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Construction, Stability and Immunogenicity of Recombinant BCG Expressing HIV-1 Subtype C Gag Under the Control of *MtrA* Promoter, with or without the Leader Sequences

University of Cape Town

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Thesis Presented for the Degree of Master of Science in the Department of Clinical Laboratory Sciences, Division of Medical Virology, Faculty of Health Sciences at University of Cape Town

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LIST OF ABBREVIATIONS

α	alpha
β	beta
Δ	delta
γ	gamma
µ	micro
µg	microgram
µl	microlitres
Ω	ohms
ACP	acyl carrier protein
ADP	adenosine diphosphate
Ag	antigen
AIDS	acquired immune deficiency syndrome
APC	antigen presenting cell
ATP	adenosine triphosphate
ART	anti-retroviral therapy
BCG	bacilli Calmette Guerin
BSA	bovine serum albumin
CA	capsid protein
CAT	chloramphenicol acetyltransferase
CD8/4	cluster of differentiation
CFE	cell free extract
CFP-10	culture filtrate protein, 10kDa
CFU	colony forming units
CoA	coenzyme A
ConA	concanavaline A
CTL	cytotoxic T-lymphocyte
DMSO	dimethyl sulphur-oxide
DNA	deoxyribonucleic acid
dNTP's	dinucleotide triphosphates
E. coli	*Escherichia coli*
EDTA	ethylene diamine tetra-acetic acid
ELISA	enzyme-linked immune-sorbant assay
Env	envelope protein
ESAT-6	early secretory antigen, 6kDa
FACS	fluorescence activated cell sorting

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FBS fecal bovine serum
FCS fecal calf serum
g grams
GFP green fluorescent protein
GIS geographical information systems
GM-CSF granulocyte macrophage colony stimulating factor
gp glycoprotein
HIV Human immunodeficiency virus
HLA human leukocyte antigen
HPLC high performance liquid chromatography
HRP horse radish peroxidase
Hsp heat shock protein
IFN-γ interferon gamma
IL interleukin
katG catalase peroxidase gene
Kb kilobases
kDa kilodaltons
kV kilovolts
L liter
M molar concentration
M. avium *mycobacterium avium*
M. smegmatis *mycobacterium smegmatis*
M. tuberculosis *mycobacterium tuberculosis*
MA matrix protein
MB middle brooks
ME mercaptoethanol
MHC major histocopmatibility complex
MIP-1α macrophage inflammatory protein 1α
ml milliliter
mg milligram
mRNA messenger RNA
MVA Modified Vaccinia Ankara
NC nucleocapsid protein
ng nanogram
NHLS National Health Laboratory Service
nm nanometer
OADC oleic acid dextrose complex

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OD	optical density
PBS	phosphate buffered saline
pg	picogram
pmol	picomole
RBC	red blood cells
RCS	relative colony size
RNA	ribonucleic acid
rpm	revolutions per minute
SDS	sodium dodecyl sulphate
SA	South Africa
sfu	spot forming units
SIV	simian immune-deficiency virus
SodA	super-oxide dismutase gene
TAT	twin arginine transferase system
TBE	tris borate EDTA
TCA	tri-carboxylic acid
TCR	T-cell receptor
Th	T-helper cell
TMAO	tri-methyl amine N-oxide reductase
TDM	α-α`-trehalose monomycolate
TMM	α-α`-trehalose dimycolate
Tyl	tyloxapol
U	units (restriction enzyme)
UNAIDS	joint United Nations program on AIDS
UK	United Kingdom
USA	United States of America
UV	ultra violet
V	volts
VLPs	virus-like particles
WHO	world health organization

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ABSTRACT

The Human immunodeficiency virus (HIV) epidemic continues to spread globally. Approximately 33.4 million people were living with HIV in 2008, with Sub-Saharan Africa remaining the most heavily affected region worldwide. This highlights the need for the development of an effective prophylactic HIV vaccine, most especially for developing countries. Despite more than 20 years of research, only a few HIV vaccines have reached phase III clinical trials, with only one of them showing modest protection against infection. For these reasons, multiple strategies for the production of an immunogenic HIV vaccine have been explored. The focus of this project is the use of the tuberculosis vaccine, *Mycobacterium bovis* bacilli-Calmette-Guerin (BCG), as an HIV vaccine vector. BCG has a proven safety record and is currently the most widely used vaccine, it stimulates a powerful immune response, is easy and cheap to manufacture since it needs limited purification and is one of the most heat-stable vaccines. Despite these advantages, high level expression of viral antigens in BCG can often increase the metabolic burden resulting in genetic instability and hence poor immune responses.

This study aimed to compare recombinant mycobacteria expressing HIV-1 *gag* under the control of different promoters and leader sequences. This was done to determine whether the genetic stability of the recombinant mycobacteria could be improved by modification of these vector features and to gain insight into what types of immune responses may be elicited in mice.

The following steps were taken to achieve the aims of this study; firstly, the optimal vector backbone was selected; secondly, a series of shuttle vectors were constructed to compare i) different promoters - *hsp60, psmcyc* or *mtrA* ii) different leader sequences - α-antigen leader sequence, 19kDa signal peptide or no signal peptide and iii) HIV-1 *gag* codon optimised for mycobacteria or un-codon optimized *gag*.

In this study it was shown that pEM19 was an optimal vector backbone, also that addition of the *mtrA* promoter; leader sequences and codon optimized HIV-1 *gag* did not have an effect on vector fitness. However, the codon optimisation of *gag* was shown to enhance vector fitness (as compared to un-codon optimised *gag*). Furthermore, the use of leader peptides resulted in differential expression levels of Gag as shown by p24 assays.

The last part of the study aimed at assessing the effect of fusing the 19kDa or α-antigen leader sequences to HIV-1 *gag* on the immunogenicity of recombinant BCG Pasteur ΔpanCD vaccines.

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Therefore the following vaccines were prepared: BCG Pasteur ΔpanCD [pEMαGag$_2$V3SV5] (Gag is fused to the α-antigen leader sequence), BCG Pasteur ΔpanCD [pEM19Gag$_2$V3SV5] (Gag is fused to the 19kDa leader sequence) and BCG Pasteur ΔpanCD [pEMGag$_2$V3SV5] (Gag with no leader sequence). The vaccination strategy employed a prime with the aforementioned rBCG vaccines followed by a boost (28 days post priming) with SAAVI MVA-C to determine cellular immune responses elicited. SAAVI MVA-C is a recombinant modified vaccinia Ankara (MVA) vaccine that expresses an HIV-1 subtype C polyprotein Gag-RT-Tat-Nef (Grttn) and a truncated Env (gp150CT). A boost of the response to the V3CTL peptide (1.8 fold above the control) was achieved with a BCG Pasteur ΔpanCD [pEMαGag$_2$V3SV5] prime and SAAVI MVA-C boost but no boost of the response of the Gag peptides was observed. For a prime with the BCG Pasteur ΔpanCD [pEM19Gag$_2$V3SV5] vaccine, a boost of the response to the Gag CD8 peptide was observed (5 fold above the control). No boost response was detected when BCG Pasteur ΔpanCD [pEMGag$_2$V3SV5] was used as the priming vaccine.

Despite the generally low immune responses elicited by the rBCG vaccines, the BCG Pasteur ΔpanCD [pEMαGag$_2$V3SV5] maybe a promising vaccine as seen by increased immune responses to the V3 CTL epitope. *M. smegmatis* [pEMαGag$_2$V3SV5] also showed higher expression levels of Gag p24 than the other recombinants. In this study various signal peptides and the *mtrA* promoter were included in the vectors, therefore future studies could include the comparison of Gag expression and localization from these rBCG vaccines upon infection of macrophages in order to better understand the relationship between antigen presentation and the nature of the immune response elicited.

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CHAPTER 1: INTRODUCTION

1.1. Global impact of HIV and AIDS

The human immunodeficiency virus (HIV) epidemic continues to spread globally; whereby there were approximately 33.4 million people were living with HIV in 2008. Sixty per cent, of HIV-positive people were female, with Sub-Saharan Africa remaining the most heavily affected region worldwide. This region accounted for over two thirds (67%) of all people living with HIV and for nearly three quarters (72%) of AIDS-related deaths in 2008. In Sub-Saharan Africa, there were 2.2 million new infections and an estimated 2.1 million adult and child deaths due to HIV in 2007**,** resulting in reduced life expectancy, slowed economic growth and increased poverty (Spearman et al. 2009, UNAIDS 2009). In 2008, 14 million children had lost one or both parents due to AIDS. With introduction of highly active anti-retroviral therapy (HAART), adult (14-49 years) prevalence rates in Sub-Saharan countries declined from 5.8% in 2006 to 5.2% in 2008. About 7 million people in the sub-Saharan region were in need of ART in 2007, however, only 44% of this population had access to treatment by the end of 2008 (WHO, Public Health Mapping and GIS Map Library 2009). Furthermore, the improved quality of life and life expectancy of HIV-infected individuals, as well as reduction in incidences of opportunistic infections has been correlated with a widespread use of ART (d'Arminio Monforte et al. 2005, Depairon et al. 2001, Detels et al. 2001, Zuniga et al. 2007). However, adverse side effects of ART, complex and prolonged dose schedules have lead to incomplete adherence and subsequent emergence of drug-resistant viral strains (Burda et al. 2010, Chaplin et al. 2010, Castelbranco et al. 2010, Descamps et al. 2010, Kandathil et al. 2009, Santoro et al. 2008), which may also be transmitted to ART-naïve individuals (Descamps et al. 2010). For instance, it has been shown that 60% of protease inhibitor users contracted diseases caused by metabolic changes such as dyslipidemia, insulin resistance and diabetes Miletus, which are the risk factors for cardiovascular disease (Depairon et al. 2001, Christeff et al. 2002).

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Several factors, including the continuous genetic and antigenic diversity of HIV, integration of HIV proviral DNA into the host-cell genome, and the early establishment of latent viral reservoirs are some of the factors that hamper treatment of HIV infections (Ross et al. 2010). Generally, viruses associated with chronic infections co-exist with the host's immune system for years and only cause disease at the late stage. Therefore, to avoid the late stage disease incident, an immune response can be induced prior to infection by means of vaccination. This

phenomenon has been successfully exploited in prevention of other chronic infections such as human papillomavirus infections (FUTURE I/II Study Group et al. 2010) and herpes zoster (Hambleton et al. 2008). Taken together, these highlight the need for development of an effective HIV vaccine, most especially for the developing countries, with greater disease burden.

1.2. The value of induction of both humoral and cellular immune responses in HIV vaccine development

While education and behavioral modifications are amongst the important aspects in controlling the HIV epidemic, there is still an urgent need for the development of an effective HIV-1 vaccine. Despite more than 20 years of research, Rv144 vaccine (priming with ALVAX-HIV; a canarypox vector expressing HIV-1 Gag and Protease as well as Gp120 from Clade E and boosting with AIDSVAX B/E; rgp120 subunit vaccine) is the only HIV-1 vaccine that shows limited but significant efficacy to prevent HIV acquisition in human clinical trials (Rerks-Ngarm et al. 2009). These limited successes of HIV-1 vaccines have emphasized the need for increased knowledge with regard to fundamental immunity against HIV-1 and have provided insights for vaccine strategies that may be implemented for designing more effective HIV-1 vaccines in the future.

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Over the years, the HIV vaccine development community has followed the three overlapping routes of vaccine development. The first being the use of candidate vaccines aimed at inducing neutralizing antibodies. Such vaccines were typically subunit vaccines. It was however discovered that neutralizing antibodies failed to neutralize primary HIV isolates (Ching et al. 2008). These vaccines also failed to protect healthy individuals as well as non-human primates from infection (Cho et al. 2001, Cho et al. 2001, Flynn et al. 2005, Pitisuttithum et al. 2006, Gilbert et al. 2005). The second route focused on the use of HIV vaccines that elicited mainly T-cell responses, which were found to be vital in early and subsequent control of both HIV and SIV in humans and non-human primates respectively (Manrique et al. 2008, Manrique et al. 2009, Sircar et al. 2010, Valor et al. 2008). However, this type of immunity is deemed to control infection, but does not result in complete clearance of HIV. Furthermore, mutations in the T-cell epitopes may arise, thus allowing for viral escape from the immune response (Barouch et al. 2003, Kemal et al. 2008). Finally, heterologous prime-boost regimes have been employed to induce both humoral and cellular immune responses (Rerks-Ngarm et al. 2009). The prime-boost

regime has also been attributed to an increased CD4+ response, thus mounting the long term memory response by the CTL and resulting in high neutralizing antibody titres (Amara et al. 2002).

Recent studies have shown that important factors governing the induction of protective immunity against HIV-1 include: robust mucosal immunity (Sircar et al. 2010), polyfunctional T cells (Valor et al. 2008), and broadly neutralizing antibodies from highly conserved glycoprotein epitopes, which have been mostly unable to neutralize heterologous viruses, but have emphasized the possible use of polyvalent, genetically diverse immunogens (Cho et al. 2001, Powell et al. 2010)

One of the important factors to consider when developing a vaccine for the developing countries is the issue of helminth infections, which result in CD4+ T-cell population skewed towards T-helper type 2 (Th2) as well as expansion of T-regulatory cells and some level of immune suppression/anergy (Da'dara, Harn 2010, Da'Dara et al. 2006, McKee, Pearce 2004, Taylor et al. 2006). Therefore, such infections need to be addressed before vaccination in order to induce protective immunity against HIV. Optimal delivery methods of DNA vaccines (Liu et al. 2008, Luckay et al. 2007) and also understanding the core immune responses mediating protection against simian immunodeficiency viruses (SIV) and HIV-1 in animal models following vaccination (Manrique et al. 2009, Hansen et al. 2009), are also the key aspects to be regarded for designing more effective HIV-1 vaccines in the future.

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A clear understanding of HIV-1 biology and viral characteristics has enlightened the development of safe and effective HIV-1 vaccines (Devadas et al. 2007). Cell associated viral infections are mostly cleared by the cellular immune responses, while humoral responses are mainly elicited by extra-cellular parasites (Rouse et al. 1988, Ziegelbauer, Overath 1993). The HIV life cycle entails both intracellular and extracellular stages and is mainly transmitted via the mucosal surfaces; therefore an effective vaccine against HIV should be capable of inducing systemic and mucosal immunity including both cellular and humoral responses (Spearman et al. 2009, Fauci et al. 2008).

1.3. Phase III trials and their implications in HIV vaccine development

Despite more than 20 years of research, only a few HIV vaccines have reached the phase III clinical trials, with only one of them showing modest protection against infection (Rerks-Ngarm

et al. 2009, Flynn et al. 2005, Pitisuttithum et al. 2006). Viral characteristics as well as poorly understood correlates of protection are some of the factors that hamper the development of a safe and immunogenic vaccine. Several pilot (phase IIB) studies have been conducted in order to evaluate vaccine safety and efficacy in humans. Due to the vast breadth of the topic, only a few of these vaccine trials will be reviewed.

The phase IIB trial (Merck (STEP) study) where a recombinant adenovirus vector (Ad5) expressing HIV-1 *gag, pol* and *nef* was used as vaccine was conducted in 2007, where about 3 000 HIV-1 negative individuals who were at high risk of infection in America and Australia were enrolled. Despite the elicited cell mediated responses, the vaccine did not prevent HIV-1 infection (Buchbinder et al. 2008). Unfortunately, the Merck study (STEP) was stopped due to possible higher rates of HIV-1 infection in vaccinees (Buchbinder et al. 2008). Graham et al. (1996) also evaluated the safety and immunogenicity of the envelope based (gp120 from HIV-1 Subtype B) vaccine formulated in emulsifier MFP59, with or without a biological response modifier MTP-PE. In this phase II study, 49 healthy adults who were at low risk of exposure to HIV-1 were enrolled with the aim of providing pilot data for anticipated phase III trials in HIV-1 infected pregnant women and to also investigate the role of vaccination schedule on immune responses. This group discovered that individuals who received vaccination with modifier showed increased local and systemic side effects. The vaccine was also able to induce $CD8^+$ T-cell responses that were vital for HIV-1 clearance (Graham et al. 1996). From this study, it was discovered that vaccination schedule played an important role in induction of antibody responses; frequent (monthly) vaccination may result in diminished antibody responses as well as attenuated cellular immune responses. On the contrary, longer vaccination intervals resulted in higher antibody titers and lymphoproliferative activity (Graham et al. 1996). However, the serum from the participants was unable to elicit antibody response with the neutralizing effect on HIV-1 primary isolates grown in PBMCs, irrespective of its safety and immunogenicity (Graham et al. 1996). Graham and his co-workers (1996) postulated that the vaccine could be formulated in another adjuvant to lower the side effects, the envelope based vaccine could be used as a booster in heterologous prime-boost regime to increase the breadth and magnitude of both humoral and cell mediated immune responses, and finally bivalent instead of monovalent gp120 vaccine may be ideal.

From these data, the world's first phase III vaccine efficacy trials were carried out on bivalent glycoprotein gp120 (AIDSVAX B/B and AIDSVAX B/E). The AIDSVAX B/B contained two

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recombinant gp120 HIV-1 subtype B antigens that were adsorbed to alum. This vaccine (VAX004) advanced to phase III clinical trials in 1998-1999 and was administered to healthy individuals in North America and the Netherlands (Flynn et al. 2005). The participants were at high risk of infection via either homosexual or heterosexual exposure. In total, 5417 participants were enrolled in this study, with 94% of these being males. Administration of the vaccine lead to mild to moderate reactogenicity occurring in the first 3 days post vaccination (Flynn et al. 2005). This is in contrast to the pilot study done by Graham et al. (1996), where participants who received monovalent gp120 vaccine with adjuvant (MFP-PE) experienced increased local and systemic side effects, thereby highlighting the possible importance of using a better adjuvant in vaccine formulations.

A 100% of all vaccinees developed type-specific neutralizing antibody responses against the SF-2 strain of HIV-1 and CD4 blocking antibodies (Graham et al. 1996), thus indicating the vaccine's immunogenicity. However, the bivalent vaccine was ineffective in preventing HIV-1 infection or in modifying markers of disease progression. Flynn et al. 2005 postulated that failure of this vaccine maybe attributed to lack of induction of antibodies capable of neutralizing genetically diverse HIV-1 primary isolates, which has also been shown by a monvalent gp120 vaccine (Graham et al. 1996). Another bivalent gp120-based vaccine candidate was in the pipeline. This vaccine was made from gp120 proteins from genetically diverse HIV-1 subtypes B and E isolates (Berman et al. 1999). From this pilot study, the following observations were made. Firstly, the use of genetically diverse subtypes may expand the breadth of neutralizing antibodies as compared to monovalent as well as bivalent vaccines with antigens isolated from the same subtype (Flynn et al. 2005, Graham et al. 1996). Secondly, the quality and magnitude of antibody responses elicited by bivalent vaccines are similar to those observed in sera from HIV-1 infected people, and lastly, antisera to gp120 vaccine can elicit antibodies neutralizing both T-cell and macrophage tropic viruses.

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This vaccine (VAX003) then advanced to phase III trials that were conducted in injection drug users (IUDs) in Thailand. In this study, 2546 IUDs were enrolled between 1999 and 2000, with 93% being males. Similar to the AIDSVAX B/B; the vaccine was administered with alum as an adjuvant. In terms of safety, the vaccinees showed mild tenderness at the site of injection. Despite the advantages of AIDSVAX B/E as observed by Berman et al. (1999), just like the AIDSVAX B/B, the vaccine did not prevent HIV-1 infection or delay disease progression (Pitisuttithum et al. 2006). Comparing the two trails, in the VAX004, an interesting trend toward

modest efficacy was observed as opposed to the VAX003 trial, with the vaccine efficacy of the former and latter being 6% and 0.1% respectively (Flynn et al. 2005, Pitisuttithum et al. 2006), thereby implying that both vaccines were ineffective in preventing HIV-1 infection.

Despite their safety, inability of these monovalent and bivalent gp120 based vaccines to neutralize primary HIV-1 isolates brought forward a number of postulations with regards to use of glycoprotein vaccines; the use of oligemeric glycoproteins as opposed to either monovalent or bivalent ones (Thongcharoen et al. 2007), the use of better adjuvant or immunoglobulins to enhance immunogenicity as well as their use as boosters in prime-boost regimen to elicit both B- and T-cell mediated immunogenicity (Rerks-Ngarm et al. 2009, Nkolola et al. 2010, Shimada et al. 2010).

The last strategy was employed in the world's largest phase III trial (RV144), where a canary pox-based (ALAVAC-HIV) and AIDSVAX B/E vaccines were used in the prime-boost regime. A total of 16 402 HIV-1 negative individuals were enrolled in Thailand in 2003, with 63% being men. The aim of the study was to evaluate the vaccines' ability to prevent HIV-1 infection and to evaluate its effect on viral loads post acute infection. There were no significant differences in viral loads or $CD4^+$ T-cell counts post infection between vaccine and placebo groups. Most importantly, the study showed a significant, modest reduction in the rate of HIV-1 infection in vaccinees as compared with placebo, with a vaccine efficacy of 26.4% (Rerks-Ngarm et al. 2009), as compared to 6% and 0.1% following vaccination with AIDSVAXB/B and AIDSVAX B/E respectively (Flynn et al. 2005, Pitisuttithum et al. 2006, Gilbert et al. 2005). This study is therefore the first successful phase III clinical trial, whereby protection from infection was seen. Rerks-Ngarm et al. (2009) proposed certain issues to be addressed concerning the results obtained from this study; evaluation of the vaccine efficacy in low risk groups (which may be increased) and the long-term effects of vaccination.

Taken together, these studies highlight, firstly, the importance of choice of better adjuvants to enhance glycoprotein immunogenicity, secondly, the shift from use of monovalent to either bivalent or oligomeric recombinant gp120 vaccines to increase the breadth of neutralizing antibodies. Lastly and most importantly the use of these bivalent or oligomeric vaccines as boosters in the heterologous prime-boost vaccination regimen, which in turn may elicit broadly neutralizing antibodies as well as T-cell mediated immune responses that are needed for HIV-1

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infection. This limited success of HIV-1 vaccines has also highlighted the importance of understanding the correlates of protection in finding safe and effective HIV-1 vaccines.

1.4. Elite controllers and their information on control of HIV infection

Following the discovery of HIV-1 as a cause for AIDS, a small group of individuals with HIV were found to maintain high $CD4^+$ T-cell counts over many years in the absence of ART and therefore did not progress towards AIDS. This group was termed “long-term nonprogressors” (Buchbinder et al. 1994). Most studies have now focused on individuals who maintain undetectable (<50copies/ml) or low (<2000copies/ml) viral loads, termed “elite or viremic controllers” respectively (Grabar et al. 2009, Okulicz et al. 2009). Postulations of mechanisms by which such individuals control viral replication may be informative in the HIV-1 vaccine efforts and these include; lack or reduced $CD4^+$ T-cell susceptibility to infection (Saez-Cirion et al. 2006), infection of individuals by replication-deficient viruses (Saez-Cirion et al. 2009) and efficient control of viral replication by the immune response (Saez-Cirion et al. 2006, Julg et al. 2010).

To test the above hypotheses, Julg et al. (2010) purified $CD4^+$ T-cells (depleted $CD8^+$ T-cells) and showed that the elite controllers had $CD4^+$ T-cells that were readily infected by X4 tropic viruses to a similar degree to those from the uninfected individuals, thereby highlighting lack of resistance to infection of $CD4^+$ T-cells from elite controllers. Studies also revealed that replication-competent viruses could only be isolated from minority (3 out of 14) of elite controllers, suggesting that the majority of these individuals may be infected with replication-deficient viruses or there may be a limited availability of HIV-1 infected $CD4^+$ T-cells in the PBMCs of these elite controllers, thus making it difficult to capture infected cells in a given PBMC sample (Julg et al. 2010).

To show that elite controllers may have reduced frequency of susceptible $CD4^+$ T-cells, the proviral DNA levels in elite controllers’ PBMCs was compared to that of PBMCs from viraemic controllers and chronic progressors (Julg et al. 2010). This study showed low viral reservoirs in elite controller $CD4^+$ T-cells, rather than infection with replication-defective viruses. This was supported by another study whereby replication-competent viruses were isolated from elite controllers (Blankson et al. 2007).

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These studies therefore indicate that the postulations that in elite controllers there is lack of or limited susceptible CD4 cells and also that these individuals may be infected with mostly replication-deficient viruses may not entirely be true, thereby implying that cellular restriction factors may not be the major cause of long-term nonprogression. Therefore another possible mechanism may be that elite controllers exhibit efficient and durable immune control of the virus also CTL selection pressure on gag-protease alters viral replication capacity (Julg et al. 2010), and that HIV-specific CTLs inducing escape mutations with fitness costs in this region may be important for strict viremia control (Saez-Cirion et al. 2009, Miura et al. 2009). Soon after HIV-1 infection, elite controllers are able to down-regulate viral replication in lymphoid tissue, thus reducing the number of infected cells, which in turn will result in low plasma viral RNA levels, very few circulating HIV-1 infected CD4 cells (as seen by low proviral DNA levels), thus leading to slow rates of disease progression (Julg et al. 2010).

How do these findings help in the quest for finding an effective HIV-1 vaccine? Firstly, they shed the light on how elite controllers manage to control their infection. Secondly, the relationship between ongoing replication and disease progression is enlightened. Lastly, these studies provide strong support for emerging efforts to manipulate the immune system to achieve a long-term control of HIV replication similar to what is seen in elite controllers in the absence of antiretroviral therapy.

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1.5. HIV vaccine vectors and vaccination strategies

During the past two decades, multiple strategies for the production of an immunogenic HIV vaccine have been explored. Various organisms have therefore been used as antigen delivery systems and have been implemented in prime-boost vaccination regimes and in single dose vaccinations. When used in single dose regimen, these vectors elicit mostly only one arm of the immune response (either cellular or humoral immune responses), thus limiting the immunogenicity of the expressed antigens, thereby emphasizing the importance of heterologous prime-boost regimes. Induction of broadly neutralizing antibodies is one of the important factors governing the induction of protective immunity against HIV-1. It has been shown that monomeric glycoprotein immunogens do not elicit broadly neutralizing antibodies in animal models and humans (Flynn et al. 2005, Pitisuttithum et al. 2006), thus several groups have focused on generating trimer proteins (Nkolola et al. 2010, Li et al. 2007). Some of the HIV vaccine vectors and vaccine strategies are briefly reviewed in Sections 1.5.1 to 1.5.6.

1.5.1. Viral vectors

The highly attenuated recombinant MVA, NYVAC and canarypox vectors are replication deficient in non-avian hosts. These poxvirus-based vaccines are extremely safe and well tolerated in animal models (Garcia-Arriaza et al. 2010, Mooij et al. 2008) and human volunteers (Rerks-Ngarm et al. 2009).

Replicating and persistent herpes viral vectors such as cytomegalovirus and adenovirus have been used to express SIV antigens. These have been shown to induce strong effector memory T-cell responses, which may potentially decrease HIV acquisition after sexual exposure (Hansen et al. 2009). Macaque studies have shown that vaccination with replicating cytomegalovirus and adenovirus vectors resulted in increased immune responses that reduced viral loads and slowed disease progression following challenge with SIV (Hansen et al. 2009, Patterson et al. 2008).

The measles virus has also been used to express SIV genes. This vector induces long-lasting specific responses in mice (Liniger et al. 2009) and non-human primates (Zuniga et al. 2007), and because the vector is based on the traditional live-attenuated measles vaccine, it has the benefit of a well-established safety profile and can be used as an injectable or aerosol vaccine (Dilraj et al. 2007). Additionally, MV genome is not translocated into the nucleus which avoids viral genome integration into host cell genome, adding to the safety profile of the vaccine.

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1.5.2. Virus like particles

Virus-like particle vaccines (pseudovirion vaccines) have also been developed as non-infectious HIV pseudovirions that may encode one or more, but not all HIV proteins, such as Gag. These virus-like particles allow for the antigens to be presented in a non-infectious form and may also induce neutralizing antibodies against homologous primary isolates (Hammonds *et al*., 2003). However, low antigen-specific and antibody responses have been attributed to use of VLPs alone, thus emphasizing the requirement of a boosting vaccine to elicit high antibody titres and broad magnitude antigen-specific immune responses (Chege et al. 2008, Chege et al. 2009).

1.5.3. Envelope-based vaccines

Envelope-based vaccines are mostly derived from HIV surface antigens such as gp120, gp140 or gp160 (Falk et al. 2000, Hamajima et al. 1997). These vaccines are not infectious, so they can safely be given to immunocompromised people and are less likely to induce unfavorable immune reactions provided the extraction procedure and detoxifying methods yield a pure product (Liljeqvist, Stahl 1999). However, expressed antigens do not usually stimulate as strong immune responses as organisms and therefore mostly require the use of adjuvants to enhance their immunogenicity (Spearman et al. 2009, Nkolola et al. 2010).

1.5.4. DNA vaccines

Similarly, lack of optimal delivery of the DNA into cells particularly in larger animals and the inability to induce robust antigen specific immune responses in non-human primates hampers the choice of these vaccines (Luckay et al. 2007). Therefore the potency of DNA vaccines has been improved by use of adjuvant technology (plasmid cytokine as well as chemokine adjuvant), improved delivery methods (*in vivo* electro-poration) and plasmid optimization (foreign gene codon optimization) (Liu et al. 2008, Shimada et al. 2010). Since the host organism expresses the foreign DNA, DNA vaccines are postulated to be extremely safe and devoid of side effects, thereby highlighting the importance and relevance of this technology (Tenbusch et al. 2010).

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1.5.5. Viral sequences used in HIV vaccines

Apart from focusing on development of the best HIV vaccine vector or improved vaccination strategy, the HIV vaccine development community has also sought to determine viral antigens that would elicit protective immune responses (Doria-Rose et al. 2005, Kothe et al. 2006). Ancestral sequences, which are re-created parental sequences for a particular lineage, as well as alignment of circulating primary HIV isolates in order to select for most common residue at each position to generate consensus sequences, are some of the strategies employed to identify viral antigens that elicit protective immune responses (Doria-Rose et al. 2005, Kothe et al. 2006). Ancestral sequences may include recently fixed escape mutations (Gaschen et al. 2002), whereas post translational modifications may arise from consensus sequences (Doria-Rose et al. 2005) thus making such technologies prone to sampling bias. This in turn may limit antigenicity and immunogenicity of these sequences (Gao et al. 2005, Kothe et al. 2007).

A broad T-cell response is said to be important in control of viral replication (Kiepiela et al. 2007), therefore polyvalent 'mosaic' antigens, which are assembled natural sequences from a defined viral population optimized to contain a high number of potential T-cell epitopes (PTE) is a promising approach in the HIV vaccine development community (Fischer et al. 2007). These full-length proteins are designed such that the natural antigen expression and processing is maintained. Because these PTE peptides include overlapping sequences that reflect naturally occurring HIV polymorphisms (Li et al. 2006), their responses entail both the recognition of a particular epitope (breadth) and the cross-recognition of variants of that epitope (depth) (Barouch et al. 2010). For example, when encoded by recombinant adenovirus, such sequences (mosaic Gag, Pol and Env sequences) induced $CD8^+$ and $CD4^+$ T-cell responses that were almost four times higher than those elicited by consensus or natural sequences. Moreover, their magnitude was comparable to that of both natural and consensus sequences, thereby emphasizing that the mosaic antigens expanded cellular immune breadth and depth without compromising the magnitude of individual epitope-specific responses (Barouch et al. 2010).

1.5.6. Prime-boost regimes

The heterologous prime-boost regimes have also been employed as vaccination strategies in order to induce both the humoral and cellular immune responses (Nkolola et al. 2010, Garcia-Arriaza et al. 2010). Since this in study the prime-boost regime was used, Table 1.1, which shows some of the recent prime-boost vaccination strategies using vectors that express one or more of the HIV genes as well as the use of glycoproteins as HIV-1 vaccines, is included in this review. The immunogenicity of these vaccines or vaccination strategies was assessed in animal models.

