depleted in HIV-1 infection they are not altogether absent, however, abnormalities in $CD8^+$ T cell response development does occur (179).

Not all HIV-infected individuals progress to AIDS in the absence of ARV treatment. About 1–5% of HIV-1-infected individuals do not experience a decline in $CD4^+$ T cell counts. They can control virus replication to levels below 1000 HIV RNA copies/ml for at least 7–10 years without ART (reviewed by Walker and Korber 2001 (180)) and are referred to as long term non-progressors (LTNPs; (155)). Another group of HIV-1 infected individuals, generally <1%, can actually control the levels of HIV-1 replication to below undetectable levels (<50 HIV-1 RNA copies/ml). They are referred to as Elite Controllers (ECs; reviewed by Deeks *et al*., 2007 (181)). Ferrando-Martinez and colleagues carried out an intriguing study on 40 untreated HIV-1 infected individuals in Spain. LTNPs and ECs always had HIV-1-specific CD4 and CD8 T cell responses, while a third of HIV-1 progressors had undetectable T cell responses to the virus (182). Thus,

studies of both LTNPs and ECs have led to key insights into T cell correlates of viral control that should be considered in HIV-1 vaccine development.

An association of Gag targeting by $CD8^+$ T cells and lower viral loads has been observed in HIV-1-infected adults (183-185), HIV-1-infected children (186-188), HIV-2-infected individuals (189), and ECs ((182); reviewed by Genovese *et al*., 2013; (190)). Early in 2014, Riou and colleagues identified highly functional Gag- and Nef-specific $CD8^+$ T cell responses as the best determinants of a low viral set point from a South African cohort of HIV-1-infected individuals (191). In a different study, the immune responses elicited by HIV-1-infected individuals during the chronic phase of infection indicated that Gag targeting was associated with lower viral load (183,184,192). In contrast, responses targeted towards Env or the accessory proteins were associated with higher viral loads (184,193). Gag-specific $CD8^+$ T cells also play an important role in slowing down HIV-1 disease progression (Figure 1.5). These responses are also cross reactive for diverse strains of HIV-1 (194,195).

1.7 THE VACCINE EVALUATION PROCESS

The evaluation of HIV vaccines is a lengthy but necessary process. Preclinical evaluation in animal models is an essential step for this. When a new vaccine concept is developed, studies are done in animal models to determine its safety and immunogenicity. The animals used include, but are not limited to mice, rabbits, guinea pigs, and non-human primates. This evaluation aims to predict the outcome in humans should the candidate vaccine reach clinical trials. Evaluating HIV-1 vaccine efficacy, however, can only be done in people since only humans get infected by HIV-1 that can develop into AIDS, and the immune systems of animals does differ from that of humans (196). From vaccine conceptualisation to Phase I clinical trials it can take at least 9 years. Only after the Phase 1 and 2 trials can Phase 3 or efficacy trials take place. After the completion of efficacy trials, it will take a further 1- 2 years for a vaccine to be licenced for distribution and use (Figure 1.6). This is mostly due to ethical regulations that need to be put into place and reviewed at each stage to ensure the safety and protection of volunteers (196,197).

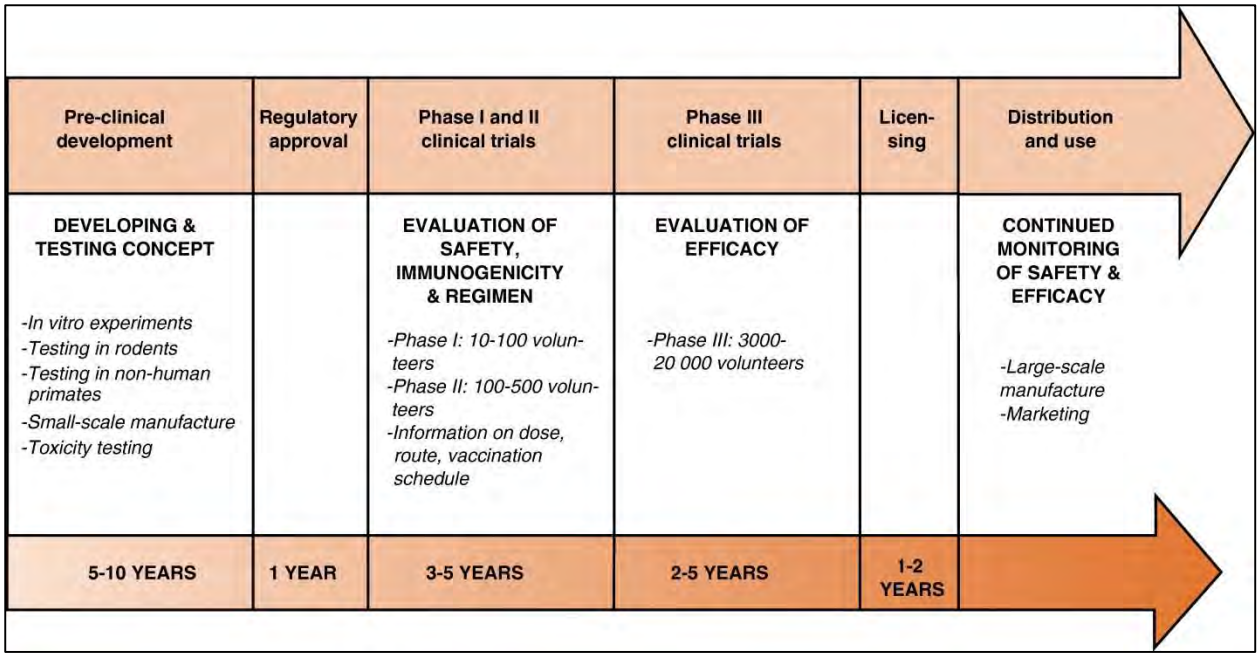


Figure 1.6: The vaccine development pipeline. Taken from Burgers *et al*., 2005 (198).

1.8 CHALLENGES ASSOCIATED WITH HIV-1 VACCINE DEVELOPMENT

From the time HIV-1 was discovered (199), it seemed that its eradication was going to be straightforward. This impression was mostly due to the vaccine development track records of other infectious diseases (200,201) as well as the development of antibody-based vaccines against HIV-1 at that time (Section 1.9; Table 1.2; (202,203)). However, the vaccine development proved to be a mammoth task despite technological advancement and efforts being collaborated globally. Results from the RV144 Thai trial (Section 1.9; Table 1.2; (204)) have given the scientific community the hope that an effective HIV-1 vaccine is achievable. However, there are still some challenges in making that hope a reality.

1.8.1 HIV-1 high mutation rate and diversity

There are several factors associated with the challenges of developing an effective HIV vaccine. HIV-1 has a high mutation rate due to non-specific recombination events and a reverse transcriptase enzyme that incorporates errors during viral replication (205). HIV-1 mutates faster than any other known RNA virus, including influenza. Any developed vaccine will therefore need to target multiple conserved epitopes within the virus to prevent mutational escape.

Viral sequences of Env within a clade vary by 15%, and by more than 35% between clades (206,207). The diversity of HIV-1 in an individual can equate to that observed in a country in a year for an influenza outbreak (207). The most common means of vaccine protection is the development of binding or broadly neutralising antibodies against the infectious agent. HIV-1 diversity makes it difficult to obtain a single prototype virus to represent the genetic and geographical range of viral isolates (208-212) or to obtain a vaccine that will elicit such antibody responses towards all clades and CRFs. HIV-1 is also heavily glycosylated, making it difficult for neutralising antibodies to access the virus to prevent infection (213).

1.8.2 The neutralisation antibody challenge

Most viral infections are controlled by vaccines that induce NAbs (reviewed by Delany *et al*., 2014 and Rappuoli and Aderem 2011; (214,215)). The first HIV-1 vaccines were designed to induce NAbs; this was however unsuccessful due to the immense diversity of circulating viral strains in comparison to laboratoty-adapted strains (Section 1.9.1; (216)). Production of bNAbs by vaccine candidates in clinical trials has been equally disappointing (217-219). Therapeutic means of passively administering non-human primates (NHPs) with NAbs (220-222) and bNAbs (223) block infection from SHIV challenge, indicating that sterilizing immunity is achievable. Moog and colleagues (2014; (222)) showed that passive administration of NoNAbs reduced viral loads in challenged NHPs. However, passive administration of anti-HIV-1 antibodies does not offer lasting immunity. Nonetheless, this approach would be too expensive to apply on a large scale, particularly in resource-limited areas where the HIV-1 pandemic is most severe. Some non-human primate studies (224) and early phase clinical trials (225,226) have, however, shown induction of durable bNAbs. Efficacy trials will indicate the ability of the generated bNAbs to neutralise the diverse circulating HIV-1 strains. Research is also underway to develop vaccines that induce neutralising antibodies based on the mutational changes of the virus (227), and the clinical significance of such vaccines is yet to be determined. The RV144 clinical trial (Table 1.2; Section 1.9.3) demonstrated that NoNAbs-mediated means of protection are also important for preventing HIV-1 infection (218).

1.8.3 Latent reservoir establishment

HIV-1 is a retrovirus, it therefore integrates within the human genome upon infection of host cells (Figure 1.2 stage 6; reviewed by Haase *et al*., 2010; (228)). HIV-1 can therefore rapidly establish latent reservoirs within days following infection (229,230). The reservoirs are difficult to eliminate. With the exception of the "Berlin" patient who got cured of HIV-1 through bone-marrow transplant (231), there has not been any reported case of elimination of HIV-1 infection, even in patients taking ARVs. This makes HIV-1 infection life-long. For an HIV-1 vaccine to achieve sterilising immunity and overcome the viral reservoir hurdle, it will need to generate highly effective immune responses soon after infection. These could be in the form of nAbs and bNAbs to neutralise the virus, NoNAbs to induce antibody dependant cell-mediated immune responses, and/or effector memory T cells to efficiently kill any infected cells.

1.8.4 Animal models to test HIV vaccine candidates

Although they have less complex immune systems than those of humans, mice make suitable models to test HIV-1 vaccines as a first test of vaccine stability and immunogenicity. They are small, have a short gestation period, require small amounts of space, and are relatively affordable. A number of mice strains are available for use in HIV-1 vaccine immunogenicity studies. The HLA binding peptides can also be easily ordered for use in *in vitro* assays that evaluate T cell responses following vaccination. Our laboratory has used laboratory-bred Bagg albino (BALB)/c mice named after their founder and phenotype (232). The "c" refers to their genotype of coat colour. We therefore chose this animal model to compare the results to those previously obtained by our group and others.

HIV-1 vaccine efficacy can only be evaluated in humans, however, preclinical studies to evaluate vaccine safety and immunogenicity must first be carried out (Section 1.7). Indian or Chinese rhesus macaque monkeys are the preferred model of choice (233,234). HIV-1, however, does not cause immune-deficiency syndrome in macaques. An ancestor of HIV-1, SIV, or hybrids of SIV and HIV-1 (SHIVs) are used instead for challenge experiments and these cause clinical symptoms similar to those of HIV-1 infected individuals (reviewed by Silvestri *et al*; 2007 and Morgan *et al*., 2008 (235,236)). The major drawback is that the SIV and SHIV isolates are limited to represent the immense

diversity of the HIV-1 strains (reviewed by Girard *et al*., 2011; (146)). Chimpanzees are the only non-human primates that can become infected with HIV (237,238). However, they seem not to develop AIDS following HIV infection; thus a more pathogenic strain would need to be used for challenge following vaccination (239). Unfortunately, chimpanzees are facing the risk of extinction and they are very closely related to humans, making it ethically questionable to do challenge experiments on them (240). NHPs are still the preferred animal model for preclinical evaluation of candidate HIV-1 vaccines. However, it is imperative that the results from these studies are carefully designed and interpreted before advancing the candidate vaccines to clinical trials (241).

Bosma and colleagues described an immuno-deficient mouse that did not reject transplanted human tissue (242). These humanised mice have lymphoid systems derived from human beings, they get infected with HIV-1, the virus persists and causes disseminated infection, dissemination of disease occurs, and the mice have low $CD4^+$ T cell counts following HIV-1 infection (243-245). Various models have since been generated as reviewed by Nischang and colleagues (246). This model is still not the best as various components of the human immune system are lacking (247). Some groups have "knocked-in" genes to circumvent these problems and the mice have been manipulated in a way that suits the research being carried out (248-250).

1.9 SUMMARY OF HIV-1 VACCINE EFFICACY TRIALS

There have been over 256 HIV-1 vaccine trials conducted to date involving nearly 50 000 volunteers. The majority of the trials are Phase I and II, and these include ongoing studies (23,251). It is essential to do many clinical trials as correlates of protection can be identified from clinical trials that display partial effectiveness (252). Despite such a huge number of clinical trials, only six have reached Phase IIb or III clinical efficacy stages, one of which showed modest protection (Table 1.2).

1.9.1 VAX003 and VAX004 trials

The first two vaccines evaluated in efficacy trials aimed to induce neutralizing antibodies against HIV-1 to prevent infection (Table 1.2; (202,203)). Although the

Table 1.2: HIV-1 vaccine efficacy trials conducted to date

Trial	Period	Phase	Vaccine type and doses administered	Desired immune response	Result	Reference
VAX004	1998-2003	III	7 X gp120 (subtype B) in alum	Humoral	No efficacy	(202)
VAX003	1999-2003	III	7 X gp120 (subtype B and E) in alum	Humoral	No efficacy	(203)
STEP	*2004-2007	IIb	3 X rAd5 *gag/pol/nef* (subtype B)	Cellular	Increased HIV-1 infection risk in uncircumcised men with pre-existing rAd5 immunity	(253,254)
Phambili	§2007	IIb	3 X rAd5 *gag/pol/nef* (subtype B)	Cellular	No efficacy	(255,256)
RV144	2003-2009	III	4 X canarypox *gag/pro* (subtype B) *env* (subtype B and CRF01_AE) + 2 X gp120 (subtype B and E) in alum	Cellular + Humoral	31.2% efficacy at 42 months. ADCC means of protection. No effect on plasma viral load and CD4 count	(204,218)
HVTN 505	‡2009-2013	IIb	3 X DNA plasmids *gag/pol/nef* (subtype B) and *env* (subtypes A, B and C) + 1 X rAd5 *gag-pol* (subtype B) env (subtype A, B and C)	Cellular + Humoral	No efficacy	(219)

*****Trial terminated for possible increase in susceptibility to HIV-1 infection in vaccine recipients
§Terminated due to the STEP trial results. Long term follow up indicated increased risk to HIV-1 infection in vaccine recipients
‡Prematurely terminated due to initial results that showed the vaccine to be ineffective in preventing HIV infections and lowering viral load in participants that did become infected
Adapted from Lema *et al*., 2014 and Kim *et al.* , 2014 (257,258)

vaccines neutralised HIV-1 isolates from the laboratory, this was not the case with circulating strains (216). No efficacy was detected in these clinical trials.

1.9.2 The STEP, Phambili, and HVTN 505 trials

The second "wave" of HIV-1 vaccines to be tested for efficacy induced cellular mediated immune responses. This was evaluated in the STEP and Phambili trials (Table 1.2). The hope was to induce T cell responses that would recognise and kill HIV-1 infected cells, providing partial or complete protection. T cell-based vaccines may not necessarily provide sterility against HIV-1 infection, but instead control the clearance of the virus. In the STEP trial, there was evidence of increased responses induced by the vaccine in some participants, particularly by the induction of HIV-1-specific $CD8^+$ T cells. A total of 77% (258/354) of the male vaccine recipients had HIV-1-specific T cells secreting IFN-γ and 62% (218/354) recognised two or three HIV-1 proteins (Gag, Pol, and Nef) as detected by ELISPOT assays (259). Using multiparameter flow cytometric studies, similar proportions of participants had virus-specific cytokine- secreting cells. The $CD4^+$ T cell responses were Gag-specific and were characterised by secretion of interleukin 2 (IL-2) alone, or in combination with IFN-γ or TNF-α. The $CD8^+$ T cell responses were Pol-specific. They were more predominant than the $CD4^+$ T cell responses and were characterised by predominant secretion of IFN-γ alone, or in combination with IL-2 and TNF-α. The STEP trial was terminated prematurely. Male participants who were not circumcised and had pre-existing immunity to adenovirus serotype 5 (Ad5) had a higher rate of HIV-1 infections than did placebo recipients. It is noteworthy that the male volunteers who had no pre-existing immunity to Ad5 had significantly greater $CD8^+$ T cell numbers than volunteers who did have anti-vector immunity (259). This study therefore showed that HIV-1 vaccines that elicit cellular immune responses induce virus-specific responses; however, there is need to use vectors to which vaccine recipients will not have pre-existing immunity. The results also showed a limited breadth (number of epitopes recognised) and future vaccines will need to account for this, especially through the use of improved immunogens.

The Phambili study was a sister trial to the STEP study conducted in South Africa. The trial was prematurely terminated following the futility of the STEP study. At the time of

termination, there were no signs of increased risk of HIV-1 infection in the vaccine recipients (255). However, long term follow ups done 42 months post vaccination suggested otherwise with 16% and 9% of the vaccine and placebo recipients getting HIV-1 infections respectively (256). These findings caused major concerns regarding the use of rAd5 as an HIV-1 vaccine vector.

The HVTN 505 trial recruited male or transgender women participants that were Ad5-seronegative and circumcised (219). The vaccination regimen included priming with DNA vaccines and boosting with rAd5-based vaccines. An Env immunogen was also included in both the priming and boosting vaccines to elicit humoral immune responses against HIV-1. 28 weeks post the first vaccination, 27 and 21 participants were infected with HIV-1 from the vaccine and placebo arms respectively. In April 2013, the trial was terminated due to lack of efficacy.

Pre-existing immunity to the Ad5 vaccine vector may have increased the numbers of activated $CD4^+$ T cells in the STEP, Phambili, and HVTN505 trials (259), the very target for HIV-1 infection. A non-human primate study was conducted to investigate this (260). Rhesus macaques were chronically infected with a mutant Ad5 prior to vaccination with a rAd5 expressing Gag, Pol and Nef from a SIVmac239 strain. There was an increased magnitude of activated $CD4^+$ T cells and particularly in the gastrointestinal mucosa. The animals that received the vaccine also had increased SIV acquisition post challenge. The animals in the control group that were not infected with the mutant Ad5 did not have increased SIV acquisition post challenge (260). Biopsies obtained from the guts of the HVTN 505 trial participants had increased numbers of activated $CD4^+$ T cells. What was unexpected was that high levels of the HIV-1 co-receptor, CCR5, were detected on the surface of these cells (261).

1.9.3 The RV144 study

The RV144 trial was conducted in Thailand. The HIV-1 gp120 protein, used in VAX003 trial, was used to boost recombinant canarypox prime vaccinations (Table 1.2). At 42 months, the results indicated a 31.2% level of protection from HIV-1 acquisition; ironically,

analysis of the Step trial indicated a 33% higher HIV-1 infection rate for vaccinees compared to the individuals who received the placebo (253,254).

The study suggested that vaccine-induced IgG1 and IgG3 antibody subclasses to the variable regions 1 and 2 (V1V2) of gp120 may be involved in protection against HIV-1 infection. Vaccine-induced IgA antibodies directed towards HIV-1 Env, on the other hand, correlated with the HIV-1 infection rate (218). The rate of HIV-1 infections in the RV144 placebo group was ten-fold lower than that of all the other efficacy trials tested to date (reviewed by Kim *et al*., 2014; (258)). This highlights the relevance of including pox-vectored vaccines in clinical trials.

Plans are underway to increase the durability and effectiveness of the RV144 vaccine (262). The same vaccination regimen is currently being evaluated in 100 high risk volunteers in South Africa (HVTN097: (263)). Preliminary results indicate that T cell responses are similar to those observed in the RV144 trial and immunoglobulin G (IgG) responses to V1V2 regions of HIV-1 Env are not influenced by body mass index, age or gender. Thus, the group of South African volunteers is responding well to the vaccination regimen, and the immune responses seem slightly better than those reported in Thailand. Plans are also underway to vaccinate a South African cohort using the same vaccination strategy as in the RV144 trial, but using subtype C based immunogens (264).

1.10 DESIRABLE RESPONSES OF AN EFFECTIVE T CELL-BASED VACCINE

Ideally, an HIV-1 vaccine should induce both humoral and cellular immunity. The NAbs and/or bNAbs should neutralize transmitted virus, while NoNAbs induce antibody-dependant cell-mediated immunity. The immense diversity of HIV-1 results in some virus escaping neutralisation, infecting cells, and becoming inaccessible to NAbs and bNAbs. The T cell immunity, together with NoNAb responses, will therefore be necessary to control breakthrough virus by killing infected cells. Thus, T cell-based vaccines alone will not necessarily eliminate HIV-1 in infected individuals. They will however lower the viral load (Figure 1.7), preferably to undetectable levels. This approach, with or without

simultaneous ARV administration, offers the advantage of prolonging the time it will take for HIV-1-infected individuals to progress to AIDS as well as reduce transmission rates.

For a T cell-based vaccine, desired immune read-outs include: breadth, magnitude, specificity, polyfunctionality, proliferative capacity/cytotoxicity, avidity, memory

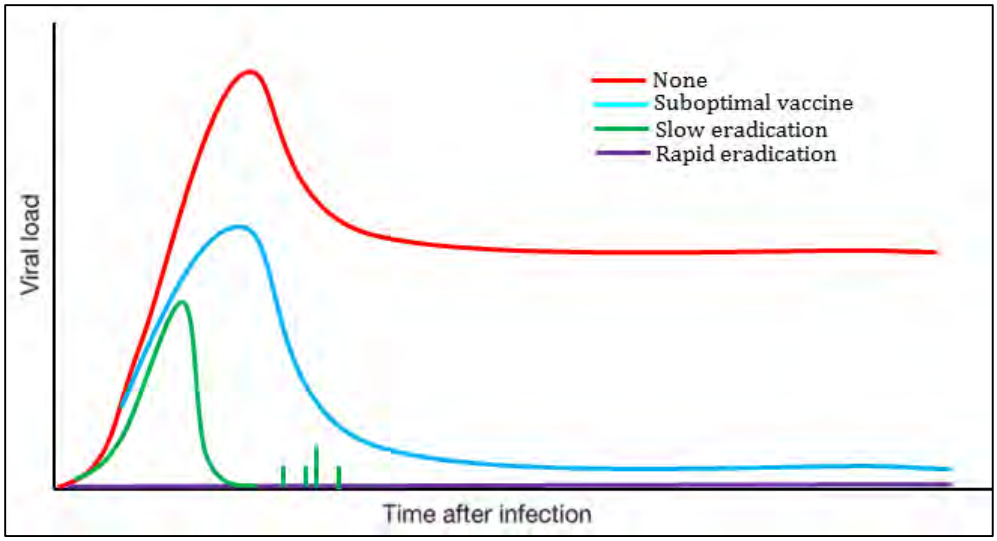


Figure 1.7: Control of SIV or HIV-1 by vaccines that stimulate cytotoxic T cells (CTL). Effect of various T cell–stimulating vaccines (key) on viral load over time (with infection on day 0) during natural infection with HIV or SIV, showing the decrease in viral load achieved without a vaccine (None), by CTL responses (suboptimal vaccine; Barouch *et al*., 2000 (265)), by the rhesus cytomegalovirus (RhCMV) vaccine (Slow eradication; Hansen *et al*., 2009, 2011, and 2013; (266-268)) and by a hypothetical vaccine that targets the virus at the site of infection (Rapid eradication). Adapted from Barouch 2008 and McMichael *et al*., 2014 (269),(270).

phenotype, and persistence. In addition, such a vaccine induced immune response will need to have the capacity to abrogate $CD4^+$ T cell destruction, and the ability to prevent viral escape. Each of these properties has been reviewed below.

1.10.1 Breadth

The breadth of T cell responses to an antigen (the capacity of a vaccine to induce an immune response that recognises multiple viral epitopes) is beneficial for clearing virus in HIV-1-infected individuals. HIV-1 is immensely diverse. If a vaccine can induce responses to multiple epitopes, it will in turn be able to elicit an immune response to multiple HIV-1

strains. McElrath and colleagues (2008; (259)) showed that virus-specific T cells generated by the MRKAd5 vaccine in the STEP trial (Section 1.9.2) had limited breadth. Despite the large amount of virus-specific T cells recorded, the breadth and specificity (Section 1.10.1) were only focused on limited epitopes that were different from those in transmitting strains. Kiepiela and colleagues (2007) discovered that an increased breadth of Gag-specific responses correlated with decreased viremia in HIV-1-infected individuals who were not under treatment (184). Other studies have also recorded similar findings (271-273). An added advantage of the elicitation of increased breadth of T cell responses induced by an HIV-1 vaccine is that some of the mutations within the virus will not result in immune escape.

1.10.2 Magnitude

To destroy virus-infected cells, there needs to be a sufficient number/magnitude of killer cells. These include cytotoxic and memory $CD8^+$ T cells and $CD4^+$ T cells (148). CD4 helper cells that stimulate the function of CD8 T cells and B cells are also necessary to have in high magnitudes, although increased $CD4^+$ T cells may mean increased targets for HIV-1 infection (261). NHPs give a good indication that the magnitude of $CD8^+$ and $CD4^+$ T cells can prevent progressive systemic dissemination of SIV infection (266) and can reduce viral levels to almost undetectable levels (268). When $CD8^+$ T cells are depleted in NHPs by the use of CD8-specific antibodies, the animals lost early viral control upon SIV infection (170,274). In another study, high magnitudes of $CD4^+$ T cells prior to SIV infection correlated with lower peak and viral set points compared to animals that had lower $CD4^+$ T cells numbers (275). In the STEP study (Section 1.9.2), 77% of vaccinated individuals generated HIV-1-specific T cells to HIV-1 before infection. Compared to HIV-1-specific T cells induced by LNTPs, the STEP experimental vaccine induced a 43% lower $CD8^+$ T cell response. Thus, there may be a threshold magnitude of T cells that probably needs to be reached for sufficient protection against HIV-1 infection (unpublished data in McElrath *et al.,* 2008 (259)).

1.10.3 Specificity

The specificity of an HIV-1 vaccine refers to an immune response that is targeted towards a particular region of the virus. This is largely a property of the vaccine immunogen and is discussed below where immunogens can be derived from the conserved or variable regions of the virus (Section 1.11). The induction of immune responses to vaccine epitopes is meaningless if the epitopes are not present in circulating viruses. Thus, the vaccine immunogen needs to closely match circulating viruses. Initial studies of HIV-1 infected individuals using the IFN-γ ELISPOT assay suggested that the specificity of the immune response obtained following vaccination may be critical for viral control (276,277).

1.10.4 Polyfunctionality

Polyfunctional T cells have been shown to possess superior capacity to control/suppress HIV-1 replication in LTNPs. In an effort to compare the capacity of $CD8^+$ T cells from HIV-1-infected progressors and non-progressors to produce different cytokines, Betts and colleagues (2006) measured five different functions of $CD8^+$ T cells; secretion of IFN-γ, IL-2, TNF-α, MIP-1β, and expression of CD107a as a marker for degranulation. Interestingly, polyfunctionality (the capacity to simultaneously produce three or more cytokines) was associated with control of viral replication (278) and is determined by how sensitively the antigen is recognised as well as viral sequence diversification (279). IFN-γ facilitates the up-regulation of HLA class I and II molecules as well as stimulate the loading of peptides onto HLA class I molecules, thus up-regulating the antigen-presenting function of this molecule (280-282). IFN-γ can also facilitate the induction of other cytokines, thus promoting the cytotoxic function of $CD8^+$ T cells (148). IL-2 is an essential growth factor for T cells, TNF-α induces apoptosis of cells infected by virus (148), and MIP-1β activates granulocytes and attracts more T cells to sites of infection (283). In Betts' study (278), LTNPs consistently maintained such functional $CD8^+$ T cells. Polyfunctional T cell responses have also been implicated in the immunological control of other viral infections (reviewed by Seder *et al*., 2008; (284)). It has also been noted that polyfunctional cells secrete increased levels of cytokine on a per-cell basis (285). An effective T cell-based vaccine should generate such responses, particularly in the acute stages of HIV-1 infection.

1.10.5 Cytotoxicity

Cytotoxicity (the ability to kill virus-infected cells) is equally important for the control of HIV-1 replication. Various studies have associated cellular cytotoxicity with reduced viral loads. Hersperger and colleagues (2010) detected an up-regulation of perforin, production of cytokines, and degranulation following stimulation with peptide pools to all HIV-1 proteins in various HIV-1-infected groups, including ECs, LTNPs, and progressors. Interestingly, $CD8^+$ T cells from elite controllers produced significantly more perforin and granzymes than those from progressors (172). Further characterisation of the generated HIV-1-specific T cells highlighted that highly cytotoxic HIV-1-specific T cells were distinguished by up-regulation of perforin and IL-2 (172). An effective HIV-1 vaccine will therefore need to show production of these markers of cellular cytotoxicity.

1.10.6 T cell Avidity

The functional avidity (the capability of T cells to respond to low concentrations of antigen) of HIV-1-specific T cells may also be of importance for a T cell-based vaccine. High avidity $CD8^+$ T cells can effectively clear HIV-1 and potentially suppress viral replication (286). A study by Mothe *et al*. (2012) on T cell responses to HIV-1 Gag p24 in controllers and non-controllers showed comparable breadth and magnitude between the two groups. However, significantly higher avidity responses were observed in controllers but not in non-controllers (287).

1.10.7 Maturation phenotype

The phenotype of $CD8^+$ T cells is a key parameter of viral control. Fully differentiated HIV-1-specific $CD8^+$ effector cells were more frequently detectable in individuals who controlled the virus compared to those who did not (288-290). Central memory $CD8^+$ T cells were associated with lower viral set points in acute phases of HIV-1 infection. Findings from the studies of Hansen and colleagues (266-268) mentioned below further emphasise the importance of effector functions in a T cell-based vaccine. However, it is critical that these T cells do not express any exhaustion markers like PD-1 (291-294).

1.10.8 Persistence of effector CD8$^+$ T cells

Live attenuated HIV-1 vaccines are not acceptable in humans (Section 1.12). However, administration of live attenuated SIV vaccines in non-human primates followed by viral challenge indicated that protection against viral infection might be a result of memory CD8$^+$ T cell persistence in the lymph nodes (295,296). Studies of the RhCMV vaccine vector indicated that persisting CD8$^+$ T cell responses correlated with reduction of viral load (266-268). A total of 13 out of 24 animals given a recombinant live CMV-SIV vaccine restricted viral loads to below detection levels following challenge with the virulent SIVmac239. Although sterilising protection was not achieved, strong and persistent effector memory CD8$^+$ T cells were responsible for controlling and maintaining the viral load through persistent antigen presentation. What was astonishing was that there was no rebound of viremia following the depletion of CD8$^+$ T cells in monkeys that had cleared the virus (266-268). The vaccine also had incredible breadth, targeting 34 epitopes in Gag alone, while the targets recorded for other vectors have been 9 or 10 (297). This vaccine platform has not yet entered clinical trials. However, safer CMV vectors are under development (298).

Live viral vectors have been considered for HIV-1 vaccine development. Such vaccines persistently express heterologous antigens inducing a durable and broadly effective immune protection (299). Such vectors will additionally need to induce robust immune responses at mucosal sites, which are the entry and replicative sites for HIV-1 (300). Preferential targeting of lymphoid tissues by such vaccines will also be an added advantage (296,301) as these organs are the sites of antigen presentation to T cells where the adaptive immune response is initiated (148).

Some replication competent viral-vectored vaccines are currently under evaluation in Phase I and II clinical trials worldwide as reviewed by Parks and colleagues (302). These include the Tian Tan vaccinia virus (TT), Sendai virus (SeV), Vesicular stomatitis virus (VSV), a derivative of New York vaccinia virus (NYVAC; NYVAC-C-KC), and the measles virus (264,303). The TT vaccine expressing HIV-1 Gag, Pol, and Env was used to boost a DNA prime in a murine model. The heterologous prime-boost regimen showed a significant induction of HIV-1-specific antibody and cellular immune responses compared to

homologous vaccination regimens (304). A Phase I trial in China was completed using this vaccination regimen. The vaccine was safe and tolerable in study participants. The vaccine has now advanced to a Phase II clinical trial (303,305).

Although replicating vectors seem attractive, there is a need to use safe vectors that do not cause anti-vector immunity or disseminated infection, particularly in resource-limited regions already burned with various infectious diseases. Thus, although there are desirable immune read-outs, some of them may have to be compromised for the safety of vaccine trial participants.

1.11 IMMUNOGENS FOR T CELL-BASED VACCINES

An optimal immunogenic antigen for a T cell-based HIV-1 vaccine will need to initiate an immune response to many diverse HIV-1 strains.

1.11.1 First and second generation HIV-1 immunogens

Initially, HIV-1 immunogens were derived from sequences of circulating or more virulent HIV-1 isolates. Isolates were also obtained from recent seroconverters. To obtain cross-reactivity to all circulating viruses would be impossible with this approach, given a 35% amino acid difference in the HIV-1 Env protein between subtypes (206). It is noteworthy that the only vaccine trial that has shown some efficacy (204) included immunogens obtained from different natural HIV-1 strains, including the first strain of HIV-1 to be isolated (199,306). If the immunogens can be improved the vaccines should be more efficacious.

Taking HIV-1 diversity into consideration and in an attempt to reduce sequence differences between the antigen and circulating viruses, a second generation of immunogens was developed. This approach involved constructing the HIV-1 immunogens based on 1) consensus sequences of amino acids derived from aligned natural isolates (206,307), 2) a most recent common ancestral sequence derived from an appropriate evolutionary model (206), or 3) a centralised isolate on a phylogenetic tree, centre of the tree, where the

evolutionary distance to all isolates on the tree is minimised (308). The drawback of these types of artificial vaccine immunogens is that they will need to be constantly updated as long as there are more entries of sequences in a database. Constructing Env immunogens in this way is also a challenge.

At the University of Cape Town, Williamson *et al*., 2003 (309) obtained a consensus sequence from 111 HIV-1 strains isolated from individuals with acute and chronic stages of infection from different provinces in South Africa. The sampling cohort included female sex workers, women attending an antenatal clinic, and men who were attending a sexually transmitted disease clinic. A naturally occurring isolate that had the closest sequence similarity to the generated consensus sequence was then selected for use in vaccine immunogens. A Phase I clinical trial (HVTN 073) was conducted in South Africa and USA using DNA and MVA vectors expressing genes from this isolate (303,310). An extended trial (HVTN 086) with 184 volunteers was initiated in 2011. This extension included a gp140 protein component to the vaccine with an adjuvant administered in different combinations with the MVA and DNA vaccines described above (303). The vaccines were safe, tolerable, and immunogenic in both studies. The results also indicated that the more vaccinations individuals were given, the better and prolonged the immune response. Unfortunately, and as in the RV144 Thai trial (204), the antibody responses waned after 6 months from the last vaccination.

1.11.2 Third generation immunogens

1.11.2.1 Mosaic immunogens

In an effort to address the tremendous diversity of HIV-1, new generation immunogens can be designed computationally. Sequences from naturally occurring HIV-1 isolates are entered into a computer program for repeated *in-silico* recombination. The recombination mimics that which happens naturally in HIV-1 evolution. The resultant artificial sequence is made up of fragments of naturally occurring HIV-1 proteins. The process is bioinformatically optimised using computer algorithms and the output recombinant protein used as an immunogen (Figure 1.8). Mosaic immunogens are bioinformatically optimised to increase the coverage of both $CD8^+$ and $CD4^+$ T cell epitopes from natural

sequences with the hope of blocking the escape pathways of HIV-1 (1,311-316). Although the recombination is artificial, the natural processing and expression of the epitopes is preserved. Infrequent epitopes are also excluded through this process. The recombination can be done on subtype-specific or on group M sequences and the number of input sequences is up to the investigator. One can also chose to have a monovalent, bivalent, trivalent or whatever number of desired output sequences from the artificial recombination. The chosen number of output sequences should however be used as one "vaccine cocktail" (1).

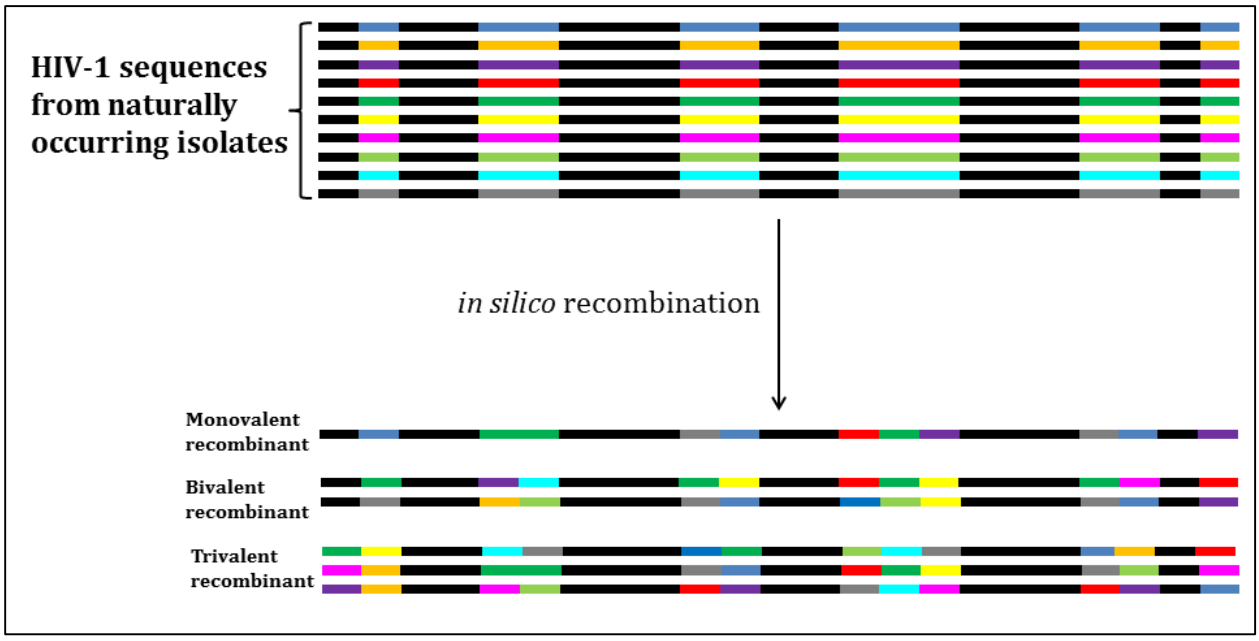


Figure 1.8: Schematic for the generation of mosaic immunogens. Viral sequences (top single coloured lines with black regions) are obtained from natural isolates. The coloured regions represent highly variable regions within HIV-1. The black regions represent less variable regions of HIV-1. Artificial recombination using computer algorithms generates the mosaic sequences. Mosaic immunogens include the most commonly occurring T cell epitopes from the input sequences. The resultant recombinants can have the variable regions in a mono-, bi-, trivalent, or more output sequences. Adapted from McMichael *et al.*, 2014 and Fischer *et al.*, 2007 (1,270).

A summary of studies done using mosaic immunogens is given in Table 1.3. It is interesting that although mosaic immunogens were initially designed to elicit T cell immune responses they induce antibody responses too [237,324]. It is suggested that this may be a result of better cross-presentation of the Envs during the selection of antibodies and affinity maturation. The presence of a third Env in a trivalent immunogen would have also

Table 1.3: Preclinical studies done using HIV-1 mosaic immunogens

HIV-1 clade/group and Immunogens	Valency	Vaccine vector	Study subjects	Key findings	Reference
Group M Env	1, 2 and 3	DNA	mice	Bi-and tri-valent mosaic vaccines elicited an increased breadth (8 peptide pools) compared to the monovalent mosaic vaccine and vaccines with immunogens obtained from natural sequences (2 peptide pools). Mosaic vaccines elicited a predominant CD8 T cell response	(317)
Group M Gag, Pol, Env	2	rAd5 and rAd26	Rhesus macaques	Vaccines expressing mosaic immunogens elicited 4 times more immune responses compared to those expressing consensus or natural sequence immunogens. Mosaic vaccines induced T cell responses with greater depth* than antigens obtained from natural or consensus sequences.	(318)
Group M Gag, Nef	4	DNA prime-Vaccinia virus boost	Rhesus macaques	Increased breadth, depth, and magnitude of responses were observed to the mosaic Gag immunogen compared to consensus immunogens. Cellular immune responses to Nef were comparable between the two types of immunogens.	(319)
Groum M Gag	2	rAd26	Human PBMCs	Mosaic immunogens elicited cross-clade immune responses that were superior that those induced by natural antigens. Mosaic immunogens are processed in a manner that causes epitope recognition by human $CD8^+$ T cells	(320)
Group M Env	2 and 3	DNA prime rAd5 boost	Rhesus macaques	The trivalent vaccine induced T cell responses that were comparable to those induced by the bivalent vaccine. Neutralising antibody responses elicited by the trivalent vaccine were significantly higher than those elicited by the bivalent vaccine. The polyvalent mosaic immunogens elicited stronger cellular immune responses compared to those elicited by consensus and natural immunogens.	(312)
Group M Gag, Pol, Env	2	rAd26, rAd35, and MVA	Rhesus macaques	Challenge using a high dose of a difficult-to-neutralise heterologous virus (SHIV-SF162P3) lowered the risk of infection by approximately 90%. Correlates of protection included nAbs, ADCD, and ADCP.	(224)
Group M Gag and Env independently administered in separate animal groups	2	DNA prime-rAd5 boost	Rhesus macaques	T cell immune responses elicited by the mosaic vaccines were robust compared to those elicited by vaccine expressing natural antigens. Immune responses elicited by SIV Env immunogens are essential for protecting non-human primates against SIV infection. However, mosaic vaccine-elicited humoral responses were lower and very distinct from those elicited by vaccines expressing natural immunogens	(321)
Group M Env	1 of 2	Trimeric protein with CpG/ Emulsigen adjuvants	Guinea pigs	Higher neutralising antibody titres were elicited against subtype B viruses than did a clade C trimeric Env did. The subtype C Env however elicited more neutralising antibody titres against subtype C and A viruses. Vaccination of guinea pigs with the two Env trimmers elicited superior antibody responses than the Envs did alone.	(316)

*depth – ability to recognise more variants within a given epitope; PBMCs – peripheral blood mononuclear cells; nAb – neutralising antibody; ADCD – antibody-dependent complement deposition; ADCP – antibody-dependent cellular phagocytosis

increased the number of Env epitopes to elicit such a response [324]. Increasing the number of output sequences simultaneously increases the coverage of epitopes from the input sequences. Unfortunately, this also means that the number of rare epitopes is included. Thus, di- or tri-valent output sequences are regarded as optimal [1,324].

Mosaic immunogens can also been used for making vaccines against other diverse viruses. Bi- and tri-valent adenoviral-vectored vaccines expressing Hepatitis C mosaic antigens induced potent cellular immune responses in mice. These were superior to the immune responses elicited by mice immunised with vaccines expressing natural immunogens [329]. Fenimore and colleagues computationally assessed the potential of using mosaic vaccines against filoviruses like Ebola [325]. They reported that the mosaics "substantially outperformed" vaccines designed from natural strains. An extension of the study using DNA vaccines expressing the filoviral mosaic envelope antigens was done by Shedlock and colleagues in a murine model [326]. Both humoral and cellular potent immune responses were induced by the vaccines and the antibody responses were strong enough to protect animals against virus challenge. Clinical trials to evaluate the safety and immunogenicity of these immunogens are being planned (303). It will be through such trials that the utility and potential of these immunogens can be further evaluated. It will be exciting to see if the results obtained in animal studies can also be duplicated in humans. Clinical trials will also be able to inform on the ability of HIV-1 to escape immune pressure exerted by mosaic vaccines.

1.11.2.2 Conserved immunogens

HIV-1 has functionally conserved regions within the Env, Gag, Pol, and RT (194,315,322). As the name suggests, conserved immunogens are derived from the most functionally invariable regions of HIV-1 (Figure 1.9). The output is a single chimeric protein sequence comprised of consensus amino acid regions from HIV-1 isolates [337,338]. The rationale is to circumvent any viral escape mutations which are often within the variable regions of the virus. Should there be any escape mutations due to the immune pressure elicited by conserved antigens, it will come at a cost to the virus [339,340]. An added advantage of

using conserved immunogens is that the processed epitopes can be targeted by different HLA types [337,341].

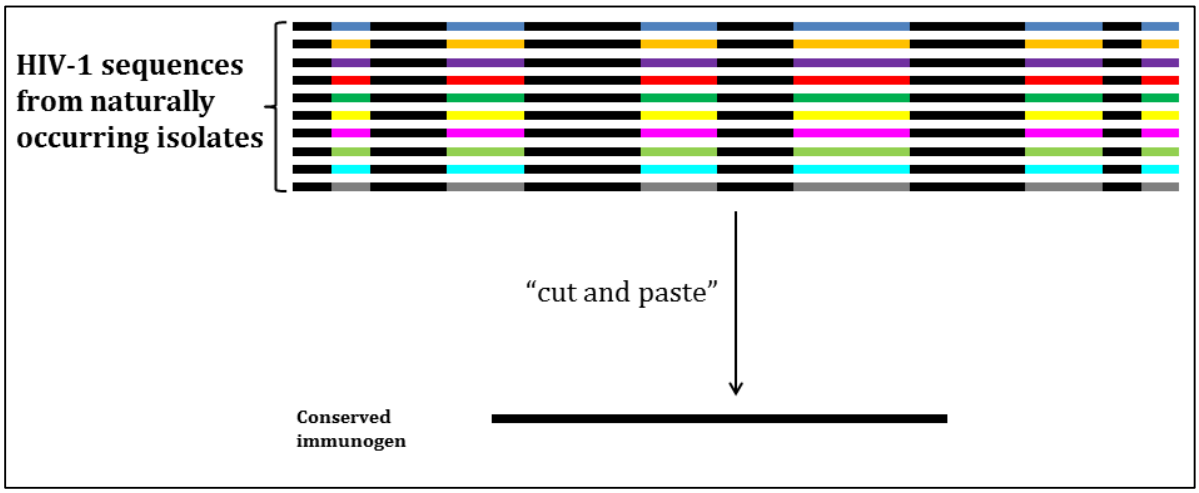


Figure 1.9: Schematic for the generation of conserved immunogens. Viral sequences (top single coloured lines with black regions) are obtained from natural isolates. The coloured regions represent highly variable regions within HIV-1. The black regions represent less variable/conserved regions of HIV-1. The conserved immunogen can be translated from the whole HIV-1 genome or from specific genes (323). The resultant recombinant is a single sequence made only of the conserved regions.

It has also been demonstrated that during the transition from acute to chronic HIV-1 infection, $CD8^+$ T cell targeting of conserved regions correlates with reduced levels of virus than targeting of more variable regions (324). Like other HIV-1 antigens, conserved immunogens can be expressed from different types of vectors for use in prime-boost vaccinations, and this has been done both in animal models (325-327) and clinical trials (328) with encouraging results. In the study by Borthwick and colleagues, T cells induced by immunisation with conserved antigens were able to inhibit HIV-1 replication in a viral inhibition assay.

In a recent study, participants received two doses of adenoviral-vectored vaccines expressing clade A *env* as well as a *gag*, *rt*, *int* and *nef* fusion immunogen. Blood was drawn and PBMCs isolated. Using a viral inhibition assay, they showed a correlation between HIV-1 replication control and cellular immune responses to the conserved regions of the virus (329). In another study involving 980 untreated HIV-1 infected patients, Mothe and colleagues (2014) used 410 overlapping peptides to determine epitopes associated with

virological control. Interestingly, the majority of the epitopes were highly conserved and were in Pol or Gag p24. Moreover, their participants were from Peru, Spain, USA and South Africa and infected with either subtype B or C virus (330) yet the results were similar despite the different geographical sampling and virus clade differences.

While conserved immunogens look promising, their coverage is not as extensive as that of mosaic immunogens.

1.12 VECTORS FOR T CELL-BASED HIV-1 VACCINES

Most vaccines against infectious diseases use whole killed, or attenuated versions of the infectious agent. Whole inactivated SIV administered in conjunction with a formalin adjuvant protected macaques against SIV challenge (331,332). These approaches cannot be applied for making an HIV-1 vaccine. There are risks involved, including that of reversion to infectious virions, recombination between the attenuated and infecting strain, modifications of the host genome due to provirus integration, as well as bad batches that may contain live virus (reviewed by Whitney *et al*., 2004 (333)) . There is therefore a need to use host vectors as "carriers" of the HIV-1 antigens (reviewed by Paris *et al*., 2010 (334)). The most common vectors used, particularly for the induction of T cell responses, are plasmid DNA, replication competent viruses, attenuated viruses, and bacterial vectors. In this thesis, *Mycobacterium (M) bovis* Bacille Calmette Guérin (BCG), DNA, and modified vaccinia Ankara (MVA), were used as vaccine vectors and will be reviewed in more depth.

