RECOMBINANT BCG EXPRESSING HIV-1 C GAG: SELECTION OF THE VACCINE GENE AND CONSTRUCTION AND EVALUATION AS A VACCINE CANDIDATE

University of Cape Town

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ABSTRACT

This study was undertaken as part of the efforts of the South African AIDS Vaccine Initiative (SAAVI) to develop a locally relevant HIV-1 vaccine for southern Africa. The use of recombinant bacille Calmette-Guerin (rBCG) as a vaccine vector was followed for this work.

The first part of this project was the selection of an HIV-1 isolate for use in the development of HIV-1 Gag vaccines targeted for South Africa. This was done following a sequence survey of representative, recently transmitted isolates obtained from different sites in South Africa. After deriving a South African subtype C *gag* gene consensus sequence from the viral sequences, isolate Du422 was chosen because it was most closely related to this consensus sequence. Du422 was found to be 98.7% related to the South African consensus sequence. In addition, it was 96% similar to the derived ancestral sequence and 97% similar to the derived consensus sequences for the whole of subtype C. An updated analysis using a larger database of sequences and an updated consensus sequence did not alter the close relationship it has to the consensus, implying that the selection was still relevant. Immunologically, Du422 was also a good choice for vaccine strain since the amino acid sequence contains T-cell epitopes recognized by southern African individuals infected with subtype C HIV-1. These epitopes were in regions of the *gag* gene with a limited ability to change and thus important for inclusion in a vaccine.

The rBCG model was chosen for this study because it is inexpensive to produce, is heat stable and can induce long lived immunity. Shuttle vectors for use in rBCG were constructed using the Du422 *gag* gene and evaluated for stability and Gag expression *in vitro* to determine which candidate(s) would be best suited for immunogenicity testing in mice. The *gag* gene from isolate Du422 was amplified and cloned in 8 different *E.coli*/*Mycobacterium* shuttle vectors which were either integrated into the host cell genome or were maintained episomally. The shuttle vectors tested four promoters – *hsp60*, *M. leprae* 18kDa, *mtrA* and *katG* – for Gag expression in combination with either the α-antigen signal sequence or the 19kDa lipoprotein signal sequence. Since genetic stability and good expression levels are paramount for generating a good immune

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response from rBCG vaccines, the genetic stability of the shuttle vectors and expression of Gag were studied in *M.smegmatis*. Southern blotting of genomic DNA demonstrated that recombinant *M.smegmatis* (rMS) carrying integrative vectors pRT101, pRT102, pRT103 and pRT104 were highly stable over ~ 40 generations, with no shuttle vector rearrangements identified. Restriction enzyme mapping of the shuttle vectors from recombinants carrying the episomal vectors pRT105, pRT106, pRT107 and pRT108 revealed that they were less stable over the same time period. Shuttle vectors contained deletions in the promoter/signal sequence region and in the *gag* gene. rMS:RT108 was found to be the most stable, while rMS:RT106 displayed moderate stability. rMS:RT105 lost stability completely within the same time period while rMS:RT107 retained only 40% integrity.

The Gag expression levels in these rMS cultures were also tracked and the results indicated that the integrative constructs had a low level of expression that was maintained. Three patterns were observed for the episomal constructs: high initial expression followed by a rapid decline in the levels of Gag in the culture, a moderate expression which dropped more slowly and a low but fairly constant expression level. The higher genetic stability of the integrative constructs was associated with the low level of expression. In general, the higher expression levels in the episomal constructs were associated with a more rapid accumulation of deletions and low genetic stability. These results were supported by an indirect evaluation of the constructs investigating the metabolic load placed on the recombinants by the expression of Gag. A high metabolic load/high expression levels yielded smaller colonies and allowed the bacteria to survive heat shock at 42°C compared to recombinants carrying an empty vector. The reverse was true for the low expression levels. Based on these studies, the medium stability/high-moderate expresser pRT106 (*mtrA* promoter + 19kDa signal sequence) and the high stability/low expresser pRT108 (*katG* promoter + 19kDa signal sequence) were selected for rBCG studies.

The pRT106 and pRT108 shuttle vectors were introduced into BCG and the expression of Gag from the recombinants was evaluated in culture and in infected macrophages. Gag levels formed

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a small percentage of the total protein in culture but transport of Gag to the cell surface was demonstrated for both constructs. Expression of Gag from rBCG:RT106 and RT108 Tokyo in macrophages was also successfully demonstrated.

The immunogenicity of both rBCG:RT106 and rBCG:RT108 were evaluated in the BALB/c mouse model. A single inoculation of rBCG:RT106 (Pasteur) at 10^3 or 10^5 cfu induced BCG-specific IFN-γ and IL-4 responses in vaccinated mice. However, no Gag-specific IFN-γ or IL-4 production or anti-p24 antibodies were detected.

A DNA:Gag prime followed by a rBCG (RT106 or HPV-16 L1) boost at 10^3 or 10^5 cfu, also yielded BCG-specific IFN-γ and IL-4 production. Anti-p24 antibodies were detected in all groups of mice but production of these antibodies was not enhanced by rBCG boosting. Although IFN-γ and IL-4 were produced after Gag-specific peptide stimulation in all groups of mice, no significant difference was seen when comparing rBCG:RT106 boosted mice to rBCG:119L1e boosted mice when measured in an *ex vivo* ELISPOT assay. However, when the memory response in these mice was tested in a cytokine bead assay (CBA), both CD8 and CD4 peptides elicited Gag-specific IFN-α and TNF-α production and IL-4 and IL-5 were produced on stimulation with CD4 peptides. The rBCG:Gag vaccine was an effective CD8 and CD4-specific boost for the DNA prime, particularly at the 10^3cfu dose of rBCG:Gag. Also apparent from both the ELISPOT assay and the CBA was that rBCG inoculation had a suppressive effect on the response.

rBCG:RT106 or rBCG:RT108 (Tokyo) were also able to generate both a cellular and a humoral response in the BALB/c mice model. Mice receiving 5 inoculations of these recombinants generated Gag-specific T cells that produced IFN-γ when stimulated with rp24 or Gag VLPs. In addition, anti-p24 antibodies were detected in the mouse sera.

The results presented here are the first to demonstrate antigen-specific cellular and humoral immune responses in mice to rBCG expressing a subtype C *gag* gene. In addition, they demonstrate that rBCG:RT106 and rBCG:RT108 are good candidates for further evaluation.

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ABBREVIATIONS

AAV	adeno-associated virus
Ad	adenovirus 5
AIDS	acquired immunodeficiency virus
ARV	AIDS-associated retrovirus
BCG	Bacille Calmette-Guerin
bp	base pair
°C	degrees Celsius
CA	capsid
CD	complementarity determining
cDNA	complimentary deoxyribonucleic acid
cfu	colony forming units
COI	cutoff index
COT	centre of tree
CRFs	circulating recombinant forms
CTL	cytotoxic T-lymphocytes
DNA	deoxyribonucleic acid
dNTP	deoxynucleoside triphosphate
ELISA	enzyme-linked immunosorbant assay
ELISPOT	enzyme-linked immunosorbant spot assay
ER	endoplasmic reticulum
g	gravitational acceleration
g	grams
GM-CSF	granulocyte macrophage colony stimulating factor
HIV	human immunodeficiency virus
hr	hour
HTLV-III	human T-lymphotrophic virus III
IAVI	International AIDS Vaccine Initiative
IFN	interferon
IgA/G	immunoglobulin A/G
IL	interleukin
IN	integrase
kb	kilobase
kDa	kiloDalton
l	litres
LAV	lymphadenopathy-associated virus
LTNP	long-term non-progressor
LTR	long terminal repeat
m-	milli-
MA	matrix
mAb	monoclonal antibodies
min	minutes
mm	millimetres
MV	measles virus
MVA	modified vaccinia virus Ankara
n-	nano-
NC	nucleocapsid
OD	optical density
ORF	open reading frame
PBS	phosphate buffered saline
PBMC	peripheral blood mononuclear cells
PCR	polymerase chain reaction
PND	principle neutralization determinant
PPD	purified protein derivative

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PR	protease
r	recombinant
rBCG	recombinant Bacille Calmette-Guerin
rMS	recombinant *Mycobacterium smegmatis*
RNA	ribonucleic acid
rpm	revolutions per minute
RT	reverse transcriptase
RT-PCR	reverse transcription polymerase chain reaction
SA	South Africa
SAAVI	South African AIDS Vaccine Initiative
SDS	sodium dodecyl sulphate
sec	seconds
SHIV	simian/human immunodeficiency virus
SIV	simian immunodeficiency virus
SU	gp120 surface protein
Th	T helper
TM	gp41 transmembrane protein
TNF	tumour necrosis factor
μ-	micro-
UNAIDS	United Nations AIDS Organization
VEE	Venezuelan equine encephalitis
VLPs	virus-like particles
VRP	Venezuelan equine encephalitis replicon particle
W	watts
YT	yeast tryptone

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CHAPTER 1

PART A: HIV VACCINES

1.A.1. BACKGROUND

The virus that we now know as human immunodeficiency virus or HIV-1 was first identified in the early 1980s (Barre-Sinoussi *et al.*, 1983; Popovic *et al.*, 1984). HIV-1 exhibited morphological and genetic characteristics typical of the *Lentivirus* genus of the *Retroviridae* family. Clavel *et al.* (1986) discovered a related but immunologically distinct human retrovirus in individuals residing in several West African countries. This virus was less pathogenic than HIV-1 and was named HIV-2. Other lentiviruses have been isolated from several nonhuman primates (Hirsch *et al.*, 1993). These viruses do not produce disease in the natural host and have been designated simian immunodeficiency virus (SIV). This literature review will focus on HIV-1, the virus responsible for the global pandemic.

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1.A.2. CLASSIFICATION OF HIV-1

HIV-1 belongs to the *Lentivirus* genus, of the Family *Retroviridae*. HIV-1 is further subdivided into 3 groups: M (major), O (outlier) and N (non-M, non-O). The M group accounts for more than 95% of infections and consists of a number of genetic subtypes or clades (A, B, C, D, F, G, H, J and K) based on the sequence of complete viral genomes. Viruses from group O have been isolated from individuals in Cameroon, Gabon and Equatorial Guinea and their genomes share less than 50% nucleotide identity with group M viruses (Subbarao and Schochetman, 1996). Group N viruses were more recently identified in Cameroonians in 1998 (Simon *et al.*, 1998). A significant portion of group M viruses consists of intersubtype recombinants. These circulating recombinant forms (CRFs) make a significant contribution to circulating HIV-1 strains and are commonly identified in areas with multiple subtypes circulating such as Kenya, Uganda and Tanzania (Janssens *et al.*, 1997) and are responsible for major epidemics in regions such as Thailand

(CRF01_AE; Subbarao *et al.*, 2000) and West African (CRF02_AG; Morison *et al.*, 2001). An M/O intergroup recombinant has also been identified (Peeters *et al.*, 1999).

1.A.3. ORIGIN OF HIV-1

The oldest known case of HIV-1 infection was from a 1959 plasma sample from the Democratic Republic of Congo (DRC; Zhu *et al.*, 1998). Phylogenetic analysis of the viral sequence placed this virus near the ancestral node of subtypes B and D in group M and indicates that these subtypes (and possibly all group M viruses) evolved from a common ancestor in Africa. Using comprehensive full-length sequence alignment of the *env* gene, Korber *et al.* (2000) estimated that the last common ancestor of group M HIV-1 to be 1931 (range 1915-1941). HIV-1 and HIV-2 have been identified as zoonotic infections (Gao *et al.*, 1999). The primate reservoir of HIV-2 has been identified as *Cercocebus atys*, the sooty mangabey (Hirsh *et al.*, 1989; Gao *et al.*, 1992; Chen *et al.*, 1996 and 1997). Gao *et al.* (1999) identified two SIV_{cpz} infected chimpanzee subspecies (*Pan troglodytes troglodytes* and *P. t. schweinfurthii*) infected with highly divergent, subspecies-specific lineages. They also showed that all HIV-1 strains are phylogenetically closely related to the SIV_{cpz} strains that infect *P.t. troglodytes*. Their results argue that *P.t. troglodytes* is the primary reservoir for HIV-1 Group M. There have been at least 3 introductions of SIV_{cpz} into humans from chimpanzees to yield Group M, N and O.

1.A.4. VIRION STUCTURE AND REPLICATION

HIV-1 virions are spherical and about 110nm in diameter (fig 1.1). In the mature capsid the outer lipid envelope and associated glycoproteins surround the conical core, the Mg^{2+}-dependent reverse transcriptase (RT) and the RNA genome. Retroviruses carry two copies of the single-stranded RNA genome that are each approximately 9.2kb in size. The three major coding regions on the genome encode structural proteins that are synthesized as polyprotein precursors (fig 1.2). These are processed by proteases (viral or cellular) into mature proteins (Barré-Sinoussi, 1996). The matrix (MA), capsid (CA), nucleocapsid (NC) and p6 proteins are derived from the 55kDa

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Gag precursor, $Pr55^{Gag}$. Autocatalysis of the $Pr160^{Gag-Pol}$ polyprotein yields the protease (PR), reverse transcriptase (RT) and the integrase (IN) proteins, while processing of the 160kDa Env precursor (gp160) by cellular enzymes gives rise to the gp120 surface (SU) and gp41 transmembrane (TM) proteins (Barré-Sinoussi, 1996). In addition to these, the genome also encodes a number of accessory and regulatory proteins (Tat, Rev, Nef, Vif, Vpr and Vpu) that are the primary translation products of spliced mRNAs (Barré-Sinoussi, 1996). The functions of the different HIV genes are summarized in fig 1.3.

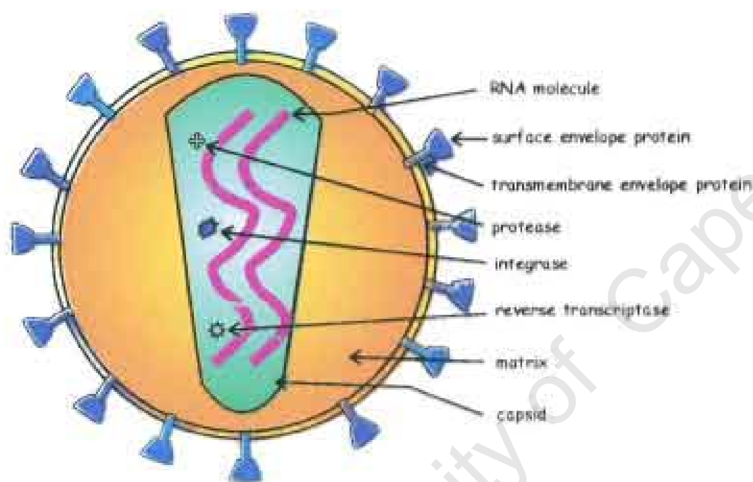

Fig.1.1. Schematic diagram of an HIV virion

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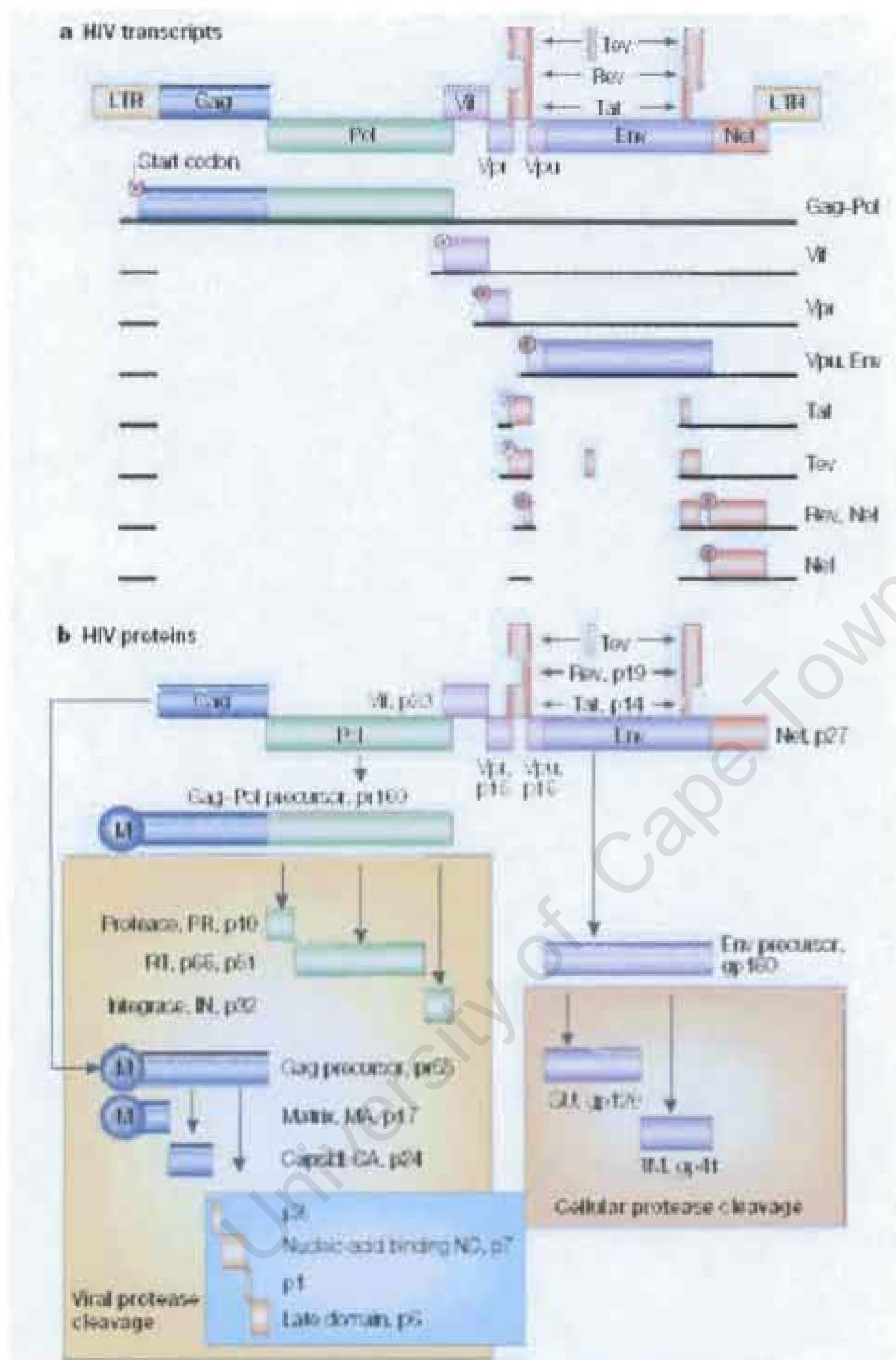

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Fig.1.2. HIV-1 genome organization and protein transcripts (taken from Peterlin and Trono, 2003)

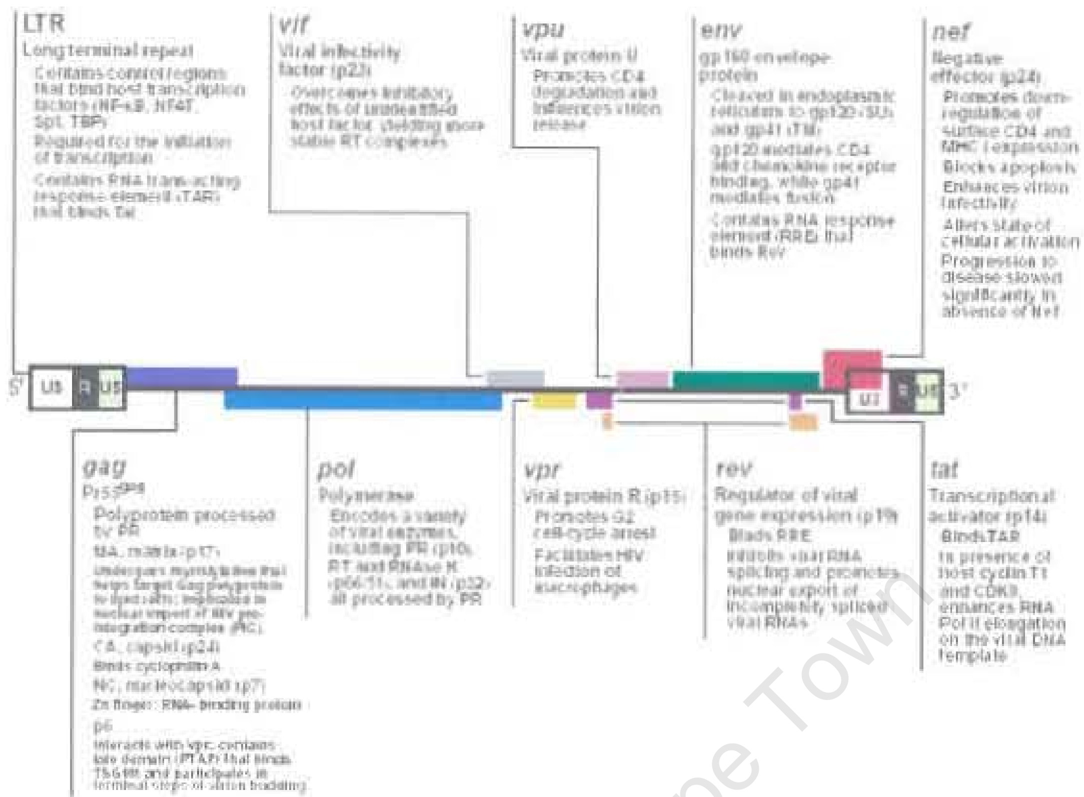

Fig. 1.3. Overview of HIV-1 gene functions (taken from Greene and Peterlin, 2002)

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HIV infects T helper (Th) cells, macrophages, dendritic cells (DCs) and, to some extent, microglial cells; all of which express CD4, the primary receptor for HIV (Peterlin and Trono, 2003). Viral tropism is further constrained by the requirement for co-receptors, CC-chemokine receptor 5 (CCR5) and CX-chemokine receptor 4 (CXCR4) (Doms and Trono, 2000). HIV can also utilize a number of other minor co-receptors *in vitro* including CCR1, CCR2b and CCR3 (Deng *et al.*, 1997), however, the relevance of these receptors *in vivo* is unknown.