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Table 1.1: Some of the key publications of the prime-boost vaccination strategies using vectors that express one or more of the HIV genes

Vector/vaccination strategy	HIV/SIV genes/antigens	Experimental design	Animal model	Key findings	reference
DNA prime, Double deletion mutant (MVA-B ΔA41L/ΔB16R) boost	HIV-1 subtype B *gp120* and polypeptide genes; *gag-pol-nef*	i.m priming of BALB/c mice with DNA-B (100μg), i.p boosting with MVA-B recombinant virus (1x10^{7}pfu/mouse). Control mice were primed with sham DNA and boosted with wt MVA.	BALB/c mice	Both vectors triggered CD8$^+$ and CD4$^+$ T-cell responses, with over 91.9% of these being CD8$^+$ T-cell responses. MVA-B mutant elicited polyfuctional, broad magnitude CD8$^+$ T-cell responses. Both vectors elicited polyfunctional env-specific CD4$^+$ T-cell responses.	(Garcia-Arriaza et al. 2010)
DNA prime, Vaccinia virus (NYVAC89.6P-SIVGPN) or Modified Vaccinia Ankara (MVA89.6P-SIVGPN) boost	SIV$_{mac239}$ *gag-pol-nef* and SHIV$_{89.6P}$ *env*, driven by the two early/late promoters in back-to-back orientation individually	i.m DNA vaccine (4mg) administered at 0 and 4wks. Control animals were given empty vector DNA vaccine. At wks 20 and 24, animals were boosted with either NYVAC89.6P-SIVGPN or MVA89.6P-SIVGPN (5x10^{8}pfu/mouse). Control groups received an empty NYVAC vector as a boost. Macaques were challenged with pathogenic SHIV$_{89.6P}$ (MID$_{50}$)	Rhesus macaques	Dominant (CM and EM phenotypes) env-specific responses elicited by DNA priming in both groups. MVA boost resulted in both polyfuctional CD8$^+$ and CD4$^+$ T-cell responses, while NYVAC elicited mainly CD4$^+$ T-cell responses specific to Gag. Similar levels of protection were seen in both groups, where the animals were able to control VL and maintained CD4$^+$ T-cell numbers.	(Mooij et al. 2008)
Adenovirus (Ad5hr) prime, gp120 peptide boost	HIV *env*$_{89.6P}$ *gp140ΔCFI*, SIV*gag*$_{239}$/SIV*nef*$_{239}$, HIV$_{89.6P}$ peptomer	At wks 0 and 12, animals were primed with Ad5hr i.n/i.t/orally at a total recombinant of 1.5x10^{9}pfu/macaque/ route. Animals were boosted with various peptides with/without adjuvant at wks 24 and 36 post priming. Control group received empty vector (Ad5hrΔE3)	Mamu-A*01 negative macaques	Significant reduction in VL was seen in gp140-boosted group. There was also better protection in this group due to persistent binding Abs as well as anamnestic SIV-specific T-cell responses. A siginificant reduction in chronic viremia following challenge was attributed to Ad recombinant priming alone. Detectable env-	(Patterson et al. 2008)

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				specific proliferative responses, which gradually peaked 32wks post priming without boosting.	
Vesicular somitatis virus (VSV) prime, rMVA boost	Both vectors expressed $SHIV_{89.6P}$ *env, gag and pol*	Animals were primed with rVSV and boosted with rMVA ($2x10^7$pfu/ml). The vaccination routes were i.m/i.t/oral	Rhesus macaques	Prime-boost with VSV/MVA generated solid control of SHIV challenge for at least 5 years. These animals remained healthy, had normal $CD4^+$ T-cell counts and their VL fell below detection limit by 5 months. SHIV neutralizing Abs were maintained in the serum out to 5 years post challenge, thus possible contributing to control of challenge.	(Schell et al. 2009)
DNA (pTHgag) priming, recombinant baculovirus $Pr55^{gag}$ VLPs boosting	HIV-1 subtype C *gag* of Du422	Animals were primed with pTHgag (5mg/dose) and boosted with Gag VLPs (29µg/dose). Some groups were vaccinated with either pTHgag or GagVLPs only.	Chacma baboons	Low Gag-specific and Ab responses to vaccinations with DNA/VLPs alone. Increased DNA inoculations did not have a positive effect on cellular or humoral immune responses. DNA/VLPs regime resulted in high Ab titers as well as broad magnitude Gag-specific responses. Recall responses of baboon PBMCs following boost were to peptides containing epitopes recognized by HIV-1 subtype C-infected individuals.	(Chege et al. 2008)
Env gp120Ig (HIV Env fused to immunoglobulin)	HIV gp120 with immunoglobulin attached to the N-/C-terminus, CMV-1E enhancer, driven by chickβ –actin promoter	The following plasmids; pgp120, pgp120Ig and pIg-gp120 were constructed. Animals were injected (i.m) with the expression plasmids (100µg). Control groups received mock plasmid (pCAGGS).	BALB/c mice	DNA vaccine encoding Gp120 with Ig fused at the C-terminus resulted in increased expression levels of Gp120 in both cell lysate and supernatant, enhanced CD8+ T-cell responses and provided effective protection against chimeric virus (as seen	(Shimada et al. 2010)

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		The animals were then boosted with the same plasmids. In some groups, mice were subjected to electric pulses at injection sites by a gene gun. At wk 6, mice were challenged with vaccinia virus vPE16 ($1x10^7$pfu) expressing HIV-1 gp160.		by the decline in VL). DNA vaccination was dose dependant (decreased DNA resulted in increased immune responses).	
Clades A and C Trimeric Env gp140 with Ribi adjuvant prime, rAd26 expressing the same trimers boost (DNA vaccine was also used as a prime).	Clade A and C gp140 trimmers with the C-terminal T4 bacteriophage fribritin trimerisation domains and poly-His mitifs	The trimmers were extracted and purified using nickel columns and concentrated. Animals were immunized (100µg/animal) with trimmers at 4/5-wk intervals with adjuvant (s.c and i.p). In some experiments, DNA/trimmers or DNA/Ad26 prime-boost regimen were followed.	Guinea pigs	Trimmers with adjuvant elicited potent, cross-Clade NAbs responses against tier 1 viruses as well as low, but detectable NAbs to tier 2 viruses of Clades A, B and C (most especially Clade C trimmers). DNA/trimmers or DNA/Ad26 prime-boost regimen did not show enhanced magnitude and breadth of NAbs.	(Nkolola et al. 2010)

i.m: intra mascular, i.n: intra nasal, i.p: intra peritoneal, i.t: intra tracheal, s.c: subcutaneous, VL: viral loads, Abs: antibodies, NAbs: neutralizing antibodies, VLPs: virus-like particles, CMV: cytomegalovirus, wk: week, poly-His: poly histidine.

These vaccination strategies differentially elicit one or both arms of the immune system. Some of these may also trigger mucosal immunity, which is ideal for the development of HIV vaccines.

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1.6. Development of rBCG as an HIV vaccine vector

BCG was developed as an anti-tuberculosis vaccine at the Institut Pasteur de Lille in France between 1904 and 1908 by serial passaging of a virulent strain of *Mycobacterium bovis* to attain an attenuated strain named bacille-Calmette-Gu`erin (BCG) (Hoft 1996). Several features make BCG a good candidate as an antigen delivery system for heterologous antigens. BCG has a proven safety record and is currently the most widely used vaccine. Over 3 billion people have been vaccinated with BCG since 1921, with very low occurrences of severe complications (Hoft 1996). As a live bacterial vaccine, BCG is easy and cheap to manufacture because it needs limited purification. This vaccine is one of the most heat-stable vaccines. It is a strong adjuvant

that can be administered at or any time after birth and is least affected by the maternal antibodies, with a single dose sensitizing to tuberculosis proteins for 5 to 50 years (Ohara, Yamada 2001). In the early studies, BCG has been extensively exploited as a delivery system for various viral, bacterial, human and non-human primate antigens (Falk et al. 2000, Badell et al. 2009, Bastos et al. 2002, Kanekiyo et al. 2005).

Earlier studies have also shown its ability to induce both humoral and cellular immune responses in animals when used in heterologous prime-boost regimens. Heterologous prime-boost with rBCG and various other vectors like MVA have resulted in increased immunogenicity compared to vaccination with rBCG alone (Ami et al. 2005, Vordermeier et al. 2004). Vordermeier et al. (2004) showed that heterologous prime-boost vaccination using rBCG and rMVA expressing Ag85A resulted in increased antigen specific CD8 T-cell responses in comparison to vaccination with rBCG vaccination alone. Furthermore, priming with rBCG-SIV_{gag} and boosting with recombinant vaccinia virus expressing HIV Gag elicited protective immunity against mucosal challenge with pathogenic SIV for a year (Ami et al. 2005).

Recent studies have shown that priming with rBCG and boosting with viral vectors such as vaccinia both expressing HIV antigens may result in induction of $CD4^+$ and $CD8^+$ T-cell-specific cytokines (Promkhatkaew et al. 2009). Similarly the rBCG prime and $Pr55^{gag}$ boost regime elicited predominant $CD8^+$ T-cell responses as well as antigen-specific humoral immune responses (Chege et al. 2009). BCG has also been used to express SIV antigens and its immunogenicity has been evaluated in animal models. This resulted in more persistent, polyfuctional SIV-specific cellular responses in rhesus macaques boosted with rAd5 (Cayabyab et al. 2009), the production of long-lasting SIV-Gag-specific serum IgG in guinea pigs (Kawahara et al. 2002). Rosario et al. (2010) evaluated the safety and immunogenicity of novel rBCG-HIV and rMVA-HIV vaccines in neonate rhesus macaques. All the vaccines were safe, with mild local reactions at the site of injection and showed no systemic adverse effects or abnormality. These vaccines also induced high frequencies of BCG-specific INF-γ secreting lymphocytes, thereby highlighting the importance of the development of possible infant vaccines. The results indeed show that heterologous prime-boost effectively optimizes both humoral and T-cell immune responses. Despite these advantages, there are several issues to be addressed when using BCG as an antigen delivery system.

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1.6.1. Disadvantages of using rBCG as a vaccine

BCG is slow-growing and the cells tend to aggregate, thus making it difficult to manipulate in the laboratory. Prior exposure to live environmental mycobacteria primes the immune system against antigens shared with BCG. The resultant memory response results in accelerated clearance of rBCG upon vaccination, thus limiting its effect as a TB vaccine in areas with environmental mycobacteria (Demangel et al. 2005). Recombinant BCG expressing foreign antigens can also be unstable *in vitro* and *in vivo* (Cayabyab et al. 2009, Joseph et al. 2010)

Increased incidences of disseminated BCG disease in HIV+ newborns has lead to urgent need for development of safer vaccines to replace BCG based vaccines. This has also lead to the recommendation of World Health Organisation against vaccination of HIV+ newborns with BCG (Hesseling et al. 2007, Hesseling et al. 2009, Nuttall et al. 2008). Several strategies have been implemented to increase the safety of such vaccines and these include generation of *M. tuberculosis* and BCG auxotrophic mutants as well as replication-limited organisms (Sambandamurthy et al. 2006, Tullius et al. 2008, Sampson et al. 2011).

For instance, studies have shown that *M. tuberculosis* pantothenate auxotroph (panCD) was highly attenuated in severe combined immune-deficient (SCID) mice and in immunocompetent BALB/c mice. SCID mice infected with this auxotroph allowed significant and prolonged survival as compared to mice infected with either *M. bovis* BCG or virulent *M. tuberculosis* (Sambandamurthy et al. 2002). Similarly, *M. bovis* BCG Pasteur ΔpanCD, a slow growing pantothenic auxotroph, was constructed as a double-deletion mutant of the *panC* and *panD* genes that are involved in the *de novo* biosynthesis of pantothenate (Bardarov et al. 2002, Tullius et al. 2008). Hence Tullius et al. 2008 showed that rBCG (Δ*panCD*) 30 (the pantothenic auxotroph that over-expresses *M. tuberculosis* antigen 85B) is more attenuated than BCG in SCID mice and provided similar protection to BCG in immunocompetent guinea pigs. Having shown such promise in animal models, such vaccine candidates may be usefull in humans, most especially immune-comprosed individuals and newborns.

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1.6.2. Stability of rBCG vaccines

Over-expression of foreign antigens in BCG is one of the factors that can limit rBCG vaccine stability. The choice of vectors also has an influence in antigen stability. Integrating vectors have been shown to be more stable than episomal vectors (Mederle et al. 2002). For instance, Stover et al. (1991) were unable to express HIV-1 gp120 under the constitutive promoter *hsp60* episomally, but the same product was successfully expressed from an integrating vector. Al-Zarouni, Dale 2002 reported low level expression of β-lactamase in BCG as a result of deletions in the plasmids. Infrequent mutations of DNA that is integrated into the chromosome and low copy (one copy) number, which leads to reduction in foreign antigen expression, have contributed significantly to making the integrating vectors more stable. However, decrease in expression due to a single copy rather than 5 copies of standard episomal vectors may result in poor immune responses (Falk et al. 2000). For this reason, foreign antigens have often been expressed episomally (Bastos et al. 2002, Langermann et al. 1994).

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1.6.3. Plasmid shuttle vector features

E. coli-mycobacterial shuttle vectors have been widely used for the delivery of foreign antigens. The level and timing of antigen expression, together with the type of antigen presentation and processing during an immune response are largely determined by the features of such vectors. Promoter elements and leader peptides are amongst these features as they regulate the level of expression and presentation pathway of expressed foreign antigen to the immune system.

Pathways of antigen presentation and processing are determined by antigen localization. Antigens can be directed to various cell compartments; including the cytoplasm, extracellular matrix and cell membrane by the use of various leader peptides such as the 19kDa lipoprotein and alpha antigen signal sequences (Al-Zarouni, Dale 2002, Stover et al. 1993). These signal peptides often function in the protein translocation systems.

1.6.4. Protein translocation systems in mycobacteria

Many mature cellular proteins are found remote from their site of synthesis and therefore require translocation to various compartments of the cell for maturation and assumption of functionality. For this reason, proteins need specialized translocation systems. Such mechanisms include

amongst others the Sec-mediated (Osborne et al. 2005) and the Sec-independent twin-arginine-specific translocation (Tat) systems (Zhang et al. 2009).

1.6.4.1. Sec-mediated system

Sec-mediated system is responsible for translocation of newly synthesized proteins out of the cytosol across the membranes before they attain their mature structure and functionality (Osborne et al. 2005, den Blaauwen et al. 1997). This system comprises of SecB; a molecular chaperone dedicated to protein export, SecA; an ATPase that aids in translocation of protein into and across membrane and the heterotrimeric complex SecYEG, which constitutes a 'channel' for protein movement. Initiation of translocation is powered by ATP and proteins are directed to the system by signal that lack sequence similarity, but share common physical-chemical structure (Rigel et al. 2009, Sargent et al. 1998). The signal peptide is an approximately 24 amino acid sequence organized in a tripartite manner; a positively charged amino-terminus, a hydrophobic core region and a hydrophilic carboxy-terminal domain containing a recognition sequence for peptidase cleavage (Posey et al. 2006). Gram negative signal sequences are approximately 20 amino acids long, while Gram positive bacteria as well as mycobacteria may possess signal peptides of length of up to 60 residues (Wiker et al. 2000).

Mycobacteria lack the SecB chaperone and therefore utilize other cytosolic chaperones to translocate proteins (Fekkes et al. 1997). Mycobacteria, in particular *M. smegmatis* and *M. tuberculosis,* have two SecA proteins (SecA1 and SecA2) (Braunstein et al. 2001). SecA1 is the 'housekeepiung' protein, whereas SecA2 is the accessory secretory factor that aids in the ATP driven secretion of superoxide dismutase (SodA) and catalase peroxidase (KatG), which in turn detoxify reactive oxygen and nitrogen species (Braunstein et al. 2003, Kurtz et al. 2006). This implies that SecA2 is functional in promoting early extracellular growth of *M. tuberculosis* in murine infection and inactivated macrophages. Studies using a *secA2* mutant of *M. tuberculosis* indicated that this accessory secretory factor is essential in inhibition of both innate and adaptive immune responses, which is important for bacterial survival within the host and for its pathogenesis (Braunstein et al. 2003, Kurtz et al. 2006).

1.6.4.2. TAT system

Trimethylamine N-oxide (TMAO) reductase and dimethyl sulfoxide (DMSO) reductase; periplasmic and membrane-bound multi subunit enzymes respectively are translocated through

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to the periplasmic space and membrane respectively via the Sec-independent twin-arginine-specific translocation (TAT) system. These precursor enzymes are directed to the TAT system by a long peptide sequence (leader peptide) of consecutive, invariant arginine residues organized in a tripartite manner similar to that of the Sec signal sequence (Posey et al. 2006). Such translocation is via the trans-membrane proton electro-chemical gradient (Zhang et al. 2009, Sargent et al. 1998). This system serves to translocate mostly mature folded proteins and is a virulence determinant in some pathogenic bacteria such as *Vibrio cholerae* (Zhang et al. 2009). The TAT system has been widely studied in *E. coli* and is functionally as well as structurally related to the pH-dependent protein import pathway found in thylakoid membranes of plant chloroplasts (Berks et al. 2000). Co-factor carrier enzymes involved in respiratory pathways and photosynthetic electron transport chain are mainly translocated via the TAT pathway (Berks et al. 2000). The TAT system is encoded by the three genes; *tatA*, *tatB* and *tatC*.

Posey et al. (2006) used the TorA-GFP reporter system to determine if mycobacteria posses the TAT system. Part of *torA* is a 150bp fragment, which has the TAT-dependant signal peptide as well as the first four amino acids of the mature protein (Posey et al. 2006). This group discovered that signal peptide coupled GFP expression was directed to the periphery of the cells as opposed to the cytoplamic localization of uncoupled GFP in *M. smegmatis*. It was also evident by individual disruption of *tat* genes that they were all essential in the TAT system. Furthermore, studies in both *E.coli* and *M. smegmatis h*ave shown that such disruptions have negative effects on physiology, including metabolism of specific substrates and colony morphology (Posey et al. 2006, Braunstein et al. 2001, Ize et al. 2004, Ochsner et al. 2002).

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1.6.4.3. Snm/ESX-1 system

Mycobacteria use the snm/ESX-1 translocation system as an equivalent to type I-V translocation systems. This system is used to translocate ESAT-6 (early secretory antigen, 6kDa) and CFP-10 (culture filtrate protein, 10kDa) antigens in mycobacteria (Guinn et al. 2004, Stanley et al. 2003). This was shown by the loss of ability of mycobacteria to export ESAT-6 and CFP-10 antigens following disruption of individual genes (*M. tuberculosis snm1/Rv3870, snm2/Rv3871* and *snm4/Rv3877*), suggesting that these genes encode for a secretion system (Guinn et al. 2004, Stanley et al. 2003). The homologs of these genes in *M. smegmatis* are *Sm3866*, *Sm3869*, *Sm3882c* and *mycP1* (Converse, Cox 2005). In *M. tuberculosis,* Rv3870 interacts with Rv3871, which function as an ATPase.

It was postulated that both ESAT-6 and CFP-10 form a tight dimmer, which is translocated by Rv3871 to the transmembrane protein formed by Rv3870 and Rv3877, which make a cytosolic membrane-spanning pore (Stanley et al. 2003, Champion et al. 2006). Studies have also indicated that CFP-10 has a seven amino acid signal sequence on the C-terminus, necessary for targeting of the antigen dimers for secretion (Stanley et al. 2003). This unstructured signal peptide is in a form of a handle that allows for ESAT-6/CFP-10 dimer translocation by Rv3871 (Champion et al. 2006). This system modulates early events such as control of macrophage cytokine response during *M. tuberculosis* infection.

1.6.5. Characteristics and use of signal peptides

In as much as amino terminal signal peptides show little homology apart from the cleavage site for signal peptidase, the interactions between these sequences and their receptors appear to be selective for their physical characteristics rather than the primary amino acid sequence (Chou, Gierasch 2005). There exist distinct domains in these sequences. The N-domain contains at least one arginine or lysine, which are not necessarily essential for protein export. This positively charged N-domain is associated with the translocation machinery and the negatively charged phospholipids with the lipid bilayer of the membrane during translocation. The α-helical conformation of the amino acids forms the H-domain, which is important for the signal peptide function (Chou, Gierasch 2005, Akita et al. 1990, Wehrl et al. 2000). The centre of the hydrophobic domain has glycine and proline residues, which allow the signal peptide to form a hair pin loop that inserts into the membrane (Wehrl et al. 2000). Cleavage of the signal sequences by specific peptidases is enhanced by the helix breaking residues found at the end of the H-domain. Lastly the C-domain contains the recognition site for peptidases, which cleave the signal peptide from the mature protein during or just after translocation (Chou, Gierasch 2005, Akita et al. 1990, Wehrl et al. 2000, Horwich et al. 1985). Such leader peptides have been shown to independently transport proteins and the efficiency of pre-protein translocation is dependent on their properties (Chou, Gierasch 2005). They have therefore been used in various vaccine vectors to translocate foreign antigens thereby possibly enhancing vaccine immunogenicity (Badell et al. 2009, Henao-Tamayo et al. 2007, Tjalsma et al. 2000).

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1.6.6. Examples of signal peptides

1.6.6.1. The 19kDa signal sequence

Recent studies and DNA analysis have shown that 19kDa lipoprotein sequences from both *M. bovis* and *M. tuberculosis* are identical and have 75% homology to *Mycobacterium avium* (Ashbridge et al. 1989, Collins et al. 1990, Huntley et al. 2005). Figure 1.1 shows the 19kDa DNA sequence and the corresponding amino acid sequence. Lipoproteins have conserved short amino acid sequences, which signal translocation of lipoproteins into the extracellular matrix (Badell et al. 2009, Henao-Tamayo et al. 2007, Tjalsma et al. 2000). Amongst a variety of these sequences is the 19kDa signal peptide that is responsible for the translocation of the 19kDa lipoprotein. Biochemical studies indicate that the 19kDa antigen undergoes post-translational acylation and glycosylation prior to lodging of the mature 19kDa lipoprotein protein into the cell membrane and these modifications have been shown to be essential in the modulation of innate immune responses in humans (Stewart et al. 2005, Wilkinson et al. 2009).

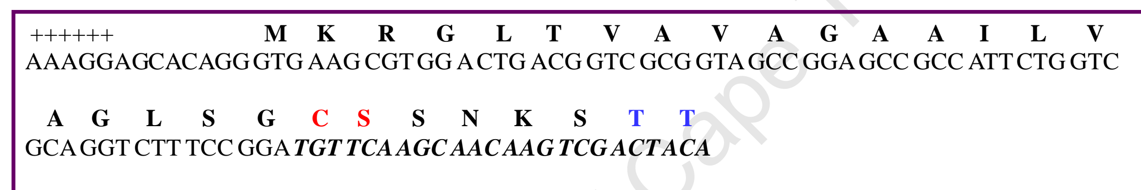

Figure 1.1: DNA sequence and corresponding amino acid sequence of the 19kDa signal peptide and part of the 19kDa lipoprotein. +++++ indicates the putative ribosome binding site. Italicized and bolded sequence corresponds to part of the 19kDa lipoprotein nucleic acid sequence. Red and blue depict the acylation and glycosylation sites on the protein respectively (Adapted from (Ashbridge et al. 1989).

This lipoprotein has been shown to be immunodominat in both mice (Erb et al. 1998) and humans (Harris et al. 1993) and has been shown to stimulate $CD4^+$ and $CD8^+$ T-cell proliferation as well as production of cytokines such as IL2, IFN-γ and IL-12 (Stewart et al. 2005, Wilkinson et al. 2009, Brightbill et al. 1999). Furthermore, studies have shown that an increased proportion of antibodies against *M. tuberculosis* were specific to the 19kDa lipoprotein thus emphasizing its immunogenicity (Prestidge et al. 1995). Deletion of the 19kDa lipoprotein rendered *M. tuberculosis* unable to replicate *in vivo* (Wilkinson et al. 2009). However this mutant strain remained immunogenic and protected mice against aerosol challenge to a similar degree to a BCG vaccine. This mutant strain was well able to grow *in vitro*, but not *in vivo* (in macrophages), suggesting that the 19kDa lipoprotein may be pivotal in the host cell phagosome (Henao-Tamayo et al. 2007). Furthermore, evidence from confocal microscopy, electron microscopy and sub-cellular fractionation indicated that this lipoprotein is exported from the

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phagosomes into the host cell within the first hour of phagocytosis of live mycobacteria (Neyrolles et al. 2001).

1.6.6.2. The α-antigen signal sequence

The α-antigen 85B (α-Ag85B) is a putative virulence factor that falls under a family of exported fribronectin-binding proteins. This family consists of Ag85A, Ag85B and Ag85C, of which Ag85A and B are the most prominent (Armitige et al. 2000). These antigens are encoded by *fbpA*, *fbpB* and *fbpC* respectively and the resultant proteins allow for the organisms to bind selectively to fibronectin and other extracellular matrix proteins thus enhancing virulence of these pathogens (Patti et al. 1994). This binding capability may be correlated to adherence and dissemination in tissue (Armitige et al. 2000). Studies have shown that the antigen 85 complex may be secreted as well as being present on the mycobacterial cell walls (Abou-Zeid et al. 1988, Peake et al. 1993, Rambukkana et al. 1992). These proteins transfer mycolic acids to α-ά-trehalose monomycolate (TMM) thus forming α-ά-trehalose dimycolate (TDM) (Belisle et al. 1997).

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Although genetically distinct, these proteins show sequence homology both intraspecially and interspecially. Studies have shown that Ag85B has three binding sites to fibronectin (Peake et al. 1993). Genes encoding Ag85B, a 30kDa protein termed MBP59 or alpha antigen (α-Ag) have been isolated from many mycobacterial species such as *M. leprae* and *M. bovis* BCG as well as non-tuberculous mycobacteria (Matsuo et al. 1988). The α-Ag has been expressed in *E. coli* and was shown to be a 323amino acid protein, consisting of 40 amino acid residues proposed to encode a signal peptide together with 283 amino acids encoding the mature protein (Matsuo et al. 1988). Figure 1.2 shows the DNA sequence and the corresponding amino acid sequence of the α-Ag signal peptide.

M T D V S R K Ile R A W G R R L M I G T A
ATG ACA GAC GTG AGC CGA AAG ATT CGA GCT TGG GGA CGC CGA TTG ATG ATC GGC ACG GCA

A A V V L P G L V G L A G G A A T A G A
GCG GCT GTA GTC CTT CCG GGC CTG GTG GGG CTT GCC GGC GGA GCG GCA ACC **GCG GGC GCG**

Figure 1.2: DNA sequence and corresponding amino acid sequence of the α-Antigen signal peptide. Bolded and underlined codons indicate the signal peptidase recognition sequence; Ala-X-Ala (Matsuo et al. 1988)

1.6.7. Use of leader peptides to determine the types of immune responses elicited

Leader sequences direct antigens to various compartments of the cells. Such peptides may lodge the protein in the membrane or may facilitate extracellular secretion of the antigens from the cell, subsequently influencing the type of immune response elicited by such vaccines. Both $CD4^+$ and $CD8^+$ T-cells have been associated with expression of foreign antigen fused to the 19kDa signal peptide, which directs the protein to the cell membrane, whereas only $CD4^+$ T-cell responses are correlated to secreted antigens fused to the α–antigen signal sequence (Grode et al. 2002). Grode et al. (2002) reported the differential induction of protective cell-mediated immunity elicited by different recombinant BCG strains expressing *Listeria monocytogenes* p60 antigen in secreted, cytosolic, or membrane-attached form. Both the membrane-linked lipoprotein and the secreted p60 antigens offered nearly equal protection from listeriosis, whereas cytosolic p60 failed to protect mice from disease. On the contrary, Dennehy et al. (2007) demonstrated that oral or intraperitoneal vaccination of mice with rBCG expressing the α antigen leader sequence and the mature α antigen fused to the rotavirus VP6 protein from the 18 kDa antigen promoter were not protected against rotavirus. However, this group argued that lack of protection by this vaccine may be related to lack of secretion or, if secretion did occur, to the type and magnitude of immune response induced by the secreted protein. On the other hand, similar to Grode et al. 2002, Dennehy et al. (2007) showed protection of mice from rotavirus infection following vaccination with BCG Pasteur containing either episomal or integrative vectors expressing the VP6 protein fused to the 19kDa antigen membrane-anchoring signal sequence. Himmelrich et al. 2000 constructed a number of different rBCG expressing secreted or membrane targeted or cytosolic forms of the MalE protein. They found that levels of expression of the secreted form of MalE were far higher than that of the cytosolic form even when expressed from the same promoter. In addition rBCG expressing the highest levels of secreted MalE induced stronger and more rapid immune responses suggesting there is a correlation between antigen expression levels and the induction of immune responses. These results therefore highlight the importance of the use of signal peptides in the expression levels and immunogenicity of antigens expressed by rBCG.

1.6.8. Mycobacterial promoters

For the development of an effective HIV vaccine based on mycobacterial vectors, use of defined mycobacterial promoters has been shown to be beneficial in driving foreign gene expression.

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Activity of such promoters has been evaluated using reporter genes such as *β-lactamase* (Al-Zarouni, Dale 2002), *β-galactosidase* (Dellagostin et al. 1995)and *gfp* (Zahrt, Deretic 2000), amongst others.

Although there are exceptions, mycobacterial promoters generally function poorly in *E. coli* (Das Gupta et al. 1993). To gain an insight into the divergence of mycobacterial promoters as compared to those of *E. coli*, Das Gupta et al. (1993) analyzed the activity of mycobacterial promoters in *E. coli* DH5α by isolating several promoters from *M. tuberculosis* H37Rv and *M. smegmatis* using a promoter selection vector that was constructed for the identification of promoter elements in a homologous environment. This vector consisted of a promoterless chloramphenicol acetyltransferase gene (CAT); a kanamycin resistance gene for positive selection in mycobacteria as well as extra-chromosomal origins of replication for *E. coli* and mycobacteria. Different plasmids (100 each) from both *M. smegmatis* and *M. tuberculosis* consisting of a variety of mycobacterial promoters fused to the promoter-less CAT gene were transformed into *E. coli*. This group discovered that only 12 plasmids containing *M. smegmatis* DNA conferred chloramphenicol resistance on *E. coli*. Furthermore, none of the promoter plasmids derived from *M. tuberculosis* conferred chloramphenicol resistance on *E. coli*. This implied that most of mycobacterial promoters were found to function poorly in *E. coli*. The *hsp60* and *mtrA* promoters were used in this study and are reviewed in more detail in the following two sections.

Constitutive expression from the *hsp60* promoter results in high levels of expression of foreign antigen which can lead to protein toxicity and instability. For instance, attempts by Stover et al. 1991 to clone the HIV *env* gene under the control of the *hsp60* promoter using a mycobacterial plasmid vector were unsuccessful; suggesting that expression of this gene was toxic. Al-Zarouni, Dale (2002) found out that the *hsp60* driven expression of β-lactamase was markedly low in rBCG. Plasmid integrity checks in *E. coli* indicated that approximately 95% of the plasmids contained deletions, which explained the low level expression of the reporter gene. Taken together, these studies do emphasize the deleterious effect of *hsp60* promoter on the expression of some foreign genes in mycobacteria. This may pose a disadvantage relative to the 18 kDa antigen and *mtrA* promoters, which have lower activity during *in vitro* growth and are induced on infection of macrophages (Dellagostin et al. 1995), thus driving the expression of a foreign antigen at a time when the antigen could be exposed to the immune system. The 18kDa antigen and *mtrA* promoters have been widely used in *E. coli*-mycobacterium shuttle vectors.

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1.6.9. The *hsp60* promoter

The Hsp60 protein belongs to a large family of heat shock proteins, induced at high levels under conditions of stress thereby allowing the bacteria to survive hostile environments. The mycobacterial *hsp60* promoter has two transcriptional start sites in BCG, *tsA* and *tsB* (Stover et al. 1991, Dellagostin et al. 1995). Only *tsA* is recognized in *M. smegmatis* (Dellagostin et al. 1995). This promoter shows high constitutive activity in mycobacterial cultures and in vivo. However, in *M. avium, hsp60* driven expression showed low activity during exponential growth in standing culture, but a considerable increase in activity during late exponential to late stationary growth phases (Batoni et al. 1998).

1.6.10.The *mtrA* promoter

Two component signal transduction systems are a class of well documented ubiquitous bacterial regulatory elements associated with signal recognition and response regulation (Zahrt, Deretic 2000). These systems are involved in regulation of transient adaptations, cellular functions, production of secondary metabolites and virulence, as well as disease progression determinants in case of pathogens. These are comprised of a membrane bound sensor kinase, which upon stimulus encounter, is phosphorylated at the histidine residue. The phophoryl group is then transferred to the cognate cytosolic response regulator, which initiates responses to stimuli (Via et al. 1996).

One of the well characterized two component signal transduction systems is the sensor kinase, MtrB, and its cognate response regulator MtrA. Studies have shown that MtrA but not MtrB is essential for *M. tuberculosis* viability *in vitro* (Zahrt, Deretic 2000). This group found that the *mtrA* promoter was expressed differentially in *M. tuberculosis* and BCG during infection of macrophages. In BCG, *mtrA* promoter expression was low immediately after infection of macrophages, but by day 3 post infection, *mtrA* promoter activity was comparable to that of *hsp60*. On the contrary, in *M. tuberculosis, mtrA* promoter activity resulted in constitutive expression upon infection of macrophages. Via et al. (1996) also showed that the *mtrA* promoter was induced in BCG on infection of macrophages.

The use of inducible mycobacterial promoters has been employed in the expression of HIV genes using BCG as a vector. This lowers the *in vitro* metabolic stress and subsequently allows

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for induction of expression of rBCG genes only upon infection of macrophages, thus driving the expression of a foreign antigen at a time when the antigen could be exposed to the immune system.

1.7. Codon optimization of foreign genes

The level of foreign antigen expression may be partly influenced by codon usage. Williams et al. (1988) first evaluated the effect of codon optimization on heterologous protein expression in *E. coli*. This group showed that there was an 8-10 fold increase in synthetic codon optimized IL-2 expression compared to the expression of native cDNA sequence driven by the *trc* promoter in *E. coli* JM101 cells. Therefore, to optimize expression levels, foreign antigen codons have been matched with those of their respective host to attain such levels. Kanekiyo et al. 2005 have evaluated the effect of codon optimization in rBCG vector-based vaccines. This group showed that there was a 40 fold increase in p24 expression levels in rBCG expressing codon optimized HIV-1 p24 as compared to non codon optimized p24. Mycobacterial genomes have a high G+C content (more than 60%), as opposed to the HIV genome, which has a high A+T content. This implies that if such genes were inserted into mycobacteria, there will be low expression levels of such genes. However, codon optimization counteracts this by lowering the A+T content in the HIV genes, thus increasing mRNA stability and translation efficiency following their insertion into BCG. This in turn increases the expression levels. Codon optimization has also been shown to reduce the risk of recombination in HIV infected individuals due to decreased sequence homology between the natural HIV viral genome and the codon optimized gene (Andre et al. 1998).

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1.8. Aim

The long-term aim of our research group is to construct stable rBCG expressing HIV-1 antigens as candidate vaccines. The aims of this study were:

- To select a stable plasmid backbone for the construction of rBCG expressing HIV-1 Gag
- To determine if BCG codon optimization of the *gag* gene impacted on the stability of the vaccine shuttle vectors
- To compare the stability rBCG expressing HIV-1 *gag* under the control of different promoters and with different leader sequences

- To compare the immunogenicity of rBCG expressing HIV-1 *gag* under the control of different promoters and with different leader sequences in a mouse model.

CHAPTER 2: MATERIALS AND METHODS

2.1 Study design

The project was divided into three parts; 1) plasmid shuttle vector construction and mapping, 2) evaluation of stability in model organism *M. smegmatis* by colony sizes and growth patterns and 3) preparation of rBCG vaccine stocks and analyses of rBCG vaccine immunogenicity in mice.

2.2 Materials and general methods for the growth and culturing of Escherichia coli

This section will detail the specific materials and methods employed in the application /use of *E. coli* in the study.