1.12.1 BCG as an HIV-1 vaccine vector

BCG is a live attenuated vaccine against tuberculosis (TB) that was developed in the early 20th century at the Pasteur Institute (335), (336). The vaccine was generated following more than 200 passages of a virulent tubercule bacillus strain on glycerin-bile-potato media (337,338) and intensive *in vitro* and *in vivo* safety evaluation (339,340). Since then, BCG has been administered to over 4 billion infants and is accessible to 80% of infants globally (341-343). This makes it the most widely used vaccine worldwide, and BCG

vaccination does correlate with a TB non-specific reduction in infant mortality (344,345). This, together with other reasons listed in Table 1.4 make BCG a potential vector for an HIV-1 vaccine. The most attractive feature for using BCG as an HIV-1 vaccine vector is its affordability. This is associated with ease of manufacturing the vaccine and that an extensive and costly cold chain to maintain efficacy is not a prerequisite. Approximately 70.8% of global HIV-1 infections are from resource limited countries. A cost effective vaccine is therefore desirable in the regions most affected by the HIV-1 epidemic. However, there are also some limitations associated with the use of BCG as a vaccine vector for HIV-1, and these are listed in Table 1.4.

Vaccinating immunocompromised individuals against TB with BCG is a major setback. Children on highly active antiretroviral therapy (HAART) develop BCG complications including ipsilateral axillary lymphadenitis and disseminated BCG infection which increases mortality (346). Strategies have however been developed for the production of safer strains of BCG, particularly auxotrophic BCG strains that have received much attention. These strains have mutations in genes required for the production of essential growth compounds, and so cannot replicate unless supplemented with the necessary growth compounds (347,348)-(349,350). Safety profiles of auxotrophic recombinant (r) BCG strains have been demonstrated in immune compromised animal models (347,351-353). Attenuated BCG strains have also been shown to not interfere with the tuberculin skin test (351). In the event that HIV-1-infected and immunocompromised individuals might unintentionally get enrolled in BCG-vectored HIV-1 vaccine campaigns, it is essential to use the safer BCG strains as vectors (354). Furthermore, since safer strains of BCG are being developed against childhood TB, it is important to use these strains as HIV-1 vaccine vectors for use as dual vaccines. Methods of generating better BCG vaccines include deleting the urease gene and incorporating a membrane-perforating listeriolysin (*hly*) gene from *Listeria monocytogenes*. This rBCGΔUreC::*hly* TB vaccine is currently under Phase II clinical assessment as rBCG VPM1002 (355-358). BCG localizes within the phagosomes of antigen-presenting cell where it prevents maturation and phagolysosome fusion by neutralizing the phagosomal compartment. Phagosomes that harbor rBCGΔureC::*hly*, however, have a low pH due to the urease deletion which catalyses

Table 1.4: Advantages and disadvantages of using BCG as an HIV-1 vaccine vector

Advantages	**Reference**
Low cost of manufacturing	(359)
Can be stored at room temperature	(359-362)
Commercial production is well established	(363)
Can prime immune system for VLP, protein, or viral vectored vaccine boost	(354,364-371)
Safe for healthy individuals. Safer strains have been developed for immunocompromised individuals	(372,373)
Elicits cellular immune responses that can last up to 50 years as it infects and persists within antigen-presenting cells	(372-374)
BCG has adjuvant properties within its cell wall	(375)
Can be administered at birth or anytime thereafter	(376)
Can be administered orally, targeting the gastrointestinal mucosa which, together with vaginal mucosa, are the primary sites of entry in HIV-1 natural infections	(367,376)
Large foreign genes can be incorporated into the BCG genome for expression	(368,377,378)
Not affected by maternal antibodies	(372)
Has been tested as a vector for other vaccines	(379-383)
It can be genetically modified to make it potent as a vaccine vector	(347,384,384-387)
Antigen can be expressed as a membrane-anchored or secreted protein to improve immunogenicity	(356,382,388-390)
Mucosal vaccination induces strong immune responses to HIV-1 and SIV antigens	(391,392)
Disadvantages	
Causes disseminated disease in immunocompromised individuals	(338,393-396)
Sensitizes for the tuberculin skin test	(351,397)
Causes BCG—induced immune reconstitution inflammatory syndrome in children receiving ARVs	(346,398)
Protective immunity against TB wanes over time	(399,400)
BCG grows very slowly *in vitro* and can form clumps in liquid vaccine cultures which can compromise vaccine stocks	(401)
rBCG expressing full-length HIV-1 Env is unstable. Truncated forms of HIV-1 Env have to be used	(361,370,377,402,403)
It is effective against childhood TB but not so effective in adults. It is also less effective in tropical regions like India and Africa	(340,404-409)
There is lack of an animal model that truly reflects the use of BCG as a suitable vector for HIV-1	(410)

ammonia production. The resultant acidic environment is optimal for the *hly* activity (411) and phagosome-lysosome fusion, which in turn enhances apoptosis, releasing rBCG-derived antigens into the cytosol and potentially improving the immunogenicity of the vaccine (358,412,413). The HLY protein is in turn degraded in the non-acidic cytosol, preventing host cell damage (414). Results of the clinical trial could provide platforms for improved BCG vectors for HIV-1 antigen delivery.

Many rBCG vaccines have been tested extensively to express antigens from bacteria, parasites and viruses (356,379-383,390,415-418), highlighting the potential of using it for delivery and expression of HIV-1 antigens. Studies of rBCG expressing HIV-1 antigens in prime-boost vaccination regimens have been conducted in various animal models to date, including mice, guinea pigs, and baboons ((419)reviewed by Chapman *et al*., 2010 (362,362)). The results obtained indicate that rBCG can be used as a vaccine vector that can induce both humoral and antigen-specific cellular immune responses to HIV-1. Ami and colleagues (2005; (371)) used recombinant BCG Tokyo (rBCG-SIVgag) and replication incompetent vaccinia virus expressing SIV Gag (rVV-SIVgag) to vaccinate cynomolgus macaques using a prime-boost regimen. In animals that received a rBCG-SIVgag prime and rVV-SIVgag boost regimen, high levels of Gag-specific IFN-γ responses were induced. The vaccinated animals were challenged with pathogenic SHIV strains, but were protected from infection for up to a year. The alternative prime-boost regime or either vaccine alone neither induced similar immune responses nor protected animals against SIV challenge. Thus, BCG-vectored HIV-1 vaccines elict potent immune responses when used as a prime in heterologous prime-boost vaccinations. Such regimens can protect non-human primates against SHIV challenge. In another study, Jensen and colleagues (2013 and 2014; (420,421)) orally vaccinated neonatal macaques with a *Mycobacterium tuberculosis* (*Mtb*) H37Rv prime at birth expressing SIV Gag and Env. They used a strain with a *panCD*, *leuCD* and *secA2* triple deletion. The *panCD* and *leuCD* deletions are known to reduce the replicative capacity of the vector, thus increasing safety. The *secA2* deletion on the other hand, improves immune recognition and reduces immune evasion, thus increasing the vaccine immunogenicity (420,422-424). The animals were boosted with MVA expressing the same antigens. Although the vaccination did not prevent SIV infection, animals that produced antibodies to SIV Env had significantly lower viral loads and set points than animals that

were not vaccinated (420,421). In the current study, an auxotrophic BCG mc²6000 strain with a *panCD* deletion (BCGΔ*panCD*; constructed in the same way as *Mtb*Δ*panCD*; (425)) was used as a vaccine vector and is described in Section 2.1. BCG-vectored HIV-1 vaccines are also yet to be evaluated in clinical trials.

1.12.2 DNA as an HIV-1 vaccine vector

DNA-based vaccines are the simplest means of inducing immunity that is broad. Their construction involves cloning one or more viral antigens into a DNA plasmid vector under the expression of a transcriptionally efficient promoter. The antigen presenting cells (APCs) within sites of vaccination are directly transfected with the vaccine or uptake the immunogen from transfected non-immune cells. The APCs then migrate to primary lymphoid organs, cross-present the target antigen, and initiate immune responses (426-430). The first of such vaccines was described in 1993 for the expression of an influenza protein in a murine model (431). DNA vaccines have since been used as vaccine vectors for cancer (432,433), bacterial, viral, and parasite infections (reviewed by Alarcon *et al*., 1999 (434)), including hepatitis B (435), malaria (436), and notably HIV-1 (437-440). Some advantages and disadvantages of using DNA as HIV-1 vaccine vectors are listed in Table 1.5.

Table 1.5: Advantages and disadvantages of using DNA-based HIV-1 vaccines

Advantages	**References**
Relatively easy and affordable to produce; thermostable which is important for storage and shipping	(441-445)
Versatility. Can be easily manipulated to enhance expression of foreign antigens	(441)
Stable; foreign genes rarely get knocked out during *in vitro* vaccine production	(441)
Safe, with no risk of virulence or anti-vector immunity and can be administered multiple times	(446,447)
In vivo antigen expression results in post-translational modification. The expressed protein therefore, closely resembles the normal structure of the infectious agent	(434)
They can generate both cellular and humoral immune responses	(448)
They can induce persistent immunogenicity	(434)
Multiple plasmid vaccines can be mixed as used as a broad spectrum vaccine	(443)
Disadvantages	
Insertion of DNA vaccine into the host genome may results in cancerous cells	(443)
Conventional and affordable vaccination routes result in sub-optimal immunogenicity in clinical trials	(449,450)

Adapted from Khan 2013 (439)

DNA-based vaccines expressing HIV-1 antigens induce antigen-specific T cell responses in non-human primate models. The elicited immune responses are even strong enough to partially or completely protect the animals against SIV challenge (451,452).

When used in homologous vaccination regimens in clinical studies, DNA vaccines elicit suboptimal immune responses that are short-lived, of low magnitude, and biased towards $CD8^+$ T cell responses (reviewed by Lu *et al*., 1998 (453)). This could be a result of one or more of the following; low transfection efficiency, insufficient transcription of the antigen, insufficient DNA uptake by non-APCs, and low antigen presentation. DNA-based HIV-1 vaccines, however, prime cellular immune responses to candidate viral vectors very efficiently. Thus, they have been used extensively in heterologous prime-boost vaccinations with MVA (225,310,328,454-456), NYVAC (457,458), fowlpox (459), and adenoviruses (219,460); (reviewed in (225,334,440,457)). DNA vaccines also elicit a more balanced immune response between $CD4^+$ and $CD8^+$ T cell responses in heterologous vaccination regimens (440).

The potency of DNA-based HIV-1 vaccines can be improved by electroporation means of delivery (448,461-465), bioinjector or gene gun means of immunisation (466-468), combined vaccination with adjuvants and cytokines like IL-15, IL-2, and IL-12 (469-471), as well as co-administration with proteins (472-474). Delivery and immunogenicity of antigens by DNA vaccines can also be enhanced by use of improved promoters, use of enhancer elements (475-477), codon optimisation of the immunogen, and incorporation of signal peptides that aid trafficking of expressed antigens (478,479).

Over 80 clinical trials have been conducted using DNA as an HIV-1 vaccine vector, alone or in combination with protein or viral vectors (303). The majority of the trials were Phase 1 safety studies. Efficacy trials with DNA prime vaccines included the terminated STEP, Phambili, and HVTN 505 trials (Table 1.2; Section 1.9). While DNA vaccines are safe and immunogenic, they do need to be used in combination with the right vector to boost the primed immunity.

1.12.3 Poxviral vaccine vectors

Poxviruses are divided into the *Chordopoxviridae* and *Entomopoxviridae* subfamilies. Poxviruses that can complete their replication cycle in humans belong to the *Chordopoxviridae* subfamily (Figure 1.10; (480)).

Poxviruses are large, brick-shaped enveloped viruses that have a dumb-bell shaped core within which lies viral enzymes and a double-stranded DNA genome of up to 360kb. The genomes have over 200 open reading frames reviewed by Brave *et al*., 2007 and Moss 2013 (481,482)**.** The poxvirus genome has a central conserved region and two external variable regions on either side of the conserved region (Figure 1.11A and B).

Replication takes place in the cytoplasm as shown in Figure 1.12. They code for almost all of their viral replication machinery, thus requiring minimal host assistance for DNA replication and transcription. The replication cycle can be divided into the pre-replicative stage (controlled by early gene products), intermediate stage, and a post replicative stage (controlled by late gene products) (483).

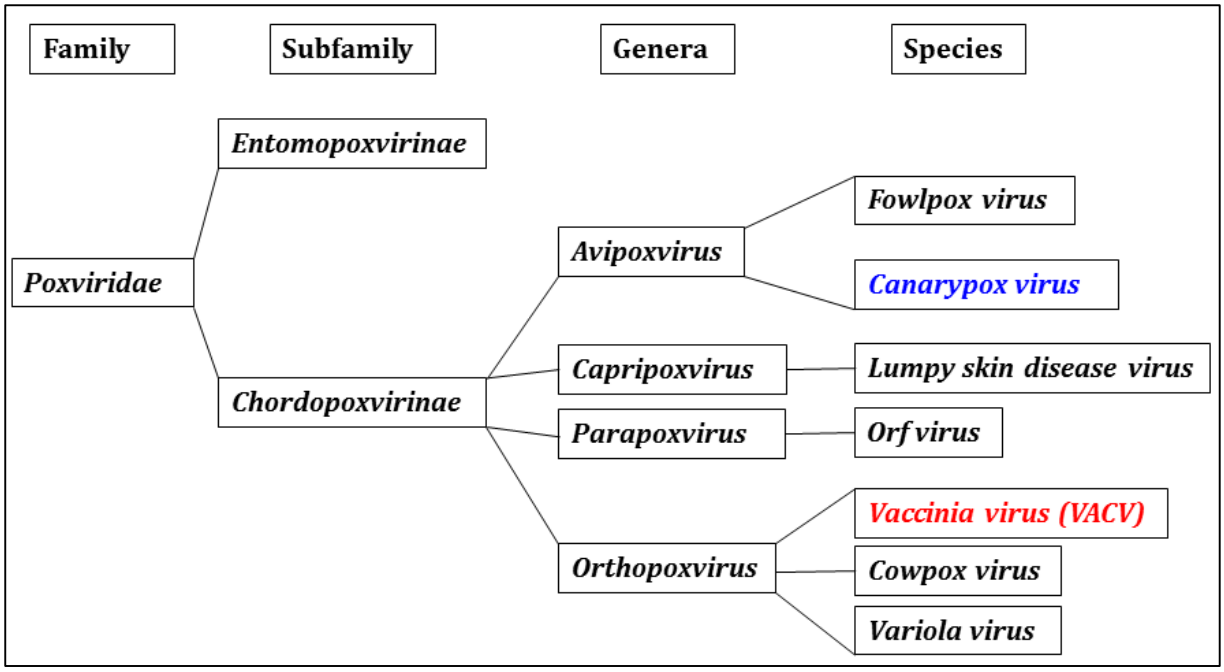
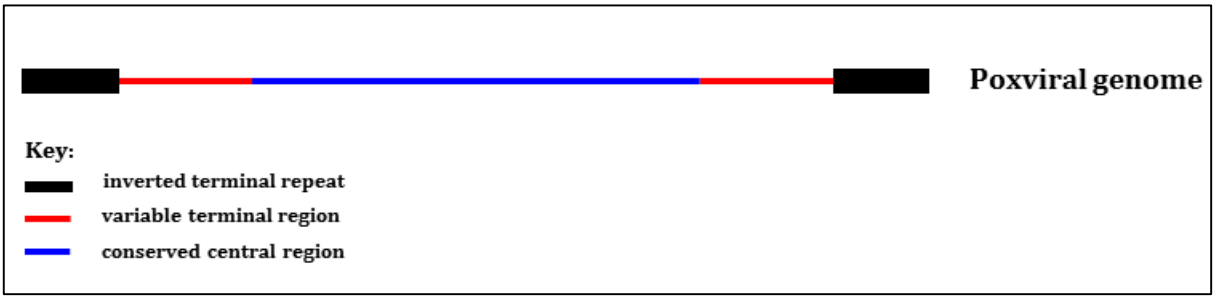


Figure 1.10: Phylogenetic schematic of the Poxviridae family. The most common genera and species are included. *Vaccinia virus*, from which MVA and NYVAC are derived, is highlighted in red. *Canarypox virus*, the vector in which the RV144 ALVAC vaccine was used is highlighted in blue

A.



B.

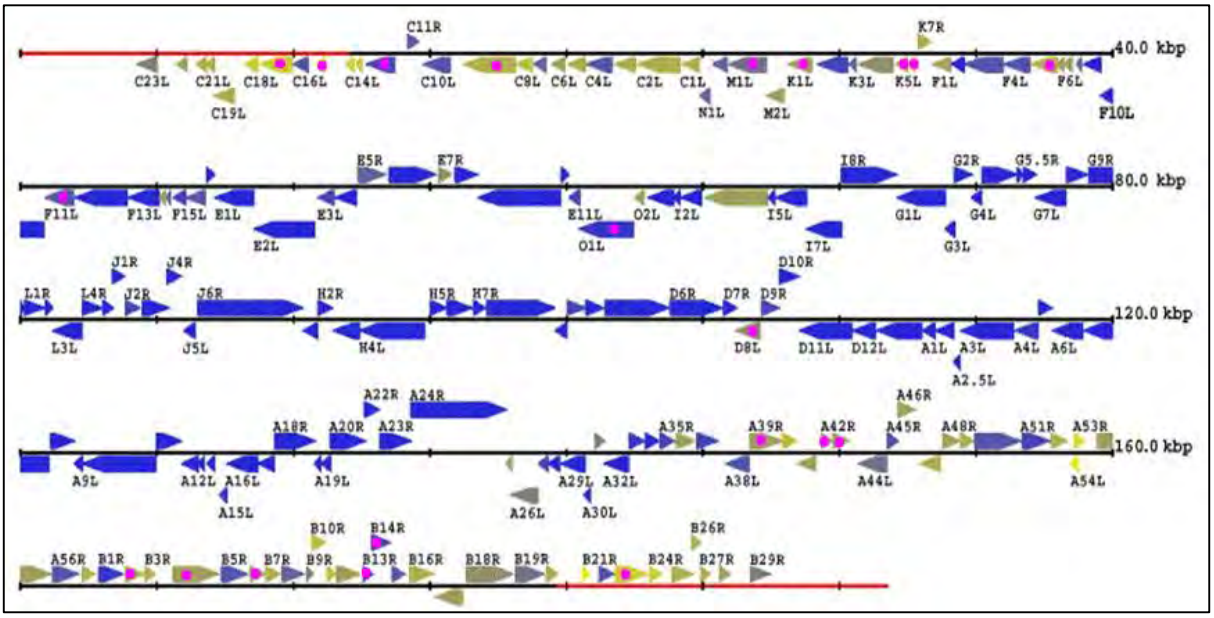


Figure 1.11: A. Schematic of a poxviral genome (not drawn to scale). Adapted from Mercer *et al*., 2007 (484) **B. ORFs of *Vaccinia virus* strain Copenhagen.** Genes are coloured using a sliding scale; dark blue – genes present in all pox viruses; green – genes present in 1 -3 poxviruses; pink dots – genes absent or mutated in modified vaccinia Ankara (MVA). The arrows represent the direction of transcription. Adapted from Antoine *et al*., 1998 and Lefkowitz *et al*., 2006 (485,486).

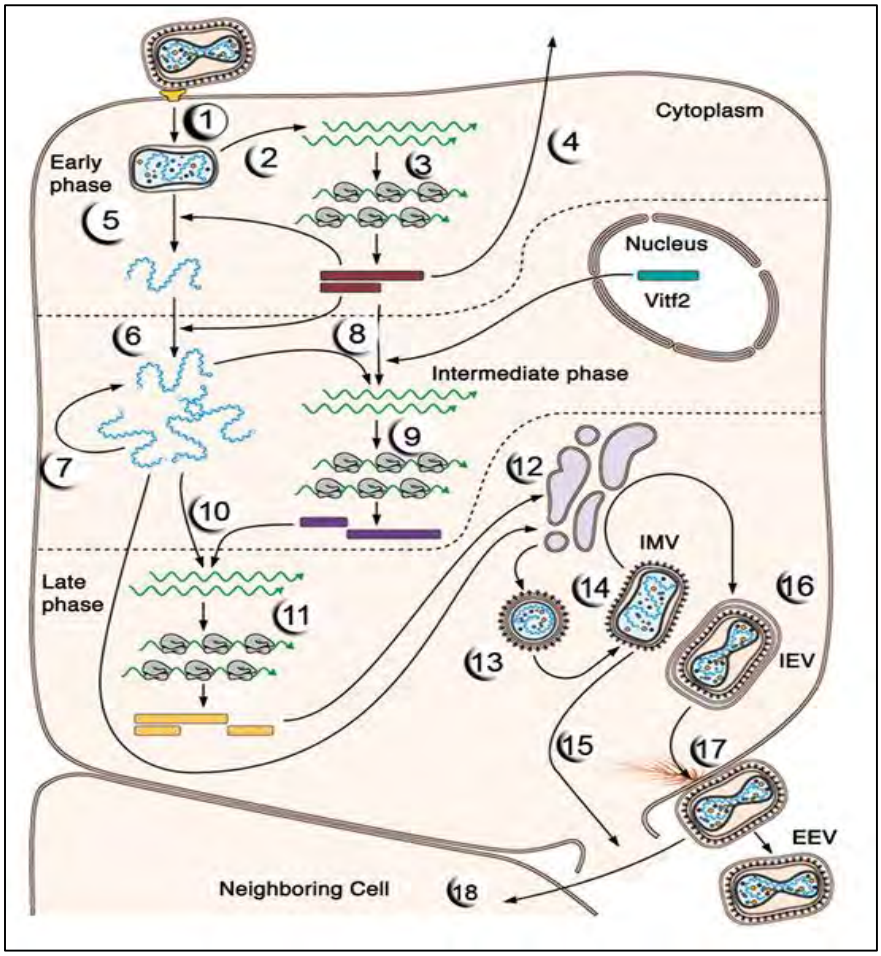


Figure 1.12: The reproductive cycle of vaccinia virus. Infectious extracellular enveloped virus (EEV) attaches and infects a susceptible host cell (**1**). Primary uncoating takes place, and the viral core is released into the cytoplasm and early mRNAs synthesised (**2**). Early mRNAs exhibit features typical of cellular mRNAs and are translated by the cellular protein-synthesizing machinery (**3**). Some early proteins are secreted from the cell and have sequence similarity to cellular growth factors, which can induce proliferation of neighbouring host cells, or are proteins that counteract host immune defense mechanisms (**4**). Synthesis of early proteins induces a second uncoating reaction in which the core wall opens and a nucleoprotein complex containing the genome is released from the core **(5)**. Other early proteins catalyze the replication of the viral DNA genome (**6**). Newly synthesized viral DNA molecules can serve as templates for additional cycles of genome replication (**7**) or as templates for transcription of viral intermediate-phase genes as required host cell factors like Vitf2 (**8**). The proteins encoded by intermediate mRNAs (**9**) include those necessary for transcription of late-phase genes (**10**)**.** The latter genes encode the proteins from which virions are built as well as the virion enzymes and other essential proteins that must be incorporated into virus particles during assembly (**11**). Viral membrane proteins are unglycosylated, and the role of cellular membranes in early stages of assembly is controversial (**12**). The initial assembly reactions result in formation of the immature virion (IV; **13**). This is a spherical particle delimited by a membrane that may be acquired from an early compartment of the cellular secretory pathway. The IV matures into a brick-shaped intracellular mature virus (IMV; **14**) which is released only when cells are lysed (**15**). However, the IMV can acquire a second, double membrane from a trans-Golgi or early endosomal compartment to form the intracellular enveloped virion (IEV; **16**). The IEVs move to the cell surface on microtubules where fusion with the plasma membrane forms cell-associated virions (CEV; **17**). These CEV induce an actin polymerization that promotes a direct transfer to surrounding cells (**18**) or they can dissociate from the membrane as extracellular enveloped virus (EEV). About 85% of the virions remain within infected cells and therefore lack the outer external membrane naturally found on released virions. Taken from Flint *et al*., 2003 (487).

Several factors make poxviruses attractive as vaccine vectors. 1) There is a lack of genomic integration into the host DNA due to independent cytoplasmic replication; 2) they are able to induce humoral and cellular immune responses to heterologous antigens; 3) recombinant poxviral-vectored vaccines can be freeze-dried. Such vaccines are thermostable, and there are therefore low costs associated with ease of manufacturing, delivery, and administration; 4) large and multiple foreign DNA sequences can be inserted into poxvirus genomes; 5) high titre vaccine stocks can be prepared from chicken embryo fibroblasts (CEFs) and baby hamster kidney (BHK) cells.

Various poxviruses (MVA, NYVAC, Fowlpox, and Canarypox) have been used as vectors for HIV-1 vaccines in 85 completed, 12 ongoing, and 5 scheduled clinical trials (303). Of 6 trials involving high risk individuals in Phase IIb and III trials, only one consisting of a canarypox-based vector prime and a protein bost was modestly protective (Table 1.2; (204)). There is therefore now much interest in using poxviruses as vaccine vectors. Our lab has used MVA (310,488) and Lumpy skin disease virus (489-491) as vaccine vectors for HIV-1 immunogens. We have also recently characterised local isolates of Avipoxviruses which are potential vaccine vectors (492-494). MVA was used as a vaccine vector in this project, and is reviewed in the next section.

1.12.3.1 MVA as a vaccine vector

Vaccination of individuals with VACV resulted in the eradication of smallpox in 1979 (54). Infrequent, but serious and unacceptable complications were associated with vaccination using some VACV strains. This led the scientific community to develop safer derivatives of smallpox vaccines, which are now being used as vectors for cancer (495-498) and other infectious diseases including tuberculosis (499), respiratory illnesses (500,501), and HIV (reviewed by Gomez *et al*., 2008 and 2012 (497,502)).

MVA is a highly attenuated strain of a Turkish VACV that is severely host restricted. In an effort to eradicate smallpox by producing host restricted and safer vaccines, Mayr and colleagues produced MVA following 572 passages of chorioallantois vaccinia virus (CVA) in CEFs at the Institute of Infectious diseases and Zoonoses (previously known as Institute of Medical Microbiology, Infectious and epidemic diseases) at the University of Munich, Germany (503). In 1974, over 120 000 individuals were vaccinated with MVA against smallpox in Germany. Strikingly, none of the MVA-immunised individuals had

severe adverse reactions as had been previously reported with VACV vaccinations (504,505). The molecular basis associated with MVA attenuation and host restriction was only uncovered about two decades later. It was discovered that independent deletions and mutations in six large genomic regions representing 15% of the CVA genome were lost during the 572 passages (Figure 1.11; (485,506)). The majority of the genes deleted affect VACV host interaction and immune evasion (485,507).

Although MVA efficiently infects most mammalian cells, its replication is abortive soon after the formation of immature virions (Figure 1.12, stage 13), meaning the virus does not replicate or spread to neighbouring cells (508). However, viral and foreign genes can still be efficiently expressed. MVA is therefore a safe and effective antigen delivery platform (508). There are additional features of MVA that make it an attractive vaccine vector. Advantages and disadvantages of using MVA as a vaccine vector are listed in Table 1.6.

Table 1.6: Advantages and disadvantages of using MVA-based HIV-1 vaccines

Advantages	**References**
MVA cannot complete its replication cycle in humans, therefore there is no risk of it spreading to neighbouring cells following vaccination. Its safety record was evident in the smallpox vaccination campaign. It can be safely administered to immune-compromised individuals and neonates	(505,508-510)
Deletion of immune-modulatory genes results in a rapid local immune response at the point of infection. MVA thus has adjuvant properties and has the potential of inducing long-lasting immunity	(485,511)
Large and multiple foreign DNA sequences can be inserted into MVA genomes	(512)
Able to induce humoral and cellular immune responses to heterologous antigens	(513,514)
Mucosal vaccination induces site-specific immune responses	(515)
High titre vaccine stocks can be prepared from CEFs and BHK cells	(484,509)
Disadvantages	
Recombinant MVA construction is a lengthy process	Personal experience; (484,516)
Transgenes inserted into the delI and del III regions can be unstable	(517); reviewed in (518)

Since smallpox was eradicated and vaccination ceased in 1979, people up to 35 years of age would not have been exposed to either *Variola virus* or *vaccinia virus*. The majority of the target population for HIV-1 vaccination would be individuals who were not immunised against smallpox; thus, there would be a low prevalence of anti-vector

immunity. MVA is therefore unique in that it provides the safety of an attenuated or killed virus vaccine because of its limited capacity to replicate in human cells, yet provides the immunogenicity of a live virus vaccine with its ability to express foreign antigen.

There are three types of promoters that have been identified in VACV, early, intermediate, and late (519). Antigens that are expressed by strong late promoters induce a strong humoral immune response (520) while antigens transcribed by early promoters seem to induce mostly cytotoxic T lymphocyte responses (521,522). The commonly used poxvirus promoters have early and/or late activity and include the p7.5, modified (m) H5, and the short synthetic promoter, pSS (523-525).

In murine studies, correlation has been shown between the magnitude of immune responses and the levels of foreign antigen expressed by recombinant MVA (526). Mice immunised with a vaccine that highly expressed an HIV-1 Env had antibody titres that were 15 times more than in mice immunised with a weaker Env expressing recombinant vaccine. The Env-specific T cell responses were also many magnitudes greater in the higher expressor. In the same study, the Env responses did not negatively affect the Gag responses induced by the same vaccine. In a more recent study, Isshiki and colleagues compared Env expression from a recombinant VACV under the expression of two different promoters, a moderate early/late p7.5 and a strong early /late pSFJ1-10 promoter (527). Interestingly, the p7.5 promoter induced stronger antigen-specific T cell responses, while the pSFJ1-10 promoter induced stronger Env-specific antibody responses. Thus, a desired immune response can be induced by manipulating poxviral vector promoters.

Recently, a naturally occurring promoter, MVA13.5L, was identified (528). It can induce potent immune responses of foreign antigens and drives very early gene expression. Another VACV transcriptional promoter derived from the pS promoter was designed *in silico*, Late-Early Optimised (LEO; (529)). Enhanced gene expression, which correlated to $CD8^+$ T cell immune responses in mice, could be detected within an hour of infection. Using an appropriate promoter in recombinant poxvirus vaccine studies is therefore crucial in eliciting the desired immune responses.

There have been 24 Phase I and 5 Phase II clinical trials that have been completed worldwide using MVA alone or in combination with other vaccine vectors expressing different HIV-1 subtype genes. Of the ongoing clinical trials, there are 6 in Phase I trials, and 2 in Phase II (303). One of these is being done in South Africa (HVTN 086, SAAVI 103) with 184 adult participants. Vaccines that were previously made in our lab, SAAVI DNA-C2 and SAAVI MVA-C (488,530), together with a Novartis subtype C gp140 in MF59 adjuvant are being evaluated for their safety and immunogenicity.

Recently, a Phase IIa clinical trial (HVTN 205) was conducted using a DNA and MVA prime boost regimen (225,226). The vaccines produced VLPs *in vitro* that displayed membrane-bound HIV-1 Env in its trimeric form (531-533) and induced predominant gp41-specific antibodies. This study demonstrated antibody responses that were durable and only declined by <3-fold 6 months after completion of vaccination. When the gp120 protein was used to boost a canarypox prime vaccination in the RV144 vaccine efficacy trial (Table 1.2; (204)), and when it was used with an adjuvant in a prime/boost regimen (534), the magnitudes of binding antibodies had 10-fold decreases 6 months after vaccination (535). These results are exciting since the durable Env-specific antibody responses have the greatest capability of preventing HIV-1 infection. The magnitude of T cell responses in the HVTN 205 trial was also durable, declining by 1.6 – 2.1- fold 6 months after the last vaccination (225). The T cell responses generated largely targeted Gag, and not Env as in the case of the DNA/rAd5 prime/boost regimen (HVTN 204; (536)), or Pol as in the case of the STEP study (Table 1.2;(259)). Over 70% of responding T cells induced by the DNA/MVA vaccinations in this study produced 2 or 3 cytokines. This was greater breadth compared to those obtained using rAd5-based vaccines (259,536). The vaccines are now being considered for an efficacy trial.

1.13 SUMMARY

HIV-1 infections are among the leading causes of death in low income countries (Section 1.3; (25)). Current measures of preventing HIV-1 infection are largely dependent on patient adherence. Vaccination has proven to be the most efficient and economical

means of preventing and eradicating viral infections as well as saving millions of lives (reviewed by Rappuoli and Aderem 2011; (215)). The RV144 Thai trial has given the scientific community hope that an effective HIV-1 vaccine is possible to obtain and free the human population of this devastating disease (204). A vaccine that elicits bNAbs towards HIV-1 would be desirable to achieve sterilising immunity (Sections 1.6 and 1.8.2). There is also a need for the vaccine to induce cellular immune responses as these have been shown to lower viral load and slow disease progression in natural infection by LTNPs and ECs (reviewed by McMichael and colleagues 2010; (149)). Notably, these responses are targeted towards Gag (Section 1.6). T cell immune responses have also been associated with disease control in SIV and SHIV vaccine studies using non-human primates (reviewed by Genovese *et al*., 2013; (190)). If the T cell read outs are appropriate (Section 1.10), SIV infections can also be cleared from non-human primates (266-268). Cellular immune responses are also essential for killing cells infected with any virus that may escape neutralisation by antibodies (reviewed by Wei *et al*., 2003 and Moore *et al*., 2009; (537,538)). T-cell based HIV-1 vaccines are often limiting as the virus can also evade the immune pressure through escape mutations ((154,157); reviewed by McMichael *et al*., 2001; (169)). The mosaic vaccine approach is designed to curb the hurdle of HIV-1 diversity. This is achieved by maximising T cell epitopes, and excluding those that rarely occur in nature by the artificial recombination of naturally occurring sequences done *in silico* (1). This approach also generates variants that are likely to be generated during viral mutational escape (1). This concept has been evaluated in non-human primates using M group-based immunogens which have been shown to process and present T cell epitopes generated during natural HIV-1 infection (320). HIV-1 vaccines expressing M group mosaic immunogens are scheduled to be evaluated in clinical trials (539). The mosaic vaccine concept, however, has not been evaluated using subtype-specific immunogens. HIV-1 Gag also makes a good target for a T cell vaccine since it is the main structural protein of HIV-1 (Section 1.5.1) and is relatively conserved across HIV-1 strains. Escape mutations in Gag will affect viral fitness (160).

1.14 PROJECT MOTIVATION

In an effort to produce affordable vaccines suitable for the regions most affected by HIV-1, our research group at the University of Cape Town made subtype-specific vaccines that entered Phase I clinical trials (303,540). Although the results were encouraging, the vaccine immunogenicity and stability could be improved. The aim of this project was to develop stable subtype-specific HIV-1 vaccines - based on BCG, DNA, and MVA vectors - expressing an HIV-1C mosaic Gag immunogen. We also aimed to assess the vaccine immunogenicity and to compare it to that elicited by a natural Gag immunogen.

To enhance the immunogenicity of the vaccines, we used a mosaic antigen that is designed *in silico* to specifically increase the breadth and depth of T cell responses to the highly diverse HI virus (1). Since this was a proof of concept study, only HIV-1C Gag was included. The mosaic immunogen is also designed to exclude rare epitopes which are often incorporated in consensus sequence-based immunogens. Matching vaccine epitopes to circulation infectious HIV-1 strains has been shown to induce potent immune responses (322,541,542). As the dominant HIV-1 subtype in Southern Africa is subtype C a subtype-specific HIV-1 mosaic Gag immunogen was selected for this project.

Three vaccine vectors were used to deliver the mosaic Gag immunogen (Gag^{M}); BCG*ΔpanCD*, DNA, and MVA; for their safety record, affordability, and/or ease of construction. Various studies have reported plasmid instability when expressing transgenes in rBCG (reviewed by Chapman *et al*., 2010; (362)). We therefore used an *mtrA* promoter (543-545) and a 19kD leader sequence to reduce metabolic load on rBCG, which in turm helps to maintain vaccine integrity. Furthermore the BCG strain we used, BCG*ΔpanCD*, has a proven safety track record in immune-compromised SCID mice (353) and is known to improve transgene immunogenicity (354,364,365). DNA-based HIV-1 vaccines are known to be immunogenic in animal studies in prime-boost vaccination regimens (310,546). This does not always translate to clinical trials and so equires multiple vaccinations, delivery of large doses, co-immunisation with adjuvants, or delivery by electroporation which are expensive (Section 1.12.2). To improve the immunogenicity of the DNA-based vaccine, we used a vector with a novel enhancer element which results in much greater gene expression levels, and was developed at the University of Cape Town (475). MVA is known to be a safe vaccine vector (Section

1.12.3.1). Insertion of transgenes into the delII and delIII regions of MVA is known to cause vaccine instability (517). We explored the use of the MVA conserved region for insertion of Gag^M to improve stability as has been described by others (517,547). The construction of these vaccines (BCG-Gag^M, DNA-Gag^M, and MVA-Gag^M) is detailed in Chapter 2. In addition, expression of Gag^M from all three vectors is investigated.

The desirable immune readouts for a T cell-based HIV-1 vaccine are known from studies on ECs, LTNPs, and animal studies. These have been described in Section 1.10. Since the mosaic immunogens are expected to be more immunogenic than natural immunogens, the optimal MVA-Gag^M dose to boost a BCG- Gag^M and DNA-Gag^M prime first had to be determined. The vaccines we made were tested for immunogenicity to Gag^M in mice (Chapter 3). This included measuring the magnitude of T cells responding to Gag-specific peptide, the production of cytokine-positive cells, and determination of T cell memory phenotype. We also investigated whether the different vaccine vectors gave different Th1/Th2 responses. Comparisons were made between the three different vectors and different prime-boost regimens.

CHAPTER 2: CONSTRUCTION AND CHARACTERISATION OF BCG, DNA, AND MVA VACCINES EXPRESSING AN HIV-1C MOSAIC GAG

Contents **Page**

2.1 INTRODUCTION

In this chapter, the construction of BCG, DNA, and MVA vaccines expressing a full length HIV-1C mosaic Gag (Gag^M) immunogen is described. The functional properties of HIV-1 Gag have been reviewed in Section 1.5.1 and mosaic immunogens have been described in Section 1.11.2.1. This chapter also describes the characterisation of the Gag^M immunogen expression since it was computationally generated (1) and, to our knowledge, has not been characterised before.

The use of BCG as an HIV-1 vaccine vector was described in Section 1.12.1. In this study, we used the BCG $mc^2$6000 (BCGΔ*panCD*) strain (constructed in the same way as *Mtb*Δ*panCD*; (364,425). BCGΔ*panCD* is a pantonthenic acid (vitamin B5) auxotrophic strain of BCG Pasteur 1172 P2 that is non-virulent, yet protective against *Mtb* challenge in guinea pigs and immunocompromised mice (347,352,353,353,386,425). Pantothenic acid is essential for coenzyme A and acyl carrier protein synthesis which are vital for several intracellular processes in mycobacteria including polyketide biosynthesis, fatty-acid metabolism, and the tricarboxylic acid cycle (548). BCGΔ*panCD* has also been shown to be a better HIV-1 vaccine candidate than wild type BCG in mice. Previous studies carried out in our laboratory have indicated reduced inflammation and an improved $CD8^+$ T cell response to HIV-1 Gag expressed by BCGΔ*panCD* in comparison to the parent BCG Pasteur strain (364,365). In a separate mouse study, Chapman and colleagues (2013; (365)) observed potent antigen-specific T cell responses induced by rBCGΔ*panCD* vaccines expressing HIV-1 Gag, RT, and Gp120. The T cell responses were also characterized by IFN-γ, TNF-α, and IL-2 production, which are characteristic of viral control in ECs (182). Heterologous antigens in rBCG vaccines have often been under the transcriptional control of mycobacterial *hsp60* and *hsp70* promoters which result in high antigen expression (reviewed by Chapman *et al*., 2010; (362)). However, the increased expression levels result in plasmid instability (549-551). Use of the *Mtb mtrA* and *M. leprae* 18kD antigen promoters on the other hand reduce transgene expression *in vitro*, but upregulate their expression *in vivo* (543-545). Furthermore, the accumulation of heterologous proteins in the cytoplasm of BCG may be toxic to the cell. Targeting the expressed proteins to the bacterial cell wall or into the extracellular space may reduce the metabolic load. This is achieved by fusing the transgenes to leader sequences within the shuttle vectors (reviewed by Chapman *et al*., 2010; (362)).

Targeting transgenes to the cell wall or extracellular space has resulted in improved immunological responses by rBCG vaccines (reviewed by Bastos *et al*., 2009; (337)).

Construction of DNA-based HIV-1 vaccines is simple and not time-consuming. Using DNA as an HIV-1 vaccine vector was reviewed in Section 1.12.2. The most commonly used DNA vaccine vectors are pTH and pVRC (477,552). Mice are usually vaccinated with doses of 50-200 μg of these vaccines when immune responses to the DNA vaccines are being assessed. Our lab has recently patented a novel pTHpCapR DNA vaccine vector backbone with a porcine circovirus enhancer element upstream of the CMV promoter ((475); Appendix A1). Antigen expression and immune responses that are 3- and 2-fold greater, respectively, can be obtained in mice vaccinated with only 10μg of the novel vectored vaccine compared to mice that were vaccinated with 100μg of the standard pTH-vectored vaccine (475). We therefore used pTHpCapR as the vector backbone for our DNA vaccines in this study.

MVA was used as a vaccine during the smallpox eradication campaign (54). Since then, it has been used as a vaccine vector for other infectious diseases and cancer (reviewed in Section 1.12.3.1). Recombinant MVA vaccines have often utilised the del II and del III regions which lie within the variable terminal regions as sites of insertion (Figure 1.11). These regions are often prone to deletions and other mutational changes. Inserting a foreign gene into the variable terminal region makes it prone to such deletion mutations. To increase transgene stability, foreign genes have been inserted between transcriptionally convergent genes where no possible transcriptional promoters could be disrupted (517,547). To detect partial MVA recombinants, marker (colour or fluorescence), or selection genes (antibiotic resistance or host range extension) have been used when making recombinant poxviruses (508,553-561). To ensure stringent selection of recombinants, a combination of marker and selection genes have been used by some groups (547,562,563). For this project, green fluorescent protein (GFP; which causes cells to fluoresce green) was chosen as a reporter gene and blasticidin (BSD) as a selection gene. BSD kills mammalian cells, however, if the *bsd* resistance gene (*bsd*^{R}) is expressed, the cells survive.

Generating recombinant MVA involves designing a transfer vector which is a plasmid with MVA flanking sequences on each side of the gene of interest (Figure 2.1). The

transfer vector is transfected into cells infected with MVA. The flanking sequences in the transfer vector homologously and sequentially recombine with the MVA genome in the regions that match the flanking sequences. A positive selection gene can be placed between or outside the viral flanking sequences. In the former, the positive selection gene is retained in the final recombinant poxvirus and in the latter the final recombinant loses the selection gene through transient selection (557). Having the positive selection gene eventually lost through homologous recombination is however recommended for vaccines that will eventually be used in clinical trials. VACV-specific promoters are included upstream of the foreign gene and the selectable marker (Section 1.12.3.1). These are often different promoters to reduce the chances of recombination within the transfer vector and subsequent loss of the gene of interest.

A permissive cell line is infected with MVA, followed by transfection with the transfer vector. Using the positive selection marker, the recombinant MVA can be preferentially grown and detected in cultured cells. If transient selection is employed the fluorescing intermediate recombinant is purified before a final recombinant is isolated. The final recombinant can then be isolated by selecting nonfluorescing foci for further growth under conditions permissive to the desired final recombinant virus. Figure 2.1 diagrammatically shows the generation of a recombinant poxvirus by transient selection. Both intermediate and final recombinants are identified by polymerase chain reaction (PCR) and the final recombinant is confirmed to be correct by DNA sequencing across the insertion site. Expression of the transgene is then determined.

2.2 MATERIALS AND METHODS

2.2.1 Design and synthesis of the HIV-1C Gag^{M} immunogen

HIV-1 vaccine design requires the use of an immunogen inserted into a vaccine vector (Section 1.12). In this study, the mosaic immunogen approach was used (Section 1.11.2.1). A monovalent HIV-1C Gag^{M} amino acid sequence was obtained from the supplementary data of Fischer *et al*., (2007; (1)). Two full length *gag^{M}* genes were synthesised by the company GeneArt (USA). One *gag* gene was codon optimized for expression in BCG (Appendix B1) and the other for expression in humans for use in the

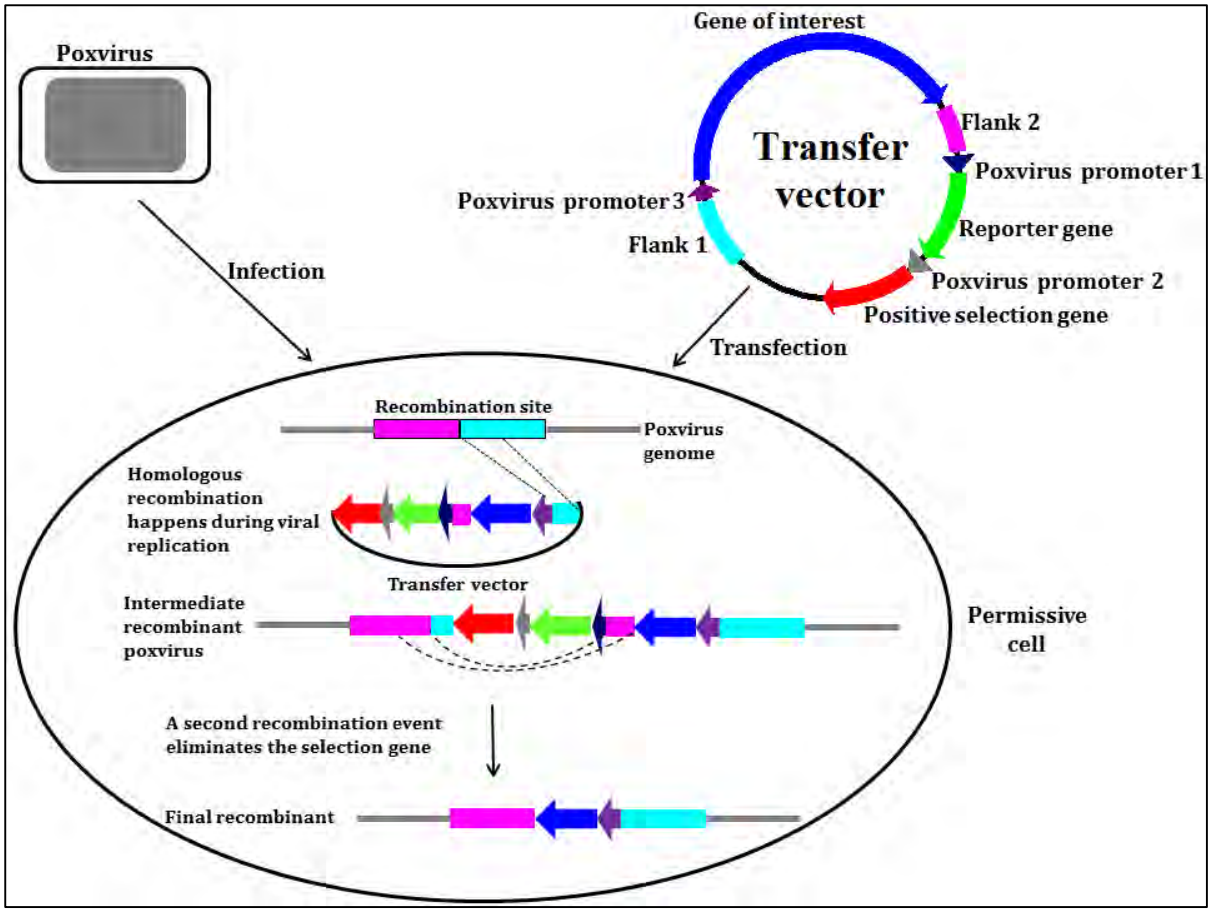


Figure 2.1: Schematic representation of the production of recombinant poxviruses. DNA recombination occurs between the transfer vector and the insertion site of interest in the MVA genome within a permissive cell line. The transfer vector has a positive selection gene, reporter gene, and the gene of interest, all with suitable poxvirus promoters. The gene of interest is flanked by DNA sequences that are homologous to the insertion site within the poxvirus genome (flank 1 and flank 2). The recombinant poxvirus is generated by infection and simultaneous transfection of cells with the transfer vector plasmid DNA. Recombination occurs between the homologous sequences of the transfer vector and poxvirus during DNA replication at either of the two flanks to form an intermediate recombinant poxvirus. This diagram depicts recombination at flank 1. In a similar manner, recombination could take place at flank 2. The intermediate recombinant poxvirus can be detected and selected for by means of the reporter and positive selection gene respectively. A second recombination step with the second flank results in the formation of the final recombinant. Adapted from Schnierle *et al*., 2007 (516) and Shen 2010 (564).