The replication cycle of HIV is illustrated in fig. 1.4. Briefly (as described by Peterlin and Trono, 2003), the virus initially binds CD4, inducing a conformational change in the envelope proteins that allows binding of the coreceptor (either CCR5 or CXCR4) and allows it to enter the cell via fusion of the cellular and viral membranes. Once inside the cell, the virus is uncoated and the pre-integration complex (PIC) is formed. The PIC consists of newly synthesized viral complementary

DNA (cDNA), the IN protein, the MA protein, RT, Vpr and host proteins. IN, MA and Vpr have nuclear localization signals that direct the PIC to the nucleus and ensure that the viral genome enters via the nuclear pores. The linear viral cDNA integrates into the host genome, usually in active gene regions. Once integrated, RNA polymerase II (RNAPII) initiates transcription at the 5'-long terminal repeat (LTR) that contains the promoter and enhancer sequences. The viral Tat protein enhances full-length transcription of the provirus. Rev transports both singly spliced and unspliced genomic transcripts to the cytoplasm. In the cytoplasm, the viral structural and enzymatic proteins are synthesized and transported to the cell membrane where immature virions are assembled on glycolipid-enriched lipid rafts. Gag recruits multivesicular bodies to the membrane that allow progeny virions to bud from the cell. Processing of Gag and Gag-Pol yields the final mature virus particle.

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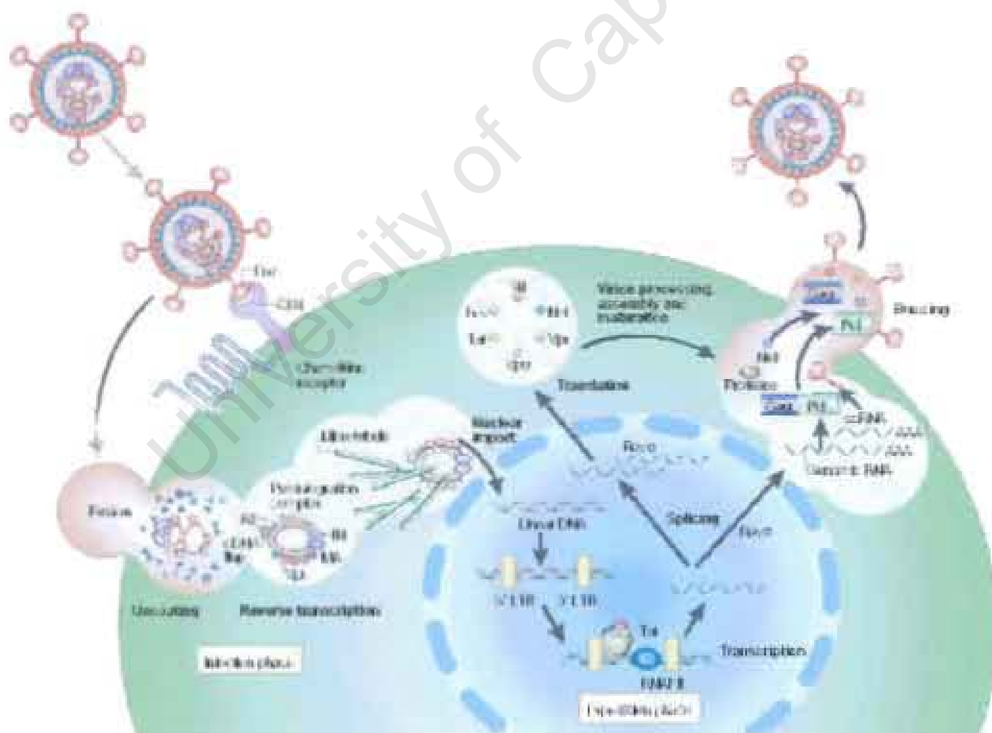

Fig.1.4. The HIV replication cycle (taken from Peterlin and Trono, 2003).

1.A.5. THE HIV EPIDEMIC

1.A.5.1. THE SOUTH AFRICAN HIV-1 EPIDEMIC

The estimated number of people living with AIDS in 2003 was 38 million (UNAIDS, 2004). Sixty-six percent of infected people reside in sub-Saharan Africa with the highest prevalence occurring in the southern African region. In 2003, there were 4.8 million new HIV infections worldwide and 2.9 million AIDS deaths (UNAIDS, 2004). The global epidemic is not homogenous and each country has wide variations in infection levels between different regions.

Sub-Saharan Africa accounts for only 10% of the world's population, however more than 60% of all the people living with HIV are from this region (AIDS epidemic update: http://www.unaids.org). South Africa has the highest number of people living with HIV, estimated at 5.3 million; 2.9 million of which are women (Department of Health, SA, 2004). From 1990 to 1998, there was an exponential increase in the prevalence of HIV in the South African population, however, the 5 years following that period has seen a stabilisation of the prevalence (figure 1.5; Department of Health, SA, 2004). In 2003, the results from the 14th annual survey of pregnant women attending government antenatal clinics showed that the prevalence of HIV in SA was 27.9%. This increase from 26.5% in 2002 was not statistically significant and the report concluded that although the infection rates remain high, the epidemic is still in a stabilization phase and are therefore not declining. The UNAIDS report cautions that epidemics that appear to be stable or in a stabilisation phase may in fact be indicative of more or less equal numbers of newly infected people and people dying of AIDS (one-third of AIDS deaths world-wide occur in southern Africa; AIDS epidemic update: http://www.unaids.org)

There is a regional variation in the prevalence of infection with three provinces having prevalence rates higher than 30% - KwaZulu-Natal (37.5%), Mpumalanga (32.6%) and Free State (30.1%). The rate was between 13 and 17.5% in the Western Cape, Limpopo and Northern Cape provinces and it was found that the rate had risen in all but two provinces (Free State at 27.1% and Gauteng at 29.6%) since 2001.

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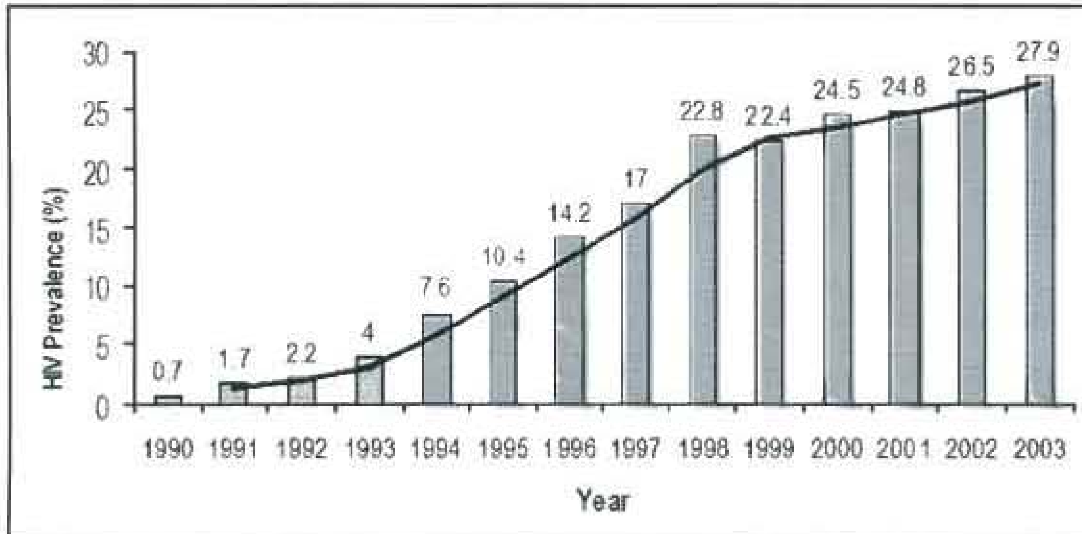

Fig 1.5. Prevalence of HIV among antenatal clinic attendees in South Africa from 1990 to 2003 (Department of Health, SA, 2004).

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There have been no significant increases in prevalence in teenagers for the last five years but other age groups have shown increases, particularly the 25 to 29 year old group with a prevalence of 35.4% (Table 1.1). The lowest prevalence was in women under 20 (15.8%). The only age group to show no increase since 2002 was the group of women older than 40 (17.2% in 2002 to 15.8% in 2003).

Table 1.1. HIV prevalence by age-group among antenatal clinic attendees in South Africa, 2001 to 2003 (Department of Health, SA, 2004).

Age Group (years)	HIV prev (CI 95%) 2001	HIV prev (CI 95%) 2002	HIV prev (CI 95%) 2003
<20	**15.4** (13.8-16.9)	**14.8** (13.4-16.1)	**15.8** (14.3-17.2)
20-24	**28.4** (26.5-30.2)	**29.1** (27.5-30.6)	**30.3** (28.8-31.8)
25-29	**31.4** (29.5-33.3)	**34.5** (32.6-36.4)	**35.4** (33.6-37.2)
30-34	**25.6** (23.5-27.7)	**29.5** (27.4-31.6)	**30.9** (28.9-32.9)
35-39	**19.3** (17.0-21.5)	**19.8** (17.5-22.0)	**23.4** (20.9-25.9)
40+	**9.8** (7.0-12.6)	**17.2** (13.5-20.9)	**15.8** (12.3-19.3)

1.A.5.2. GLOBAL DISTRIBUTION OF HIV-1

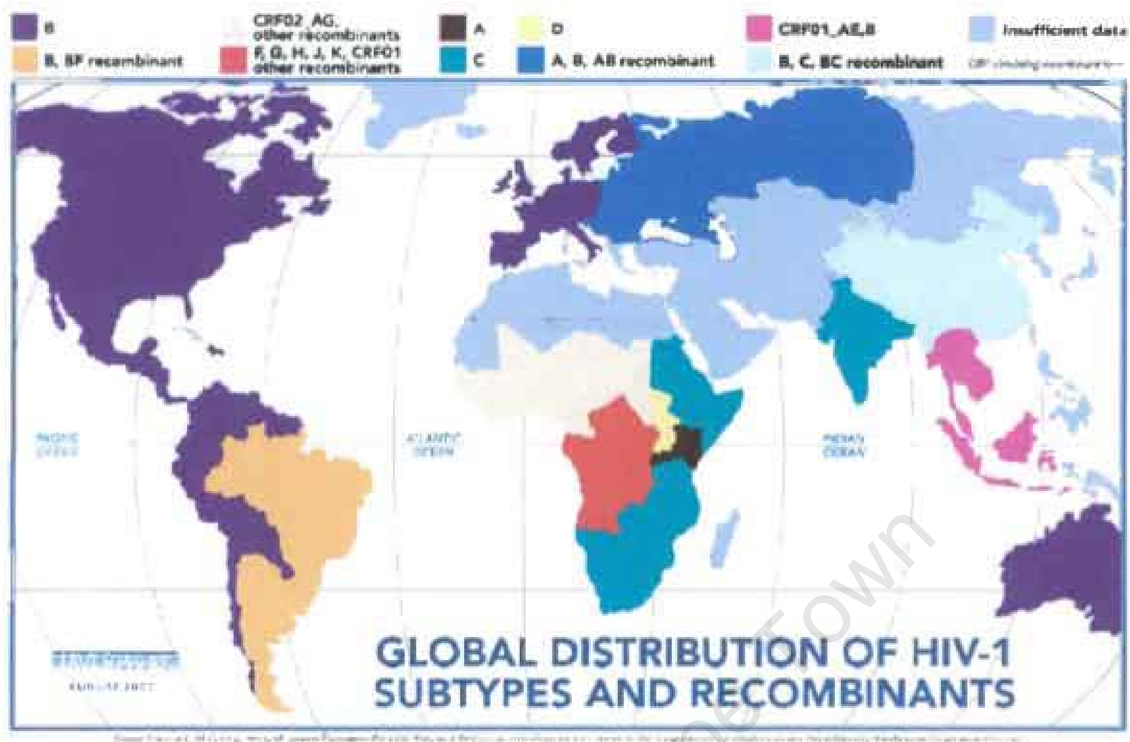

Fig 1.6. The global distribution of HIV-1 subtypes and recombinants (taken from IAVI Report August 2004)

Most countries have predominant circulating subtypes or circulating recombinant forms (CRFs; Fig 1.6) and 10 different HIV-1 epidemic patterns have been identified (McCutchan, 2003). In North America, Western Europe and Australia subtype B is the predominant strain of HIV-1. South America has subtype B predominating only in the western portion of the continent, while a BF recombinant epidemic is occurring in the rest of the continent. Eastern Europe has a mixed epidemic consisting of subtypes A and B and AB recombinants. The Asian distribution has three different patterns:

- subtype C,
- a mixture of subtype B and C and BC recombinants and
- a mixture of subtype B and CRF01_AE.

Since the HIV epidemic has its origin in Africa, it is not surprising that the greatest diversity is found here. The southern and eastern parts of Africa are dominated by subtype C, while eastern

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Central Africa have both subtype A and subtype D foci. Although the western and western Central regions are mainly CRF02_AG, a number of other recombinants are also circulating at a lower level. Central Africa has the most complex epidemic with no clear predominant subtype in circulation.

1.A.5.3. THE SUBTYPE C EPIDEMIC

The rapid emergence and escalation of HIV-1 subtype C infections means that it now accounts for more than 56% of all global HIV infections (Esparza and Bhamarapravati, 2000). The HIV epidemic in southern Africa is predominantly a subtype C epidemic, with some regions having prevalence rates as high as 40% in pregnant women (Department of Health, SA, 2004). Over 95% of infections in South Africa are a result of subtype C (SA; see section 2.1.2) and this subtype has been associated with major epidemics in Botswana (Bredell *et al.*, 1998; Novitsky *et al.*, 1999 and Ndung'u *et al.*, 2000), Zambia (Handema *et al.*, 2001; Morison *et al.*, 2001 and Trask *et al.*, 2002), Zimbabwe (Obi *et al.*, 1997a and 1997b and Batra *et al.*, 2000), Malawi (Candotti *et al.*, 2001 and McCormack *et al.*, 2002), Swaziland (Bredell *et al.*, 1998), Lesotho (Bredell *et al.*, 1998) and Mozambique (Bredell *et al.*, 1998; Engelbrecht *et al.*, 1998 and Downing *et al.*, 2000). Studies also suggest that subtype C has begun spreading further north into countries like the Congo, Tanzania, Burundi and Kenya (Zachar *et al.*, 1996; Janssens *et al.*, 1997 and Koch *et al.*, 2001; Rodenburg *et al.*, 2001).

The epidemic in India is also predominantly subtype C (Shankarappa *et al.*, 2001), while Brazil (Gao *et al.*, 1998 and Soares *et al.*, 2003a and 2003b) and China (Yu *et al.*, 1998 and Rodenburg *et al.*, 2001) are isolating subtype C viruses with increasing frequency. Intersubtype recombinants with subtype C have also been isolated in several countries: C/D in Tanzania (Hoelscher *et al.*, 2001) and C/B in India (Rodenburg *et al.*, 2001) and China (Yang *et al.*, 2002 and 2003).

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Subtype C Epidemic in South Africa

In the early 1980s the homosexual population was the group most affected by the AIDS epidemic with HIV moving into the heterosexual in the late 1980s (Sher, 1989; Kustner, *et al.* 1994). In 1995, Williamson *et al.*, found that the homosexual men recruited into their study were infected with mainly subtype B viruses (15 of 20), while the majority of heterosexual individuals were infected with subtype C viruses (28 of 33). This apparent association between mode of transmission and HIV-1 subtype was confirmed by van Harmelen *et al.* (1997).

Isolates from migrant workers in three SA gold mines were all shown to be subtype C but the heterogeneity of the sequences suggested that multiple introductions of subtype C occurred in this population (Bredell *et al.*, 1998). In a study using 107 samples from 4 different provinces in South Africa, it was concluded that subtype C was responsible for the vast majority of heterosexual infections and no evidence of a clonal epidemic could be detected (van Harmelen *et al.*, 1999). Full-length genome sequencing studies have also shown that there is little intersubtype recombination (van Harmelen, 2001; Papathanasopoulos, 2002).

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The predominance of subtype C in SA has been well established however non-subtype Cs have been identified, including some intersubtype recombinants. Among ten viruses studied in the *gag* and *env* region, Bredell *et al.* (2002) identified four recombinants of which one was unclassified *env* (C/A, C/D, G/A and D/un). There was evidence of a local recombination event since a subtype A and a subtype G fragment were shown to be in combination with subtype C. Also, three viruses containing subtype G sequences and a CRF02_AG-like virus not previously isolated in South Africa were identified. Another virus contained a sequence fragment that could not be identified. Papathanasopoulos *et al.* (2002) identified a novel recombinant between subsubtype A2 and subtype C as well as a complex recombinant containing segments from subtypes A, C, D, G and K. The novel A2/C recombinant was isolated from a female sex worker in Durban and thus requires careful monitoring since it is in a high transmission setting.

Dual infections with two phylogenetically distinct strains of HIV-1 subtype C have been identified in South Africa. Grobler *et al.* (2004) showed that within 3 months of infection 19% of the women in the study were dually infected and that this dual infection was associated with an increased viral load set point.

Possible factors aiding the spread of HIV-1 subtype C

The factors allowing for this devastating spread of subtype C are not completely understood but are thought to relate to the host, the virus and socioeconomic conditions in affected countries (Gordon *et al.*, 2003). Viral factors which have been suggested for the rapid spread of subtype C include an extra NFkB binding site in the promoter region of the LTR which may represent an adaptive advantage for subtype C viruses since it represents an increased capacity for TNF-α responsiveness (Montano *et al.*, 2000). TNF-α is a proinflammatory cytokine that has been shown to enhance HIV-1 gene expression (Matsuyama *et al.*, 1991). Montano *et al.* (2000) speculate that this TNF-α-linked transcription may increase the viral load in transmission fluids and thereby increase the probability of transmission and the pathogenesis of subtype C viruses. Velazquez-Campoy *et al.* (2001) have suggested that subtype C viruses are more viable since their proteases have greater catalytic efficiency than other subtypes.