2.2.1 *E. coli* strains, growth media and culture conditions

E. coli DH5α and SCS110 cells were used in this study. *E. coli* recombinants were spread plated on 2YT agar (16g Tryptone, 10g Yeast extracts, 5g NaCl and 15g agar per litre of water) supplemented with with 25µg/ml of kanamycin [Sigma-Aldrich, SA] and incubated overnight at 37°C. The resultant recombinant colonies were cultured in 2YT broth (16g Tryptone, 10g Yeast extracts and 5g NaCl per litre of water) supplemented with 25µg/ml of kanamycin or in Luria Bertani broth (10g Tryptone, 5g Yeast extracts and 5g NaCl per litre of water), supplemented with kanmycin for large scale plasmid isolations. The cultures were grown at 37°C overnight with vigorous shaking. Recombinant *E. coli* glycerol stocks were prepared after culturing in 2YT broth, cultures were re-suspended in sterile glycerol to a final concentration of 15% and stored at -80°C until needed for further studies.

2.2.2 Preparation of competent *E. coli* cells

E. coli competent cells were prepared using a modification of the dimethyl sulphur-oxide (DMSO) method by Chung, Miller (1988). A loop-full of a glycerol stock of *E. coli* cells was streaked onto a 2YT agar plate and incubated overnight at 37°C. A single colony was selected, inoculated into 5ml of 2YT broth and incubated at 37°C overnight with shaking to generate a

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starter culture. The starter cultures was diluted 1/100 in 2YT broth and grown to early log phase (OD_{600} 0.2 – 0.4). The cells were harvested by centrifugation at 5000 rpm for 5 minutes at 4°C, and then re-suspended in 10ml of ice-cold TSB (10% w/v PEG, 5% v/v DMSO, 10mM $MgCl_2$, and 10mM $MgSO_4$). The cells were held on ice for 10 minutes and either transformed immediately or stored in ice-cold sterile glycerol to a final concentration of 10% v/v in 100µl aliquots at -80°C.

2.2.3 Transformation of *E. coli* with shuttle plasmid vectors

The heat-shock procedure was followed as described by Sambrook et al. (1989), with some modifications, for transformation of *E. coli* competent cells with plasmid vectors. Plasmid DNA (approximately 10ng) or ligation reactions were gently mixed with 100µl of competent cells that were previously thawed on ice. The mix was incubated on ice for 10 minutes and then heat-shocked in a 42°C water bath for 2 minutes. 2YT broth (900µl) was added and the mixture was incubated at 37°C for 60 minutes to allow for the expression of the antibiotic marker. The transformation mix was plated onto 2YT agar containing the appropriate antibiotics and then incubated at 37°C overnight.

2.2.4 Enzymatic reactions

2.2.4.1 Agarose gel electrophoresis of DNA

Plasmid DNA and DNA fragments were separated according to size using agarose gel electrophoresis. Agarose was prepared at a concentration of 0.8% (0.8g agarose per 100ml of buffer, with 0.025% ethidium bromide), in 1X TBE buffer (24.2g Tris, 2.75g Boric acid and 10ml 0.5M EDTA, pH8.0). The method followed was adopted from Sambrook et al. (1989). A 6X loading buffer [Fermentas, SA] was added to the DNA samples (at 1x final concentration) which were then loaded onto the agarose gel together with a 1kb DNA molecular weight marker [Promega, SA]. TBE buffer was used as the running buffer and 0.05% ethidium bromide was added to allow for visualisation of DNA samples over UV light. The gels were electrophoresed for 120 minutes at 60 to 120 V in standard gel cast trays (75mm x100mm or 155mm x 200mm) and for 15 minutes at 40V for slide gels (75mm x 50mm).

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2.2.4.2 Restriction endonuclease digestion, DNA extraction and purification

Restriction endonuclease digestion of plasmids was performed according to the manufacturer's instructions [Roche, SA Diagnostics or Fermentas, SA Life Sciences] in a final volume of 20µl, apart from the restriction endonuclease digestions performed for cloning purposes. For cloning purposes, 4µg of plasmid DNA was digested with 10 units of restriction enzyme and 4µl of buffer in a final volume of 40µl. Restriction enzyme digests were incubated at 37°C (unless otherwise specified) for 1 to 3 hours and stopped by the addition of 6X loading dye [Fermentas, SA]. Plasmid DNA was purified from reaction mixes using a Qiaquick Purification Kit [Qiagen, SA] and DNA fragments were purified from agarose gels using the Qiaquick Gel Extraction Kit [Qiagen, SA] according to the manufacturer's instructions.

2.2.4.3 Blunting of restriction sites by T4 DNA polymerase

Where necessary 5' and 3' DNA overhangs resulting from restriction endonuclease digestion were blunted by the use of T4 DNA polymerase [Roche, SA Diagnostics], which fills in the DNA template in a 5' – 3' direction and also nibbles back in a 3' - 5' direction. The latter process was employed to blunt the 3' overhangs on plasmids generated in this study. For blunting reactions, the plasmid DNA purified from agarose gels was mixed with 10nM dNTP's, 5U of T4 DNA polymerase and the appropriate volume of water to make up a final reaction volume of 50µl and incubated at 37°C for 8 minutes. The reaction was stopped by the addition of 3µl of 0.5M EDTA and placed on ice. The reaction was then cleaned up with a Min-Elute Reaction Cleanup Kit [Qiagen, SA] according to the manufacturer's instructions. The final concentration of the DNA sample was confirmed by comparison to lambda DNA of known concentration following slide gel electrophoresis.

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2.2.4.4 DNA ligation

Ligations were performed using T4 DNA ligase [Promega, SA] at ambient temperature for 4 to 5 hours. Following blunting reactions and reaction clean ups, the remainder of the blunting reaction mix (linear plasmids) were ligated using 10U of T4 DNA ligase, 1X ligase buffer and water to make up the total volume of 12µl.

2.2.5 Small scale plasmid DNA isolation

The plasmid DNA was isolated on a small scale as follows: A single colony was inoculated into 800µl of 2YT broth containing the appropriate antibiotic and incubated at 37°C overnight. Reference plates were prepared simultaneously by stabbing each selected transformant onto a numbered grid on a 2YT agar plate containing the appropriate antibiotic. Small scale plasmid isolation was performed on the overnight cultures using the modified alkaline lysis method from Sambrook et al. (1989). The isolated plasmids were re-suspended in 50µl of High Pure Plasmid extraction Kit elution buffer (10mM Tris-HCl, pH 8.5) [Roche, SA].

2.2.6 Large scale plasmid isolation

To perform large scale plasmid isolation, a single colony from the reference plate of transformants was inoculated into 100ml of LB broth containing the appropriate antibiotic and incubated at 37°C overnight with shaking. Large scale plasmid isolation was performed using Geno-pure Plasmid Midi Kits [Roche, SA Applied Science] according to the manufacturer's specifications. A Nanodrop® ND-1000 Spectrophotometer was used to quantify the plasmid DNA. The machine was initialised using 2µl of distilled water, blanked with 2µl of HPLC water and the absorbance at A_{260} and A_{280} of the samples were recorded. The calculation of DNA concentration was based on the theory that 1OD is equivalent to 50µg/ml of double stranded DNA (Sambrook et al. 1989). Screening and selection process for candidate recombinant clones carrying the six recombinant plasmid shuttle vectors with or without the *gag* gene is outlined in Figure 2.1.

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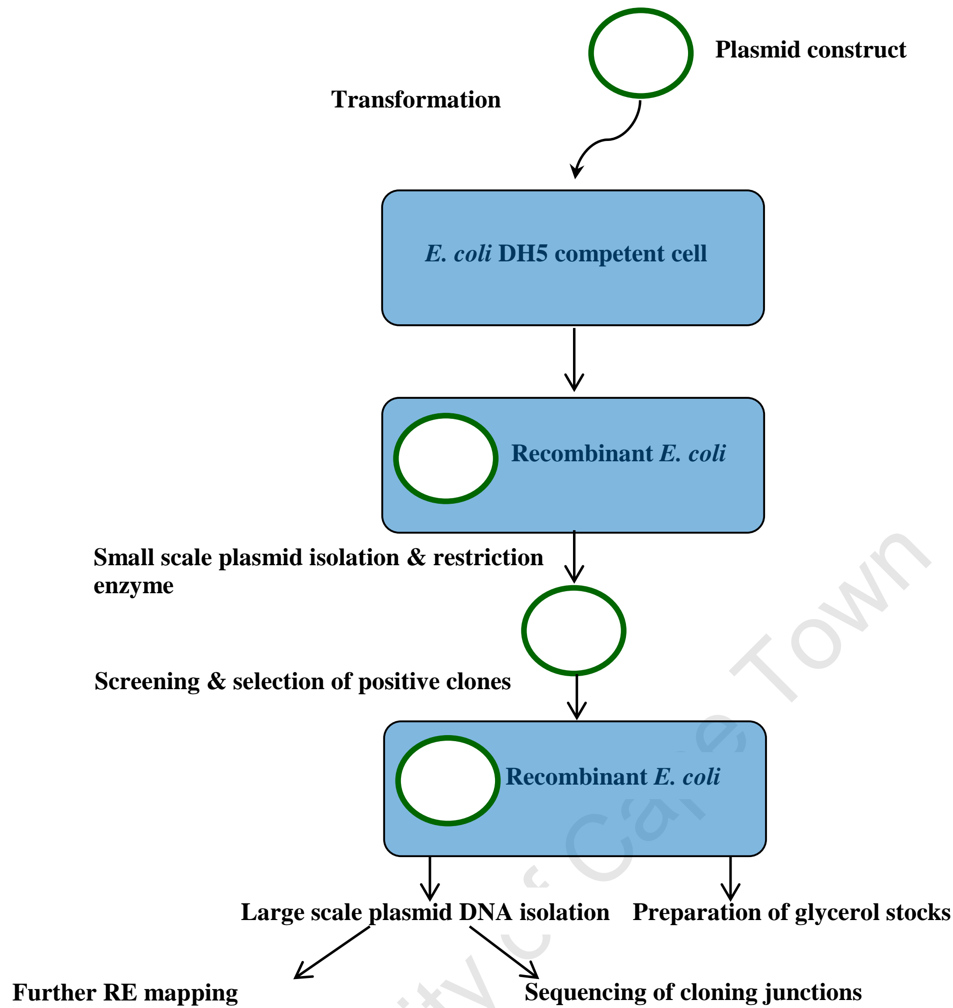

Figure 2.1: Schematic diagram of the screening and selection process for candidate recombinant clones

2.3 Construction of *E. coli*/mycobacterial shuttle vectors

The plasmids pEMαGag$_2$(V3SV5)gp33-41 and pEMGag$_2$(V3SV5)gp33-41 and pEM19Gag$_2$(V3SV5)gp33-41 were kindly supplied by Dr Helen Stutz (University of Cape Town, South Africa). These plasmids were derived from pMV261 (Stover et al. 1991) which contains a kanamycin resistance gene, an *E. coli* plasmid origin of replication, a low copy number mycobacterial plasmid origin of replication and a transcriptional terminator downstream of an expression cassette. The expression cassette contains: the *M. tuberculosis mtrA* promoter for induction of antigen expression in the host; no leader sequence or either the *M. tuberculosis* 19kDa or antigen 85B (α) signal sequences for export of antigen from the cell; an HIV-1 subtype C Gag (Du151) gene; the 10 amino acid V3 CTL epitope derived from the HIV-1 subtype B envelope protein; the paramyxo-virus derived SV5 monoclonal antibody tag and the gp33-41

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epitope from Lymphocytic Choriomeningitis Virus (LCMV). The HIV-1 *gag* gene, V3, SV5 and gp33-41 epitope tags were codon optimized for optimal expression in mycobacteria. The 1689bp DNA fragment, containing the HIV-1 *gag* and the epitope tags, shown below in Figure 2.2, was designed to contain unique restriction enzyme sites for ease of cloning and was synthesized by GeneArt [Germany]. The V3 CTL tag is a CD8 T cell epitope and was included to allow the comparison of the cellular immunogenicity of the different proteins (Burgers et al. 2006). The SV5 tag is a B cell epitope that has been shown to react with a monoclonal antibody when fused to the C terminus of various proteins (Hanke et al. 1992). These tags were used to allow the different proteins used in this study to be tracked and compared with one another. The LCMV gp33-41 tag was included for the comparison of T cell responses between different vaccine vectors used in another study.

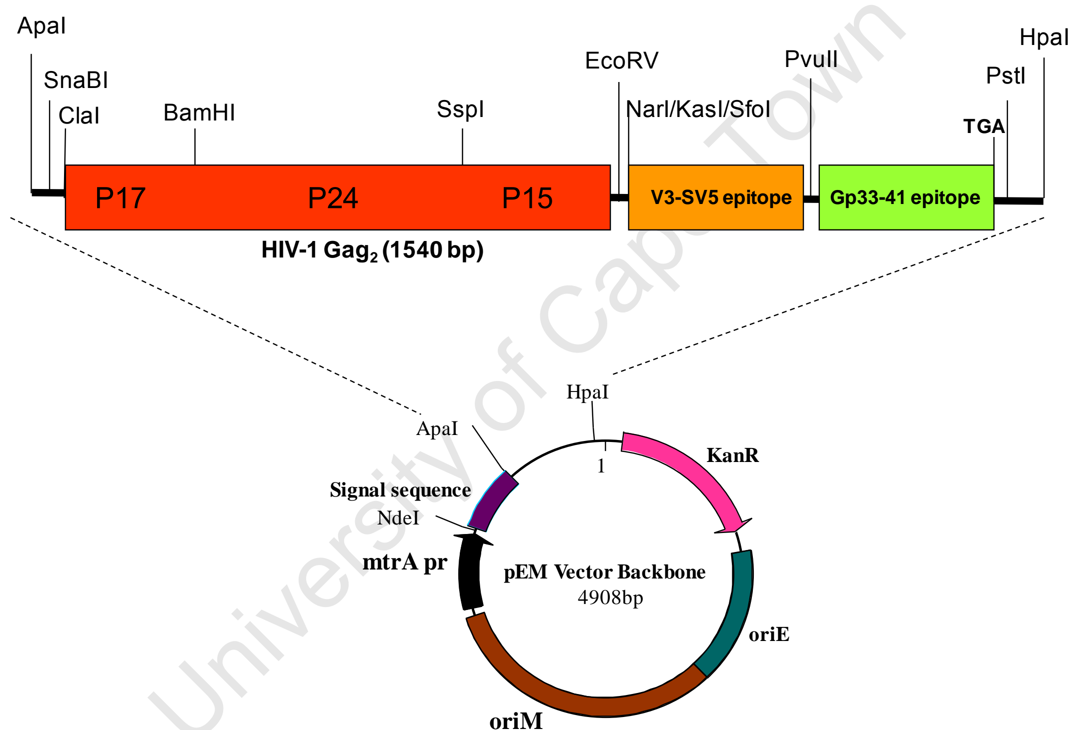

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Figure 2.2: The schematic diagram of the Gag expression cassette synthesized by GeneArt and the strategy for cloning into the pEM plasmid backbone to yield the three parental plasmids (pEMαGag_2(V3SV5)gp33-41, pEM19Gag_2(V3SV5)gp33-41 and pEMGag_2(V3SV5)gp33-41). This figure was supplied by Dr Helen Stutz.

Six plasmid shuttle vectors were constructed, from the three plasmids described above, to produce a combination of vectors with or without signal peptides and with or without the HIV-1 *gag*.

2.3.1 Construction of 1st phase plasmids

Plasmids; pEMαGag$_2$(V3SV5), pEM19Gag$_2$(V3SV5) and pEMGag$_2$(V3SV5) were constructed from plasmids: pEMαGag$_2$(V3SV5)gp33-41, pEM19Gag$_2$(V3SV5)gp33-41 and pEMGag$_2$(V3SV5)gp33-41 respectively by excising the gp33-41 epitope tag. This was done as the gp33-41 tag had been found to cause plasmid instability in recombinant BCG (personal communication with Dr Helen Stutz). In addition the tag was not required in this study.

Plasmids pEMαGag$_2$(V3SV5)gp33-41, pEM19Gag$_2$(V3SV5)gp33-41 and pEMGag$_2$(V3SV5)gp33-41 were digested with *Pst*I [Roche, SA]. Linear DNA fragments were isolated, following gel electrophoresis using the QIAGEN, SA Min-Elute Gel Extraction Kit [QIAGEN, SA] according to the manufacturer's instructions. The eluted DNA was digested with *Pvu*II [Roche, SA] and the reaction was cleaned up using the QIAGEN, SA Min-Elute Reaction Clean up Kit [QIAGEN, SA] according to the manufacturer's instructions. The *Pst*I 5' overhangs were then blunted using T4 DNA polymerase [Promega, SA] and the relative plasmid DNA loss was assessed by comparing the band intensities of the blunted and unblunted DNA fragments following electrophoresis using a slide gel. The remainder of the blunted DNA was ligated using T4 DNA ligase [Promega, SA]; thereafter half of the ligation mix was used to transform *E. coli* DH5α competent cells. Expression mixes were plated on 2YT agar containing kanamycin (25µg/ml) and potential positive recombinants screened for by restriction enzyme mapping of plasmid DNA that had been isolated by a small scaled DNA isolation process. Selected recombinants were picked off reference plates prepared during the small scale plasmid isolation, cultured in 100 ml Luria Bertani broth containing kanamycin (25µg/ml) overnight at 37^0C with shaking and plasmid DNA isolated using a Geno-pure Plasmid Midi Kit [Roche, SA Applied Science]. The plasmid DNA was quantified using a Nanodrop Spectrophotometer and subjected to further restriction endonuclease enzyme mapping. The *Pst*I/*Pvu*II cloning junctions were sequenced to confirm that the ligation reaction had generated in frame fusions using primers pFRF7 5'-CGCAAGAAGGGCTGCTGG-3' and pEM R, 5'- AGCAGACAGTTTTATTGTTC-3'. The sequencing reactions were done at Stellenbosch University Sequencing Facility [Western Cape, South Africa]. DNAMAN [Lynnon Biosoft, Canada] and Chromos v2.3 [Technelysium, Australia] software were used to analyze the sequences.

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2.3.2 Construction of 2nd phase plasmids

Plasmids; pEMα(V3SV5), pEM19(V3SV5) and pEM(V3SV5) were constructed from the plasmids generated in the previous section: pEMαGa_{g2}(V3SV5), pEM19Ga_{g2}(V3SV5) and pEMGa_{g2}(V3SV5) respectively. These plasmids were constructed by removing the HIV-1 *gag* gene.

The plasmids were digested sequentially with *SnaB*I [Roche, SA Diagnostics] and *EcoR*V [Roche, SA Diagnostics]. These restriction endonuclease sites span the *gag* (1.5kb) and both generate blunt ends. The reaction mix was electrophoresed to separate the DNA fragments. The larger ~ 5kb fragment was isolated, gel purified and plasmid loss assessed, as described previously. The linear plasmid was then ligated using T4 DNA ligase in a total volume of 12µl, thereafter 5µl of the ligated product was transformed into *E. coli* DH5α competent cells as previously described. Screening and selection of candidate recombinant clones was then performed as described in the previous section. The *SnaB*I/*EcoR*V cloning junctions were sequenced to confirm that the ligation reaction had generated in frame fusions using primers; pCONEPI F 5'- TGGCGAACTCCGTTGTAGTG-3' and pEM R, 5'-AGCAGACAGTTTTATTGTTC-3'. The sequencing reactions were done at Stellenbosch University Sequencing Facility [Western Cape, South Africa] and the sequences were analyzed as previously mentioned.

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2.4 Materials and methods for the growth and culturing of Mycobacteria

This section will detail the specific materials and methods employed in the application /use of mycobacteria in the study. The plasmids used in this chapter were constructed as outlined in Section 2.3.

2.4.1 Mycobacterial strains, growth media and culture conditions

M. smegmatis mc^2155 (obtained from the BioVac institute, Cape Town, S.A) was used in this study as a model organism since it is a fast growing mycobacterium. This strain was used to evaluate the stability of the six constructed shuttle vectors by colony size and growth patterns. *M. bovis* BCG Pasteur Δ*panCD* mc^26000 (BCG Pasteur ΔpanCD), a pantothenic acid auxotroph

obtained from Dr. William Jacobs Jr. (Albert Einstein College of Medicine, New York) was also used in this study. The attenuated auxotroph has been shown to possess a superior safety profile in immunocompromised mice in comparison to the current BCG vaccine and therefore was better suited for use as the rBCG vaccine (Tullius et al. 2008).

All mycobacterial strains were grown on selective Middlebrook 7H9 (MB7H9) [Difco^{TM}, USA] with 0.025% v/v tyloxapol (Tyl) [Sigma-Aldrich, SA] and 10% v/v oleic-acid albumin dextrose complex (OADC) [BioLab] (MB7H9-OADC-Tyl) or Middlebrook 7H10 (MB7H10) [Difco^{TM}, USA] with 10% v/v OADC (MB7H10-OADC). Since BCG Pasteur ΔpanCD strain carried a hygromycin marked deletion of the *panCD* genes required for pantothenate biosynthesis, the propagation of this strain required the additional supplementation with 24µg/ml pantothenate as well as 25µg/ml hygromycin for positive selection.

Mycobacterial starter cultures were generated by inoculation of a single colony from solid agar plates or an aliquot of a thawed (on ice) 10% glycerol stock stored at -80°C, into 5ml of MB7H9-OADC-Tyl. These starter cultures were incubated in 50ml conical sterile tubes [Sterilin, UK] standing at 37°C with loosened lids for approximately 2 days for *M. smegmatis* and 5 or more days for the BCG strains. The starter culture volumes were subsequently increased to 10ml with the same M7H9-OADC-Tyl media and further incubated at 37°C until an OD_{600} of approximately 0.5 was obtained. The 10ml starters were then used to inoculate 100ml of the same media in 1L Schott bottles. BCG cultures were first incubated standing for 48hours at 37°C with loosened lids before being placed on rollers until the required cell density was obtained, while *M. smegmatis* cultures were placed on rollers straight away.

For determination of growth patterns, cultures were propagated as outlined above and OD_{600} readings were recorded every 8 hours for *M. smegmatis* and every 24 hours for BCG Pasteur ΔpanCD on a spectrophotometer [Beckmann DU-40, USA] using cell density as an indicator of bacterial growth. For accuracy, cultures were diluted to read between OD_{600} 0.1 and 0.5 where necessary.

2.4.3 Preparation of electro-competent mycobacterial cells

The two mycobacterial strains used in this project were made competent by methods described by Parish, Stoker (1998). After starter cultures were established, inoculums were propagated in

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100ml of M7H9-TYL-OADC media in the absence of antibiotic selection, except for BCG Pasteur ΔpanCD, which required culturing in the presence of Hygromycin B, and pantothenic acid as previously described to an OD_{600} of 0.8-1 units. The cells were harvested as follows; the cultures were divided into 25ml aliquots, placed in centrifuge tubes and placed on ice for 2hours. These were then centrifuged at 10000rpm for 10 minutes at 4°C, then re-suspended in 12ml ice-cold sterile 10% glycerol and centrifuged again at 10000rpm for 10 minutes at 4°C. This step was repeated once more and the pellet was finally re-suspended in 1ml ice-cold sterile 10% glycerol, after which the tubes were pooled together and the electro-competent cells were aliquoted into 0.4ml aliquots in 1.5ml cryo-tubes and stored at -80°C.

2.4.4 Electro-transformation of shuttle plasmid vectors into mycobacteria

M. smegmatis and BCG Pasteur ΔpanCD competent cells were thawed on ice, centrifuged at 4000rpm for 1 minute and gently re-suspended in 0.6ml sterile 10% glycerol and then placed on ice. Constructed and confirmed plasmid DNA at a concentration of 0.5-1µg in a 5µl volume of HPLC water was added to 50µl of competent cells and gently well mixed to ensure a homogenous solution. This was transferred into a pre-chilled, 0.1mm electro-poration cuvette [Bio-Rad], tapped on paper towel to bring contents to the bottom of the cuvette, and placed on ice for 1minute. The cuvette was wiped dry with paper towel, placed in the Gene Pulser™ [Biorad, SA] and pulsed at a voltage of 1.8kV, capacitance of 25µF and resistance of 1000Ω. Time constants were noted and 1ml MB7H9-OADC-Tyl (supplemented with pantothenate for BCG Pasteur ΔpanCD samples) was added immediately. The expression mix was incubated at 37°C for 4hours for *M. smegmatis* or overnight for BCG Pasteur ΔpanCD. Expression mixes were plated onto MB7H10-OADC agar plates containing 10µg/ml kanamycin. In case of BCG Pasteur ΔpanCD, the medium was supplemented with 24µg/ml pantothenate and 25µg/ml hygromycin. The plates were inverted and incubated at 37°C for 3 to 4 days for *M. smegmatis* or 3 to 4 weeks for BCG Pasteur ΔpanCD, before the colonies could be visualised. Plasmid DNA (provided by Dr Helen Stutz) was used as a positive control and a no DNA sample served as a negative control for transformation.

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2.4.5. Total protein extraction

Recombinant mycobacterial colonies were cultured in liquid media as described in Section 2.4.1 to yield recombinant cultures from which cell free extract was obtained. Total protein or cell free extract was prepared for determination of basal level HIV-1 Gag expression from *M. smegmatis* cultures using the HIV-1 p24 capture ELISA. The bead beating method of cell free extract is described in the next section.

2.4.5.1 Bead beating method

This is the mechanical method of extraction of total protein from mycobacterial cultures. It employs the use of 0.1mm silicon/zirconia beads [BioSpec] and the FastPrep machine [Bio 101] for mechanical disruption of the cell wall thus releasing the cell free extract. A 30ml culture was harvested by centrifugation at 4000rpm for 10 minutes at 4°C and then re-suspended in 2ml of phosphate buffered saline (PBS, pH7.4). A 1ml aliquot was stored in 15% glycerol at -80°C for use in plasmid integrity checks, while the other aliquot was transferred to a FastRNA tube [Bio 101] containing 0.5g of 0.1mm silicon/zirconia beads. The samples were processed at setting 6 for 20 seconds, followed by incubation on an ice-water slurry for 2 minutes to prevent the samples from overheating. This was repeated three times. The samples were then centrifuged at 14000rpm, at room temperature for 10 minutes to pellet the cell wall debris. The supernatant (cell free extract) was recovered and placed in a new 1.5ml eppendorf^{TM} tube and further clarified by centrifugation. Three aliquots of approximately 240µl of the cell free extract were stored at -80°C. One of these aliquots was subjected to a standard DC-Biorad, SA protein quantification assay.

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2.4.6 Quantification of total protein from mycobacterial cultures

Total protein was quantified following the modified Lowry method using the DC-Biorad, SA Kit [Biorad, SA] according to the manufacturer’s instructions. Bovine Serum Albumin (BSA) [Roche, SA Diagnostics] was used as a standard.

2.5 Evaluation of vector fitness

One way of evaluating vector fitness is by growth rates on solid medium of recombinant cultures as this is indicative of the relative 'health' of the colonies. The more the recombinant antigen expressed, the greater the metabolic burden placed on the host cell, thus the slower the growth rates (the smaller the colony size) (Bourn et al. 2007, Griffin et al. 2009). However, this strategy may be relatively unreliable when used as a sole stability indicator as mycobacterial colonies tend to clump, thereby making it difficult to count and infer colony size (Parish, Stoker 1998). Therefore, growth rates on liquid medium were also used to evaluate vector fitness. Furthermore, care was taken to compare colony sizes between plates with similar cell counts since a high density of colony forming units may limit colony size. Due to its faster replication capability in relation to slow growing BCG strains, *M. smegmatis* was used as a model organism. Studies done in our laboratory have indicated that the higher the foreign antigen is expressed by the vector, the slower the growth rate of the mycobacterium harboring such a vector (Griffin et al. 2009). This is due to the inverse correlation between metabolic burden and growth rate as mentioned earlier. To test for this, we evaluated the growth rates of recombinant *M. smegmatis* cultures harboring various shuttle vectors.

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2.5.1 Indication of vector fitness by growth rates on solid medium

Plasmid DNA (0.5µg) was electroporated into the same batch of *M. smegmatis* competent cells as outlined in Section 2.4.4. The expression mix was incubated at 37°C for 4 hours, then plated (100µl, 200µl and remainder of mix) onto MB7H10-OADC agar plates containing 10µg/ml kanamycin and incubated at 37°C for 3 to 4 days. The resultant colonies were evaluated for growth rates by colony size. The resultant colonies from the same dilution of expression mix were compared in order to get an aproaximate evaluation of colony sizes. An arbitrary selected scale ranging from 1 to 5 asterisks indicating the relative sizes from smallest to largest was used to determine the sizes of the colonies. *M. smegmatis* transformants containing the control vector pCONEPI were assigned a score of 5 asterisks. The pCONEPI does not contain an expression cassette and therefore does not express Gag-V3SV5 antigen.

2.5.2 Indication of vector fitness by growth rates in liquid medium

Cultures (100ml) of recombinant mycobacteria at starting 0.005 OD units were grown on rollers at 37°C. Absorbance readings (OD_{600nm}) were taken at 8hr intervals until cultures reached stationary phase. Plasmid pCONEPI served as a plasmid control.

2.6 Evaluation of plasmid stability

One way of evaluating plasmid stability *in vitro* is by colony counts whereby cultures harbouring plasmid vectors are grown in the absence of antibiotic selection in liquid medium. These cultures are then plated onto both selective and non-selective medium to determine percentage loss of plasmid.

Following growth of recombinant mycobacterial cultures in liquid medium for evaluation of vector fitness, the cultures were stored in 15% glycerol in 1ml aliquots at -80°C for use in evaluation of plasmid stability (this was done at the end of the growth curve, after 48 hours, where cultures were grown in medium without kanamycin). The 15% glycerol stocks were thawed at room temperature and then serially diluted in MB7H9-OADC-Tyl. The serial dilutions (100µl each) of glycerol stocks were then plated on selective and non-selective MB7H10-OADC agar plates and incubated at 37°C for 3-5 days.

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2.7. Evaluation of basal level expression of HIV-1 Gag by p24 capture ELISA assay

HIV-1 Gag p24 assays were done to evaluate expression of HIV-1 Gag in recombinant *M. smegmatis* cultures. The cell free extract was collected as cultures reached OD_{600} readings ranging between 0.5 and 1. This was done by the bead beating method as previously described. One µg of the cell free extracts was sent to National Health Laboratory Service (NHLS) virology laboratories, Groote Schuur Hospital, Cape Town, South Africa for quantification of HIV-1 Gag p24 antigen. Gag p24 content in the extract was determined using the VIDAS HIV P24 II machine [bioMérieux, France], and the HIV Ag (Gag p24) kit [Roche, SA] with recombinant Gag p24 (donated by Robin Thomas) as a standard.

2.8. Preparation of rBCG vaccine stocks

The rBCG vaccine stocks were prepared in order to evaluate their stability and to test their immunogenicity in mice. The cultures were grown in selective MB7H9-OADC-Tyl media, supplemented with pantothenic acid and hygromycin, to an OD_{600} of between 0.5 and 1. A 20 ml sample was removed for protein isolation. The remaining cells were pelleted, resuspended in 20 ml resuspension buffer (10% glycerol (v/v), 0.025% v/v tyloxapol, 140 mM NaCl) and pelleted as before. The pellets were resuspended in 5 ml resuspension buffer and a sample was taken to determine the absorbance. Samples were then adjusted to a final OD_{600} of 10 using resuspension buffer, aliquoted and frozen at -80°C. Prior to vaccination aliquots were defrosted on ice, dose dilutions were made in resuspension buffer and the vaccines were passed through a 25 gauge needle 5 times. Vaccine doses were based on absorbance readings whereby one absorbance unit was taken to be equivalent to $1x10^7$cfu/100µl bacteria, comparable to the estimate of (Stover et al. 1993).

2.9 Evaluation of rBCG vaccine stability by restriction endonuclease mapping of isolated plasmid DNA

A modification of the method used in the High Pure plasmid isolation Kit [Roche, SA] was used to isolate plasmid from the recombinant BCG cultures. Exactly 200µl of rBCG vaccine stock stored at $1x10^9$cfu/ml was thawed at ambient temperature following which the cells were harvested by centrifugation at 14000rpm for 5 minutes. The pellet was re-suspended in GTE buffer (50mM glucose, 25mM Tris-HCl, pH 8.0, 10mM EDTA) and 20mg/ml lysozyme and incubated at 37°C with mixing for 3 hours. Plasmid was then isolated according to the High Pure Plasmid Isolation Kit's instructions. A 50µl of elution buffer was used to elute DNA. The sample was left at RT for 5 minutes and DNA was collected by a 14000rpm centrifugation for 60 seconds at room temperature. Five µl of eluted DNA was transformed into competent *E. coli* cells, while the remaining DNA was stored at -20°C.

The plasmids isolated from rBCG cultures vaccine stocks (20 colonies per rBCG vaccine stock) were transformed into *E. coli* as the quantity and quality of plasmid DNA isolated directly from mycobacteria is usually too low for restriction enzyme mapping. Recombinant plasmids isolated from *E. coli* were mapped with restriction enzymes *Xba*I/*KspA*I (*Hpa*I) to confirm their general structure.

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2.10 Vaccination of BALB/c mice with the rBCG vaccines and SAAVI-MVA-C

The rBCG vaccines expressing HIV-1 Gag, with various signal sequences were prepared as described in Section 2.8 and subsequently tested for their immunogenicity in BALB/c mice. The vaccines, vaccination schedules and protocols are described in Tables 2.1 and 2.2 as well as Section 2.10 below. Female BALB/c mice were purchased from South Africa Vaccine Producers Pty Ltd (Johannesburg, South Africa). These were housed in groups of 5 in the Animal Unit (University of Cape Town, South Africa) for a minimum of 10 days before vaccinations to allow them to adapt to their environment. All the animal procedures were approved by the University of Cape Town Animal Ethics Committee (AEC), approval number AEC 07-017a.

The mice were primed with the rBCG vaccine ($1x10^7$ cfu/200µl resuspension medium) by intra-peritoneal vaccination on day 0 and boosted with $1x10^4$ pfu/100µl of SAAVI MVA at day 28 by intra-muscular injection of half the dose (50ul) into each hind leg muscle. On day 40, the mice were killed by cervical dislocation without anaesthesia and mouse spleens were harvested for isolation of a single cell suspension of splenocytes prepared as described in Section 2.10.1. The vaccines used and the outline of the vaccination schedule are shown in Tables 2.1 and 2.2 respectively. SAAVI MVA-C is the South African AIDS Vaccine Initiative Modified Vaccinia Akara that containes a fusion gene comprising of portions of the HIV-1 subtype C *gag, reverse transriptase, tat* and *nef* genes (*grttnC*) and the truncated (by removal of 124 amino acids from the cytoplasmic tail of gp160) HIV-1 subtype C *Env* (*gp150*) and the V3 loop CTL epitope from the HIV-1 subtype B envelope protein (Burgers et al. 2006).