DNA and MVA vaccines (Appendix B2). During the optimization process, the following sequences were avoided: typical promoter-like and ribosomal binding site sequences to avoid nonsense translation events, AT- and GC-rich sequence stretches, chi-sites, repeat sequences, RNA instability motifs, and RNA secondary structures. TTTTTNT poxvirus termination sequences were also excluded in the human codon optimised Gag^{M} immunogen. Restriction enzyme sites used for cloning purposes were avoided within the immunogen coding sequence. The HIV-1C gagM DNA sequences were cloned into pUC18/19-based plasmid vector backbone (Appendix A2) to make pGag-BCG and pGag-

MVA (Figure 2.2; Appendix A3 and A4, respectively). The additional features of pGag-MVA are described in Section 2.2.6.1.

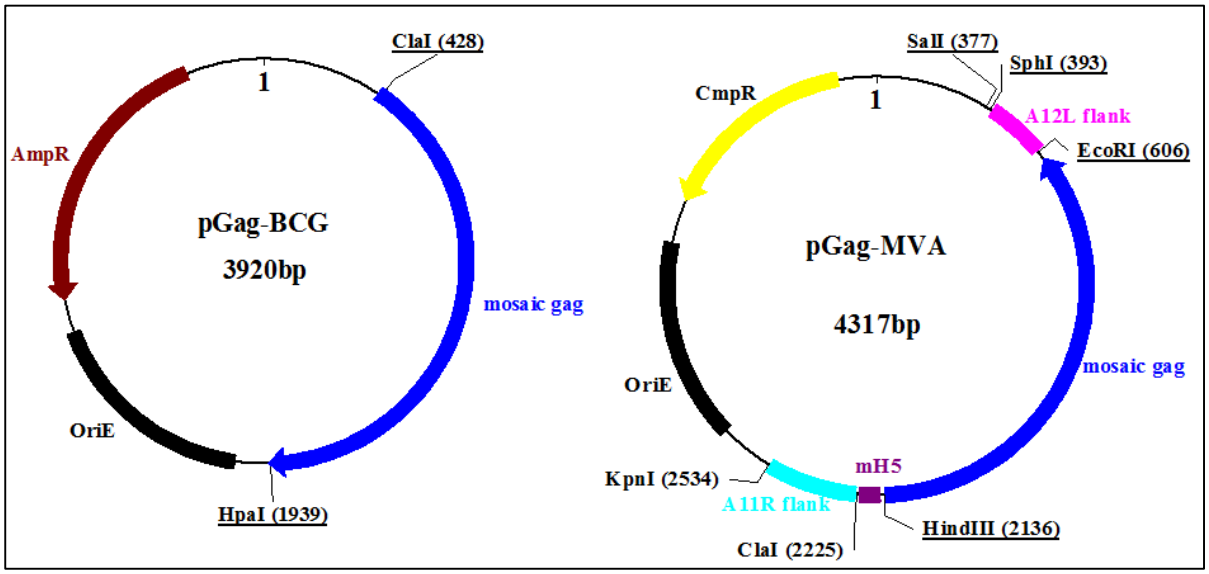


Figure 2.2: Schematic representation of the plasmids pGag-BCG and pGag-MVA. The plasmids were designed as part of this project and synthesised commercially by GeneArt (USA). Restriction sites used for subsequent cloning are indicated in black bold and underlined type. OriE – *E. coli* origin of replication; AmpR – ampicillin resistance; CmpR – chloramphenicol resistance gene; mH5 – modified H5 promoter; A11R and A12L flanks – partial MVA ORFs; the *gag*^{M} gene is indicated in dark blue and the *gag* sequences shown in Appendix B1 and B2.

2.2.2 Source of plasmids, bacteria, viruses, and cell lines

The plasmids, bacteria, viruses and cell lines used in this study are listed in Table 2.1.

2.2.3 Recombinant BCG vaccine construction

2.2.3.1 BCG shuttle vector construction

The BCG*ΔpanCD* was used as one of the vaccine vectors in this study. To facilitate foreign gene expression using BCG, the gene of interest is cloned into a plasmid shuttle vector. The shuttle vector is then transformed into competent BCG cells by means of electroporation. The shuttle vector backbone, pHS400 (Table 2.1; Appendix A5), which has been used previously in our lab for vaccine evaluation in mice and non-human primates (354,364,365) was also used in this study. The cloning procedure used to construct the BCG shuttle vector is outlined in Figure 2.3. To generate the BCG shuttle vector pTJBCG3 (Appendix A7), which contained the *gag*^{M} gene, pGag-BCG was digested with *Cla*I and *Hpa*I (Appendix C1). A 1511bp HIV-1C *gag*^{M} insert was excised from an agarose gel (Appendix C2) using the QIAquick® Gel Extraction kit (Qiagen, Germany)

Table 2.1: Plasmids, bacteria, viruses and cell lines used in this study

	Description	**Source/Reference**
Plasmids		
pHS400	This vector has i) an *Escherichia (E). coli* origin of replication for growth in this bacteria, ii) a kanamycin resistance gene for selection of clones that have the recombinant plasmid, iii) a mycobacterial origin of replication for propagation in *M. bovis* BCG, iv) a *mtrA* promoter which in mycobacteria expresses the immunogen weakly *in vitro* and strongly *in vivo*, and v) a 19kD signal sequence which should result in the expressed immunogen being targeted to the *M. bovis* BCG cell wall and prevent accumulation of the HIV immunogen within the host cell, and to improve antigen recognition in the immunised individual (390). (Appendix A5)	(364)
pTHpCapR	A pTH DNA vaccine with a novel enhancer element for improved antigen expression. (Appendix A1)	(475)
Bacteria		
E. coli DH5α	Has deletions in the following genes to make it highly efficient for transformation procedures: *fhuA2, lac(del)U169, phoA, glnV44, Φ80' lacZ(del)M15, gyrA96 recA1, relA1, endA1, thi-1, hsdR17.* Bethesda Research Laboratories in Taylor *et al*., 1993 (565)	Laboratory stocks
BCGΔpanCD	Pantothenic auxotrophic strain derived from an *M. bovis* Pasteur 1172 P2 strain	Kindly provided by William R Jacobs (364)
Viruses		
Wild type (wt)MVA	A highly attenuated strain of a Turkish VACV	(503). Kindly provided by Bernard Moss, National Institute of Health
MVA-Gag^{N}	MVA expressing a truncated natural HIV-1C Gag (Gag^{N}). The natural HIV-1 isolate was obtained from a commercial sex worker, Du422, in South Africa (309) and the truncation was in the p6 domain. P6 is the most variable region of HIV-1 Gag (124) and has previously been deleted to stabilise the expression and purification of Gag (64,566). Gag^{N} in MVA-Gag^{N} was under the transcriptional control of a VACV mH5 promoter and inserted into the delIII region of MVA.	Constructed by Nicolette Johnston, a former member of our laboratory (364).
Cell lines		
Human embryonic kidney (HEK) - 293 cells	Easily transformable epithelial cells, derived from the kidneys of a human embryo	American Type Culture Collection (ATCC®, USA), CRL-1573™; (567)
Baby hamster kidney (BHK)-21 cells	Fibroblast cells derived from the kidneys of the Syrian golden hamster, *Mesocricetus auratus*	ATCC® (USA), CCL-10™; (568)
HeLa cells	Human epithelial cells derived from the cervix of a patient who had cervical cancer	ATCC® (USA), CCL-2™; (569)

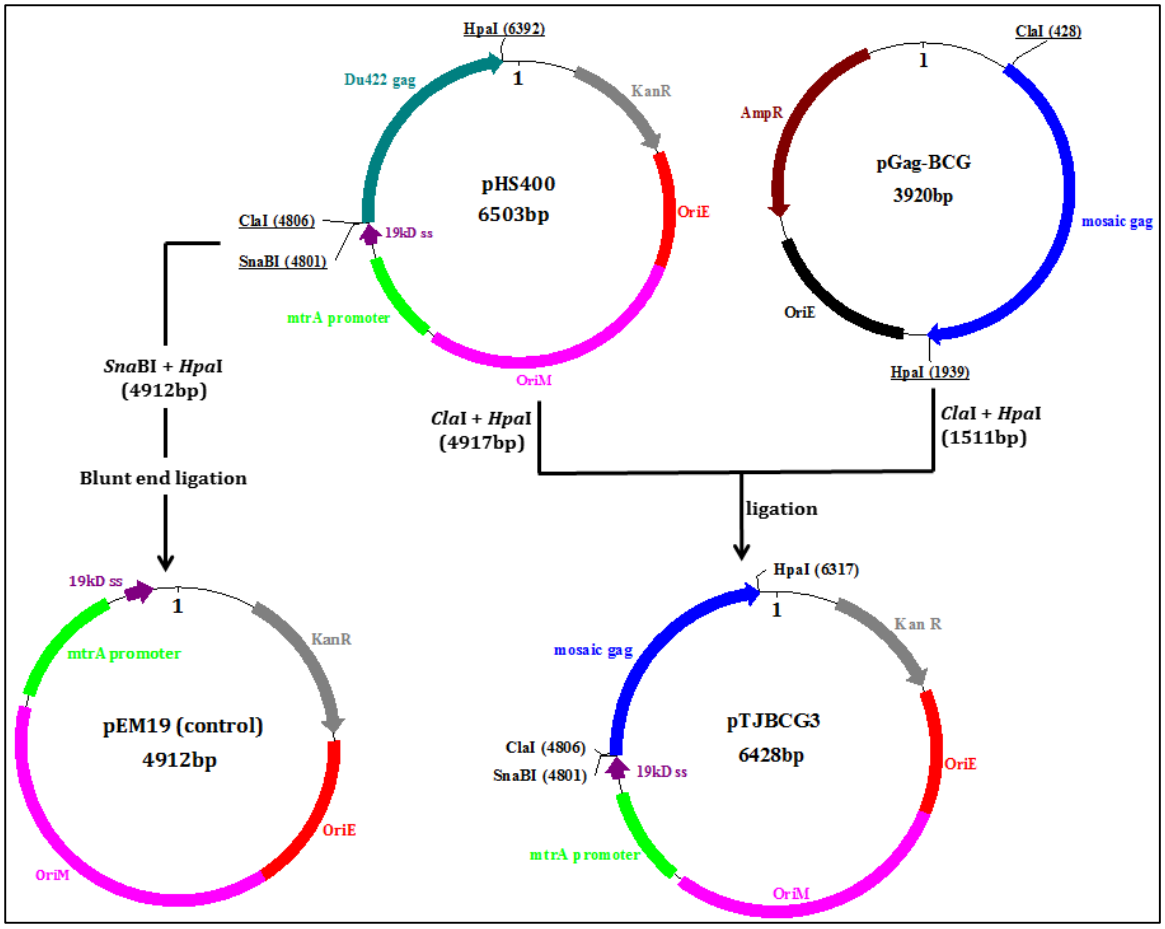


Figure 2.3: Schematic representation of the cloning procedures used to construct the BCG shuttle vectors, pTJBCG3, and pEM19. Restriction sites used for subsequent cloning are indicated in black bold and underlined type. OriE – *E. coli* origin of replication; AmpR – ampicillin resistance; OriM – mycobacterial origin of replication, 19kD ss – 19kD signal sequence; KanR – kanamycin resistance gene. *mtrA* promoter – a promoter of *M. tuberculosis* origin. pGag-BCG was designed as part of this project. pEM19 and pTJBCG3 were constructed as part of this project.

following the manufacturer's instructions and recommendations. The plasmid pHS400 ((364); Appendix A5) was similarly digested and the 4917bp vector backbone ligated to the 1511bp mosaic HIV-1C gag insert. T4 DNA ligase (New England Biolabs, UK) was used for the ligation according to the manufacturer's instructions. A control plasmid that had no insert in it (pEM19; Figure 2.3; Appendix A8) was included in the study. The plasmid pHS400 was digested with *Sna*BI and *Hpa*I (Appendix C1) to generate a plasmid of 4912bp with blunt ends. The plasmid was then used for a blunt end ligation using T4 DNA ligase (New England Biolabs, UK) according the manufacturer's recommendations. Following BCG shuttle vector construction, the plasmids were transformed into competent *E. coli* cells (Appendix C3). To map the plasmids, restriction enzyme digests were carried out on 5µl of plasmid DNA that had been extracted on a small scale

(Appendix C4). The plasmids were then extracted on a larger scale using the High Pure Plasmid Isolation™ kit (Roche, Switzerland) following the manufacturer's instructions and recommendations. To confirm the cloning sites and to confirm that no additional DNA sequence changes were introduced, the shuttle vectors were sequenced using the ABI Prism® BigDye™ Terminator Cycle Sequencing kit (Applied Biosystems, USA) according to the manufacturer's instructions. This was done as a service offered by the Central Analytical Facilities laboratory (Stellenbosch University, South Africa).

2.2.3.2 Preparation of BCG competent cells

Insertion of the shuttle vector into BCG requires that the cells are competent. BCGΔ*panCD* competent cells were prepared as described by Parish and Stoker (2001; (570)) from a culture grown in Middlebrooks 7H9 media (Appendix D1) supplemented with 10% oleic acid-albumin-dextrose-catalase (OADC), 48μg/ml vitamin B5, 0.2% glycerol, and 0.05% Tween-80. A 0.1 volume of 2M glycine (final conc 1.5%) was added to the cell culture 24 hours before harvesting to improve transformation efficiency (571,572). The competent cells were aliquoted into 200 μl aliquots and either used immediately for transformation with shuttle vectors or stored at –80˚C until required.

2.2.3.3 Electroporation of BCG competent cells

Electroporation of BCG competent cells is required to facilitate insertion of the shuttle vector into the cells. Thawed and washed competent BCG cells were mixed with 1μg of recombinant or control plasmid DNA as recommended by Parish and Stoker (2001; (570)). The mixture was placed in a 0.1cm electrode-gap electroporation cuvette (Biorad, USA) and electroporated using the Gene Pulser™ (BioRad, USA) set at voltage: 2.5 kV, Capacitance: 25 μF and resistance: 1000 Ω. The time constants to determine the efficiency of the electroporation were noted. The transformation mix was incubated at 37˚C after adding 1ml of expression media (7H9 media supplemented with 10% OADC, 48μg/ml vitamin B5, and 0.05% Tween-80). Cultures of recombinant BCG were plated on the appropriate selective Middlebrooks 7H10 agar (Appendix D2) containing 10% OADC, 48μg/ml vitamin B5, 0.5% glycerol, 25μg/ml hygromycin, and 10μg/ml kanamycin. Control plates did not contain kanamycin. The plates were sealed in plastic bags to prevent dehydration and incubated at 37˚C for 3 weeks. Recombinant BCG

vaccine stocks were made as previously described (364) and stored at -80˚C until required.

2.2.3.4 Preparation of crude BCG cell lysates

The stability of transgenes in recombinant BCG can be compromised *in vitro* and *in vivo* due to metabolic load. This can be caused by not codon optimising the transgenes (364) or high expression of the recombinant antigen due to the promoter used or strong translational signals (550,573). To evaluate the integrity of the shuttle vector in the vaccine stocks, vaccine stocks were plated onto 7H10 agar (Appendix D2) with antibiotics and incubated at 37°C. Colonies were picked and crude lysates prepared from the obtained BCG colonies or directly from thawed vaccine stocks using a protocol previously developed in our laboratory. To prepare the crude lysate, a recombinant BCG colony was resuspended in 100µl high performance liquid chromatography (HPLC) water. Alternatively, a thawed aliquot of a BCG vaccine stock was centrifuged at 14000rpm (Eppendorf Centrifuge 5417C, Germany) for 2 minutes and resuspended in 100µl HPLC water. The cell suspension was lysed using a FastPrep FP120 machine (Savant, USA) on speed 6 for 20 seconds in the presence of 0.1mm silica beads. The crude extraction cycle was repeated two more times with 2 minute incubations on ice between cycles. Genomic DNA obtained from the supernatant following a 5 minute centrifugation step at 14000rpm (Eppendorf Centrifuge 5417C, Germany) was stored at -20˚C or 5µl used for electroporating into *E. coli* DH5α electro-competent cells (Section 2.2.3.5).

2.2.3.5 Electroporation of E. coli electro-competent cells

The quantities of extracted plasmid from mycobacterial species are often very low (384,574). Thus, the shuttle vectors obtained from the crude lysate (Section 2.2.3.4) may not be sufficient for subsequent analysis. To obtain sufficient quantities of the shuttle vector from crude BCG lysates, propagation in more amenable species like *E.coli* is recommended. The method described by Parish and Stoker (2001; (570)) was used for this. A 5µl aliquot of crude BCG lysate was added to 100µl of thawed *E. coli* electro-competent cells and incubated on ice. The cell and DNA mixture was added to a 0.2cm electroporation cuvette and electroporation was carried out under the following conditions: 200Ω, 2.5kV, and 25µF. Time constants between 4.5 and 5.5 were recorded

as a measure of transformation efficiency. Super Optimal broth with Catabolite repression (SOC) media (900 µl; Appendix D3) was added and the cells were incubated at 37°C with shaking for an hour for expression of the antibiotic resistance genes. The transformation mix was plated onto LB agar plates (Appendix D4) containing the appropriate antibiotic at the recommended concentration and incubated overnight at 37°C. Shuttle vectors were recovered from *E. coli* colonies by small scale plasmid extraction (Appendix C4) and mapped by restriction enzyme digests (Appendix C1).

2.2.3.6 Evaluating plasmid DNA integrity from BCG-vaccinated mice

Since plasmid integrity in BCG can be compromised *in vivo*, it was essential to determine the shuttle vector integrity post vaccination. Thus, to determine the integrity of pTJBCG3 (Appendix A7) and pEM19 (Appendix A8) following mice vaccination with the BCG vaccines (Table 3.1; Section 3.2.1), we used a protocol previously developed in our laboratory. Lymph nodes from each vaccine group were pooled and left over splenocytes from each mice group (Section 3.2.2.1) were homogenised in 1ml BCG resuspension buffer (Appendix D9) using an OMNI tissue homogeniser (OMNI international, USA). A 1 in 10 dilution was made and 100µl plated in triplicate on 7H10 agar plates (Appendix D2). The plates were incubated at 37°C for 18 – 21 days sealed in plastic bags. BCG crude lysates (Section 2.2.3.4) were obtained from a total of 10 individual colonies (5 from the splenocyte homogenate and 5 from the lymph node homogenate) per vaccine group. An aliquot of 5µl DNA from the crude lysate was used as template in a PCR reaction. The PCR components were 25µl PCR ImmoMix Red (Bioline, USA), 10µM each primer (pCB119F: 5' – CAT ATG AAG CGT GGA CTG AC – 3' and pEMRev: 5' – AGC AGA CAG TTT TAT TGT TC - 3') and 10µl distilled water. A negative control was included with no DNA to detect possible contamination of any of the reagents. The PCR reaction conditions were initial denaturation at 95°C for 10 minutes, followed by 30 cycles of DNA denaturation at 95°C for 30 seconds, annealing of primers at 56°C for 30 seconds, and DNA extension at 72°C for one minute with a 5 second increment per cycle. A final extension step at 72°C for 4 minutes completed the reaction. The PCR products were stored at 4°C or analysed by agarose gel electrophoresis (Appendix C2) immediately. PCR products were also sequenced to confirm shuttle vector integrity. This was done as a service offered by the Central Analytical Facilities laboratory (Stellenbosch University, South Africa).

2.2.4 DNA vaccine construction

DNA vaccines are simple to make and manufacture (Section 1.12.2). To make such vaccines, a gene of interest is cloned into a chosen vector backbone. Three DNA vaccines were constructed for this project; pTJDNA4, pTJDNA5, and pTJDNA6 (Appendix A9, 10, and 11 respectively), and cloning procedure is illustrated in Figure 2.4. The pTHpCapR vector backbone was obtained from an *Eco*RI/ *Hin*dIII digest (Appendix C1) of pTHpCapRgrttnC ((475); Appendix A1). The 5008bp vector backbone fragment was excised from an agarose gel using a dark reader spot lamp (Clare Chemical Research, USA) and recovered using the QIAquick Gel Extration kit (Qiagen, Germany) following the manufacturer's instructions and recommendations.

To generate plasmid pTJDNA4 which contained the *gag*^{M} gene, pGag-MVA was digested with *Eco*RI, *Hin*dIII, and *Kpn*I (Appendix C1). The DNA was purified as detailed in Appendix C5 and used for ligation with the gel extracted pTHpCapR vector backbone. The *Kpn*I restriction site lies upstream of the A11R gene in pGag-MVA, outside of the mosaic HIV-1 *gag* sequence. The *Kpn*I restriction enzyme was therefore included to reduce the chances of pGag-MVA re-ligating to itself and to reduce the chances of the pGag-MVA backbone ligating into the pTHpCapR vector backbone.

pTJDNA5 contains no insert and was used as control. It was obtained by blunting the *Eco*RI and *Hin*dIII sites of the gel extracted pTHpCapR vector backbone using T4 DNA polymerase (Thermo Scientific, USA) following the manufacturer's instructions. The DNA polymerase was inactivated for 10 minutes at 75°C, and 20ng of plasmid used for a blunt end ligation using T4 DNA ligase (New England Biolabs, UK) according the manufacturer's recommendations.

The plasmid pTJDNA6 contains a full length *gag*^{N} gene derived from the HIV-1C strain Du442 (309). The full length *gag*^{N} gene was previously cloned into pTHgagMr (Appendix A13) by Dr Nyasha Chin'ombe, a former member of the Medical Virology division at UCT. pTHgagMr was digested with *Eco*RI, *Hin*dIII, and *Bgl*II (Appendix C1). The DNA was purified as detailed in Appendix C5 and used for ligation with the gel extracted 5008bp pTHpCapR vector backbone. The *Bgl*II restriction site lies downstream of the AmpR gene in pTHgagMr, outside of the HIV-1 *gag*^{M} sequence.

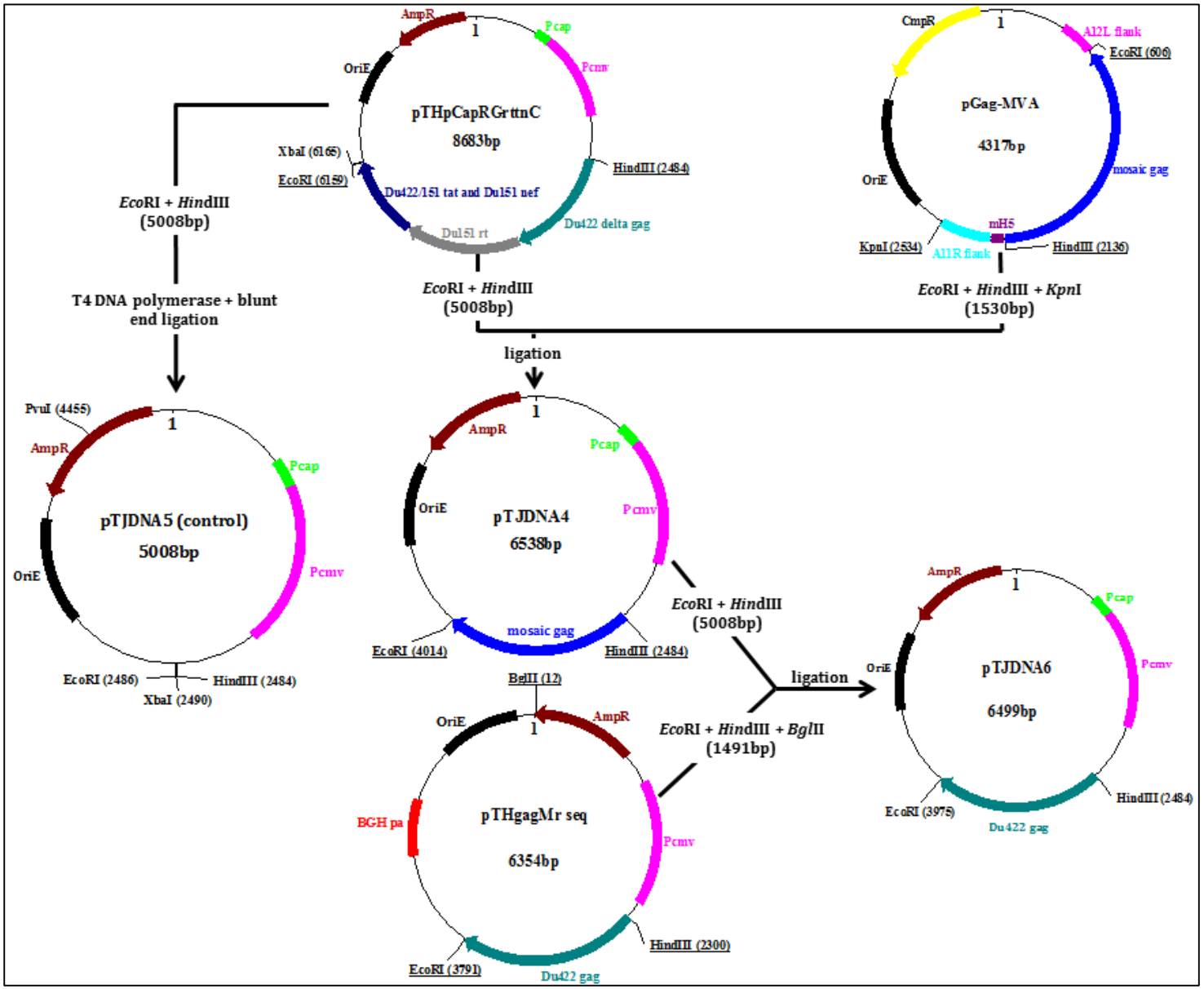


Figure 2.4: Schematic representation of the cloning procedures used to construct the DNA vaccines pTJDNA4, pTJDNA5, and pTJDNA6. The pTHpCapR vector backbone [45] was used for constructing the DNA-based vaccines used in this project. pTJDNA5 has no insert and was used as a negative control, pTJDNA4 has the *gag*^{M} and pTJDNA6 has a natural Du422 *gag* cloned in the *Eco*RI and *Hin*dIII sites of the pTHpCapR vector backbone. Pcap – porcine circovirus enhancer element; Pcmv – CMV promoter; CmpR – Chloramphenicol resistance gene; AmpR – ampicillin resistance gene; OriE; *E. coli* plasmid origin of replication; A11R and A12L flanks – partial MVA ORFs. pGag-MVA was designed as part of this project. pTJDNA4, 5, and 6 were constructed as part of this project.

The BglII restriction enzyme was therefore included to reduce the chances of pTHgagMr re-ligating to itself and to reduce the chances of the pTHgagMr backbone ligating into the pTHpCapR vector backbone. The extracted fragments were ligated overnight using T4DNA ligase (New England Biolabs, UK) according the manufacturer's recommendations. The DNA was purified as detailed in Appendix C5 and the 1530bp fragment used for ligation with the gel extracted pTHpCapR vector backbone.

Following the ligation experiments, the DNA plasmids were transformed into *E. coli* DH5α cells (Appendix C3). To map the plasmids, restriction enzyme digests (Appendix C1) were carried out on plasmid that had been extracted on a small scale (Appendix C4). The plasmids were then extracted on a larger scale using the EndoFree Plasmid Maxi kit® (Qiagen, Germany) following the manufacturer's instructions and recommendations. This plasmid extraction kit was used to exclude endotoxin contamination for subsequent cell culture experiments (Section 2.2.5). To confirm the cloning sites and to confirm that no additional DNA sequence changes were introduced, the plasmids were sequenced using the ABI Prism® BigDye™ Terminator Cycle Sequencing kit (Applied Biosystems, USA) according to the manufacturer's instructions. This was done as a service offered by the Central Analytical Facilities laboratory (Stellenbosch University, South Africa). DNA vaccine stocks were prepared commercially by Aldevron (USA) and aliquots stored at -80˚C until required.

2.2.5 Cell culture

2.2.5.1 Thawing and culturing of cell lines

Various cell lines (Table 2.1) were used for different assays in this study. The cells were stored at -80˚C and required thawing and maintenance. The cells were thawed by incubation in a 37˚C water bath as recommended by our laboratory. This was followed by resuspension in 10ml of pre-warmed (37˚C) Dulbecco's Modified Eagle's medium (DMEM)-10 (DMEM supplemented with 10% fetal calf serum (FCS; Biochrom, England), 1000U/ml penicillin (Lonza, Belgium), 1000U/ml streptomycin (Lonza, Belgium), and 10µg/ml fungin (Invivogen, USA)) as soon as the cells had thawed. Cells were subjected to centrifugation at 1500rpm (Eppendorf Centrifuge 5810, Germany) for 5 minutes and re-suspended in DMEM-10. The cells were seeded into tissue culture (TC) flasks and

incubated at 37˚C in a 5% humidified CO_2 incubator until the required confluence was reached.

2.2.5.2 Trypsinizing, counting, maintaining, and freezing cell lines

After cells reached 80 – 90% confluence, they needed to be detached from the TC flasks by trypsinizing for maintenance, counting, or to make frozen stocks. To trypsinize the cells, the protocol described by Doyle and Griffith (1998; (574)) was followed. The cells were washed twice with phosphate buffered saline (PBS) and incubated with a trypsin/EDTA solution (Lonza, Belgium; diluted 1 in 10 in PBS) for 5 minutes at 37˚C. The trypsin was deactivated by adding DMEM-10. A 1 in 2 dilution of the cells was stained with Trypan Blue (Life Technologies, USA) and counted using a Neubauer cell counting chamber (Marienfield, Germany) and an Olympus Optical C011 microscope (Olympus, Japan). Cell numbers were adjusted to seed flasks or TC plates and the cells were incubated at 37˚C, 5% CO2 in DMEM-10.

For general maintenance and growth, cell lines were cultured in DMEM-10. To make frozen cell stocks, the cells were detached from TC flasks using a trypsin/EDTA solution. The cells were then harvested by centrifugation at 1500rpm (Eppendorf Centrifuge 5810, Germany) for 5 minutes. The pellet was resuspended in FCS with 10% dimethyl sulfoxide (DMSO). Cells were counted and readjusted to 2 x 10^6 cells/ml with FCS containing 10% DMSO. The cells were stored in 1ml aliquots at -80˚C.

2.2.6 Recombinant MVA construction

2.2.6.1 Transfer vector construction

Recombinant (r) MVA vaccine construction requires the gene of interest to first be inserted in a transfer vector (Figures 2.1 and 2.5). The transfer vector pTJMVA2 was constructed from two plasmids, pGag-MVA and pGFP-BSD. The transfer vector was designed to insert the *gag*^{M} gene into the conserved region of MVA in a two-step recombination process between the A11R (encoding a 36.1kD putative protein; (485)) and A12L (which encodes a 20kD virion protein; (485)) transcriptionally convergent open reading frames. The vaccinia virus mH5 transcriptional promoter (484) was

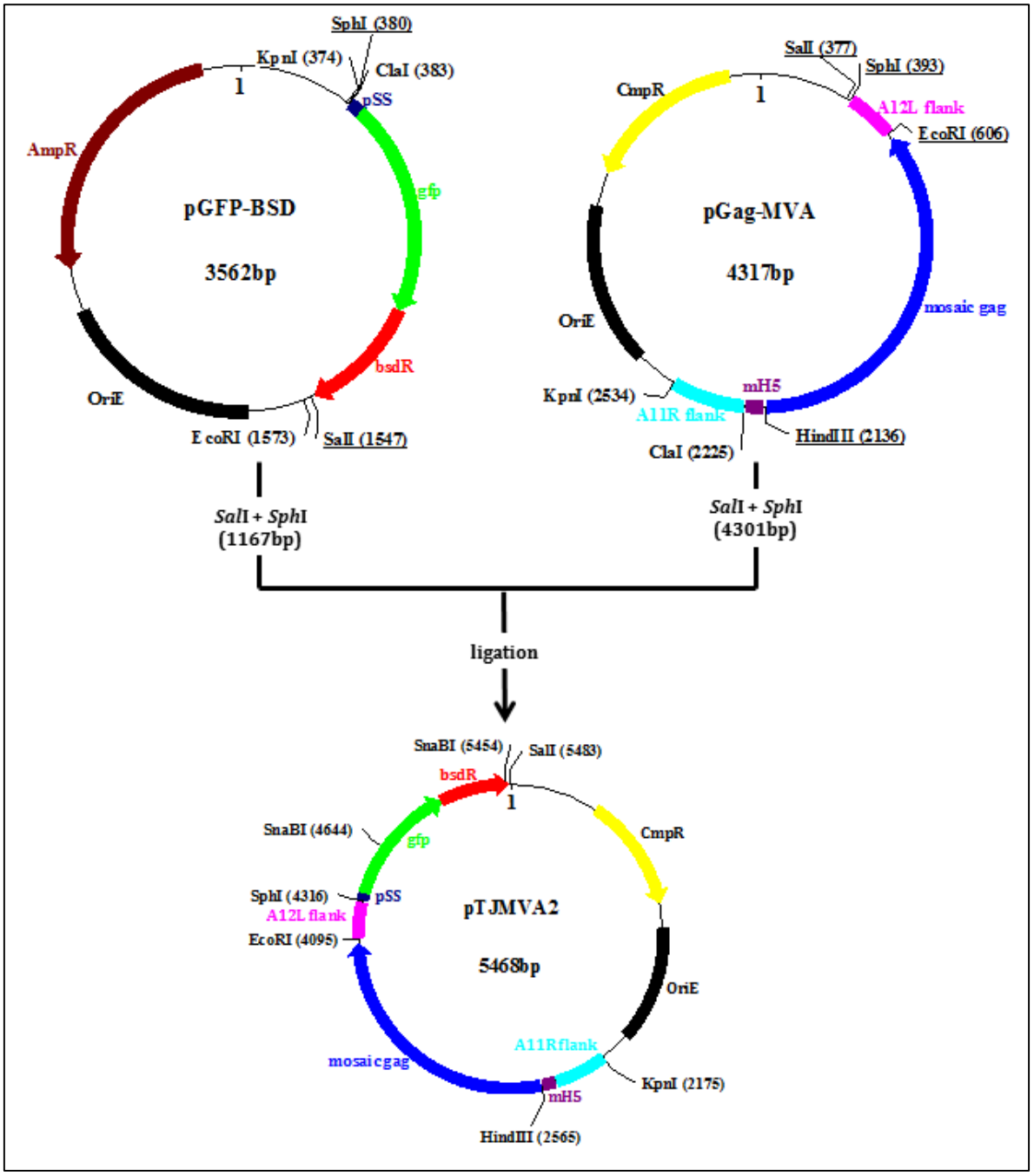


Figure 2.5: Schematic representation of the construction of the transfer vector pTJMVA2 from the plasmids pGFP-BSD and pGag-MVA. Following an *Sph*I/*Sal*I restriction digest of pGFP-BSD, the 1167bp *gfp-bsd* fusion gene was cloned into a similarly digested pGag-MVA. pSS – MVA synthetic promoter promoter; gfp –green fluorescent protein gene; bsdR – blasticidin resistance; OriE – *E. coli* plasmid origin of replication; AmpR – ampicillin resistance; CmpR – chloramphenicol resistance gene; bsdR – blasticidin resistance gene; mH5 – modified H5 promoter; A11R and A12L flanks– partial MVA ORFs. pGag-MVA and pGFP-BSD were designed as part of this project. pTJMVA2 was constructed as part of this project.

included upstream of the gene. For cloning purposes, unique restriction enzyme sites were included upstream and downstream of the mH5 promoter, and flanking sequences. The transfer vector pTJMVA2 was obtained from cloning the *gfp-bsd* fusion gene from pGFP-BSD into the *Sal*I/*Sph*I sites of pGag-MVA (Figure 2.5) and was

designed in such a way that the selection and marker genes would be deleted following the final recombination event. BSD expression resulted in the selection of the first recombinant which expressed GFP. The final recombinant could be isolated as a non-fluorescing plaque.

Following the ligation experiments, the transfer vector was transformed into *E. coli* DH5α cells (Appendix C3). To map the plasmid, restriction enzyme digests (Appendix C1) were carried out on plasmid that had been extracted on a small scale (Appendix C4). The plasmids were then extracted on a larger scale using the EndoFree Plasmid Maxi kit® (Qiagen, Germany) following the manufacturer's instructions and recommendations. This plasmid extraction kit was used to exclude endotoxin contamination for subsequent cell culture experiments (Section 2.2.5). To confirm the cloning sites and to confirm that no additional DNA sequence changes were introduced, the transfer vector was sequenced using the ABI Prism® BigDye™ Terminator Cycle Sequencing kit (Applied Biosystems, USA) according to the manufacturer's instructions. This was done as a service offered by the Central Analytical Facilities laboratory (Stellenbosch University, South Africa).

2.2.6.2 Generation of a BSD kill curve

The BSD antibiotic (Invitrogen, USA) was used for the positive selection of intermediate recombinants during the construction of MVA expressing the HIV-1C (MVA-Gag^{M}). Any cells containing the intermediate recombinant would express the *bsd* gene and survive in the presence of the antibiotic (575). In order to optimise this selection, it was necessary to determine the optimal concentration that would prevent the growth of cells without the presence of the selection gene. To determine the optimal BSD concentration, 1 x 10^5 BHK-21 cells per well were seeded in a 12 well TC plate. BSD was added at various concentrations (2.5, 5, 7.5, 10, and 15µg/ml in DMEM-2 (DMEM supplemented with 2% FCS (Biochrom, England), 1000U/ml penicillin (Lonza, Belgium), 1000U/ml streptomycin (Lonza, Belgium), and 10µg/ml fungin (Invivogen, USA)) in triplicate. BHK -21 cells with no BSD were included as a negative control. Cells were observed daily for viability using a light microscope (Olympus Optical CK2, Japan) up to 3 days. The optimal concentration was determined as the minimum concentration required to kill all cells within 3 days.

2.2.6.3 MVA virus infection of cells

Adherent cell monolayers were used for MVA or recombinant MVA infection. When cells were 60-70% confluent, as estimated by visual examination using an inverted Olympus Optical CK2 microscope (Olympus, Japan) in TC plates, they were infected with wtMVA (multiplicity of infection (MOI) = 0.1) diluted in DMEM-0 (DMEM supplemented with 1000U/ml penicillin (Lonza, Belgium), 1000U/ml streptomycin (Lonza, Belgium), and 10µg/ml fungin (Invivogen, USA) only; 100µl/well for 12-well plates and 500µl/well for 6-well plates) and left to adsorb for 2 hours at 37˚C. The inoculum was then removed and fresh DMEM-2 was added. Cells were incubated at 37˚C in a 5% CO_2 humidified incubator for 72 hours post infection (h.p.i.), unless otherwise stated.

2.2.6.4 Transfection

Transfection was carried out to fascilitate the recombination of wtMVA with the transfer plasmid pTJMVA2 within a permissive cell line as depicted in Figure 2.1. A total of 5 x 10^4 cells were seeded per well in 6 well plates and adhered to the TC plates overnight at 37˚C in a 5% CO_2 humidified incubator. The cells were infected with MVA (Section 2.2.6.3) and then transfected with a 1:1 ratio of 4µg of recombinant plasmid DNA: 4µl X-treme Gene HP® transfection reagent obtained from Roche (Switzerland) according to manufacturer's instructions. Cells were incubated at 37˚C in a 5% CO_2 humidified incubator for 48 hours.

2.2.6.5 Plaque purification

Following cell infection (Section 2.2.6.3) and transfection (Section 2.2.6.4), individual plaques were picked to initially obtain intermediate recombinants (single cross-over), and then eventually to obtain final recombinants (with double cross-over). The intermediate recombinants contained the transfer vector and formed fluorescent plaques. The final recombinants, on the other hand do not contain the selection or marker genes and the plaques do not fluoresce. Intermediate fluorescing plaques were initially picked and passaged in the presence of 10µg/ml BSD to eliminate any contaminating wild type MVA virus. Before picking plaques, the plates were viewed using a fluorescence microscope (Zeiss, Germany) to select plaques with rMVA. The positions of plaques showing green fluorescence were marked from beneath the plate with a permanent marker. Medium was aspirated from infected cells in TC plates. The

cells were then washed twice with 500µl PBS and the marked plaques were picked by scraping the cells from the marked positions with a pipette tip and transferred into 300µl of DMEM-0. The recombinant MVA virus was released from the cells by doing three freeze thaw cycles and vortexing after each cycle. The lysates were stored at -20˚C or used immediately, using 10-fold serial dilutions, to infect a fresh monolayer of cells. The use of BSD in the culture media was stopped after an intermediate recombinant was identified by PCR. After this, non-fluorescent plaques were picked and lysates prepared for PCR (Section 2.2.6.6). At this stage, recombination produces either wild type MVA or the desired recombinant MVA virus containing the Gag^{M} insert (See Section 2.3.3 in Results). The lysate was passaged three more times until a final recombinant was identified by PCR.

2.2.6.6 Polymerase chain reaction of recombinant MVA

The polymerase chain reaction (PCR) was carried out to determine the presence of the intermediate and final MVA recombinants in cell lysates of infected cells. A final recombinant, MVA-Gag^{M}, was isolated after a two-step procedure of isolating an intermediate recombinant containing the entire transfer vector (pTJMVA2), followed by the deletion of the *gfp-bsd* gene fusion to give a final MVA recombinant containing the HIV-1C gag^{M} sequence only. The presence of the intermediate recombinant MVA was confirmed by PCR amplification using a *gag*-specific primer in combination with a primer which bound adjacent to the insertion site in the MVA genome (Table 2.2; Figure 2.14 in Results section). The presence of the final recombinant was confirmed by PCR using primers that were located on either side of the insertion site in MVA (Table 2.2; Figure 2.14 in Results section). The cell lysate for PCR was obtained using a method described by David Tscharke (personal communication). BHK-21 cell monolayers in 12-well TC plates were infected (Section 2.2.6.3) using 100µl of 10- fold dilutions of freeze/thawed suspensions of picked non-fluorescent plaques (Section 2.2.6.5). After two to three days at 37˚C in a 5% CO_2 humidified incubator, when plaques were visible, the culture medium was aspirated and the cells gently washed with 1ml PBS/well. A volume of 250µl 1 X PCR buffer (Appendix D5) with proteinase K (10µg/ml; Sigma-Aldrich, USA) was added to the cells. The cells in the TC plate were incubated at -80˚C until frozen, and then thawed at 37˚C. Cell lysates were transferred to clean and well labelled eppendorf tubes and incubated in a 56˚C water bath for 20 minutes.

Table 2.2: Primers and PCR assays used for detecting intermediate and final MVA recombinants

Primer sets and MVA recombinant detected	**Primer name**	**Primer sequence (5 ' → 3')**	**Primer target**	***PCR cycle (X 25)**	**Product size (bp)**
Set 1: Intermediate 1	A11R*for* gfp*rev*	ACA AAC ACC ATC CTT GGG AGTA AAA GTT CTT CAC CCT TAG ACG CC	A11R (upstream of flank) gfp	95˚C (30'') 60˚C (30'') 72˚C (40'')	1100
Set 2: Final recombinant/ Intermediate 1	gag*for* A12L*rev*	CCC TAG AAA GAA AGG CTG CTG GAA AAT CGG TGG AGA TGC AGC CGT CAA	gag A12L (downstream of flank)	95˚C (30'') 60˚C (30'') 72˚C (40'')	791
Set 3: Final recombinant/ wtMVA	A11R*for* A12L*rev*	ACA AAC ACC ATC CTT GGG AGTA AAT CGG TGG AGA TGC AGC CGT CAA	A11R (upstream of flank) A12L (downstream of flank)	95˚C (30'') 60˚C (30'') 72˚C (2')	Final recombinant 3042/ wtMVA 1014
Set 4: Final recombinant/ Intermediate 2	A11R*for* gag*rev*	ACA AAC ACC ATC CTT GGG AGTA TTC TTT CCG CCA GGC CTC AGT	A11R (upstream of flank) gag	95˚C (30'') 60˚C (30'') 72˚C (40'')	724
Set 5: Intermediate 2	colE1*for* A12L*rev*	GCG TGA GCT ATG AGA AAG CGC CA AAT CGG TGG AGA TGC AGC CGT CAA	*E. coli* origin of replication A12L (downstream of flank)	95˚C (30'') 60˚C (30'') 72˚C (40'')	1211

Initial denaturation was 95˚C for 10 minutes and final elongation was 72˚C for 5 minutes for all assays. Final elongation time was 7 minutes for set 3 PCR to detect the final recombinant. 25 PCR cycles were performed as indicated in column 4.

The proteinase K activity was inactivated by incubating the lysates in an 85°C water bath for 10 minutes. Cell debris was removed by centrifuging the lysate for 10 minutes at 2000 rpm (Eppendorf Centrifuge 5417C, Germany) and transferring the supernatant to a clean, well labelled eppendorf tube. A 5 – 10µl aliquot was used for PCR using the ImmoMix Red PCR mix (Bioline, USA) according to the manufacturer's instructions. The PCR conditions for each primer set are listed in Table 2.2. The PCR product for the final recombinant was confirmed by sequencing using the ABI Prism® BigDye™ Terminator Cycle Sequencing kit (Applied Biosystems, USA) according to the manufacturer's instructions. This was done as a service offered by the Central Analytical Facilities laboratory (Stellenbosch University, South Africa).

2.2.6.7 Large scale recombinant MVA virus preparation

To generate MVA-Gag^{M} vaccine stocks for animal immunisations and vaccine characterisation, BHK-21 cells were infected with recombinant MVA at an MOI of 0.01 in T75cm^2 TC flasks. When all the cells were infected after 3 days, they were freeze-thawed three times. The media was transferred to McCartney bottles and cell debris removed by centrifugation at 1400rpm (Boeco U-320, Germany) for 30 minutes. MVA-Gag^{M} was harvested by high speed centrifugation at 15000rpm (Sorval RCSC Plus, USA), 4°C for 1 hour on a sucrose gradient (36% in PBS). The pellet was resuspended in 10ml PBS and centrifuged at high speed for an hour at 4°C on a sucrose-dextran gradient (36% sucrose; 10% dextran). The harvested MVA-Gag^{M} was resuspended in 500µl PBS and stored in aliquots at -80°C until required. MVA-Gag^{M} virus titration was done on BHK-21 cells as previously described by Chapman *et al*., 2012 (364).

2.2.7 Confirmation of HIV-1C Gag expression

The Gag^{M} immunogen sequence is computationally generated. To determine its ability to be expressed, various experiments were carried out as part of this study.

2.2.7.1 Lysate preparation and protein concentration determination

Total protein lysates from infected (Section 2.2.6.3) or transfected (Section 2.2.6.4) cells were obtained to detect HIV-1C Gag^{M} expression. A lysis buffer was made by dissolving a protease inhibitor tablet (c***O***mplete EDTA-free®; Roche, Switzerland) in 1ml distilled water and adding 400µl of the solution to 10ml of Glo Lysis buffer (Promega, USA). The protease inhibitor was added to prevent protein degradation from protease enzymes. A

volume of 200µl of the lysis buffer was added to infected or transfected cell monolayers that had been washed twice with 1ml PBS/well. After a 5 minute incubation at room temperature with the lysis buffer, the lysate and debris was transferred to clean, well-labelled eppendorf tubes, and centrifuged at 4°C for 10 minutes at 13 000rpm (Eppendorf Centrifuge 5417C, Germany). The supernatant was used immediately or stored at -80°C in 50µl aliquots. The protein concentration of samples was determined by using the Bio-Rad DC protein assay kit (Bio-Rad, USA) using the manufacturer's recommendations, and the absorbance readings were measured at 750nm using a VERSAmax microplate reader (Molecular Devices, USA).