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The *ex vivo* fitness of 9 subtype B strains with 6 subtype C strains were compared by measuring the infection rate/host cell entry in a number of cell types using a pairwise competition experiment (Ball *et al.*, 2003). The authors found that in PBMC, CD4+ lymphocytes and blood-derived macrophages, subtype B isolates outcompeted the subtype C isolates. In skin-derived Langerhans cells, however, the rate of host cell entry was the same for both the C and B isolates. Infection of Langerhans cells provides a model for primary HIV-1 infection in the genital tract and thus virus transmission, while T cells and macrophages are cells that become infected in later stages of disease (Ball *et al.*, 2003). This implies that subtype C isolates may be transmitted as efficiently as subtype B isolates (Langerhans cell data) but that they are less fit during further disease progression (T-cell and macrophage data).

Another difference between subtype C and other subtypes is that subtype C isolates use the CCR5 coreceptor almost exclusively throughout infection and that CXCR4 coreceptor usage is rare (Abebe *et al.*, 1999; Ping *et al.*, 1999; Cecilia *et al.*, 2000 and Morris *et al.*, 2001a). These reports implied that subtype C viruses may have envelope proteins that are atypical in structure or function compared to viruses of other subtypes and that these differences could provide an explanation as to why subtype C viruses are spreading so rapidly. However, it has been shown more recently that subtype C envelope proteins can accommodate the necessary amino acid changes to use CXCR4 as a coreceptor and, in patients with advanced AIDS, CXCR4-using subtype C viruses could be isolated (Cilliers *et al.*, 2003).

Subtype C viruses have other characteristics that differ from the non-C subtypes including a subtype-specific insertion in the transmembrane region of Vpu (McCormick-Davis *et al.*, 2000), a prematurely truncated second exon in *rev* (Rodenburg *et al.*, 2001) and increased natural amino acid variability in protease cleavage sites (de Oliveira *et al.*, 2003).

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Immune response to HIV-1 subtype C in southern African individuals

The genetic background of the local population, as well as the immunogen, can impact the immune response to the virus and to a vaccine candidate. It is thus important for vaccine design to characterize the circulating viruses on an epidemiological level and to investigate the immune responses to these viruses in infected individuals. Many of the early studies investigating the immune response to HIV-1 have been in individuals infected with subtype B viruses. However, there have been a number of recent reports from groups working on subtype C in southern Africa that have shed light on the immune response to subtype C viruses.

Goulder *et al.* (2000) studied Gag-specific CTL responses in subtype B infected Caucasoids versus subtype C infected African and found that the two-thirds of the dominant responses mapped to 3 peptides in the p17 and p24 region, irrespective of the subtype, ethnicity or age of

the group studied. However, they did find that the Caucasoid response was predominantly in p17 while the predominant response in Africans was in p24. This difference is a result of different dominant HLAs in the different populations. Detailed analysis of subtype C-specific CTL responses in infected blood donors in Botswana allowed the identification of immunodominant regions within Gag, Tat, Rev and Nef (Novitsky *et al.*, 2001). As in the study by Goulder *et al.* (2000), the dominant Gag-specific responses were in the p24 region. The data on Tat- and Rev-specific responses indicated distinctive differences in the pattern of immunodominance in these regions when comparing responses among subtype B infected people with subtype C infected people from Botswana (Novitsky *et al.*, 2001). When the CTL responses were investigated for the entire genome, the analysis showed that the magnitudes and frequencies of the responses were spread unevenly (Novitsky *et al.*, 2002a). The strongest responses were found in the p24, Vpr, Tat and Nef regions with less robust responses distributed in other regions of p24, Tat and Nef as well as in Pol, Vif and Env.

Defining responses associated with control of viral replication is important for designing immunogens which should be included in candidate vaccines. An association between plasma viral load and HIV-specific CTL responses has been demonstrated for subtype C infections (Novitsky *et al.*, 2003). For p24, better CTL responses were detected in individuals with lower viral loads but the opposite was true for Nef-specific responses. These results were confirmed in a study to determine the relationship between the breadth and magnitude of T-cell responses and viral loads in subtype C-infected individuals in southern Africa (Masemola *et al.*, 2004a). In this study, Nef was targeted in almost a third of the responses across the genome, while the next most frequently targeted was p24, which comprised of 17% of the responses. Although there was no correlation between the breadth of the responses and the viral load, there was a weak positive correlation between the total magnitude of the responses and the viral load. When investigating the hierarchy of the responses, they found that preferential targeting of Gag correlated with viral control. Thus targeting of Gag during early stages of infection with subtype C virus is associated with viral control and may be a useful marker of immune efficacy and in vaccine assessment.

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When screening 38 individuals from KwaZulu-Natal, South Africa with recent subtype C infections, 74% of them recognized at least 1 Gag peptide pool and 10 epitopic regions were identified across p17, p24 and p2p7p1p6 (Masemola *et al.*, 2004b). These regions were found to be highly conserved and fine epitope mapping revealed that 5 of the identified optimal Gag epitopes were novel.

Novitsky *et al.* (2003) reported that lower viral loads were associated with the HLA B44 supertype and, in the study by Masemola *et al.* (2004b), the defined optimal epitopes were restricted by more than one HLA allele. The epitopes did overlap with regions that contain previously described epitopes from subtype B infections and the authors suggest that these results add weight to the hypothesis that HLA diversity can shape epitope identity but may not be a constraint for universal recognition of Gag in highly conserved domains (Masemola *et al.*, 2004b).

The neutralizing activity of sera from HIV-1 infected individuals seems to be independent of genetic subtype implying that, in general, there is no relationship between subtype and neutralization serotype (Kostrikis *et al.*, 1996 and Weber *et al.*, 1996). The neutralization properties of subtype C viruses from South Africa and Malawi were shown to be similar to subtype B virus isolates in both their resistance to inhibition by soluble CD4 and their sensitivity to neutralization by the G1b12 monoclonal antibody (Bures, *et al.*, 2002). However, 2F5, another monoclonal antibody that neutralizes subtype B viruses, was only effective against two of sixteen subtype C isolates in this study. In addition, all sixteen subtype C isolates were shown to be resistant to neutralization by the 2G12 monoclonal antibody.

Although the neutralizing antibody response was slow to mature following primary infection with subtype C viruses in South Africa, the eventual response not only neutralized autologous virus but showed extensive cross-neutralization of heterologous subtype C viruses from South Africa. This implies a geographic clustering of neutralization serotypes in South Africa meaning that

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there are a number of shared neutralization determinants at a regional level in South Africa (Bures, *et al.*, 2002).

While most of the serum samples had subtype-specific neutralization activity, two of them neutralized a broad range of both subtype B and C isolates; indicating that subtypes B and C could be made up of multiple neutralization serotypes with varying levels of subtype-specificity. The observation that SA subtype C isolates have clusters of shared neutralization determinants could mean that vaccine-induced neutralizing antibodies will have a lower level of epitope diversity to overcome at a regional level (Bures, *et al.*, 2002).

1.A.6. CORRELATES OF PROTECTION

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HIV establishes a persistent and chronic infection. HIV infection is not cleared by the host immune system nor does natural immunity to reinfection by HIV develop. It has been suggested, however, that since superinfection seems to be rare, natural immunity due to an initial HIV infection might inhibit the establishment of a superinfection by another HIV (Gottlieb *et al.*, 2004). Natural control of infection and control by vaccination are due, in general, to adaptive immune responses in the form of neutralizing antibodies and virus-specific T cells ($CD4^+$ and $CD8^+$). While neutralizing antibodies protect from infection, cell-mediated immunity protects from disease or controls the infection.

1.A.6.1. CYTOTOXIC T LYMPHOCYTES (CTLs)

The focus has shifted in recent years to producing anti-HIV CTL or CD8+ T cell inducing vaccines, due in part to the difficulties facing researchers in the development of AIDS vaccines that elicit a strong neutralizing antibody response. The first phase III trial which tests T-cell inducing vaccine prime (canary pox vaccine vector) and gp120 monomer boost is currently underway in Thailand and results from these trials may determine whether this line of reasoning is sound (see section 1.7), bearing in mind that responses to canary pox vaccines are not very immunogenic (Cao *et al.*, 2003).

The natural HIV CTL response and control of the virus

HIV-specific CTLs are able to lyse target cells via apoptosis (Yang *et al.*, 1996), secrete cytokines such as IFN-γ and TNF-α that induce an antiviral effect in infected cells or secrete chemokines such as CCL3 and CCL4 that block coreceptor molecules and prevent HIV entry into cells (Rubbert *et al.*, 1997; Wagner *et al.*, 1998; Garzino-Demo *et al.*, 1998 and Appay *et al.*, 2000).

Although high levels of HIV-specific CTLs are associated with control of viral load following acute infection (Koup *et al.*, 1994 and Borrow *et al.*, 1994), they fail to control the virus effectively during the entire course of an untreated infection. A significant inverse correlation was observed between HIV Gag- and Pol-specific CTL frequency and plasma RNA viral load in individuals at different stages of disease (Ogg *et al.*, 1998). However, more recent analysis of total HIV-specific T-cell responses indicated that there is actually a positive correlation between the plasma viral load and the total HIV-specific CTL frequency (Betts *et al.*, 2001). A comprehensive epitope analysis of T-cell responses directed against the entire expressed HIV-1 genome also showed that neither the breadth nor the magnitude of the total HIV-1-specific CTL responses correlated with plasma viral load (Addo *et al.*, 2003). HIV-specific CD8+ T cell proliferation and enhanced effector function in asymptomatic infected people is coupled to perforin expression (Migueles *et al.*, 2002). However, most antiviral CD8+ T cells do not express high levels of perforin during chronic viral infection and are not directly cytotoxic (Zhang *et al.*, 2003). These results imply that not all HIV-specific CTLs that are detected *in vitro* have *in vivo* functionality and may point to an explanation for the fact that certain viral epitopes remain unchanged in infected individuals despite the *ex vivo* detection of persistent responses to that epitope (Altman and Feinberg, 2004).

There are a number of possible reasons why the HIV-specific CTLs seen during acute infection are unable to maintain control of replication at later stages of infection. One of the reasons is the plasticity of the HIV genome – the virus readily generates CTL escape mutants carrying changes in immunodominant epitopes and their flanking regions (Phillips *et al.*, 1991; Borrow *et al.*, 1997;

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Goulder *et al.*, 1997; Evans *et al.*, 1999; Allen *et al.*, 2000; Barouch *et al.*, 2002 and Draenert *et al.* 2004). Primary cells infected with HIV-1 are not lysed efficiently by CTLs in the presence of the Nef protein, since its expression results in the downregulation of HLA class I expression (Collins *et al.*, 1998). HIV-1 Rev protein activity may be associated with a decreased susceptibility of HIV-infected cells to CTL-mediated killing (Bobbit *et al.*, 2003). Although, the majority of circulating HIV-specific CD8(+) T cells were able to the secrete of antiviral cytokines in response to antigen, they are impaired in cytolytic activity (Appay *et al.*, 2000). Also maturation of HIV-specific memory CD8+ T cells during HIV infection is defective (Champagne *et al.*, 2001). Further compounding the problem and of extreme importance is the fact that CD4+ T cells are preferentially infected and killed by HIV which means there is insufficient T cell help to maintain CD8+ T cell function (McCune, 2001, Ribeiro *et al.*, 2002 and Douek *et al.*, 2002).

A vaccine-induced CTL response could control the virus

There is evidence that supports the contention that a CTL-response is an important correlate of protection against HIV and that it will be important for an effective vaccine to generate such responses. These are reviewed below:

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Long-term non-progressors (LTNPs)

Virus-specific CTLs that are present in LTNPs maintain a high proliferative capacity which is linked to enhanced effector function (Migueles *et al.*, 2002). This is in contrast to the situation in normal progressors. Although early studies by Harrer *et al.* (1996a and b) suggested that a vigorous *in vivo* HIV-1-specific CTL response was responsible for the control of viral replication in stable nonprogressive HIV-1 infection, Addo *et al.* (2004), found that when responses in 80 LTNPs were compared to 24 untreated HIV-1 progressors, no statistically significant differences were observed in breadth and magnitude of HIV-1-specific CTL responses. Additionally, some of the LTNPs with lowest viral loads also exhibited the lowest levels of CTLs. What these results imply is that the virus-specific CTL responses were related to the level of antigen rather than functioning to suppress virus replication.

However, the importance CTL is implied in studies which should an association between HLA and disease progression. Delayed progression to AIDS is also linked to a greater HLA class I gene diversity and furthermore certain HLA alleles have been associated with slow or rapid progression (Tang *et al.*, 1999; Carrington *et al.*, 1999 and Costello *et al.*, 1999). A significantly greater number of CTL responses were HLA-B-restricted, compared to HLA-A in 375 HIV-1 infected individuals from southern African (Kiepiela *et al.*, 2004). The rate of disease progression in the cohort was strongly associated with HLA-B but not HLA-A allele expression and more selection pressure was exerted on HIV-1 by HLA-B alleles than HLA-A alleles. The group concluded that the main focus of HIV-specific activity was thus at the HLA-B locus and that HLA-B gene frequencies in the population were most likely to be influenced by HIV disease. This is consistent with the observation that B alleles evolve more rapidly than A alleles.

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Highly exposed seronegative individuals

Studies on cohorts of highly HIV-exposed but seronegative individuals lend support to the relevance of the CTL-vaccine effort. Low-level HIV-specific CTL responses are detected in these individuals: in a group health-care workers (HCW) exposed to HIV contaminated blood, lack of subsequent infection appeared to result in the activation of cellular immunity without activation of antibody production (Clerici *et al.*, 1994); in another group of exposed, seronegative HCW, Env-specific CTL responses were detected (Pinto *et al.*, 1995); CTL responses to HIV epitopes were also detected in exposed seronegative women from Nairobi and Gambia (Rowland-Jones *et al.* 1995 and 1998 and Kaul *et al.*, 2000 and 2001a). It is suggested that these CTL responses are conferring relative resistance to infection in these people. In one of these cohorts – a group of highly exposed, seemingly HIV-resistant female sex workers from Nairobi – late seroconversion occurred after longer term follow-up (Kaul *et al.*, 2001b). This late seroconversion may be associated with the waning of HIV-specific CTL responses due to reduced antigenic exposure as a result of a break from sex work (Kaul *et al.*, 2001b). In two other cohorts of exposed but persistently seronegative men, virus-specific CTL responses were not detected and the protective action of these responses remains unconfirmed (Schmechel *et al.*, 2001 and Yang *et al.*, 2002).

CTLs and viral load

The viral load set point is a strong predictor of the rate of progression to AIDS after HIV-1 seroconversion, with the higher the set point the more rapid the progression (Mellors *et al.*, 1995). The viral load is also the chief predictor of the risk of heterosexual transmission of HIV-1 (Quinn *et al.*, 2000 and Gray *et al.*, 2001). Studies in HIV-infected humans and SIV-infected macaques have demonstrated that HIV-specific CTL activity is associated with initial control of primary virus replication (Koup *et al.*, 1994; Borrow *et al.*, 1994; Reimann *et al.*, 1994 and Kuroda *et al.*, 1999). The selection of CTL-escape mutants in HIV and SIV infection also occurs at the time of maximal CTL responses which is during primary viraemia (Phillips *et al.*, 1991; Borrow *et al.*, 1997; Price *et al.*, 1997; Evans *et al.*, 1999 and Allen *et al.*, 2000).

Compelling support for the role of CTLs in the control of HIV replication comes from experiments in SIV-infected macaques that have been depleted of CD8+ T cells, during either acute or chronic infection. These animals were unable to control infection and a dramatic increase in the level of viraemia was observed (Matano *et al.*, 1998; Schmitz *et al.*, 1999 and Jin *et al.*, 1999). Vaccine-induced responses in the macaque model have been shown to be partially protective against a SHIV-89.6P challenge (Amara *et al.*, 2001 and Shiver *et al.*, 2002). The reduction in viral load in these animals was inversely correlated to the virus-specific CTL response and the magnitude of reduction in viremia in the animals was predicted by the magnitude of the vaccine-elicited CTL response prior to SIV challenge (Seth *et al.*, 2000 and Ourmanov *et al.*, 2000). Importantly, it was reported that 22 out of 23 of the vaccinated animals (Amara *et al.*, 2001) were able to successfully control their viremia until their time of euthanasia at 200 weeks postchallenge (Sadagopal *et al.*, 2005). The animals had low to undetectable viral loads, normal CD4 counts and stable T-cell responses. In addition, high titers of binding and neutralizing antibody persisted throughout the postchallenge period and long-term control was effective in macaques of diverse HLA types (Sadagopal *et al.*, 2005).

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What to expect from an HIV-1 CTL-inducing vaccine

It is necessary to determine the difference between natural and vaccine-elicited responses in order to facilitate the development of an effective AIDS vaccine. In humans, the virus preferentially infects and kills HIV-specific CD4+ T cells resulting in a lack of appropriate T-cell help, which in turn cripples the virus-specific CTL response (McCune *et al.*, 2001, Ribeiro *et al.*, 2002 and Douek *et al.*, 2002). Vaccination with a CTL-based vaccine is expected to yield a pool of fully functional HIV-specific CD4+ T helper cells prior to infection that would result in the maintenance of CD8+ T cell proliferation and function in the post-infection state. The immune system would thus receive a head start in its efforts to control viral replication since the recall response would be quicker compared to a naïve response.

It will also be important for a vaccine to elicit a sizable pool of long-lasting memory CD4+ and CD8+ T cells. The murine LCMV infection model has demonstrated that CD8+ T memory cells are more protective against a second LCMV challenge than CD8+ T effector cells and, importantly, that a break from antigen exposure is necessary to achieve complete differentiation of virus-specific memory T-cells (Wherry *et al.*, 2004). However, an HIV vaccine would induce HIV-specific CD4+ T cell production and the exposure to HIV antigen would be transient in vaccinees, so if there is a higher probability that virus-specific memory T cells would develop. A pool of vaccine-induced HIV-specific memory CD8+ T cells of sufficient size and consisting of fully-differentiated cells available at the time of natural infection should limit viral replication and damage – this would lead to increased levels of virus-specific CD8+ T cells with enhanced antiviral capacity being available at the chronic infection stage (Garber *et al.*, 2004a).

1.A.6.2. NEUTRALIZING ANTIBODIES

The HIV envelope is a difficult target for antibody neutralization and the efforts to develop immunogens that elicit high titre neutralizing antibodies have not borne fruit thus far. Although there are conserved structures on the viral envelope glycoproteins, the virus has evolved to evade the neutralizing antibody response (Wyatt and Sodroski, 1998). The gp160 glycoprotein

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precursor is proteolytically cleaved to produce the envelope glycoproteins, gp120 (surface) and gp41 (transmembrane) (Earl, *et al.*, 1991). The envelope is composed of homotrimer consisting of gp120-gp41 heterodimers (Earl, *et al.*, 1991). The surface glycoprotein consists of five hypervariable loops interspersed with five conserved regions and its surface is heavily glycosylated (Freed and Martin, 2001). The conserved Env core, containing critical neutralization epitopes such as the binding site for the CD4 and CCR5 receptors, are shielded by these surface exposed hypervariable loops and the glycosylation of gp120 (Wyatt *et al.*, 1998). Kwong *et al.* (2002) demonstrated that conformational changes are necessary to expose the CCR5 binding site on gp120 and that this conformational masking is a further mechanism preventing antibody binding to that region of gp120.

Natural antibody responses to HIV

During the course of an HIV infection, neutralizing antibodies directed against the envelope are made (Wei *et al.*, 2003 and Richman *et al.*, 2003). However, there is immediate selection for viral escape mutants and neutralization-resistant viruses eventually predominate, therefore the effects of these antibodies are transient (Wei *et al.*, 2003 and Richman *et al.*, 2003). Human monoclonal antibodies have been isolated from the sera of a number of HIV-infected individuals and these antibodies are capable of neutralizing a variety of primary isolates and laboratory-adapted strains (Posner *et al.*, 1991; Ho *et al.*, 1991a and 1991b; Thali *et al.*, 1992 and Trkola *et al.*, 1996). These monoclonal antibodies recognize conserved epitopes of gp120 and gp41 using various structural features. The monoclonal antibody b12 has a protruding, finger-like domain that penetrates the recessed CD4-binding site of gp120 (Saphire *et al.*, 2001). Another monoclonal antibody, 2G12, neutralizes a broad range of HIV-1 isolates by binding an unusually dense cluster of carbohydrate moieties on gp120 using a unique interdigitation of Fab domains (Calarese *et al.*, 2003). Other monoclonal antibodies that are broadly neutralizing target relatively conserved domains in gp41. Examples include 4E10 and 2F5 which inhibit the virus-cell fusion process (Zwick *et al.*, 2001 and Muster *et al.*, 1993).