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Table 2.1: Description of the vaccines used to vaccinate mice

Vaccine code	vaccine	Description of plasmids
BCG1	**BCG Pasteur ΔpanCD [pEMαGag$_2$V3SV5]**	shuttle vector; *mtrA* promoter, α-signal sequence, codon-optimised *gag* and V3SV5 epitope tag in a stable vector backbone
BCG2	**BCG Pasteur ΔpanCD [pEM19Gag$_2$V3SV5]**	shuttle vector; *mtrA* promoter, 19kDa-signal sequence, codon-optimised *gag* and V3SV5 epitope tag in a stable vector backbone
BCG3	**BCG Pasteur ΔpanCD [pEMGag$_2$V3SV5]**	shuttle vector; *mtrA* promoter, no signal sequence, codon-optimised *gag* and V3SV5 epitope tag in a stable vector backbone
BCG4	**BCG Pasteur ΔpanCD [pEM19Gag$_2$V3SV5] HS**	Same as pEM19Gag$_2$V3SV5, used as a control vaccine (provided by Dr Helen Stutz)
BCG 5	**BCG Pasteur ΔpanCD [pCONEPI]**	empty shuttle vector backbone (control without gag and V3SV5 tag)
MVA	**SAAVI-MVA**	Recombinant vaccinia virus expressing the HIV-1 *grttnC* genes as well as *gp150CT* containing the BALB/c CD8+T-cell epitope at the 3` end.

Table 2.2: The vaccination schedule

Mouse group (# mice per group)	Day 0 Prime rBCG 10^7 cfu/200µl (i.p.)	Day 28 Boost SAAVI MVA-C 10^4 pfu/100µl (i.m. 50µl each tibialis muscle)	Day 40 End of experiment
1 (5)	BCG 1	MVA	Kill, remove spleens
2 (5)	BCG 2	MVA	Kill, remove spleens
3 (5)	BCG 3	MVA	Kill, remove spleens
4 (5)	BCG 4	MVA	Kill, remove spleens
5 (3)	BCG 5	MVA	Kill, remove spleens
6 (5)	-	MVA	Kill, remove spleens

i.p: interperitoneal vaccination. **i.m:** intramascular vaccination.

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2.10.1 Preparation of Mouse splenocytes

Mouse spleens were placed in 10ml RPMI 1640 [Gibco/Invitrogen, SA].The spleens were then poured into a sterile cell strainer, placed in a petri dish and meshed with a 2ml syringe rubber plunger. The cell strainer was washed with 10ml RPMI and the cell suspension was transferred to a 50ml tube. This splenocyte cell suspension was topped up to 50ml with RPMI and then centrifuged at 1500rpm for 5 minutes. The supernatant was discarded and the pellet was re-suspended in 50ml RPMI. The suspension was washed twice with 30ml of RPMI (before centrifuging the cells at the last wash, any fibrin clots were removed using a Pasteur pipette). The supernatant was removed and the pellet re-suspended in a final volume of 50ml RPMI. The red blood cells (RBC) were lysed with 1ml of the RBC lysis buffer, the suspension was mixed gently, centrifuged as before and supernant removed. The RBC free splenocytes were then re-suspended in 50ml RPMI. The cells were diluted 1:10 in Trypan Blue and Turks and counted in a 0.002mm^2 Neubauer counting chamber [Superior, Germany]. The required number of cells for ELISPOT and FACS were removed and the procedures carried out as described below.

2.10.2 Fluorescence-activated cell sorting (FACS) analysis to determine cell population in mouse spleens

FACS was done using antibodies to cell surface markers on the isolated splenocytes to determine the composition of T cells and B cells in the splenocyte population. Cell surface receptors were stained with a fluorescent antibody mix (CD3-APC, CD4-FITC, CD8-PerCP and CD19-PE). Blocking solution (25µl) (FACS Buffer, normal rat serum, normal mouse serum and CD16/CD32) was added into a tube containing $1x10^6$ splenocytes and mixed. The tubes were incubated on ice for 20 minutes. The cells were then washed with 1ml FACS Buffer (PBS containing 1% FCS and 0.1% NaN_3) and centrifuged at 1500 rpm for 5 minutes. The supernatant was discarded and the pellet re-suspended in the residual (small amount of buffer left after centrifugation) FACS buffer. The fluorescent antibody mix (50µl) was added into the tubes and placed on ice for 30 minutes in the dark. The cells were washed once in 2ml of FACS buffer on ice and re-suspended in the residual volume of FACS Buffer. About 400µl of FACS buffer was added into tubes and acquired on FACS Caliber Multiple Auto Sampler flow cytometer [BD BioSciences, USA].

2.10.3 Assessment of CD8 and CD4 T-cell responses in spleens

IFN-γ and IL-2 enzyme-linked immunospot (ELISPOT) assays were used to determine the frequencies of antigen-specific T-cells in the spleen (Section 2.10.4). The following peptides/antigens (Table 2.3) were used as stimulants during the IFN-γ and IL-2 ELISPOT assays.

Table 2.3: The stimulating peptides/antigens for the IFN-γ and IL-2 ELISPOT assays

Stimulatory peptide	Amino acid sequence	Description
No peptide	-	(Media only; background control)
Irrelevant peptide	TYSTVALSSL	Irrelevant amino acid sequence
AMQM (Gag CD8)	AMQMLKDTI	HIV-1 p24 Gag (65-73)
MRC13 (Gag CD4)	NPPIPVGDIYKRWIILGLNK	HIV-1 p24 Gag (121-140)
MRC17 (Gag CD4)	FRDYVDRFFKTLRAEQATQE	HIV-1 p24 Gag (161-180)
ConA	-	(Positive control [Sigma, MO, USA])

2.10.4 Mouse IFN-γ and IL-2 ELISPOT assays

ELISPOT assays to quantify Gag-specific IFN-γ and IL-2-producing mouse splenocytes was performed using an ELISPOT Set/Kit [BD Biosciences, USA] according to the manufacturer's

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instructions. The 96 well plates [BD Biosciences, USA] were pre-wet with 35% ethanol (15µl/well) for 60 seconds, before coating with capture antibody; these were then washed three times with 200µl of PBS. To coat the plates, 100µl of the diluted purified anti-mouse IFN-γ or IL-2 antibody solution was added into each well of the ELISPOT plates and these were covered with parafilm and incubated at 4°C overnight. The coating antibody was removed and the wells were washed once with 200 µl/well of blocking solution (R10 medium containing RPMI 1640 with Glutamax, 10% Penicillin-Streptomycin, 10% FBS, 0.001% 2-ME). Blocking solution (200 µl/well) was added into each well and plates were incubated for 2 hours at ambient temperature.

While blocking, red blood cells (RBC) were lysed in the splenocyte suspension prior to use. Per spleen cell pellet, 1ml of RBC lysing buffer (0.15M NH_4Cl. 10mM $KHCO_3$, 0.1mM EDTA and distilled water [Sigma-Aldrich, SA]) was added at room temperature. The tube was gently shaken for 1 minute. This was followed by addition of 45ml RPMI 1640 and centrifugation for 7 minutes at 5 000rpm. The medium was decanted and pellet re-suspended in fresh RPMI 1640 with 10%FCS. The cells were counted and adjusted to a cell concentration of 5 x 10^6 splenocytes /ml R10. Blocking solution was discarded from the wells of the ELISPOT plate. The following (100µl per well) were then added to triplicate wells: medium to determine background responses; irrelevant peptide, peptide stimulants (V3CTL, AMQM, MRC13, MRC17) (Table 2.3); BCG lysate (1.871µg protein/ml BCG lysate) to determine responses to BCG and ConA (0.1µg/ml ConA) as a positive control stimulant. Splenocytes were then added to each well (100µl containing 0.5x10^6 splenocytes). The plates were covered with aluminum foil and incubated at 37°C, 5% CO_2, humidified incubator for 23-24 hours. The well contents were decanted and wells were washed twice with 200µl/well of deionized H_2O (wells were allowed to soak for 3-5 minutes at each wash step). Wells were washed three times with wash buffer I (PBS, 0.05% Tween 20) (the washes were programmed on the EL_{x50} Auto Strip Plate Washer [Analytic and Diagnostic Products, SA]). Excess wash buffer from the wells was removed by blotting the plate on a piece of paper towel.

For detection purposes, Biotinylated anti IFN-γ or anti IL-2 detection antibody [BioCom Biotechnology, SA] was prepared in dilution buffer (PBS, 10% FBS) according to the manufacturers' instructions and 100 µl was added into each well. The plates were incubated for 2 hours at room temperature. Detection antibody solution was discarded from plates, and wells were washed three times with wash buffer I. Avidin-horseradish peroxidase (Avidin-HRP) [BioCom Biotechnology, SA] was diluted in dilution buffer as indicated by manufacturers and

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100 µl of this was added into each well. The plates were covered and incubated for 1 hour at room temperature. The avidin-HRP solution in each well was decanted and plates were washed three times with wash buffer I as before. Wells were then washed three times with wash buffer II. Vector Nova Red Substrate sk-4800 solution [Southern Cross Biotechnology, USA] (100 µl/well) was added. Spot development was monitored for 5 to 10 minutes in the dark; making sure not to allow spots to overdevelop, to prevent high background responses. The substrate reaction was stopped by washing wells with H_2O (5 to 6 washes under a running cold water tap). The plastic tray under the plate was removed to facilitate air-drying at room temperature overnight. Plates were stored in a dry place in the dark until analysis. The plates were scanned using the CTL Immunospot ELISPOT Reader Analyzer [Cellular Technology Ltd, Europe]. The spot forming units were enumerated using the ImmunoSpot Software, version 3.2. The mean number of spots in triplicate wells was calculated and background spots were subtracted. The values were expressed as net antigen-specific IFN-γ spot forming units (net sfu) per million splenocytes.

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2.10.5 Validation of vaccination dose and rBCG recovery from mouse spleens

The appropriate dilutions of rBCG vaccine stocks were plated on MB7H10-OADC-VitB$_5$-Hygr with and without kanamycin to confirm that the correct doses had been used and that the vaccines were stable. Also following the mice sacrifice, the rBCG cells were recovered from the splenocytes. The splenocytes in 35ml RPMI 1640 left over from the ELISPOT assay were centrifuged at 1 200rpm for 10 minutes and re-suspended in 1ml of re-suspension buffer. These were then plated onto MB7H10-OADC-VitB$_5$-Hygr plates with and without kanamycin (0.5ml of each group onto each 90mm petri dish). The petri dishes were then inverted, incubated at 37°C for 4 weeks. At the end of this period, the rBCG colonies were counted.

CHAPTER 3: RESULTS

3.1 Summary of results

E. coli-mycobacterium shuttle vectors have been widely used for the delivery of foreign genes into BCG. Critical issues to be considered in developing such vectors include the choice of promoter; codon optimization of the foreign gene for expression in mycobacteria and the use of leader sequences to facilitate localization of the foreign antigen.

The following steps achieved the aim of this study, which was to compare rBCG expressing HIV-1 *gag* under the control of different promoters and leader sequences. Firstly, the optimal vector backbone was selected based on colony sizes. Secondly, a series of shuttle vectors with differing features, were constructed in order to assess the effect of these features on vector "fitness" *in vitro*. These features include promoters (*hsp60, psmcy and mtrA*), signal peptides (α-antigen and 19kDa signal peptides), codon optimization of HIV *gag* and its subsequent addition into the vector. The last section of the study focused on the preparation of BCG Pasteur ΔpanCD vaccines with the best shuttle vectors based on vector stability. These vectors contained various leader peptides and their immunogencity was then assessed following vaccination of BALB/c mice.

In this study, it was shown that the addition of various vector features such as leader sequences and codon optimized HIV-1 *gag* did not have an effect on vector stability. Furthermore, the use of leader peptides resulted in differential expression levels of Gag as shown by p24 assays and lastly the use of such peptides may also have influenced the immunogenicity of Gag. The flow diagram of the summary of what was done in the study as well as selection criteria for vectors to be used in the immunogenicity studies is shown in Figure 3.1 and Table 3.1, which also indicates which vectors were constructed and/or used in this study.

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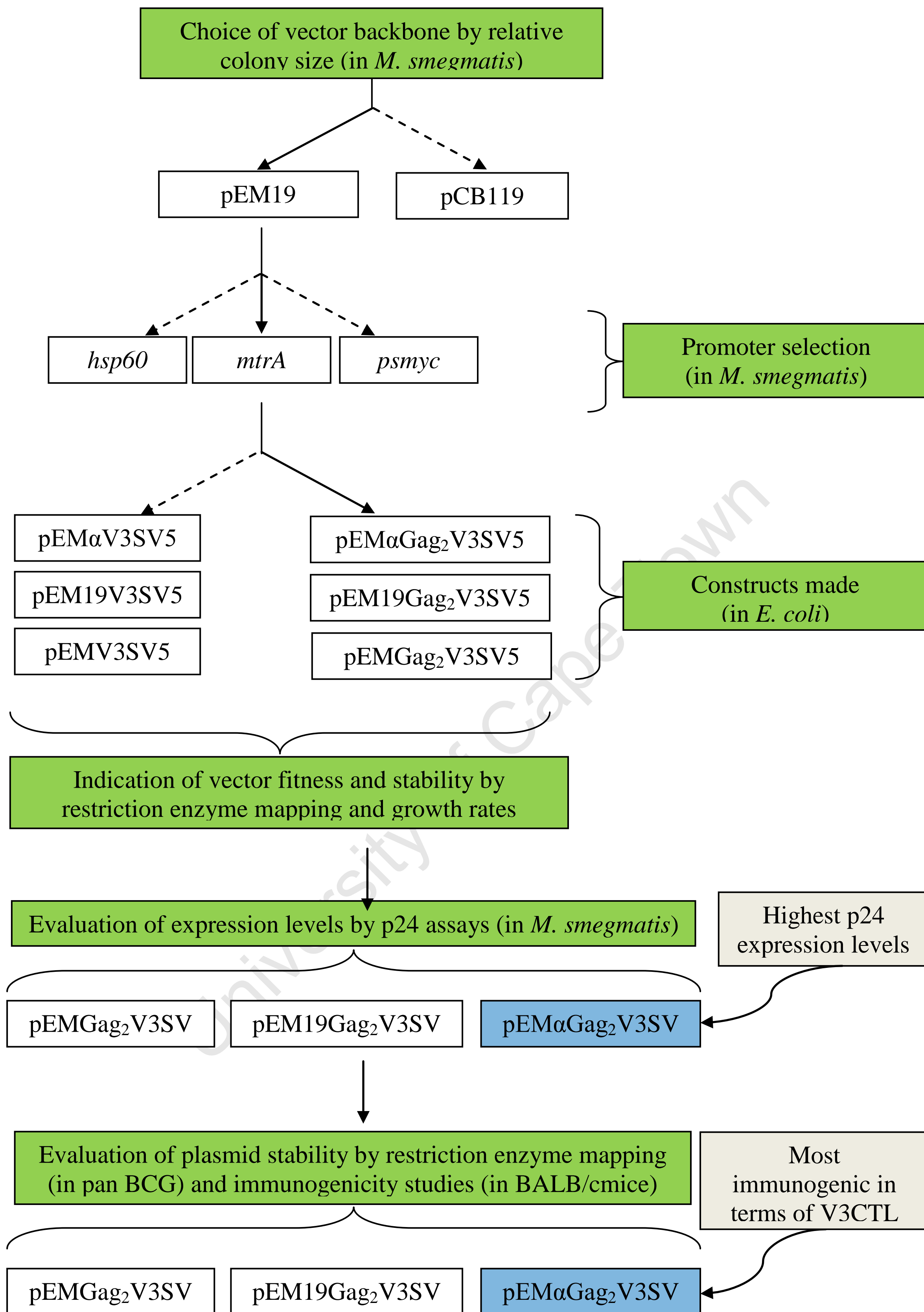

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Figure 3.1: The flow diagram of the summary of what was done in the study as well as selection criteria for vectors to be used in the immunogenicity studies

Table 3.1: Summary of the vectors constructed in the study and experiments carried out

Name of construct	Features			Made in this study	Plasmid checked by RE mapping	Sequencing of cloning junctions	Growth rates on media		backbone fitness
	promoter	Signal sequence	V3SV5				Solid	Liquid	
pCB119Gag	*hsp60*	19kDa	None	X	X	X	√	√	N
pEM19Gag	*mtrA*	19kDa	None	X	X	X	√	√	Y
pCB119Gag$_1$	*hsp60*	19kDa	None	X	X	X	X	√	X
pEM19Gag$_1$	*mtrA*	19kDa	None	X	X	X	√	√	X
pESDGag$_2$V3SV5	psmyc	None	√	X	X	X	√	X	X
pEHDGag$_2$V3SV5	*hsp60*	None	√	X	X	X	√	X	X
pEMαGag$_2$V3SV5	*mtrA*	α	√	√	√	√	√	X	X
pEM19Gag$_2$V3SV5	*mtrA*	19kDa	√	√	√	√	√	√	X
pEMGag$_2$V3SV5	*mtrA*	None	√	√	√	√	√	√	X
pEMαV3SV5	*mtrA*	α	√	√	√	√	X	X	X
pEM19V3SV5	*mtrA*	19kDa	√	√	√	√	√	X	X
pEMV3SV5	*mtrA*	None	√	√	√	√	√	X	X
pF10	*hsp60*	None	None	X	X	X	X	X	X
pCONEPI	None	None	None	X	X	X	√	√	Y

The constructs used in this study were with or without HIV-1 *gag*, where **Gag**: non-codon optimised, **Gag$_1$**: first codon optimisation, **Gag$_2$**: second codon optimisation. √: indicates what was done or feature present, while **X**: indicates what was not done and **none**: shows feature that was not present in vector. **N** or **Y**: show lack of or vector fitness

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3.2 Development of stable vaccine shuttle vectors

Instability of episomal vectors expressing foreign antigens in rBCG is often reported (Joseph et al. 2010). This may be due to the overexpression of foreign proteins, which are in some way toxic to mycobacteria. In BCG, foreign antigen can be expressed within the cell, secreted or linked to the mycobacterial cell membrane. Expression of high levels of foreign antigen in the cytoplasm of BCG could also result in instability of rBCG, thus the use of leader sequences targeting the antigen to the mycobacterial membrane or out of the cell maybe beneficial. The location of the foreign antigen may also determine the pathway by which the antigen is presented to the immune system. In this study a more stable shuttle vector was selected and the effect of the use of different leader peptides assessed in terms of vector fitness, antigen expression and immunogenicity.

3.2.1 Selection of optimal shuttle vector

Choice of stable vector backbones may also enhance expression of foreign antigens. In order to address this, the relative vector fitness of two *E. coli*/mycobacterial shuttle vectors pCB119Gag and pEM19Gag were compared in order to select the best vector in terms of its fitness as indicated by colony sizes. Both plasmids contain the *M. tuberculosis mtrA* promoter, the 19 kDa

leader sequence fused to the *gag* gene, *E. coli* and mycobacterial origins of replication and the *aph* gene which confers kanamcyin resistance. In addition pCB119Gag contains the *lys*A gene, which encodes meso-diaminopimelate decarboxylase, an enzyme catalyzing the last step in lysine biosynthesis, expressed from the *hsp60* promoter. In our laboratory, it was discovered that the *hsp60* expressed *lysA* gene, encoded on the original vector backbone (pCB119) and designed for selection in lysine auxotrophic strains, imposed additional metabolic stress resulting in slower growth rates and hence leading to vaccine instability. Therefore, to improve plasmid stability, the *hsp60* promoter and the *lysA* gene were removed from the vector to generate plasmid pEM19Gag.

The relative fitness of the two vectors was assessed by comparing the colony size of *M. smegmatis* transformed with plasmids pCB119Gag (8146bp) and pEM19Gag (6412bp). A scale was selected by means of asterisks (*), with pCONEPI transformants being assigned five asterisks (*****), as the largest colony size. The pCONEPI was used as an empty vector control in this study. It contains the *E. coli* and mycobacterial origins of replication and the *aph* gene which confers kanamcyin resistance but does not contain the *mtrA* promoter, 19 kDa leader sequences or the *hsp60* promoter – *lysA* gene cassette.

The recombinant *M. smegmatis* colonies harbouring pCB119Gag were very small as compared to *M. smegmatis* [pEM19Gag] colonies which were slightly larger and were assigned a score of two asterisks (Figure 3.2 and Table 3.2). These results indicated that the pCB119 vector backbone contributed to impaired recombinant mycobacterial fitness as compared to pEM19. The pEM19 vector backbone was therefore chosen as the better vector for all further studies. A more detailed vector fitness and stability assessment is shown in Section 3.2.3

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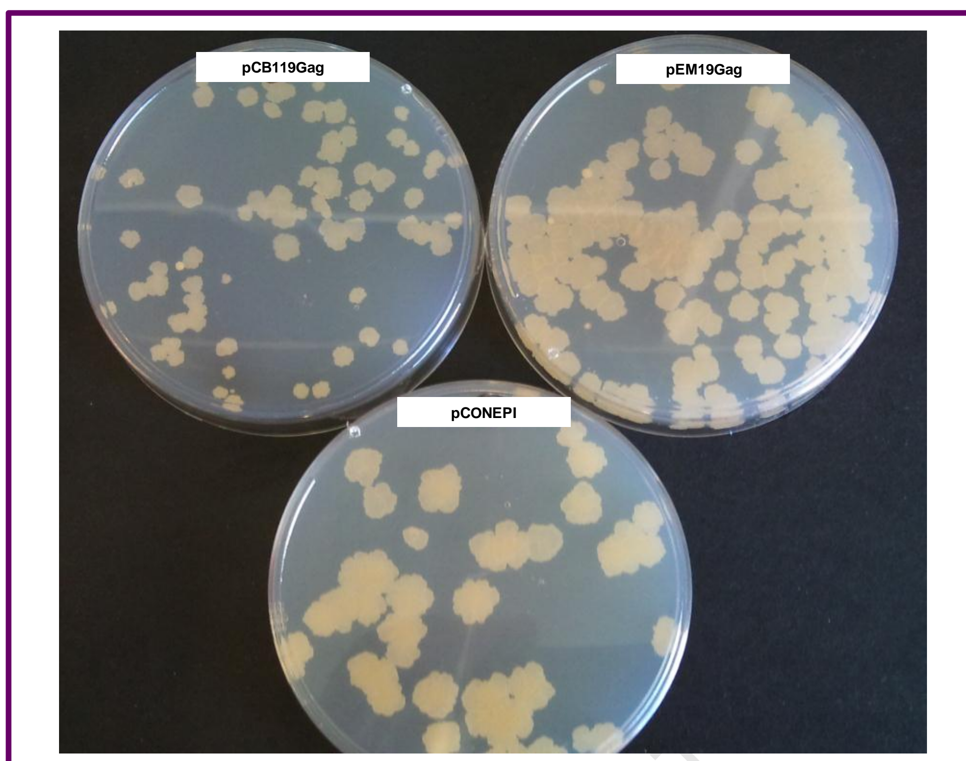

Figure 3.2: Determination of plasmid stability by recombinant *M. smegmatis* colony sizes. Comparison of *M. smegmatis* colonies harbouring the following plasmids; pEM19Gag; pCB119Gag and the positive control pCONEPI.

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Table 3.2: Determination of plasmid stability by comparison of recombinant *M. smegmatis* colony sizes. Relative colony size (RCS) was described using an arbitrary scoring system by means of asterisks ranging from * to *****, from smallest to largest.

Plasmid	RCS
pEM19Gag	***
pCB119Gag	**
pCONEPI	*****

3.2.2 Construction of plasmid shuttle vectors for rBCG-HIV vaccines

Having selected the most "fit" episomal vector backbone; pEM19 in the previous section, we designed and constructed six vectors with the *mtrA* promoter, either the α signal sequence, the 19kDa signal sequences or no signal sequence, with or without the HIV-1 *gag* and the V3SV5 tag (pEMαGag$_2$(V3SV5), pEM19Gag$_2$(V3SV5), pEMGag$_2$(V3SV5), pEMα(V3SV5), pEM19(V3SV5) and pEM(V3SV5)). Table 3.11 shows the features of these plasmid shuttle vectors. The *in vitro* stability of these vectors was evaluated by means of growth rates in the fast growing model organism *M. smegmatis*. Three of these were then selected in order to determine

the influence of the signal peptides on the immunogenicity of HIV-1 Gag antigen following vaccination of BALB/c mice with such rBCG vaccines.

3.2.2.1 Construction of pEMαGag$_2$(V3SV5), pEM19Gag$_2$(V3SV5) and pEM Gag$_2$(V3SV5)

In order to evaluate the effect of α and 19kDa signal peptides on vector fitness as well as their immunogenicity, we constructed three plasmids; pEMαGag$_2$(V3SV5), pEM19Gag$_2$(V3SV5) and pEMGag$_2$(V3SV5) from the following parental plasmids; pEMαGag$_2$(V3SV5)gp33-41, pEM19Gag$_2$(V3SV5)gp33-41 and pEMGag$_2$(V3SV5)gp33-41 respectively. The resultant constructs were similar to the parentals, but lacked the gp33-41 epitope, which was removed by a sequential restriction endonuclease digestion with *Pvu*II and *Pst*I restriction enzymes as mentioned in Section 2.3.1. Figure 3.3 shows schematic diagram of the cloning strategy employed in the construction of these vectors.

Restriction endonuclease mapping with *Ssp*I and *Pst*I confirmed that the plasmids were of correct sizes. *Ssp*I sites are located in the HIV-1 *gag* gene and also on kanamycin resistance gene 3` downstream of the gp33-41, while *Pst*I was lost during construction of the plasmids. Therefore no digestion of pEMαGag$_2$(V3SV5), pEM19Gag$_2$(V3SV5) and pEMGag$_2$(V3SV5) constructs with *Pst*I endonuclease was expected. As seen in lanes 2 of Figures 3.4 to 3.6, the pEMαGag$_2$(V3SV5), pEM19Gag$_2$(V3SV5) and pEMGag$_2$(V3SV5) plasmid DNA digested with *Pst*I had the same banding pattern as undigested controls in lanes 3, indicating that the *Pst*I site had been lost as expected. In contrast, the original plasmids still contained the *Pst*I restriction site (lanes 4). As expected, a small difference in the size of the smaller DNA fragment (68bp) could be seen following digestion with restriction enzyme *Ssp*I (Table 3.3, Figures 3.4, 3.5 and 3.6, lanes 6 and 7). This showed that the constructed vectors were indeed of correct sizes and had lost the gp33-41 epitope as expected.

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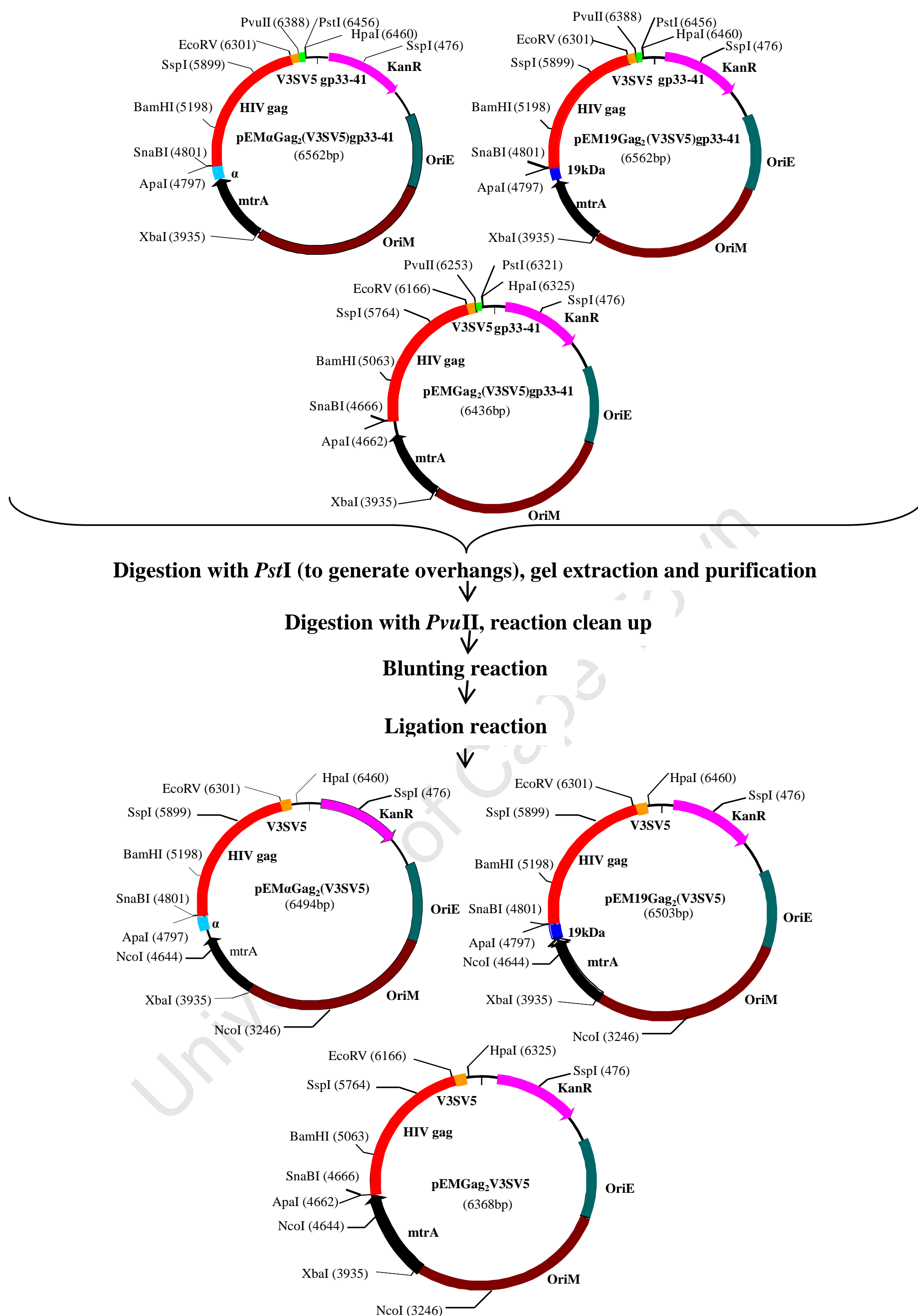

Figure 3.3: Schematic diagram of the cloning strategy employed in order to remove the gp33-41 tag from the three recombinant plasmid shuttle vectors containing the *gag* gene: pEMαGag$_2$(V3SV5)gp33-41, pEM19Gag$_2$(V3SV5)gp33-41 and pEMGag$_2$(V3SV5)gp33-41. The gp33-41 tag is shown in green, following the V3SV5 tag (yellow).

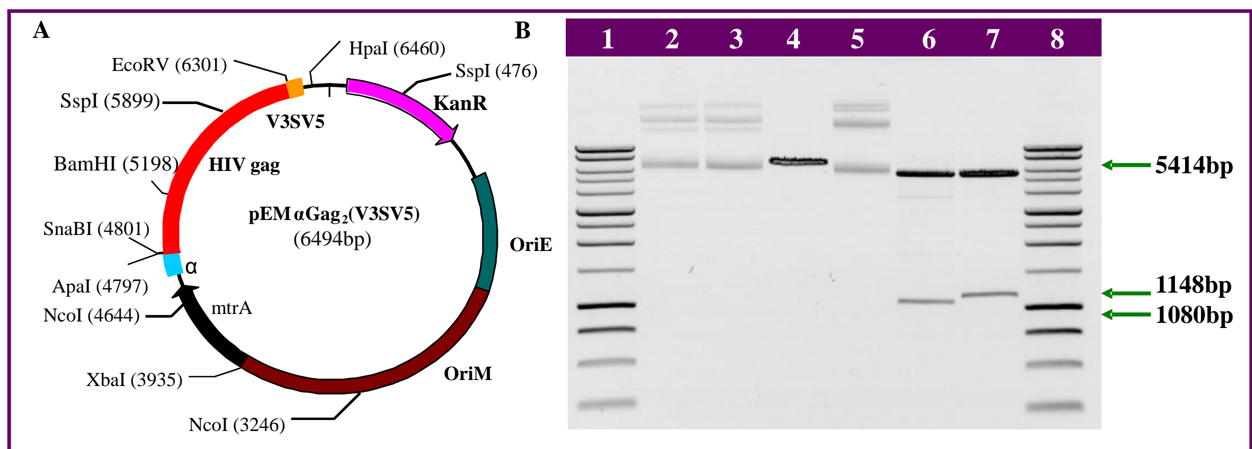

Figure 3.4: Restriction enzyme mapping of plasmid pEMαGag$_2$(V3SV5) by digestion with *Pst*I and *Ssp*I. Lanes 1 and 8: 1kb DNA molecular weight marker; lane 2: pEMαGag$_2$(V3SV5) digested with *Pst*I; lane 3: undigested pEMαGag$_2$(V3SV5) plasmid; lane 4: pEMαGag$_2$(V3SV5)gp33-41 plasmid control digested with *Pst*I; lane 5: undigested pEMαGag$_2$(V3SV5)gp33-41; lane 6: pEMαGag$_2$(V3SV5) digested with *Ssp*I; lane 7: pEMαGag$_2$(V3SV5)gp33-41 digested with *Ssp*I.