2.2.7.2 Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) and Western blot analysis

To confirm the expression of the correct sized protein (55kD) from the DNA and MVA vaccines made, proteins obtained from cell lysates (Section 2.2.7.1) were separated by SDS-PAGE using 12.5% denaturing polyacrylamide gels (Appendix C6; BioRad, USA) and subjected to Western blot analysis as outlined in Appendix C7. This was done using cell lysates obtained from DNA-transfected HEK-293 (Section 2.2.6.4) and from MVA-Gag^{M}-infected BHK-21 cells (Section 2.2.6.3) and Gag was detected using HIV-1 gag p24-specific anti-serum (ARP432, NIBSC Centralised Facility for AIDS Reagents, MRC, England).

2.2.7.3 Immunostaining

To detect the presence of Gag in infected cells and to titrate wt and recombinant MVA, immunostaining was performed. Immunostaining of BHK-21 and HeLa cells infected at an MOI of 1 with wtMVA, MVA-Gag^{M} , or MVA-Gag^{N} was done as described by Chapman *et al*., 2012 (364). This was done to determine the expression of the HIV-1C Gag^{M} in permissive (BHK-21) and non-permissive (HeLa) cells at 12, 18, 24, 30, and 48h.p.i. The experiment was also done to determine the optimal time point to prepare samples for electron microscopy (Section 2.2.7.5).

2.2.7.4 p24 ELISA assay

The p24 ELISA assay was carried out as described by Tanzer *et al*., 2011 (475) to confirm expression of the HIV-1C Gag^{M} and to determine the optimal time to set up

samples for electron microscopy (Section 2.2.7.5). HeLa cells ($0.2x10^6$ cells/well) and BHK-21 cells ($0.3x10^6$ cells/well) were infected at an MOI of 1 with wtMVA, MVA-Gag^N or MVA-Gag^M in 6-well TC plates (Section 2.2.6.3). Uninfected cells were included as controls. Supernatants and lysates were obtained at 3, 6, 9, 12, 18, 24, and 30h.p.i. to detect HIV-1 p24. Protein concentrations were determined by using the Bio-Rad DC protein assay kit (Bio-Rad, USA) using the manufacturer's recommendations. The absorbance readings were measured at 750nm using a VERSAmax microplate reader (Molecular Devices, USA). A total of 1µg protein from the lysates in 200µl distilled water was used for p24 detection. Capture ELISA was done using a MiniVidas machine (bioMérieux, France) and an Elecsys HIV Ag® (p24) kit (Roche, Switzerland). This was done as a service offered by the National Health Laboratory Service (NHLS, Cape Town). A 200µl aliquot of the supernatant was used for p24 detection. Where necessary, 1 in 10 dilutions of the samples were made to obtain readings within the limits of the p24 detection kit.

2.2.7.5 Electron microscopy

HIV-1 $Pr55^{Gag}$ can self-assemble and form VLPs without other HIV-1 proteins. The VLPs look like immature HIV-1 and bud from the membrane of the infected cell into the culture medium (Section 1.5.1; Figure 1.4). Electron microscopy was used to determine the ability of the HIV-1C Gag^M immunogen to bud and form VLPs in permissive and non-permissive cell lines. Cells were infected (Section 2.2.6.3) with an MOI = 5 of wtMVA, MVA-Gag^N or MVA-Gag^M in duplicate. A negative control of uninfected cells was also included. A total of $2x10^5$ BHK-21 cells/well and $3x10^5$ HeLa cells/well in 6-well TC plates were used for the infection. At 6, 12, and 48h.p.i, the cells were pooled and pelleted at 13000rpm (Eppendorf Centrifuge 5417C, Germany) for 3 minutes. The cells were washed in 1 ml PBSand then fixed *in situ* at 4˚C overnight with 2.5% ice cold gluteraldehyde (Merck, Germany) diluted in PBS, pH 7.4, at 10X the volume of the pellet. Fixed cells were pelleted at 5000rpm (Eppendorf Centrifuge 5417C, Germany), washed twice in 200µl PBS, and resuspended in 10-20µl of 2% low melting point agarose (Lonza, Belgium) depending on the pellet size. To enhance the electron density of the protein material, samples were incubated in 500µl of 0.5% Tannic acid (Sigma-Aldrich, USA; diluted in distilled water) for 1hour at room temperature and washed once in 1 X PBS prior to the post-fixation step with osmium tetroxide (Sigma-Aldrich, USA).

Samples were washed in 500µl of 2 X PBS for 5 minutes and once in 500µl of distilled water for 5 minutes. This was followed by dehydration in an ethanol gradient (30%, 50%, 70%, 80%, 90%, 95%, 100%) for 10min in each ethanol dilution. Samples were incubated a second time in 100% ethanol for 10 minutes, and twice in acetone for 10 minutes. The samples were incubated overnight in 400µl of a 1:1 acetone: Agar Low Viscosity Resin (Agar Scientific, UK) mixture, followed by an 8 hour incubation in a 1:3 acetone: resin mixture. After an overnight incubation in 100% resin, the samples were incubated for a further 2hours on in fresh 100% resin, oriented into moulds, topped up with more resin, and polymerised at 60^{o}C for 24 hours. Thin sections were cut using a diamond knife as a service provided by the UCT Electron Microscope Unit (UCT, South Africa), stained in 1% uranyl acetate (Agar Scientific, UK) and lead citrate (Agar Scientific, UK), and viewed using a FEI Tecnai 20 transmission electron microscope (FEI, Netherlands).

2.2.8 Summary of vaccines made in this project

A list of the plasmids and vaccines made in this project is given in Table 2.3.

Table 2.3: Vaccines made and used in this study

Vaccine name	Vector	Insert	Vector name
BCG-Gag^{M}	BCGΔ*panCD*	HIV-1C *gag*^{M}	pTJBCG3
BCG^{E}	BCGΔ*panCD*	None	pEM19
MVA-Gag^{M}	MVA	HIV-1C *gag*^{M}	pTJMVA2
MVA-Gag^{N}	*MVA	HIV-1C Du422 Δ*gag*	*
DNA-Gag^{M}	DNA (pTHpCapR)	HIV-1C *gag*^{M}	pTJDNA4
DNA^{E}	DNA (pTHpCapR)	None	pTJDNA5
DNA-Gag^{N}	DNA (pTHpCapR)	HIV-1C *gag*^{N}	pTJDNA6

*This vector and vaccine was previously made by Nicolette Johnston a former member of the Medical Virology division at UCT (364).

2.3 RESULTS

2.3.1 BCG shuttle vector construction and the confirmation of plasmid integrity of BCG vaccines prior to and post vaccination

The BCG shuttle vectors, pTJBCG3 (expressing a monovalent HIV-1C Gag^{M} immunogen) and a control vector pEM19 lacking the *gag* gene were constructed (Figure 2.6; Appendix A7 and A8), electroporated into competent BCGΔ*panCD*, and vaccine stocks

generated. As the stability of recombinant BCG (rBCG) can be compromised *in vitro* and *in vivo* (reviewed by Chapman *et al.*, 2010; (362)), the genetic integrity of the rBCG vaccine stocks was assessed by restriction enzyme mapping of the shuttle vectors using *Xho*I (pTJBCG3) and *Sma*I (pEM19). Plasmid DNA isolated from vaccine stocks of BCG-Gag^{M} and BCG^{E} gave DNA fragments of the expected size, following restriction digestion (Figures 2.7A and B).

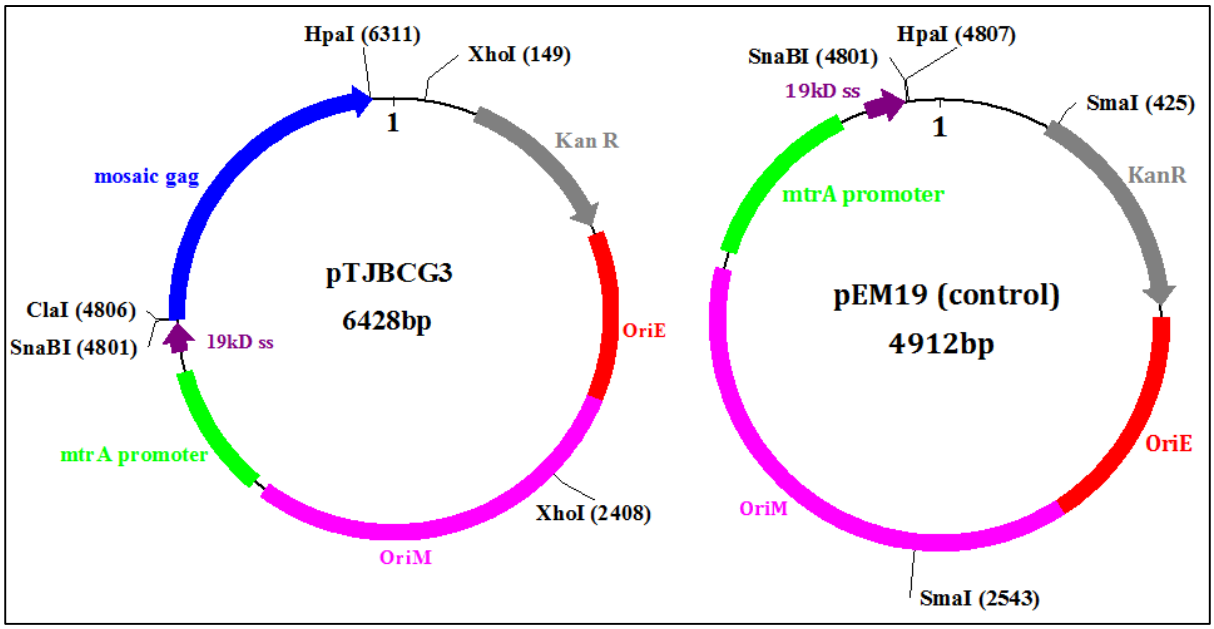


Figure 2.6: Schematic representation of the BCG shuttle vector pTJBCG3 and the control vector pEM19. Restriction sites used for cloning and restriction mapping analysis are indicated in black bold type.OriE – *E. coli* origin of replication; OriM – mycobacterial origin of replication, 19kD ss – 19kD signal sequence; Kan R – kanamycin resistance gene.

The genetic integrity of the BCG shuttle vectors *in vivo* was determined by isolating mice spleens and lymph nodes 11.5 weeks post vaccination. The organs were homogenised and plated onto MiddleBrook 7H10 agar plates (Appendix D2) supplemented with vitamin B, hygromycin and OADC. Plasmid DNA was isolated from rBCGΔ*panCD* colonies (Section 2.2.3.4) and the *gag*^{M} gene amplified by PCR to determine plasmid integrity (Section 2.2.3.6). The primers pCB119F and pEMRev that bind to the 19kD leader sequence and upstream of the kanamycin resistance gene, respectively (365), were used in the PCR amplification. PCR products of the expected sizes of 344bp and 1869bp were obtained from pEM19 and pTJBCG3 respectively. Eight PCR products were also sequenced to confirm genetic integrity and no mutations were observed (data not shown). The shuttle vectors within the recombinant BCGs were found to have maintained their integrity before and after immunisation (Figures 2.7C and D).

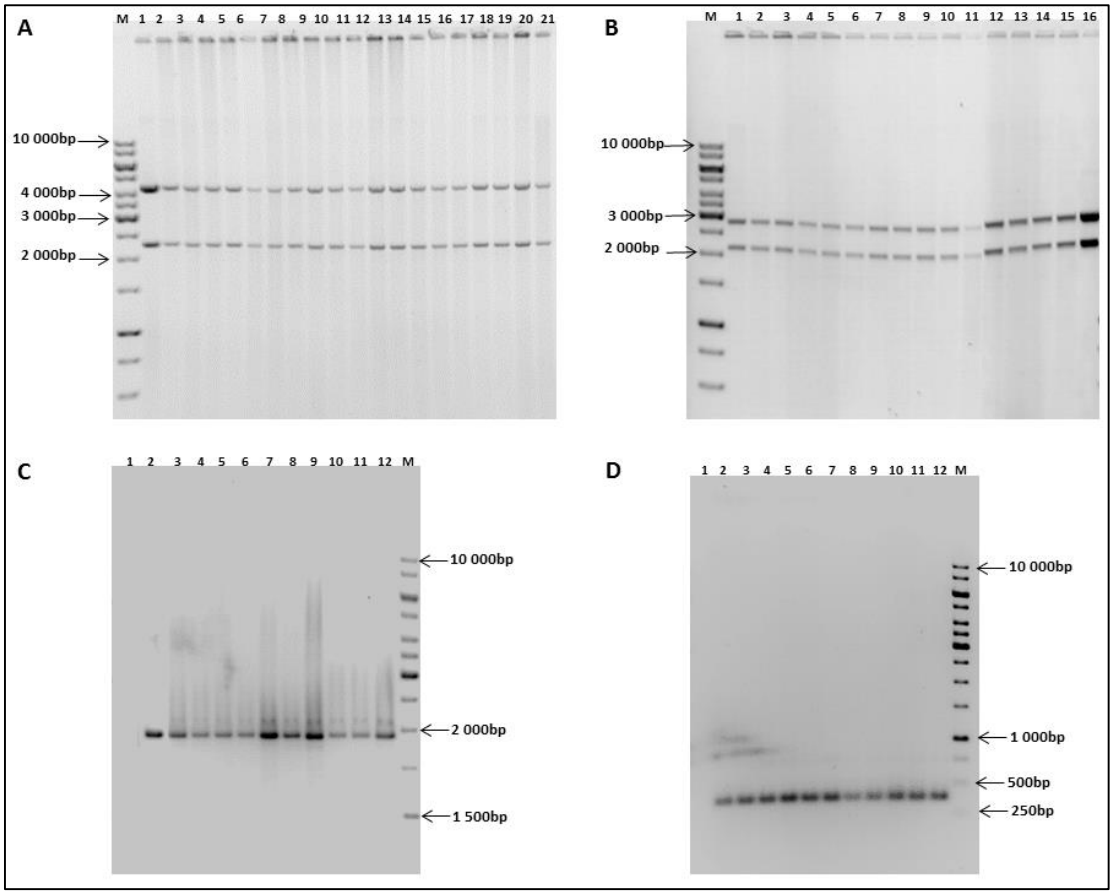


Figure 2.7: Genetic integrity of plasmids pTJBCG3 and pEM19 isolated from recombinant BCG *in vitro* and *in vivo*. (**A**) pTJBCG3 digested with *Xho*I, to yield fragments of 2259bp and 4169bp. Lane 1 is a positive control of pTJBCG3 DNA prior to transformation into BCGΔ*panCD*. Lanes 2-21 contain pTJBCG3 DNA obtained from recombinant BCG-Gag^{M} vaccine stocks. (**B**) pEM19 digested with *Sma*I. Fragments of 2118bp and 2794bp were obtained. Lanes 1–15 contain pEM19 plasmid DNA isolated from recombinant BCG^{E} vaccine stocks, and lane 16 pEM19 plasmid DNA isolated prior to transformation into BCGΔ*panCD* (positive control). PCR amplification from plasmid DNA isolated from rBCG found in the spleens and lymph nodes of mice vaccinated with BCG-Gag^{M} (**C**) or BCG^{E} (**D**). Lane 1 – negative control; Lane 2 – positive control; Lane 3 – 12 are PCR products from the shuttle vectors of randomly picked rBCG from previously vaccinated mice obtained from homogenised spleen (3-7) or lymph nodes (8-12). Lanes M in **A** - **D** contain the molecular weight marker O'GeneRuler™ 1kb DNA ladder (Appendix E1) and the sizes are indicated.

2.3.2 DNA vaccine construction and immunogen expression

The plasmid pTHpCapR contains the commonly used pTH DNA vaccine vector backbone (552) and has a porcine circovirus enhancer element upstream of the cytomegalovirus (CMV) AD169 immediate-early promoter to facilitate increased expression of the immunogen, thus enabling dose sparing of the vaccine (475). In this study, three DNA vaccines were constructed using the pTHpCapR backbone (Figure 2.8). The vaccine

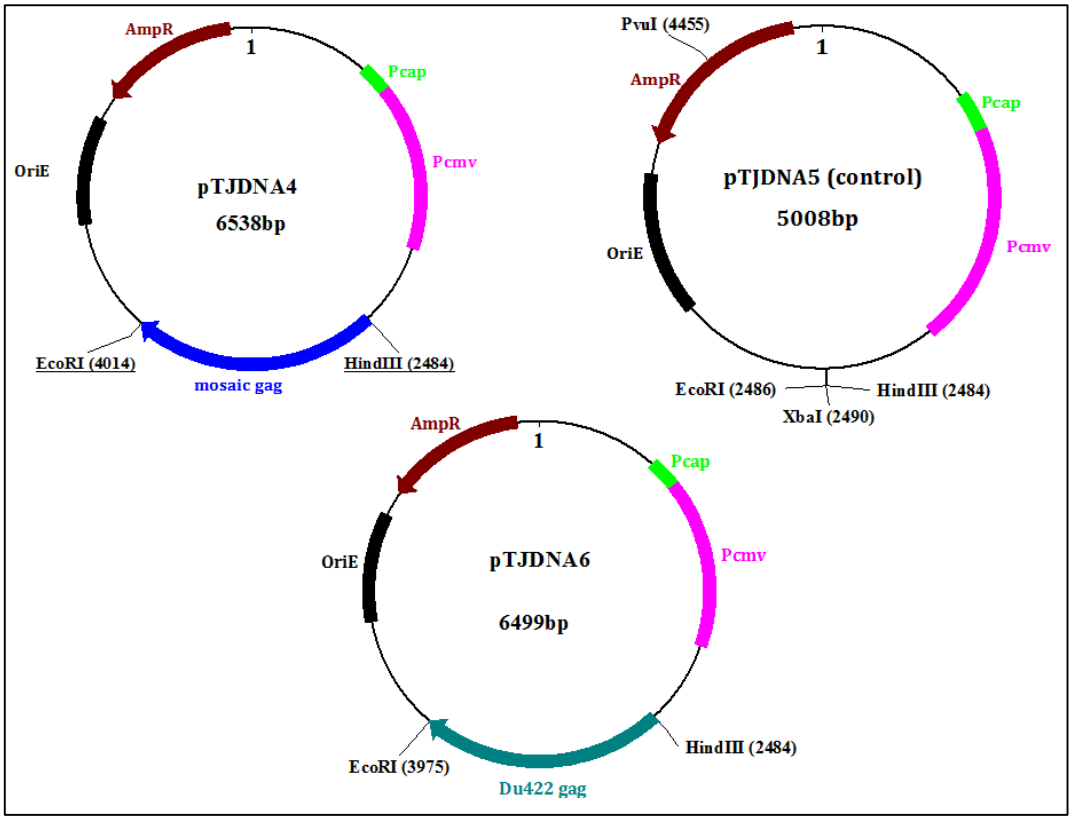


Figure 2.8: Schematic representation of the DNA vaccines pTJDNA4, pTJDNA5, and pTJDNA6. The pTHpCapR vector backbone (475) was used for constructing the DNA-based vaccines used in this project. pTJDNA5 has no insert and was used as a negative control, pTJDNA4 has the *gag*^{M} and pTJDNA6 has a *gag*^{N} cloned in the *Eco*RI and *Hin*dIII sites of the pTHpCapR vector backbone. The *gag*^{N} was obtained from a natural HIV-1C isolate in a previous study (309).Pcap – porcine circovirus enhancer element; Pcmv – CMV promoter; AmpR – ampicillin resistance gene; OriE; *E. coli* plasmid origin of replication.

vector, pTJDNA5, was made for use as an empty vector negative control (DNA^{E}). pTJDNA4 contains the HIV-1 subtype C *gag*^{M} insert (DNA-Gag^{M}), and pTJDNA6 has a *gag*^{N} insert (DNA-Gag^{N}; Figure 2.8). The natural HIV-1 subtype C *gag* gene was isolated from an HIV-1 positive sex worker, Du422, in South Africa and had the closest sequence similarity to the consensus sequence following a subtype C Gag alignment (309).

Cloning of all three DNA vaccines was verified by restriction enzyme digestion (Figure 2.9) and sequencing (data not shown) of plasmid prepared on a large scale. Gag expression from the constructed plasmids was determined by SDS PAGE and Western blot analysis of lysates obtained from HEK-293 transfected with the individual plasmids (Section 2.2.7.2). Western blots of cell lysates were probed with a rabbit anti-HIV-1-p24 Gag antibody (ARP432), followed by an anti-rabbit antibody conjugated to alkaline phosphatase (Sigma-Aldrich, USA). A HEK-293 cell lysate transfected with a plasmid

known to express full length Gag was used as a positive control. The presence of a 55kD fragment indicated the expression of a full length Gag (Figure 2.10).

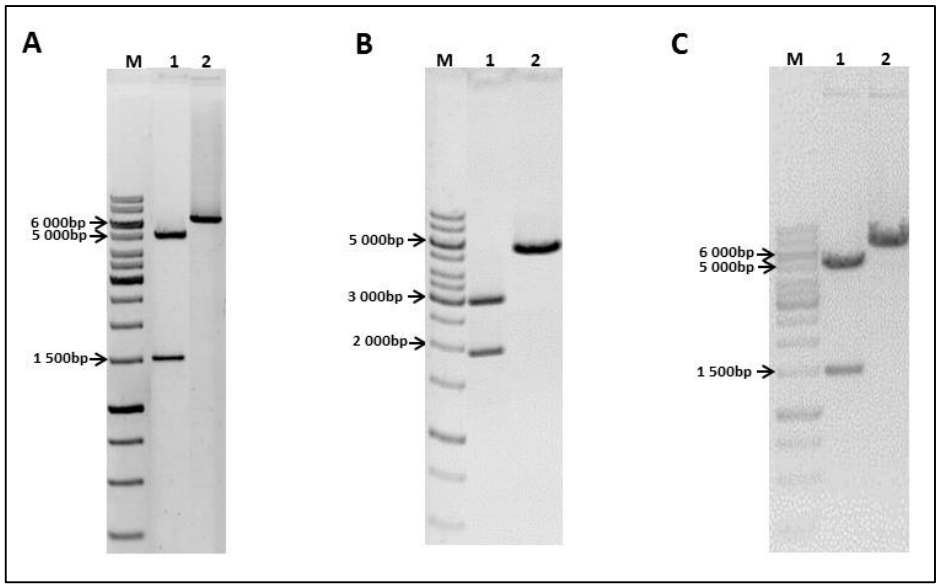


Figure 2.9: Restriction enzyme digests of DNA vaccines. (**A**) pTJDNA4 digested with *Eco*RI and *Hin*dIII (lane 1; 1530bp and 5008bp fragments), and *Eco*RI only (lane 2; 6538bp). (**B**) pTJDNA5 digested with *Xba*I and *Pvu*I (lane 1; 1965bp and 3043bp fragments), and *Pvu*I only (lane 2; 5008bp). (**C**) pTJDNA6 digested with *Eco*RI and *Hin*dIII (lane 1; 1491bp and 5008bp fragments), and *Eco*RI only (lane 2; 6499bp). Lanes M in (**A**), (**B**) and (**C**) contain the molecular weight marker O'GeneRuler™ 1kb DNA ladder (Appendix E1) and the sizes are indicated to the left.

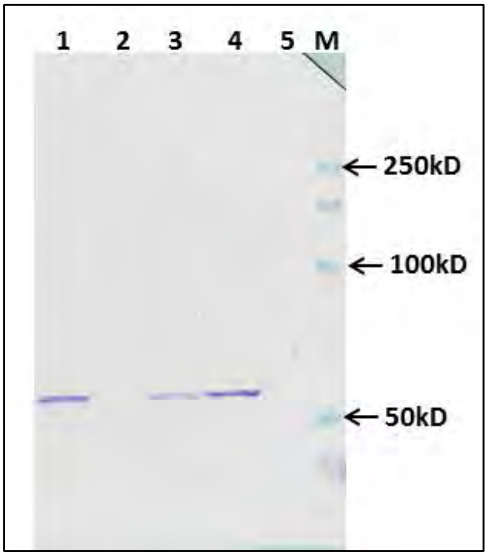


Figure 2.10: *In vitro* expression of HIV-1 Gag in HEK-293 cells transfected with DNA vaccines. Cell lysates were prepared from HEK-293 cells transfected with pTJDNA4 (DNA-Gag^{M}; lane 1), pTJDNA5 (DNA^{E}; lane 2), pTJDNA6 (DNA-Gag^{N}; lane 3), and from untransfected cells (lane 5). HEK- 293 cells transfected with a plasmid known to express full length Gag was used as a positive control (lane 4). A Precision Plus Protein Kaleidoscope pre-stained standard (lane M; Appendix E3) was used and the sizes are indicated to the right. The expression of full length Gag is indicated by a 55kD fragment.

2.3.3 MVA vaccine construction and immunogen expression

The MVA transfer vector used in this study, pTJMVA2, was designed to insert the HIV-1C Gag^{M} into a conserved region of the MVA genome, between the A11R and A12L ORFs as described in Section 2.2.6.1 (Figure 2.11A). The transfer vector pTJMVA2 was constructed and shown to produce bands of the correct size upon *Sna*BI digestion Figure 2.11B.

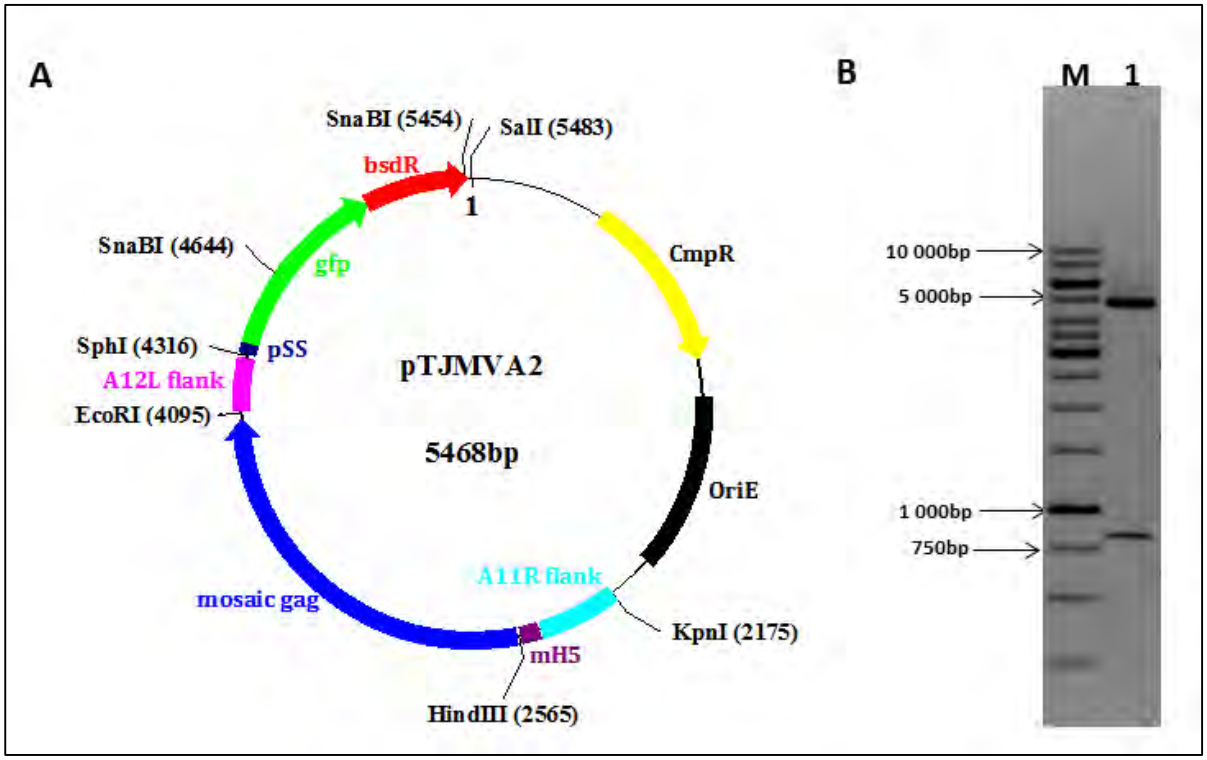


Figure 2.11: The transfer vector pTJMVA2 used for making recombinant MVA. (**A**) Schematic representation of the transfer vector pTJMVA2 (**B**) Mapping of pTJMVA2 by restriction enzyme digestion. Fragments of 810bp and 4658bp were obtained following a *Sna*BI digest (lane 1). Lane marked M has the molecular weight marker O'GeneRuler™ 1kb DNA ladder (Appendix E1) and the sizes are indicated to the left. pSS – MVA synthetic promoter promoter; gfp –green fluorescent protein gene; bsdR – blasticidin resistance; OriE – *E. coli* plasmid origin of replication; AmpR – ampicillin resistance; CmpR – chloramphenicol resistance gene; mH5 – modified H5 promoter; A11R and A12L flanks – partial MVA ORFs.

To determine the minimum BSD concentration required to kill BHK-21 cells within 3 days, a kill curve was carried out (Section 2.2.6.2; Figure 2.12). BHK-21 cells with the transfer vector, pTJMVA2, expressing the *bsd* resistance gene would therefore be selected for during the recombinant MVA construction process. After 3 days cell death was observed with 2.5-15µg/ml BSD (Figure 2.12, panels F, I, L, O, R). Live cells were still detectable when incubated with 2.5, 5 and 7.5µg/ml BSD (Figure 2.12, panels F, I

and L). No live cells were detectable at 10 and 15µg/ml BSD (Figure 2.12, panel O and R). We therefore used 10µg/ml BSD for the subsequent experiments.

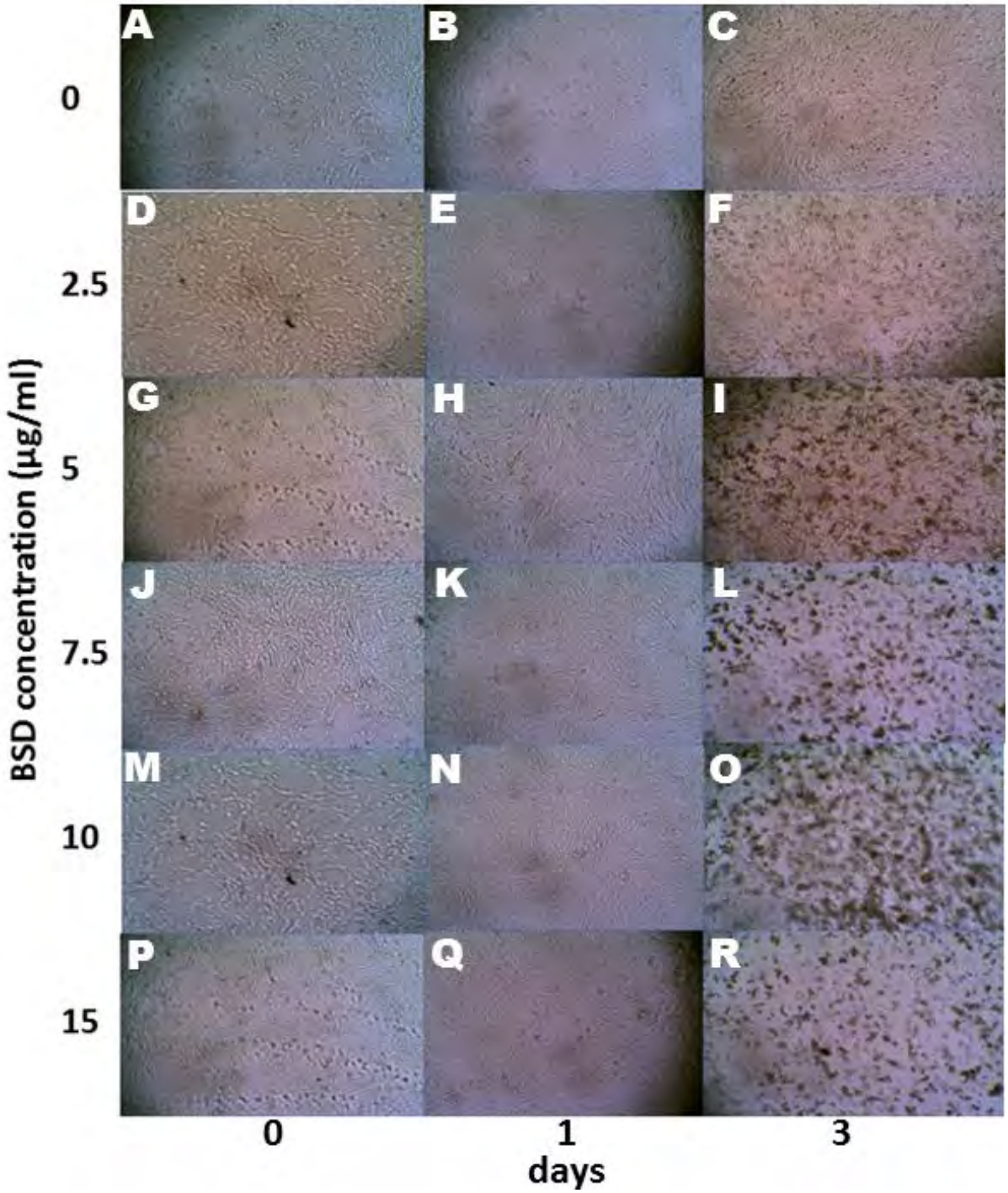


Figure 2.12: Determination of the optimal BSD concentration required to kill all cells by day 3. BHK-21 cells were seeded with various concentrations of BSD (0-15µg/ml). The concentration required to kill all cells by day 3 was determined by viewing the cells using a light microscope.

Recombinant MVA was isolated as described in Section 2.2.6. Initially, a fluorescing single cross-over recombinant containing the entire plasmid was purified (Figure 2.13).

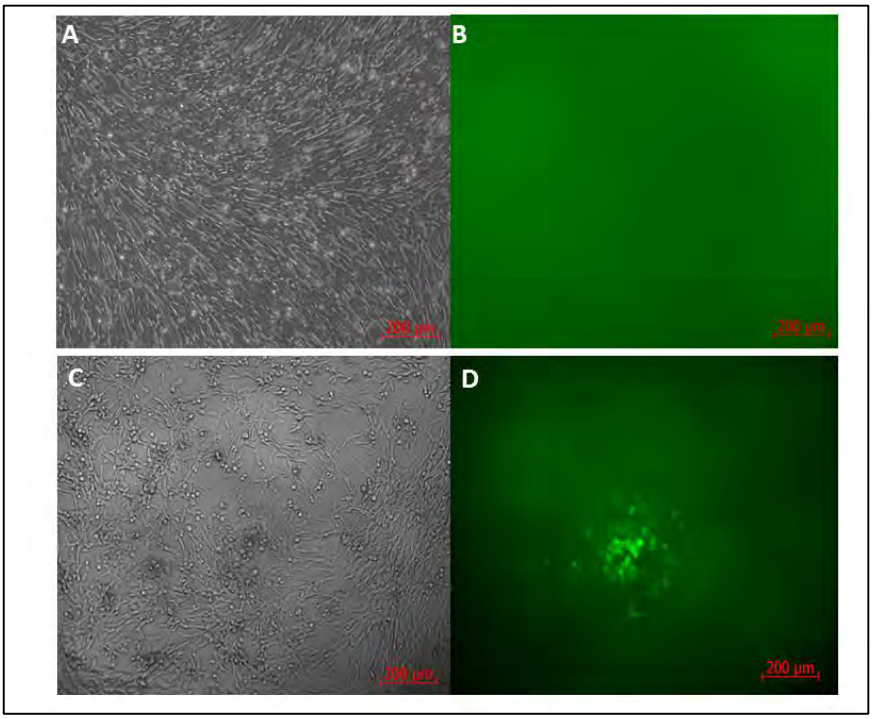


Figure 2.13: Expression of GFP in BHK-21 cells infected with recombinant MVA. BHK-21 cells were infected with MVA (MOI = 1) and transfected with 4µg pTJMVA2. MVA virus was harvested and passaged onto fresh monolayers of BHK-21 cells in the presence of 10µg/ml BSD for 4 passages. The phase contrast (**A**) and fluorescence (**B**) images of uninfected cells were included as a negative control. Panels **C** and **D** are phase contrast and fluorescence images, respectively, of cells infected with rMVA after 4 passages in BHK-21 cells, 48h.p.i. Individual fluorescing plaques (**D**) were picked and passaged further. Scale bars are shown on each image.

PCR was used to detect the presence of this intermediate recombinant using the primers and conditions described in Table 2.2 (Section 2.2.6.6). Primers were designed to distinguish between insertion into either the A11R or the A12L ORF. Figure 2.14 shows two scenarios of how the transfer vector pTJMVA2 could homologously recombine into the MVA genome. Recombination could initially take place at the A12L site (pink; Figure 2.14C). Primer sets 1 and 2 would detect the presence of this recombinant with 1100bp and 791bp PCR amplicons respectively. A second recombination event would then take place between the A11R flank (light blue) within pTJMVA2 and within the MVA genome to generate a final recombinant with the *gag*^{M} gene (dark blue), under the control of an mH5 promoter, inserted between the A11R and A12L ORFs (Figure 2.14E).

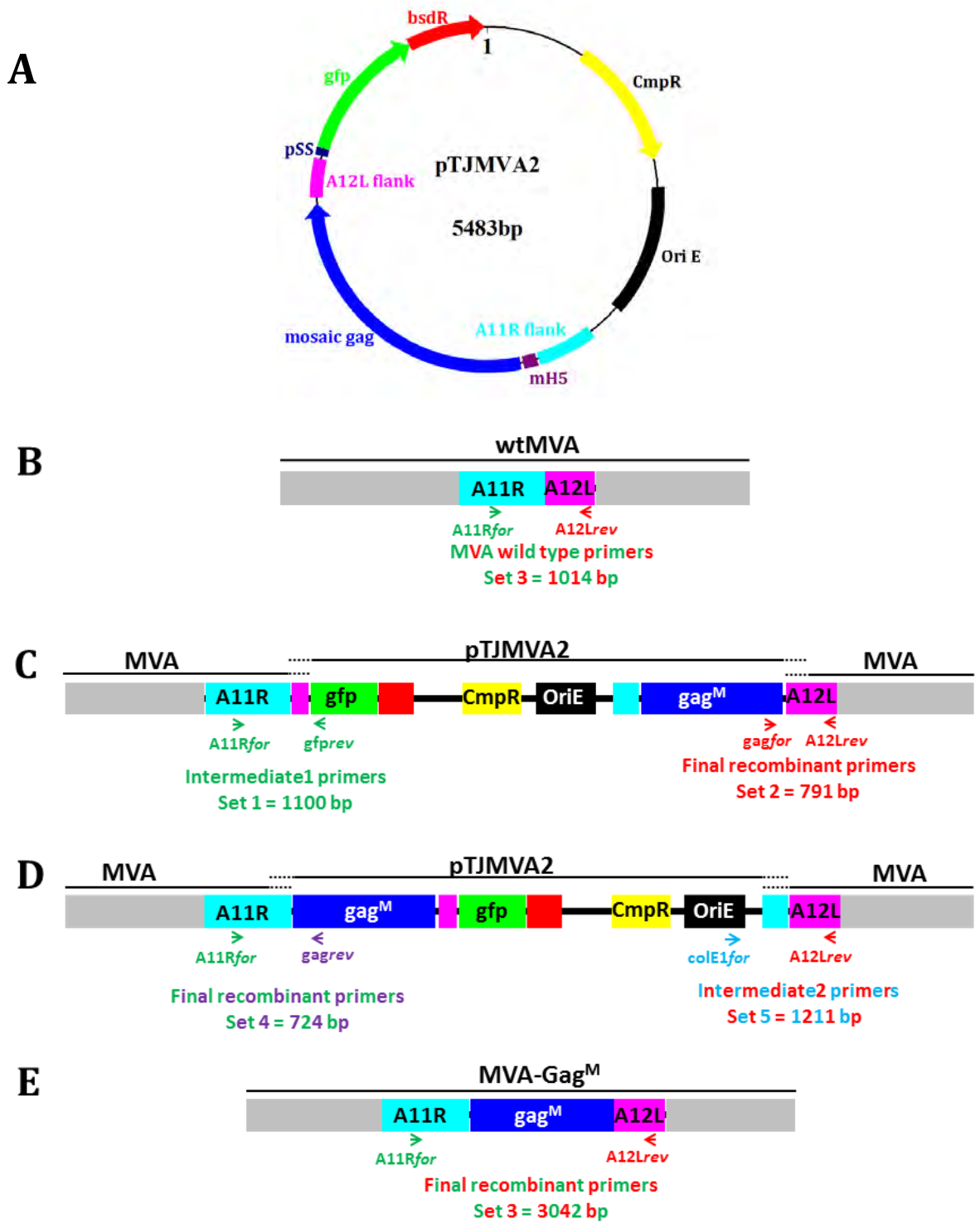


Figure 2.14: Transfer vector and primer design used to construct and detect a recombinant MVA expressing HIV-1C Gag^{M}. (**A**) The transfer vector pTJMVA2 designed for insertion of *gag*^{M} into the MVA genome between ORFs A11R and A12L. pSS – MVA synthetic promoter promoter; gfp –green fluorescent protein gene; bsdR – blasticidin resistance gene; CmpR – chloramphenicol resistance gene; OriE – *E. coli* plasmid origin of replication; A11R and A12L flanks – partial sequences of MVA ORFs; mH5 – modified H5 promoter. (**B**) Schematic of a partial wtMVA genome showing positions of the A11R and A12L ORFs. (**C**) Schematic of the transfer vector inserted into A12L. (**D**) Schematic of the transfer vector inserted into A11R. (**E**) Final MVA-Gag recombinant with the Gag^{M} inserted between the A11R and A12L ORFs. Arrows indicate the position and direction of primers used to detect the intermediate recombinants and the final recombinant by PCR. The expected PCR product sizes are indicated for each primer set.

A 791bp PCR product using primer set 2 would also confirm the presence of a final recombinant. Alternatively, recombination could have also taken place at the A11R site first (light blue; Figure 2.14D). Primer sets 4 and 5 would detect the presence of this recombinant by generating 724bp and 1211bp PCR amplicons respectively. A second recombination event would then take place between the A12L flank within pTJMVA2 and within the MVA genome to generate a final recombinant with the gene of interest inserted between the A11R and A12L ORFs. A 724bp PCR product using primer set 4 would also suggest the presence of a final recombinant. In both scenarios, primer set 3 was used to detect the presence of wild type MVA (1014bp; Figure 2.14B) and the final recombinant (3042bp; Figure 2.14E).

PCR amplification of DNA isolated from fluorescing plaques obtained from the intermediate in Figure 2.13D is shown in Figure 2.15. PCR with primer sets 1 and 2 yielded no PCR products (results not shown). PCR with primer set 3 (Figure 2.15A) yielded a PCR product of 1014bp suggesting the presence of wild type MVA. PCR with primer set 4 yielded a 724bp product suggesting the presence of a final recombinant and/ or an intermediate where the initial recombination was in the A11R ORF of MVA (Figure 2.14D; Figure 2.15B). There was, however, no final recombinant present at this stage of passaging since primer set 2 did not give a 791bp product (result not shown). A PCR product of 1211bp was obtained using primer set 5 and confirms the presence of an intermediate that recombined into the A11R ORF of MVA.

The purified intermediate was passaged 4 times in the absence of BSD, and non-fluorescing plaques were picked and screened using PCR until a final recombinant was obtained. After 7 rounds of plaque-picking in the absence of BSD, a final recombinant was identified by PCR using primer set 3 which bind on either side of the insertion site in MVA (Figure 2.14E). Figure 2.16A shows that there was a mixture of wild type and recombinant MVA viruses. The recombinant MVA was passaged three more times at which stage no wtMVA could be detected by PCR (Figure 2.16B). Figure 2.16B shows that the MVA virus isolated is the desired final recombinant, MVA-Gag^{M} as the expected PCR product of 3042bp was generated using primer set 3. This PCR product was sequenced and confirmed the composition of MVA-Gag^{M}.

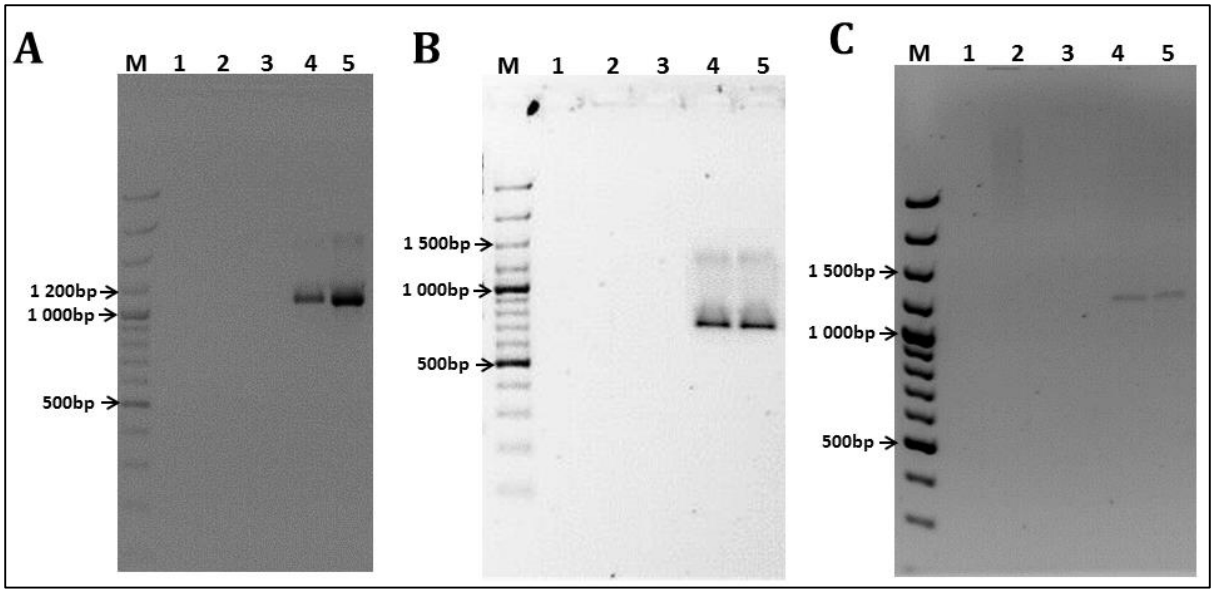


Figure 2.15: PCR detection of an MVA intermediate recombinant. A 1% agarose gels showing PCR products amplified from primer sets 3 (**A**), 4 (**B**) and 5 (**C**) of lysates obtained from the plaque in Figure 2.13. The same template was used for each gel. Lane 1 is empty; Lane 2 is a negative control and has no lysate; Lane 3 has lysate from uninfected cells; Lane 4 has lysate from the fluorescing plaque in Figure 2.13; Lane 5 has lysate from another fluorescing plaque in the same well as the plaque in Figure 2.13. Lanes marked M have the molecular weight marker O'GeneRuler™ 100bp Plus DNA ladder (Appendix E2) and the sizes are indicated to the left.