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Protection from infection by passive transfusion of neutralizing antibodies

Convincing evidence of the protective effect of neutralizing antibodies has been provided by a non-human primate prophylactic treatment model whereby recombinant forms of monoclonal antibodies were passively delivered prior to challenge. Purified immunoglobulin from chimpanzees infected with several different HIV-1 isolates was used for passive immunization of macaques (Shibata *et al.*, 1999). The immunized animals were able to completely block infection after a challenge with pathogenic SHIV. Similarly, passive intravenous transfer of the monoclonal antibody b12 was shown to protect macaques from a vaginal challenge with a CCR5-using SHIV (Parren *et al.*, 2001). Topical vaginal administration of the b12 antibody was also shown to protect macaques from SHIV infection through the vagina in a dose-dependent manner (Veazey *et al.*, 2003).

Potentially high manufacturing costs for the amount of antibody and the number of doses required to achieve protection, and longevity of this protection will likely limit the practicality of these approaches in humans (Garber *et al.*, 2004a). Current vaccine efforts have been undertaken to design immunogens that can elicit similarly broadly neutralizing antibodies *in vivo* (Burton *et al.*, 2004). Designing immunogens which mimic the mature trimeric form located on the virions, as well as modified versions which mimic the 'open conformation' or 'triggered' env structure which exposes hidden neutralizing epitopes, has proven problematic. A number of approaches are under investigation including stripping glycosylations sites or deleting variable loops that shield useful epitopes (Johnson *et al.*, 2001 and Srivastava *et al.*, 2003), presenting on surface of pseudovirions or proteoliposomes (Yang *et al.*, 2001; Grundner *et al.*, 2002), cross-linking to generate stable intermediates of the entry process (Fouts *et al.*, 2002) and linking to CD4 mimotopes (Dorgham *et al.*, 2005). To date, none of these strategies have managed to elicit broadly neutralizing antibodies.

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1.A.7. CURRENT PROSPECTS FOR AN AIDS VACCINE

1.A.7.1. POSSIBLE OUTCOMES OF VACCINATION AGAINST HIV

Vaccination against HIV could result in a number of potential outcomes. Since the virus integrates into the host cell genome, complete elimination of the virus or sterilizing immunity may not be possible. For first generation vaccines, a more realistic target for a successful vaccine may be long-term control of the virus. This would be either in the form of a transient infection or a controlled infection. With a transient infection, low levels of HIV would be detected following exposure but virus replication would be suppressed after an initial peak. In a controlled infection scenario, the viral set point would drop to a low or undetectable level after the acute phase and remain there. Vaccination with these outcomes would hopefully reduce the viral load in infected individuals to a level that results in a slower disease progression and also reduces the rate of viral transmission.

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Massad *et al.* (2001) developed a mathematical model for the impact of partially protective HIV vaccines on the incidence of infection. This model predicted that even the poorest vaccine candidates should have an impact on the reduction of virus transmission even when high risk behaviour increased. Davenport *et al.* (2004) developed an age-structured epidemiological model of the effects of a disease-modifying vaccine. Their results suggest that the key predictor of vaccine efficacy is the extent of viral load reduction in vaccinated individuals – a one log reduction in viral load would significantly reduce HIV-associated mortality.

1.A.7.2. ONGOING CLINICAL TRIALS OF HIV VACCINES

The developing world carries the weight of the AIDS pandemic burden and antiretroviral therapy does not provide a long term solution to controlling the epidemic. There is thus an urgent need to develop an effective and inexpensive prophylactic vaccine against HIV-1. Recently, the results of the first two phase III trials of a gp120 subunit vaccine (AIDSVAX) were reported (Cao *et al.*,

2003). These studies showed the lack of protective efficacy of the envelope vaccine, but this was not unexpected since AIDSVAX does not induce neutralizing antibodies. There are, however, more than 30 ongoing clinical trials of HIV-1 preventive vaccines (Table 1.2; http://www.iavi.org/). These trials are phase I for the most part and are thus designed to test safety and to generate preliminary immunogenicity data for the different candidates.

The trials which are underway are a clear indication of the current breadth of innovation in vector design and vaccine approaches. Much progress has been made in eliciting HIV-specific CD4+ and CD8+ responses using these candidate vaccines in animal models (Amara *et al.*, 2001; Shiver *et al.*, 2002; Barouch *et al.*, 2000 and 2001, Matano *et al.*, 2001; Chen *et al.*, 2001; Rose *et al.*, 2001 and Davis *et al.*, 2002) and human trials are necessary to test the validity of the CTL-based vaccines against HIV infection.

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Table 1.2. Ongoing Trials of Preventive HIV Vaccines [Last updated: 7 February, 2005; http://www.iavi.org]

Trial No.	Sponsor Manufacturer	Start Date	Sites (No.)	Vaccine	Antigen	Clade	Comment
Phase III (Large-size trials in high-risk populations; test vaccine efficacy)							
N/A	WRAIR, AFRIMS, MoH; Aventis, VaxGen	October 2003	Thailand (several)	ALVAC vCP1521 AIDSVAX B/E	*env* (E), *gag/pol* (B) *env* (B, E)	B, E B, E	16,000 healthy normal HIV negative adult volunteers
Phase II (Mid-size trials in low- & high-risk populations; test vaccine safety, immunogenicity)							
HVTN 502/ Merck 023	HVTN, Merck; Merck	December 2004	US (12), Canada (1), Peru (2), Dominican Republic (1), Haiti (1), Puerto Rico (1), Australia (1)	MRKAd5 HIV-1 gag/pol/nef	*gag, pol, nef*	B	To test whether cellular immune response generated by Merck's vaccine is potent enough to impact infection with HIV in 1,500 at-risk volunteers
ANRS VAC 18	ANRS; Aventis	September 2004	France (6)	LIPO-5	5 lipopeptides containing CTL epitopes (from Gag, Pol, Nef)	B	CompareCD8 response of 3 doses of LIPO-5 versus placebo
IAVI 010	IAVI; KAVI	February 2003	UK; Kenya	DNA.HIVA MVA.HIVA	*gag* + 25 CTL epitopes *gag* + 25 CTL epitopes	A A	HIV-DNA +/-MVA boost
Phase I/II (Mid-sized trials in low-risk populations; test vaccine safety, immunogenicity)							
HVTN 042/ ANRS VAC 19	HVTN, ANRS; Aventis	June 2004	US (13)	LIPO-5 ALVAC-HIV (vCP1452)	See above See above	B B	To evaluate safety and immunogenicity of LIPO-5 alone, vCP1452 alone, and ALVAC prime/LIPO-5 boost
GTU-MultiHIV	FIT Biotech	February 2004	Finland	GTU-MultiHIV B clade	*nef, rev, tat, gag, pol, env,* CTL epitopes	B	Immunogenicity of GTU-MultiHIV clade B DNA after intradermal and intramuscular injection.
HVTN 052	HVTN; Vical	December 2003	US (10)	VRC-HIVDNA-009-00-VP	*gag, pol, nef env*	B A,B,C	Phase IB, safety, immunogenicity of multiclade DNA Vaccine
N/A	UNSW; AVC	June 2003	Australia	pHIS-HIV-B rFPV-HIV-B	*gag*, RT, *rev, tat, vpu, env gag*, RT, *rev, tat, vpu, env*	B B	DNA Vaccine + fowlpox boost
Phase I (Small trials in low-risk populations; test vaccine safety, immunogenicity)							
IAVI C002	IAVI; IDT	January 2005	US (2)	ADMVA	*env/gag-pol, nef-tat*	C	Safety, immunogenicity of an MVA vector vaccine
HVTN 057	NIAID, VRC	November 2004	US (12)	VRC-HIVADV014-00-VP	*gag/pol* polyprotein *env*	B A,B,C	Safety, immune response to VRC-HIVADV014-00-VP, when given as a booster to already vaccinated adults
HVTN 059	NIAID, AlphaVax	October 2004	US (5)	AVX101 (VEE)	*gag*	C	Safety, immunogenicity of an alphavirus replicon

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Table 1.2. Ongoing Trials of Preventive HIV Vaccines, continued

Trial No.	Sponsor Manufacturer	Start Date	Sites (No.)	Vaccine	Antigen	Clade	Comment
VRC 007 (04-I-0254)	NIAID/VRC	August 2004	US (1)	VRC-HIVDNA016-00-VP	*gag, pol, nef env*	B A, B, C	Safety, immunogenicity of a 6-plasmid multiclade HIV-1 DNA vaccine
HVTN 055	NIAID; Therion	July 2004	US (6)	TBC-M358 (MVA) TBC-M335 (MVA) TBC-F357 (FPV) TBC-F349 (FPV)	*env, gag tat, rev, nef*, RT *env, gag tat, rev, nef*, RT	B B B B	Safety, immunogenicity of MVA-HIV and rFPV-HIV alone or in combination.
ANRS VAC 16	ANRS; Biovector SA	July 2004	France (6)	LIPO-4T (LPHIV-1)	4 lipopeptides containing CTL epitopes (from Gag, Pol-RT, Pol, Nef)	B	Safety and immunogenicity of lipopeptides LIPO-4T, by two administration routes.
VRC 006 (04-I-0172)	NIAID; GenVec	May 2004	US	VRC-HIVADV014-00-VP	*gag/pol* polyprotein *env*	B A,B,C	Safety, tolerability, immune response of a multiclade HIV adenoviral vector vaccine in uninfected adults.
N/A	AVANT; NIAID; WRAIR	May 2004	US	LFn-p24	Anthrax-derived polypeptide LFn *gag* p24 protein	B	18 health volunteers. Aim: inducing strong and persistent HIV-1 gag specific CD8 T Cell responses.
HVTN 056	NIAID, Wyeth	April 2004	US (7)	HIV CTL MEP	CTL epitopes from *env* or *gag*	B	Safety of and immune response to a new HIV vaccine: HIV CTL MEP
N/A	UMMS; ABL	April 2004	US	DNA Proteins	*gag* + 5 *env* 5 recombinant gp120	A,B,C,E A,B,C,E	DNA prime: 1 gag gene (C) + 5 env genes (A, 2 B, C, E). Boost: 5 gp120 (same isolates as DNA). Adjuvant: QS21.
HVTN 050/ Merck 018	NIAID; Merck	January 2004	Thailand, Brazil, Haiti, Puerto Rico	MRKAd5 HIV-1	*gag*	B	Replication defective Ad-5 vector
Phase I (Small trials in low-risk populations; test vaccine safety, immunogenicity) (continued)							
IAVI A001	IAVI; Targeted Genetics	December 2003	Belgium (2); Germany (2); India (1)	tgAAC09 AAV	*gag*, protease, rt	C	Recombinant AAV vector; single shot
IAVI C001	IAVI; ADARC; Vical	December 2003	US (2)	ADVAX DNA	*gag, env, pol, nef, tat*	C	Multi-gene approach
HVTN 049	HVTN; Chiron	December 2003	US (8)	Gag and Env DNA/PLG Oligomeric gp140/MF59	*gag, env* DNA/PLG; Oligomeric *gp140*	B B	Safety, Immunogenicity of DNA/PLG and env DNA/PLG prime, oligomeric gp140/MF59 boost
HVTN 044	HVTN; Vical	December 2003	US (3)	VRC-HIVDNA-009-00-VP	*gag, pol, nef env*	B A,B,C	Safety, immunogenicity of of multiclade DNA Vaccine with IL-2/Ig DNA adjuvant
IAVI 011	IAVI, SAAVI IDT	November 2003	South Africa (2), UK (1), Switzerland (1)	MVA-HIVA	*gag* + 25 CTL epitopes	A	Dose response
EnvPro	St Jude's	September	US	EnvPro protein	*gp140*	D	Purified env

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Table 1.2. Ongoing Trials of Preventive HIV Vaccines, continued

		2003					protein
Trial No.	**Sponsor Manufacturer**	**Start Date**	**Sites (No.)**	**Vaccine**	**Antigen**	**Clade**	**Comment**
ISS P-001	ISS; Excell	September 2003	Italy (4)	HIV-1 Tat protein	Tat	B	Safety, immunogenicity of the recombinant HIV-1 Tat protein in healthy HIV-negative volunteers.
N/A	Merck; Aventis Pasteur	2003	US (17)	MRKAd5 HIV-1; ALVAC vCP205	*gag env, gag, pol*	B	MRKAd5 HIV-1 prime, ALVAC vCP205 boost
HVTN 040	NIAID; SAAVI	July 2003	US (4); South Africa (2)	AVX101 VEE	*gag*	C	Safety and immunogenicity of VEE vector
ANRS VAC 14	ANRS; Aventis	June 2003	France (2)	gp160MN/LAI-2	*gp120* (MN strain), *gp41* (LAI strain)	B	Safety and immunogenicity, using several routes
HVTN 048	NIAID; Epimmune	April 2003	US (2); Botswana	EP HIV-1090 DNA	21 CTL epitopes from *gag, pol, env, nef, rev, vpr*	All	Safety and immunogenicity
VRC 004 (03-I-0022)	NIAID/VRC; Vical	November 2002	US	VRC-HIVDNA009-00-VP	*gag, pol,nef* (clade B); *env* (clades A, B, C)	A, B, C	Safety and immunogenicity of a multiclade vaccine
B011; RV 138	WRAIR; Aventis	July 2002	US	ALVAC-HIV vCP205	*env, gag, pol*	B	Response to vaccine subcutaneously (via dendtritic cells), intradermally, or intramuscularly
N/A	Merck	2002	US	gag DNA	*gag*	B	Dose response
01-I-0079	NIAID/VRC; Vical	January 2001	US	VRC4302 DNA	*gag, pol*	B	Dose and immune response
N/A	Merck	2001	US	gag DNA Ad5 gag	*gag gag*	B B	Evaluation of DNA vs. Ad5 prime + Ad5 boost

KEY:
ABL: Advanced BioScience Laboratories, Inc.; ADARC: Aaron Diamond AIDS Research Center; AFRIMS: Armed Forces Research Institute of Medical Sciences, Bangkok, Thailand, is a joint U.S.-Royal Thai Army Command; AlphaVax: AlphaVax Human Vaccines Inc.; ANRS: Agence Nationale de Recherche sur le SIDA; AVANT: AVANT Immunotherapeutics, Inc.; AVC: Australian Vaccine Consortium; Aventis: Aventis Pasteur; CAN: Canada; Chiron: Chiron Corporation; CTL: cytotoxic T-lymphocyte; Epimmune: Epimmune Inc.; Excell: Excell Biotech; GSK: GlaxoSmithKline; HVTN: HIV Vaccine Trials Network; IAVI: International AIDS Vaccine Initiative; IDT: Impfstoffwerk Dessau Tornau GmbH; ISS: Istituto Superiore di Sanità; KAVI: Kenyan AIDS Vaccine Initiative; MoH: Ministry of Health (Thailand); MRC: UK Medical Research Council; NIAID: U.S. National Institute Allergy and Infectious Diseases; NL: Netherlands; PACTG: Pediatric AIDS Clinical Trials Group; PR: Puerto Rico; SAAVI: South African AIDS Vaccine Initiative; St Jude's: St Jude's Childrens Hospital; Therion: Therion Biologics Corporation; TT: Tetanus Toxoid; UMMS: University of Massachusetts Medical School; UNSW: University of New South Wales; US: United States; UVRI: Uganda Virus Research Institute; VEE: Venezuelan equine encephalitis; Vical: Vical Inc.; VRC: Vaccine Research Center; WRAIR: Walter Reed Army Institute of Research

1.A.7.3. HIV VACCINE STRATEGIES

The kinds of vaccine strategies being employed include live virus vectors, live bacterial vectors, DNA vaccines, pseudovirions, subunit vaccines and peptide vaccines (Table 1.3). The South African AIDS Vaccine Initiative (SAAVI) has funded a group working at the University of Cape Town to develop candidate vaccines using a number of these approaches. These include recombinant bacterial [bacillus Calmette Guerin (BCG) and *Salmonella*] and viral [modified

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vaccinia Ankara (MVA)] vaccines, DNA vaccines (van Harmelen *et al.*, 2003) and protein subunit vaccines (Jaffray *et al.*, 2004). The major vaccine strategies are reviewed briefly below.

Table 1.3. HIV vaccine strategies (adapted from Bojak *et al.*, 2002 and www.niaid.nih.gov/vaccine/concepts.htm)

VACCINE STRATEGY	DESCRIPTION	ADVANTAGES	DISADVANTAGES	STATUS
Live viral vectors	Recombinant viruses expressing HIV genes (eg. MVA, Canarypox)	Can control amount and kind of viral protein produces	Complicated to prepare; current vaccines elicit only modest immune responses	In phase II trials
Replicon vaccines	Viruses that are replication incompetent expressing HIV genes (eg. VEE and AAV)	Increased expression of the viral protein	Complicated to prepare	In phase I trials
Live bacterial vectors	Recombinant bacteria expressing HIV genes (eg. BCG and *Salmonella*)	Simple and inexpensive to produce	Can only accommodate a limited amount of genetic material; stability concerns	Close to phase I trials
DNA vaccines	Plasmid DNA expressing HIV genes	Simple and inexpensive to produce	Worries about integration of HIV genes into human cells; elicit only modest immune responses	In phase I trials
Pseudovirions	Non-replicating VLPs	Present HIV proteins in a relatively natural conformation	Difficult to produce	In phase I trials
Subunit vaccines	Recombinant viral proteins such as gp120, p24	Safe and simple to produce	Vaccine-elicited antibodies have failed to recognize primary HIV isolates	In phase II and phase II trials
Peptide vaccines	Chemically synthesized protein fragments and/or epitopes	Simple and inexpensive to produce; safe	Can only accommodate a limited amount of genetic material	In phase I trials
Combined vaccines	Combinations of the above strategies in prime-boost modalities (eg. DNA prime-rBCG boost)	Should stimulate both humoral and cellular immune responses	Current combinations elicit modest immune responses; antibodies are not neutralizing	In phase II trials

MVA, modified Vaccinia Ankara; VEE, Venezuelan equine encephalitis; AAV, Adeno-associated virus; VLP, virus-like particles

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Live viral vectors

A number of viral vectors in the form of replication-defective or live-attenuated viruses have been developed for use in vaccine research and are currently in phase I, II and III clinical trial (Table 1.2 and 1.3).

Poxviruses have been studied the most and have proceeded furthest in human trials (Table 1.2). An early study by Hu *et al.* (1992) demonstrated that macaques immunized with recombinant vaccinia virus expressing SIV gp160 and boosted with soluble protein was able to protect the animals from challenge with homologous virus. Later studies showed that the protective response was of limited breadth since macaques vaccinated with vaccinia virus expressing SIV Gag, Pol and Env and boosted with SIV particles were not protected from challenge with a low dose of

heterologous SIV_{mac} (Daniel *et al.*, 1994). Complete protection could only be achieved when the challenge virus was matched to the vaccine (Polacino *et al.*, 1999). Macaques immunized with recombinant MVA expressing Gag-Pol and Env were infected upon SIV challenge; however, there was a significant reduction in viraemia and an increase in survival compared to control animals (Seth *et al.*, 2000 and Ourmanov *et al.*, 2000). Importantly, the reduction in viral load was inversely correlated to the Gag-specific CTL response and the magnitude of the CTL response was a predictor of the magnitude of viral load reduction.

A number of poxvirus vectors have entered human trials with the most data available on the canarypox vaccines. Both humoral and cell-mediated immune responses have been elicited in human volunteers inoculated with canarypox vaccines expressing Gag-Pol and Env (Belshe *et al.*, 2001, Gorse *et al.*, 2001, Gupta *et al.*, 2002 and Cao *et al.*, 2003). In these studies, CTL responses and neutralizing antibody responses to T-cell laboratory-adapted isolates (Belshe *et al.*, 2001) and primary isolates (Cao *et al.*, 2003) were induced in most of the volunteers and the magnitude of these responses was increased by the administration of recombinant gp120 (Belshe *et al.*, 2001, Gorse *et al.*, 2001 and Gupta *et al.*, 2002). Despite low responses these approaches has moved into phase III clinical trials (Table 1.2).