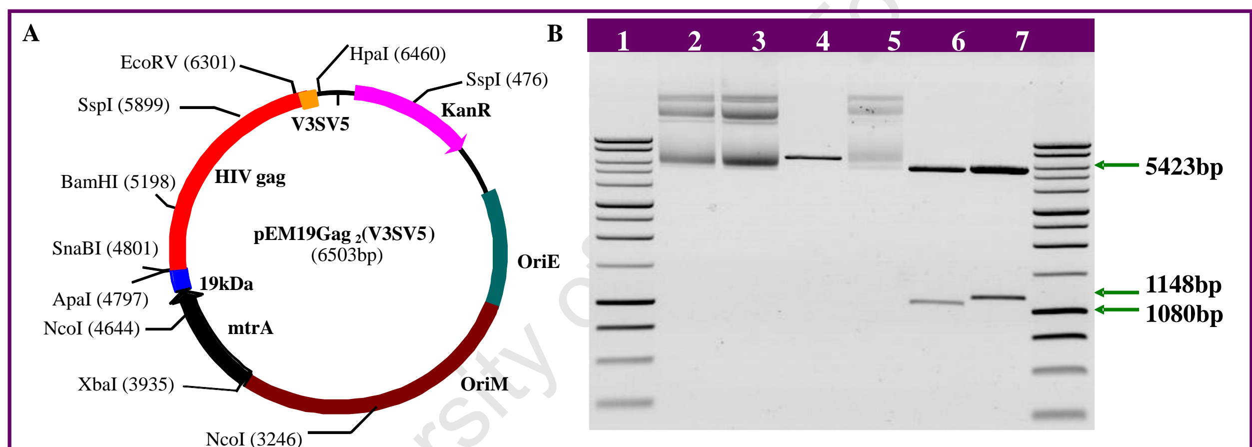

Figure 3.5: Restriction enzyme mapping of pEM19Gag$_2$(V3SV5)by digestion with *Pst*I and *Ssp*I. Lanes 1 and 8: 1kb DNA molecular weight marker; lane 2: pEM19Gag$_2$(V3SV5) digested with *Pst*I; lane 3: undigested pEM19Gag$_2$(V3SV5) plasmid; lane 4: pEM19Gag$_2$(V3SV5)gp33-41 plasmid control digested with *Pst*I; lane 5: undigested pEM19Gag$_2$(V3SV5)gp33-41; lane 6: pEM19Gag$_2$(V3SV5) digested with *Ssp*I; lane 7: pEM19Gag$_2$(V3SV5)gp33-41 digested with *Ssp*I.

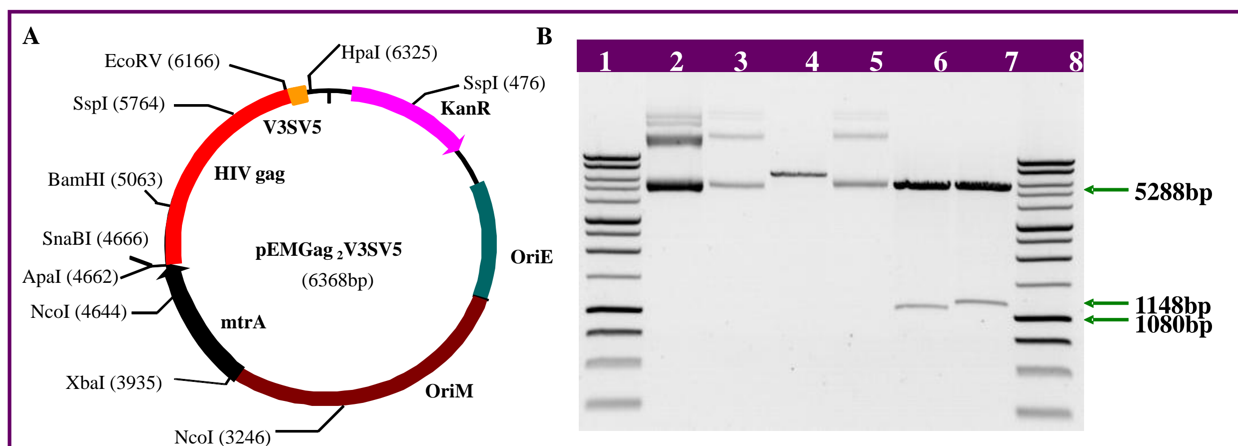

Figure 3.6: Restriction enzyme mapping of plasmid pEMGag$_2$(V3SV5) by digestion with *Pst*I and *Ssp*I. Lanes 1 and 8: 1kb DNA molecular weight marker; lane 2: pEMGag$_2$(V3SV5) digested with *Pst*I; lane 3: undigested pEMGag$_2$(V3SV5) plasmid; lane 4: pEMGag$_2$(V3SV5)gp33-41 plasmid control digested with *Pst*I; lane 5: undigested pEMGag$_2$(V3SV5)gp33-41; lane 6: pEMGag$_2$(V3SV5) digested with *Ssp*I; lane 7: pEMGag$_2$(V3SV5)gp33-41 digested with *Ssp*I.

Table 3.3: Expected fragment sizes (bp) resulting from restriction endonuclease digestion of the plasmid DNA

Plasmid Name	Expected fragment size	
	***Ssp*I**	***Pst*I**
pEMαGag$_2$(V3SV5)gp33-41	1148, 5414	6562
pEM19Gag$_2$(V3SV5) gp33-41	1148, 5423	6571
pEMGag$_2$(V3SV5) gp33-41	1148, 5288	6436
pEM αGag$_2$(V3SV5)	1080, 5414	Uncut
pEM19Gag$_2$(V3SV5)	1080, 5423	Uncut
pEMGag$_2$(V3SV5)	1080, 5288	Uncut

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The plasmids were sequenced using the pCONEPI forward 5’-TGGCGAACTCCGTTGTAGTG-3’ and pEM reverse 5’- AGCAGACAGTTTTATTGTTC-3’ primers to confirm that the V3SV5 epitope-tag was in frame with the stop codon. The *Pvu*II and *Pst*I junctions were in frame with the stop codon in all the constructs. Having established that the constructs were of correct sizes by restriction endonuclease mapping and confirmed that the cloning junctions were in frame; these plasmids were subsequently used for generation of the last three constructs that lacked the HIV-1 *gag* gene.

3.2.2.2 Construction of pEMα(V3SV5), pEM19(V3SV5) and pEM(V3SV5)

The last set of three plasmids pEMα(V3SV5), pEM19(V3SV5) and pEM(V3SV5) were constructed such that they lacked the HIV-1 *gag* gene, but still maintained the other features similar to the first three constructs. These constructs were to be used as negative controls, most especially during the evaluation of Gag expression by HIV-1 p24 assays. Figure 3.7 shows schematic diagram of the cloning strategy employed in the construction of these vectors.

The following plasmids; pEMα(V3SV5), pEM19(V3SV5) and pEM(V3SV5) were constructed from; pEMαGag_2(V3SV5), pEM19Gag_2(V3SV5) and pEMGag_2(V3SV5) respectively by the removal of the HIV-1 *gag* following a sequential restriction endonuclease digestion with *SnaB*I and *EcoR*V as mentioned in Section 2.3.2. The restriction enzymes *Nco*I and *EcoR*V, which lie adjacent to *gag* gene were selected as suitable sites to confirm that the plasmids had indeed lost the 1.5 kb HIV-1 *gag* gene and were therefore of correct sizes. Plasmids digested with *EcoR*V and undigested plasmids; pEMα(V3SV5), pEM19(V3SV5) and pEM(V3SV5) were loaded in lanes 2 and 3 respectively. The similar banding pattern was observed in lanes 2 and 3 as plasmids pEMα(V3SV5), pEM19(V3SV5) and pEM(V3SV5) had lost the *EcoR*V site following the removal of *gag* gene as expected (Figure 3.8 to 3.10). This is as expected since all the three plasmids had lost the *EcoR*V site during their construction (lanes 3). As expected, a 1.5kb band difference of larger fragments in lanes 6 and 7 following digestion with *Nco*I indicated that the *gag* gene had been removed.

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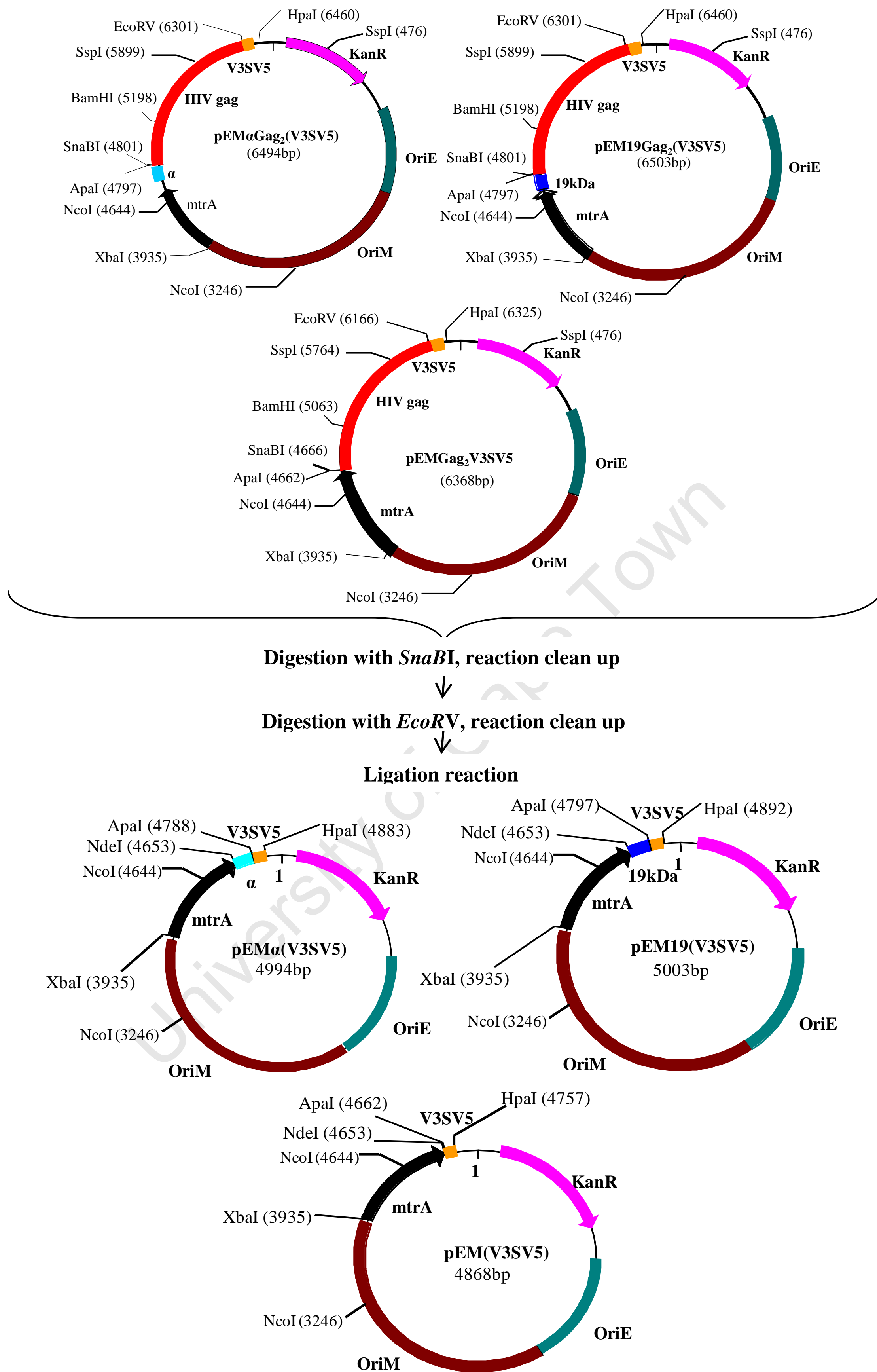

Figure 3.7: Schematic diagram of the cloning strategy employed for the construction recombinant plasmid shuttle vectors without the gag gene: pEMα(V3SV5), pEM19(V3SV5) and pEM(V3SV5).

Table 3.4: Expected fragment sizes (bp) resulting from restriction endonuclease digestion of the plasmid DNA

Plasmid Name	**Expected fragment size**	
	***Nco*I**	***Eco*RV**
pEMαGag$_2$(V3SV5)	1398, 5096	6494
pEM19Gag$_2$(V3SV5)	1398, 5105	6505
pEMGag$_2$(V3SV5)	1398, 4970	6398
pEMα(V3SV5)	1398, 3596	Uncut
pEM19(V3SV5)	1398, 3605	Uncut
pEM(V3SV5)	1398, 3470	Uncut

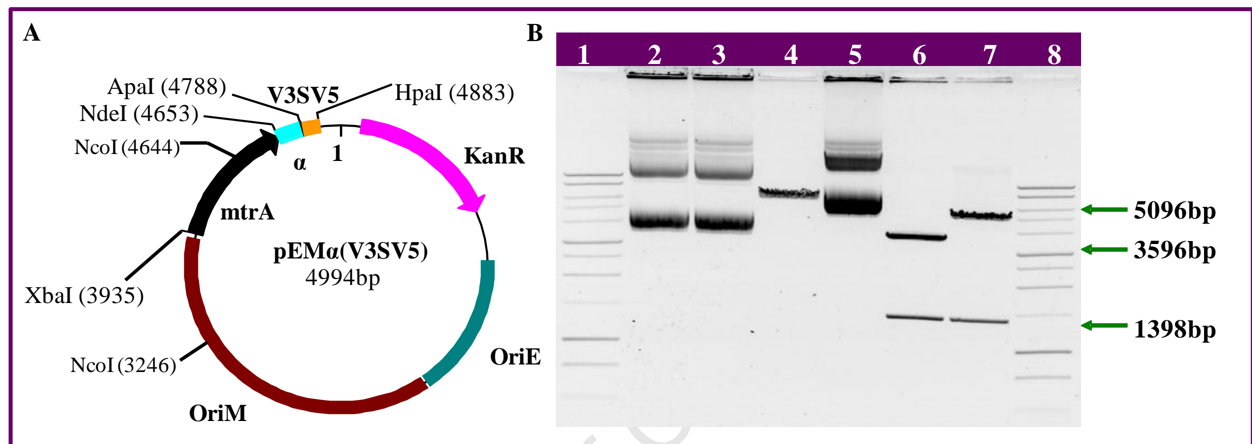

Figure 3.8: Restriction enzyme mapping of plasmid pEMα(V3SV5) by digestion with *Eco*RV and *Nco*I. Lanes 1 and 8: 1kb DNA molecular weight marker; lane 2: pEMα(V3SV5) digested with *Eco*RV; lane 3: undigested pEMα(V3SV5) plasmid; lane 4: pEMαGag$_2$(V3SV5) plasmid control digested with *Eco*RV; lane 5: undigested pEMαGag$_2$(V3SV5); lane 6: pEMα(V3SV5) digested with *Nco*I; lane 7: pEMαGag$_2$(V3SV5) digested with *Nco*I.

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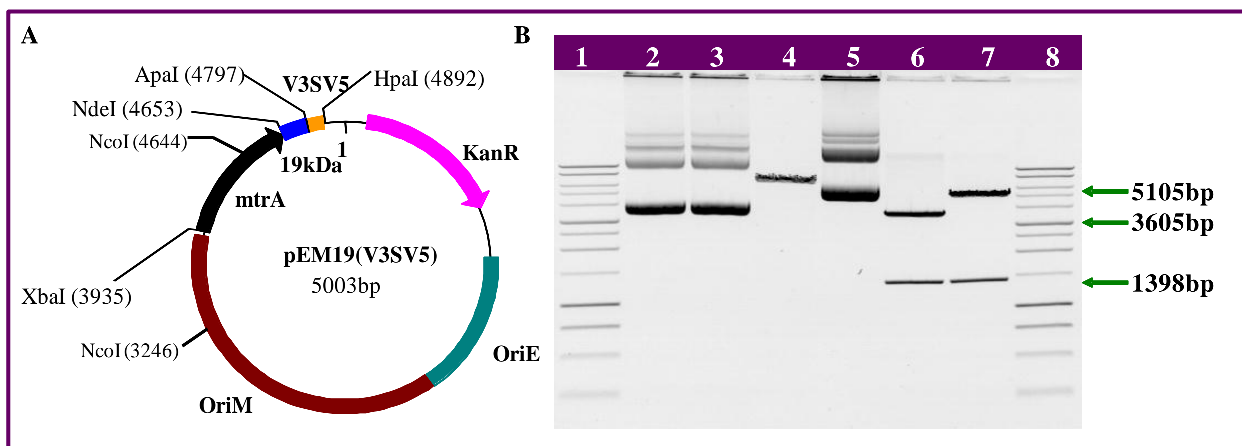

Figure 3.9: Restriction enzyme mapping of plasmid pEM19(V3SV5) by digestion with *Eco*RV and *Nco*I. Lanes 1 and 8: 1kb DNA molecular weight marker; lane 2: pEM19(V3SV5) digested with *Eco*RV; lane 3: undigested pEM19(V3SV5) plasmid; lane 4: pEM19Gag$_2$(V3SV5) plasmid control digested with *Eco*RV; lane 5: undigested pEM19Gag$_2$(V3SV5); lane 6: pEM19(V3SV5) digested with *Nco*I; lane 7 pEM19Gag$_2$(V3SV5) digested with *Nco*I.

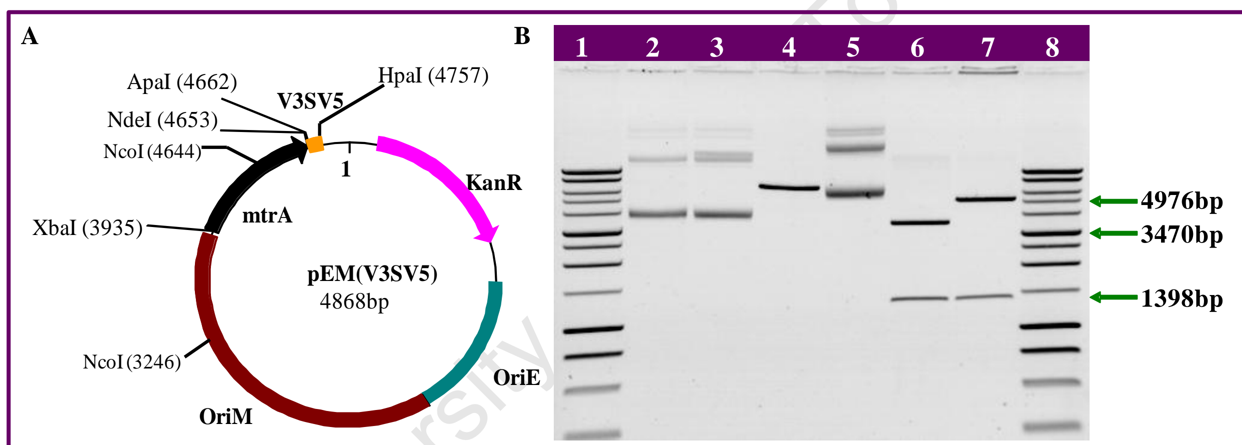

Figure 3.10: Restriction enzyme mapping of plasmid pEM(V3SV5) by digestion with *Eco*RV and *Nco*I. Lanes 1 and 8: 1kb DNA molecular weight marker; lane 2: pEM(V3SV5) digested with *Eco*RV; lane 3: undigested pEM(V3SV5) plasmid; lane 4: pEMGag$_2$(V3SV5) plasmid control digested with *Eco*RV; lane 5: undigested pEMGag$_2$(V3SV5); lane 6 pEM(V3SV5) digested with *Nco*I; lane 7: pEMGag$_2$(V3SV5) digested with *Nco*I.

To confirm that the cloning junctions were in frame with the V3SV5 epitope-tag and the stop codon, the plasmids were sequenced using the pCONEPI forward 5'-TGGCGAACTCCGTTGTAGTG-3' and pEM reverse 5'- AGCAGACAGTTTTATTGTTC-3' primers. Figure 3.11 shows the schematic summary of the expression cassettes cloned into the stable episomal plasmid vector backbone pEM. The resultant vectors were then evaluated for their fitness *in vitro* using *M. smegmatis*.

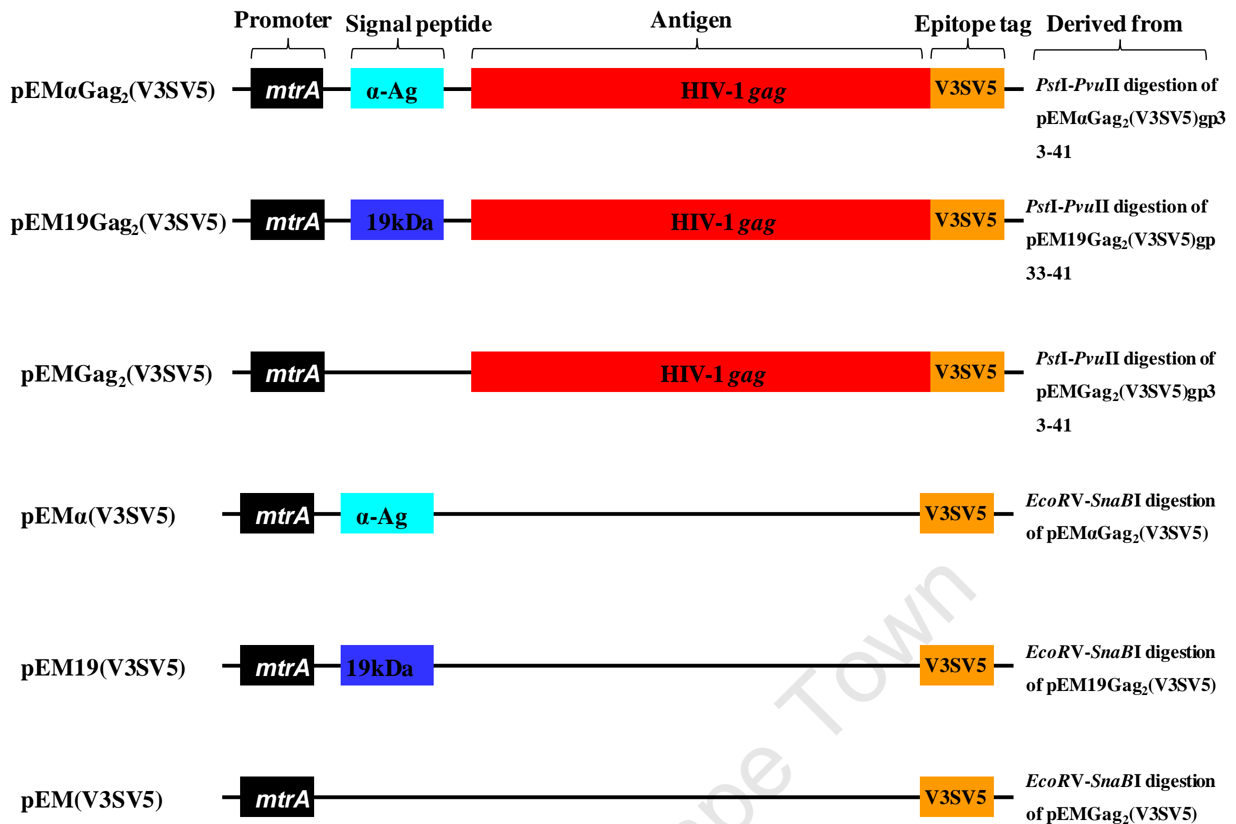

Figure 3.11: Schematic summary of the expression cassettes cloned into the stable episomal plasmid backbone pEM vector backbone. These vectors have various features such as the *MtrA* promoter, α or 19kDa signal sequences, with or without the HIV-1 gag and the V3 CTL-SV5 monoclonal tag. The figure also shows where these were derived.

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3.2.3 Determination of vector fitness by growth rates

A large number of *E. coli*-mycobacterial shuttle vectors have been developed for the expression of foreign antigens in BCG. Key features of these vectors influence the stability as well as the extent and nature of the immune response that can be elicited by recombinant BCG. Promoter elements have received particular attention, as these regulate the level of expression of a foreign gene as a function of promoter strength and the timing of expression according to promoter activity**.** Other vector features that also influence expression levels include leader sequences used to target the protein within the BCG cell and inserted foreign DNA sequences (Langermann et al. 1994, Himmelrich et al. 2000). These shuttle vector features influence stability of recombinant BCG antigen delivery systems.

For this reason, we sought to evaluate shuttle vector fitness using *M. smegmatis* as a model organism due to its faster replication capability in relation to slow growing BCG strains. Thus as

an indicator of vector fitness, *M. smegmatis* was transformed with equal quantities of the various shuttle vector constructs and colony sizes were compared to a control, *M. smegmatis* containing an empty plasmid backbone pCONEPI, (the smaller the colonies the more unstable the shuttle vector). Furthermore, care was taken to compare colony sizes between plates with similar cell counts since a high density of colony forming units may limit colony size. Cultures were also evaluated for stability by growth curves; whereby relative growth rates were compared.

3.2.3.1. Indication of fitness by growth rates on solid medium

The recombinant *M. smegmatis* cultures were generated by electro-poration of constructed plasmid shuttle vectors into electro-competent *M. smegmatis* cells. The cells were plated onto selective MB7H10-OADC agar plates. The resultant colonies were evaluated for vector fitness by relative colony sizes (RCS) after addition of various shuttle vector features.

3.2.3.2 Selection of a suitable promoter (*hsp60*/*psmyc* vs *mtrA*)

Promoters regulate the expression of foreign genes. Certain promoters are only induced upon an encounter with stimulus, while others constitutively express genes. The use of *hsp60* promoter has been shown to result in high levels of constitutive transcription, which could, at times be deleterious to rBCG, whereas the *mtrA* promoter has low *in vitro* activity but is induced to similar levels to *hsp60 in vivo* (Zahrt, Deretic 2000). This allows for the expression of foreign antigen in rBCG at the time of exposure to the immune system.

In this study, we sought to select the best promoter by electro-porating the following plasmids into *M. smegmatis*; pEMGag$_2$(V3-SV5), pEHDGag$_2$(V3-SV5), pESDGag$_2$(V3-SV5), which have the *M. tuberculosis* *mtrA* and *hsp60* promoters , and *M. smegmatis* p_{smyc} promoter respectively. The latter two constructs were kindly donated by Dr Ros Chapman (Unversity of Cape Town). The recombinant cells harbouring these plasmids were compared to the positive control pCONEPI by means of colony sizes. Figure 3.12 indicated that expression driven by the p_{smyc} promoter (pESDGag$_2$(V3-SV5)) resulted in poor vector fitness as no transformants were obtained. This experiment was repeated three times and the same results were obtained. The constitutive expression of *gag* by the *hsp60* promoter (pEHDGag$_2$(V3-SV5)) also resulted in very small pin prick size colonies, thus indicating lack of vector fitness. This could be due to

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high levels of HIV-1 Gag expression, which appears to be toxic to mycobacteria. On the contrary, inducible expression by the *mtrA* promoter (pEMGag$_2$(V3-SV5)) resulted in stable colonies, which were comparable to those harbouring pCONEPI. From these results, it is evident that high levels of constitutive expression driven by both the *hsp60* and p_{smyc} promoters contributed to reduced vector fitness *in vitro* as depicted by the small colony size scores of cells harbouring plasmids with such promoters or lack of transformants. In contrast, the inducible promoter (*mtrA*) enhanced vector fitness *in vitro*.

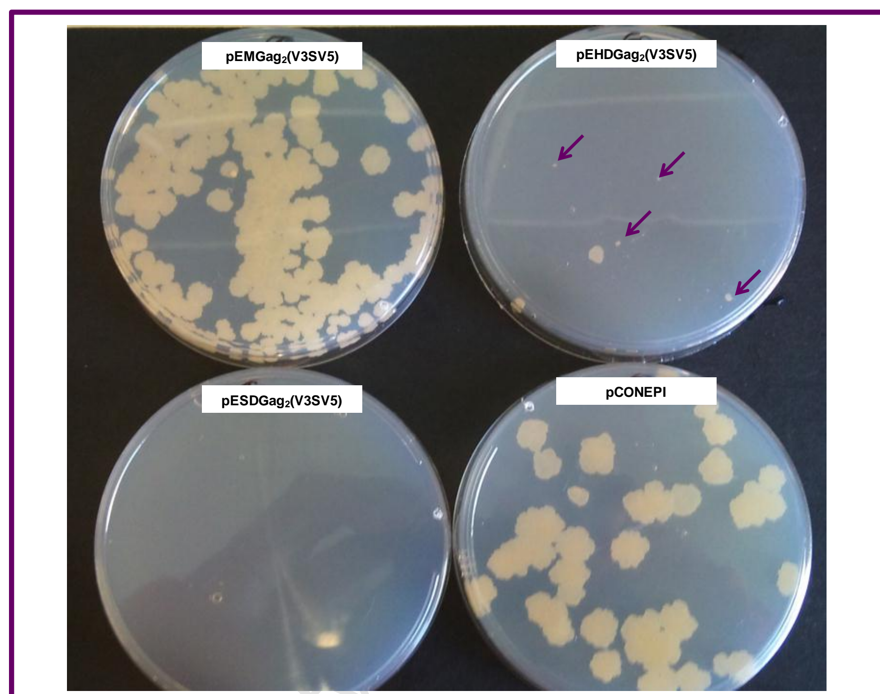

Figure 3.12: Evaluation of vector fitness following use of various promoters.Comparison of *M. smegmatis* colonies harbouring the following plasmids; pEMGag$_2$(V3SV5); pEHDGag$_2$(V3SV5); pESDGag$_2$(V3SV5) and the control pCONEPI. The arrows indicate the pinprick colonies.

Table 3.5: The relative colony size scores (RCS) of recombinant M. smegmatis colonies. RCS was assigned an arbitrary scoring system by means of asterisks ranging from * to *****, from smallest to largest.

Plasmid	RCS
pEMGag$_2$(V3SV5)	****
pESDGag$_2$(V3SV5)	No colonies
pEHDGag$_2$(V3SV5)	*
pCONEPI	******

3.2.3.3 The use of leader peptides

Leader sequences direct antigens to various compartments of the cells. Such peptides may lodge the protein in the membrane or may facilitate extracellular secretion of the antigens from the cell.

The colonies of *M. smegmatis* cells harbouring pEMαGag$_2$(V3SV5), pEM19Gag$_2$(V3SV5) and pEMGag$_2$(V3SV5), which have the α-antigen signal sequence, the 19kDa signal peptide and no signal sequence respectively were compared to those harbouring an empty vector pCONEPI. There was no difference in the relative colony sizes of the *M. smegmatis* cells (Figure 3.13 and Table 3.6), thus indicating that the signal peptides had no effect on vector fitness. The colony sizes were comparable to *M. smegmatis* [pCONEPI] colonies (Figure 3.13). These results showed that the use of either α-antigen or 19kDa signal peptides did not reduce stability.

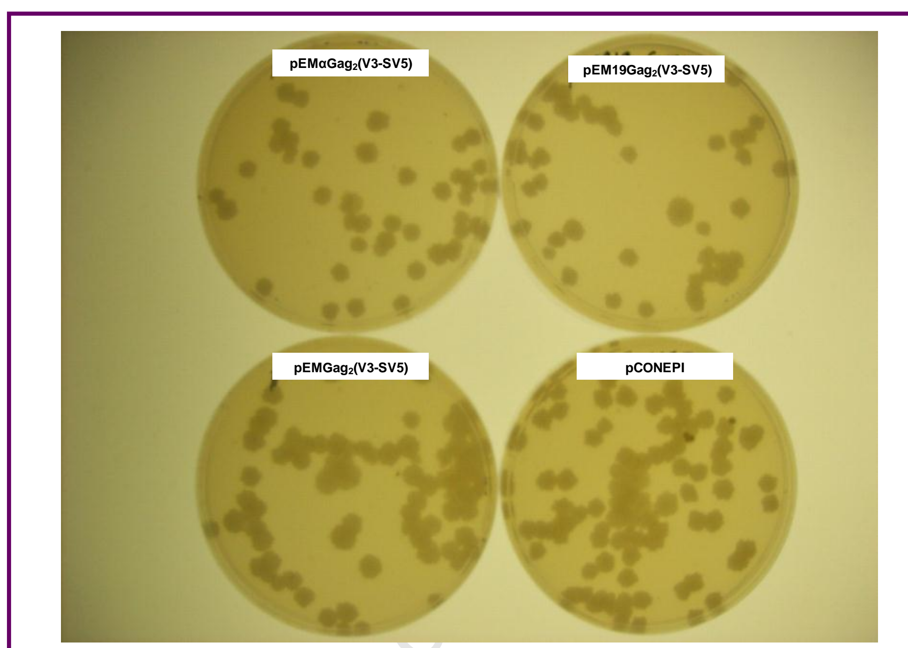

Figure 3.13: Evaluation of vector fitness following use of various leader peptides. Comparison of *M. smegmatis* colonies harbouring the following plasmids; pEMαGag$_2$(V3SV5); pEM19Gag$_2$(V3SV5); pEMGag$_2$(V3SV5) and the positive control pCONEPI.

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Table 3.6: The relative colony size scores (RCS) of recombinant M. smegmatis colonies. RCS was assigned an arbitrary scoring system by means of asterisks ranging from * to *****, from smallest to largest.

Plasmid	RCS
pEMαGag$_2$(V3SV5)	*****
pEM19Gag$_2$(V3SV5)	*****
pEMGag$_2$(V3SV5)	*****
pCONEPI	*****

3.2.3.4 Effect of HIV-1 *gag* codon optimisation on vector fitness

There has been a strong correlation between codon usage and the level of expression in highly expressed antigens (Kanekiyo et al. 2005). Therefore, in this study, we evaluated the effect of

codon optimisation on vector fitness using the following plasmids: pEM19Gag$_1$ and pEM19Gag, which contain codon optimised and non-codon optimised HIV-1 *gag* respectively. There was a slight increase in colony size between *M. smegmatis* [pEM19Gag] (RCS = ***) and *M. smegmatis* [pEM19Gag$_1$] (RCS of ****), indicating that codon optimisation may slightly improve relative vector fitness (Figure 3.14).