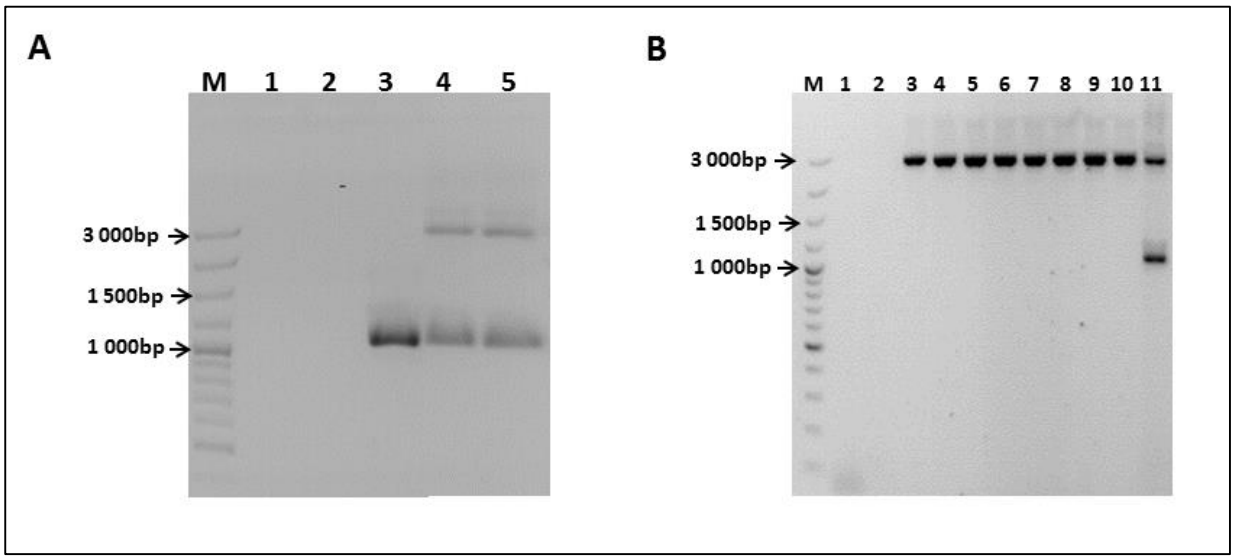


Figure 2.16: PCR detection of MVA-Gag^{M}. (**A**) Agarose gel showing PCR products from the lysates of BHK-21 cells (lane 2), MVA-infected BHK-21 cells (lane 3), and lysate from cells passaged 4 times in the presence of BSD, followed by 7 plaque purification steps in the absence of BSD to obtain the potential recombinant (lane 4 and 5). Lane 1 was included as an additional negative control and had no lysate added. The final recombinant is detected by the 3042bp fragment, and wild type MVA is detected by a 1014bp PCR product. Both samples screened (lane 4 and 5) had both wild type MVA and MVA-Gag^{M} present. (**B**) Agarose gel of PCR products from the lysates of uninfected BHK-21 cells (lane 2) and cell lysates obtained after 4 passages on BHK-21 cells followed by 10 plaque pick purification steps (lanes 3 - 10). Lane 11 had lysate from cells passaged 4 times, followed by 7 plaque picks. Lane 1 was included as a negative control and had no lysate added. Lanes marked M have the molecular weight marker O'GeneRuler™ 100bp Plus DNA ladder (Appendix E2) and the sizes are indicated to the left.

Vaccine stocks of MVA-Gag^{M} were generated by infecting BHK-21 cells in multiple large TC flasks. MVA-Gag^{M} was then harvested, and titrated on BHK-21 cells (Section 2.2.6.7). The titre was determined by averaging results of immunostaining using both an anti-HIV-1 p24 Gag antibody (ARP432) and anti-vaccinia virus antibody (AbSerotec, UK). Uninfected cells and cells infected with wild type MVA were used as negative controls. As a positive control, we included BHK-21 cells that were infected with MVA-Gag^{N} (309,364) (Figure 2.17). MVA-Gag^{M} virus titres of 7.8 x 10^{7} pfu/ml and 9.4 x 10^{7} pfu/ml were obtained on BHK-21 cells by immunostaining using the anti-Gag and anti-MVA antibodies, respectively. The titre was averaged to 8.6 x 10^{7} pfu/ml. The anti-Gag immunostain results also confirmed the expression of the Gag^{M} immunogen. The similar values obtained for the two titrations indicates that all the MVA-Gag^{M} virus expresses Gag^{M} and there is no residual wtMVA present in the vaccine stock.

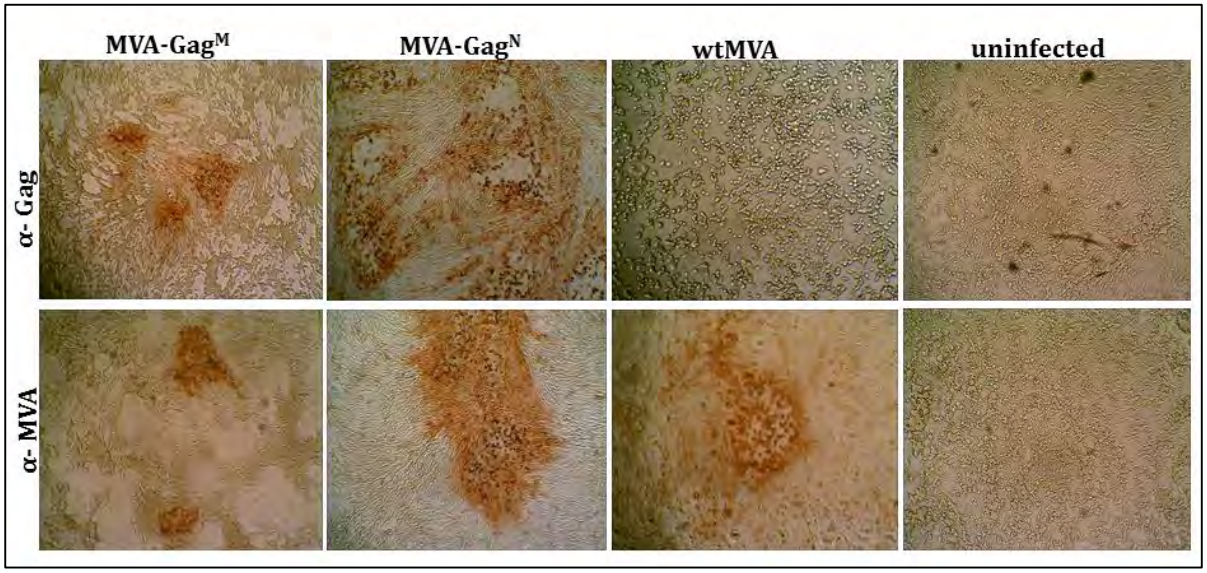


Figure 2.17: Titration and *in vitro* expression of Gag by BHK-21 cells infected with MVA-Gag^{M} as determined by immunostaining. BHK-21 cells were infected with 10 fold serial dilutions of MVA-Gag^{M}, MVA-Gag^{N}, wild type MVA (wtMVA), or left uninfected for 72 hours. HIV-1 Gag was detected with anti-Gag antibody ARP432 (α-Gag; top panels) as well as an anti-vaccinia virus antibody (α-MVA; bottom panels), followed by an anti-rabbit HRP-conjugated antibody. The titre was determined by averaging the number of stained foci using the anti-Gag and anti-vaccinia antibodies, and factoring in the dilution factor and infection volume.

In vitro expression of the Gag^{M} protein in the vaccine stocks was confirmed by Western blot analysis following SDS PAGE. Cell lysates derived from BHK-21 cells infected with MVA-Gag^{M}, when probed with a Gag-specific antibody, showed expression of a protein of the correct size of 55kD (Figure 2.18, Lane 4). Lysates from uninfected cells were used as a negative control and a HeLa cell lysate transfected with a plasmid known to

express full length Gag was used as a positive control This experiment confirmed the expression of the Gag^M protein from MVA-Gag^M.

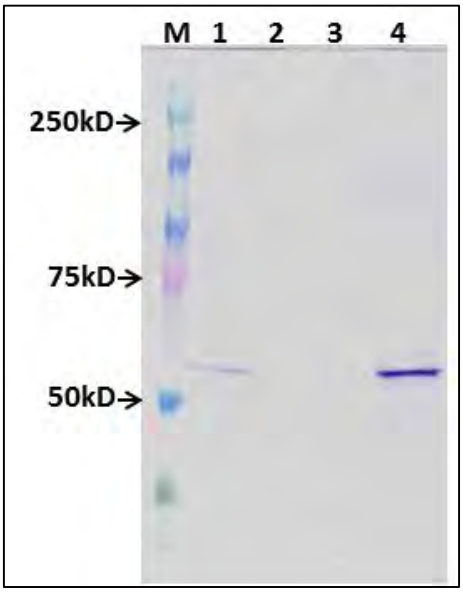


Figure 2.18: *In vitro* expression of a Gag^M immunogen from lysates of BHK-21 cells infected with MVA-Gag^M. Cell lysates were prepared from BHK-21 cells infected with MVA-Gag^M (lane 4), wild type MVA (lane 3), or left uninfected (lane 2). HeLa cells transfected with a plasmid known to express full length Gag was used as a positive control (lane 1). Western blots were probed with a rabbit anti-HIV-1-p24 Gag antibody (ARP432), followed by an anti-rabbit antibody conjugated to alkaline phosphatase (Sigma-Aldrich, USA). A Precision Plus Protein Kaleidoscope pre-stained standard (lane M; Biorad, USA; Appendix E3) was used and the sizes are indicated to the left.

Due to the numerous deletions in MVA, compared to the parent vaccinia virus (485,506), the host range has been compromised, particularly in mammalian cell lines (508,509). MVA can, however, complete its replication cycle in BHK-21 cells (509) and rat IEC-6 cells(576). To assess the expression of the Gag^M in a non-permissive cell line, HeLa cells were infected with MVA-Gag^M at an MOI of 1 (Section 2.2.6.3). An immunostaining assay using a rabbit anti-HIV-1-p24 Gag antibody (ARP432) followed by a peroxidase-conjugated anti-rabbit antibody, (AbSerotec, UK) was carried out at different time points post infection ((364); Section 2.2.7.3). MVA-Gag^N-infected, and uninfected HeLa cells were included for comparison (Figure 2.19). The presence of stained cells indicated the expression of Gag from MVA-Gag^M in a non-permissive cell line (panels A-E) from as early as 12 hours post infection (panel A; Figure 2.19). Although the same MOI and number of cells were used for the infection, there were more brown-stained cells in the Gag-immunostain of cells infected with MVA-Gag^M (panels A-E) than those infected with MVA-Gag^N (panels F-J). In the latter, only a few

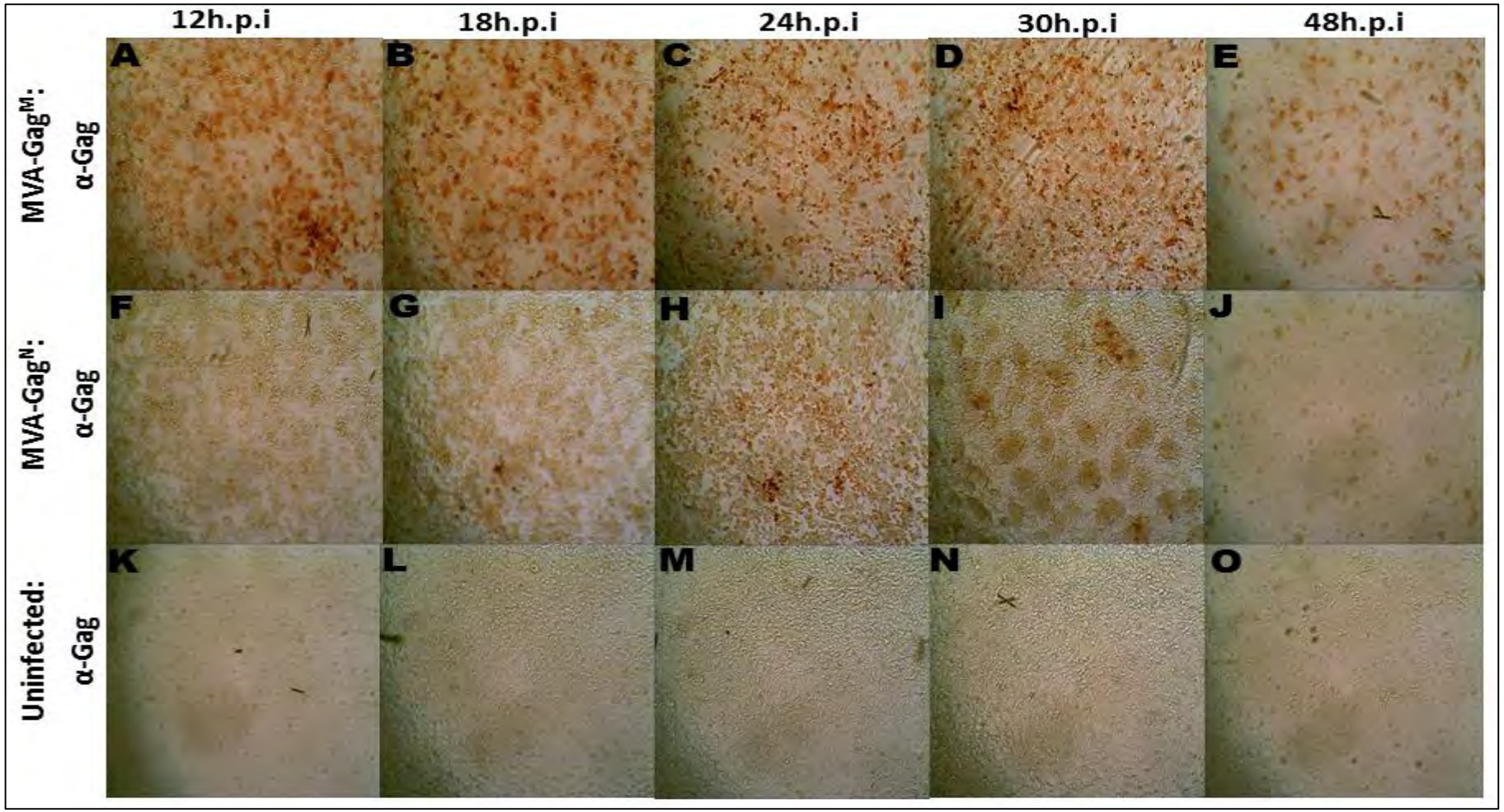


Figure 2.19: *In vitro* expression of Gag in a non-permissive cell line infected with recombinant MVA. HeLa cells were infected at an MOI of 1 with MVA-Gag^{M} (panels **A-E**), MVA-Gag^{N} (panels **F-J**), or left uninfected (panels **K-O**). HIV-1 Gag was detected with an anti-Gag antibody ARP432 (α-Gag) followed by an anti-rabbit HRP-conjugated antibody. Expression of Gag was detected as brown-stained cells. Camera settings were the same at for time points.

stained cells were visible by 48 hours as most cells had rounded and lifted (panels E, J, and O). To further confirm the expression of Gag^M in permissive and non-permissive cell lines, a p24 ELISA assay was done on supernatants and lysates of permissive (BHK-21) and non-permissive (HeLa) cell lines infected with MVA-Gag^M or MVA-Gag^N at various time points (Figure 2.20). Since Gag expression was detected from as early as 12h.p.i with the immunostaining, the p24 ELISA assays were done from 3h.p.i. Gag p24 production from cells infected with MVA-Gag^M was detected from as early as 3h.p.i in both lysates and the supernatants of both cell lines (Figure 2.20A) at values ≥ 400pg/ml. The amount of p24 increased over time in the cell supernatants, reaching 7480pg/ml at 30h.p.i in infected BHK-21 cells. However, detectable p24 decreased in the cell lysates over time, dropping to below 200pg/ml in HeLa cells by 12h.p.i and in BHK-21 cells by 18h.p.i.

Overall, there was more p24 detected in the supernatants than lysates from both cell lines infected with MVA-Gag^M (Figure 2.20A). In contrast, for cells infected with MVA-Gag^N, there was a large difference in the amount of detectable p24 in the supernatant BHK-21 compared to HeLa cells (Figure 2.20B). The peak p24 levels in the supernatant of MVA-Gag^M-infected BHK-21 cells (maximum 7480pg/ml at 30h.p.i.) were 25.7-fold higher than those of MVA-Gag^N-infected BHK-21 cells (maximum 291.3pg/ml at 30h.p.i.). Interestingly, at the same time point, the p24 detected in the supernatant of BHK-21 cells infected with MVA-Gag^M was already dropping. Detectable p24 from cell lysates of MVA-Gag^N infected BHK-21 and HeLa cells did not exceed 50pg/ml until after 30 hours in BHK-21 cell lysates.

The presence of p24 Gag in the supernatant of infected cells suggests the formation of VLPs. Transmission electron microscopy on permissive BHK-21 and non-permissive HeLa cells infected with MVA-Gag^M (panel C; Figures 2.21 - 2.24) was carried out. Cells infected with MVA-Gag^N (panel D; Figures 2.21 - 2.24) were included to compare VLP production, and cells infected with wtMVA (panel B; Figures 2.21 - 2.24) were included to compare the viral morphogenesis at 6 and 12h.p.i. Uninfected cells were included as a negative control, and no signs of viral infection were detectable (panel A; Figures 2.21 - 2.24). VLPs were detected in both permissive and non-permissive cell lines infected with MVA-Gag^M from as early as 6h.p.i (panels C and E in Figures 2.21 -2.24). Budding particles were also evident in HeLa cells 12h.p.i (panel C and E in Figure 2.24).

Immature poxviruses (IV) and/or viral crescents (VCs) which indicate that MVA morphogenesis occurred were detected in both cell lines infected with MVA-Gag^{N}. Surprisingly, no VLPs were detected in both cells lines at 6 and 12h.p.i. (panel D; Figures 2.21 - 2.24).

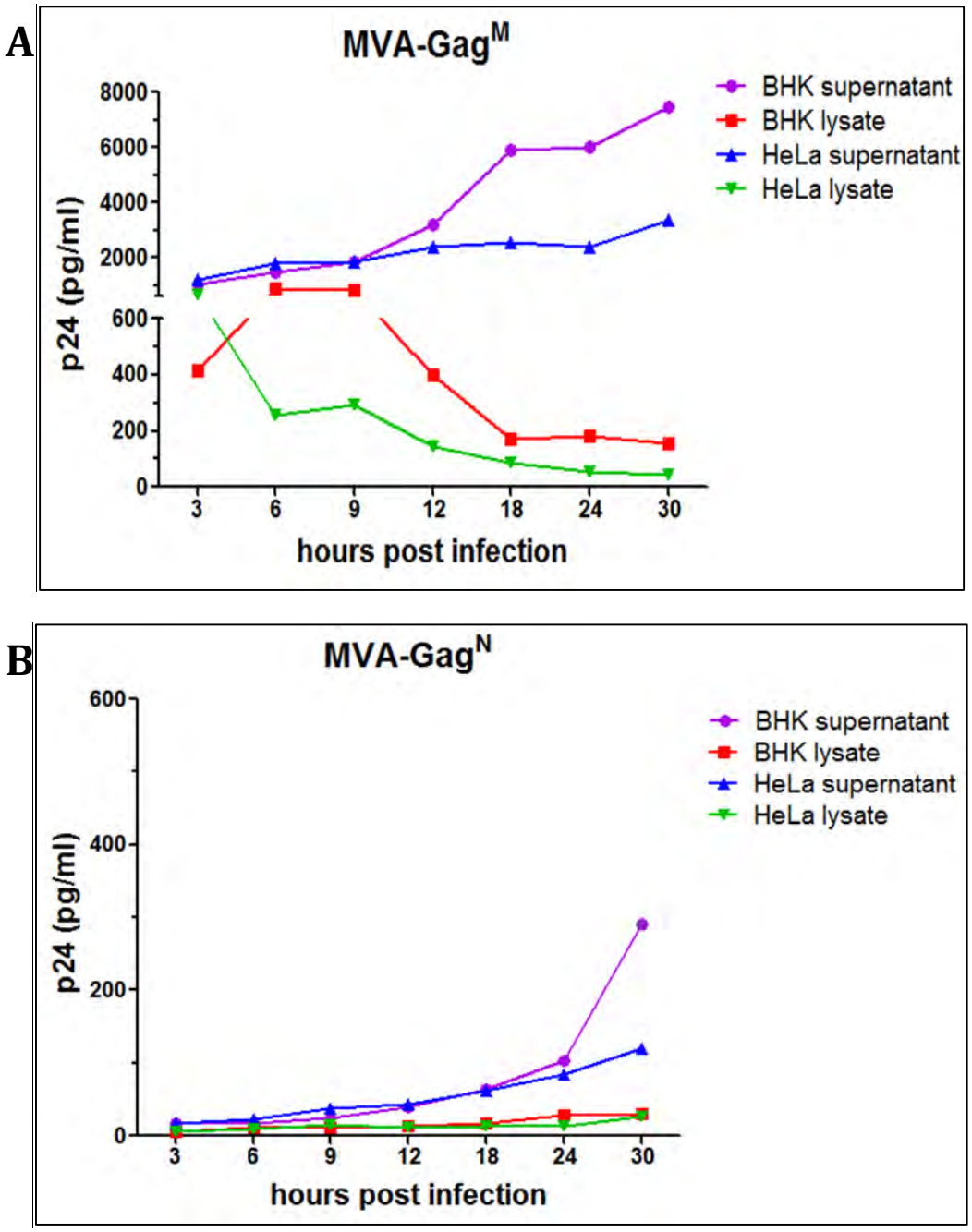


Figure 2.20: p24 Gag production and release by recombinant MVA in BHK-21 and HeLa cell lines. Gag p24 was detected using an ELISA assay. Permissive (BHK-21) and non-permissive (HeLa) cell lines were infected at an MOI of 1 with recombinant MVA and samples taken at the indicated time points. (**A**) Gag p24 production by MVA-Gag^{M} (**B**) Gag p24 production by MVA-Gag^{N}. **Note**, the Y axis scale is different between A and B.

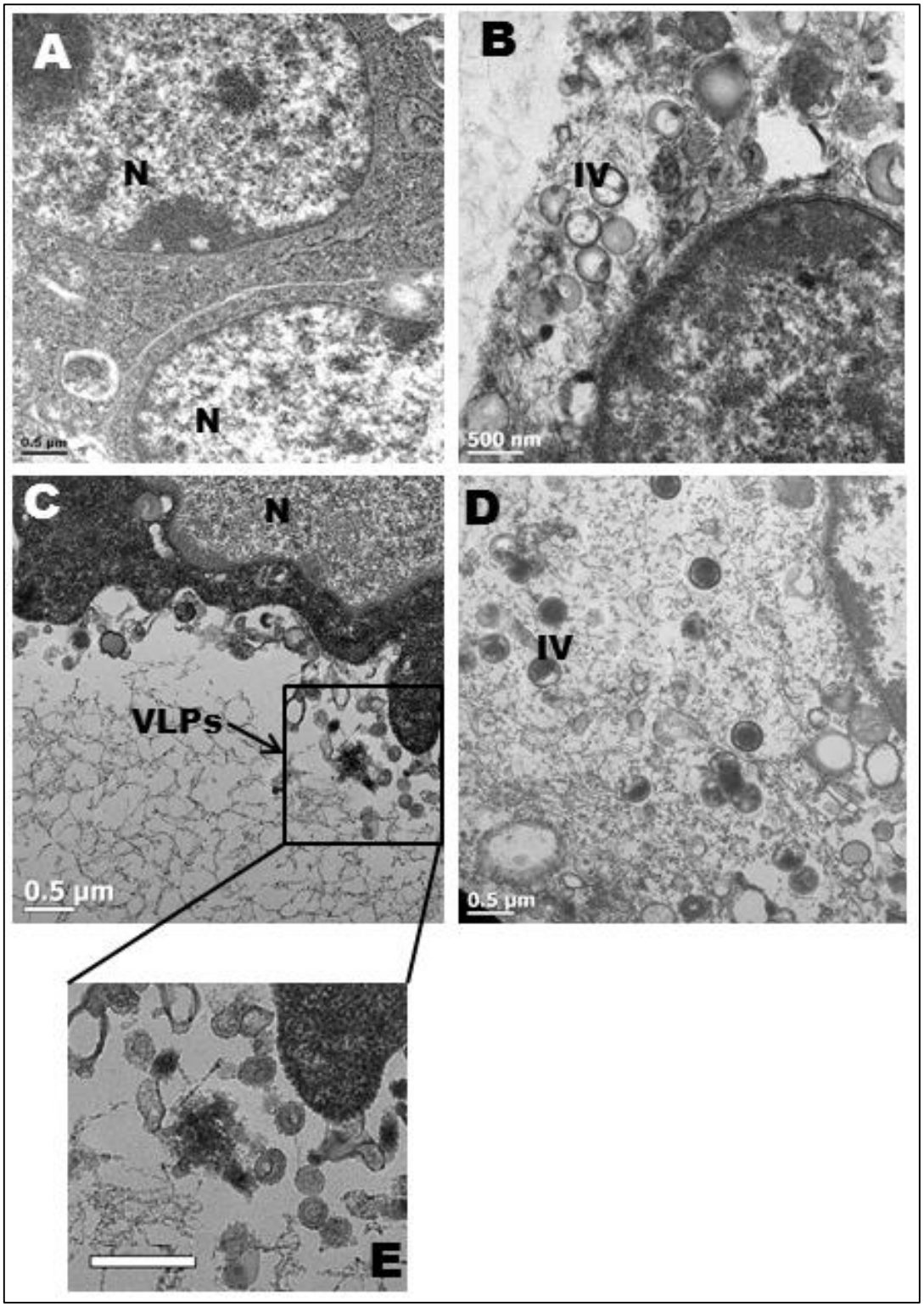


Figure 2.21: Electron micrographs of VLP formation in permissive BHK-21 cells infected with recombinant or wtMVA 6.h.p.i. BHK-21 cells were either left uninfected (**A**) or infected with wtMVA (**B**), MVA-Gag^{M} (**C**), or MVA-Gag^{N} (**D**) with an MOI of 5. Cells were processed for the detection of VLPs by transmission electron microscopy. The nucleus (N), extracellular VLPs, and immature virus (IV) are labelled. The insert (**E**) is a magnification of extracellular VLPs in the selected area of cells infected with MVA-Gag^{M} (C). Scale bars represent 500nm.

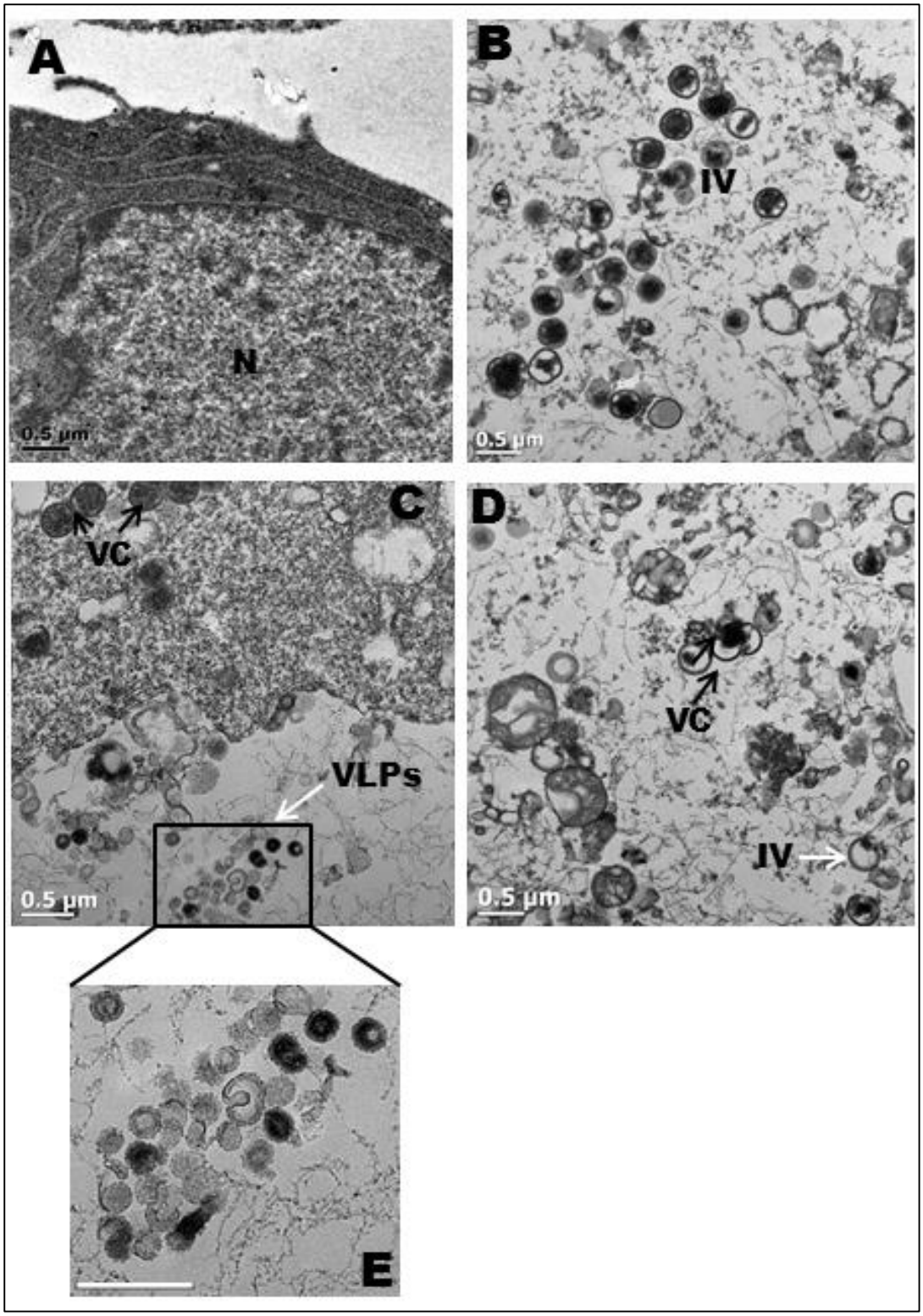


Figure 2.22: Electron micrographs of VLP formation in permissive BHK-21 cells infected with recombinant or wtMVA 12.h.p.i. BHK-21 cells were either left uninfected (**A**) or infected with wtMVA (**B**), MVA-Gag^{M} (**C**), or MVA-Gag^{N} (**D**) with an MOI of 5. Cells were processed for the detection of VLPs by transmission electron microscopy. The nucleus (N), extracellular VLPs, viral crescents (VC), and immature virus (IV) are labelled. The insert (**E**) is a magnification of extracellular VLPs in the selected area of cells infected with MVA-Gag^{M} (C). Scale bars represent 500nm.

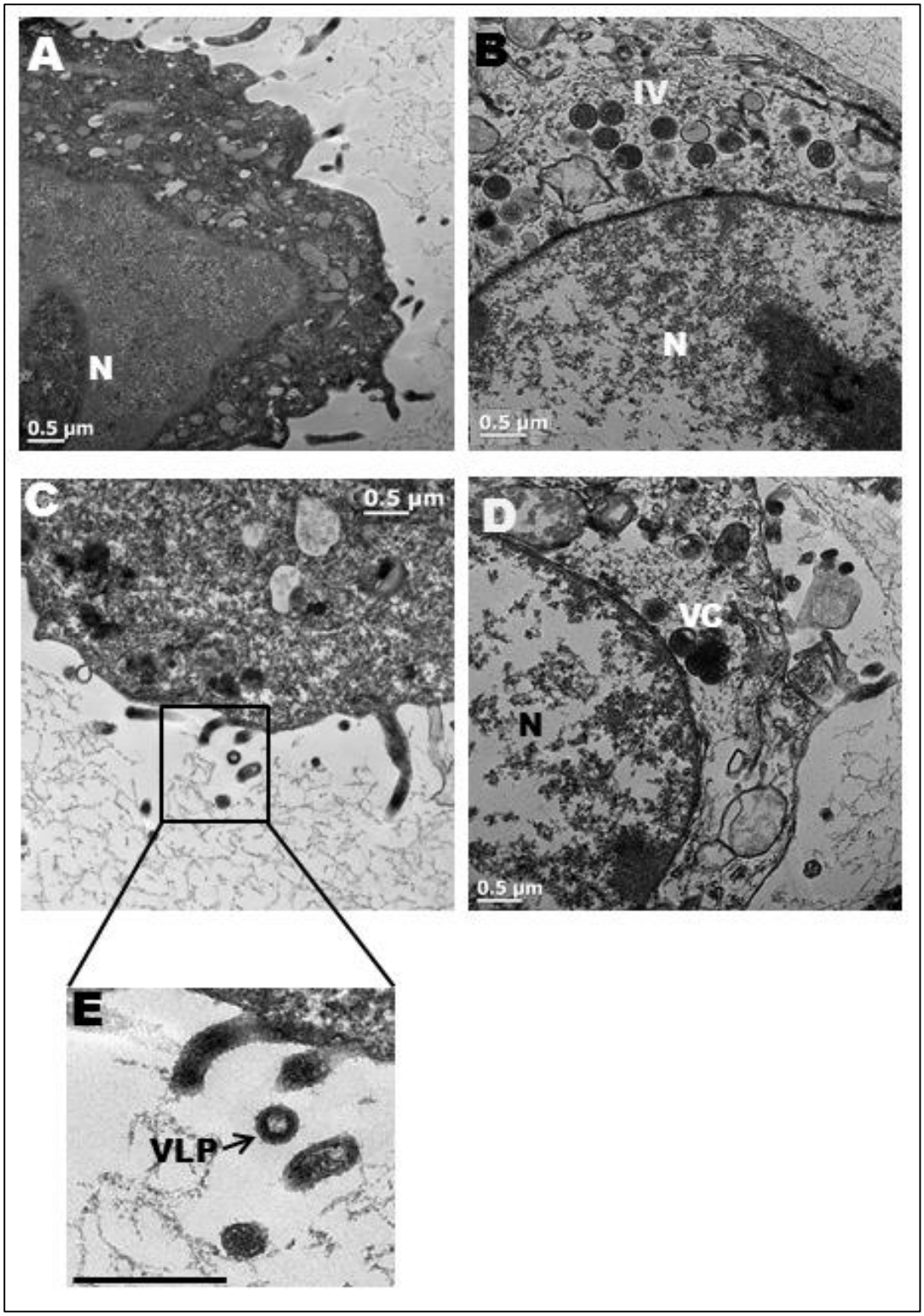


Figure 2.23: Electron micrographs of VLP formation in non-permissive HeLa cells infected with recombinant or wtMVA 6.h.p.i. HeLa cells were either left uninfected (**A**) or infected with wtMVA (**B**), MVA-Gag^{M} (**C**), or MVA-Gag^{N} (**D**) with an MOI of 5. Cells were processed for the detection of VLPs by transmission electron microscopy. The nucleus (N), extracellular VLPs, and viral crescents (VC) are labelled. The insert (**E**) is a magnification of extracellular VLPs in the selected area of cells infected with MVA-Gag^{M} (C). Scale bars represent 500nm.

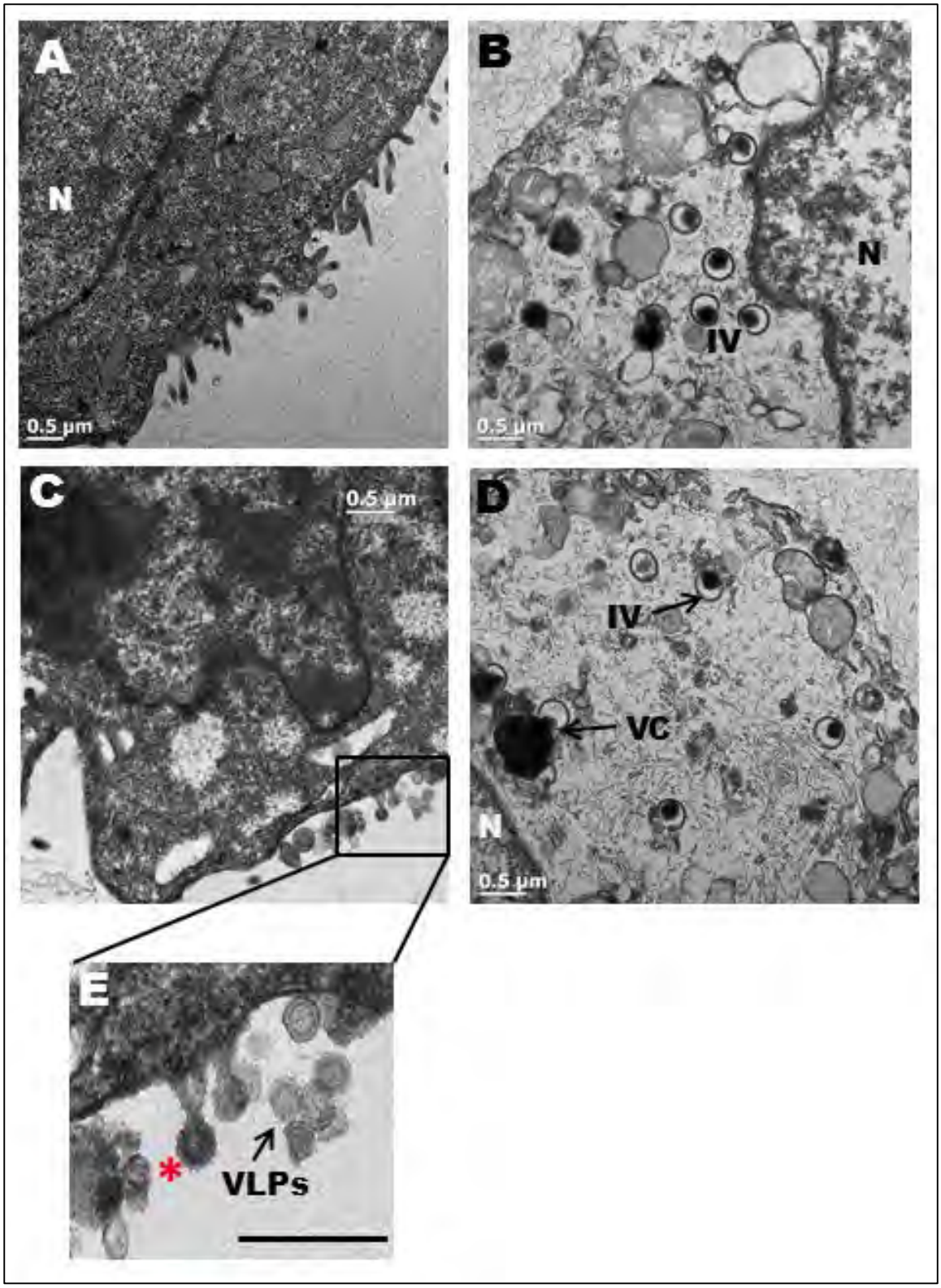


Figure 2.24: Electron micrographs of VLP formation in non-permissive HeLa cells infected with recombinant or wtMVA 12.h.p.i. HeLa cells were either left uninfected (**A**) or infected with wtMVA (**B**), MVA-Gag^{M} (**C**), or MVA-Gag^{N} (**D**) with an MOI of 5. Cells were processed for the detection of VLPs by transmission electron microscopy. The nucleus (N), extracellular VLPs, viral crescents (VC), and immature virus (IV) are labelled. The insert (**E**) is a magnification of extracellular VLPs in the selected area of cells infected with MVA-Gag^{M} (C). The asterisk (*) indicates a budding VLP. Scale bars represent 500nm.

At 48h.p.i almost all MVA-Gag^{M}–infected BHK-21 cells were lysed and dead, although a few VLPs were detectable (Figure 2.25C). VLPs were also detectable in MVA-Gag^{N}–infected BHK-21 cells at the same time point (Figures 2.25A and B) and appear to be the same size as those formed by MVA-Gag^{M} under the same TC conditions. Thus, the computationally generated HIV-1C Gag^{M} is expressed in mammalian cells, buds and forms extracellular VLPs. The expression of Gag^{M} appears to be more efficient than that of Gag^{N} (in the DNA vaccines) as evidenced by Western blot analysis of transfected HEK-293 cells (Figure 2.10), and immunostaining (in the MVA vaccines; Figure 2.19) and p24 ELISA (in the MVA vaccines; Figure 2.20) analysis of rMVA-infected BHK-21 and HeLa cells. However, MVA-Gag^{N} does not have the p6 domain which is known to influence VLP formation. The expression of Gag^{M} in non-permissive HeLa cells suggests that it would be expressed in humans who are the ultimate target for the generated vaccine.

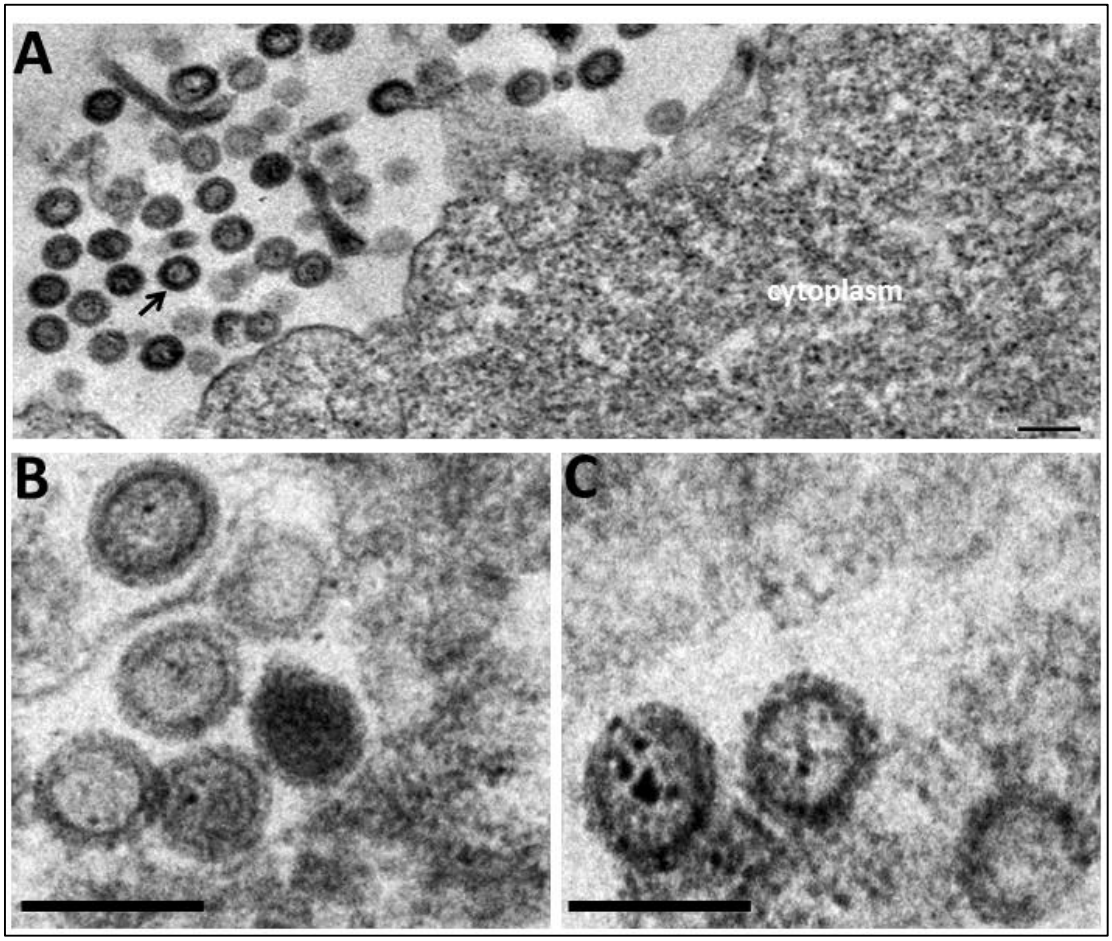


Figure 2.25: Electron micrographs of VLP formation in permissive BHK-21 cells infected with rMVA 48.h.p.i. BHK-21 cells were infected with MVA-Gag^{N} (**A** and **B**), or MVA-Gag^{M} (**C**) with an MOI of 1. Cells were processed for the detection of VLPs by transmission electron microscopy. The cytoplasm is labelled. The VLPs are indicated by an arrow in A and the scale bar represents 200nm.

2.4 DISCUSSION

The stability and expression of transgenes is critical in vaccine development. This is essential for memory cells to elicit a correct and potent immune response to the antigen in the event of an infection. In this study, three vaccines expressing a monovalent HIV-1C Gag^{M} were constructed (BCG-Gag^{M}, DNA-Gag^{M}, and MVA-Gag^{M}).

Recombinant BCG vaccines expressing heterologous antigens can be unstable (364,550,551,573). This can be a result of not codon optimising the transgenes (364), their over expression and cytoplasmic accumulation (550,573), or due to the use of a strong promoter ((573); reviewed by Chapman *et al*., 2010; (362)). These, in turn, can cause metabolic overload on the recombinant BCG and results in vaccine instability. To overcome the BCG-Gag^{M} vaccine instability, the *gag*^{M} DNA sequence in our study was codon optimised for maximal expression in BCG, which would also potentially translate to increased immunogenicity. A 19kD signal sequence was also included upstream of *gag*^{M}, to direct the immunogen to the *M. bovis* BCG*ΔpanCD* cell wall and prevent accumulation of the HIV immunogen within the host cell. Gag^{M} expression from the BCG-Gag^{M} vaccine was however not assessed *in vitro*. This was due to the presence of the *mtrA* promoter which downregulates antigen expression *in vitro*, and upregulates antigen expression *in vivo* (543,550). Thus, the Gag^{M} would only be expressed at high levels *in vivo*, and not during the generation of vaccine stocks. This modification was also done so that vaccine stocks could be bulked up without compromising the shuttle vector or immunogen integrity. The shuttle vectors in the BCG-Gag^{M} and BCG^{E} vaccines we constructed here were detectable in peripheral lymphoid organs (spleen and lymph nodes) of vaccinated mice 11.5 weeks post vaccination. This was encouraging, as these are sites where adaptive immune responses are initiated (148). Furthermore, the *gag*^{M} DNA sequence obtained from BCG-Gag^{M} in the peripheral lymphoid organs was unaltered as determined by PCR and sequencing.

The expression of a correctly sized 55kD Gag^{M} protein was determined from the DNA-Gag^{M} and MVA-Gag^{M} vaccines (Figures 2.10 and 2.18, respectively). Western blot analysis for Gag expression shows a fainter band for DNA-Gag^{N} (Figure 2.10; lane 3) in comparison to the band obtained from the lysates of HEK cell transfected with DNA-Gag^{M} (Figure 2.10; lane 1). This suggests that Gag^{M} is expressed better than Gag^{N} from

the pTHpCapR DNA vaccine vector backbone. Pilot HIV-1 Gag p24 ELISA assays were carried out on lysates of HEK-293 cells and expression of the Gag^M immunogen was 3-fold greater than that of the Gag^N immunogen. Both genes were confirmed to be full length *gag* by sequencing. For both the Western blot and p24 ELISA assays, the transfection experiments for the two DNA vaccines were conducted in parallel, using the same DNA concentrations and other conditions. There are various DNA sequence features that influence protein expression levels during transcription (e.g. polymerase slippage sites), mRNA processing (e.g. the free energy of stable RNA and secondary structure of the mRNA), translation (e.g. ribosomal binding sites) and protein folding (e.g. translation pause sites (577)). It would therefore be interesting to investigate further if the mosaic sequence somehow causes more efficient translation or production of a more stable protein. Gag^M was successfully cloned into the conserved region of MVA, between ORFs A11R and A12L. MVA-Gag^M titration on BHK-21 cells using antibodies to Gag and MVA gave similar results (Figure 2.17), showing that the gag^M insert was maintained and not lost during vaccine scale up in BHK cells.

Mosaic immunogens are designed computationally to increase the coverage of T cell epitopes of vaccines (1). We have shown that an HIV-1C Gag^M budded and formed stable VLPs (Figures 2.20-2.25). The amount of p24 detected by the ELISA assay continued decreasing between 18 and 30 hours in the lysates of MVA-Gag^M-infected cells (Figure 2.20A), suggesting cell death. However, the amount of p24 detected in the culture supernatants did not decrease. However, this could also be a result of the produced VLPs being stable and remaining in the supernatant, a desirable property for candidate HIV-1vaccines expressing Gag. The experiment could be repeated with additional time points.

The control gag^N gene had been previously cloned into MVA by Nicolette Johnston, a former member of our laboratory. Although it was inserted in a different region (delIII) from gag^M (A11R-A12L), it was expressed from the same mH5 transcriptional promoter and was shown to be stable (364). The use of an MVA vaccine expressing a full length Gag^N inserted between the A11R and A12L ORFs would have been an ideal control for comparative purposes in this study. The differences in Gag expression levels, as determined by immunostaining on HeLa cells (Figure 2.19), reduced p24 detection for gag^N (Figure 2.20B), and delayed VLP formation for gag^N (Figures 2.21-2.25), could be

due to the differences in the *gag* gene sequences, gene transcription, mRNA stability, or protein translation or folding as suggested above. RSV studies have shown that VLP production is dependent on a threshold expression of Gag (578). When expressed at low concentrations, HIV-1 Gag is known to remain in the cytoplasm (579,580). However, as the Gag concentration increases in the cytoplasm of a host cell, its concentration at the plasma membrane also increases, and so does VLP formation. This is most likely to be due to an increase in the Gag multimerisation process (580). Furthermore, the gag^N gene in MVA-Gag^N was truncated and lacked the p6 domain. This region of HIV-1 Gag affects virus particle budding and subsequent release from the host cell into the extracellular space (132,133). The presence of Gag^M p24 in supernatants and lysates of DNA-Gag^M-transfected HEK-293 cells was determined 48 hours post transfection. A p24 assay on lysates and supernatants of DNA-Gag^M and DNA-Gag^N transfected HEK-293 cells would give a more quantitative comparative measure of Gag expression by the two DNA vaccines.