The results of phase I testing of an MVA vaccine alone or as a boost following a DNA prime was found to be safe and was able to stimulate HIV-specific T-cell responses in most of the vaccinees (Mwau *et al.*, 2004). MVA alone induced stronger and more frequent responses than DNA alone and the prime-boost regimen was found to induce similar levels to MVA alone.

One of the most promising vaccine approaches used attenuated Adenovirus vector. An adenovirus-SIV envelope vaccine followed by a recombinant gp120 boost has been tested in macaques (Buge *et al.*, 1997 and 1999). SIV-specific humoral and cellular responses were detected and although infection resulted after challenge, vaccinated animals had a lower viral load at setpoint. In chimpanzees, the adenovirus-HIV envelope vaccine with a recombinant gp120

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boost elicited neutralizing antibodies which were effective against clinical and laboratory strains of HIV (Lubeck *et al.*, 1997). The chimpanzees also developed HIV-specific CTL responses. Casimiro *et al* (2003) tested the immunogenicity of replication-incompetent Adenovirus type 5 (Ad5) and DNA-based HIV-1 codon-optimized Gag vaccines in baboons. The Ad5 vaccine was able to elicit strong CTL responses, while the DNA vaccine induced weak responses that were moderately enhanced by chemical adjuvants. However, the DNA vaccine was an effective prime for both Ad5 and MVA vaccine boosts.

Replicon vaccines

These vaccines make use of replication incompetent viruses which can copy their genome. The HIV genes are inserted into the genome for expression and the viruses are used to deliver the HIV genes to the cells.

Venezuelan equine encephalitis virus (VEE) replicon particles carrying SIV Gag and Env induced humoral and cellular immune responses in vaccinated macaques (Davis *et al.*, 2000). After challenge with a pathogenic uncloned stock of SIV, all four of the vaccinated animals were protected against disease for 16 months. Vaccinated animals also had a mean peak viral load that was 2 orders of magnitude lower than unvaccinated controls and the viral load after the acute infection phase was 750-fold lower. This strength of the protective response and the reduction in viral load correlated with the magnitude of SIV-specific antibody and CTL responses.

Other replicon viral vectors include sindbis virus (Vadjy *et al.*, 2001), semliki forest virus (Hanke *et al.*, 2003) and adeno-associated virus (AAV; Xin *et al.*, 2001)

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Live bacterial vectors

BCG, *Salmonella* and *Listeria* have been developed for use as HIV vaccine vectors. Immunogenicity testing of live bacterial vectors vaccines is not as far along as viral vector testing, but results in various preclinical animal models have been encouraging.

The recombinant BCG approach is the subject of this thesis and is discussed in further detail in Chapter 1: Part B.

Salmonella spp. have been attenuated for use as potential vectors to stimulate immune responses in the gastrointestinal mucosa (Schodel and Curtiss, 1995). Berggren *et al.* (1995) were able to show proliferative responses in mice immunized with *S. typhimurium* expressing HIV gp120. Lymphoproliferative responses have also been shown in macaques vaccinated with *S. typhimurium* expressing the SIV capsid p27 (Steger *et al.*, 1999). Macaques inoculated with *S. typhimurium* expressing HIV-2 *gag* were not protected from a challenge infection with HIV-2 (Franchini *et al.*, 1995). *S. typhi* and *S. typhimurium* expressing fragments of SIV Gag fused to the type III-secreted protein SopE were shown to effectively prime a CTL response in vaccinated macaques (Evans *et al.*, 2003). Although only low-level responses were detected after *Salmonella* inoculations, boosting with MVA Gag yielded strong Gag-specific responses. However, there animals did not show an improved control of virus replication following an SIV challenge.

Listeria monocytogenes has been attenuated for use as a vaccine vector (Frankel *et al.*, 1995 and Lieberman and Frankel, 2002). *L. monocytogenes* expressing HIV *gag* was shown to induce strong and long-lasting CTL responses in immunized mice (Lieberman and Frankel, 2002). These CTL responses were able to protect mice from infection in a vaccinia virus challenge model. Peters *et al.* (2003) were also able to show that mucosally administered *L. monocytogenes* expressing HIV *gag* elicited HIV-specific CD8+ T cells in the spleens of vaccinated mice and that

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this response could be maintained by boosting. Safety and immunogenicity studies in macaques are ongoing (Lieberman and Frankel, 2002).

DNA vaccines

Extensive testing of HIV DNA vaccines performed in small animals and non-human primates (Robinson, 1997). DNA vaccines have been shown to elicit neutralizing antibodies to laboratory-adapted viruses and CTL responses against both HIV and SIV antigens (Boyer *et al.*, 1997; Letvin *et al.*, 1997; Robinson *et al.*, 1997; Barouch *et al.*, 2001; Akahata *et al.*, 2003 and Bazhan *et al.*, 2004). In some cases these responses have been protective (Boyer *et al.* 1997) or have resulted in a significant decrease in viral load at set point and prevention of disease (Boyer *et al.*, 1997; Letvin *et al.*, 1997; Barouch *et al.*, 2000; Kumar *et al.*, 2002 and Akahata *et al.*, 2003).

DNA vaccines alone are poorly immunogenic and a number of approaches have been used to optimize of antigen expression by DNA vaccine and improve immune responses to the antigens: optimizing codon usage (Andre *et al.*, 1998; zur Megede *et al.*, 2003; van Harmelen *et al.*, 2003 and Ramakrishna *et al.*, 2004); including immunomodulatory cytokine genes in the vaccine (Barouch and Letvin, 2000; Billaut-Mulot *et al.*, 2000; Boyer *et al.*, 2000 and Kim *et al.*, 2001) and formulation of the DNA vaccine with adjuvants or novel delivery systems (Ara *et al.*, 2001; Denis-Mize *et al.*, 2003 and Otten *et al.*, 2004). Prime-boost strategies using the DNA vaccine as the prime or the boost has also been investigated as a way to enhance the responses (see **Combined vaccines** below).

Clinical trials of a number of DNA vaccines on their own or as part of a prime-boost regimen are underway (Table 1.2). The results of a therapeutic DNA vaccine phase I trial using a plasmid expressing HIV-1 Env and Rev indicated that the vaccine was safe in the HIV-infected individuals. There were modest increases in anti-gp120 antibodies and gp160-specific CTL and lymphoproliferative activity (MacGregor *et al.*, 1998). When this DNA vaccine was administered to HIV seronegative volunteers, antigen-specific CD4+ T helper responses, lymphoproliferative

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responses and antigen-specific production of both IFN-γ and β-chemokine were detected (Boyer *et al.*, 2000 and MacGregor *et al.*, 2002). Another more recent trial evaluated a DNA vaccine and an MVA expressing HIV-1A p24/p17 fused to a string of CTL epitopes either separately or in a DNA prime-MVA boost combination. All three regimens were found to be safe and induced HIV-specific responses in the majority of the vaccinated people (Mwau *et al.*, 2004).

Pseudovirions

Non-infectious virus-like particles (VLPs) which are morphologically similar to immature HIV virions have been produced in a number of recombinant systems via expression of $Pr55^{Gag}$ (Gheysen *et al.*, 1989; Overton *et al.*, 1989; Shioda & Shibuta, 1990; Vernon *et al.*, 1991; Royer *et al.*, 1991, 1992; Jowett *et al.*, 1992; Mergener *et al.*, 1992 and Nermut *et al.*, 1994, 2003). Other epitopes and complete proteins are often incorporated into the Gag VLPs. Type I VLPs have small epitopes fused or integrated within the particle, while Type II VLPs incorporate the foreign proteins on the outer surface (Deml *et al.*, 2005). VLPs have been shown to stimulate both humoral and cellular immune responses in vaccinated animals (Table 1.4.).

A number of clinical trials in human volunteers have been conducted using HIV-1 p17/p24:Ty VLPs (p24-VLP) which is expressed in yeast (Adams *et al.*, 1987). In HIV-seronegative volunteers, the vaccine was shown to be safe and to induce production of anti-p24 antibodies and proliferative T-cell responses (Martin *et al.*, 1993 and Weber *et al.*, 1995). Although the vaccine was shown to be safe in HIV-seropositive individuals as well, there was no induction of antibodies against p24 (Veenstra *et al.*, 1996; Peters *et al.*, 1997 and Kelleher *et al.*, 1998). However, at higher doses, marginal Gag-specific immune responses were elicited in limited numbers of HIV-1-seropositive individuals, with some showing transient elevation of HIV-1 viral load (Klein *et al.*, 1997). When p24-VLP was administered in combination with zidovudine to HIV-positive individuals, the antibody levels did not change significantly and no proliferative responses were induced, although the CTL activity was augmented (Kelleher *et al.*, 1998 and Benson *et al.*,

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1999). In a long-term follow-up of HIV seropositive subjects vaccinated with p24-VLP, it was found that the vaccine did not slow progression to AIDS (Lindenburg *et al.*, 2002).

Combined vaccines

Although a single vaccine against HIV would be preferable, a number of studies have shown that the prime-boost regime induces stronger and broader immune responses. Several studies have shown that a heterlogous prime-boost strategy can yield protection from challenge in non-human primate models. A DNA prime-fowlpox virus boost was shown to contain viral challenges in macaques (Robinson *et al.*, 1999). A multigene DNA prime-vaccinia virus boost or vaccinia virus prime-multigene DNA boost regimens protected vaccinated macaques from CD4+ T cell loss and decreased the viral load at setpoint after SHIV89.6P challenge (Doria-Rose *et al.*, 2003). Bertley *et al.*, 2004 demonstrated anti-SHIV humoral and cellular responses in macaques using a DNA prime-MVA boost strategy that reduced CD4+ T cell loss and progression to AIDS after challenge with SHIV89.6P. A mucosal challenge with SHIV89.6P was controlled in macaques that received a DNA prime (Gag-Pol_Env) followed by an equivalent MVA boost (Amara *et al.*, 2001). In baboons, Casimiro *et al.* (2004) demonstrated that a replication-defective adenovirus prime-poxvirus boost regimen elicited responses that were significantly greater than either vaccine alone or by poxvirus prime-adenovirus boost. Leung *et al.* (2004) used a DNA prime-protein boost strategy to achieve antigen-specific antibody and lymphoproliferative responses in vaccinated baboons.

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Several human clinical trials based on the prime-boost approach are ongoing (Table 1.2). Examples include an Australia phase I/II trial testing a DNA prime-fowlpox boost regimen and a US phase I trial of a adenovirus prime and boost strategy.

Table 1.4. Induction of humoral and cellular immune responses by recombinant VLP immunogens (taken from Deml *et al.*, 2005)

Immunogen	Animal model	Antibodies		Neutralisation	CTL		Protection	Reference
		Carrier	Foreign polypeptide		Carrier	Foreign polypeptide		
Naked Gag-VLP	Rhesus macaques	n.d.	-	n.d.	Strong	-	n.d.	Paliard *et al.*, 2000
Type I VLPs								
HIV-2 Gag/V3, Gag/CD4BR, Gag/V3+CD4BR	Rabbits	Detectable	Detectable	Weak	n.d.	n.d.	n.d.	Luo *et al.*, 1992
HIV-1 Gag/V3	BALB/c mice, rats	Strong	Weak	Weak	n.d.	Strong	n.d.	Griffiths *et al.*, 1993
HIV-1 Gag/V3	BALB/c mice	Strong	Weak to medium	Weak	n.d.	Strong	n.d.	Wagner *et al.*, 1996
Type II VLPs								
VLP/gp160	New Zealand White Rabbits	n.d.	Strong	Medium	n.d.	n.d.	n.d.	Haffar *et al.*, 1991
VLP/chimeric HIV-$1_{LAI/MN}$ gp 160	SJL/L mice	Strong	Strong	Medium	n.d.	n.d.	n.d.	Rovinski *et al.*, 1992
VLP/EHV-1 gp14	BALB/c mice	n.d.	Good	n.d.	n.d.	n.d.	Complete protection	Osterrieder *et al.*, 1995
VLP/gp120TM variants	BALB/c mice	Strong	Medium	Weak, depending on gp120 variant	n.d.	n.d.	n.d.	Deml *et al.*, 1997a and b
VLP/gp160 +QS21	Rhesus macaques	Detectable (WB)	Detectable (WB)	Weak	Weak; transient	n.d.	n.d.	Montefiori *et al.*, 2001
VLP(GagPolNef)/ gp120TM	BALB/c mice	Strong	Strong	Weak	Strong	Strong	n.d.	Buonaguro *et al.*, 2002

Unless otherwise noted, all polypeptides were derived from HIV-1 isolates. Abbreviations: VLP: virus-like particle; n.d.: not defined; Gag: group-specific antigen; HIV: human immunodeficiency virus; gp: glycoprotein; V3: third variable loop of HIV-1 gp120; gp120: external HIV-1 glycoprotein; CD4BR: CD4-binding region of HIV-1 gp120; gp160: uncleaved HIV-1 envelope protein precursor; EHV-1: equine herpesvirus type-1; TM: transmembrane domain; QS1: natural saponin adjuvant derived from the tree Quillaja saponaria Molina; Pol: polymerase; Nef: negative-regulatory factor.

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PART B: RECOMBINANT BCG VACCINES

1.B.1. BACKGROUND

Bacille Calmette-Guérin (BCG), the vaccine against *Mycobacterium tuberculosis,* is the most widely administered vaccine worldwide. Calmette and Guérin developed the original strain of BCG from a virulent *M.bovis* strain isolated by Nocard (Oettinger *et al.*, 1999). From 1908 to 1919, the "lait Nocard" strain was passaged 230 times over glycerinated bile potato medium until they declared that organism was safe in guinea pigs, cows, horses, hamsters, mice and rabbits and provided protective immunity to virulent *M.tuberculosis* challenge (Guérin and Rosenthal, 1957). In 1921, after this preliminary animal testing, Weill-Hallé administered the vaccine orally to infants (Calmette, 1931).

Over the ensuing years, based on the reported protection of 90% shown in the vaccinated children, BCG was distributed all around the world. Different laboratories passaged and maintained their cultures and seed lots differently and there are now BCG variants that have emerged during production (Behr and Small, 1999). The substrains that are used most extensively today include Connaught, Danish, Glaxo, Moreau, Pasteur and Tokyo (Oettinger *et al.*, 1999). Behr and Small (1999) worked out an evolutionary framework of the substrains based on the variability of IS*6110* and mpt64 typing (fig.1.7.).

BCG has numerous advantages associated with its use: it can be given to neonates; a single vaccination can lead to long-lasting immunity; it has a proven safety record; it is heat stable and it is inexpensive to produce (Bloom and Fine, 1994). However, the reported protective efficacy of BCG varies widely in different regions. Colditz *et al.* (1995) performed a meta-analysis of published literature on the efficacy of the vaccine and concluded that BCG vaccination reduced the risk of tuberculosis in infants and neonates by over 50% on average. BCG vaccination also protected against pulmonary TB and disseminated TB (78% protective effect), tuberculous meningitis (64% protective effect) and reduced the risk of death by 71% (Colditz *et al.*, 1995).

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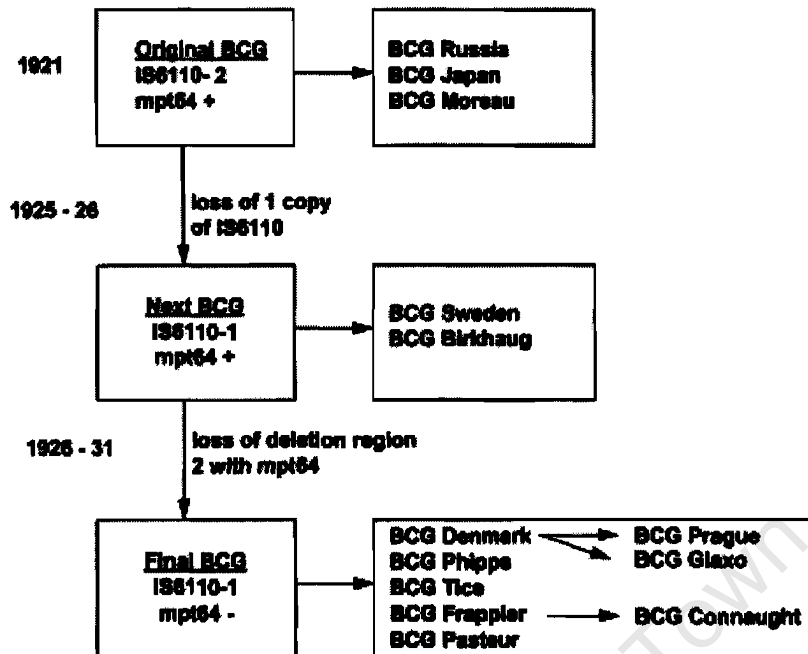

Fig.1.7. Evolutionary framework of BCG strains based on variability of IS*6110* mpt64 typing showing the BCG variants that have emerged during production. (Behr and Small, 1999)

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1.B.2. RECOMBINANT BCG TECHNOLOGY

BCG is a challenging organism to manipulate genetically. It has a slow growth rate with a long *in vitro* doubling time of 20-24 hours – it takes 2-4 weeks to form colonies on solid media. The lipid-rich cell wall means that BCG will not form single colonies on solid medium and it forms clumps in liquid culture unless a detergent is added to the growth medium (O' Donnell, 1997). DNA transformation of BCG is inefficient. Other properties such the high GC content of the genome and *Mycobacterial* promoters and origins of replication have necessitated the development of genetic manipulation tools that are *Mycobacterium*-specific.

Jacobs *et al.* (1987) constructed the first shuttle vectors that allowed the introduction of foreign DNA by infection into *M.smegmatis* and BCG. These recombinant shuttle phasmids were chimaeras that contained mycobacteriophage DNA and an *E.coli* cosmid; they were thus able to replicate as plasmids in *E.coli* and as phages in *Mycobacteria*. A plasmid transformation system

was developed by randomly inserting an *E.coli* plasmid expressing a kanamycin resistance gene into the *M.fortuitum* pAL5000 plasmid (Snapper *et al.*, 1988). This shuttle vector expressed kanamycin resistance and was capable of replication in *E.coli, M.smegmatis* and BCG. Husson *et al.* (1990) described a method of gene replacement in *M.smegmatis* that made use of homologous recombination. Ranes *et al.* (1990) constructed an *E.coli-Mycobacterium* shuttle vector that expressed kanamycin resistance in both *M.smegmatis* and BCG, while Matsuo *et al.* (1990) used the extracellular α-antigen of *M.kansasii* to establish a foreign antigen secretion system in *Mycobacteria.* With this system, BCG was shown to secrete a B-cell epitope of HIV-1 p17 along with the α-antigen. Lee *et al.* (1991) made use of mycobacteriophage L5 to construct plasmid vectors that transformed *M.smegmatis, M.tuberculosis* and BCG by site-specific integration of the plasmid into the genome. These initial forays into shuttle vector construction lead to further studies where recombinant BCGs (rBCG) were manufactured that expressed foreign antigens that induced immune responses. Recombinant BCGs have been produced which express bacterial, parasitic and viral antigens, as well as rBCG expressing cytokines (see section 3.1.5).

1.B.3. IMMUNE RESPONSE TO FOREIGN ANTIGENS EXPRESSED IN rBCG

Tables 1.5. to 1.6. summarize the results of experiments investigating the immune response to foreign antigens expressed in rBCG in various animal models. Below is a brief review of the results.