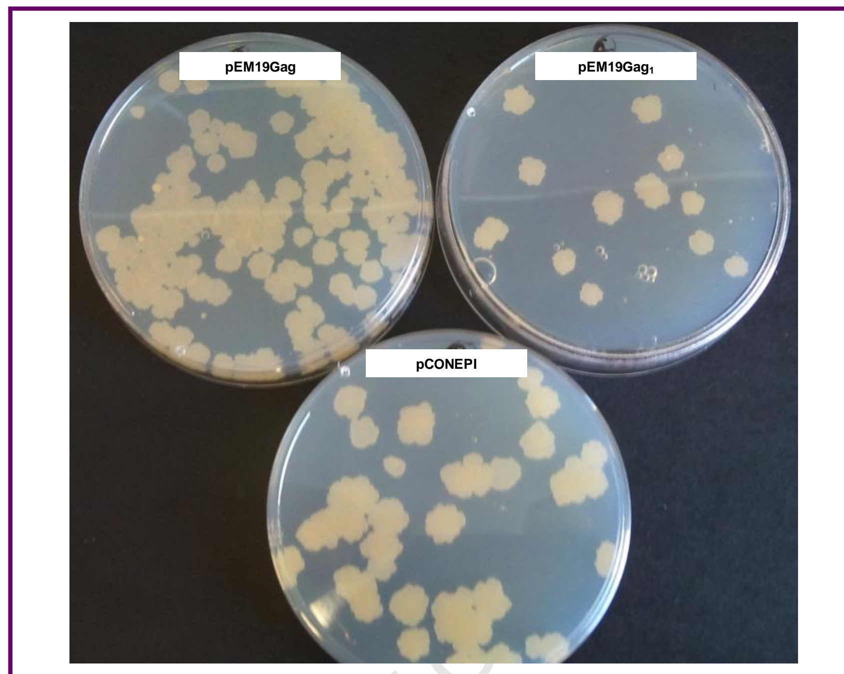

Figure 3.14: Comparison of *M. smegmatis* colonies harbouring the following plasmids; pEMαGag, pEM19Gag$_1$ and the positive control pCONEPI.

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Table 3.7: The relative colony size scores (RCS) of recombinant *M. smegmatis* colonies. RCS was assigned an arbitrary scoring system by means of asterisks ranging from * to *****, from smallest to largest.

Plasmid	RCS
pEM19Gag	***
pEM19Gag$_1$	****
pCONEPI	*****

3.2.3.5 The effect of HIV-1 *gag* on vector fitness

To evaluate the effect of addition of the codon optimised HIV *gag* on vector fitness, the *M. smegmatis* colonies harbouring pEM19Gag$_2$(V3SV5), pEMGag$_2$(V3SV5), pEM19(V3SV5) and pEM(V3SV5) were compared. All the constructs were stable as shown by the high relative colony size scores (Figure 3.15), thus the addition of codon optimised *gag* did not have any effect on the relative vector fitness *in vitro* when expressed from the *mtrA* promoter.

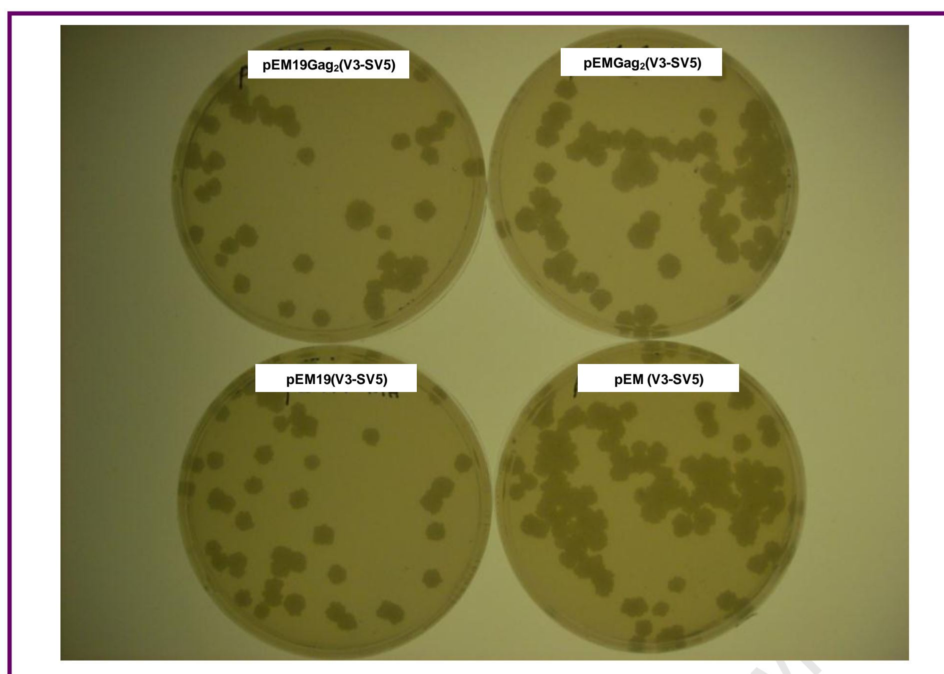

Figure 3.15: Evaluation of vector fitness following addition of codon optimised HIV-1 *gag* gene. Comparison of *M. smegmatis* colonies harbouring the following plasmids; pEM19Gag$_2$(V3SV5), pEMGag$_2$(V3SV5), pEM19(V3SV5) and pEM(V3SV5).

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Table 3.8: The relative colony size scores (RCS) of recombinant M. smegmatis colonies. RCS was assigned an arbitrary scoring system by means of asterisks ranging from * to *****, from smallest to largest.

Plasmid	RCS
pEM19Gag$_2$(V3SV5)	****
pEMGag$_2$(V3SV5)	****
pEM19(V3SV5)	****
pEM(V3SV5)	****

In essence, change of plasmid backbone from pCB- to pEM- resulted in more "fit" recombinant mycobacteria. Furthermore, addition of the inducible *mtrA* promoter, leader sequences, and codon optimised *gag* as well as epitope-antibody tags did not interfere with the plasmid fitness.

3.2.4 Indication of fitness by growth rates on liquid medium

As mentioned earlier, one way of evaluating vector fitness is by growth rates of recombinant cultures on solid medium. However, this strategy may be relatively unreliable when used as a sole stability indicator as mycobacterial colonies tend to clump, thereby making it difficult to count and infer colony size. Therefore, growth rates in liquid medium were also used to evaluate vector fitness. When grown in selective medium, cultures may exhibit faster growth rates due to

vector stability. To test for this, the growth rates of recombinant *M. smegmatis* cultures harbouring various vectors were evaluated. These cultures were grown in selective and non-selective MB7H9-OADC-Tyl medium and the growth rates of these cultures were evaluated by monitoring the OD_{600} readings every 8 hours (every second doubling time for *M. smegmatis*) for a period of up to 48 hours (Figure 3.16).

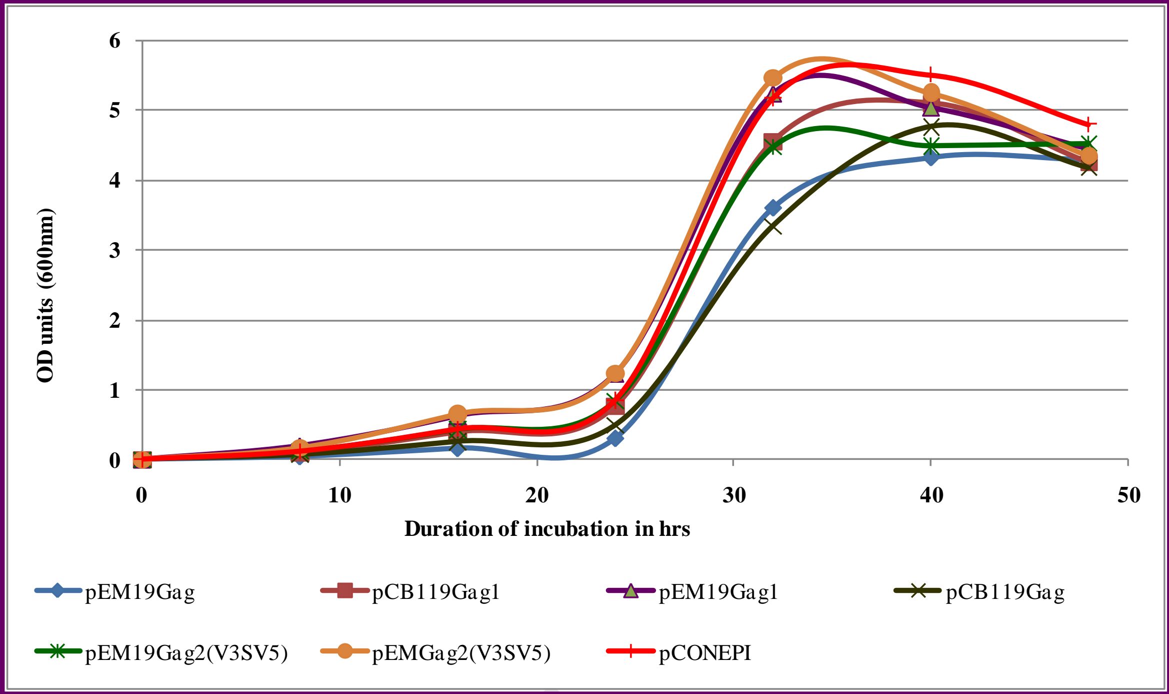

Figure 3.16A: Growth curves of *rM. smegmatis* cultures grown in the presence of kanamycin

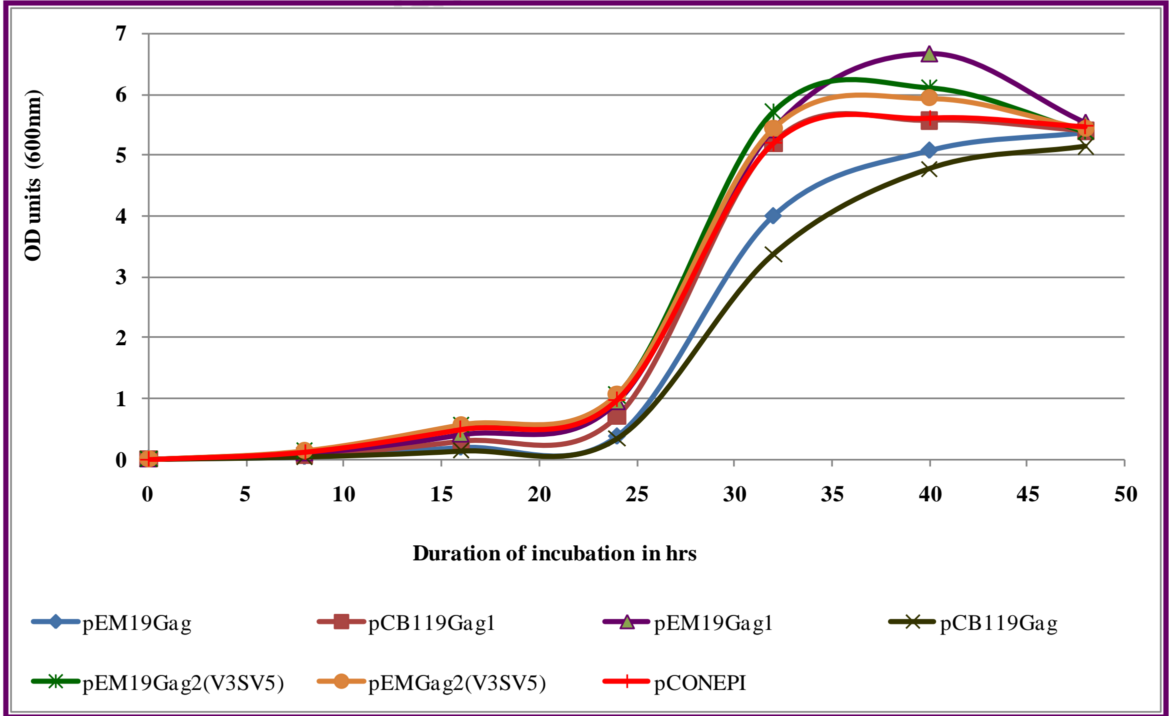

Figure 3.16B: Growth curves of *rM. smegmatis* cultures grown in the absence of kanamycin

Both the *M. smegmatis* [pEMGag$_2$(V3SV5)] and *M. smegmatis* [pEM19Gag$_1$] cultures grew faster than all the other cultures, when grown in media containing kanamycin. This is evident by their short lag phases and the fast exponential growth. On the other hand, *M. smegmatis* [pEM19Gag$_2$(V3SV5)] and *M. smegmatis* [pCB119Gag$_1$] showed intermediate exponential growth rates, whereas *M. smegmatis* [pEM19Gag] and *M. smegmatis* [pCB119Gag] were the slowest growing cultures (Figure 3.16A). When grown in non-selective medium (without kanamycin), all the cultures harbouring vectors with the codon optimised gag showed the faster exponential growth as compared to both non-codon optimised *M. smegmatis* [pCB119Gag] and *M. smegmatis* [pEM19Gag] cultures (Figure 3.16B).

3.2.5 Evaluation of plasmid stability

One way of evaluating plasmid stability *in vitro* is by colony counts whereby cultures harbouring plasmid vectors are grown in the absence of antibiotic selection in liquid medium (Section 3.2.4). These cultures are then plated onto both selective and non-selective medium to determine percentage loss of plasmid. In this study, plasmid stability was evaluated in the similar manner using duplicate cultures of r*M. smegmatis*.

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Table 3.9: The plasmid stability evaluation by colony counts following culturing in the absence of selection

Plasmid transformed	With kanamycin		Without kanamycin		% plasmid loss
	Average number of colonies	SD	Average number of colonies	SD	
pCB119Gag$_1$	163.5	20.5	133	5.6	**0**
pEM19Gag$_1$	155	25.5	142	45.3	**0**
pCB119Gag	127	9.9	276.5	50.2	**54**
pEM19Gag	211	15.6	230.5	33.2	**8**
pEM19Gag$_2$(V3SV5)	137.5	27.6	115.5	17.7	**0**
pEMGag$_2$(V3SV5)	94	21.2	106	21.1	**11**
pCONEPI	98	5.6	98.5	2.1	**0.5**

Gag: non-codon optimised, **Gag$_1$**: first codon optimisation, **Gag$_2$**: second codon optimisation. SD: standard deviation

The *M. smegmatis* [pEM19gag$_1$], *M. smegmatis* [pCB119Gag$_1$] *M. smegmatis* [pEM19Gag$_2$(V3SV5)] and *M. smegmatis* [pCONEPI] cultures showed no plasmid loss, whereas *M. smegmatis* [pCB119Gag] culture (culture with pCB119 backbone) experienced 54% plasmid loss. To note the *M. smegmatis* [pEMGag$_2$(V3SV5)] and *M. smegmatis* [pEM19Gag] cultures had 11% and 8% plasmid loss, which was a not a great loss as compared to 54% loss, therefore indicating that the plasmids were also stable. These results indicate that generally codon

optimisation of genes contributes to plasmid stability to a certain degree, whereas vector backbone has a significant effect on vector fitness.

3.3. Basal level expression of Gag from recombinant *M. smegmatis*

HIV-1 Gag p24 assays were done to evaluate expression of HIV-1 Gag in recombinant *M. smegmatis* cultures harbouring plasmids with different leader peptides. This was done by the use of p24 capture Enzyme-Linked Immunosorbant Assay (ELISA). The cell free extract was collected as cultures reached OD_{600} readings of approximately 1. This was harvested by bead beating method. Approximately 1μg of the cell free extract in HPLC water was analysed.

M. smegmatis transformed with pEMαGag_2(V3SV5) seamed to express more Gag as compared to *M. smegmatis* [pEM19Gag_2(V3SV5)] and *M. smegmatis* [pEMGag_2(V3SV5)] (64pg/ml as compared to 39 and 17 respectively). As expected, the three recombinants harbouring vectors without the *gag* showed no Gag expression. The <12pg/ml value reflects the background as observed with the negative control *M. smegmatis* [pCONEPI]. The three recombinants expressing *gag* from the inducible *mtrA* promoter expressed far lower levels of p24 *in vitro* as compared to the recombinant expressing *gag* from the constitutive *hsp60* promoter; *M. smegmatis* [pF10]). There was a clear difference in p24 expression levels. The expression levels of both the constitutive and inducible expressers were comparable only when the former was at a tenfold lower concentration. These results indeed showed that the Gag expression levels were low during *in vitro* growth when the *mtrA* promoter was used. Table 3.10 below summarises the findings from this part of the study. The next part of the study was to prepare rBCG vaccine stocks and to evaluate their immunogenicity following vaccination of BALB/c mice.

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Table 3.10: Summary of the evaluation of vector fitness and stability in the model organism *M. smegmatis* by means of relative colony sizes and growth rates

Name of construct	***Evaluation of Vector fitness***		***Stability after 10 generations***	***HIV-1 Gag p24 expression***
	Colony size	**Growth rate in liquid media**		
pEMαGag$_2$(V3SV5)	**	X	Stable	69.7
pEM19Gag$_2$(V3SV5)	**	Intermediate	Stable	39.4
pEMGag$_2$(V3SV5)	***	Fast	Stable	17.4
pEMα(V3SV5)	X	X	X	<12
pEM19(V3SV5)	**	X	X	<12
pEM(V3SV5)	***	X	X	<12
pCONEPI	****	Fast	Stable	<12
pF10	X	X	X	>400

*: depicts the relative colony sizes, **X**: indicates what was not done on the vector.

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3.4 Recombinant BCG Pasteur ΔpanCD vaccine stock preparation and stability checks by restriction enzyme mapping

These results show that removal of *hsp60-lysA*, addition of signal peptides, codon optimisation of gag for expression in mycobacteria as well as use of inducible *mtrA* promoter for controlled expression of *gag* improve the relative vector fitness. Thus shuttle vectors containing the pEM backbone, codon optimised *gag* and *mtrA* promoter were chosen for further studies in BCG. Table 3.11 shows the constructs used in this part of the study as well as which vectors were chosen for immunogenicity studies.

Table 3.11: Summary of the vectors constructed in the study and experiments carried out

Name of construct	**Features**			**rBCG vaccine stock**	**Evaluation of stability by RE mapping**	**Vaccination of BALB/c mice**
	promoter	**Signal sequence**	**V3SV5**			
pEM αGag$_2$(V3SV5)	*mtrA*	α	√	√	√	√
pEM19Gag$_2$(V3SV5)	*mtrA*	19kDa	√	√	√	√
pEM Gag$_2$(V3SV5)	*mtrA*	None	√	√		√
					√	
pEM α(V3SV5)	*mtrA*	α	√	√	√	X
pEM19(V3SV5)	*mtrA*	19kDa	√	√	√	X
pEM(V3SV5)	*mtrA*	None	√	√	√	X
pCONEPI	None	None	None	√	X	√

The constructs used in this study were with or without HIV-1 *gag*, where **Gag**: non-codon optimised, **Gag$_1$**: first codon optimisation, **Gag$_2$**: second codon optimisation. √: indicates what was done or feature present, while **X**: indicates what was not done and **none**: shows feature that was not present in vector.

To assess the effect of fusing different leader sequences to HIV-1 Gag on the immunogenicity of our recombinant BCG Pasteur ΔpanCD vaccines stocks, the following vaccines were prepared:

BCG Pasteur ΔpanCD [pEMαGag$_2$V3SV5], BCG Pasteur ΔpanCD [pEM19Gag$_2$V3Sv5] and BCG Pasteur ΔpanCD [pEMGag$_2$V3SV5].

Studies have shown that *M. tuberculosis* pantothenate auxotroph (ΔpanCD) was highly attenuated in severe combined immune-deficient (SCID) mice and in immunocompetent BALB/c mice. This auxotroph allowed significant and prolonged survival of SCID mice than mice infected with either bacille Calmette-Guerin (BCG) vaccine or virulent *M. tuberculosis* (Sambandamurthy et al. 2002). Pantothenic acid (vitamin B5) is an essential molecule required for the synthesis of coenzyme A and acyl carrier protein (ACP), that play important roles as acyl-group carriers in fatty-acid metabolism, the tricarboxylic-acid cycle, biosynthesis of polyketides and several other metabolic reactions. Similarly, *M. bovis* BCG Pasteur ΔpanCD a slow growing pantothenic auxotroph was constructed as a double-deletion mutant of *M. tuberculosis* in the *panC* and *panD* genes that are involved in the *de novo* biosynthesis of pantothenate (Bardarov et al. 2002, Tullius et al. 2008). Tullius et al. (2008) showed that rBCG (Δ*panCD*) 30 (the pantothenic auxotroph that over-expresses *M. tuberculosis* antigen 85B) is more attenuated than BCG in SCID mice and provided similar protection to BCG in immunocompetent guinea pigs.

To confirm that the BCG Pasteur ΔpanCD vaccine stocks were stable before vaccination of mice, plasmid DNA was isolated from the rBCG vaccine stocks. The plasmid DNA was then transformed into *E. coli* and plasmid DNA again extracted and then mapped using restriction endonucleases; *Xba*I and *KspA*I (*Hpa*I). The plasmid DNA was transformed into *E. coli* and re-isolated as the yield of plasmid DNA from mycobacteria is very poor. All plasmid DNA isolated from the BCG vaccine stocks showed expected banding patterns following restriction endonuclease digestion (Table 3.12, Figures 3.17 to 3.19).

Prior to vaccination of BALB/c mice with the rBCG vaccines, sequencing of plasmids (pEMαGag$_2$(V3SV5), pEM19Gag$_2$(V3SV5) and pEMGag$_2$(V3SV5) from these vaccines was done to further confirm that these vaccines were stable. Large scale plasmid isolation was performed and 100ng/µl of the resultant plasmids was sent for sequencing at Stellenbosch University, Western Cape, South Africa. The entire 1687 fragment (GeneArt codon optimised) was sequenced and all the plasmids had no deletions, indicating that they were stable (data not shown). This implied that all the vaccine stocks were 100% stable. Following these experiments,

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these r BCG Pasteur ΔpanCD vaccines were then used to vaccinate mice in order to evaluate their immunogenicity.

Table 3.12: The expected number and sizes of DNA fragments following double digestion of plasmid DNA with *Xba*I and *KspA*I

Plasmid	Expected no. of bands	Expected band sizes in bp
pEM αGag$_2$(V3SV5)	2	4006, 2488
pEM19Gag$_2$(V3SV5)	2	4046, 2457
pEM Gag$_2$(V3SV5)	2	4046, 2322

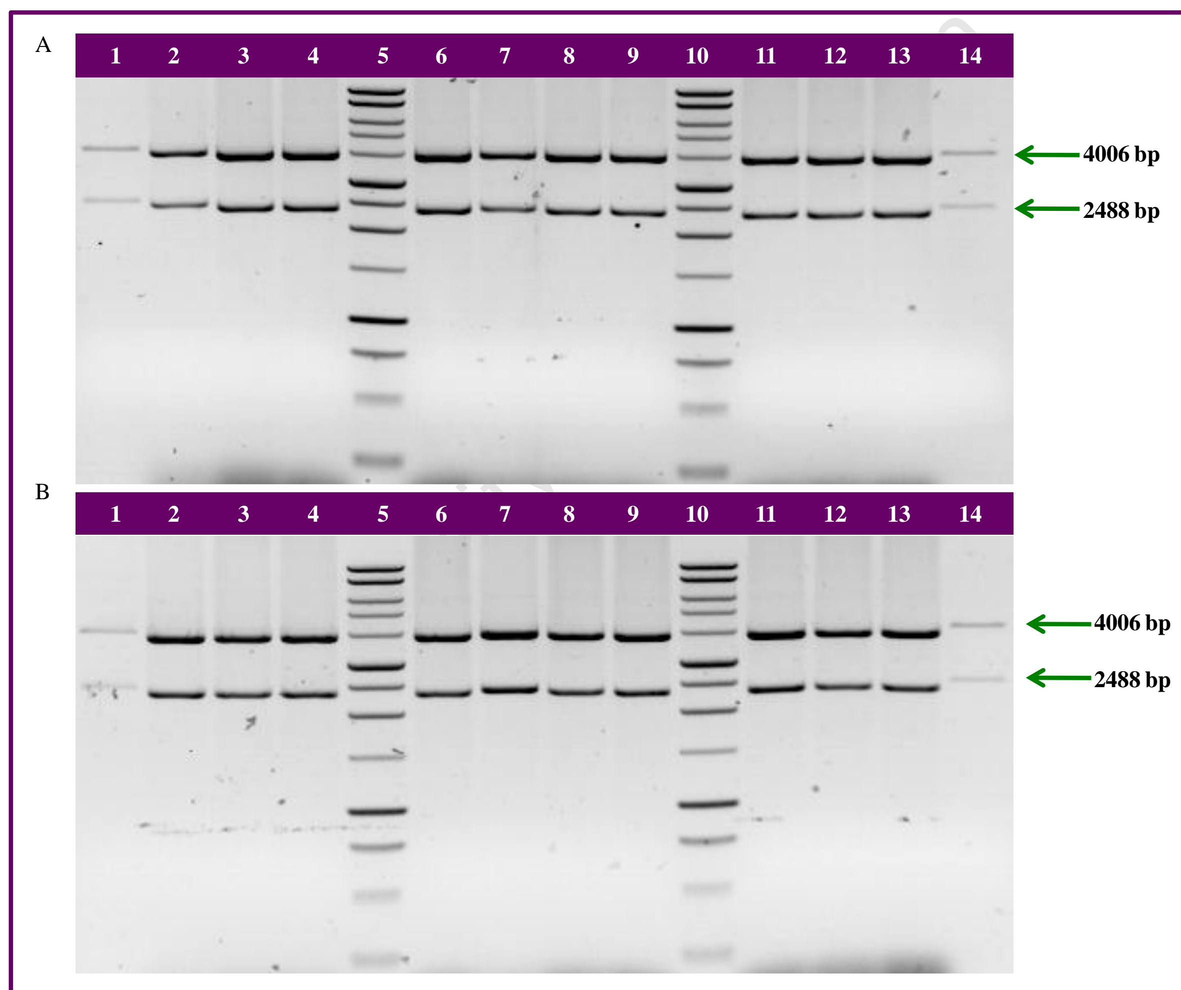

Figure 3.17: Restriction enzyme mapping of pEMαGag$_2$(V3SV5) plasmid DNA isolated from BCG vaccine stocks. Plasmid DNA was digested with restriction enzymes *Xba*I and *KspA*I (*Hpa*I). A & B: Lanes 1 and 14: positive control, original, untransformed DNA; lanes 2 – 4, 6 – 9 and 11 -13: plasmid DNA isolated from BCG vaccine stocks; lanes 5 and 10: 1kb DNA molecular weight marker.

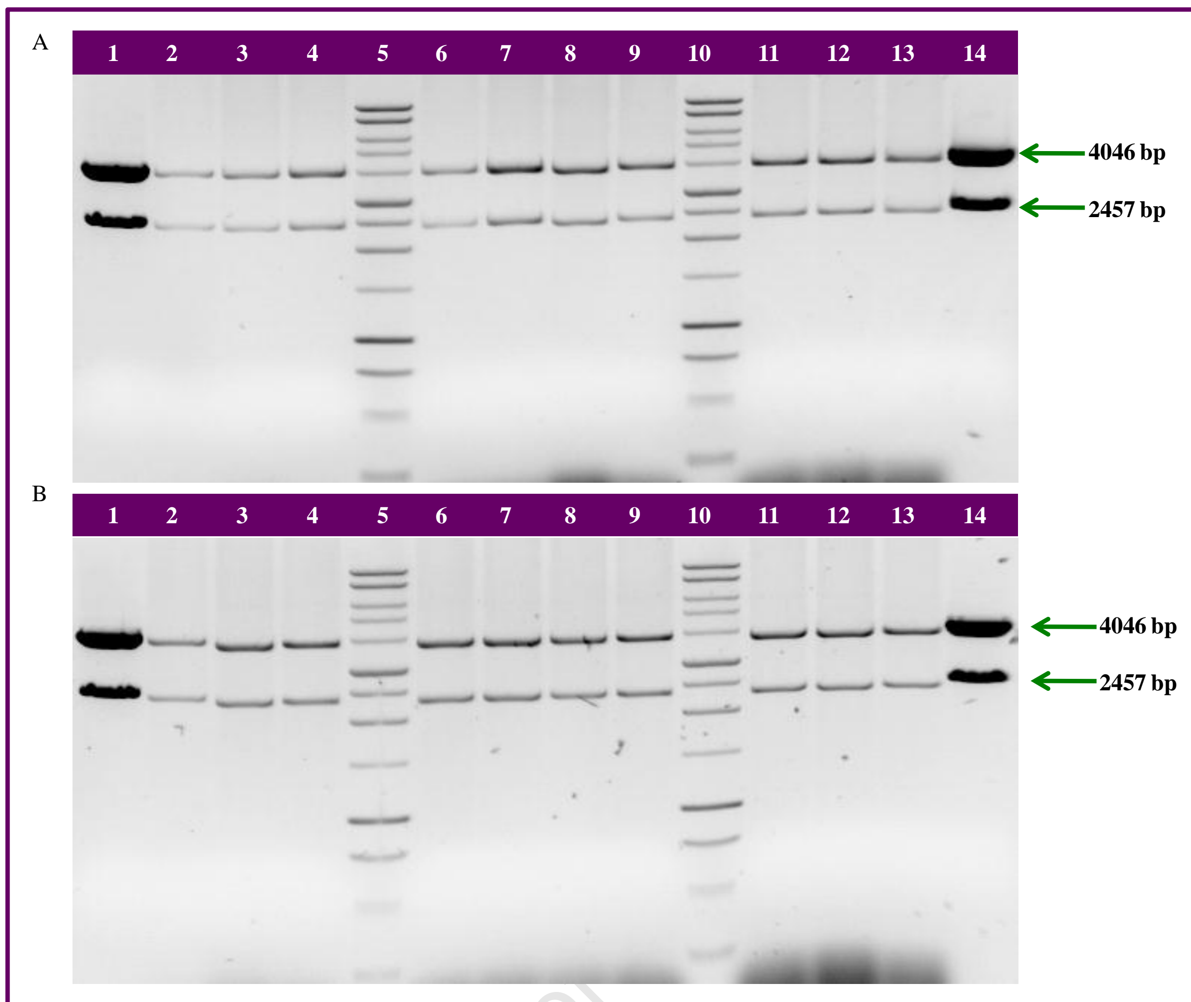

Figure 3.18: Restriction enzyme mapping of pEM19Gag$_2$(V3SV5) plasmid DNA isolated from BCG vaccine stocks. Plasmid DNA was digested with restriction enzymes *Xba*I and *KspA*I (*Hpa*I). A & B: Lanes 1 and 14: positive control, original, untransformed DNA; lanes 2 – 4, 6 – 9 and 11 -13: plasmid DNA isolated from BCG vaccine stocks; lanes 5 and 10: 1kb DNA molecular weight marker.

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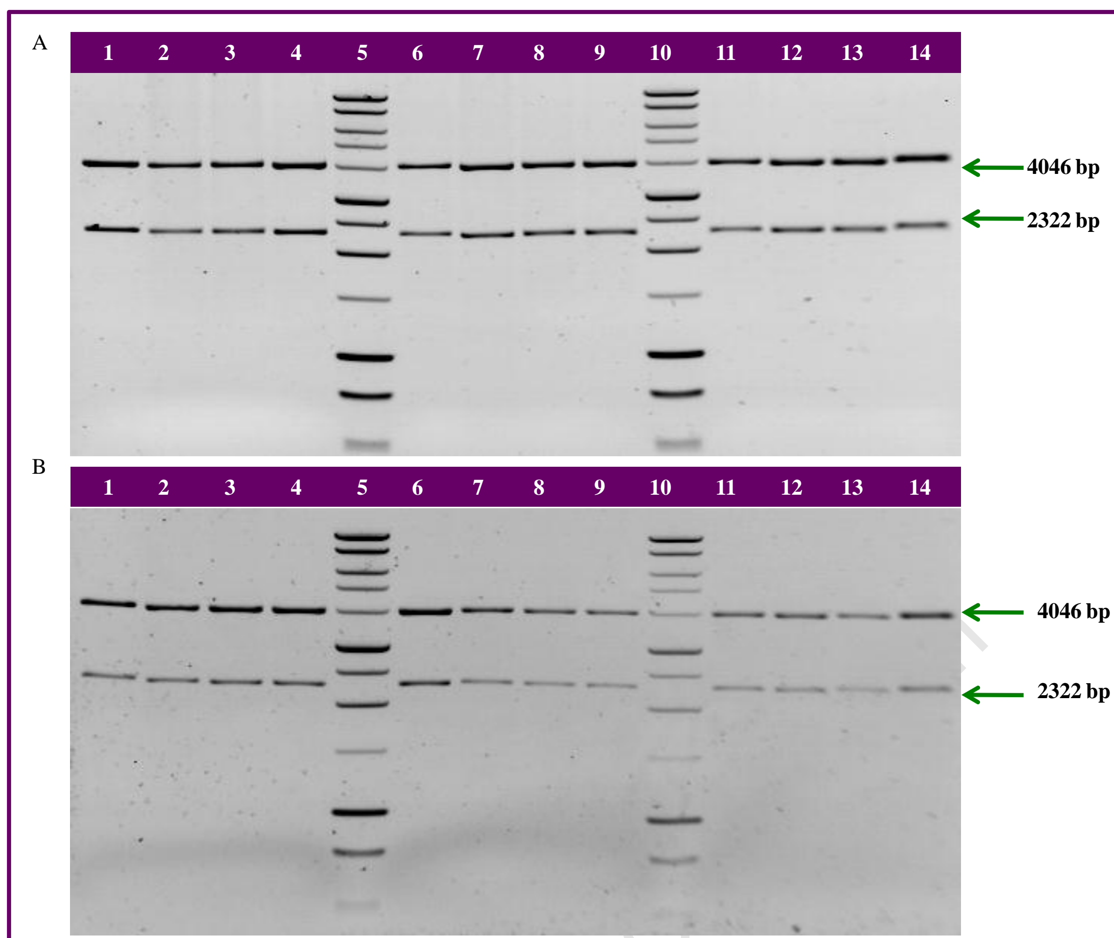

Figure 3.19: Restriction enzyme mapping of pEMGag$_2$(V3SV5) plasmid DNA isolated from BCG vaccine stocks. Plasmid DNA was digested with restriction enzymes *Xba*I and *KspA*I (*Hpa*I). A & B: Lanes 1 and 14: positive control, original, untransformed DNA; lanes 2 – 4, 6 – 9 and 11 -13: plasmid DNA isolated from BCG vaccine stocks; lanes 5 and 10: 1kb DNA molecular weight marker.