Studies done by others have shown HIV-1 Group M mosaic vaccines to be more immunogenic than the vaccines expressing antigens derived from natural or consensus HIV-1 sequences (312,318,319). High immunological responses to candidate vaccines have been also been correlated to stability and increased expression levels of the antigen (365,526). The biochemical properties of HIV-1 mosaic antigens have not been described before; we speculate that they may be responsible for the increased expression/accumulation of Gag we observed in our study. Immunological evaluation of the HIV-1C Gag^M vaccines constructed here was determined in female BALB/c mice and is described in the next chapter.

CHAPTER 3: IMMUNOLOGICAL EVALUATION OF HIV-1C MOSAIC GAG VACCINES IN BALB/C MICE

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3.1 INTRODUCTION

This chapter describes the assessment of the immunogenicity of the BCG, DNA and MVA vaccines expressing Gag^{M} that were described in the previous chapter. Pilot studies were carried out to determine the optimal dose of MVA for use in a BCG or DNA prime MVA boost regimen. Using the determined optimal dose of MVA, the immunogenicity of the vaccines expressing Gag^{M} was compared in both heterologous and homologous prime boost combinations. In addition DNA and MVA vaccines expressing Gag^{N} protein derived from HIV-1C strain Du422 were included in the comparisons. The Gag protein from HIV-1C strain Du422 was selected previously on the basis of its similarity to a derived South African consensus sequence (309,530).

It is essential to determine the immunogenicity and safety of candidate vaccines prior to advancing a vaccine to clinical trials. Thus the immunological evaluation of the vaccines constructed in this study was carried out in BALB/c mice (reviewed in Section 1.8.4). Mice are suitable animal models for the initial evaluation of HIV-1 vaccines because their HLA type is well studied and there are generally low costs associated with their use. Preclinical studies will be carried out in non-human primates once the immunogenicity of these vaccines has been confirmed in mice.

Prime-boost vaccination regimens using attenuated vaccines induce more potent immune responses than single dose vaccinations.. In particular, the heterologous prime-boost vaccinations delivering the same antigen but in different vectors elicit immune responses of better quality and magnitude than homologous prime-boost vaccinations (reviewed by Ondondo *et al*., 2014, Garcia-Arriaza *et al*., 2014, and Girard *et al*., 2011; (146,440,581)). The ideal HIV-1 vaccine will need to elicit T cell and humoral immune responses; and heterologous prime-boost vaccinations have been shown to do this in non-human primates and in clinical trials (reviewed by Hutnick *et al*., 2011; (582)). Heterologous prime-boost vaccinations also offer the advantage of boosting immunity to the immunogen and not the vaccine vector, unlike homologous vaccinations (583). The frequency of memory T cells has also been shown to increase with heterologous prime-boost vaccinations, with effector memory T cells being generated with an increased number of vaccinations (reviewed by Nolz *et al*., 2011; (584)). Priming with a DNA-vectored vaccine is advantageous for focusing the immune response to the

immunogen, unlike priming with viral or bacterial vectors that have multiple other antigens in their backbone (reviewed by Hutnick *et al*., 2011; (582)). Roederer and colleagues showed that rhesus macaques were significantly protected from a SIV_{smE660} strain challenge following vaccination with three DNA and one rAd5 vaccine expressing SIV mosaic Gag and Env genes (321). The RV144 study used a canarypox-based vaccine for priming a protein boost. This is the only efficacy clinical trial that has shown some promise towards an HIV-1 vaccine (204). It supports the use of heterologous prime-boost vaccinations and this study showed that potent immune responses can be elicited when priming with a viral vector.

For T cell-based candidate HIV-1 vaccines, the immunological evaluation of pre-clinical studies involves the use of assays that determine the responses desirable for such vaccines (reviewed in Section 1.10). These are based on studies done on ECs and LTNPs where such immune responses correlate with lower viral load and slow disease progression (reviewed by McMichael and colleagues 2010; (149)). The functions of some of the cytokines evaluated in response to viral infection have also been reviewed in Section 1.6. IFN-γ secretion by $CD4^+$ and $CD8^+$ T cells is associated with the suppression of HIV-1, SIV, and SHIV replication during viral infections (585). The production of IL-2 is essential for viral control. This phenotype is also typical of ECs (182) who can control HIV-1 levels to below undetectable levels (reviewed by Deeks *et al*., 2007; (181)). $CD4^+$ and $CD8^+$ T cell responses associated with simultaneous secretion of IFN-γ, IL-2, and TNF-α are also associated with viral control in ECs (586-589). The IFN-γ ELISPOT assay is used to measure the number of cells secreting IFN-γ (590). The cytometric bead array (CBA) assay quantifies cytokine production from a given number of cells per test. Each test can measure the production of 3 or more cytokines. The cytokines secreted can then be used to determine if the immune response elicited is Th1 or Th2 biased. IFN-γ, IL-2, and TNF-α are associated with a Th1 response, while IL-4, IL-6, and IL-10 are associated with a Th2 response (148).*- Intracellular cytokine staining (ICS) coupled with flow cytometry is used to detect multiple parameters of immune cells including size, granularity, and the expression of surface and intracellular markers at the single cell level (591,592).

3.2 Materials and Methods

The immunogenicity of the vaccines made in this study was evaluated in mice.

3.2.1 Mice vaccinations

The vaccines used in this study are listed in Table 2.3 (Section 2.2.8). Groups of four or five 6–8 weeks old female BALB/c mice were used for each experiment and mice were vaccinated as detailed in Table 3.1. BALB/c mice have the H-2K^{d} gene in their HLA which allows them to respond to specific HIV-1 Gag epitopes.

The BCG vaccine inoculums were administered intraperitonially by needle injection in 200µl BCG re-suspension buffer (Appendix D9). Prior to all intramuscular vaccinations, the mice were anaesthetised with a mixture of ketamine hydrochloride (120mg/kg) and xylazine (16mg/kg) in saline (injection water). The DNA vaccines were re-suspended in 100µl PBS and administered intramuscularly by needle injection, 50µl in the tibialis muscle of each hind leg. The MVA vaccines were re-suspended in 100µl PBS and 50µl administered by needle injection in the tibialis muscle of each hind leg.

3.2.2 Immunogenicity assays to evaluate mosaic vaccines

3.2.2.1 Isolation of splenocytes and lymph nodes

On sacrifice dates (Table 3.1), spleens from mice that received the same vaccination regimen were pooled and so were the lymph mesenteric nodes from the BCG vaccinations. A single cell suspension from the spleens was prepared as described (364,564) for use in the IFN-γ ELISPOT assay (Section 3.2.2.2), intracellular cytokine staining (Section 3.2.2.3), and for the cytometric bead array assay (Section 3.2.2.4). Mesenteric lymph nodes and left over splenocytes from the BCG vaccinations (Table 3.1) were stored at -80˚C in BCG resuspension buffer (Appendix D9) until required for evaluating the integrity of the shuttle vectors (Section 2.2.3.6).

3.2.2.2 ELISPOT assay

Secretion of IFN-γ by CD4^{+} and CD8^{+} T cells, from vaccinated mice, in response to stimulation with HIV-1 Gag peptides was determined by ELISPOT assays. The Mouse IFN-γ ELISPOT kit (BD Bioscience, USA) was used according to manufacturer's instructions (590) and as described by our group (310,365).

Table 3.1: Vaccination schedule used on female BALB/c mice for this study

Group	Prime	Prime	Boost	Sacrifice	Mice per group
A. Determination of the optimal MVA dosage to boost a BCG prime vaccination					
1	**Day 0:** $2x10^7$ BCG-Gag^M	-	**Day 70:** 10^2 pfu MVA-Gag^M	**Day 82**	**4**
2	**Day 0:** $2x10^7$ BCG-Gag^M	-	**Day 70:** 10^4 pfu MVA-Gag^M	**Day 82**	**4**
3	**Day 0:** $2x10^7$ BCG-Gag^M	-	**Day 70:** 10^6 pfu MVA-Gag^M	**Day 82**	**4**
4	**Day 0:** $2x10^7$ BCG^E	-	**Day 70:** 10^2 pfu MVA-Gag^M	**Day 82**	**4**
5	**Day 0:** $2x10^7$ BCG^E	-	**Day 70:** 10^4 pfu MVA-Gag^M	**Day 82**	**4**
6	**Day 0:** $2x10^7$ BCG^E	-	**Day 70:** 10^6 pfu MVA-Gag^M	**Day 82**	**4**
B. Determination of the optimal MVA dosage to boost a DNA prime vaccination					
7	**Day 0:** 10µg DNA-Gag^M	**Day 28:** 10µg DNA-Gag^M	**Day 56:** 10^2 pfu MVA-Gag^M	**Day 68**	**5**
8	**Day 0:** 10µg DNA-Gag^M	**Day 28:** 10µg DNA-Gag^M	**Day 56:** 10^4 pfu MVA-Gag^M	**Day 68**	**5**
9	**Day 0:** 10µg DNA-Gag^M	**Day 28:** 10µg DNA-Gag^M	**Day 56:** 10^6 pfu MVA-Gag^M	**Day 68**	**5**
10	**Day 0:** 10µg DNA^E	**Day 28:** 10µg DNA^E	**Day 56:** 10^2 pfu MVA-Gag^M	**Day 68**	**5**
11	**Day 0:** 10µg DNA^E	**Day 28:** 10µg DNA^E	**Day 56:** 10^4 pfu MVA-Gag^M	**Day 68**	**5**
12	**Day 0:** 10µg DNA^E	**Day 28:** 10µg DNA^E	**Day 56:** 10^6 pfu MVA-Gag^M	**Day 68**	**5**
C. Vaccination groups used for comparison					
13	**Day 0:** 10µg DNA-Gag^N	**Day 28:** 10µg DNA-Gag^N	**Day 56:** 10^4 pfu MVA-Gag^N	**Day 68**	**5**
14	-	**Day 0:** 10µg DNA-Gag^N	**Day 28:** 10µg DNA-Gag^N	**Day 40**	**5**
15	-	**Day 0:** 10µg DNA-Gag^M	**Day 28:** 10µg DNA-Gag^M	**Day 40**	**5**
16	**Day 0:** $2x10^7$ BCG-Gag^M	-	**Day 70:** $2x10^7$ BCG-Gag^M	**Day 82**	**4**
17	-	-	**Day 0:** 10^4 pfu MVA-Gag^M	**Day 12**	**5**
18	-	**Day 0:** 10^4 pfu MVA-Gag^M	**Day 28:** 10^4 pfu MVA-Gag^M	**Day 40**	**5**

Peptides that correspond to known $CD4^+$ and $CD8^+$ T cell epitopes in HIV-1 Gag (Table 3.2) were synthesized at Bachem (Switzerland), diluted in complete medium and used to stimulate splenocytes at a final concentration of 2µg/ml. An irrelevant peptide was also included as a negative control (Table 3.2) while ConA, a polyclonal stimulant, (0.1µg/ml; Sigma-Aldrich USA) was included as a positive control. Each peptide or control were added in triplicate for each group of mice with 500 000 cells/well. Spots were developed using the Nova red (Vector Laboratories, USA) substrate according to manufacturer's instructions. ELISPOT plates were analysed with a CTL ImmunoSpot system (Cellular Technology Limited, USA).

Table 3.2: Control and peptide stimulants used in the ELISPOT, ICS and CBA assays

Stimulant/control	**Description**	**Peptide sequence**
ConA	Assay positive control	N/A
Irrelevant peptide	H-2K^d binding peptide (negative peptide control)	TYSTVASSL
GagCD8	Gag H-2K^d – restricted class I peptide (CD8 peptide)	AMQMLKETI
GagCD4(13)	Gag MHC class II-restricted peptide (CD4 peptide)	NPPIPVGDIYKRWIILGLNK
GagCD4(17)	Gag MHC class II-restricted peptide (CD4 peptide)	FRDYVDRFFKTLRAEQATQE

Adapted from Shen 2010 (564).

For each group of vaccinated mice, the mean number of spots and SD of the mean were calculated and expressed as spot forming units per 1 x 10^6 splenocytes (sfu/10^6). The background response per group was determined as the mean number of spots and its SD in the absence of peptide. Responses were considered as positive if the sfu/10^6 splenocytes was greater than the background plus two standard deviations (SDs). Responses below this background valuewere assigned a value of 0 sfu/10^6 splenocytes. The background responses were subtracted from the HIV-1 Gag specific responses and the final response expressed as net sfu/10^6 splenocytes.

3.2.2.3 Intracellular cytokine staining (ICS)

The production of IFN-γ, TNF-α, and IL-2 in response to peptide stimulation was measured by ICS and flow cytometry. Splenocytes pooled from 4 or 5 mice per group were adjusted to 2 x 10^6 cells/ml with wash buffer (PBS with 1% normal mouse serum) with or without HIV-1 Gag stimulants and incubated for 6 hours at 37°C. Each group consisted of a negative unstimulated control (cells and 0.02µg/µl brefaldin A (BFA; Sigma-Aldrich, USA)), positive control (cells, 0.02µg/µl BFA and 4µl Leukocyte activation cocktail (phorbol 12-myristate 13-acetate and ionomycin; BD Biosciences, USA) and the test (cells, 0.02µg/µl BFA, 2µg/ml Gag CD8 peptide, 2µg/ml Gag CD4(13) peptide, 2µg/ml Gag CD4(17) peptide – (Table 3.2). Cells were washed in 1 ml of wash buffer and blocked in 50µl blocking solution (0.12µl normal mouse serum, 0.12µl normal rat serum, 0.16µg CD16/32(BD Biosciences, USA)) for 15 minutes at 4°C. Cells were stained in 98.75µl stain buffer (BD Biosciences, USA) with 0.05µg of each fluorochrome-conjugated surface antibody (CD3-Alexa 700, CD4-PE-Cy7, CD8-APC-Cy7, CD62L-APC, and CD44-FITC). Unbound antibodies were removed by a wash step before adding 100µl fixation/permeabilization buffer (BD Biosciences, USA) and incubating at 4°C for 30 minutes. The fluorochrome-conjugated cytokine antibodies (0.2µg TNF-PE, 0.06 µg IL-2-PE, 0.06 µg IFN-γ-PE) were diluted in Perm/Wash buffer (BD Biosciences, USA). A 100µl aliquot of the master mix was added to each tube and the cells incubated at 4°C for 20 minutes. Cells were washed twice in 1ml wash buffer before being resuspended in 200µl stain buffer and incubated at 4°C until acquisition on a BD LSRII (BD Biosciences, USA). The gating strategy is illustrated below (Figure 3.1), and a representative plot shown in Appendix B3). The results were analysed using FlowJo. Experiments were done in triplicate for each mice group. Cells were positive for cytokine production if the proportion was ≥ 0.05% after subtracting the background. A response was considered positive if >10 cytokine-positive memory T cells were detected.

3.2.2.4 Cytometric bead array assay (CBA)

Quantification of extracellular antigen-specific cytokine production was determined by cytokine bead array (BD Bioscience, USA) on aliquots of splenocytes pooled from 4 or 5 mice a group cultured with HIV-1 Gag peptides. The splenocytes were adjusted to 15 X

10^6 splenocytes/ml with complete medium and 100µl (1.5 X 10^6 splenocytes) added to each well in a 96 well round-bottomed micro-titre plate. The responses to each peptide

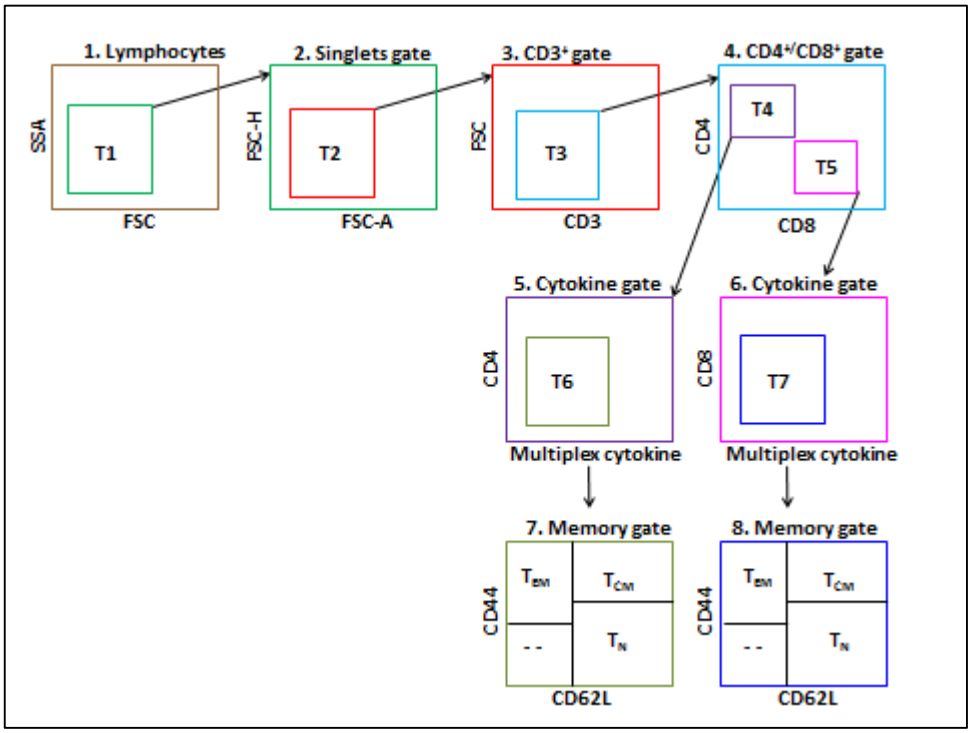


Figure 3.1: Gating strategy used for flow cytometry analysis of Gag-specific $CD8^+$ and $CD4^+$ cytokine - producing cells. Progressive gating strategy: lymphocytes (plot 1, T1), singlets (plot 2, T2), $CD3^+$ cells (plot 3, T3), $CD4^+$ and $CD8^+$ T cells (plot 4, T4 and T5), cytokine-producing $CD4^+$ and $CD8^+$ cells (plot 5, T6 and plot 6, T7, respectively), effector (T_{EM}) and central (T_{CM}) memory cytokine detection.

or control (Table 3.2) were done in triplicate for each group of mice. Complete media, and (1.5 X 10^6 splenocytes) were added to each well in a 96 well round-bottomed micro-titre plate. Each peptide or ConA or the stimulant peptides (100µl) were added to the allocated wells. The plate was incubated at 37˚C in a humidified incubator with 5% CO_2 for 48 hours. The supernatants were harvested (150µl) without disturbing the cell pellets and stored at -20˚C until the cytokine content was determined using a CBA mouse Th1/Th2 cytokine kit (BD Biosciences, USA) that measures IFN-γ, TNF-α, IL-2, IL-4, IL-6, and IL-10. The method was performed according to the manufacturer's instructions. The samples were analysed by flow cytometry on a FACS Calibur (Beckton Dickinson, USA). Results were calculated as picogram (pg) of cytokine per 1ml. The background response per group was determined as the mean amount of cytokine released and its SD in the absence of peptide. The background responses were subtracted from the HIV-1 Gag specific responses and the final response was expressed

as pg/ml. After subtracting the background, a cytokine level of 8pg/ml was considered a cut off for a positive response.

3.2.3 Statistical analysis

Data was statistically analysed using Prism version 5.0 (Graphpad Software, San Diego, CA). The *t* test for independent unpaired non-parametric comparisons was applied to assess the level of significance of comparisons between means. All tests were two-tailed. *P* values ≤ 0.05 were considered significant.

3.3 RESULTS

3.3.1 Pilot experiment to determine the optimal MVA-Gag^{M} dosage to boost a BCG prime

To determine the optimal MVA dose required to effectively boost the BCG vaccines mice were primed with 2 x 10^7 cfu of either the recombinant BCG-Gag^{M}, which expresses the HIV-1C mosaic Gag immunogen, or the mock BCG^{E}, which contained a plasmid with no *gag* insert, and boosted on day 70 with 10^2, 10^4, or 10^6 pfu of MVA-Gag^{M} which expresses the HIV-1C mosaic Gag immunogen (see table insert in Figure 3.2A). The BCG vaccine dose of 2 x 10^7 cfu was previously determined as optimal in our lab (Dr Ros Chapman, personal communication).

3.3.1.1 Magnitude of HIV-1 Gag-specific IFN-γ ELISPOT responses

As previously determined (364), twelve days following the MVA-Gag^{M} boost, mice spleens were pooled from each group for immunological assays (Section 3.2.2.1). An IFN-γ ELISPOT assay was used to quantify induced Gag specific CD4 and CD8 T cells (Figure 3.2B). Results from cells stimulated with an irrelevant peptide or with no peptide have not been included.

Priming with the recombinant or mock BCG vaccines and boosting with 10^2 pfu MVA-Gag^{M} elicited no Gag-specific responses in mice (Figure 3.2B; Groups 1 and 4 respectively). High cumulative HIV-1 Gag-specific IFN-γ ELISPOT responses were induced in mice primed with BCG-Gag^{M} and boosted with 10^4 and 10^6 pfu MVA-Gag^{M},

with response evenly balanced between CD4 and CD8 T cells (Figure 3.2B, Groups 2 and 3 respectively).

Priming with BCG-Gag^{M} and boosting with a dose of 10^4 pfu MVA-Gag^{M} (Group 2) elicited responses of 534 and 739 sfu/10^6 splenocytes to CD8 and CD4 peptides respectively. These responses were 5.4 and 1.6 fold higher, respectively, compared to those induced in mock-primed mice boosted with a 10^4 pfu dose of MVA-Gag^{M} (Group 5). Priming with BCG-Gag^{M} and boosting with a dose of 10^6 pfu MVA-Gag^{M} (Group 3) elicited cumulative Gag-specific responses of 2023 sfu/106 splenocytes, which were 4.1 fold higher than those induced in mock-primed mice boosted with a 10^6 pfu dose of MVA-Gag^{M} (496 sfu/10^6 splenocytes, Group 6). Thus, MVA-Gag^{M} efficiently boosts a BCG-Gag^{M} prime at a dose of 10^4 pfu or 10^6 pfu but not at a dose of 10^2 pfu.

Background responses in mice boosted with 10^2 and 10^4 pfu MVA-Gag^{M} (Groups 1, 2, 4 and 5) were below 20 sfu/10^6 splenocytes in the presence of an irrelevant peptide and when not stimulated. Background responses in mice boosted with 10^6 pfu MVA-Gag^{M} (Groups 3 and 6) however, were between 273 and 367 sfu/10^6 splenocytes when not stimulated, and between 292 and 420 sfu/10^6 splenocytes in the presence of an irrelevant peptide (results not shown). A dose of 10^6 pfu MVA-Gag^{M} was regarded as too high a dose for experimentation, and a dose of 10^4 pfu MVA-Gag^{M} was regarded as optimal.

3.3.1.2 Cytokine production and phenotype

To further characterise the immune responses induced by boosting BALB/c mice with different doses of MVA-Gag^{M} following a BCG prime, ICS followed by flow cytometry was carried out (Section 3.2.2.3). The cytokine response profile and the memory phenotype of the cytokine-producing cells were assessed on mice splenocytes. IFN-γ, TNF-α, and IL-2 antibodies were conjugated to a PE fluorochrome and the combined responses recorded (Figures 3.2C and D).

Priming with BCG-Gag^{M} or with the mock BCG, and boosting with 10^2 pfu MVA-Gag^{M} did not elicit any detectable HIV-1 Gag-specific cytokine-producing CD8^{+} T cells (Figure

A

Group	Prime	Boost	Sacrifice	Number of mice
1	Day 0: $2x10^7$ cfu BCG-Gag^M	Day 70: 10^2 pfu MVA-Gag^M	Day 82	4
2	Day 0: $2x10^7$ cfu BCG-Gag^M	Day 70: 10^4 pfu MVA-Gag^M	Day 82	4
3	Day 0: $2x10^7$ cfu BCG-Gag^M	Day 70: 10^6 pfu MVA-Gag^M	Day 82	4
4	Day 0: $2x10^7$ cfu BCG^E	Day 70: 10^2 pfu MVA-Gag^M	Day 82	4
5	Day 0: $2x10^7$ cfu BCG^E	Day 70: 10^4 pfu MVA-Gag^M	Day 82	4
6	Day 0: $2x10^7$ cfu BCG^E	Day 70: 10^6 pfu MVA-Gag^M	Day 82	4

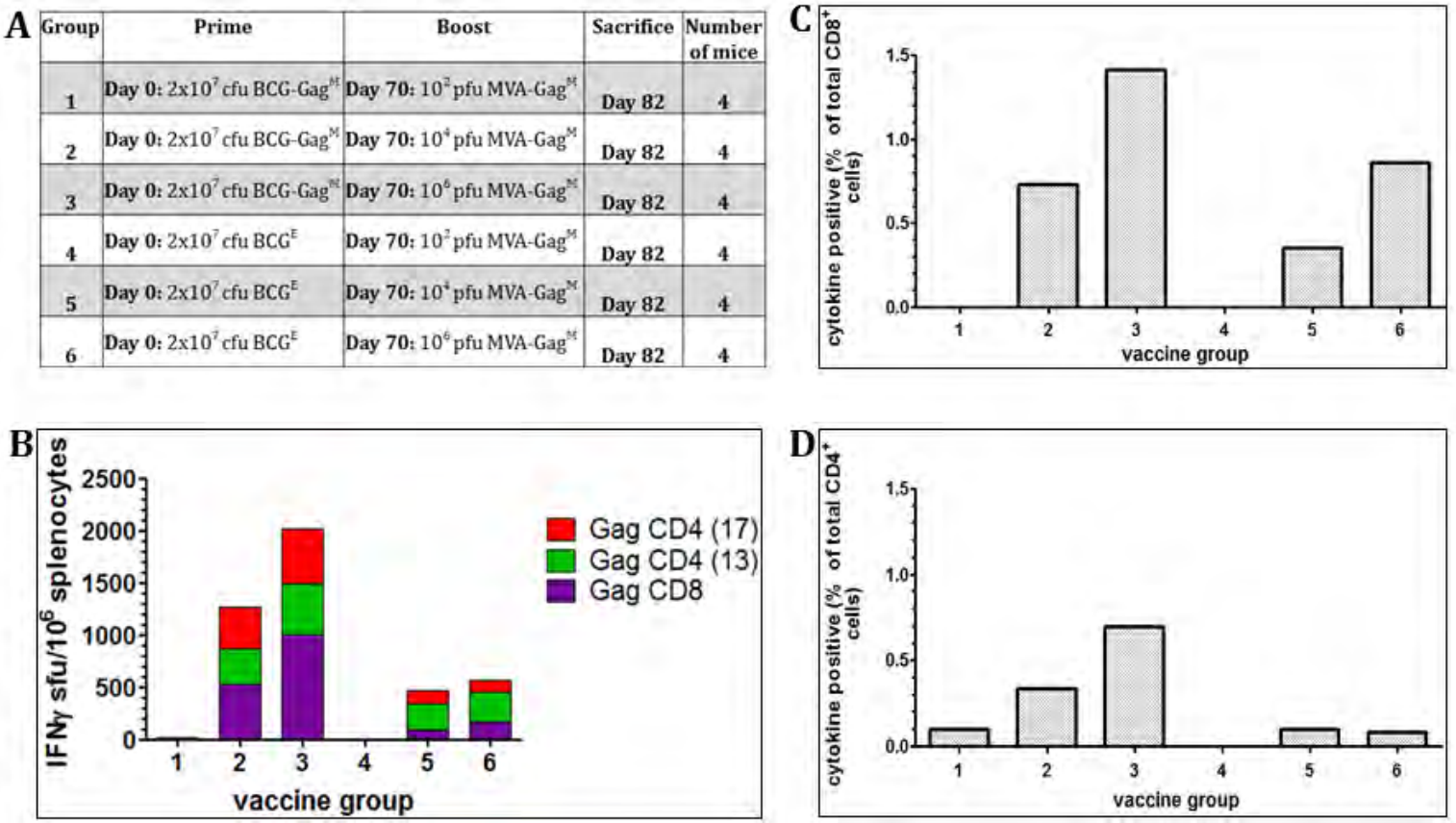


Figure 3.2: Determination of the optimal dosage of MVA-Gag^M to boost a BCG-Gag^M prime. (**A**) Mice were primed on day 0 with 2 x 10^7 cfu BCG-Gag^M (Group 1 - 3) or BCG^E (Group 4 - 6) and boosted on day 70 with 10^2 (Group 1 and 4), 10^4 (Group 2 and 5), or 10^6 (Group 3 and 6) pfu MVA-Gag^M. as indicated in the table insert. (**B**) Cumulative IFN-γ ELISPOT CD8$^+$ and CD4$^+$ responses of vaccinated mice to HIV-1 Gag peptides to determine the optimal MVA-Gag^M dosage to boost a BCG prime. The ELISPOT assay was carried out using three Gag-specific peptides for stimulation of pooled splenocytes that were isolated 12 days post the MVA-Gag^M boost. Bars represent the magnitude of net responses to individual peptides, expressed as sfu/10^6 splenocytes after subtracting the background. (**C**) and (**D**) Total frequency of T cells producing IFN-γ, IL-2, and/or TNF-α, after subtracting the background, in response to HIV-1 Gag peptide stimulation following a BCG prime and an MVA-Gag^M boost at different doses. Cells were positive for cytokine production if the proportion was ≥0.05% after subtracting the background.

3.2C, Groups 1 and 4). Mice primed with BCG-Gag^{M} and boosted with 10^4 pfu MVA-Gag^{M} (Group 2) elicited almost double the frequency of HIV-1 Gag-specific cytokine-producing CD8$^+$ T cells as compared to mock-primed mice that were similarly boosted (Group 5). Cytokine-producing CD8$^+$ T cells from mice that received a BCG-Gag^{M} prime and a boosting dose of 10^6 pfu MVA-Gag^{M} (Group 3) were almost double those of the control group (Group 6), suggesting an effective BCG prime.

The percentages of cytokine positive CD4$^+$ T cells were generally lower than those of cytokine positive CD8$^+$ T cells, with all values being less than 1%, irrespective of the vaccination regimen (Figure 3.2D). Both a dose of 10^4 and a dose of 10^6 pfu MVA-Gag^{M} efficiently boosted a BCG-Gag^{M} prime.

3.3.2 Immune responses in BALB/c mice elicited by BCG prime-MVA boost vaccines expressing a Gag^{M} immunogen

While the greatest cumulative immune response to the Gag peptides was detected from mice boosted with 10^6 pfu MVA-Gag^{M} (Figures 3.2; Group 3), the amount of background responses in the IFN-γ ELISPOT assay were high (up to 420 sfu/10^6 splenocytes). An MVA-Gag^{M} boost of 10^4 pfu for BCG-primed mice was therefore chosen as the optimal dose to compare immune responses to different BCG prime vaccinations. To validate the results obtained from a BCG prime and an MVA boost of 10^4 pfu, the experiments were repeated two more times. Mice vaccinated with a single dose of BCG-Gag^{M} or MVA-Gag^{M} and homologous prime-boost vaccinations with BCG-Gag^{M} or MVA-Gag^{M} were also included to compare the immune responses elicited (Figure 3.3). The inoculation schedule is detailed in Figure 3.3A. Vaccinations were done in three separate experiments to produce data that could be assessed statistically. Spleens were pooled from each group 12 days after the last vaccination.

3.3.2.1 Magnitude of HIV-1 Gag-specific IFN-γ ELISPOT responses

An IFN-γ ELISPOT assay was used to determine the magnitude of Gag specific CD4$^+$ and CD8$^+$ T cells responses (Figure 3.3B). Cells stimulated with an irrelevant peptide or with no peptide produced less than 40 sfu/10^6 splenocytes and have not been included. There was also no detectable response from the BCG-Gag^{M} single vaccination (mice sacrificed on day 82), and this result has not been included. Mean cumulative responses

to Gag-specific $CD4^+$ and $CD8^+$ T cell peptides for Group 2 mice (BCG-Gag^M/MVA-Gag^M) reached a magnitude of 1143 ± 117 sfu/10^6 splenocytes with almost equally balanced responses to Gag CD8 (475 ± 55 sfu/10^6 splenocytes) and Gag CD4 (668 ± 32.7 sfu/10^6 splenocytes; Figure 3.3B). There was a 2.8-fold difference between the cumulative responses of mice primed with BCG-Gag^M (Group 2) and those that received a mock-prime (Group 5 - 410 ± 98 sfu/10^6 splenocytes). Thus, BCG-Gag^M significantly primed an MVA-Gag^M boost ($p<0.01$).

Mean cumulative IFN-γ ELISPOT HIV-1 Gag responses of mice vaccinated with the BCG-Gag^M/MVA-Gag^M heterologous prime-boost regimen (Group 2) were 3- and 1.7-fold greater than the BCG-Gag^M/BCG-Gag^M homologous prime-boost (Group 16; 380 ± 64.7 sfu/10^6 splenocytes) and Group 18 (MVA-Gag^M/MVA-Gag^M homologous prime-boost; 656.7 ± 8.5 sfu/10^6) mice respectively. The BCG-Gag^M/MVA-Gag^M heterologous prime-boost was therefore significantly more efficient than the BCG-Gag^M/BCG-Gag^M ($p<0.001$) and MVA-Gag^M/MVA-Gag^M ($p<0.01$) homologous prime-boost vaccinations.

Mean cumulative IFN-γ ELISPOT HIV-1 Gag responses from the mice that received a homologous MVA-Gag^M prime-boost (Group 18) were 1.7-fold significantly greater than the cumulative responses elicited by a homologous BCG-Gag^M prime-boost vaccination (Group 16; $p<0.01$), and 2-fold significantly greater than the mean cumulative responses elicited by a single MVA-Gag^M vaccination (Group 17; $p<0.001$). Interestingly, responses to the CD8 Gag peptide were similar for Groups 17 (MVA-Gag^M) and 18 (MVA-Gag^M/ MVA-Gag^M; Figure 3.3C). The second MVA-Gag^M vaccination, however boosted $CD4^+$ T cells responses to Gag (Figure 3.3D).

3.3.2.2 Cytokine production and phenotype

The proportions of cytokine-producing T cells as well as the memory phenotype were determined by flow cytometry (Section 3.2.2.3; Figures 3.3 C and D). The frequency of Gag-specific $CD8^+$ T cells was 2.3-fold higher but not significantly different for mice that received a BCG-Gag^M/MVA-Gag^M heterologous prime-boost regimen (Figure 3.3C; Group 2) compared to those that received a mock prime (Group 5). The BCG-Gag^M/MVA-Gag^M heterologous prime-boost regimen (Group 2) resulted in $CD8^+$ T cells with a greater effector memory phenotype (91.6%) than those in the control group (Group 5 - 66.5%).

A

Group	Prime	Boost	Sacrifice	Number of mice
2	Day 0: $2x10^7$ BCG-Gag^M	Day 70: 10^4 pfu MVA-Gag^M	Day 82	4
5	Day 0: $2x10^7$ BCG^E	Day 70: 10^4 pfu MVA-Gag^M	Day 82	4
16	Day 0: $2x10^7$ BCG-Gag^M	Day 70: $2x10^7$ BCG-Gag^M	Day 82	4
17	-	Day 0: 10^4 pfu MVA-Gag^M	Day 12	5
18	Day 0: 10^4 pfu MVA-Gag^M	Day 28: 10^4 pfu MVA-Gag^M	Day 40	5

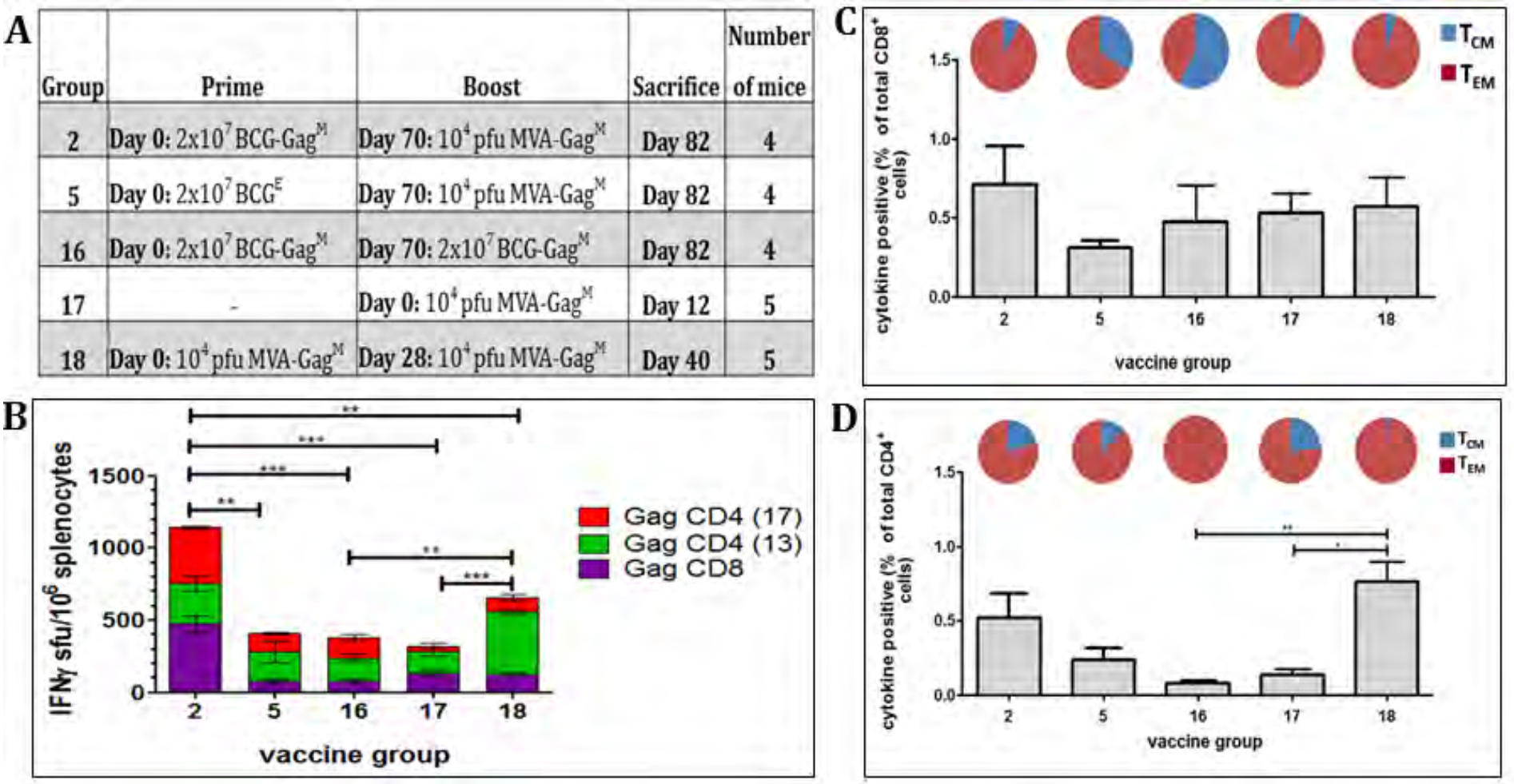


Figure 3.3: (legend on next page)

E

Cytokine	Mean ± SD (pg/ml)				
	Group 2 BCG-Gag^{M}/MVA-Gag^{M}	Group 5 BCG^{E}/MVA-Gag^{M}	Group 16 BCG-Gag^{M}/BCG-Gag^{M}	Group 17 MVA-Gag^{M}	Group 18 MVA-Gag^{M}/MVA-Gag^{M}
IFN-γ	2106 ± 180.1	859.8 ± 92.8	575.3 ± 102.5	448.3 ± 34	1202.7 ± 36.7
TNF-α	777.1 ± 107.2	157.8 ± 8	167.4 ± 4.1	63.6 ± 2.3	309.8 ± 1.8
IL-2	2839 ± 194.2	597.7 ± 26.8	848.3 ± 97	415.7 ± 19.3	2028.4 ± 114
IL-4	80.9 ± 5.1	36.1 ± 4.1	37.9 ± 8.5	17.4 ± 4.3	44.8 ± 4.5
IL-6	112.6 ± 5.9	22.9 ± 6.1	10.2 ± 1.5	0	57.4 ± 5.3
IL-10	74 ± 2.9	16.4 ± 2.2	29 ± 7.8	10.3 ± 0.6	95.3 ± 10.7

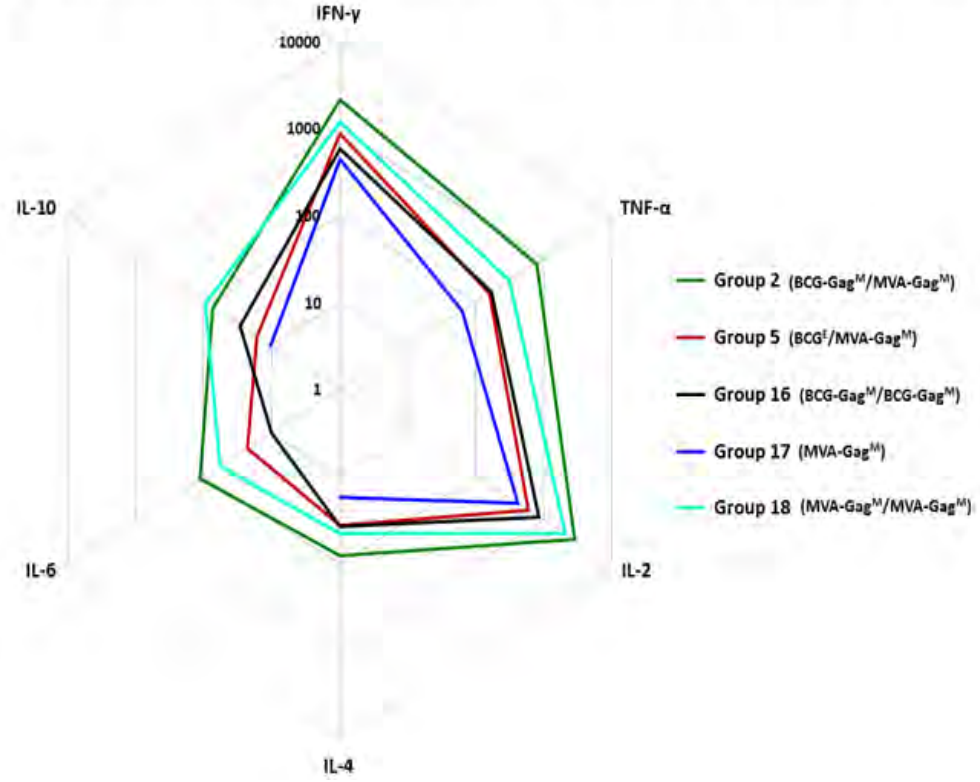


Figure 3.3: Evaluation of a BCG-Gag^{M} prime/ MVA-Gag^{M} boost in BALB/c mice. (**A**) Vaccination schedule used. (**B**) Cumulative IFN-γ ELISPOT CD8^{+} and CD4^{+} responses of vaccinated mice to HIV-1 Gag peptides. The ELISPOT assay was done using pooled spleens on the day of sacrifice using three Gag-specific peptides for stimulation. Bars are the mean and standard deviation of the mean responses for the indicated individual peptides from 3 independent experiments. Responses are expressed as sfu/10^{6} splenocytes after background subtraction. Horizontal bars with asteriks indicate statistical significance of the mean responses between the indicated groups. $^{**}p<0.01$, $^{***}p<0.001$; Student t-test of unpaired data. (**C**) and (**D**) Total frequency of T cells producing IFN-γ, IL-2, and/or TNF-α in response to HIV-1 Gag peptide stimulation. ICS and flow cytometry were carried out on pooled spleens per group using three Gag-specific peptides for stimulation. The memory distribution of the cytokine producing T- cells in the central and effector memory compartment (T_{CM} and T_{EM}) are represented as pie charts above each corresponding bar per group. Cells were positive for cytokine production if the proportion was ≥ 0.05% after subtracting the background. The cellular phenotype was positive if there were ≥ 10 cells per test. (**E**) Results from cytokine measurements in cell supernatants of mice vaccinated with different vaccine regimens using a CBA Th1/Th2 Kit and radial plot of cells producing six different cytokines in response to HIV-1 Gag peptide stimulation. The levels of cytokines in the culture supernatants were quantified using a Th1/Th2 cytokine bead array assay followed by flow cytometry. The recorded results were obtained after subtracting the background. The distance from the centre of the plot indicates a $\log_{10}$-fold change (ranging from 1 to 10 000) and cytokine levels were expressed as pg/ml.

No significant differences were detected in $CD8^+$ T cell cytokine production for any of the vaccination regimens (Figure 3.3C). The cytokine producing $CD8^+$ T cells elicited by a single MVA-Gag^M (Group 17) and double MVA-Gag^M (Group 18) vaccination had a predominantly effector memory phenotype (94% and 92% respectively). However, a BCG-Gag^M homologous prime-boost resulted in cytokine positive $CD8^+$ T cells that were predominantly of a central memory phenotype (58%; Group 16). There were less cytokine-producing $CD4^+$ T cells than there were cytokine-producing $CD8^+$ T cells for all vaccination regimens except for the Group 18 mice that received an MVA-Gag^M homologous prime-boost vaccination (Figure 3.3C and D).

The frequency of HIV-1-specific $CD4^+$ T cell immune responses was 2.2-fold higher but not significantly different for mice that received a BCG-Gag^M/MVA-Gag^M heterologous prime-boost regimen (Figure 3.3D; Group 2) compared to those that received a mock prime (Group 5). There were however less $CD4^+$ T cells with an effector memory phenotype in mice that received the BCG-Gag^M/MVA-Gag^M heterologous prime-boost regimen (Group 2 - 79.5%) than in the control group that received the mock/MVA-Gag^M prime-boost regimen (Group 5 - 87.2%).

Cytokine-producing $CD4^+$ T cells following an MVA-Gag^M homologous prime-boost (Figure 3.3D; Group 18 - 0.76%) were 9.5–and 5.4-fold significantly higher than those of the BCG-Gag^M homologous prime-boost (Group 16; 0.08% (27-43 cytokine positive cells); $p<0.01$), and the single MVA-Gag^M vaccination (Group 17 - 0.14%; $p<0.05$) respectively. A single MVA-Gag^M vaccination resulted in cytokine-positive $CD4^+$ T cells with a predominant effector memory phenotype (Group 17 - 76%), and a homologous boost increased the proportion of effector memory $CD4^+$ T cells to 99% (Group 18). Cytokine-positive $CD4^+$ T cells following a BCG-Gag^M homologous prime-boost all had an effector memory phenotype (Group 16).

3.3.2.3 Profile of cytokines secreted into the supernatant

To assess the Th1/Th2 bias of the immune response to the vaccines used, a cytokine bead array assay was used. The Th1 and Th2 cytokines were quantified from the culture medium collected from splenocytes stimulated with Gag CD4 and CD8 peptides (Figure 3.3E). IFN-γ, TNF-α, and IL-2 had the highest cumulative levels in all groups of mice, suggesting a Th1 bias. IFN-γ, TNF-α, and IL-2 were 2.4-, 4.9-, and 4.7-fold higher,

respectively, in Group 2 (BCG-Gag^{M}/MVA-Gag^{M}) compared to Group 5 mice (BCG-Gag^{E}/MVA-Gag^{M}) suggesting an efficient prime with the BCG-Gag^{M} vaccine.

The MVA-Gag^{M} vaccine also potently boosted the BCG-Gag^{M} prime. Cumulative IFN-γ, TNF-α, and IL-2 levels were 3.7-, 8.3-, and 3.3-fold higher, respectively, in mice that received the heterologous BCG-Gag^{M}/MVA-Gag^{M} prime-boost vaccination (Group 2) compared to Group 16 mice that received a BCG-Gag^{M}/BCG-Gag^{M} homologous vaccination. Splenocytes from the heterologous prime-boost vaccination (Group 2) produced high levels of IFN-γ, TNF-α, and IL-2 compared to any of the homologous prime-boost vaccinations (Group 16 and 18; BCG-Gag^{M}/BCG-Gag^{M} and MVA-Gag^{M}/MVA-Gag^{M} respectively). However, two vaccinations with MVA-Gag^{M} (Group 18) produced more IFN-γ, TNF-α, and IL-2 than two vaccinations with BCG-Gag^{M} (Group16). The cumulative cytokine levels were higher in Group 18 mice by factors of 2.1, 1.9, and 2.4, respectively.