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Table 1.5. Immune responses in vaccinated animals to rBCG expressing viral antigens under the control of different promoters with or without localization sequences

Antigen source	Antigen	Vector features: Promoter	Vector features: Gene or localization sequence fused to promoter	Animal	Immune response	Reference
HIV-1	p17 B-cell epitope	*M. kansasii* α-antigen	*M. kansasii* α-antigen signal	mice	antibodies in 3/7 mice; persisted for >14 months	Wada *et al.*, 1996
	V3 loop epitope	*M. kansasii* α-antigen	*M. kansasii* α-antigen signal	mice	CTLs produced	Kameoka *et al.*, 1994
	PND of V3 loop	*M. kansasii* α-antigen	*M. kansasii* α-antigen signal	Guinea pigs	DTH response, CTLs, antibodies; neutralization of lab and primary strains of HIV by serum antibodies; passive protection in immune deficient mice	Honda *et al.*, 1995
				mice	high titres of long-lasting antibodies; IgG detected in serum was able to neutralize primary strains *in vitro*	Hiroi *et al.*, 2001
	epitopes from PND of V3 loop (subtype E)	*M. kansasii* α-antigen	*M. kansasii* α-antigen signal	Guinea pigs	serum IgG from animals inoculated with the 12aa epitope-rBCG neutralized the WHO reference strain and primary strains of subtype E and Thai B(B`)	Chujoh *et al.*, 2002
	19aa codon-optimized V3 loop sequence from Japanese consensus	*M. kansasii* α-antigen	*M. kansasii* α-antigen signal	Guinea pigs	DTH responses, IgG and IgA serum antibodies, enhanced neutralization of HIV_{MN}, and high levels of IFN-γ and IL-2 production with combination of id and ir routes	Kawahara *et al.*, 2002
	Gp120	BCG hsp70	none	mice	MHC-I-restricted CTLs	Stover *et al.*, 1991
	Gp41 and Gp120	BCG hsp60	none	mice	anti-Gp41 antibodies and Gp120-specific CTLs	Fuerst *et al.*, 1992
	Nef	*Streptomyces albus* groES/groEL1	none	mice	proliferation	Winter *et al.*, 1991
	Gag and Env	hsp70	none	mice	IgG antibodies; IFN-γ and IL-2 production; CTLs produced	Aldovini and Young, 1991

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Table 1.5. Immune responses in vaccinated animals to rBCG expressing viral antigens under the control of different promoters with or without localization sequences, continued

Antigen source	Antigen	Vector features		Animal	Immune responsetr	Reference
		Promoter	Gene or localization sequence fused to promoter			
SIV	Gag	hsp70	first few codons of lacZ	macaques	CTLs produced	Yasutomi *et al.*, 1993
	Nef	*M.paratuberculosis* P_{AN}	ORF2	mice	proliferation and CTLS produced (subcutaneous immunization)	Winter *et al.* 1995
					proliferation and IFN-γ and TNF-α production after oral immunization	Lagranderie *et al.*, 1997
	N-terminal portion of Gp110	pBlaF*	none	mice and guinea pigs	CTLs produced in mice	Lim *et al.*, 1997
					IgG antibodies in mice and guinea pigs that could neutralize field isolates of SIVmac251	
	Nef and Gag	P_{AN}	none	mice	cocktail of all three rBCG elicited IgA and IgG antibodies; CTLs produced	Lagranderie *et al.*, 1998
	Env	pBlaF*				
				macaques	same cocktail elicited CTLs and IFN-γ production; challenge with SIVmac251 elicited cellular and antibody responses but did not prevent infection	Méderlé *et al.*, 2003
	Nef-Gag fusion	pBlaF*	none	mice	IFN-γ production after Gag protein boost; antibodies	Méderlé *et al.* 2002
	Gag, Pol, Env, Nef	hsp70	none	macaques	cocktail of all four rBCG elicited IgA and IgG antibodies; CTLs produced; proliferation	Leung *et al.*; 2000
Measles virus	N protein	hsp60	influenza virus NS protein	C3H/He mice	proliferation; antibodies; low levels neutralizing antibodies; after challenge: reduction in virus titre, decrease in encephalitis incidence and decrease in mortality	Fennelly *et al.*, 1995
		hsp60	BCG 19kDa signal			
		Streptomyces coelicolor A3 agarase protein	*Streptomyces coelicolor A3* agarase protein signal			

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Table 1.5. Immune responses in vaccinated animals to rBCG expressing viral antigens under the control of different promoters with or without localization sequences, continued

Antigen source	Antigen	Vector features		Animal	Immune response	Reference
		Promoter	Gene or localization sequence fused to promoter			
Measles virus	N protein	hsp60	Influenza virus NS protein	Infant macaques	proliferation but no antibodies; after challenge: did not prevent systemic infection but did reduce lung inflammation, virus titres were reduced and viral shedding was shorter	Zhu *et al.*, 1997
Human papillomavirus	L1 of HPV-6b E7 of HPV-16	hsp70	none	BALB/c and C57BL/6 mice	DTH, proliferation and antibodies; E7-specific CTLs; responses were less potent than conventional vaccine	Jabbar *et al.*, 2000
Hepatitis C virus	NS5a	*M.kansasii* α-antigen	*M.kansasii* α-antigen signal	mice	CTLs produced; reduction in virus titres following challenge	Uno-Furuta *et al.*, 2003
Rabies virus	N protein B- and T-cell epitopes	*M.leprae* 18kDa BCG hsp60	*M.leprae* 18kDa protein	mice	higher antibody titres than conventional rabies vaccine	Da Cruz *et al.*, 2002
Porcine reproductive and respiratory syndrome virus	GP5 and M proteins	hsp60	none	BALB/c mice	antibodies; IFN-γ production	Bastos *et al.*, 2002 and 2004
			BCG 19kDa signal	pigs	antibodies but no IFN-γ production; following challenge vaccinated pigs showed lower temperature, viraemiaand viral load	

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1.B.3.1. VIRAL ANTIGENS

A number of different viral antigens have been expressed in rBCG and have induced both cellular and humoral immune responses in vaccinated animals (Table 1.5.).

HIV-1 antigens

Matsuo *et al.* (1990) were the first group to express a viral antigen in rBCG. They expressed a B-cell epitope of HIV-1 p17 fused to the α-antigen from *M.kansasii* and demonstrated high titres of long-lasting antibodies to the epitope in 3 of 7 mice inoculated with the rBCG (Wada *et al.,* 1996). This α-antigen secretion system was used to express various HIV-1 Env antigens in rBCG:

- An immunodominant epitope from the V3 loop of HIV-1 *gp120*; splenocytes from mice inoculated subcutaneously with this rBCG generated a CTL response against V3-peptide pulsed P815 cells (Kameoka *et al.*, 1994)
- The principal neutralization domain (PND) of the HIV-1 V3; responses included a specific DTH response, specific lysis of V3-pulsed P815 cells and anti-PND antibodies in guinea pigs (Honda *et al.,* 1995); strong neutralization of HIV_{MN} and primary isolates by serum antibodies and protection against HIV-1 infection after passive transfer of serum antibodies into SCID/hu or SCID/PBL mice (Honda *et al.*, 1995); high titres of long-lasting antibodies after nasal inoculation in mice and high levels of prolonged serum-IgG which were able to neutralize clinical isolates of HIV-1 *in vitro* in Th1- and Th2-deficient mice (Hiroi *et al.,* 2001)
- V3 PND rBCG using epitopes (15, 12 and 11 amino acids) from a subtype E HIV-1; serum IgG from guinea pigs inoculated with the 12aa rBCG neutralized the WHO reference strain as well as field isolates of subtype E virus and Thai B(B`) strains (Chujoh *et al.*, 2001)

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- Codon-optimized DNA fragment encoding the 19 aa tip of the V3 sequence from the Japanese consensus HIV-1; significant DTH responses to V3 in guinea pigs inoculated with a combination of the intrarectal and intradermal routes; significantly higher titres of HIV-1 specific serum IgG and IgA and enhanced neutralization of HIV_{MN} compared with those immunized by the intrarectal route only; induction of high levels of IFN-γ and IL-2 in PBMC, splenocytes and intraepithelial lymphocytes was detected at least 110 weeks post-inoculation (Kawahara *et al.*, 2002)

rBCG expressing HIV-1 subtype B *gp120* from the *hsp70* or *hsp60* promoters elicited MHC-I restricted CTLs in immunized mice (Stover *et al.*, 1991 and Fuerst *et al.* 1992, respectively), while *gp41* expressed from the *hsp60* promoter elicited anti-gp41 antibodies (Fuerst *et al.*, 1992). rBCG expressing subtype B Gag and Env (*hsp70* promoter) elicited low but detectable levels of IgG antibodies against p24, p17 and gp160 in mice (Aldovini and Young, 1991). rBCG-Gag also induced substantial IFN-γ and IL-2 production and Gag-specific CTLs. Subtype B *nef* (*Streptomyces albus groES/groEL1* promoter; episomal) induced *in vitro* proliferation of lymph node cells from subcutaneously immunized mice (Winter *et al,* 1991).

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SIV antigens

Yasutomi *et al.* (1993) attempted to enhance expression from the *hsp60* and *hsp70* promoters by fusing the SIVmac251 *gag* gene to the first few codons of *lacZ*. The *hsp70*-regulated rBCG-Gag was given intradermally to macaques. No anti-Gag antibodies or Gag-specific CTL were detected. However, after a boost at 19 weeks, they were able to show MHC-I restricted lysis of vaccinia-SIVgag-infected target cells. The SIVmac251 *nef* gene fused to ORF2 in rBCG (P_{AN} promoter from *M.paratuberculosis*) induced significant proliferation in lymph node cells from mice inoculated subcutaneously (Winter *et al.*, 1995) and specific proliferation and production of IFN-γ and TNF-α in mice inoculated orally (Lagranderie *et al.*, 1997). The N-terminal portion of SIVmac *gp110* in rBCG (*pBlaF** promoter) induced CTL responses in mice and systemic IgG directed against *gp110* in mice and guinea pigs by parenteral routes of inoculation (Lim *et al.*, 1997).

These antibodies were able to neutralize primary field isolates of SIVmac251. Oral immunization of guinea pigs also produced significant levels of anti-gp110 antibodies in faeces.

Mice were inoculated with a cocktail of rBCG consisting of SIV *nef* and *gag* expressed (P_{AN} promoter) and SIV *env* (*pBlaF** promoter) via the oral, aerogenic, nasal and rectal routes (Lagranderie *et al.*, 1998). All routes induced IgA antibodies against SIV Env and Gag in bronchial lavages and faeces, as well as specific IgG in serum. Strong, specific cytotoxic responses against Nef, Env and Gag were induced in splenocytes by all mucosal routes of inoculation. Méderlé *et al.* (2003) administered this cocktail to cynomolgus macaques and after a single intradermal inoculation they found CTL responses to the 3 antigens in circulating blood cells as well as IFN-γ secretion. Rectal or oral boosting elicited anti-SIV IgA in the rectum and increased IFN-γ secretion by the circulating blood cells. After rectal challenge with pathogenic SIVmac251, anti-SIV cellular and antibody responses were higher in rBCG-inoculated monkeys than in controls, however, the viral load in plasma was comparable for all animals. This indicates that anti-SIV CD4+ T-cell memory was induced even though infection was not prevented. rBCG expressing the entire SIV *gag*, *pol* and *nef* genes and a truncated *env* gene in separate shuttle vectors (*hsp70* promoter) were given to rhesus macaques in a single intravenous inoculation of all four rBCG (Leung *et al.*, 2000). SIV-specific IgA and IgG antibodies, CTL responses and helper T cell proliferation were induced.

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A nef-gag (SIVmac251) fusion in rBCG expressed under the control of the pBlaF* promoter in both integrative and episomal vectors was developed by Méderlé *et al.* (2002). They found that a high level and persistent expression of the *gag* gene was required for the generation of memory T cells in mice inoculated with this rBCG. However, after a Gag protein boost, similar levels of IFN-γ production were detected from mice inoculated with either the episomal or the integrative vectors. This suggested that only an initial high level of gag expression in the rBCG was critical for the induction of cellular immune responses. This is further confirmed by the fact that they could not detect cellular responses to Nef, which was expressed at a lower level. Despite a low level of Gag

expression in one of the integrative vectors, however, high levels of anti-Gag antibodies were detected. This implies that the same is not true for the humoral response in this system.

Measles virus antigens

Fennelly *et al.* (1995) attempted to develop an immunization strategy that would permit priming for T cell memory for measles virus (MV) in infants using rBCG expressing the full-length nucleocapsid (N) protein. They constructed three rBCGs with the MV N protein fused to: the influenza virus non-structural (NS) protein under the control of the *hsp60* promoter; the *Mycobacterial* 19kDa signal sequence also under *hsp60* promoter control; the *Streptomyces coelicolor A3* agarase protein (Dag) under the control of its own promoter. T cells from C3H/He mice immunized with the three rBCGs were able to proliferate when stimulated with recombinant N antigen and with MV. Anti-MV antibodies were also generated in immunized mice, while low levels of neutralizing antibodies were detected after intracranial infection with the CAM/R40 strain of MV. They also reported a considerable reduction in virus titre recovered from brain homogenates, a decrease in encephalitis incidence and severity and a decrease in mortality in rBCG-immunized mice compared with controls.

The same group tested rBCG expressing MV N protein in rhesus macaques (Zhu *et al.*, 1997). Infant monkeys were immunized with the rBCG and then challenged with MV. Although, N protein-specific lymphoproliferation was detected, no anti-N protein antibodies were. The rBCG vaccination did not prevent systemic MV infection but did reduce the lung inflammation after challenge. In addition, virus titres were significantly reduced in the lymph nodes and viral shedding was shorter in some of the monkeys.

Human papillomavirus antigens

The immunogenicity of rBCG vaccines expressing the L1 protein of human papillomavirus (HPV) 6b and the E7 protein of HPV-16 (*hsp70* promoter) was evaluated in BALB/c and C57BL/6 mice (Jabbar *et al.*, 2000). Both rBCG-L1 and rBCG-E7 elicited specific immune responses – DTH,

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proliferation and antibody responses were demonstrated for both and rBCG-E7 induced specific lysis of E7-expressing tumour cells. However, the observed responses were at a lower level than those elicited by the convential recombinant protein/adjuvant approach and the authors felt that the rBCGs would only be useful to prime or retain memory responses to antigens.

Hepatitis C virus antigens

An rBCG vaccine expressing a CTL epitope of the hepatitis C virus (HCV) nonstructural protein 5a (NS5a) fused to the *M.kansasii* α-antigen was tested in mice (Uno-Furuta *et al.*, 2003). NS5a-specific cytotoxic T lymphocytes that were MHC-I restricted were produced and when these mice were challenged with recombinant vaccinia virus expressingNS5a, they showed a significant reduction in virus titre compared with control mice.

Rabies virus antigens

Rabies virus nucleoprotein B-cell and T-cell epitopes were cloned into the *M.leprae* 18kDa antigen gene and the fusion protein was expressed under the control of either the *hsp60* promoter or the 18kDa promoter in rBCG (Da Cruz *et al.*, 2001). Both rBCG were able to induce a higher antibody titre in vaccinated mice than the conventional rabies vaccine – the higher response was elicited by the rBCG expressing the epitopes from the 18kDa promoter.

Porcine reproductive and respiratory syndrome virus antigens

Bastos *et al.* (2002 and 2004) used rBCG to express a truncated form of GP5 and M protein of porcine reproductive and respiratory syndrome virus (PRRSV) under the control of the *hsp60* promoter. Four different rBCG were constructed with both proteins being expressed either in the cytosol or as fusion proteins with the 19kDa protein on the cell membrane. BALB/c mice immunized with these rBCG were able to produce anti-GP and anti-M antibodies 30 days post-inoculation. At 60 and 90 days post-inoculation, PRRSV-specific IFN-γ was detected in the splenocytes of these mice. When these rBCG vaccines were given to pigs, they were also able to produce anti-GP and anti-M antibodies, but were not able to generate PRRSV-specific IFN-γ

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production. However, following challenge with a pathogenic PRRSV strain, rBCG-immunized pigs showed a lower temperature, viraemia and virus load in bronchial lymph nodes than control animals. This was suggestive of partial protection against PRRSV infection.

1.B.3.2. BACTERIAL ANTIGENS

Various bacterial antigens expressed in rBCG have also been shown to induce cellular and humoral immune responses in vaccinated animals (Table 1.6.).

***E.coli* antigens**

Stover *et al.* (1991) were the first to report expression of a bacterial antigen in rBCG. They expressed *E.coli* β-galactosidase (β-gal) from the BCG *hsp60* promoter in both an integrative and an episomal vector. BALB/c mice inoculated intravenously with rBCG:*lacZ* produced high levels of antibodies to β-gal, even after inoculation with as few as 200 cfu. Strikingly, the antibody response to β-gal was not only higher than the response to BCG antigens but also persisted for longer. IFN-γ production and cytotoxic activity was also demonstrated in mice receiving this rBCG.

The *lacZ* gene fused to part of ORF2 from IS*900* and expressed from the *M.paratuberculosis* P_{AN} promoter elicited proliferative responses, IFN-γ production and antibody responses that were β-gal specific in mice inoculated subcutaneously with this rBCG (Murray *et al.*, 1992). In guinea pigs inoculated by the oral, respiratory and intradermal routes (Lagranderie *et al.*, 1993), antibody responses to β-gal were induced. The rBCG:*lacZ* induced a mucosal IgA antibody response when administered orally and a DTH response to β-gal was also shown. Strong β-gal-specific T-cell proliferative responses were seen in the spleen and lymph node cells and the induction of TNF was seen in the sera of the guinea pigs.

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Table 1.6. Immune responses in vaccinated animals to rBCG expressing bacterial antigens under the control of different promoters with or without localization sequences

Antigen source	Antigen	Vector features		Animal	Immune response	Reference
		Promoter	Gene or localization sequence fused to promoter			
E.coli	β-gal	hsp60	none	BALB/c mice	long-lasting antibodies; IFN-γ production, cytotoxic activity	Stover *et al.*, 1991
		P_{AN}	none	BALB/c mice	antibodies; proliferation, IFN-γ production	Murray *et al.*, 1992
		P_{AN}	none	Guinea pigs	antibodies, DTH, proliferation	Lagranderie *et al.*, 1993
		α-antigen	α-antigen signal	BALB/c mice	Thi1-like response with IFN-γ and IL-2 production	Kumar *et al.*, 1999
	MalE			BALB/c mice	antibody and proliferative responses highest for secreted form	Himmelrich *et al.*, 2000
Tetanus toxin	ToxC	hsp60	none	NIH Swiss mice	antibodies; protection from challenge	Stover *et al.*, 1991 and Fuerst *et al.*, 1992
Pertussis toxin and tetanus toxin	S1-TTC	hsp60		mice	antibodies to tetanus toxin; neutralized toxins in challenge; IL-2 production	Abomoelak *et al.*, 1999
		Ag85A	Ag85A signal		none	
Pertussis toxin	S1PT	pBlaF*	β-lactamase	BALB/c mice	low-level antibodies	Nascimento *et al.*, 2000
				Swiss mice	IFN-γ production; protection from challenge	
Diptheria toxin	CRM_{197}	pBlaF*		BALB/c mice	antibody response; no neutralization	Miyaji *et al.*, 2001
					in combination with rBCG-FC neutralization was shown	Miyaji *et al.*, 2001 and Mazzantini *et al.*, 2004
Tetanus toxin	FC	pBlaF*		BALB/c mice	antibodies	Mazzantini *et al.*, 2004
					in combination with rBCG-CRM_{197} also elicited antibodies and induced protection	
				Guinea pigs	antisera from animals neutralized tetanus and diptheria toxin	

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Table 1.6. Immune responses in vaccinated animals to rBCG expressing bacterial antigens under the control of different promoters with or without localization sequences, continued

Antigen source	Antigen	Vector features		Animal	Immune response	Reference
		Promoter	Gene or localization sequence fused to promoter			
E.coli heat labile enterotoxin	LT-Bh	hsp60	none	BALB/c mice	IgG and IgA antibodies with all three	Hayward *et al.*, 1999
		19kDa antigen	19kDa antigen signal		mucosal and serum IgA antibodies with all three but greater for secreted and membrane-associated	
		hsp60	α-antigen signal			
Cholera toxin	CTB	hsp60	α-antigen signal	mice	anti-BCG IgA responses that correlated with TGF-β production	Biet *et al.*, 2003
Borrelia burgdorferi	OspA	hsp60	none	BALB/c; C3H/HeJ and Swiss Webster (outbred) mice	protective antibody responses (highest for the membrane-associated form)	Langermann *et al.*, 1994a
			membrane			
			secreted		IgA response better than ip IgA response; disseminated throughout the mucosal immune system	
		hsp60	membrane	Humans	initially PPD-skin test and OspA antibody negative; converted to PPD-skin test positive but didn't develop anti-OspA antibodies	Edelman *et al.*, 1999
Streptococcus pneumoniae	PspA	hsp60	none	BALB/c and C3H/HeJ mice	antibodies with comparable titres; protective responses for membrane-associated and secreted forms; antisera showed passive protection in immunodeficient mice	Langermann *et al.*, 1994b
			membrane			
			secreted			
Listeria monocytogenes	p60	hsp60	none	BALB/c mice	membrane-associated and secreted forms protected from challenge	Grode *et al.*, 2002
			19kDa antigen signal			
			Ag85B signal			
M.tuberculosis	30kDa major secretory protein	30kDa major secretory protein	none	Guinea pigs	better protection from challenge than wild-type BCG	Horwitz *et al.*, 2000

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Table 1.6. Immune responses in vaccinated animals to rBCG expressing bacterial antigens under the control of different promoters with or without localization sequences, continued

Antigen source	Antigen	Vector features: Promoter	Vector features: Gene or localization sequence fused to promoter	Animal	Immune response	Reference
M.tuberculosis	ESAT-6	hsp60	Hsp60 α-antigen signal	BALB/c mice	Hsp60 fusion yielded higher antibody responses and IFN-γ production; both strains conferred protection against challenge but not better than wild-type BCG	Bao *et al.*, 2003
Mycobacteria	19kDa antigen; 38kDa antigen and ESAT-6	Trm	none	BALB/c mice	Th2 response from rBCG-19kDa (IL-10 production) Th1 response from rBCG-38kDa (IFN-γ production) mixed Th1/Th2 response from ESAT-6 (~IL-10 and IFN-γ levels)	Rao *et al.*, 2003
	Ag85A; Ag85B and MPB51	Native promoters	Native signals	BALB/c and C57BL/6 mice	multiplication of *M.leprae* in mouse footpads was inhibited to larger extent than with wild-type BCG	Ohara *et al.* 2000 and 2001
M.avium	Ag85B	*M.leprae* hsp65 T31 S16	none	BALB/c mice	BCG-specific immune responses that were predominantly Th1 in nature	Dhar *et al.*, 2003
	35kDa protein	pBlaF*	none	C57BL/6 mice	proliferation and IFN-γ production; inhibition of *M.avium* growth after challenge but not better than wild-type DNA vaccine induced similar proliferation and IFN-γ production, which was enhanced by a boost; reduced *M.avium* growth DNA prime-BCG boost better than BCG alone but not better than DNA alone	Martin *et al.*, 2001

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Splenocytes taken from mice vaccinated with an rBCG that secreted *E.coli* β-gal secreted under the control of the BCG α-antigen promoter and signal sequence produced larger amounts of β-gal-specific IFN-γ and IL-2 and lower amounts of IL-5 compared with controls (Kumar *et al.*, 1999). They also had significantly lower β-gal-specific serum IgE, indicating that rBCG secreting antigens could be used to induce a Th1-like response.