3.5. Immunogenicity of rBCG vaccines

3.5.1 Immunogenicity of HIV-1 Gag

The immunogenicity of HIV Gag is briefly reviewed in the following section and indicates the justification of the use of Gag in this study as a model HIV antigen. As viral set point after infection predicts disease progression, an effective vaccine should elicit strong immune responses to lower steady-state viral load upon infection (Manrique et al. 2008, Manrique et al. 2009, Sircar et al. 2010, Valor et al. 2008). One of the main strategies for HIV vaccines is the induction of a CTL immune response to highly conserved or cross-reactive epitopes of structural proteins such as Gag (Falk et al. 2000). Kanekiyo et al. (2005) demonstrated that rBCG expressing codon optimized HIV Gag p24 antigen was capable of inducing strong long-lasting

cell mediated immune responses even at low vaccine doses. Honeyborne et al. (2007) analyzed the role of HLA-B*13 restricted Gag epitopes, which is associated with low viral load in HIV infection. The group discovered that HLA-B*13 positive individuals had low viremia as opposed to HLA-B*13 negative participants, who had high viral loads. The group also showed that, of the Gag peptides targeted, only the p24 Gag specific $CD8^+$ T-cell responses were significantly associated with decreasing viral loads. On the contrary, Env or accessory protein-specific CD8+ T-cell responses were associated with increased viral loads (Kiepiela et al. 2007, Novitsky et al. 2003). These results collectively show the strong correlation between increasing Gag-specific immune responses and decreased viral load.

3.5.2 Immunogenicity of rBCG vaccines using a prime-boost vaccination regimen

Heterologous prime-boost vaccination regimens employing bacteria and viruses as vectors delivering common T cell based immunongens induce stronger responses than multiple repeated dosing with homologous vaccine modalities (Rerks-Ngarm et al. 2009). HIV candidate vaccines have been shown in pre-clinical and clinical trials to induce better immune responses when used in prime - boost regimens (Paris et al. 2010, Brown et al. 2010). Support for use of prime-boost vaccination regimens was achieved with the results from the RV144 trial, a phase 3 efficacy trial conducted in 2009 in Thailand. Modest protection from HIV infection was achieved with this trial that evaluated a prime with a recombinant canary poxvirus vector vaccine (ALVAC-HIV [vCP1521] (expressed HIV-1 subtype B Gag and protease) and a boost with a recombinant glycoprotein 120 subunit vaccine (AIDSVAX B/E) (Gray et al. 2010). HIV-specific $CD4^+$ and $CD8^+$ T cells resulting from the prime-boost regimen express long term memory markers which may be required for participation in further recall responses during HIV infection (Jabbari, Harty 2006). Data from studies comparing vaccines suitable for use as booster vaccines have indicated that recombinant poxviruses vectors are most suitable for development as booster vaccines (Hovav et al. 2007, Geiben-Lynn et al. 2008).

Studies evaluating CD8 T cell priming by rBCG indicate excellent memory CD8 T cells are generated to the insert. (Russell et al. 2007, van Faassen et al. 2005). These insert-specific cells differentiate directly to the central memory phenotype which precludes their detection in an IFN-γ ELISPOT assay. Such memory cells proliferate in response to a booster vaccine and the generated effector cells can then be detected in the IFN-γ ELISPOT assay.

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3.5.3 Immunogenicity study objectives

In this study, the vaccination strategy employed a prime with the rBCG vaccines expressing Gag and the V3CTL tag followed by a boost with SAAVI MVA-C to determine cellular immune responses induced by the rBCG. Effector cells generated by the regimen were detected in an IFN-γ ELISPOT assay that used isolated BALB/c mouse splenocytes and Class I and Class II specific peptides as specific stimuli. The BCG vector used is a *panCD* auxotroph engineered to express HIV-1 Gag fused to either the α-antigen or the 19kDa signal sequences under the *mtrA* promoter (described in Section 2.10). SAAVI MVA-C is a multi-gene HIV-1 subtype C recombinant MVA vaccine that was developed by UCT and SAAVI then manufactured by Therion and is now in Phase I Clinical Trials. This vaccine was made available for this study for use as a booster vaccine. SAAVI MVA-C expresses HIV subtype C polyprotein Gag-RT-Tat-Nef (Grttn) and truncated Env (gp150CT) proteins (Burgers et al. 2008). The *grttnC* gene has been inserted into one insertion site in MVA and the *gp150CT* gene into a second insertion site in the same MVA. The gp150CT is a truncated Env protein with a V3CTL tag (T) for detection of immune responses to Env in BALB/c mice and was thus considered to be a suitable vaccine to boost the primary immune response induced by the rBCG vaccines. The rBCG prime-SAAVI MVA-C boost vaccine regimens were performed as detailed in Table 3.13. A total of 3 experiments were performed.

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Table 3.13: The vaccination schedule

Mouse group (# mice per group)	*Day 0 Prime rBCG 10^7 cfu/200µl (i.p.)*	*Day 28Boost SAAVI MVA-C10^4 pfu/100µl (i.m. 50µl each tibialis muscle)*	*Day 40 End of experiment*
1 (5)	**BCG Pasteur ΔpanCD [pEMαGag$_2$V3SV5]**	MVA	Kill, remove spleens
2 (5)	**BCG Pasteur ΔpanCD [pEM19Gag$_2$V3SV5]**	MVA	Kill, remove spleens
3 (5)	**BCG Pasteur ΔpanCD [pEMGag$_2$V3SV5]**	MVA	Kill, remove spleens
4 (5)	**BCG Pasteur ΔpanCD [pEM19Gag$_2$V3SV5] HS**	MVA	Kill, remove spleens
5 (3)	**BCG Pasteur ΔpanCD [pCONEPI]**	MVA	Kill, remove spleens
6 (5)	-	MVA	Kill, remove spleens

i.p: interperitoneal vaccination. **i.m:** intramascular vaccination.

3.5.4 Splenocyte numbers and lymphocyte phenotype after vaccination

Following isolation of mouse splenocytes, FACS was done using antibodies to cell surface markers for identifying the phenotype of the lymphocytes in the spleen (see Section 2.10.2). Table 3.14 shows the splenocyte numbers and lymphocyte phenotype for the spleens harvested for Experiment 3.

Table 3.14: The proportion (as % of events in the lymphocyte gate) of B and T cells in the spleens of the mice groups vaccinated with the respective rBCG vaccines (experiment 3)

Mouse group	**Neg**	**CD3**	**CD3/CD4**	**CD3/CD8**	**CD4/CD8 ratio**	**CD19**	**cells/spleen ($x10^6$)**
Group 1	13.1	39.2	27.9	10.6	2.6	47.7	**62**
Group 2	13.0	41.9	30.9	10.5	2.9	45.1	**83**
Group 3	13.9	40.8	29.6	10.4	2.8	45.3	**75**
Group 4	12.9	45.6	32.3	12.1	2.7	41.5	**91**
Group 5	25.8	44.0	30.2	12.9	2.3	30.2	**84**
Group 6	17.8	49.3	33.5	14.9	2.2	32.9	**49**
Naïve mice	**15.9**	**41.4**	**28.3**	**12.3**	**2.3**	**42.7**	**45**

Data on the Naïve mice was obtained from the Vaccine Immunology Laboratory, University of Cape Town.

From these data, comparing the cell populations of the vaccinated animals and the naïve mice, it can be concluded that BCG vaccination does not change the composition of the lymphocyte population but does increase total cell numbers in the spleen, whereas SAAVI MVA-C does not alter spleen numbers.

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3.5.5 Magnitude of V3 CTL-specific and Gag-specific IFN-γ producing CD8 and CD4 T cells induced by the prime-boost vaccination regimen

In order to assess the immune responses elicited by our vaccines, three experiments were performed. Mice were primed with the rBCG vaccines. Control vaccine BCG Pasteur ΔpanCD [pCONEPI] (empty vector) was included. The prime was followed 28 days later with a boost with SAAVI MVA-C as indicated in Table 3.14. For all the prime-boost experiments, spleens were harvested on day 40. A single cell suspension of splenocytes was then used in an IFN-γ ELISPOT assay with BALB/c mouse restricted Gag CD4 and CD8 peptides and the V3CTL peptide. Responses to BCG were also determined using a lysate of BCG as the stimulant. The assay included responses in the absence of peptide (medium control) and an irrelevant peptide so that background responses for the IFN-γ ELISPOT assay could be determined (Section 2.10.4). Data from these background responses were used to determine whether responses to a specific peptide were or were not positive. For all three experiments the mean background responses were 10 ± 5 sfu/10^6 splenocytes. Therefore a response of ≥ to this mean background response ± 1 standard deviation (SD) of this response (i.e. 15 sfu/10^6 splenocytes) was considered to be a positive response. A mean response to the Gag or V3CTL peptides in the IFN-γ ELISPOT assay ≥ 1.5 fold above that of a control BCG prime and SAAVI MVA-C boost was considered a boosted response.

In this study three experiments were done. In the first experiment, the immune responses were assessed only 7 days following vaccination of mice with rBCG vaccines. In the second and third experiments, the prime-boost strategy listed above was followed.

Experiment 1:

For this experiment no positive responses to any peptide above the background of 15 sfu/10^6 splenocytes were detected (data not shown). However there was a response to the BCG lysate (Figure 3.22).

Experiment 2:

This experiment was a repeat of the first experiment but included an additional positive control rBCG vaccine, BCG Pasteur ΔpanCD [pEM19Gag$_2$(V3SV5)], provided by the Vaccine Development Laboratory. The responses to the HIV peptides in the IFN-γ ELISPOT assay are shown in Figure 3.20. Priming with the positive control rBCG vaccine and SAAVI MVA-C boost resulted in an excellent response to the V3CTL peptide as well as the GagCD8 peptide of 7.3-fold and 3-fold respectively above that with a prime with the rBCG control (empty vector) and SAAVI MVA-C boost. For the rBCG vaccines prepared for this project the positive V3CTL peptide boost responses were only 2-3 fold above that with a prime with the rBCG control (empty vector) and SAAVI MVA-C boost. A positive GagCD8 and CD4 peptide boost response of 1.6 fold above that with a prime with the rBCG control (empty vector) and SAAVI MVA-C boost was only achieved with a prime with the BCG Pasteur ΔpanCD [pEM19Gag$_2$V3SV5] vaccine. Despite these positive responses, of concern were the low frequencies of responding cells in the IFN-γ ELISPOT assay achieved for the positive control rBCG vaccine. Of which, the responses were approximately 40% lower than that expected by the Vaccine Immunology Laboratory for this vaccine when used in combination with SAAVI MVA-C. This may have been the result of unexplained mouse health problems.

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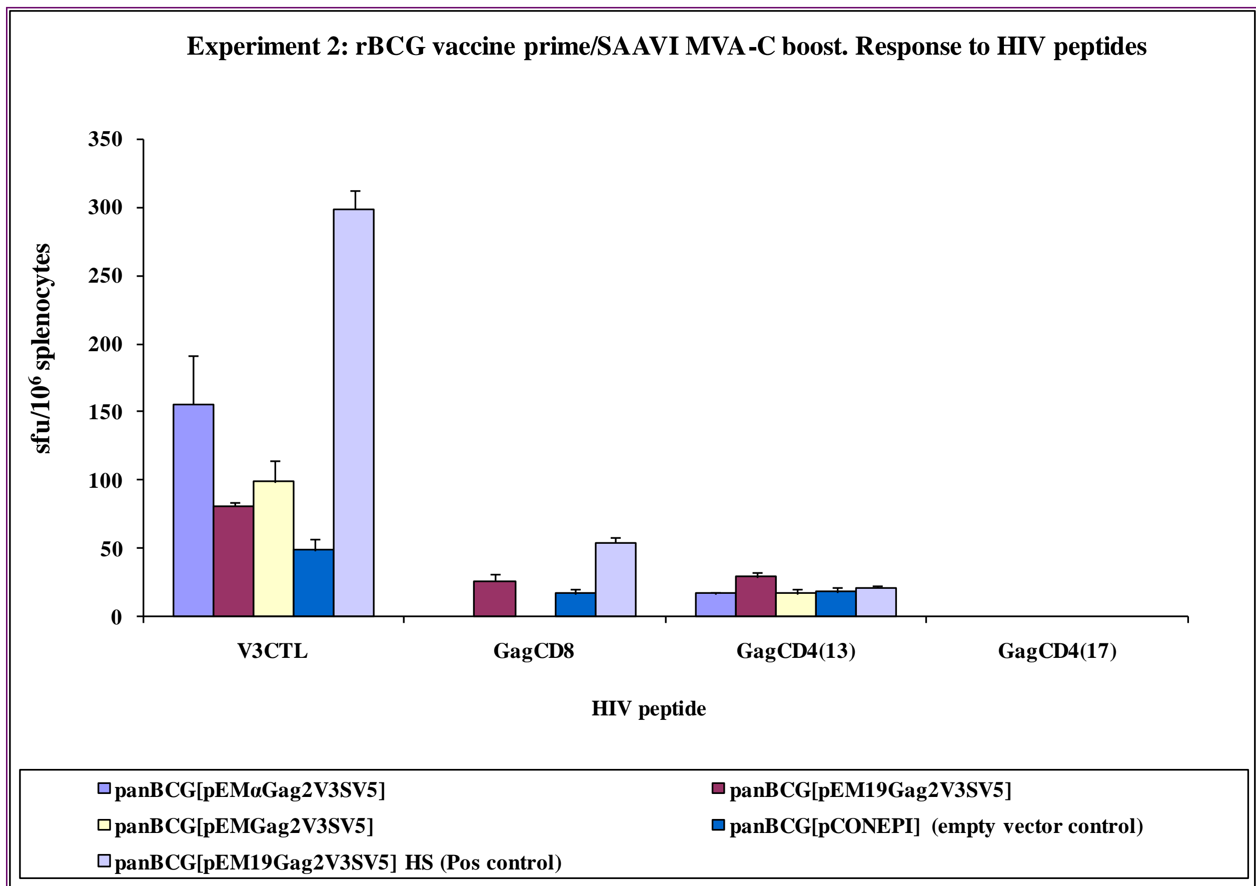

Figure 3.20: Experiment 2. The V3 CTL-specific and Gag-specific IFN- γ ELISPOT responses on day 40 to a prime with the indicated rBCG vaccines (10^7 cfu, i.p.) on day 0 and boost with SAAVI-MVA C (10^4 pfu; i.p.) on day 28. Each bar on the graph represents the average SFU/10^6 splenocytes ± SD (n=3 reactions in the ELISPOT assay) and are positive responses ≥ the background responses of 15 sfu/10^6 splenocytes. pEM19Gag$_2$V3SV5 HS (pos control) depicts the positive control.

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Experiment 3:

A third experiment was performed approximately 9 months after the second experiment. Attention was paid to mouse stock and quality of mouse food and bedding for this experiment. The responses to the HIV peptides in the IFN-γ ELISPOT assay are shown in Figure 3.21. Priming with the positive control rBCG vaccine and a SAAVI MVA-C boost, resulted in the response to the V3CTL peptide being 2.6-fold above that with a prime with the rBCG control (empty vector) and SAAVI MVA-C boost. No boost response to the Gag CD8 and CD4 peptides was observed. A boost of the response to the V3CTL peptide (1.8 fold above the control) was achieved with a BCG Pasteur ΔpanCD [pEMαGag$_2$V3SV5] prime and SAAVI MVA-C boost but no boost of the response of the Gag peptides was observed. For a prime with the BCG Pasteur ΔpanCD [pEM19Gag$_2$V3SV5] vaccine, a boost of the response to the Gag CD8 peptide

was observed (5 fold above the control). No boost response was detected when BCG Pasteur ΔpanCD [pEMGag$_2$V3SV5] was used as the priming vaccine.

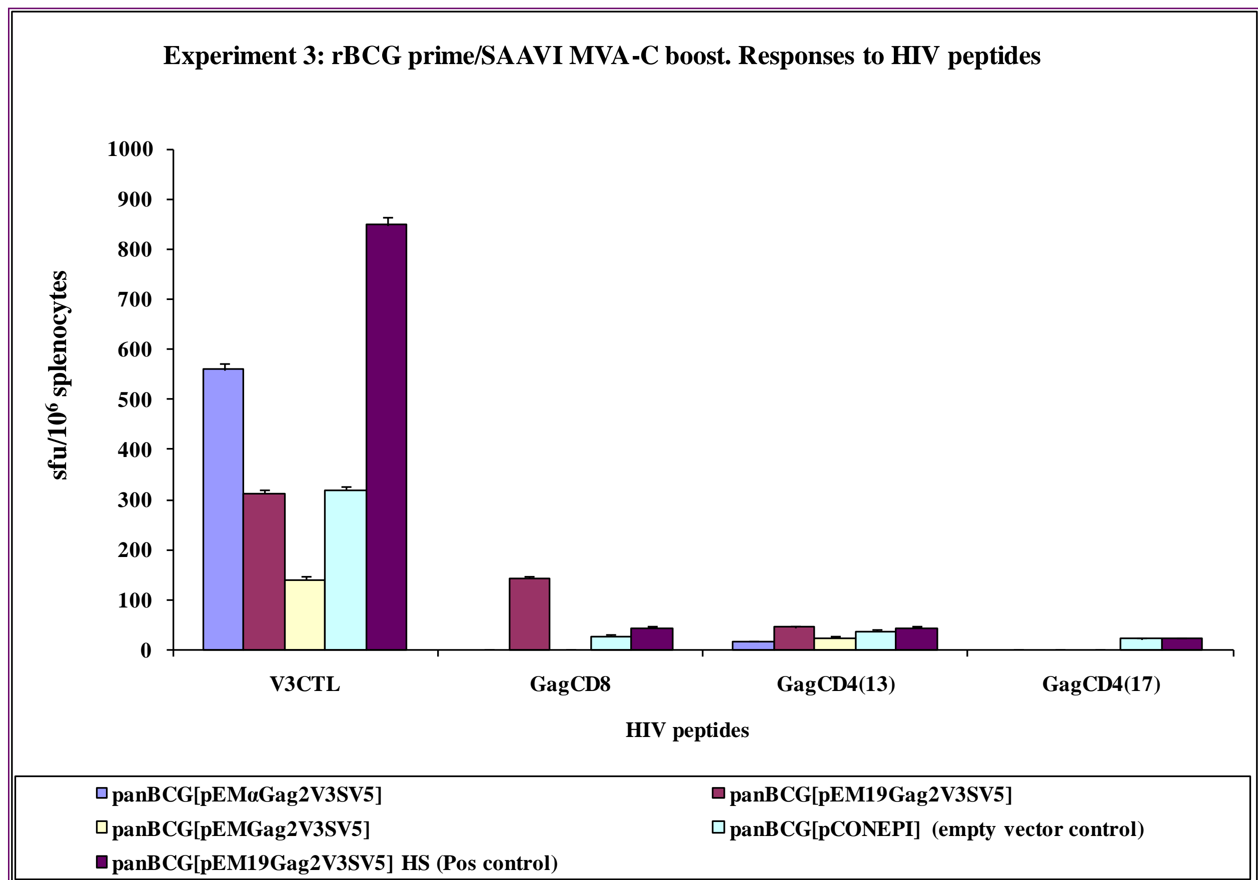

Figure 3.21: Experiment 3. The V3CTL-specific and Gag-specific IFN- γ ELISPOT responses on day 40 after a prime with the indicated rBCG vaccines (10^7 cfu, i.p.) on day 0 and boost with SAAVI-MVA C (10^4 pfu; i.p.) on day 28. Each bar on the graph represents the average SFU/10^6 splenocytes ± SD (n=3 reactions in the ELISPOT assay) and are positive responses ≥ the background responses of 15 sfu/10^6 splenocytes. pEM19Gag$_2$V3SV5 HS (pos control) depicts the positive control.

3.5.6 Responses to BCG

BCG vector responses were also evaluated in the IFN-γ ELISPOT assay using a lysate of BCG as the stimulant. All the BCG vaccines used to prime the immune system induced BCG responses (Figure 3.22). The second experiment resulted in higher BCG responses as compared to the other experiments, with the third experiment eliciting the least responses.

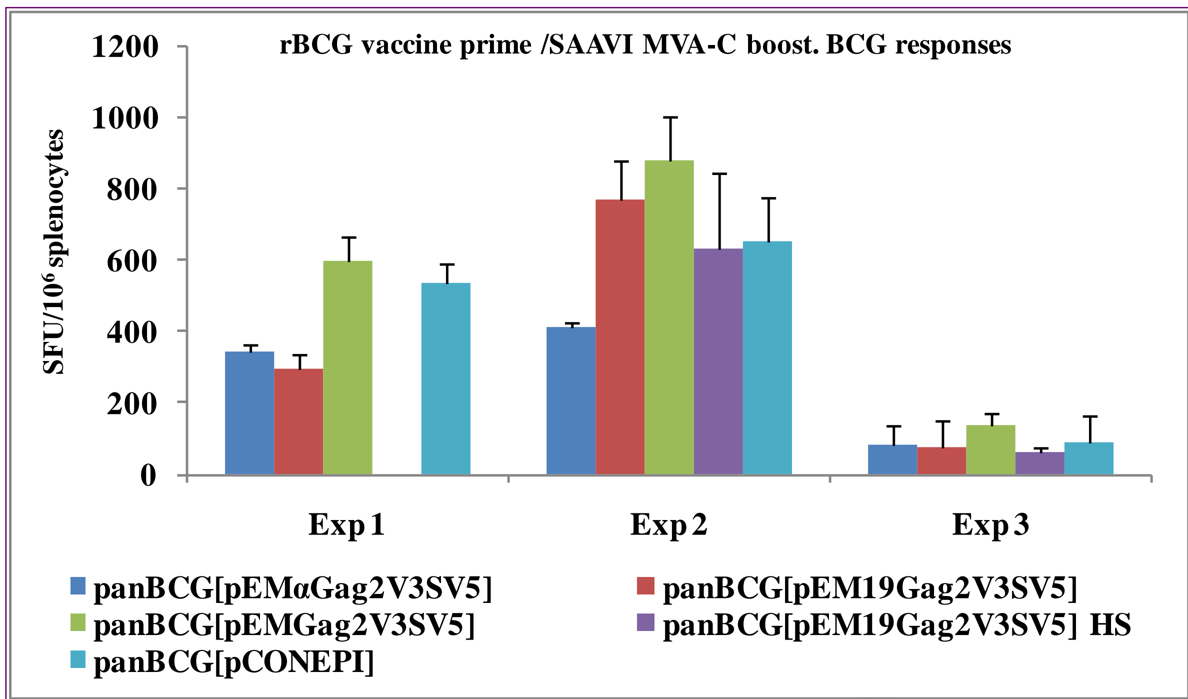

Figure 3.22: Responses to BCG lysate in the IFN- γ ELISPOT assay on day 40 after a prime with the indicated rBCG vaccines (10^7 cfu, i.p.) on day 0 and boost with SAAVI-MVA C (10^4 pfu; i.p.) on day 28. Each bar on the graph represents the average SFU/10^6 splenocytes ± SD (n=3 reactions in the ELISPOT assay) and are positive responses ≥ the background responses of 15 sfu/10^6 splenocytes. pEM19Gag$_2$V3SV5 HS depicts the positive control.

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3.5.7 Responses to SAAVI MVA-C only

In both experiment 2 and 3 a group of mice vaccinated with SAAVI MVA-C only was included. This allowed the effect of a BCG control vaccine prime on responses to SAAVI MVA-C to be evaluated. IFN-γ ELISPOT responses on day 40 to the HIV peptides after a SAAVI MVA-C vaccination on day 28 are shown in Figure 3.23. Data from Experiment 2 and 3 as well as data from the Vaccine Immunology Laboratory files is compared. The response to the V3CTL peptide for the data from Experiment 3 compares favorably with the data from the Vaccine Immunology Laboratory. For the experiments in this study responses to RT were not evaluated but the Vaccine Immunology Laboratory shows the SAAVI MVA-C vaccine induced a predominantly V3 CTL-specific and RT-specific immune responses with very low responses to the Gag peptides. The data from Experiment 2 is low and may be due to the mice used in the experiment. When the responses to HIV peptides after SAAVI MVA-C only vaccination (Figure 3.23) are compared to that with a BCG control vaccine prime and SAAVI MVA-C boost (see Fig. 3.21 & 3.22) it is clear that the immune response to BCG suppresses the response to SAAVI MVA-C.

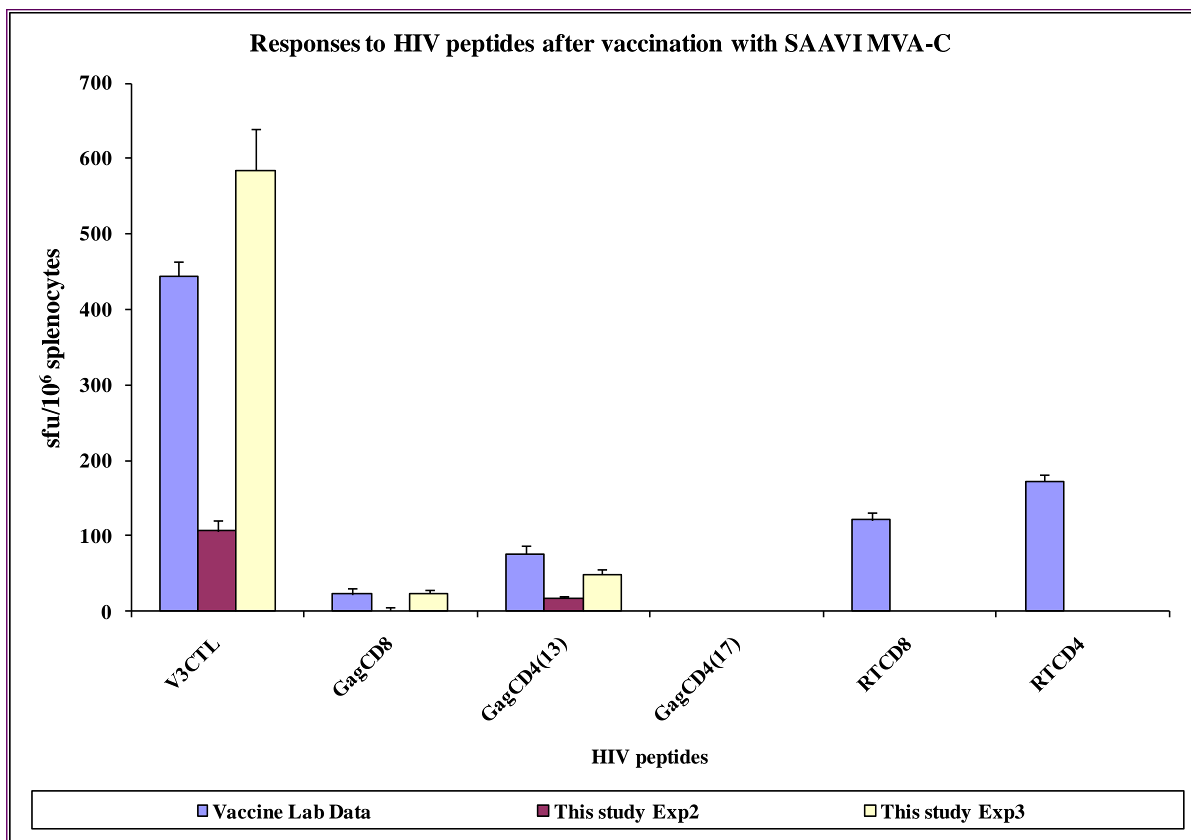

Figure 3.23: Responses to HIV peptides in the IFN- γ ELISPOT assay on day 40 after vaccination on day 28 with SAAVI MVA-C. The data from the Vaccine Immunology Laboratory are the mean ± SD of 3 replicate experiments. The data from Experiment 2 and Experiment 3 are mean values of triplicate responses in the ELISPOT assay.

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3.6 Validation of vaccination dose by plating of rBCG vaccine stocks

Following vaccination of mice, the absorbance readings of the rBCG vaccine stocks were taken in order to validate vaccine dose. These vaccine stocks were also serially diluted and plated as described in Section 2.10.5. Both experiments 1 and 2 indicated inconclusive results as the colony counts did not follow any trend within each vaccination group. However, in experiment 3 the actual readings and rBCG colony counts were lower than expected as shown in Tables 3.15 and 3.16 respectively, whereby the latter table shows that the rBCG numbers of colonies were approximately 50% lower than the expected numbers (only at high colony numbers). Both the absorbance readings and the number of colonies from plates may possibly indicate that the vaccination dose may be lower than expected, though this may be questionable as the lower dilutions yielded similar or slightly higher colony counts than expected.

Table 3.15: The actual and expected OD600nm readings of prepared vaccine stocks

rBCG vaccine colony harboring	Expected OD_{600nm}	Actual OD_{600nm}
BCG Pasteur ΔpanCD [pEMαGag$_2$V3SV5]	10.00	**6.44**
BCG Pasteur ΔpanCD [pEM19Gag$_2$V3SV5]	10.05	**6.72**
BCG Pasteur ΔpanCD [pEMGag$_2$V3SV5]	10.60	**8.20**
BCG Pasteur ΔpanCD [pCONEPI]	9.15	**7.12**

Table 3.16: The rBCG colonies of the vaccine stocks

rBCG vaccine colony harboring	Expected number of colonies	Actual number of colonies		
		Experiment 1	Experiment 2	Experiment 3
pEMαGag$_2$V3SV5	500	**TNTC**	**9**	**158**
	50	**23**	**6**	**30**
	5	**4**	**2**	**5**
pEM19Gag$_2$V3SV5	500	**TNTC**	**74**	**203**
	50	**17**	**21**	**105**
	5	**18**	**11**	**8**
pEMGag$_2$V3SV5	500	**TNTC**	**40**	**220**
	50	**TNTC**	**24**	**76**
	5	**27**	**23**	**11**
pEM19Gag$_2$V3SV5 *	500	**-**	**44**	**192**
	50	**-**	**40**	**57**
	5	**-**	**24**	**6**
pCONEPI	500	**TNTC**	**75**	**190**
	50	**TNTC**	**24**	**72**
	5	**28**	**10**	**30**

TNTC: depicts that the colonies were too numerous to count. * indicates positive control

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3.7. Evaluation of plasmid stability *in vivo*

The splenocytes remaining after the set up of the IFN-γ ELISPOT assay were plated onto selective MB7H10 agar plates to culture recovered rBCG. This was done to determine the plasmid stability *in vivo* by restriction enzyme mapping and evaluation of plasmid loss. Small scale plasmid isolations were done on the recovered rBCG colonies (20 colonies from each vaccine) from mouse splenocytes. The resultant plasmids were mapped using restriction endonuclease digestion. On occasions, large scale plasmid isolation was performed on the same plasmids and two of these were sequenced as described before.

Table 3.17: Evaluation of percentage plasmid loss following culturing of rBCG isolated from mouse splenocytes

Plasmid	***With kanamycin***		***Without kanamycin***		***% plasmid loss***
	Average number of colonies	**SD**	**Average number of colonies**	**SD**	
pEMαGag$_2$(V3SV5)	14	0.71	15	0	**6.7**
pEM19Gag$_2$(V3SV5)	49	1.41	47.5	0.35	**0**
pEMGag$_2$(V3SV5)	46	0.71	37	1.41	**0**
pCONEPI	41	0.71	39	0.71	**0**

SD: standard deviation

All the rBCG colonies recovered from mouse splenocytes carried stable plasmids as shown in Table 3.17, where the rBCG cultures with pEM19Gag$_2$V3SV5 and pEMGag$_2$V3SV5 showed 0% plasmid loss. The rBCG colonies harbouring pEMαGag$_2$V3SV5 showed a plasmid loss of 6.7%, which may be due to the low colony numbers. Therefore, the small difference in the numbers between rBCG cultures plated with or without kanamycin may make a huge difference in the percentage plasmid loss. Furthermore, the plasmids isolated from the rBCG within splenocytes from all groups of mice vaccinated with BCG Pasteur ΔpanCD [pEMαGag$_2$V3SV5], BCG Pasteur ΔpanCD [pEM19Gag$_2$V3SV5] and BCG Pasteur ΔpanCD [pEMGag$_2$V3SV5] were 100% stable (see Figure 3.24 to 3.26 and Table 3.18). Some of the plasmids isolated from the splenocytes of mice vaccinated with BCG Pasteur ΔpanCD [pEM19Gag$_2$V3SV5] (group 2) were incompletely digested by *Nco*I. This is shown by the banding pattern exhibited by a mixture of digested and uncut plasmids in lanes 8, 9 and 12 of Figure 3.25A.

Table 3.18: The expected number of fragments and band sizes of mini and maxi preps of plasmids following digestion with *Nco*I

Plasmid	**Restriction enzymes**	**Expected no. of bands**	**Expected band sizes in bp**
pEMαGag$_2$(V3SV5)	*Nco*I	2	1398, 596
pEM19Gag$_2$(V3SV5)	*Nco*I	2	1398, 5105
pEM Gag$_2$(V3SV5)	*Nco*I	2	1398, 4970

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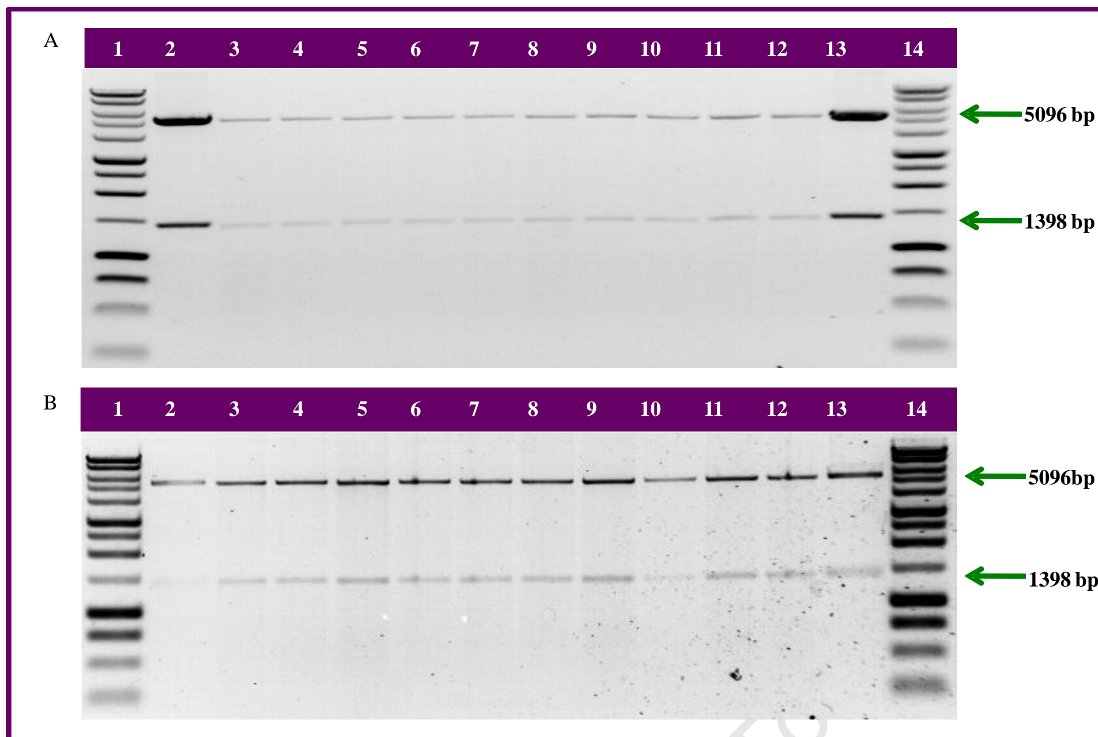

Figure 3.24: Restriction enzyme mapping of pEMαGag$_2$(V3SV5) plasmid DNA extracted from rBCG isolated from mouse splenocytes. Plasmid DNA was digested with restriction enzyme *Nco*I. A & B: Lanes 1 and 14: 1kb DNA molecular weight marker; Lanes 2 and 13: positive control, original, untransformed DNA; Lanes 3-12: plasmid DNA extracted from rBCG isolated from mouse splenocytes.