Two vaccinations of MVA-Gag^{M} (Group 18) also induced the secretion of higher levels of cytokines as compared to mice that were vaccinated with a single dose of MVA-Gag^{M} (Group 17). The IFN-γ, TNF-α, and IL-2 levels were 2.7, 4.9, and 4.9 fold higher respectively.

3.3.3 Pilot experiment to determine the optimal MVA-Gag^{M} dosage to boost a DNA prime

To determine the optimal MVA dose required to effectively boost the DNA vaccines, mice were primed with 10μg of either the DNA-Gag^{M}, which expresses the HIV-1C Gag^{M} immunogen, or the mock DNA^{E}, which has no *gag* insert. Mice were then boosted on day 56 with 10^{2}, 10^{4}, or 10^{6} pfu of MVA-Gag^{M} which expresses an HIV-1C Gag^{M} immunogen. Twelve days following the MVA-Gag^{M} boost, mice spleens were pooled from each group for immunological assays (Section 3.2.2.1).

3.3.3.1 Magnitude of HIV-1 Gag-specific IFN-γ ELISPOT responses

An IFN-γ ELISPOT assay was used to quantify the Gag specific CD4 and CD8 T cells responses (Figure 3.4B). Results from cells stimulated with an irrelevant peptide or with no peptide have not been included.

A

Group	Prime	Prime	Boost	Sacrifice	Number of mice
7	Day 0: 10µg DNA-Gag^{M}	Day 28: 10µg DNA-Gag^{M}	Day 56: 10^{2} pfu MVA-Gag^{M}	Day 68	5
8	Day 0: 10µg DNA-Gag^{M}	Day 28: 10µg DNA-Gag^{M}	Day 56: 10^{4} pfu MVA-Gag^{M}	Day 68	5
9	Day 0: 10µg DNA-Gag^{M}	Day 28: 10µg DNA-Gag^{M}	Day 56: 10^{6} pfu MVA-Gag^{M}	Day 68	5
10	Day 0: 10µg DNA^{E}	Day 28: 10µg DNA^{E}	Day 56: 10^{2} pfu MVA-Gag^{M}	Day 68	5
11	Day 0: 10µg DNA^{E}	Day 28: 10µg DNA^{E}	Day 56: 10^{4} pfu MVA-Gag^{M}	Day 68	5
12	Day 0: 10µg DNA^{E}	Day 28: 10µg DNA^{E}	Day 56: 10^{6} pfu MVA-Gag^{M}	Day 68	5

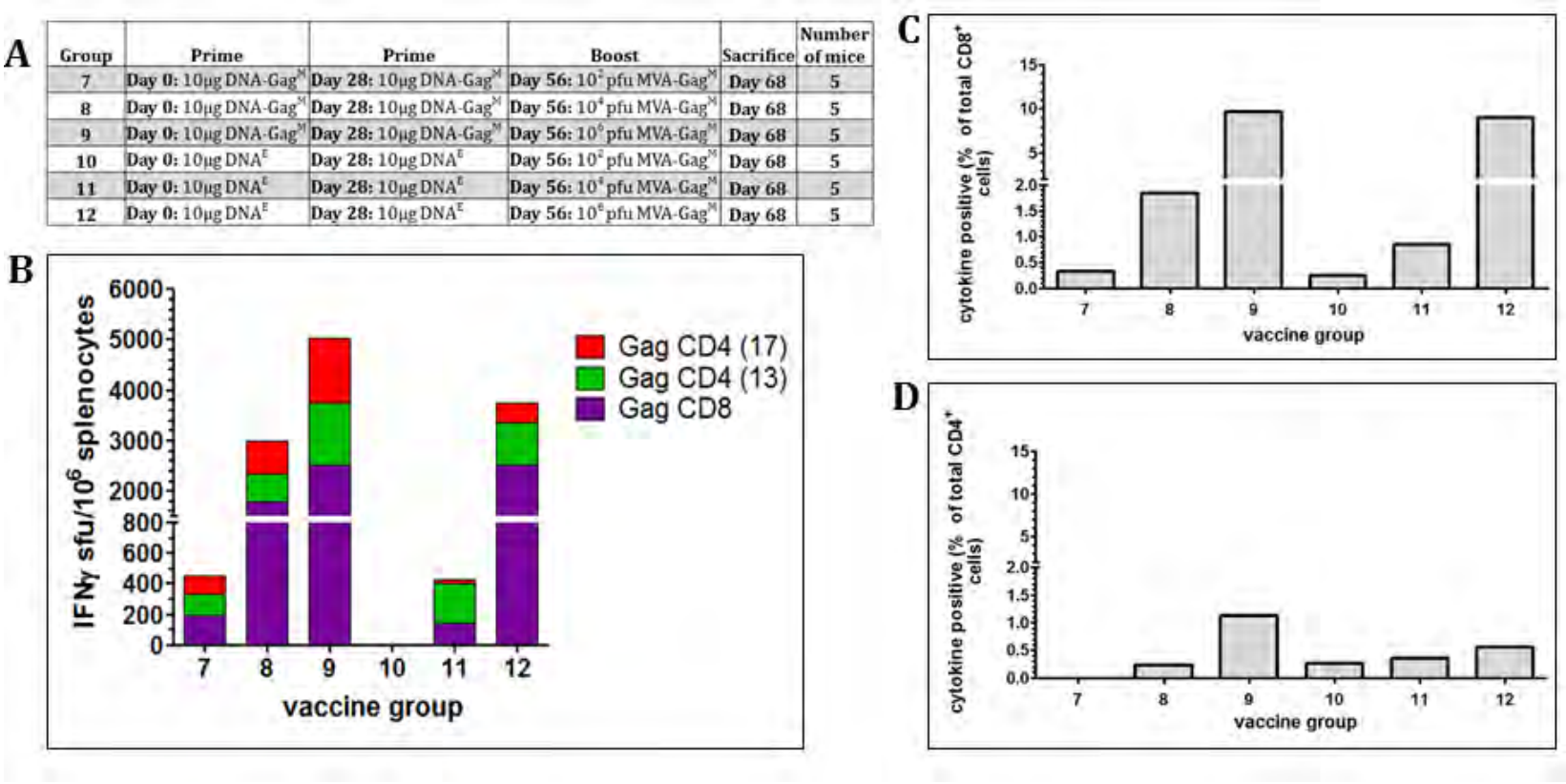


Figure 3.4: Determination of the optimal dosage of MVA-Gag^{M} to boost a DNA-Gag^{M} prime. (**A)** Mice were primed on day 0 with 10µg DNA-Gag^{M} (Group 7-9) or DNA^{E} (Group 10-11) and boosted on day 56 with 10^{2} (Group 7 and 10), 10^{4} (Group 8 and 11), or 10^{6} (Group 9 and 12) pfu MVA-Gag^{M}. as indicated in the table insert. (**B**) Cumulative IFN-γ ELISPOT CD8^{+} and CD4^{+} responses of vaccinated mice to HIV-1 Gag peptides to determine the optimal MVA-Gag^{M} dosage to boost a DNA prime. The ELISPOT assay was carried out using three Gag-specific peptides for stimulation of pooled splenocytes that were isolated 12 days post the MVA-Gag^{M} boost. Bars represent the magnitude of net responses to individual peptides, expressed as sfu/10^{6} splenocytes after subtracting the background. (**C**) and (**D**) Total frequency of T cells producing IFN-γ, IL-2, and/or TNF-α, after subtracting the background, in response to HIV-1 Gag peptide stimulation following a DNA prime and an MVA-Gag^{M} boost at different doses. Cells were positive for cytokine production if the proportion was ≥0.05% after subtracting the background.

Priming with the two doses of DNA-Gag^{M} and boosting with a dose of 10^2 pfu MVA-Gag^{M} elicited cumulative T cell responses of 454 sfu/10^6 cells (Figure 3.4B; Group 7) that were balanced between CD4 and CD8 T cells. Increasing the MVA-Gag^{M} dose to 10^4 pfu and 10^6 pfu generated cumulative T cell responses that were 6.6- and >11-fold greater respectively, than the MVA-Gag^{M} dose of 10^2 pfu (Figure 3.4B; Group 8 - 2983 sfu/10^6 cells; Group 9 - >5026 sfu/10^6 cells). These responses were predominantly CD8 T cell responses.

The DNA^{E} mock prime vaccine boosted with a dose of 10^2 pfu MVA-Gag^{M} elicited no detectable T cell responses with an IFN-γ ELISPOT assay (Figure 3.4B; Group 10). Priming with two doses of DNA-Gag^{M} and boosting with a dose of 10^4 pfu MVA-Gag^{M} (Group 8) elicited 1798 and 1185 sfu/10^6 splenocytes responses to CD8 and CD4 peptides respectively. These responses were 12.1 and 4.1 fold higher, respectively, compared to those induced in mock-primed mice boosted with a 10^4 pfu dose of MVA-Gag^{M} (Figure 3.4B; Group 11 - 148 and 287 sfu/10^6 splenocytes responses to CD8 and CD4 peptides respectively). Priming with two doses of DNA-Gag^{M} and boosting with a single dose of 10^6 pfu MVA-Gag^{M} (Figure 3.4B; Group 9) elicited 2511 sfu/10^6 splenocytes responses to Gag CD4 peptides. This was 2-fold higher compared to Gag CD4 responses induced in mock-primed mice boosted with a 10^6 pfu dose of MVA-Gag^{M} (Figure 3.4B; Group 12).

While the immune responses to Gag increased proportionally with the MVA-Gag^{M} boosting dose, it is noteworthy that the HIV-1 Gag CD8 T cell responses for mice boosted with 10^6 pfu of MVA-Gag^{M} (Figure 3.4B; Group 9 and 12) were beyond the limit of accurate detection of the IFN-γ ELISPOT kit. The recorded results are therefore similar between the groups (2515 and 2532 net sfu/10^6 splenocytes for Group 9 and 12 respectively). These responses were obtained from the highest number of spots detected for the ConA positive control responses on the ELISPOT plate in those experiments. All three doses of MVA-Gag^{M} investigated therefore efficiently boost two DNA-Gag^{M} prime vaccinations. The two doses of the DNA-Gag^{M} vaccine also potently prime the MVA-Gag^{M} boost.

Background responses in mice boosted with 10^2 and 10^4 pfu MVA-Gag^{M} (Groups 7, 8, 10, and 11) were below 20 sfu/10^6 splenocytes in the presence of an irrelevant peptide and

when not stimulated. Background responses in mice boosted with 10^6 pfu MVA-Gag^M (Groups 9 and 12) however, were between 69 and 113 sfu/10^6 splenocytes when not stimulated, and between 61 and 133 sfu/10^6 splenocytes in the presence of an irrelevant peptide (results not shown).

3.3.3.2 Cytokine production and phenotype

To further characterise and compare the immune responses induced by boosting mock- and DNA-Gag^M primed animals with different doses of MVA-Gag^M, we evaluated cytokine production and memory phenotype by flow cytometry (Section 3.2.2.3; Figures 3.4C and D).

Priming with DNA-Gag^M and boosting with a dose of 10^2 pfu MVA-Gag^M elicited low frequency cytokine-positive CD8$^+$ T cells (Figure 3.4C; Group 7 - 0.32% (246 cytokine positive CD8$^+$ T cells out of 77831 CD8$^+$ T cells after background subtraction)). Increasing the MVA-Gag^M dosage to 10^4 pfu elicited 5.8 fold more cytokine-positive CD8$^+$ T cells (Figure 3.4C; Group 8 - 1.85%). Further increasing the MVA-Gag^M dosage to 10^6 pfu elicited exceptionally high frequencies of cytokine-producing CD8$^+$ T cells (Figure 3.4C; Group 9 - 9.63%).

Mice primed with DNA-Gag^M and boosted with 10^2 pfu and 10^4 pfu MVA-Gag^M had 1.28 and 2.2 fold higher levels of cytokine-positive CD8$^+$ T cells respectively than those primed with the empty vector, DNA^E, and boosted with the same doses of MVA-Gag^M. Boosting mice primed with DNA-Gag^M or DNA^E with 10^6 pfu MVA-Gag^M elicited a very high frequency of cytokine-positive CD8$^+$ T cells for (9.63% and 9.02% respectively). A 10^6 pfu dose of MVA-Gag^M elicited such high CD8$^+$ T cell responses that differences in the DNA prime could not be distinguished.

The proportions of cytokine-positive CD4$^+$ T cells were lower than those of cytokine-positive CD8$^+$ T cells (Figure 3.4D). No detectable cytokine-positive CD4$^+$ T cells were elicited by Group 7 mice that received a DNA-Gag^M/ 10^2 pfu MVA-Gag^M heterologous prime-boost vaccination. Increasing the MVA-Gag^M dosage to 10^4 and 10^6 pfu elicited 0.4% (Group 8) and 1.12% (Group 9) cytokine-positive CD4$^+$ T cells respectively. There was a 1.5-fold increase in the amount of cytokine-positive CD4$^+$ T cells between Group 8 and 11, suggesting an effective DNA-Gag^M prime. DNA-Gag^M was also effective priming

mice boosted with 10^6 pfu MVA-Gag^M (Group 9 - 1.12%) in comparison to the mock-primed mice that received the same dose of MVA-Gag^M (Group 12; 0.55%) as there was a 2-fold increase in the proportion of cytokine-positive CD4$^+$ T cells.

3.3.4 Immune responses in BALB/c mice elicited by a DNA prime-MVA boost vaccine expressing a Gag^M immunogen and comparison to vaccines expressing natural Gag

Although the highest immune responses were obtained from mice vaccinated with the DNA-Gag^M prime/10^6 pfu MVA-Gag^M boost (Figures 3.4 B, C and D; Group 9) and mock-prime/10^6 pfu MVA-Gag^M boost (Figures 3.4 B, C and D; Group 12) regimens, these mice elicited high background IFN-γ ELISPOT responses as described in Section 3.5.3.1. The responses to the HIV-1 Gag CD8 peptide were also above the limit of accurate detection of the ELISPOT assay. A dosage of 10^4 pfu MVA-Gag^M was therefore chosen as the optimal boost for DNA-Gag^M-primed mice to further evaluate the immunogenicity of the mosaic Gag antigen and the experiments were repeated.

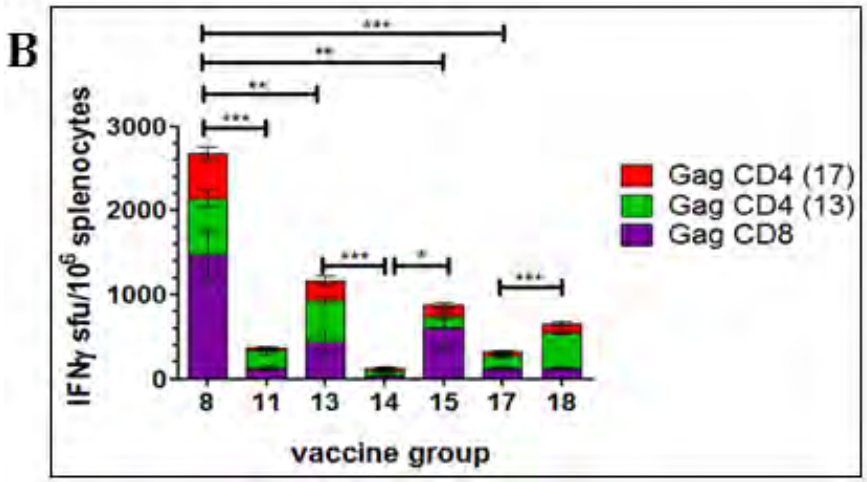
DNA and MVA vaccines expressing a natural Gag have previously been constructed in our laboratory. Included in the experiment were mice vaccinated with a DNA-Gag^N prime/10^4 pfu MVA-Gag^N heterologous prime boost regime. The DNA-Gag^N and MVA-Gag^N vaccines express a natural HIV-1C Gag immunogen that was isolated from an HIV-1 positive sex worker, Du422, in South Africa. The Gag^N had the closest sequence similarity to the consensus sequence following a subtype C Gag alignment (309). The MVA-Gag^N vaccine was previously constructed in our lab (364), and the DNA-Gag^N vaccine was constructed as part of this project. Mice were vaccinated as indicated in Figure 3.5A.

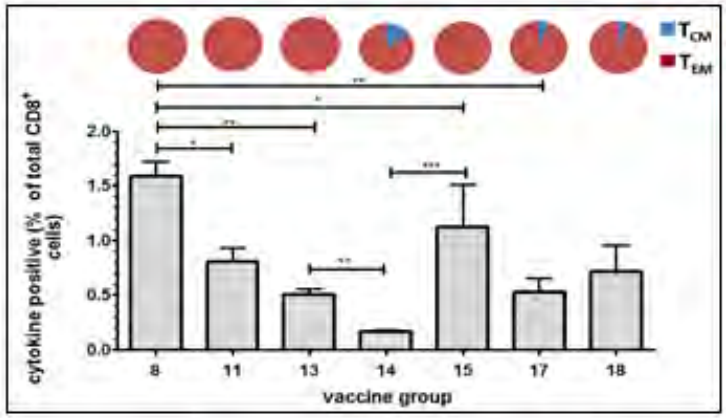
3.3.4.1 Magnitude of HIV-1 Gag-specific IFN-γ ELISPOT responses

There was a 7.1-fold significant difference ($p<0.001$) in the mean cumulative Gag-specific IFN-γ ELISPOT response of mock-primed mice boosted with 10^4 pfu MVA-Gag^M (Figure 3.5B; Group 11 - 375 ± 70.7 sfu/10^6 splenocytes) compared to mice primed with two doses of DNA-Gag^M and similarly boosted (Figure 3.5B; Group 8 - 2675.3 ± 292.8 sfu/10^6 splenocytes). Group 11 mice had a predominantly CD4 response (241.7 ± 29.7 sfu/10^6 splenocytes) to Gag, whereas a prime with DNA-Gag^M elicited a fairly balanced response to Gag CD4 and CD8 peptides (Group 8 - 1200.3 ± 183.2 sfu/10^6 and 1475 ±

A

Group	Prime	Prime	Boost	Sacrifice	Number of mice
8	Day 0: 10µg DNA-Gag^{M}	Day 28: 10µg DNA-Gag^{M}	Day 56: 10^4 pfu MVA-Gag^{M}	Day 68	5
11	Day 0: 10µg DNA^{E}	Day 28: 10µg DNA^{E}	Day 56: 10^4 pfu MVA-Gag^{M}	Day 68	5
13	Day 0: 10µg DNA-Gag^{N}	Day 28: 10µg DNA-Gag^{N}	Day 56: 10^4 pfu MVA-Gag^{N}	Day 68	5
14	-	Day 0: 10µg DNA-Gag^{N}	Day 28: 10µg DNA-Gag^{N}	Day 40	5
15	-	Day 0: 10µg DNA-Gag^{M}	Day 28: 10µg DNA-Gag^{M}	Day 40	5
17	-	-	Day 0: 10^4 pfu MVA-Gag^{M}	Day 12	5
18	-	Day 0: 10^4 pfu MVA-Gag^{M}	Day 28: 10^4 pfu MVA-Gag^{M}	Day 40	5





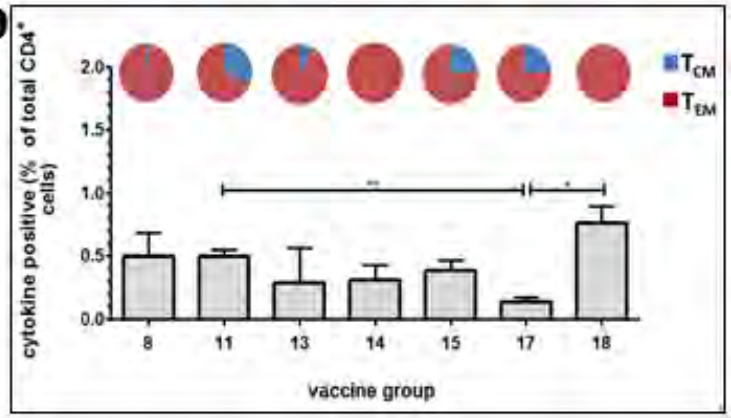


Figure 3.5: (legend on next page)

E

Cytokine	Mean ± SD (pg/ml)						
	Group 8 DNA-Gag^{M}/MVA-Gag^{M}	Group 11 DNA^{E}/MVA-Gag^{M}	Group 13 DNA-Gag^{N}/MVA-Gag^{N}	Group 14 DNA-Gag^{N}/DNA-Gag^{N}	Group 15 DNA-Gag^{M}/DNA-Gag^{M}	Group 17 MVA-Gag^{M}	Group 18 MVA-Gag^{M}/MVA-Gag^{M}
IFN-γ	15787.2 ± 914.6	714.4 ± 180.2	3561.1 ± 309.4	331.8 ± 27.6	1063.2 ± 169	448.3 ± 34	1202.7 ± 36.7
TNF-α	397.5 ± 22.6	24.6 ± 3.4	158.4 ± 15.2	105.9 ± 0.9	102.3 ± 11.6	63.6 ± 2.3	309.8 ± 1.8
IL-2	2450.9 ± 336	479.6 ± 65.2	1032.2 ± 30.5	296.3 ± 60.3	743.6 ± 28.2	415.7 ± 19.3	2028.4 ± 114
IL-4	168.8 ± 23.6	0	50.5 ± 2.3	0	47.4 ± 2.4	17.4 ± 4.3	44.8 ± 4.5
IL-6	45.6 ± 6.5	17.8 ± 1.2	15.4 ± 1.2	32.9 ± 12.8	39.2 ± 8.7	0	57.4 ± 5.3
IL-10	179.3 ± 14.5	11.7 ± 3.2	92.2 ± 3.1	19. ± 1.9	41.2 ± 6.9	10.3 ± 0.6	95.3 ± 10.7

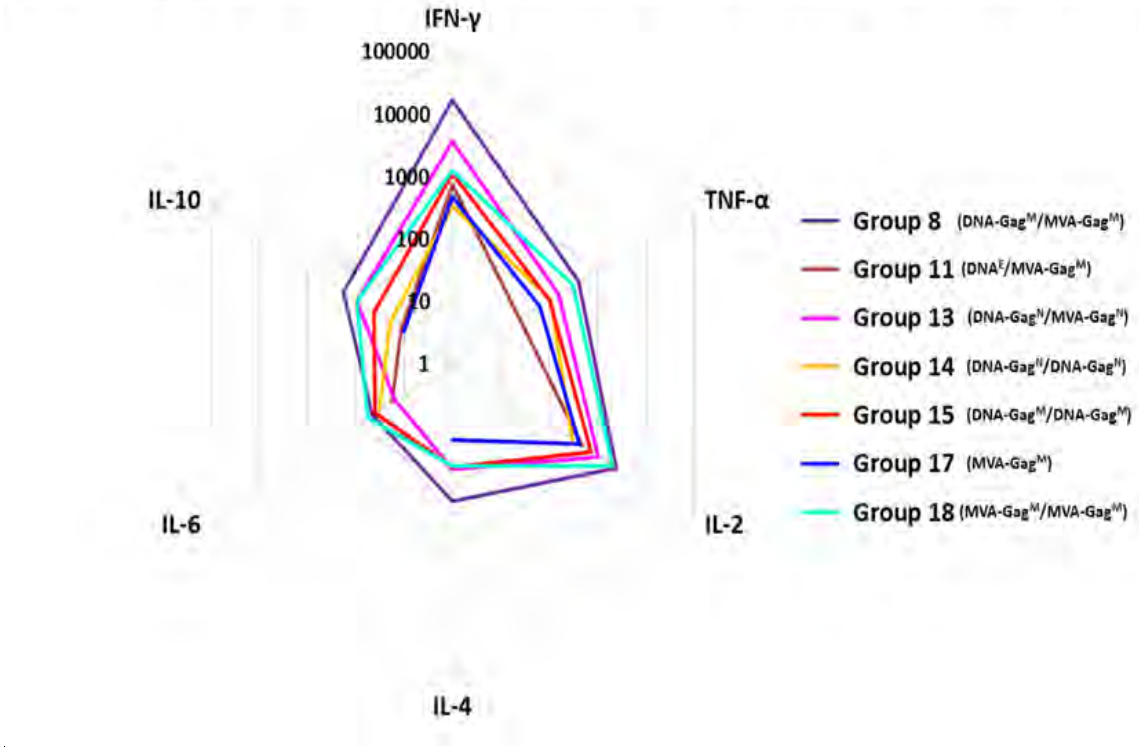


Figure 3.5: Evaluation of a DNA-Gag^{M} prime/ MVA-Gag^{M} boost in BALB/c mice. (**A**) Vaccination schedule used. (**B**) Cumulative IFN-γ ELISPOT CD8^{+} and CD4^{+} responses of vaccinated mice to HIV-1 Gag peptides. The ELISPOT assay was done using pooled spleens on the day of sacrifice using three Gag-specific peptides for stimulation. Bars are the mean and standard deviation of the mean responses for the indicated individual peptides from 3 independent experiments. Responses are expressed as sfu/10^{6} splenocytes after background subtraction. Horizontal bars with asteriks indicate statistical significance of the mean responses between the indicated groups. *p<0.05, **p<0.01, ***p<0.001; Student t-test of unpaired data. (**C**) and (**D**) Total frequency of T cells producing IFN-γ, IL-2, and/or TNF-α in response to HIV-1 Gag peptide stimulation. ICS and flow cytometry were carried out on pooled spleens per group using three Gag-specific peptides for stimulation. The memory distribution of the cytokine producing T- cells in the central and effector memory compartment (T_{CM} and T_{EM}) are represented as pie charts above each corresponding bar per group. Cells were positive for cytokine production if the proportion was ≥ 0.05% after subtracting the background. The cellular phenotype was positive if there were ≥ 10 cells per test. (**E**) Results from cytokine measurements in cell supernatants of mice vaccinated with different vaccine regimens using a CBA Th1/Th2 Kit and radial plot of cells producing six different cytokines in response to HIV-1 Gag peptide stimulation**.** The levels of cytokines in the culture supernatants were quantified using a Th1/Th2 cytokine bead array assay followed by flow cytometry. The recorded results were obtained after subtracting the background. The distance from the centre of the plot indicates a $\log_{10}$-fold change (ranging from 1 to 100 000) and cytokine levels were expressed as pg/ml.

91.6 sfu/10^6 splenocytes respectively). The efficiency of the DNA-Gag^M prime is also evident on comparison of the mean cumulative Gag-specific IFN-γ ELISPOT responses between Group 8 mice (2675.3±292.8 sfu/10^6 splenocytes) and Group 17 mice that were only vaccinated with a single dose of MVA-Gag^M (323.7 ± 23.9 sfu/10^6 splenocytes). There was an 8.3-fold significant difference between the groups ($p<0.001$). A comparison of the mean cumulative Gag-specific IFN-γ ELISPOT responses in Group 17 mice (MVA-Gag^M) and Group 11 mice (DNA^E/MVA-Gag^M) that were mock-primed and boosted with MVA-Gag^M indicated that the mock-prime did not influence the immune response elicited by the MVA-Gag^M boost (Group 11 - 375 ± 70.4 sfu/10^6 splenocytes).

The MVA-Gag^M vaccine significantly boosts two priming doses of DNA-Gag^M ($p<0.01$) as seen in the 3-fold increase in the mean cumulative Gag-specific IFN-γ ELISPOT responses between Group 8 mice (DNA-Gag^M/MVA-Gag^M) and Group 15 mice (DNA-Gag^M/DNA-Gag^M) that only received two priming does of DNA-Gag^M (Figure 3.5B; 882.3 ± 297.8 sfu/10^6 splenocytes).

There was a 2.3–fold significant difference ($p<0.01$) in the mean cumulative Gag-specific IFN-γ ELISPOT response between the heterologous DNA-Gag^M /MVA-Gag^M (vaccines expressing Gag^M) and the heterologous DNA-Gag^N prime/ MVA-Gag^N (vaccines expressing the natural Gag) prime-boost vaccinations (Figure 3.5B; Group 8 and Group 13 - 1171 ± 124.2 sfu/10^6 splenocytes). Group 8 vaccination regimen elicited higher Gag-specific CD8 T cell responses compared to Group 13 mice (1475 ± 295.3 sfu/10^6 and 444 ± 144.9 sfu/10^6 splenocytes respectively). Thus, the heterologous vaccination using vaccines that express a mosaic Gag immunogen elicited more potent IFN-γ ELISPOT immune responses compared to the vaccine expressing a natural Gag immunogen.

Mice that were vaccinated with two doses of DNA-Gag^M only (Figure 3.5B; Group 15) had mean cumulative Gag-specific IFN-γ ELISPOT responses that were 6.5–fold greater than mice vaccinated with two doses of DNA-Gag^N (Figure 3.5B; Group 14 - 135.7 ± 14 sfu/10^6 splenocytes). These results indicate that the mosaic Gag is significantly more immunogenic than the natural Gag ($p<0.05$). Surprisingly, two doses of DNA-Gag^M (Group 15) elicited mean cumulative Gag-specific IFN-γ ELISPOT responses that were

1.3–fold greater than mice vaccinated with two doses of MVA-Gag^{M} (Figure 3.5B; Group 18 - 656.7 ± 8.5 sfu/10^6 splenocytes). Group 15 mice had higher responses to the CD8 than the CD4 Gag peptide (604 ± 239.2 sfu/10^6 and 278.4 ± 32.6 sfu/10^6 splenocytes respectively). On the other hand, Group 18 mice had more responses to the CD4 than the CD8 Gag peptide (530 ± 20.5 sfu/10^6 and 126.7 ± 11.7 sfu/10^6 splenocytes respectively).

3.3.4.2 Cytokine production and phenotype

The proportions of cytokine-producing T cells as well as the memory phenotype induced by boosting BALB/c mice with a dose of 10^4 pfu MVA-Gag^{M} following a DNA prime were determined by flow cytometry (Section 3.2.2.3; Figures 3.5C and D). All vaccine regimens elicited cytokine-producing CD8$^+$ and CD4$^+$ T cells. However, the proportion of CD8$^+$ T cells producing cytokines was greater than that of CD4$^+$ T cells.

A DNA-Gag^{M}/MVA-Gag^{M} heterologous prime-boost vaccination elicited a cytokine-positive CD8$^+$ T cell response that was 2-fold significantly greater ($p<0.05$) than mock-primed mice that were similarly boosted (Figure 3.5C; Group 8 - DNA-Gag^{M}/MVA-Gag^{M} - 1.59%; Group 11 - DNA^{E}/MVA-Gag^{M} - 0.81% respectively). Both vaccine groups had cytokine-positive T cells with an effector memory phenotype. There was no significant difference in the proportion of cytokine-positive CD4$^+$ T cells between the two groups (Figure 3.5D; Group 8 – 0.5%; Group 11 – 0.35%). However, Group 8 mice (DNA-Gag^{M}/MVA-Gag^{M}) had more cytokine-positive CD4$^+$ T cells with an effector memory phenotype (97.5%) than Group 11 mice did (DNA^{E}/MVA-Gag^{M}; 67%). Thus, two doses of the DNA-Gag^{M} vaccine significantly primed the MVA-Gag^{M} boost by increasing the proportion of cytokine-positive CD8$^+$ T cells and the effector memory phenotype of cytokine-positive CD4$^+$ T cells.

The efficiency of the DNA-Gag^{M} prime is also demonstrated in the comparison of Group 8 and Group 17 (single MVA-Gag^{M} vaccination) mice. Cytokine-positive CD8$^+$ T cells in Group 8 mice were 3-fold significantly greater ($p<0.01$) than Group 17 mice (Figure 3.5C). The proportion of cytokine-positive CD8$^+$ T cells with an effector memory phenotype increased from 94% (Group 17), to 100% (Group 8). Cytokine-positive CD4$^+$ T cells were 3.6-fold higher in Group 8 than in Group 17 mice (Figure 3.5D). The effector

memory phenotype of these cells increased from 76% to 97.5% (Figure 3.5D; Group 17 and 8 respectively).

The efficiency of the MVA-Gag^{M} boost was determined by comparing immune responses from mice that received the DNA-Gag^{M}/MVA-Gag^{M} heterologous prime-boost regimen (Group 8) to those vaccinated with the DNA-Gag^{M}/DNA-Gag^{M} regimen (Group 15). Cytokine-positive CD8^{+} T cells from Group 8 (1.59%) mice were 1.4-fold significantly higher than those elicited by Group 15 mice (1.12%; Figure 3.5C). All the cytokine-positive CD8^{+} T cells had an effector memory phenotype in both groups. Surprisingly, there was only a 1.3-fold difference in cytokine-positive CD4^{+} T cells in mice that received the heterologous prime-boost vaccination (Group 8 - 0.8%) compared to those that only received the prime vaccination (Group 15 – 0.38%; Figure 3.5D). However the effector memory phenotype increased from 76.7% (Group 15) to 97.5% (Group 8) following the boost vaccination. Thus the MVA-Gag^{M} boost vaccination increases cytokine-positive CD8^{+} T cells significantly as well as the effector memory phenotype of cytokine-positive CD4^{+} T cells.

The immune responses elicited by vaccines expressing the mosaic Gag immunogen was compared to those vaccinated with vaccines expressing the natural Gag. There was a significant 3.1-fold difference in cytokine-positive CD8^{+} T cells between Group 8 (DNA-Gag^{M}/MVA-Gag^{M} heterologous prime-boost) and Group 13 (DNA-Gag^{N}/MVA-Gag^{N} heterologous prime-boost) mice (Figure 3.5C; 1.59% and 0.51% respectively; $p<0.01$). However, there was no difference between the memory phenotype of cytokine-positive CD8^{+} T cells in these two groups. Cytokine-positive CD4^{+} T cells in Group 8 mice were 1.7-fold higher than Group 13 mice. The effector memory phenotype of the cytokine-positive CD4^{+} T cells was very high in both groups, 92% for Group 13 and 97.5% for Group 2. Thus, the mosaic Gag immunogen seems to be more immunogenic by significantly increasing the proportion of cytokine-positive CD8^{+} T cells.

A comparison of mice that were vaccinated with two doses of the DNA-Gag^{M} vaccine (Group 15) to those given two doses of the DNA-Gag^{N} vaccine (Group 14) also indicated increased CD8^{+} T cell responses due to the mosaic Gag immunogen. Cytokine-positive CD8^{+} T cells were 7-fold significantly higher in Group 15 mice (Figure 3.5C; Group 15 – 1.12%; Group 14 – 0.16%; $p<0.001$). The effector memory phenotype in this T cell

compartment was 100% and 84.5% in Group 15 and 14 mice respectively. Cytokine-production in the $CD4^+$ T cell compartment was very similar between the two groups (Figure 3.5D; Group 15 – 0.38%; Group 14 – 0.31%). However, Group 15 mice had a lower proportion of cells that had an effector memory phenotype (76.7%).

Interestingly, mice vaccinated with two doses of the DNA-Gag^M vaccine (Group 15) had predominantly $CD8^+$ T cells responses, while those vaccinated with two doses of the MVA-Gag^M vaccine (Group 18) had predominantly $CD4^+$ T cells responses (Figures 3.5C and D). Frequencies of cytokine-positive $CD8^+$ T cells were 1.2-fold greater in Group 15 mice (1.12%) compared to Group 18 mice (0.57%). The proportion of cytokine-positive $CD8^+$ T cells with an effector memory phenotype was also higher in Group 15 mice (100%) compared to Group 18 mice (91.6%). On the other hand, cytokine-positive $CD4^+$ T cells were 2-fold greater in Group 18 mice (0.76%) compared to Group 15 mice (0.38%). The proportion of cytokine-positive $CD4^+$ T cells with an effector memory phenotype was also higher in Group 18 mice (99%) compared to Group 15 mice (76.9%).

3.3.4.3 Profile of cytokines secreted into the supernatant

To assess the Th1/Th2 bias of the immune response to the vaccines, a cytokine bead array assay was used. The Th1 and Th2 cytokines were quantified from the culture medium collected from splenocytes stimulated with Gag CD4 and CD8 peptides (Figure 3.5E). IFN-γ, TNF-α, and IL-2 had the highest cumulative levels in all groups of mice, suggesting a Th1 bias. TNF-α was the lowest secreted cytokine of these three.

The IFN-γ ELISPOT assay indicated a 7.1-fold difference in the cumulative number of cells producing this cytokine between mice that received a DNA-Gag^M/MVA-Gag^M heterologous vaccination (Group 8) and those that were mock-primed and boosted with MVA-Gag^M (Group 11; Figure 3.5B). Levels of IFN-γ secretion from cultured splenocytes differed by a factor of 22. Thus, exceptionally high cytokine levels were induced by the DNA-Gag^M/MVA-Gag^M heterologous vaccination. TNF-α, and IL-2 were 16.2- and 5.1-fold higher respectively in Group 8 mice compared to Group 11 mice, suggesting an efficient DNA-Gag^M prime. A comparison of Group 8 and Group 17 mice (single MVA-Gag^M vaccination) indicated a 35.2-, 6.3-, and 5.9- fold difference in IFN-γ, TNF-α, and IL-2cytokine production levels respectively. This also highlighted the potency of the DNA-

Gag^M prime vaccination. An MVA-Gag^M boost was necessary to elicit potent cytokine production. Group 8 mice had 14.8-fold higher levels of IFN-γ than Group 15 mice (DNA-Gag^M homologous vaccination; Figure 3.5E). TNF-α, and IL-2 were 3.9- and 3.3-fold greater respectively in Group 8 compared to Group 15.

The DNA-Gag^M/MVA-Gag^M (Group 8) heterologous prime-boost vaccination regimen elicited more potent immune responses than the heterologous DNA-Gag^N/MVA-Gag^N (Group 13) vaccination regimen. IFN-γ, TNF-α, and IL-2 cytokine production levels were 4.4-, 2.5-, and 2.4-fold greater, respectively, in Group 8 mice than in Group 13 mice. A comparison of the cytokines produced by the prime vaccinations only (Group 14; DNA-Gag^N/DNA-Gag^N and Group 15; DNA-Gag^M/DNA-Gag^M) suggests that the mosaic immunogen elicits more potent immune response than the immunogen derived from a natural HIV-1C isolate.

It was interesting to note that a DNA-Gag^M homologous vaccination induced more cytokine production than an MVA-Gag^M homologous vaccination (Figure 3.5E). This correlated positively with the IFN-γ ELISPOT assay (Figure 3.5B), but not with the ICS and flow cytometry findings (Figures 3.5C and D).

3.4 DISCUSSION

HIV-1C vaccines expressing Gag^M were evaluated in mice and shown to be very immunogenic, particularly when the BCG-Gag^M and DNA-Gag^M were boosted with MVA-Gag^M.

Recombinant BCG vaccines have been used before to efficiently prime the immune system for a boost with recombinant poxvirus, recombinant adenovirus, protein, or Gag VLPs ((366,369-371,593) and reviewed by Chapman *et al*., 2010 (362)). In the BCG prime-MVA-boost experiments, the recombinant MVA doses often used range from 10^6 - 10^8 pfu/mouse (369,594) and our group has often used 10^7pfu/mouse (310,364,365,387,488). In this study, we have shown that MVA-Gag^M at a dose of 10^4 pfu boosts a BCG prime to elicit potent immune responses in BALB/c mice. The responses we obtained were comparable or greater than those of others who boosted mice with 100 to 10000 fold higher doses of MVA. For example, Hopkins and colleagues (369) primed BALB/c mice with 10^6 cfu of BCG expressing HIVA and boosted them with 10^8

pfu of MVA expressing the same antigen 12 weeks later. HIVA contains the p24 and p17 portions of Gag fused to a string of 25 HIV-1 CTL epitopes (Hanke & McMichael, 2000). Despite using a 10000 fold higher dose of MVA they obtained similar frequencies of HIV specific $CD8^+$ T cells to ours. This suggests that full length Gag immunogens may be better for use in vaccine candidates. Recently, Chapman and colleagues (387) primed BALB/c mice with 10^7 cfu BCG expressing a modified HIV-1C Gag on day 0. Mice were boosted with 10^7 pfu MVA expressing HIV-1C Gag on day 56 and killed on day 68. Gag-specific $CD8^+$ T cell responses of 1343 ± 17 sfu/10^6 splenocytes were obtained by an IFN-γ ELISPOT assay. This was almost 3-fold higher than what we obtained for the same assay (Figure 3.3B), however, only IFN-γ was detected by the CBA assay in their study. Our study showed the secretion of IFN-γ, TNF-α, and IL-2 (Figure 3.3E), cytokines that play a role in controlling HIV-1 viral load (Section 1.6).

As discussed in Section 1.10.8 of the Literature Review, live attenuated SIV and CMV vaccines that elicited persistent $CD8^+$ T cell responses, have been shown to control viral load in macaques (266-268). In this study, we demonstrated that recombinant BCG expressing the vaccine immunogen was persistent in the tissues of vaccinated mice as determined by the presence of the shuttle vector in BCG-Gag^M isolates in the peripheral lymphoid organs (spleen and lymph nodes) of these mice 11.5 weeks post vaccination (Chapter 2; Figure 2.7). Saubi and colleagues (2012; (595)) as well as Chapman and colleagues (364,365,387) have shown *in vivo* BCG vaccine stability and persistence over periods of 9.5 to 20 weeks post vaccination. The replication of BCG *in vivo* is slow. rBCG persistence subsequently results in low antigen expression and low levels of antigen presentation (596). Low T cell immune responses to the antigen are induced, differentiate in to memory phenotype, and get stimulated when boosted with a matching antigen (597). Vaccination with a BCG-Gag^M prime MVA-Gag^M boost also generated predominantly effector memory cytokine-positive T cells, a T cell subset shown to play a role in the control of viral load after vaccination with CMV-based vaccines (266). Splenocytes were isolated from mice 12 days after the MVA-Gag^M boost, this is when the peak response to the MVA vaccination is expected to occur, thus most of the memory T cell subset would be expected to be of the effector phenotype (598). It would be interesting to see if the memory phenotype changes if samples are taken at a later time point as MVA is not a persistent vector and so the T cell response might

contract and the memory phenotype of the T cells change to a predominantly central memory phenotype.

Antigens derived from mycobacteria are processed and presented by macrophages. Antigens delivered into the phagolysosome usually get processed by the HLA class II pathway. Such antigens would induce $CD4^+$ T cell responses (reviewed by Hess *et al*., 2000; (599)). In our study, the BCG-Gag^M prime and MVA-Gag^M boost resulted in the frequency of cytokine-secreting $CD8^+$ T cells being greater than that of $CD4^+$ T cells (Figure 3.3C and D). The Gag^M antigen in our study was linked to the 19kD signal sequence in the BCG shuttle vector (Chapter 2; Figure 2.6; Appendix A7). This is meant to target Gag^M to the BCG*ΔpanCD* cell wall, making it accessible for processing by the HLA class I pathway and inducing $CD8^+$ T cell responses (550). Furthermore, the BCG*ΔpanCD* strain is known to induce mostly $CD8^+$ T cell responses to HIV-1 Gag (354,364,365).

The BCG-Gag^M vaccines in our study induced a Th1 bias (Figure 3.3E). This could be attributed to the mosaic immunogen, but this was not explored further. A Th1 immune response has been shown to be important for protection against viral challenge in mice as reported by Someya and colleagues (2004; (600)) and by Betts and colleagues (195,278). In HIV-1 natural infection, $CD8^+$ T cells function by producing IFN-γ and TNF-α which, in turn, induce antiviral activity in infected cells as reviewed in Section 1.6. Furthermore, IL-2 production increases cytotoxicity and is associated with reduced virus loads in ECs as reviewed in Section 1.10.4. It is therefore desirable for candidate vaccines to induce these cytokines as potential correlates of protection.

DNA vaccines elicit weak immune responses when administered alone in clinical trials and the response varies according to the route of immunisation (reviewed by Hutnick *et al*., 2011 (582)). Improved responses can be elicited when these vaccines are used as a prime in heterologous prime-boost regimens, co-administered with protein or an adjuvant, administered by electroporation, or when expression can be enhanced by modifying the DNA vaccine vector (Discussed in Section 1.12.2). This study explored the use of a porcine circovirus enhancer element to increase expression of mosaic Gag by our DNA vaccine vector. This strategy of improving DNA vaccines was indirectly demonstrated in a study by Tanzer and colleagues (2011; (475)) who showed high

immune responses to the target antigen using 5-20-fold lower doses than what is normally administered in mice (601-607). The use of enhancer elements to improve expression and /or immunogenicity is most likely associated with a combination of any of the following: 1) recruitment of host cell factors that improve the transcription of the transgene (608,609), 2) effective mRNA export from the nucleus and/or subsequent stabilization in the cytoplasm (610), 3) the enhancer element acting as a ribosomal entry site and so enhancing the post-transcriptional expression of the transgene (611), or 4) simply improving optimal expression in APCs which in turn improves T-cell responses specific for the transgene. Takebe and colleagues (1988; (612)) showed that an enhancer could increase *in vitro* expression levels of chloramphenicol acetyltransferase 10 – 40 times. This was achieved using a DNA vector with an enhancer element derived from the R segment of human T-cell leukemia virus type 1 (HTLV-1) upstream of an SV40 promoter. Using the same enhancer element upstream of the CMV promoter in a plasmid backbone (pCMV/R), Barouch and colleagues (2005; (477)) showed HIV-1 Env expression increased by 5 to 10 times as determined by Western blot analysis with an IgG primary antibody. Importantly, this increased expression translated to enhanced cellular immune responses in mice (≥900 sfu/10^6 splenocytes) when administered at 50μg, compared to the DNA vaccines delivered using the parental vaccine backbone at the same dose. Kong and colleagues (2009; (317)) obtained potent immune responses when they vaccinated B6D2FI/J mice with 15μg pCMV/R. The DNA vaccines expressed mono-, bi-, or tri-valent HIV-1 mosaic Env immunogens. The vaccines were administered four times at two week intervals by needle injection with no adjuvant. Although the mosaic vaccines expanded mostly the breadth of $CD4^+$ T cell responses to Env, cumulative cytokine production (IFN-γ and TNF-α) was more pronounced with the $CD8^+$ T cells as determined by ICS. The frequencies were 0.3, 0.5, and 0.8% for the mono-, bi-, and tri-valent HIV-1 Env mosaic vaccines respectively. The DNA vaccine alone in our study had a predominant response to the CD8 Gag peptide as determined by the IFN-γ ELISPOT assay (Figure 3.5B). However, the responses were balanced between Gag-specific CD4 and CD8 peptides in a DNA-Gag^M/MVA-Gag^M prime-boost vaccination regimen as determined by the IFN-γ ELISPOT assay (Figure 3.5B). These findings were in agreement with those of others (reviewed by Ondondo *et al*., 2014 and Lu *et al*., 2008 (440,453)). A balanced $CD4^+$ and $CD8^+$ T cell response is

desirable in an HIV-1 vaccine as $CD8^+$ T cells will kill viral-infected cells while $CD4^+$ T cells provide help for the cytotoxic T lymphocytes as discussed in Section 1.6.