Himmelrich *et al.* (2000) expressed *E.coli* MalE as cytosolic, membrane-associated or secreted protein in rBCG and found that both humoral and proliferative responses were highest in mice receiving rBCG that secreted MalE.

Bacterial toxins

Stover *et al.* (1991) expressed fragment C (ToxC) of *Clostridium tetani* tetanus toxin from the BCG *hsp60* promoter. Although the expression of ToxC was only 1/250th that of β-gal expressed in the same system, NIH Swiss mice receiving rBCG:*toxC* intradermally were able to mount an antibody response which increased for at least 12 weeks. Antibody responses to rBCG:*toxC* expressed from an integrative vector were able to completely protect or partially protect NIH Swiss mice (inoculated either intradermally or intraperitoneally) from challenge with 100 lethal doses of tetanus toxin (Fuerst *et al.* 1992).

Two rBCGs expressing a hybrid protein containing the S1 subunit of pertussis toxin fused to fragment C of tetanus toxin were constructed (Abomoelak *et al.*, 1999). This S1-TTC protein was expressed under the control of either the BCG *hsp60* promoter or the 85A antigen promoter and signal peptide. Although expression was obtained from both rBCGs, secretion was not obtained with the 85A antigen signal peptide and only the *hsp60* rBCG was able to induce antibodies specific to the tetanus toxin in inoculated mice. These antibodies were able to neutralize toxin activity in a challenge model. In addition, this BCG induced S1 and TTC-specific IL-2 production in the vaccinated mice.

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In the first step towards an alternative to the diptheria-pertussis-tetanus toxoid (DPT) vaccine, the detoxified S1 subunit of pertussis toxin (S1PT) was expressed under the control of the *M.fortuitum* β-lactamase promoter, *pBlaF** in fusion with either the entire β-lactamase signal sequence or the entire β-lactamase protein (Nascimento *et al.*, 2000). rBCG-S1PT induced low-level antibody production in immunized BALB/c mice, whereas Swiss mice showed elevated IFN-γ levels in their splenocytes after stimulation with pertussis toxin. These mice also had a high level of protection against intracerebral challenge with live *Bordetella pertussis.*

Next, the potential of CRM_{197}, a mutated nontoxic derivative of diphtheria toxin, was analyzed as an antigen for expression in rBCG (Miyaji *et al.*, 2001). The mutated toxin was also expressed under the control of the *M.fortuitum pBlaF** promoter. BALB/c mice immunized with this rBCG mounted an anti-diphtheria toxoid antibody response, but the antisera from these mice were unable to neutralize diphtheria toxin activity. However, when the rBCG was administered in combination with an rBCG expressing the tetanus toxin fragment C (FC), the antibodies produced did show neutralizing activity; this suggested that the rBCG-FC had an adjuvant effect on the response induced by rBCG-CRM_{197}. The rBCG-CRM_{197} has an adjuvant effect on the response induced by rBCG-FC (*pBlaF** promoter) as well (Mazzantini *et al.*, 2004). The rBCG-FC alone and in combination with rBCG- CRM_{197} induced anti-tetanus toxin antibodies in immunized mice; the combination reduced the time required for the immune response to mature and increased the levels of anti-tetanus antibodies. The combination of the two rBCGs also protected 75% of mice from challenge with 100 minimum lethal doses of tetanus toxin. Additionally, antisera from guinea pigs immunized with both rBCGs were able to neutralize both tetanus and diphtheria toxin.

Hayward *et al.* (1999) constructed three different rBCGs that expressed the B sububit of *E.coli* heat labile enterotoxin (LT-Bh) in either cytosolic (*hsp60* promoter), membrane-associated (19kDa antigen promoter and signal sequence) or secreted (*hsp60* promoter and α-antigen signal sequence) form. IgG and IgA antibodies to LT-Bh were induced in BALB/c mice inoculated intraperitoneally with these three rBCG. Oral inoculation induced both mucosal and serum IgA

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antibodies to LT-Bh but antibody responses were greater for secreted or membrane-associated LT-Bh than the cytoplamically expressed LT-Bh.

An rBCG strain producing (*hsp60* promoter) and secreting (α-antigen secretion signal) the cholera toxin B subunit (CTB) induced anti-BCG IgA responses in the bronchoalveolar lavage fluids of intranasally immunized mice that were stronger than the responses to wild-type BCG (Biet *et al.*, 2003). This IgA response correlated well with increased TGF-β production in the mice. The results indicated that the CTB-producing rBCG could be used as a powerful adjuvant for mucosal vaccines.

***Borrelia burgdorferi* OspA**

The outer surface protein A (OspA) of *Borrelia burgdorferi* was expressed under the control of the *hsp60* promoter as a cytosolic protein, as a secreted fusion protein or as a membrane-associated lipoprotein (Stover *et al.*, 1993). The membrane-associated form resulted in protective antibody responses that were 100-1000-fold higher than the responses elicited by the other forms in three different mouse strains (BALB/c, C3H/HeJ and outbred Swiss Webster) after two intraperitoneal inoculations of 10^6 cfu. When investigating the intranasal route of delivery, they found that the IgG response was similar to that in mice inoculated ip. The IgA response, however, was potent and highly sustained (Langermann *et al.*, 1994a). This secretory IgA response was disseminated throughout the mucosal immune system and was also shown to have the features of a systemic immune response by the appearance and persistence of lymphoid aggregates.

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Based on these results, the rBCG expressing OspA in membrane-associated form was used in the first phase I clinical trial involving a rBCG vaccine (Edelman *et al.*, 1999). The vaccine was administered intradermally to 24 healthy adults in four progressively higher doses (from $2x10^4$cfu to $2x10^7$cfu) over a period of 28 weeks. Initially the volunteers were both PPD-skin test negative and OspA antibody negative. Eventually, 13 of the 24 converted to PPD-skin test positive but

none of them developed antibodies against OspA. The rBCG had a safety profile that was deemed comparable to that of the parental BCG.

***Streptococcus pneumoniae* PspA**

The parental vectors described by Stover *et al.* (1993) were used to express the NH_2-terminal portion of Pneumococcal surface protein A (PspA) of *Streptococcus pneumoniae* in BCG (Langermann *et al.*, 1994b). All the rBCG:PspA strains induced PspA-specific antibodies with comparable titres in BALB/c and C3H/HeJ mice. Animals immunized with the rBCG that expressed PspA in secreted or membrane-associated form were able to mount protective responses to a lethal dose of virulent pneumococci. The antisera from these animals were shown to passively protect immunodeficient mice from challenge with three different highly virulent strain of *S.pneumoniae*.

***Listeria monocytogenes* p60**

BALB/c mice inoculated with rBCG strains expressing (*hsp60* promoter) the *L.monocytogenes* p60 Ag in secreted (Ag85B secretion signal) and membrane-associated (19kDa secretion signal) form yielded equivalent anti-listerial protection, while the cytosolic form yielded no protection (Grode *et al.*, 2002).

***Mycobacterial* antigens**

In an effort to increase the efficacy of BCG as an anti-tuberculosis vaccine, Horwitz *et al.* (2000) developed an rBCG vaccine expressing the *M.tuberculosis* 30-kDa major secretory protein. The protein was expressed from its own promoter in two BCG host strains (Connaught and Tice). They tested the efficacy in a highly susceptible guinea pig model and found that after challenge with a highly virulent strain of *M.tuberculosis* the guinea pigs that received the rBCG had 0.5 logs fewer bacilli in their lungs and 1 log fewer in their spleens than those that received conventional BCG. The animals receiving the rBCG also had fewer and smaller lesions in their lings, spleen and liver than those receiving parental BCG vaccines.

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A similar strategy was employed to express the *esat-6* gene of *M.tuberculosis* in rBCG under the control of the *hsp60* promoter either as a fusion protein linked to Hsp60 or as a secreted protein (α-antigen; Bao *et al.*, 2003). The rBCG expressing the fusion protein yielded significantly higher specific antibody responses and higher levels of antigen-specific IFN-γ production in immunized BALB/c mice, while the rBCG expressing the secreted ESAT-6 induced immunity similar to that of wild-type BCG. Although both strains of rBCG conferred protection against virulent *M.tuberculosis* challenge, this protection was not significantly better than that conferred by wild-type BCG vaccination.

The *M.tuberculosis* 19kDa antigen, 38kDa antigen and ESAT-6 antigen genes were cloned under the control of the strong *M.tuberculosis* ribosomal RNA promoter, Trrn, for expression in rBCG (Rao *et al.*, 2003). The immune response in BALB/c mice immunized with these rBCG strains was modulated significantly in comparison with the response in mice receiving wild-type BCG. The responses to the antigens, however, were markedly different: the rBCG expressing the 19kDa antigen exhibited a Th2-type response characterized by a high level of IL-10 production after BCG sonicate stimulation; the rBCG expressing the 38kDa antigen produced high levels of IFN-γ in response to stimulation and was thus polarized towards a Th1-type response; the ESAT-6 expressing rBCG exhibited a mixed Th1/Th2 response with levels of antigen-specific IFN-γ and IL-10 production more or less comparable.

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The multiplication of *M.leprae* in the footpads of BALB/c and C57BL/6 mice immunized with rBCG that overproduced two of the *Mycobacterial* antigen 85 complex of major secretory proteins – Ag85A and Ag85B – and the related protein, MPB51 along with their native signal sequences under the control of their own promoters was inhibited to a greater extent than the inhibition induced by parental BCG (Ohara *et al.*, 2000 and 2001).

Martin *et al.* (2001) compared the immune response induced by rBCG over-expressing the *M.avium* 35kDa protein to that induced by a DNA vaccine expressing the same antigen. rBCG-35, expressing the protein from the *pBlaF** promoter, induced strong antigen-specific IFN-γ-secreting and proliferative responses in the T-cell of immunized C57BL/6 mice. DNA-35 induced similar levels of proliferation and IFN-γ secretion after a single immunization and this was enhanced by a further immunization. While rBCG-35 immunization did inhibit virulent *M.avium* growth after challenge, the level was similar to that induced by wild-type BCG; DNA-35 immunization significantly reduced *M.avium* growth. DNA prime-rBCG boost strategies yielded better protection than rBCG alone but not better than DNA alone.

BALB/c mice immunized with rBCG expressing Ag85B under the control of either the *M.leprae* hsp65 promoter, the T31 or the S16 promoters (from a Mycobacterial promoter library) mounted BCG-specific immune responses that were analyzed to determine whether they were Th1 or Th2 in nature (Dhar *et al.*, 2003). Investigating the antigen-specific IFN-γ versus IL-10 and the IgG2a versus IgG1 responses indicated that, although the responses ranged from a mixed Th1/Th2 to Th1, they were predominantlyTh1 in nature.

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1.B.3.3. PARASITE ANTIGENS

rBCG technology has been used for the expression of various parasite antigens from different promoters. Animals immunized with these rBCG have been shown to mount cellular and humoral immune responses to these antigens (Table 1.7.).

Table 1.7. Immune responses in vaccinated animals to rBCG expressing parasite antigens under the control of different promoters with or without localization sequences

Antigen source	Antigen	Vector features: Promoter	Vector features: Gene or localization sequence fused to promoter	Animal	Immune response	Reference
Plasmodium falciparum	CSP	64kDa antigen	none	mice	no humoral or proliferative responses	Haeseleer *et al.*, 1993
		hsp70		BALB/c mice	antibodies; IFN-γ and IL-2 production; enhanced by boost	Zheng *et al.*, 2001a and 2002
	MSA2	hsp70	none	BALB/c mice	significant and sustained antibodies; proliferative responses; IFN-γ and IL-2 production	Zheng *et al.*, 2001b
P.yoelii	CSP B cell epitope	α-antigen	α-antigen signal	mice	long-lasting antibodies in 1/7 mice	Matsumoto *et al.*, 1996b
	MSP1	α-antigen	α-antigen signal	C3H/He mice	IFN-γ production related to clearance of parasite; antisera was shown to protect against challenge	Matsumoto *et al.*, 1998
Leishmania major	gp63	hsp60	none	BALB/c and CBA/J mice	significant protection against *L.mexicana* challenge but only CBA/J mice protected against *L.major*	Connell *et al.*, 1993
		pBlaF*	*M.fortuitum* β-lac protein	BALB/c mice	significant protection against *L.major* challenge	Abdelhak *et al.*, 1995
L.chagasi	LCR1	hsp60	none	BALB/c mice	better protection against challenge with rBCG than with LCR1 protein alone; IFN-γ production	Streit *et al.*, 2000
Schistosoma mansoni	Sm28GST	hsp60	none	BALB/c mice	significant antibodies enhanced by booster; neutralization of Sm28GST enzyme activity; IgG1, IgG2a, IgG2b and IgA isotypes produced	Kremer *et al.*, 1996
	Sm14	pBlaF*	*M.fortuitum* β-lac protein	BALB/c mice	IFN-γ production; 48% decrease in worm burden after cercarial challenge	Varaldo *et al.*, 2004
S.haematobium	Sh28GST	hsp60	none	BALB/c mice	strong antibodies; IgG1, IgG2a, IgG2b and IgA isotypes produced	Kremer *et al.*, 1998
Toxoplasma gondii	GRA1	Ag85A	Ag85A signal	OF1 outbred mice	no humoral or cellular response	Supply *et al.*, 1999
				sheep	proliferation and IFN-γ production; partial protection from challenge	

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Malarial antigens

The first attempt to induce an immune response to malarial antigens using rBCG technology was unsuccessful. Haeseleer *et al.* (1993) were able to express the first 412 amino acids of the *Plasmodium falciparum* circumsporozoite protein (CSP) under the control of the BCG 64kDa antigen promoter in an integrative shuttle vector system but were unable to induce humoral or proliferative responses in immunized mice. In a more recent study, the CSP gene from *P.falciparum* strain FCC-1/HN was expressed under the control of the *M.tuberculosis hsp70* promoter in rBCG (Zheng *et al.*, 2001a). BALB/c mice immunized with this rBCG developed a significant anti-CSP antibody response that was enhanced after a booster inoculation (Zheng *et al.*, 2002). Specific proliferative responses, IFN-γ and IL-2 production were also demonstrated after stimulation with malarial antigens and these all increased after the boost (Zheng *et al.*, 2002). In addition, the same system was used to express the merozoite surface antigen 2 (MSA2) from *P.falciparum* in rBCG (Zheng *et al.*, 2001b). The administration of rBCG-MSA2 to BALB/c mice induced significant and sustained anti-MSA2 antibodies. Specific proliferative responses, IFN-γ and IL-2 production were also induced after malarial antigen stimulation.

A B-cell epitope of the CSP from the rodent malarial parasite, *P.yoelii*, expressed under the control of the *M.kansasii* α-antigen promoter and signal sequence yielded a long-lasting antibody response in 1 mouse out of 7 inoculated with the vaccine (Matsumoto *et al.*, 1996b). Using the same system group the COOH-terminal fragment of MSP1 from *P.yoelii* was also expressed in rBCG (Matsumoto *et al.*, 1998). When comparing the protective efficacy of rBCG-MSP1 with recombinant MSP1 in RIBI adjuvant or incomplete Freund's adjuvant in immunized C3H/He mice, the rBCG yielded the highest degree of protection and mouse splenocytes were able to produce MSP-specific IFN-γ that was important in the clearance of the parasites in a challenge experiment. Antibody responses specific to MSP1 were not involved in clearance, however, after challenge, antisera carrying IgG2a antibodies were shown to be protective. The endurance of this protective response was observed for as long as nine months after the final immunization (Matsumoto *et al.*, 1999).

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***Leishmania* antigens**

The *Leishmania major* surface proteinase *gp63* gene was cloned and expressed in rBCG under the control of the *hsp60* promoter (Connell *et al.*, 1993). BALB/c and CBA/J mice were immunized with the rBCG and then challenged with promastigotes of *L.major* and *L.mexicana.* Both mouse strains demonstrated significant protection against *L.mexicana* but only the CBA/J mice were protected against *L.major* – a delay in *L.major* growth was observed in immunized BALB/c mice. Challenge with *L.mexicana* amastigotes also resulted in a strong protective immune response.

The *L.major gp63* gene expressed in rBCG as a fusion with the N-terminal region of the *M.fortuitum* β-lac protein (*pBlaF** promoter) was able to induce significant protection in immunized BALB/c mice that were challenged with *L.major* (Abdelhak *et al.*, 1995).

Another *Lesihmania* antigen, LCR1 from *L.chagasi*, was expressed in rBCG under the control of the *hsp60* promoter (Streit *et al.*, 2000). A better protective response was elicited in BALB/c mice inoculated subcutaneously with rBCG-LCR1 than in mice that received the LCR1 protein alone. These mice also produced significantly more LCR1-specific IFN-γ.

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***Schistosoma* antigens**

Kremer *et al.* (1996) developed an rBCG expressing (*hsp60* promoter) the 28kDa glutathione *S*-transferase of *Schistosoma mansoni* (Sm28GST). BALB/c mice give a single inoculation with the rBCG via the intravenous, intraperitoneal, subcutaneous or intranasal route were able to mount significant anti-Sm28GST antibody responses. These responses were enhanced by a booster dose and remained at a high level for as long as a year post-immunization. Isotyping of the antibodies showed that antigen-specific IgG1, IgG2a, IgG2b and IgA antibodies were elicited. Strong neutralization of the Sm28GST enzyme activity by the antibodies was also demonstrated.