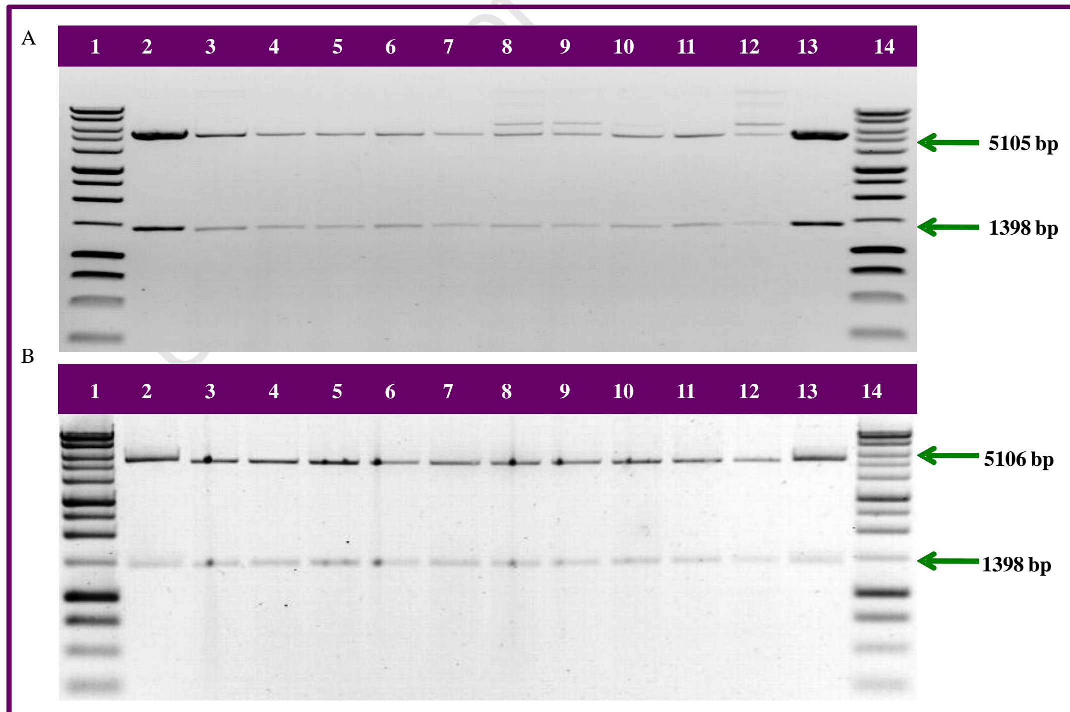

Figure 3.25: Restriction enzyme mapping of pEM19Gag$_2$(V3SV5) plasmid DNA extracted from rBCG isolated from mouse splenocytes. Plasmid DNA was digested with restriction enzyme *Nco*I. A & B: Lanes 1 and 14: 1kb DNA molecular weight marker; Lanes 2 and 13: positive control, original, untransformed DNA; Lanes 3-12: plasmid DNA extracted from rBCG isolated from mouse splenocytes.

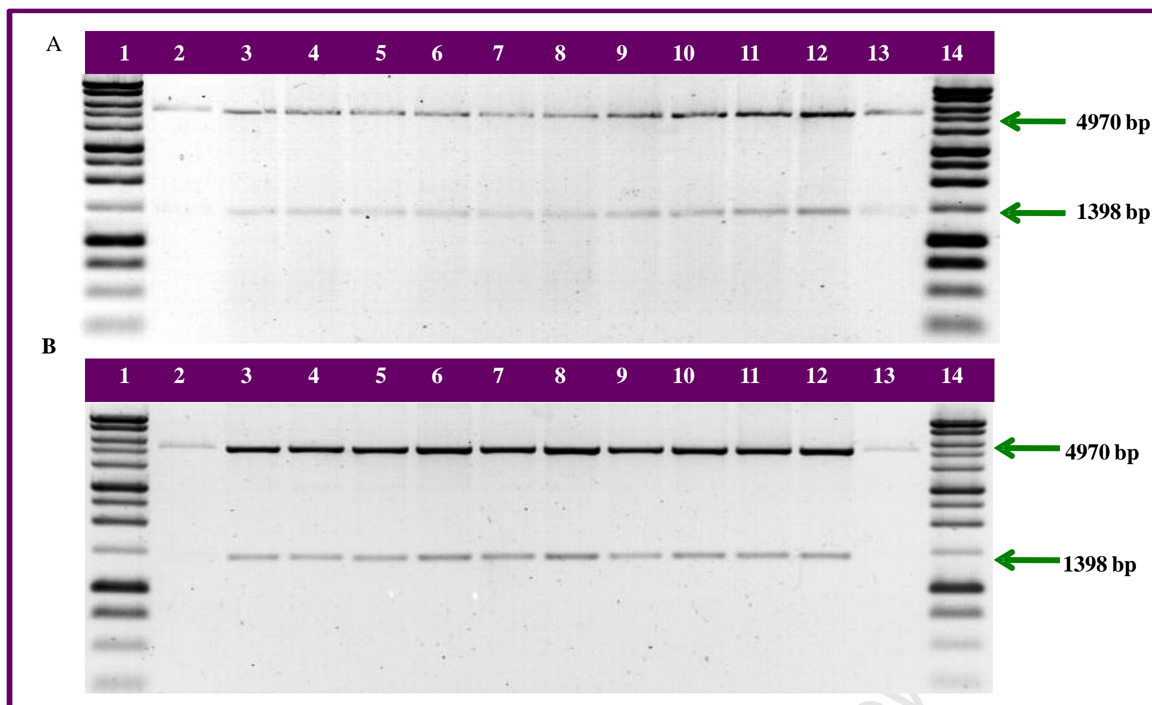

Figure 3.26: Restriction enzyme mapping of pEMGag$_2$(V3SV5) plasmid extracted from rBCG isolated from mouse splenocytes. Plasmid DNA was digested with restriction enzyme *Nco*I. A & B: Lanes 1 and 14: 1kb DNA molecular weight marker; Lanes 2 and 13: positive control, original, untransformed DNA; Lanes 3-12: plasmid DNA extracted from rBCG isolated from mouse splenocytes.

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The V3 CTL boost responses were lower than expected and the Gag CD8 responses were higher following priming with BCG Pasteur ΔpanCD [pEM19Gag$_2$V3SV5]. We therefore sought to address this by further restriction enzyme mapping of pEM19Gag$_2$V3SV5 extracted from rBCG recovered from mouse splenocytes as well as sequencing this entire insert; *mtrA*-19kDa signal sequence-HIV-1 gag-V3 CTL epitope-SV5 monoclonal antibody tag with the primers shown in Table 3.19. This was done in order to determine whether a deletion, insertion of gene rearrangement might have occurred between the Gag CD8 and the V3 CTL epitopes. The restriction enzyme mapping in Figure 3.27 shows that the recovered plasmids were 100% stable and there were no mutations in the sequenced insert (data not shown).

Table 3.19: Primers, their sequences and the sides of binding on the constructed plasmids

Primer series	Primer sequences	Binding sites on plasmids
pCONEPI-F	TGGCGAACTCCGTTGTAGTG	60bp upstream of the *Xba*I site in the vector backbone
pCB119-F	CCATATGAAGCGTGGACTGAC	About 160bp downstream of ATG of pEM19 vectors (for only pEM vector backbones)
pFRF 4	ACACCAAGGAGGCCCTGG	About 5097bp on pEM19Gag$_2$V3SV5 (suitable for Gag$_1$ or Gag$_2$ plasmids)
pFRF 6	TCCGCGACTACGTGGACC	About 5684bp on pEM19Gag$_2$V3SV5 (suitable for Gag$_1$ or Gag$_2$ plasmids)
pFRF 7	CGCAAGAAGGGCTGCTGG	About 6034bp on pEM19Gag$_2$V3SV5 (suitable for Gag$_1$ or Gag$_2$ plasmids)

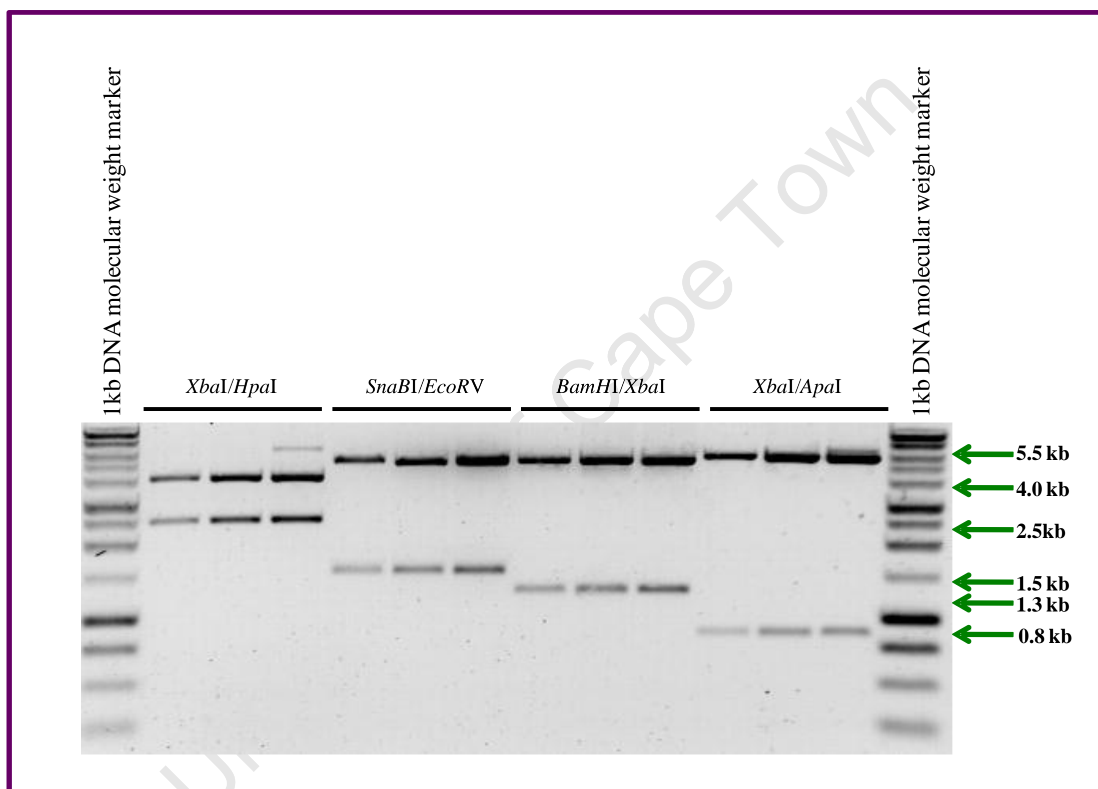

Figure 3.27: Restriction enzyme mapping of pEM19Gag$_2$(V3SV5) plasmid DNA. Lanes 1 and 14: 1kb DNA molecular weight marker; Lanes 2, 5, 8 and 11: positive control, original, untransformed DNA; Lanes 3, 6, 9 and 12: plasmid DNA extracted from rBCG isolated from mouse splenocytes (clone 8); Lanes 3, 6, 9 and 12: plasmid DNA extracted from rBCG isolated from mouse splenocytes (clone 16).

Table 3.20: The expected number of fragments and band sizes of pEM19Gag$_2$(V3SV5) plasmid following double digestion with various enzymes

Plasmid	Restriction enzymes	Expected no. of bands	Expected band sizes in bp
pEM19Gag$_2$(V3SV5)	*Xba*I/*Hpa*I	2	2457, 4046
	*SnaB*I/*EcoR*V	2	1500, 5003
	*BamH*I/*Xba*I	2	1263, 5242
	*Xba*I/*Apa*I	2	862, 5641

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CHAPTER 4: DISCUSSION

The aim of the study was to select a stable plasmid backbone for the construction of rBCG expressing HIV-1 Gag under the control of different promoters and with different leader sequences. These shuttle vectors stability and immunogenicity was then compared.

4.1 Construction of stable vaccine shuttle vectors

E. coli-mycobacterium shuttle vectors have been widely used for the delivery of foreign genes into BCG (Chege et al. 2009, Ami et al. 2005, Cayabyab et al. 2009). In developing such vectors: the choice of promoter, such as *mtrA* or *hsp60* (Zahrt, Deretic 2000); codon optimization of the foreign gene for expression in mycobacteria (Kanekiyo et al. 2005); the use of leader sequences (19kDa or α) to facilitate secretion of the foreign antigen (Al-Zarouni, Dale 2002, Himmelrich et al. 2000) and the use of episomal or integrative vectors are amongst the plasmid vector features that may affect vector stability.

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In our laboratory, it was shown that from the same pCB119 vector backbone, there was an increased expression of Gag driven by the *mtrA* promoter as compared to the *M. tuberculosis katG* promoter (Thomas 2005). It was also demonstrated that vaccination with rBCG harboring plasmid pRT106 (pCB119 backbone, 19kDa signal peptide, *mtrA* promoter and *gag*) elicited better cellular and humoral immune responses as compared to pRT108 (same vector, but with *katG* promoter) in mice. Such responses were attributed to increased *gag* expression from a relatively stable rBCG vaccine (Thomas 2005). Another study conducted in our lab in non-human primates by Chege et al. (2009) gave similar results, where immune responses following priming with the same rBCG and boosting with Gag VLPs were evaluated. The IFN-γ response to Gag peaked 2 weeks after the boost to a mean of 677 and 274 SFU/10^6 PBMC for BCG [pRT106] and BCG [pRT108] primed baboons respectively. Lower responses in the IFN-γ ELISPOT assay after the prime with RT108 and boost with Gag VLPs suggests levels of Gag expression determine the success of priming the immune system. It has therefore been speculated that the stability and increased expression levels of Gag may also be enhanced by cloning it into a more stable vector backbone.

4.2 Selection of an optimal episomal vector

High expression levels of heterologous antigens can lead to a toxic and/or a metabolic burden on bacterial cells (Joseph et al. 2010, Al-Zarouni, Dale 2002). This leads to slower growth rates and can result in genetic instability of the gene being expressed, since as soon as a mutant that does not express the heterologous antigen arises, it will have a growth rate advantage and will therefore out-compete the rest of the colony or culture. On solid media, slow growth rates are normally indicated by small colony size. This is reflected in relative colony sizes wherein; the larger the colonies, the more 'healthy' the recombinants and the smaller the colonies, the more stressed or 'unhealthy' the recombinants are. Such a phenomenon was exploited in this study whereby fitness of the shuttle vectors with different vector backbones was evaluated by growth of recombinant *M. smegmatis* on solid media.

In the current study, the colony sizes were rated relative to the positive control with the pCONEPI plasmid, as it is an empty vector only expressing the kanamycin resistance gene. The reason being this vector would experience no metabolic burden as it does not express any foreign genes such as *gag*. In this study, it was evident that *M. smegmatis* [pCB119Gag] colonies grew at a slower rate than *M. smegmatis* [pEM19Gag] colonies, resulting in slightly smaller colonies on the transformation plates. This was also observed in liquid medium and may be attributed to the metabolic burden placed on the cell by the *lysA* gene expression, thus resulting in retarded growth rate. An interesting observation from this study is that, even when the codon optimised gag was inserted into both vectors (pCB119 or pEM19), in liquid media *M. smegmatis* [pCB119Gag] cultures still grew at a slower rate than *M. smegmatis* [pEM19Gag], thus indicating vector fitness was enhancement once the *lys A* gene was removed from the backbone.

An accurate measurement of plasmid stability is hard to achieve because of the well-known fact that mycobacteria exist as clumps that are very difficult to disperse (Parish, Stoker 1998). This then makes it difficult to measure growth rates in liquid culture by means of absorbance readings. Therefore, growth rates cannot be solely used to evaluate stability, but can infer vector fitness. For this reason, it has been documented that to determine plasmid stability in mycobacteria; cultures are serially passaged by re-inoculating into fresh media in the absence of antibiotic until the bacterial cells have passed through a number of generations and then these are plated on both selective and non-selective solid media (Bourn et al. 2007, Griffin et al. 2009). This will reflect on the plasmid retention, which indicates plasmid stability. In this, study the

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recombinant *M. smegmatis* (after 10 generations) cultures were serially plated onto both selective and non-selective solid media (agar plates) and colonies were counted to evaluate percentage plasmid loss as an indicator of stability after a number of generations, as well as to infer possible rearrangements.

What was striking was that the cultures harbouring the pCB119Gag vector showed a great deal of plasmid loss (54%) as compared to those with pEM19Gag. This indeed revealed the detrimental effects of the presence of *hsp60-lysA* on vector fitness and stability. For these reasons, it was speculated that pCB119 vector backbone was not very stable and we therefore based our vectors on the pEM backbone.

4.3. Construction of rBCG shuttle vectors

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In order to assess the effect of addition of signal peptides , inducible promoters as well as codon optimised Gag on vector fitness *in vitro* and also to determine their stability and immunogenicity in *M. smegmatis* and BCG respectively six vectors with differing features were successfully designed and constructed. The first three shuttle vectors derived from the following parental plasmids; pEMαGag$_2$(V3SV5)gp33-41, pEM19Gag$_2$(V3SV5)gp33-41 and pEMGag$_2$(V3SV5)gp33-41 were successfully constructed. The other three shuttle vectors pEMα(V3SV5), pEM19(V3SV5) and pEM(V3SV5) were designed such that they lacked the HIV-1 *gag* gene and were constructed from the first three vectors. These constructs were used as controls, most especially during the evaluation of Gag expression by HIV-1 p24 assays. Three of these vectors were selected in order to determine the influence of the signal peptides on the immunogenicity of HIV-1 Gag antigen following vaccination of BALB/c mice with such rBCG vaccines. In essence the vectors were constructed such that they contained unique features that will allow for induced expression of codon optimized *gag*, which may be directed to the cell membrane, secreted or remain in the cytoplasm. The addition of these features on vector fitness and *in vitro* stability was evaluated before vaccination of mice.

4.4 Determination of vector fitness by growth rates and evaluation of plasmid stability

In this study, we evaluated the effect of addition various shuttle vector features on vector fitness. Promoter elements such as inducible *mtrA* as well as constitutive *hsp60* and p_{scmyc} promoters, have received particular attention, as these regulate the level of expression of a foreign gene as a function of promoter strength and the timing of expression according to promoter activity. Other vector features that also influence expression levels include leader sequences (19kDa or α) used to target the protein within the BCG cell (Zahrt, Deretic 2000, Via et al. 1996) and inserted foreign DNA sequences (Dennehy et al. 2007). It has been shown that stability of recombinant BCG antigen delivery systems is influenced by these shuttle vector features (Joseph et al. 2010).

Use of episomal vectors in rBCG has been shown at times to result in high levels of stability in vitro. For example, Horwich et al. (1985) showed that plasmids responsible for expressing *M. tuberculosis* α-antigen in BCG were stable over six consecutive 4-week cultures without antibiotic selection and changes in the level of expression. In addition, Kawahara et al. (2002) were able to show that rBCG could express the antigen containing the 19 amino acid V3 peptide even after at least 450 passages. However, some studies have emphasized the deleterious effect of *hsp60* promoter on the expression of some foreign genes in mycobacteria (Joseph et al. 2010, Dennehy et al. 2007), which may pose a disadvantage relative to the inducible *mtrA* promoter that has lower activity during *in vitro* growth, but is induced on infection of macrophages (Al-Zarouni, Dale 2002, Zahrt, Deretic 2000) thus driving the expression of a foreign antigen at a time when the antigen could be exposed to the immune system.

Carroll et al. (2010) showed that fluorescence from the high copy number plasmids expressing Coral *Discosoma striata* red (*Ds*Red) reporter genes under the *hsp60* promoter was not higher than for the low copy number plasmid in *M. smegmatis*. Plasmids recovered from non-fluorescent colonies had deletions in the promoter-fluorescent protein region, whereas plasmids recovered from the coloured colonies appeared intact, confirming that loss of expression was due to the acquisition of deletions in the plasmids. The group also indicated that using p_{smyc} promoter resulted in 100% of all transformants fluorescing when low copy number plasmids were transformed into *M. tuberculosis.* On the contrary, *hsp60* driven expression of the reporter gene resulted in fluorescence of only 5% of the transformants. After three passages of recombinant *M. tuberculosis,* there was no decrease in the fluorescence intensity of the Psmyc-reporter plasmids.

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However, *hsp60* promoter-driven expression decreased over time, suggesting accumulation of deletions or mutations in the plasmid (Carroll et al. 2010) thus indicating lack of vector fitness. Gall, Barker (2006) also showed that foreign antigen expression under the control of another strong mycobacterial promoter such as G13 caused deletions of upstream promoter regions, thus resulting in 20-40 fold reduction of GFP expression over all growth phases.

Similarly, in our study high levels of constitutive expression of Gag driven by either the *hsp60* or p_{smyc} promoter contributed to reduced vector fitness, thus resulting in small or no colonies. This is in contrast with the inducible expression of *mtrA* promoter that resulted in large, healthy looking colonies. This could be attributed to reduced HIV-1 Gag expression *in vitro*, which in turn leads to a lower metabolic burden on the recombinant mycobacteria. We also evaluated the *mtrA* promoter driven expression of HIV-1 Gag by culturing r*M. smegmatis* cells and then isolating the cell free extract to evaluate the Gag p24 levels. The three recombinants expressing Gag from this inducible promoter expressed far lower levels of p24 *in vitro* as compared to the recombinant mycobacteria expressing Gag from the constitutive *hsp60* promoter, thus emphasising that use of *mtrA* may result in low level *in vitro* expression of foreign antigens. In another study by Zahrt, Deretic (2000), S1 nuclease protection of total cellular RNA-ssDNA probe hybridisation products and primer extension analysis reflected that *mtrA* was expressed at low levels during *in vitro* growth.

Extracellular foreign antigen expression by signal peptides may result in higher expression levels than intracellular expression of the same antigen (Al-Zarouni, Dale 2002). This is because expressed foreign antigens, which remain within the cell, can lead to increased metabolic burden or protein toxicity, whereas extracellular secretion or lodging of these antigens by signal peptides into the membrane may result in reduced protein toxicity, thereby increasing vector fitness.

On the contrary, in this study, it was shown that the recombinant *M. smegmatis* [pEMGag$_2$V3SV5] colonies (with no signal peptide) had similar colony size as tranformants harbouring vectors with either 19kDa or α-antigen signal peptides. This was not expected and may have been because the plasmid had deletions that gave such mutants a growth advantage over the others. There was no indication of this in our vaccine stocks; however, after 10 generations in liquid of culture *M. smegmatis* [pEMGag$_2$V3SV5] showed 11% plasmid loss as compared to 0% of pEMαGag$_2$V3SV5 plasmid, which was not a great loss. Increased expression of HIV-1 p24 Gag from *M. smegmatis* [pEMαGag$_2$V3SV5] cell free extracts indicated that

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secretion of foreign antigen may result in higher expression levels. Similarly, Al-Zarouni, Dale (2002), provided evidence that the 85A (α-antigen) secretion signal peptide markedly enhanced the levels of cell-associated product, while the 19-kDa signal sequence had little effect on gene expression.

One strange observation was that *M. smegmatis* [pEM19Gag$_2$V3SV5] cultures grown with selection in liquid medium exhibited a slightly slower growth, which was in contrast with the relative 'health' seen when the same culture was grown on solid medium. One possible reason, which could not be explained, would be that the cultures harbouring such a vector (with the 19kDa signal peptide) may show differential growth patterns in either liquid or in solid media. One important factor to note is that, since the α-antigen and 19kDa direct the expression of antigens to the extracellular matrix and membrane respectively, the accurate determination of Gag expression levels may be limited to that only intracellular HIV-1 Gag p24 levels were measured.

Another possibility of different expression levels from the cultures harbouring plasmids with different signal sequences may be due to the presence or absence of 'downstream boxes' (DB). These are 12-15 base long sequences downstream of the mRNA initiation codon. This box is complementary to the 16s RNA thus enhancing the binding of the ribosome to the RNA and translation initiation. Rush, Steyn 2005 used codon optimised DB and a strong *katG* promoter driving luciferase expression to evaluate their effect on translation. This group showed increased expression of luciferase from plasmids without Shine Dalgarno (SD), but with DB in both *E. coli* and *M. smegmatis*. DB-specific effect of translation was also seen as plasmids with *E. coli* optimised DB showed increased expression in *E. coli* than in *M. smegmatis* and the reverse effect was seen as when *M. smegmatis* DB optimised plasmids were transformed into both *M. smegmatis* and *E. coli* (Rush, Steyn 2005). The specificity and efficiency of DB has previously been shown in *E. coli* (Sprengart et al. 1996). Since the constructs used in this study did not have any obvious ribosome binding sites, it was found out that only pEM19Gag$_2$V3SV5 plasmid contained a potential DB (though it was only about 60% identical to other mycobacterial down stream boxes), which did not enhance Gag expression.

We showed that the addition of HIV-1 *gag* did not have any effect on vector fitness, but its codon optimisation did enhance vector fitness as seen by larger colonies. The increase in colony size may be due to codon optimisation of the gene for expression in mycobacteria. As the *mtrA*

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promoter expresses at such a low level in culture, levels of Gag expression were probably very low and therefore this could be why little or no difference was seen when *gag* was included in the vector.

Similarly, when grown in non-selective medium (without kanamycin), all the cultures harbouring vectors with the codon optimised *gag* showed the faster exponential growth as compared to non-codon optimised *M. smegmatis* [pEM19Gag] cultures, which also only 8% plasmid loss. From these results, we observed that codon optimization of *gag* resulted in increased vector fitness and plasmid stability, which is in agreement with the studies carried out by Kanekiyo et al. (2005), whereby the group showed that codon optimization of *gag* resulted in increased expression levels.

4.5. Immunogenicity of rBCG vaccines in mice

Dennehy et al. 2007 showed the absence of antibodies against rotavirus following vaccination of mice with rBCG expressing antigens from episomal vectors with either the *M. leprae* 18kDa promoter and α- antigen signal peptide (pCB112-Edim6) or the *mtrA* promoter and *M. tuberculosis 19kDa* signal sequence (pCB119-Edim6). The group proposed that the lack of antibodies might be attributed to lack of antigen expression, low export of this antigen or inability of lodging to the membrane, and its subsequent lack of presentation to B cells. The mice were also not protected from challenge virus, which collectively indicated that levels of antigen expression might have an effect on the immune responses.

Similar results are obtained in this study whereby in this case, low cell mediated immune responses were elicited by rBCG vaccine expressing HIV-1 Gag from episomal vectors with either the 19kDa or α–antigen signal sequences as well as those without any leader peptides. This was the case with all the three experiments. In the first experiment, since the mice were not boosted with SAAVI MVA-C vaccine, the low immunogenicity of the vaccines may be accounted for by lack of or delayed immune activation of the CD8 T-cells. Russell et al. (2007) showed that CD8+ T cells that differentiate during infection of mice with BCG undergo limited activation during the first 7 days of infection, the response peaks during the third week of infection, followed by a protracted and reduced contraction phase. The group proposed that the delay in generating a rapid primary CD8+ T cell response against BCG could be due to the induction of inappropriate inflammatory signals by BCG, or due to the reduced generation of

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antigenic load *in vivo*. However, this may not entirely apply to this study as stimulation of splenocytes from vaccinated mice with a BCG lysate did induce BCG-specific responses. In the second experiment, since the BCG-specific responses as well as low magnitude Gag-specific responses were also induced and also the SAAVI MVA-C boosted the responses, the health of the mice may have been an issue that led to reduced immunogenicity of the vaccines.

Comparing our results in experiment 3, vaccination with BCG Pasteur ΔpanCD [pEMαGag$_2$V3SV5] resulted in the highest (a positive V3 CTL boosted responses), which may be due to the secreted nature of Gag. The increased Gag CD8$^+$ T-cell responses elicited by BCG Pasteur ΔpanCD [pEM19Gag$_2$V3SV5] may have been elevated because of low immune-dominant V3 CTL immune responses. *M. smegmatis* [pEM19Gag$_2$V3SV5] cultures grown with selection in liquid medium exhibited slow growth which was in contrast with the relative 'vector fitness' (larger colony size) seen when the same culture was grown on solid medium. Therefore, since the vaccines were prepared as liquid cultures, the same scenario may have been experienced, whereby the rBCG with the same plasmid may have showed slowed growth, with low Gag expression that may consequently have a negative effect on immunogenicity. This may possibly explain why vaccination with BCG Pasteur ΔpanCD [pEM19Gag$_2$V3SV5] elicited low immune responses to V3 CTL, however the disparity in the growth patterns of cultures in either liquid or solid media could not be explained.

Several other factors may have contributed to the low immunogenicity of these vaccines; firstly, vector stability *in vivo* may have been an issue. However, following isolation of plasmids from rBCG recovered from mouse splenocytes, we discovered that all the plasmids were 100% stable, thereby ruling out vector stability as a contributing factor. Another factor that may have contributed to low immunogenicity of our vaccines may be low vaccination dose. The remainder of the vaccines following vaccination showed markedly lower absorbance readings than expected and the rBCG colonies plated from the vaccine stocks before vaccination were unexpectedly low, only at the higher dilutions. These then reflect that the vaccine dose may have been lower than the expected 1 x 10^7cfu/mouse, thereby leading to reduced immunogenicity levels.

Similarly, Lesellier et al. (2006), when evaluating the safety and immunogenicity of BCG Danish strain 1331 in European badgers (*Meles meles*) showed that repeat injection of the lower BCG dose induced a response of reduced magnitude and persistence compared with the high

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dose vaccination, despite prior sensitization to BCG. On the contrary, both Phase I studies of a recombinant replication-defective adenovirus type 5 (rAd5) vector expressing HIV-1 Gag and Pol from subtype B and multiclade Env, given alone showed no significant effect of rAd5 dosage on immunogenicity endpoints (Jaoko et al. 2010, Peiperl et al. 2010). The disparity in these results may be argued that the type of vector used may be influential in the magnitude and breadth of immune response elicited. For this reason, since we used a bacterial vector, the vaccine dose may have played a critical role in the immunogenicity of our vaccines, though the statement may not be conclusive. Russell et al. (2007) also suggested that for pathogens that display poor *in vivo* growth (such as BCG), the dose of the pathogen can enormously modulate the differentiation of CD8+ T cell response.

4.6 Conclusions and future work

This study showed that the use of the pEM19 vector backbone as opposed to pCB119 vector backbone resulted in increased vector fitness and stability. In addition, the use of signal peptides to direct expression foreign antigens to either the extra-cellular matrix or the cell wall may be beneficial in enhancing the immune responses elicited by rBCG vaccine harbouring such vectors. Therefore, as seen in this study and many others, the choice of vector backbone, the use of inducible promoters, antigen secretion to enhance the immunogenicity and to prevent foreign proteins from becoming toxic to BCG and codon optimization of the foreign gene to increase expression are some of the factors that enhance vector fitness and stability. In the immunology experiment 2, a positive GagCD8 and CD4 peptide boost response was only achieved with a prime with the BCG Pasteur ΔpanCD [pEM19Gag_2V3SV5] vaccine. Despite these positive responses, of concern were the low frequencies of responding cells in the IFN-γ ELISPOT assay achieved for the positive control rBCG vaccine. In experiment 3, a boost of the response to the V3CTL peptide was achieved with a BCG Pasteur ΔpanCD [pEMαGag_2V3SV5] prime and SAAVI MVA-C boost but no boost of the response of the Gag peptides was observed. No boost response was detected when BCG Pasteur ΔpanCD [pEMGag_2V3SV5] was used as the priming vaccine. Therefore, BCG Pasteur ΔpanCD [pEMαGag_2V3SV5] maybe a promising vaccine as seen by increased immune responses to the V3 CTL epitope as well as by the increased Gag p24 levels following transformation of *M. smegmatis* with the same vector.

The use of small foreign DNA sequences (conserved epitopes) instead of the full-length *gag* may be considered in the future to increase epitope coverage in order to increase antigen specific

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immune responses. Furthermore, the detailed profiling of cell-mediated immune responses elicited by these vaccines may be a way forward, not ruling out the validation of vaccine dose before vaccination. Most importantly, in this study various signal peptides and the *mtrA* promoter were included in the vectors, therefore future studies could include the comparison of Gag expression and localization from these rBCG vaccines using *in vitro* mouse derived macrophage cultures as well as use of fluorescent microscopy in order to better understand the relationship between antigen presentation and the nature of the immune response elicited. Moreover, transcriptome analysis (mRNA profiling) would also be an informative approach in order to evaluate the different messages relayed by such vaccines in mice.

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