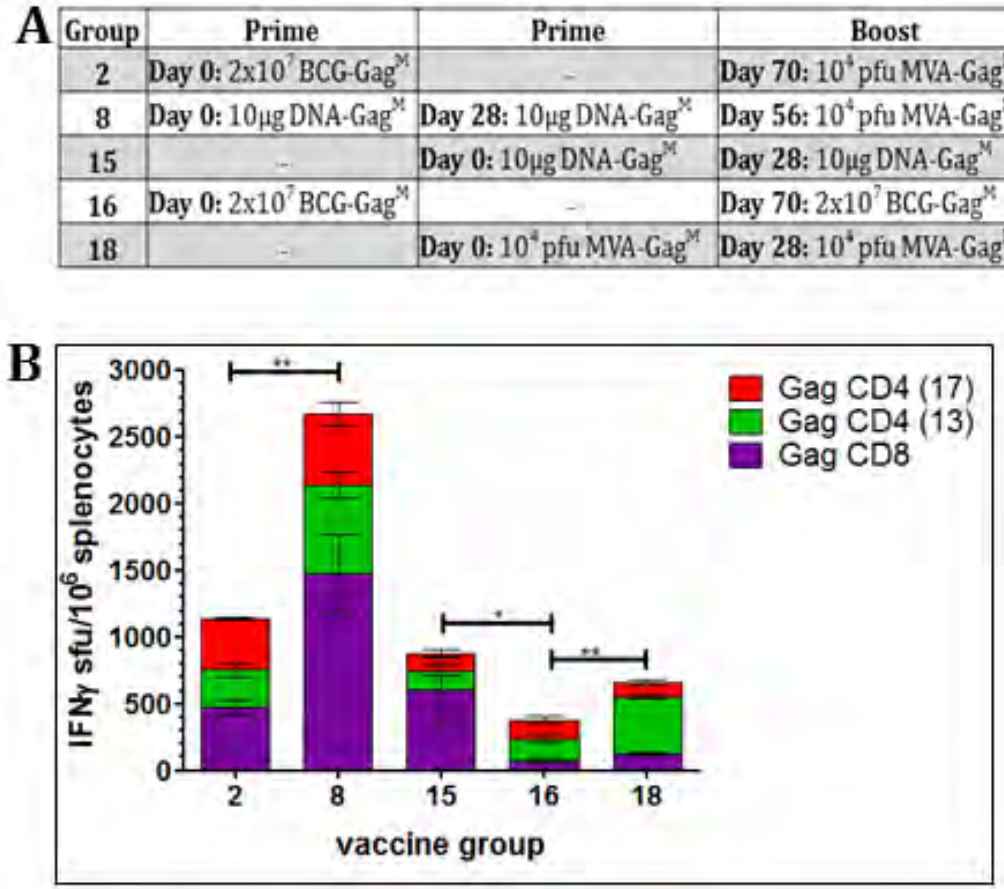
Boost vaccinations are also used to enhance the immunogenicity of DNA vaccines used to prime the immune system. When boosted with rMVA, the doses often used are 10^6 - 10^7 pfu/mouse (469,603-605,613-615). Only relative comparisons can be made for the immunogenicity of candidate vaccines made by various research groups. This is mostly because protocols differ between the groups including the selection of HIV-1 genes for immunogens in the vaccines, promoters used for immunogen expression, vaccination regimens, routes of vaccine administration, timing between vaccinations, immunological assays used, panels used for ICS, and differences in peptides used for stimulations. Someya and colleagues obtained Gag-specific IFN-γ responses of 735 ± 124 sfu/10^6 splenocytes when they primed mice with a eukaryotic expression DNA vector expressing SIV Gag-Pol. The vaccination was boosted by 10^6 pfu of vaccinia virus expressing the same genes in mice (600). Maeto and colleagues (2014; (616)) vaccinated mice with 50μg DNA and 10^7 pfu MVA expressing HIV-1 natural Env and in the presence of one or two adjuvants. They obtained Env-specific IFN-γ responses of not more than 1200 sfu/10^6 splenocytes. In both studies, their responses were lower than what we obtained using a lower dose of both the DNA and MVA vaccines, as well as a mosaic immunogen.

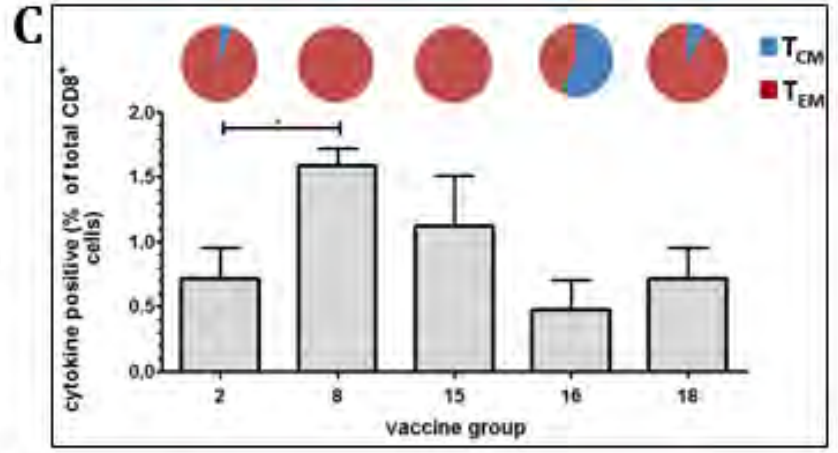
To summarise the immunology data, the results obtained from the BCG, DNA, and MVA heterologous and homologous vaccination regimens were compared. The vaccination regimens that were compared are listed in Figure 3.6A and the immune responses shown in Figures 3.6 B-E. The heterologous prime-boost vaccinations elicited more robust immune responses than the homologous prime-boost vaccinations. Of the heterologous vaccinations, the DNA-Gag^M/MVA-Gag^M (Group 8) vaccine was more potent than the BCG-Gag^M/MVA-Gag^M (Group 2) vaccination regimen.

The HIV-1 Gag^M vaccines elicited cytokine-positive T cells with a predominant effector memory phenotype. Effector memory cells act as the first line of defence at the sites of HIV-1 infection. When effector memory T cells are restimulated, they mature rapidly into effector cells that secrete large amounts of cytokines (148). Central memory T cells on the other hand take longer to differentiate into effector T cells and do not secrete as

A

Group	Prime	Prime	Boost	Sacrifice
2	Day 0: $2x10^7$ BCG-Gag^M	-	Day 70: 10^4 pfu MVA-Gag^M	Day 82
8	Day 0: 10µg DNA-Gag^M	Day 28: 10µg DNA-Gag^M	Day 56: 10^4 pfu MVA-Gag^M	Day 68
15	-	Day 0: 10µg DNA-Gag^M	Day 28: 10µg DNA-Gag^M	Day 40
16	Day 0: $2x10^7$ BCG-Gag^M	-	Day 70: $2x10^7$ BCG-Gag^M	Day 82
18	-	Day 0: 10^4 pfu MVA-Gag^M	Day 28: 10^4 pfu MVA-Gag^M	Day 40





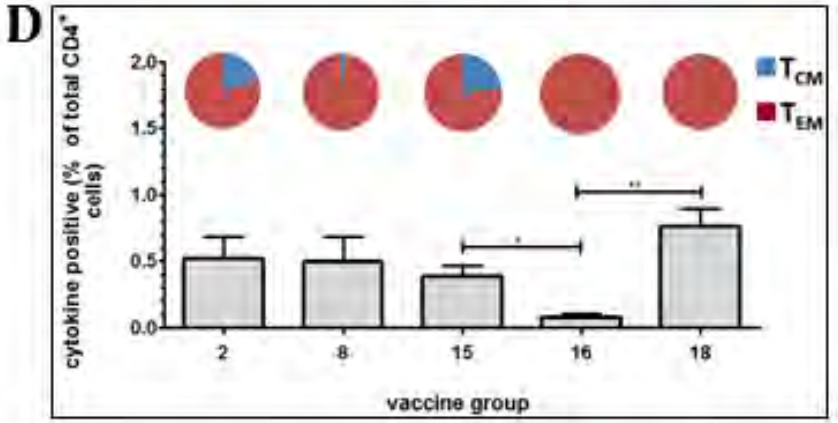


Figure 3.6: (legend on next page)

E

Cytokine	Mean ± SD (pg/10^6 splenocytes)				
	Group 2	Group 8	Group 15	Group 16	Group 18
IFN-γ	2106 ± 180.1	15787.2 ± 914.6	1063.2 ± 169	575.3 ± 102.5	1202.7 ± 36.7
TNF-α	777.1 ± 107.2	397.5 ± 22.6	102.3 ± 11.6	167.4 ± 4.1	309.8 ± 1.8
IL-2	2839 ± 194.2	2450.9 ± 336	743.6 ± 28.2	848.3 ± 97	2028.4 ± 114
IL-4	80.9 ± 5.1	168.8 ± 23.6	47.4 ± 2.4	37.9 ± 8.5	44.8 ± 4.5
IL-6	112.6 ± 5.9	45.6 ± 6.5	39.2 ± 8.7	10.2 ± 1.5	57.4 ± 5.3
IL-10	74 ± 2.9	179.3 ± 14.5	41.2 ± 6.9	29 ± 7.8	95.3 ± 10.7

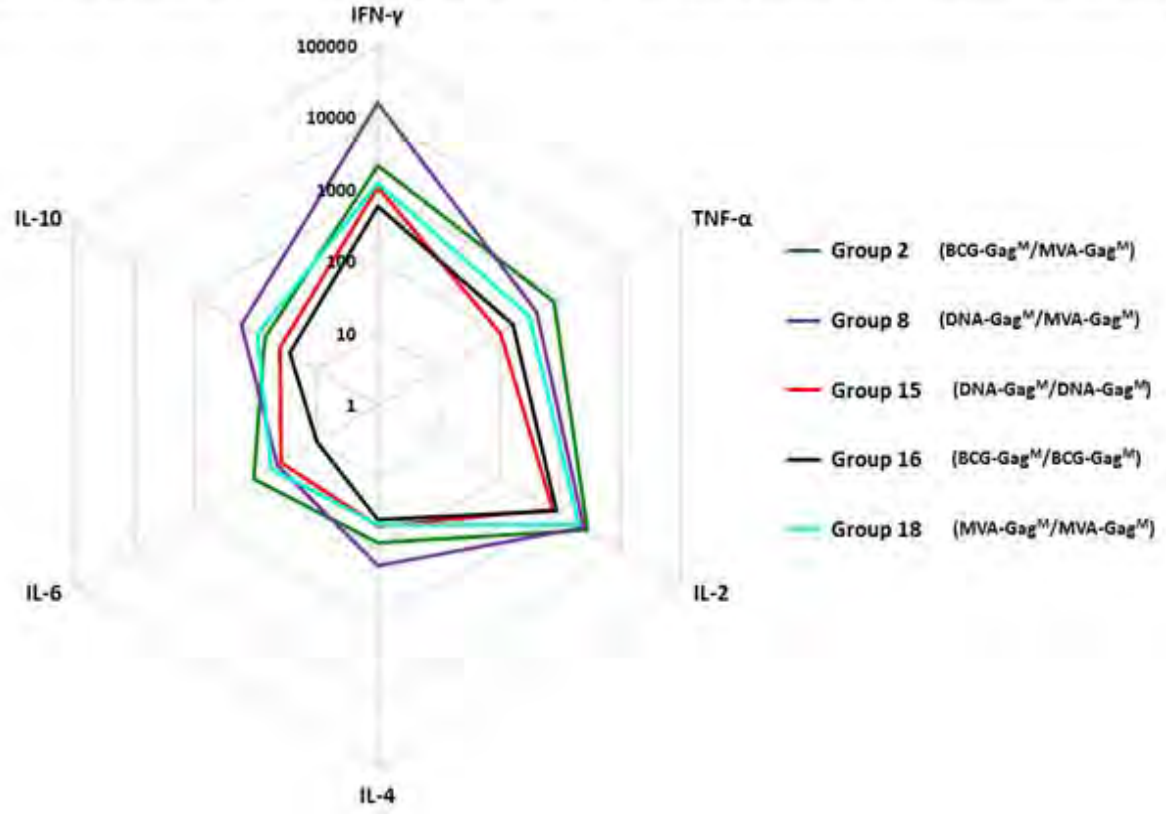


Figure 3.6: Comparison of the immunological responses elicited by different vaccination regimens expressing a mosaic *gag* immunogen. (**A**) Vaccination schedule used. (**B**) Cumulative IFN-γ ELISPOT $CD8^+$ and $CD4^+$ responses of vaccinated mice to HIV-1 Gag peptides. The ELISPOT assay was done using pooled spleens on the day of sacrifice using three Gag-specific peptides for stimulation. Bars are the mean and standard deviation of the mean responses for the indicated individual peptides from 3 independent experiments. Responses are expressed as sfu/10^6 splenocytes after background subtraction. Horizontal bars with asteriks indicate statistical significance of the mean responses between the indicated groups. *p<0.05, **p<0.01, ***p<0.001; Student t-test of unpaired data. (**C**) and (**D**) Total frequency of T cells producing IFN-γ, IL-2, and/or TNF-α in response to HIV-1 Gag peptide stimulation. ICS and flow cytometry were carried out on pooled spleens per group using three Gag-specific peptides for stimulation. The memory distribution of the cytokine producing T-cells in the central and effector memory compartment (T_{CM} and T_{EM}) are represented as pie charts above each corresponding bar per group. Cells were positive for cytokine production if the proportion was ≥ 0.05% after subtracting the background. The cellular phenotype was positive if there were ≥ 10 cells per test. (**E**) Results from cytokine measurements in cell supernatants of mice vaccinated with different vaccine regimens using a CBA Th1/Th2 Kit and radial plot of cells producing six different cytokines in response to HIV-1 Gag peptide stimulation**.** The levels of cytokines in the culture supernatants were quantified using a Th1/Th2 cytokine bead array assay followed by flow cytometry. The recorded results were obtained after subtracting the background. The distance from the centre of the plot indicates a $\log_{10}$-fold change (ranging from 1 to 100 000) and cytokine levels were expressed as pg/ml.

many cytokines as effector memory T cells do following restimulation. They do, however, act to replenish effector memory T cells (148,617). As described in Section 1.10.8, Hansen and colleagues have shown that the protection of vaccinated non-human primates from SIV challenge was due to both $CD4^+$ and $CD8^+$ effector memory T cell responses (266-268).

The mosaic vaccines used in heterologous prime-boost vaccinations also generated predominantly $CD8^+$ T cell responses as determined by ICS assays (Figures 3.6 C and D). This supports studies done earlier by Barouch and colleagues (318) and by Santra and colleagues (319) testing mosaic vaccines in non-human primates. $CD4^+$ cells are essential for providing help to $CD8^+$ T cells (173,618-620). However, $CD4^+$ T cells are also the target of HIV-1 infection (reviewed by Grossman et al., 2006 (141)). There is therefore a fine balance between inducing enough of a $CD4^+$ response to provide help to $CD8^+$ cells and inducing too many $CD4^+$ cells, which will increase the pool of target cells for HIV-1 infection.

Overall, the high immune responses observed in both heterologous vaccination arms could be attributable to the increased and early levels of Gag^M expression by MVA-Gag^M as described in Chapter 2. It has been shown that the levels of expressed Gag in a candidate vaccine correlate positively with the magnitude of immune responses (387). Furthermore, the ability of MVA-Gag^M to form VLPs as discussed in Chapter 2 may also be associated with the potent immune responses. VLPs can stimulate the immune system better than antigens that are not particulate (621). We did not test for VLP formation from the DNA-Gag^M vaccine; however, it has been shown that immune responses induced by a DNA-based HIV-1 vaccine can be elevated if the expressed antigen forms VLPs *in vitro* or is co-administered with HIV-1 VLPs (531,622). The preliminary p24 ELISA data from our DNA vaccines (Section 2.4) did suggest more VLP formation for the DNA-Gag^M, than for the DNA-Gag^N vaccine.

There were however, some limitations in this work that possibly could be addressed in future studies. Gag is a vital component of an HIV vaccine. In natural infection, Gag-specific responses are important for an effective T cell immune response. However, it is essential for an HIV-1 vaccine to induce potent bNAbs and NoNAbs, to protect from HIV virions, as well effective $CD8^+$ T cell responses to kill virus-infected cells. It will be

essential to combine our HIV-1C Gag^{M} vaccine with a vaccine expressing HIV-1C mosaic Env or with a protein boost to induce humoral immunity. Both bi- and tri-valent mosaic immunogens have been shown to increase breadth which is essential for clearing diverse strains of HIV-1 in infected individuals (Section 1.10.1). BALB/c mice have a limited number of HIV-1 epitopes that can be used to evaluate the breath of candidate vaccines. This immune readout was therefore not evaluated as part of the study. Furthermore, cytokine-positive T cells in vaccinated mice were detected using antibodies conjugated to the same fluorophore (Section 3.2.2.3). We therefore could not detect the polyfunctionality of our candidate vaccines. Cytotoxicity and T cell avidity were also not evaluated as part of this study in determining desirable immune readouts for a T cell-based vaccine. It would also be of interest in future studies to compare the expression levels of Gag from the 3 vaccine platforms *in vivo* as this could be contributing to the differences in immunogenicity of the vaccines.

Nonetheless, the novelty of this study lies in subtype-specific monovalent HIV-1 Gag^{M} vaccines being highly immunogenic in comparison to Gag^{N} when delivered as DNA vaccines. This immunogen delivered by a low dose MVA-Gag^{M} vaccine to boost a BCG-Gag^{M} prime and a low dose DNA-Gag^{M} prime had potent immune responses in BALB/c mice. This is very attractive for dose sparing and reduced costs for the targeted resource-limited regions should the vaccine get to clinical trials, licencing, and large scale distribution. Further, the DNA vaccine had a novel enhancer element described by our colleagues here at UCT (475). To our knowledge, mosaic immunogens have not been characterised before. Here we show that Gag^{M} expressed from an MVA vaccine vector forms VLPs. A homologous DNA-Gag^{M} vaccination regimen elicited a more robust immune response than the equivalent homologous DNA-Gag^{N} vaccination regimen.We did not investigate further the properties that make Gag^{M} so immunogenic apart from increased expression and formation of VLPs. Although the same doses of vaccine were administered to mice, the actual dose of the immunogen may not be the same and may be influenced by codon optimisation, mRNA stability, post translational modification, protein folding, and/or VLP stability. Phase I (303,310,488) and II (225) clinical studies using DNA and MVA-based HIV vaccines expressing natural immunogens have been conducted with very promising results. Here, we show that a novel strategy of using

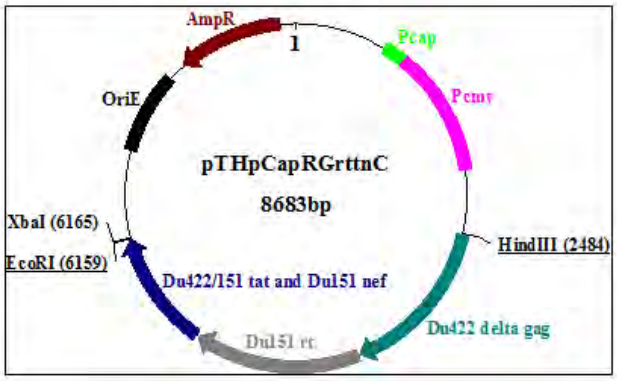
subtype-specific mosaic immunogens and a novel DNA vaccine with a Pcap enhancer element can further improve HIV-1C vaccine immunogenicity.

The promising immunogenicity data generated from the HIV-1C vaccines expressing a monovalent mosaic Gag immunogen warrants further evaluation in non-human primates, and in combination with the HIV-1C vaccines expressing mosaic Env immunogens.

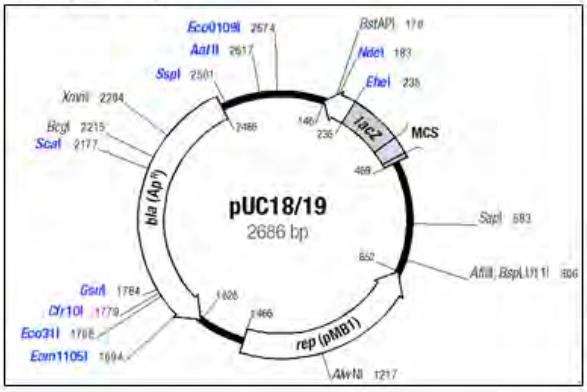
APPENDICES

APPENDIX A: PLASMIDS

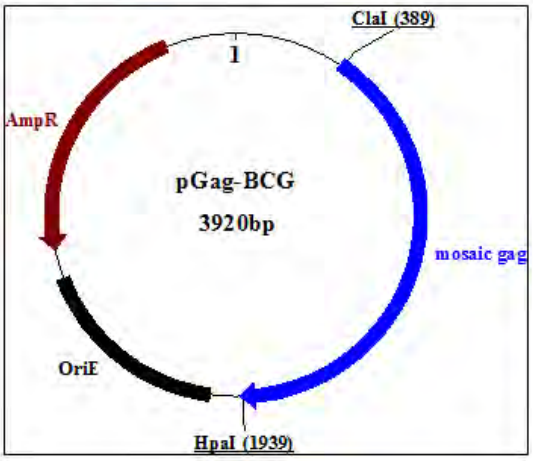
A1. pTHpCapRgrttnC



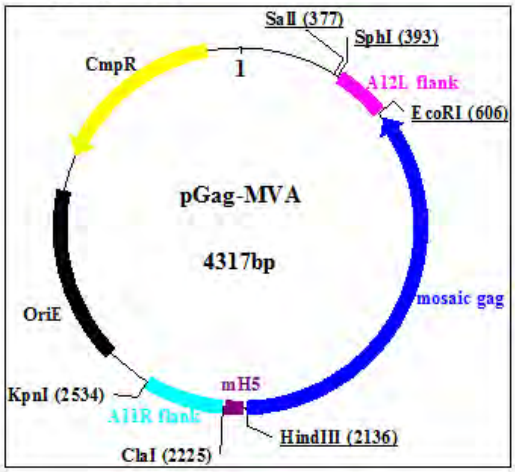
A2. pUC19/18



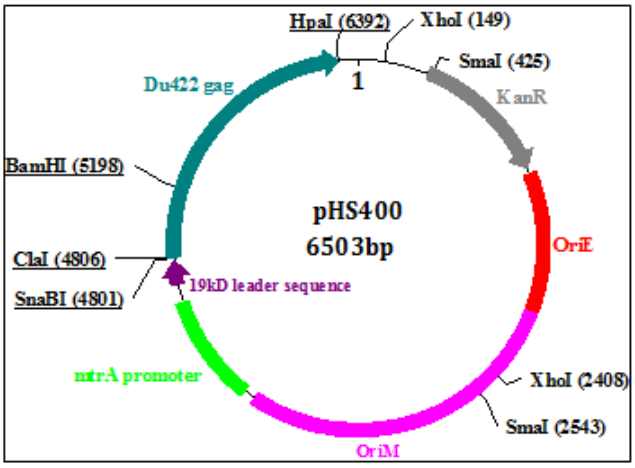
A3. pGag-BCG



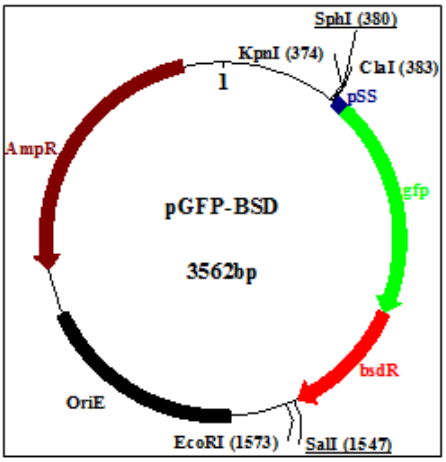
A4. pGag-MVA



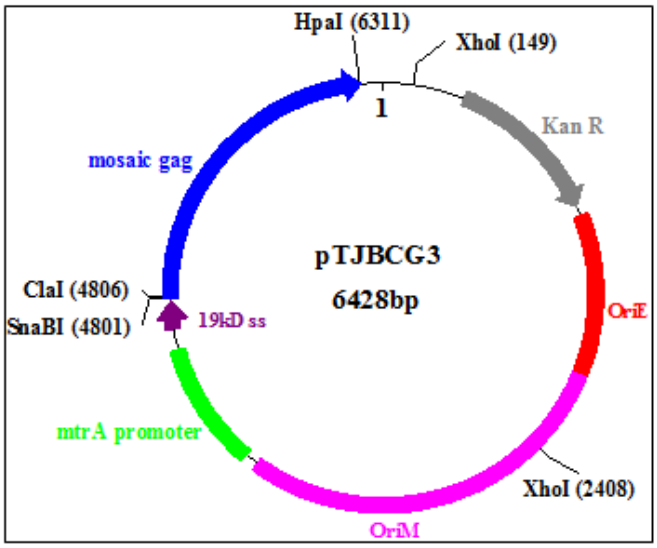
A5. pHS400



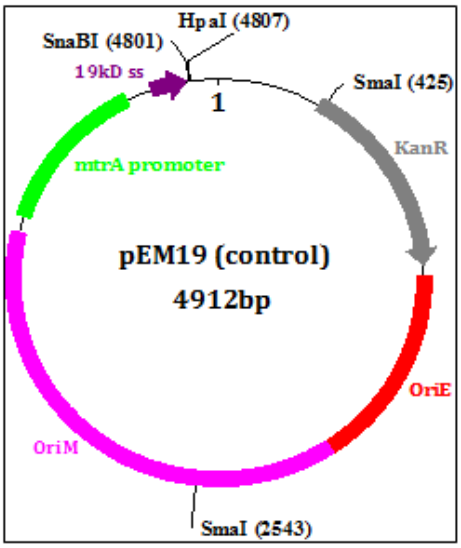
A6. pGFP-BSD



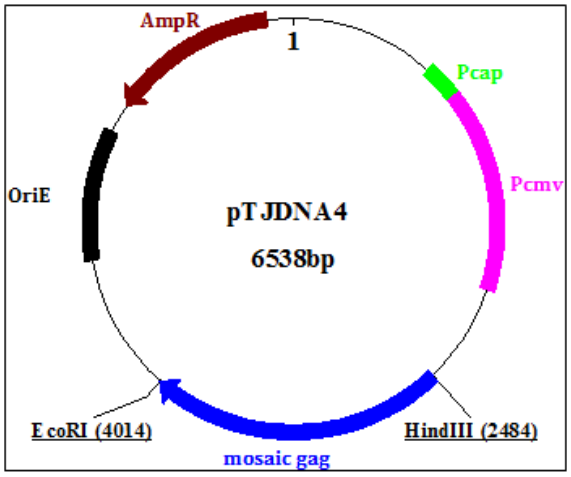
A7. pTJBCG3



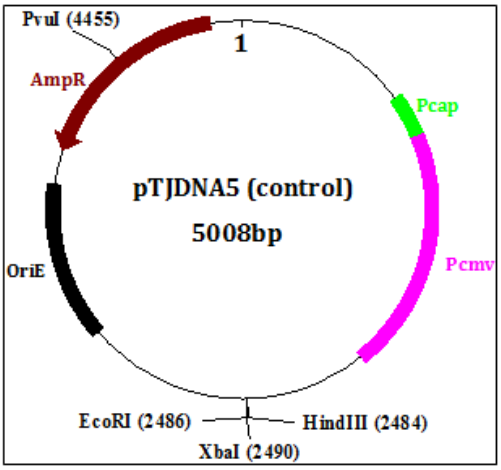
A8. pEM19



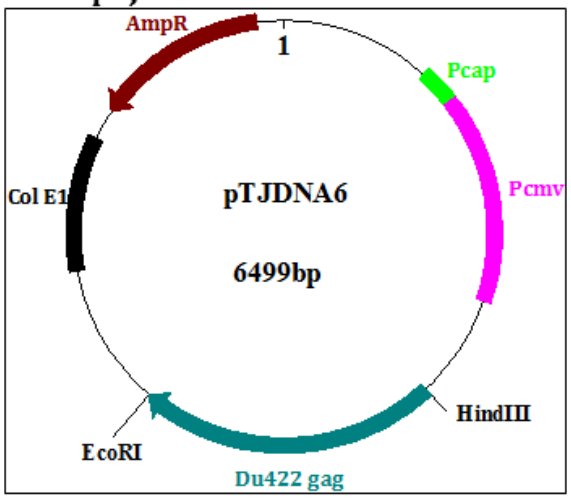
A9. pTJDNA4



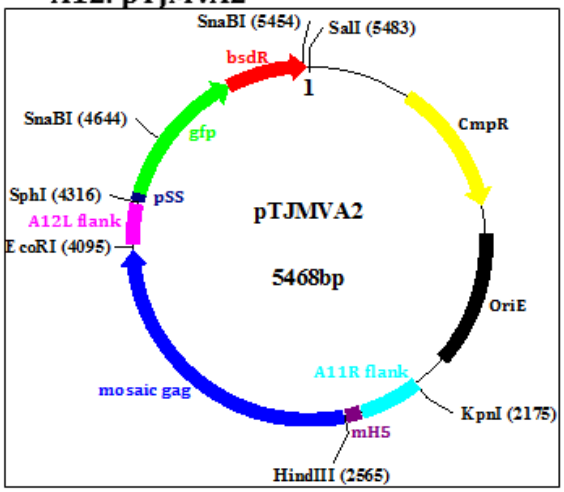
A10. pTJDNA5



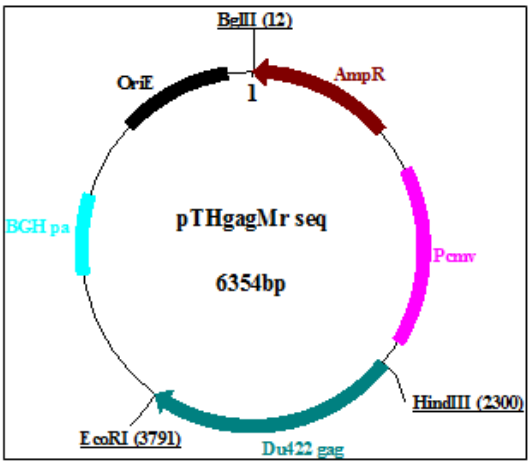
A11. pTJDNA6



A12. pTJMVA2



A13. pTHgagMr



APPENDIX B: SUPPLEMENTARY DATA

Appendix B1: HIV-1C Gag^{M} gene sequence codon optimised for maximal expression in the BCG vaccine. This sequence was used to make the BCG vaccines. Start and stop codons are in underlined bold text

ATGAGCTCGGCGCCGGGCCCATCGATATGGGCGCGCGCGCGTCGATCCTGCGCGGCGGCAAGCTGGACAAGTGGG
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TCGAAGTGCGCGACACCAAGGAAGCCCTGGACAAGATCGAAGAAGAACAGAACAAGTCGCAGCAGAAGACCCAGC
AGGCCAAGGCCGCCGACGGCAAGGTGTCGCAGAACTACCCGATCGTGCAGAACCTGCAAGGCCAGATGGTGCACC
AGGCCATCTCGCCGCGCACCCTGAACGCCTGGGTGAAGGTGATCGAAGAGAAGGCGTTCTCGCCGGAAGTGATCC
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CGGCCCGATCGCCCCGGGCCAGATGCGCGAACCGCGCGGCTCGGACATCGCCGGCACCACGTCGACCCTGCAAGAA
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TACGTGGACCGCTTCTTCAAGACCCTGCGCGCCGAGCAGGCCACCCAGGACGTGAAGAACTGGATGACCGACACC
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AGATGAAGGACTGCACCGAGCGCCAGGCCAACTTCCTGGGCAAGATCTGGCCGAGCCACAAGGGCCGCCCGGGCA
ACTTCCTGCAATCGCGCCCGGAACCGACCGCCCCGCCGGCCGAGCCGACCGCGCCGCCCGCGGAATCGTTCCGCTT
CGAAGAAACCACCCCGGCCCCGAAGCAGGAACCGAAGGACCGCGAACCGCTGACCAGCCTGAAGTCGCTGTTCGG
CTCGGACCCGCTGTCGCAGCTGCAGCGCGGCCCCGGCCGCGCCTTCGTGACCATCTGACTGCAGGGT**TAA**

Appendix B2: HIV-1C Gag^{M} gene sequence codon optimised for maximal expression in humans. This sequence was used to make the DNA and MVA vaccines. Start and stop codons are in underlined bold text

ATGGGAGCTAGGGCTAGCATCCTGAGAGGCGGAAAGCTGGATAAGTGGGAGAAGATTAGACTGAGGCCTGGCGG
AAAGAAACACTACATGCTGAAGCACCTGGTCTGGGCTAGTAGAGAGCTGGAAAGATTCGCCCTGAACCCTGGCCT
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GAGATCACTGTATAACACCGTGGCTACCCTGTACTGCGTGCACGAGAAGATCGAAGTGCGGGACACCAAAGAGGC
CCTGGATAAGATCGAGGAAGAACAGAACAAGTCACAGCAGAAAACCCAGCAGGCTAAGGCTGCCGACGGCAAAG
TGTCTCAGAACTACCCTATCGTGCAGAACCTGCAGGGCCAGATGGTGCATCAGGCTATTAGCCCTAGAACCCTGA
ACGCCTGGGTGAAAGTGATCGAGGAAAAGGCCTTTAGCCCTGAAGTGATCCCTATGTTCACAGCCCTGTCAGAAG
GCGCTACCCCTCAGGACCTGAACACTATGCTGAACACCGTGGGAGGCCATCAGGCTGCTATGCAGATGCTGAAGG
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ACTGTAGAGCCCCTAGAAAGAAAGGCTGCTGGAAGTGTGGCAAAGAAGGCCATCAGATGAAGGACTGCACCGAG
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GAGCCTACAGCCCCTCCTGCTGAGCCTACCGCTCCCCCAGCCGAGAGCTTTAGATTCGAGGAAACTACCCCTGCCC
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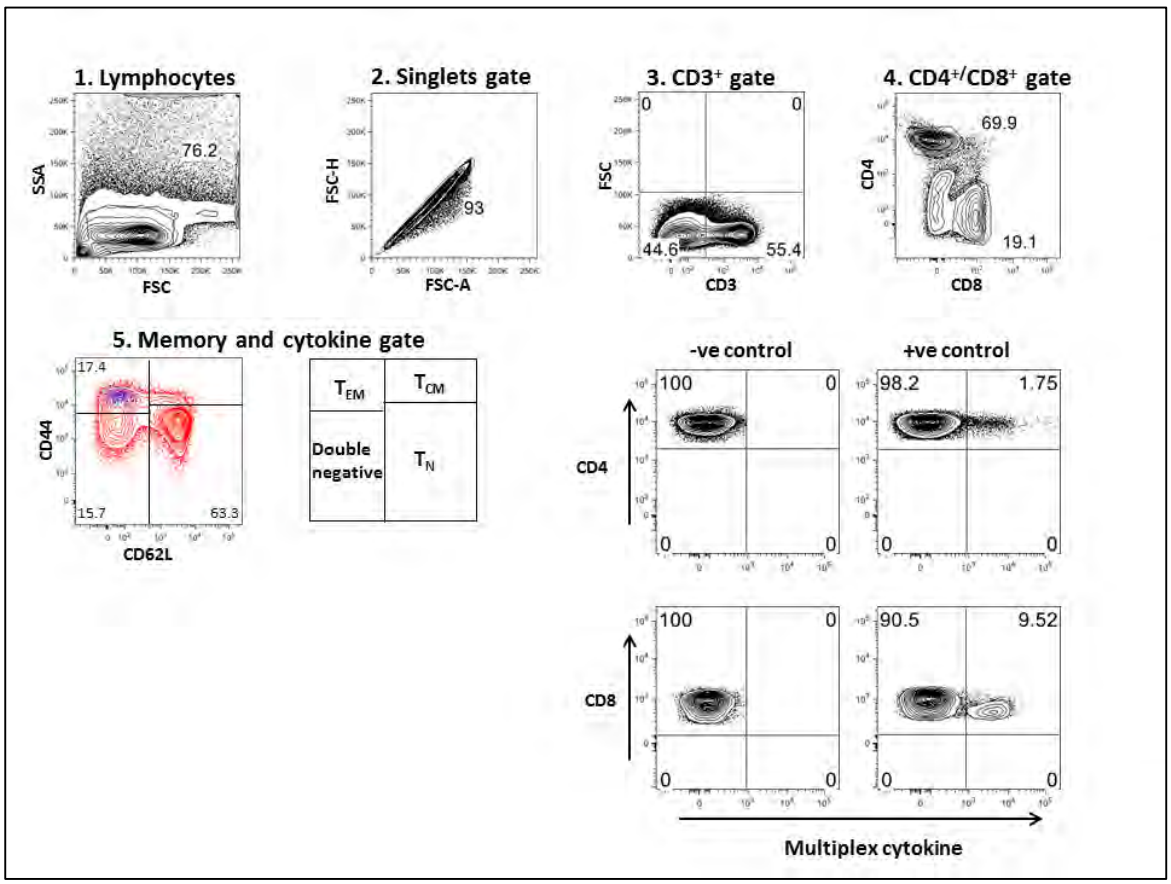
AA**



Appendix B3: Gating strategy used for flow cytometry analysis of Gag-specific CD8^{+} and CD4^{+} cytokine - producing cells. Progressive gating strategy: lymphocytes (plot 1), singlets (plot 2), CD3^{+} cells (plot 3), CD4^{+} and CD8^{+} T cells (plot 4), cytokine-producing CD4^{+} and CD8^{+} cells (plot 5 and plot 6), effector (T_{EM}) and central (T_{CM}) memory cytokine detection.

APPENDIX C: STANDARD PROTOCOLS

C1. Restriction enzyme digest

FastDigest restriction enzymes (Thermo Scientific, USA) were used for restriction enzyme digests of plasmids as recommended by the manufacturer. For cloning purposes, 2-5µg of purified plasmid DNA was used. For plasmid mapping following small scale plasmid extraction (Appendix B4), 5µl of extracted plasmid was used. Enzyme inactivation, were necessary, was done according to manufacturer's instructions.

C2. Agarose gel electrophoresis

Agarose gels were prepared in Tris-borate-EDTA (TBE) buffer (Appendix D8) and stained with 0.01µg/ml of ethidium bromide (Sigma-Aldrich, USA). DNA was electrophoresed in gels submerged in TBE buffer at 3 volts (V)/cm alongside standard molecular weight markers (Thermo Scientific, USA; Appendix D1 and D2). DNA was visualized at 302 nm by UV transillumination using an UVITEC UV light box (UK) and the results captured by an UVITEC camera (Japan).

C3. Preparation and transformation of *E. coli* competent cells

Competent *E*. coli DH5a cells were prepared using dimethyl sulphoxide as previously described by Chung and Miller (1988; (623)) with slight modifications. Briefly, a 1/200 dilution was made from an overnight 5ml starter culture in Luria Bertani (LB) broth (Appendix D4). When the cell culture reached log phase (OD_{600} = 0.2-0.4) at 37°C with shaking, the cells were harvested and resuspended in $1/10^{th}$ culture volume of ice-cold TSB (Appendix D6). After a 10 minute incubation on ice, aliquots of 100µl were made and frozen on a dry ice-ethanol bath or liquid nitrogen for storage at -80°C.

For transformation, competent cell aliquots were used immediately after making them or the frozen stocks were thawed on ice. Plasmid DNA was added to the cells and incubated on ice for 30 minutes. To facilitate DNA uptake into the cells, the mixture was heat shocked at 42°C for two minutes and then placed on ice for another two minutes to maintain the transformed plasmid within the competent cells (624). A volume of 900µl TSBG (Appendix D7) was added and the cells incubated with shaking at 37°C for an hour

to allow the expression of antibiotic resistance genes. The transformation mix was plated onto Luria agar plates containing antibiotic and incubated overnight at 37˚C.

C4. Plasmid DNA extraction and quantification

Small scale plasmid DNA extraction from *E. coli* colonies was done as previously described (625). Large scale plasmid DNA preparation from *E. coli* cells was done using the High Pure® Plasmid Isolation kit (Roche, Switzerland). Large scale extraction of endotoxin-free plasmid DNA was done using the EndoFree Plasmid Maxi® kit (Qiagen, Germany). All procedures using kits were carried out according to the manufacturer's instructions. Plasmid DNA was quantified using a Nanodrop spectrophotometer, ND-1000 UV/Vis (USA). DNA was stored at 4˚C for short term storage, and -20˚C for long term storage.

C5. DNA purification

The standard ethanol precipitation method of Ausubel and colleagues (1987; (626)) was used to clean up and recover DNA following restriction enzyme digests. Glycogen (1µg/µl; Thermo Scientific, USA) was included to give a visible pellet containing the DNA following centrifugation.

C6. Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE)

Protein samples were obtained from DNA and recombinant MVA cell lysates. The protein concentration of samples was determined by using the Bio-Rad DC protein assay kit (Bio-Rad, USA) using the manufacturer's recommendations, and the absorbance readings were measured at 750 nm using the VERSAmax microplate reader (Molecular Devices, USA). A total of 20µg of each protein was mixed with 20% Sample buffer (BioRad, USA) and 2µl Reducing agent (BioRad, USA), made up to 40µl with PBS (Lonza, Belgium). The samples were boiled for 5 minutes, cooled in ice and analyzed on a Criterion XT Tris acetate 12.5% denaturing gel (BioRad, USA). Precision Plus Protein™ Kaleidoscope™ Molecular Weight Marker (BioRad, USA; Appendix D3) was used as a standard. Purified Gag proteins from our Laboratory were used as positive controls and cell lysate from uninfected or untransfected cells were used as negative controls.

Initially the SDS-PAGE was run at 100 V for 20 minutes, and later increased to 150 V for 55 minutes using 1X Tricine Running Buffer (Biorad, USA).

C7. Western blotting analysis

Prior to protein transfer, the denaturing gels, the extra thick blotting papers and the PVDF membranes (Biorad, USA) were equilibrated in 20 ml transfer buffer in small open containers for a maximum period of 30 minutes. The PVDF membranes were soaked in 20 ml 100% methanol for 5 minutes, the methanol was poured off and the membranes rinsed in distilled H_2O with intermittent shaking. After 5 minutes, the water was discarded and 20 ml protein transfer buffer (Appendix D10) was added to the membranes and left for 20 minutes. A BioRad SD semi-dry Electroblotting apparatus (BioRad, USA) was used to transfer the protein from the denaturing gel to the PVDF membrane at 25 V for 60 minutes. The transfer sandwich was set up with the blotting paper at the bottom, followed by the gel, membrane, and more blotting paper

The membranes were rinsed with 1 X TBS (Appendix D11), and stained with Ponceau S (Appendix D12) for 2 minutes to check for proteins on the membrane and finally rinsed with distilled H_2O. The membrane was then incubated in Block /Wash buffer (Appendix D13) overnight at 4°C with gentle shaking. A rabbit anti-HIV-1 p24 Gag primary antibody (ARP432) was prepared (1 in 10000 dilution) in 30ml Block/Wash buffer. The diluted antibody was added to the membrane and incubated for 2hours at room temperature with gentle agitation. The membrane was rinsed twice in 20ml Block /Wash buffer followed by four 20 minutes washes in fresh Block /Wash buffer on a shaker at a moderate speed. The blocking buffer was discarded and an anti-rabbit antibody conjugated to alkaline phosphatase (Sigma-Aldrich, USA) diluted 1in 10000 (3µl in 19,997ml Block /Wash buffer), added to the membrane, and incubated for an hour at room temperature with gentle shaking. The membrane was rinsed and washed as described previously in Block /Wash buffer. The membrane was rinsed once in 1 X TBS and then developed using Nitro blue tetrazolium chloride/ 5-bromo-4-chloro-3-indolyl phosphate (NBT/BCIP; Roche, Switzerland) according to manufacturer's instructions.

APPENDIX D: MEDIA

D1. MB-7H9 media: 4.7 g MB-7H9 broth (Difco); 100 ml OADC (Biolabs); 0.025% v/v tyloxapol (Sigma-Aldrich); 0.25% w/v glycerol); made up to 1litre of water

D2. MB-7H10 agar: 19 g MB-7H9 agar (Difco); 100 ml OADC (Biolabs); 0.63% w/v glycerol; made up to 1litre of water

D3. SOC media: 20g Tryptone powder; 5g Yeast Extract; 2ml of 5M NaCl; 2.5ml of 1M KCl; 10ml of 1M $MgCl_2$; 10ml of 1M $MgSO_4$; 20ml of 1M glucose per litre of water

D4. LB broth/agar: 16 g Tryptone powder; 10 g Yeast extract; 5 g NaCl; (15 g agar) per litre of water

D5. 1X PCR buffer: 10 mM TrisCl; 2.5mM $MgCl_2$; 50mMKCl; pH to 8.3

D6. TSB: 16 g Peptone powder; 11 g Yeast extract powder ; 5 g NaCl; 10% w/v PEG, 5% v/v DMSO, 10 mM $MgCl_2$, 10 mM $MgSO_4$ per litre of water

D7.TSBG: 20% w/v Glucose; 100 ml TSB

D8. TBE: 0.4 M Tris; 10 mM Na_2EDTA; 10 mM Na acetate, pH 8.5.

D9. BCG resuspension buffer: 8.5% w/v NaCl; 10%glycerol; 10% Tyloxapol

D10. Protein transfer buffer: 0.303% Tris; 1.44% Glycine

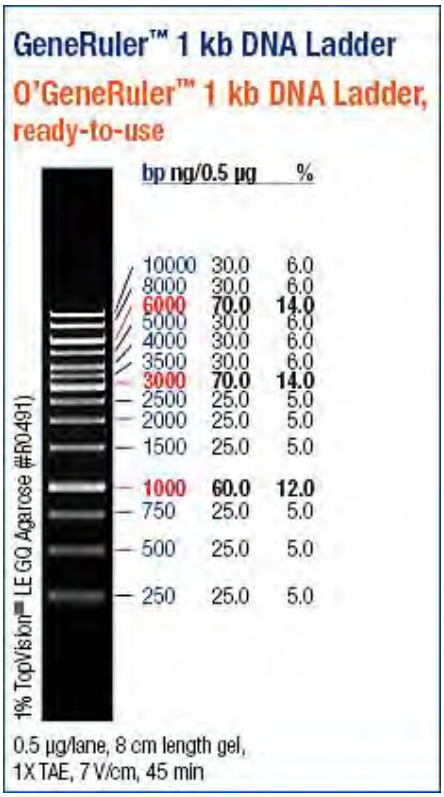
D11. 10 X TBS: 50 mM Tris; 150 mM NaCl; pH to 7.5

D12. 10X Ponceau S staining solution: 0.1% w/v Ponceau S; 5% v/v Acetic acid

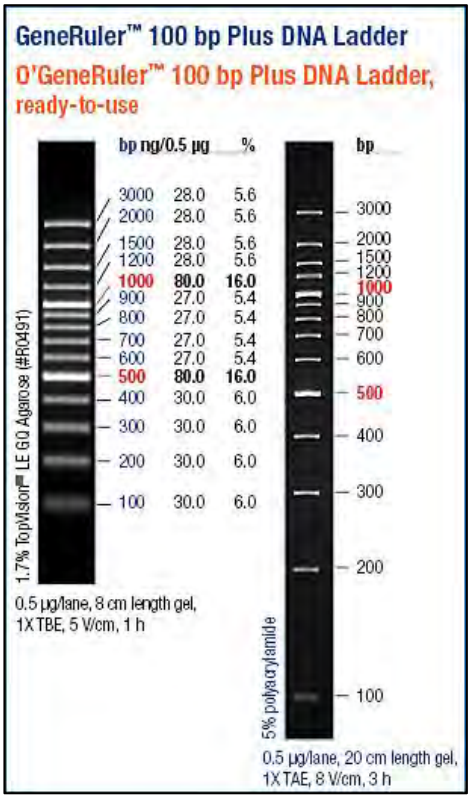
D13. Block wash buffer: 1 X TBS; 0.5% v/v Tween-20; 4% w/v Elite milk powder

APPENDIX E: MOLECUAR WEIGHT MARKERS

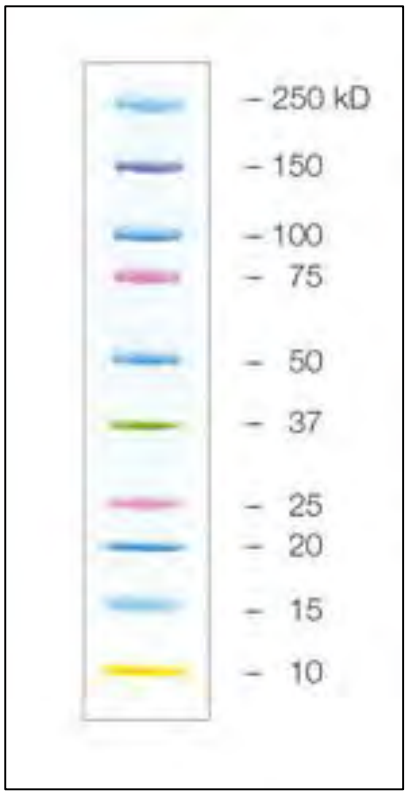
E1.



E2.



E3. Precision Plus Protein™ Kaleidoscope™ (Biorad, USA)



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“Our deepest fear is not that we are inadequate. Our deepest fear is that we are powerful beyond measure. It is our light, not our darkness that most frightens us. We ask ourselves, 'Who am I to be brilliant, gorgeous, talented, fabulous?' Actually, who are you not to be? You are a child of God. Your playing small does not serve the world. There is nothing enlightened about shrinking so that other people won't feel insecure around you. We are all meant to shine, as children do. We were born to make manifest the glory of God that is within us. It's not just in some of us; it's in everyone. And as we let our own light shine, we unconsciously give other people permission to do the same. As we are liberated from our own fear, our presence automatically liberates others.”

Marianne Williamson, A Return to Love: Reflections on the Principles of "A Course in Miracles"

"Science appears calm and triumphant when it is completed; but science in the process of being done is only contradiction and torment, hope and disappointment."

Emile Roux 1853-1933