An rBCG expressing the glutathione *S*-transferase of *S.haematobium* (Sh28GST) in the same system was also constructed (Kremer *et al.*, 1998). Intraperitoneal and intranasal immunization of BALB/c mice resulted in strong anti-Sh28GST antibody responses that were enhanced by a booster immunization. The mice inoculated intranasally produced IgG1, IgG2a, IgG2b and IgA antibodies. An interesting finding was that, although Sm28GST shares over 90% identity with Sh28GST, a much stronger humoral response was elicited by rBCG-Sh28GST after intranasal inoculation (this was not the case for intraperitoneal inoculation).

Recently, Varaldo *et al.* (2004) developed an rBCG expressing the Sm14 antigen of *S.mansoni* as a fusion with the *M.fortuitum* β-lac protein under the control of the *pBlaF** promoter. Immunization of BALB/c mice with rBCG-Sm14 did not elicit an antigen-specific antibody response, but IFN-γ was produced in the splenocytes of the mice when stimulated with rSm14. Immunized mice were challenged with live *S.mansoni* cercaria amd showed a 48% decrease in worm burden; a reduction comparable with three doses of rSm14.

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***Toxoplasma* antigens**

OF1 outbred mice immunized with rBCG expressing the GRA1 antigen of *T.gondii* in rBCG (*M.tuberculosis* Ag85A promoter and fused to its signal sequence) did not mount GRA1-specific humoral or cellular immune response (Supply *et al.*, 1999). When sheep were immunized subcutaneously and then boosted intravenously with the rBCG, GRA1-specific proliferation and IFN-γ production was induced; no antibody responses were observed. These sheep were challenged with *T.gondii* oocysts and displayed an abbreviated temperature response compared with controls; this result suggested partial protection.

1.B.3.3. IMMUNOMODULATORS

A number of cytokines have also been expressed in BCG in attempts to enhance its anti-cancer and anti-tuberculosis properties. These include IL-2 (O'Donnell *et al.*, 1994; Murray *et al.*, 1996; Yamada *et al.*, 2000), IL-4, IL-6 (Murray *et al.*, 1996) and IL-18 (Biet *et al.*, 2002). Other

immunomodulators that have been investigated are IFN-γ (Murray *et al.*, 1996; Kong *et al.*, 1997), IFN-α (Luo *et al.*, 2001) and GM-CSF (Murray *et al.*, 1996).

AIM OF THIS WORK

There is an urgent need to develop an effective and inexpensive prophylactic vaccine against HIV-1. Until recently, most vaccine development efforts have focused on subtype B strains of HIV-1, however, subtype C viruses predominate in the southern African region and worldwide. The overall aim of this project was to identifiy subtype C isolates for inclusion into candidate HIV vaccines and to evaluate BCG as an HIV vaccine vector.

- As HIV diversity may impact on vaccine efficacy, the initial aim of this thesis was to select an HIV-1 subtype C *gag* gene that is relevant to the southern African region for inclusion in vaccine candidates (Chapter 2).
- A number of integrative or episomal *E.coli/Mycobacterium* shuttle vectors for Gag expression from different combinations of promoters and cellular localization signals were constructed (Chapter 3);
- To determine which shuttle vectors were suitable candidates for further study, the stability and Gag expression levels of the shuttle vectors were compared in recombinant *M. smegmatis* (Chapter 3);
- In addition, assessment of Gag expression in rBCG cell culture and in rBCG-infected macrophages was also performed in preparation for immunogenicity studies (Chapter 3);
- Immunogenicity testing of the rBCG:Gag vaccine candidates in the mouse model using two approaches – a DNA prime-rBCG boost and 5 inoculations of rBCG – was also performed (Chapter 4).

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CHAPTER 2

SELECTION OF A HIV-1 SUBTYPE C *GAG* GENE FOR USE IN VACCINE DEVELOPMENT

2.1. INTRODUCTION

The design of early candidates HIV vaccines was restricted to genes from laboratory adapted strains of HIV-1 subtype B as these were the available reagents (Hu *et al.*, 1987; Graham *et al.*, 1992; Belshe *et al.*, 1993 and Raedelli *et al.*, 1994). However, by the early 1990s, as the diversity of the pandemic became evident, it was apparent that non-B subtypes predominated in the regions most affected by the epidemic and there was increased interest in developing vaccines based on these subtypes. It may also be of benefit to design vaccines using sequences from recently infected individuals as these may contain unique signature pattems compared to viruses from chronic infection (Derdyn *et al.*, 2004). The aim of this study was to identify a *gag* gene, of a recently transmitted virus, representative of the viruses circulating in South African

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Strains from the same HIV subtype can differ by up to 20% in Env and this can reach as high as 35% between strains from different subtypes (Gaschen *et al.*, 2002). As this variation may impact on epitope recognition, and thus vaccine effectiveness, it may be advantageous to match vaccines to the local epidemics. The influenza virus vaccine strains, for example, needs to be changed when the amino acid differences between the circulating strain and the vaccine are ~ 2% (Korber *et al.*, 2001). HIV diversity exceeds that of any other known virus (Rambaut *et al.*, 2004) with the viral diversity within an infected individual comparable to the global diversity of influenza virus in a given year (Korber *et al.*, 2001). Thus, the design and choice of immunogens to include in a candidate HIV-1 vaccine may be crucial to minimize the impact that the enormous diversity of this virus may have on vaccine efficacy.

Ideally a vaccine will need to elicit both neutralizing antibodies, as well as cellular immune responses which will protect from infection. This study is focused on vaccines that will elicit cellular immune responses. Cross-reactive CTL responses have been demonstrated in various studies (McKinney *et al.*, 2004; Barugahare *et al.*, 2005 and Currier *et al.*, 2005). CTLs from individuals infected with HIV-1 are able to mount significant cross-reactive responses to other subtypes (Coplan *et al.*, 2005) and CTLs from uninfected, vaccinated individuals are also able to respond to HIV isolates from different subtypes (Ferrari *et al.*, 1997). However, the CTL responses to peptides matched to the infecting strain tend to be stronger, more durable and have more breadth (McKinney *et al.*, 2004; Currier *et al.*, 2005 and Coplan *et al.*, 2005). It is thus unlikely that a vaccine that is genetically distant from the infecting virus, for example a subtype B vaccine for a subtype C epidemic, will provide a better immune response than a vaccine genetically matched to the circulating subtypes (Novitsky *et al.*, 2002a).

The ideal vaccine is one genetically matched to the potential infecting strains. However, as it is not possible to achieve complete homology between the vaccine and circulating viruses, strategies to minimize the genetic distance between them have been developed.

One of these strategies makes use of actual viral sequences where regional strains are selected opportunistically from viruses within the geographic area where the vaccine is intended for use (Goudsmit, 2000). In reviewing available results and sequences for subtype C, Gaschen *et al.* (2002) found that, while there were clear phylogenetic outliers, there was no single choice of a sequence most representative of the diversity in a given region. However, they suggested that it may be advantageous to select a sequence with a short branch length relative to the common ancestor which most closely resembles the sequence of the epidemic-founding strain. This central sequence would be most similar to the majority of contemporary sequences in the tree (Foley *et al.*, 2000 and Gaschen *et al.*, 2002).

Other strategies make use of artificial sequences constructed based on analysis of phylogenetic relatedness between sequences (Korber *et al.*, 2001; Gaschen *et al.*, 2002;

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Learn and Mullins, 2000; Mullins *et al.*, 2002; Novitsky *et al.*, 2002a; Nickle *et al.*, 2003 and Gao *et al.*, 2003). There are several approaches that have been proposed which derive sequences which are most similar to the contemporary circulating viruses. These include:

- A consensus sequence which is derived from the most common amino acid in each position in an alignment (Korber *et al.*, 2001 and Novitsky *et al.*, 2002a)
- An ancestral sequence reconstructed from an evolutionary model (Learn and Mullins, 2000; Mullins *et al.*, 2002)
- A centre of tree (COT) sequence which is at the point on the phylogeny where the average evolutionary distance from the COT to each tip is minimized (Nickle *et al.*, 2003)

A consensus sequence is more similar to the circulating viruses than they are to each other (Korber *et al.*, 2001). Lukashov and Goudsmit (1997) found that an HIV-1 subtype consensus was fairly stable over time since the sequences within a subtype are constrained to a space which is a fixed distance from that consensus. One use for the consensus approach would be in regions like the Democratic Republic of the Congo where there are multiple subtypes and CRFs circulating that the regional diversity is similar to the global diversity (Mokili *et al.*, 1999 and Vidal *et al.*, 2000). Here, an M-group consensus derived from a set of subtype consensus sequences might be useful (Gaschen *et al.*, 2002).

The construction of these artificial sequences is dependent on the sequences included in the analysis and the consensus sequence, in particular, will change as the available sequences accumulate. The ancestral sequences are also more similar to contemporary viruses than they are to each other (Learn and Mullins, 2000; Mullins *et al.*, 2002). Nickle *et al.* (2003) state that although consensus and ancestral vaccines minimize the distance between vaccine antigens and circulating viruses when they are derived from symmetrical phylogenies, they may not be as accurate when the phylogenies are asymmetrical (multiple subtypes or CRFs). They suggest that an ancestral sequence has more chance of generating a protein that will fold and function correctly and that it is unlikely to require much change when new sequences are added to the analysis. However, they developed another method for vaccine antigen

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design which is similar to ancestral sequences but is less sensitive to phylogenetic outliers. This Centre of Tree (COT) is derived from a phylogenetic tree at the point where the evolutionary distances between the COT and other sequences in the analysis are minimized. The COT is designed to be more similar to rapidly evolving viral subtypes than an ancestral sequence and still maintain similarity to the slower evolving lineages. It may thus be more useful when trees are asymmetrical (Nickle *et al.*, 2003).

Being artificial sequences, the expression, antigenicity and biological activity of the consensus and ancestral sequences need to be characterized before use in a vaccine (Gaschen *et al.*, 2002 and Novitsky *et al.*, 2002a). However, although structure may be important for the generation of antibody responses where discontinuous epitopes recognition occurs, this is less critical for CTL responses where recognition of linear peptides may even be improved with the use of consensus sequences (Gaschen *et al.*, 2002). The consensus and ancestral sequences have been shown to conserve both CTL epitopes and predicted immunoproteasome cleavage sites, implying that CTL responses would not be affected by their use (Gaschen *et al.*, 2002). This analysis also predicts that using a subtype B-based vaccine against a subtype C epidemic would be less effective than a subtype C-based vaccine and a subtype C consensus would be the most effective (Gaschen *et al.*, 2002).

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In a summary of the use of the COT, ancestral and consensus methods, Gao *et al.* (2003) state that the three derived sequences will only be slightly different from each other since they are subject to different biases but that the biological properties of such artificial sequences cannot be predicted. Experiments need to be conducted to test their preservation of key antigenic domains, their immunogenicity and the extent to which they induce cross-reactive protection.

Other strategies that attempt to deal with the problem of diversity in vaccine design include the design of modified envelope proteins which enhance the exposure of hidden epitopes known to induce broadly neutralising antibodies (Barnett *et al.*, 2001 and Saphire *et al.*, 2001), the use of polyvalent peptides or epitopes that span a region that induces strain-

specific neutralising antibodies and T-cells (Liao *et al.*, 2002 and Zhan *et al.*, 2004) and vaccine cocktails expressing the same gene from a number of different subtypes (Ljungberg *et al.*, 2002).

2.1.1. RATIONALE

This objective of this study was to identify a *gag* sequence, from a recently transmitted virus, that was closest to a South African consensus sequence derived from circulating viruses, for inclusion into CTL inducing vaccines. Viruses belonging to the same subtype are more closely related to each other than to viruses belonging to other subtypes and consequently viruses of the same subtype have more shared epitopes. Also, CTL responses of infected invididuals to epitopes of viruses from the same subtype are better than the responses to epitopes of viruses from different subtypes (McKinney *et al.*, 2004 and Currier *et al.*, 2005). It has been shown that there are differences in selection pressure on subtype B and C envelope genes, implying that there is lineage-specific immunogenicity (Gaschen *et al.*, 2002). Together, these data argue for the use of subtype-specific vaccines that would maximize the number of potentially cross-reactive epitopes between the vaccine and those in viruses circulating in the target population. In this study a sequencing survey of currently circulating viruses in South Africa was performed, a *gag* subtype C consensus sequence was derived, and a sequence most closely related to this consensus selected for inclusion into vaccines.

The HIV-1 *gag* gene was selected as a model immunogen to include in vaccine candidates as: the *gag* gene has been shown to be rich in CTL epitopes (HIV Molecular Immunology Database; http://hiv-web.lanl.gov/content/immunology/ctl_search); regions of the *gag* gene sequence are highly conserved across different subtypes (Korber *et al.*, 2001); some studies have shown that anti-Gag responses are associated with control of viral replication (Ogg *et al.*, 1999 and Masemola *et al.*, 2004a). As detailed in section 1.5.2, a high frequency of Gag-specific CTL-responses have been demonstrated in a number of different cohorts infected with HIV-1 subtype C (Goulder *et al.*, 2000; Novitsky *et al.*, 2001, 2002a and Masemola *et al.*, 2004a and b).

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The vaccine strain was selected based on the virus which was most closely matched to the amino acid sequence of the consensus, from an individual with acute infection and which grew in tissue culture. This approach attempts to minimise the distance between the vaccine and circulating strains while still utilizing genes from wild type virus and thus not requiring resynthesis of the gene for use in the vaccine candidate. The sequence survey was performed using representative, recently transmitted subtype C isolates from patients either within 1 year of infection (acute infection) or with CD4 counts >500 cells/cm^3. Selection of genes from individuals with acute HIV-1 infection has the advantage that they have properties close to the transmitted virus. The selected strain would be used in the development of Gag vaccines targeted for South Africa.

2.2. METHODS

2.2.1. SAMPLES

To ensure geographical representation, samples were collected from HIV-1 infected individuals from three provinces: KwaZulu Natal, Gauteng and the Western Cape. Ethical approval was obtained for sample collection from University of Cape Town Research Ethics Committee [UCT Research Ethics Committee number: 137/95]. Samples were classified into two groups based on the timing of their infection.

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Individuals near seroconversion

This group was composed of samples collected from 14 female sex workers (Du prefix) from five truck stops between Johannesburg and Durban. The demographics of the individuals and the viral characteristics are shown in Table 2.1. The women were participating in a phase III HIV-1 prevention microbicide trial (van Damme *et al.*, 2002) and were being screened monthly for HIV-1 infection. Samples were provided from individuals within 1 year of infection although two of the women had seroconverted 14-16 months before sample collection (Du174 and 179). Samples were provided by G. Ramjee (Medical Research Council, South Africa) and S. Abdool-Karim (University of KwaZulu-Natal, South Africa).

TABLE 2.1. Clinical data, viral phenotype and coreceptor usage from recently infected individuals from the Du cohort (adapted from Williamson *et al.*, 2003).

Sample number	Estimated months after seroconversion[a]	CD4+ cell count (cells/µl)	Viral load (HIV-1 RNA copies/ml)	Viral phenotype[b]	Coreceptor usage[c]
Du115	6.8	437	7 597	-	-
Du123	3.5	841	19 3331	NSI	R5
Du151	2.1	367	>500 000	NSI	R5
Du156	<1	404	22 122	NSI	R5
Du172	3.2	793	1 916	NSI	R5
Du174	14.1	634	9 454	NSI	R5
Du179	16.1	394	1 359	SI	R5X4
Du204	6.5	633	8 734	NSI	R5
Du258	6.3	433	9 114	-	-
Du281	4.7	594	24 689	NSI	R5
Du386	8.3	670	13 933	NSI	R5
Du422	2.0	409	17 118	NSI	R5
Du457	3.5	665	6 658	-	-
Du467	3.2	671	19 268	-	-

[a]All women were screened monthly for HIV-1 infection, except for DU172, whose last negative ELISA and first positive ELISA were 4 months apart. Estimated months after seroconversion was taken from the midpoint between the last negative and the first positive ELISA
[b] Negative PCR or culture; NSI = nonsyncytium inducing; SI = syncytium inducing
[c]Negative PCR or culture; Viral isolates could not be made from the PBMCs of 4 of the cohort samples

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Prevalent asymptomatic individuals

This group was composed of 2 individuals from outpatient clinics in Cape Town (CTSC prefix), 7 women attending an antenatal clinic in Johannesburg (GG prefix; samples provided by G.Gray; Chris Hani Baragwanath Hospital, South Africa) and 9 men attending a sexually transmitted disease clinic at the West Driefontein mine in Carltonville (RB prefix; samples provided by R. Ballard; Carltonville). These individuals were clinically well and had CD4+ cell counts greater than 500cells/µl (information provided by National Institute for Communicable Diseases; C. Gray).

2.2.2. RT-PCR

RNA Isolation

Viral RNA was isolated from 140µl patient plasma using QIAamp Viral RNA Kit (Qiagen, Germany); the RNA was eluted with 50µl RNase-free water.

Primers

Primers were designed to amplify 939bp of *gag*, incorporating p17 and most of p24.

Table 2.2 lists the primers used in the RT-PCR.

TABLE 2.2. PCR primer sequences

Primer name	Sequence	HXB2 numbering[a]
Outer primers:		
i-gag-f	GATCTCTCGACGCAGGAC	680-697
i-gag-r	AATACTGTATCATCTGCTCCTG	2420-2441
Inner primers:		
GAG1-UP	ATGGGTGCGAGAGCGTCAATATTA	790-813
GAG1-DN	ACATGGGTATTAGCTCTGGGCT	1282-1303
GAG2-UP	CACCTAGAACTTTGAATGCATG	1232-1253
GAG2-DN	TCTAATGAAGCCCCTGGTCCTAAT	1797-1820

[a]Numbering is according to the HXB2 sequence (www://hiv-web.lanl.gov/)

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RT-PCR

The viral RNA was heated at 95°C for 2 min and then placed on ice for 2 min. Thereafter, 3µl of the RNA was used to synthesize cDNA in a reverse transcription mastermix containing 2µl of 10x HiFi buffer with $MgCl_2$; 0.4µl of 25mM dNTPs; 1.5µl of the i-gag-r primer at 10pmol/µl; 0.25µl of 40U/µl RNASin and 0.4µl of AMV RT in a final reaction volume of 20µl. The reaction mix was incubated at 42°C for 30 min. After deactivating the AMV RT for 5 min at 95°C, the reaction was placed on ice for a further 5 min. The cDNA produced by this reaction was used in the outer PCR mix.

The 20µl of cDNA produced in the RT reaction was added to the 30µl outer PCR mix that consisted of 3µl of 10x HiFi Buffer with $MgCl_2$; 1.5µl of the i-gag-f primer at 10pmol/µl; 0.75µl of Expand High Fidelity Taq (3.5U/µl; Roche, Basel, Switzerland). The thermocycle for the outer reaction consisted of a cycle of 95°C for 2 min followed by 10 cycles of [95°C for 45 sec; 55°C for 45 sec; 68°C for 1 min], then 10 cycles of [95°C for 45 sec; 55°C for 45 sec; 68°C for 2 min], 10 cycles of [95°C for 45 sec; 55°C for 45 sec; 68°C for 3 min] and 10 cycles of [95°C for 45 sec; 55°C for 45 sec; 68°C for 4 min]. The PCR was completed with a final elongation cycle of 7 min at 72°C.

The inner PCR mix was made up of 5µl of 10x HiFi Buffer with $MgCl_2$; 0.4µl of 25mM dNTPs; 1.5µl of each primer (UP/DN) at 10pmol/µl; 0.75µl of Expand High Fidelity Taq (3.5U/µl; Roche, Basel, Switzerland) and 3µl of the outer PCR product in a 50µl reaction volume. The thermocycle for the inner reaction was the same as that for the outer reaction.

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2.2.3. SEQUENCING AND SEQUENCE ANALYSIS

The inner PCR amplification products (514bp and 589bp) were purified using the QIAquick PCR purification kit (Qiagen, Germany). The purified products were quantitated on agarose gels by comparing band intensities to bands of known concentration and 20-30ng of each product were used in sequencing reactions. The inner PCR primers (Table 2.1) were used in sequencing reactions performed on both strands, carried out on an ABI-Prism 377 DNA sequencer (Applied Biosystems, Foster City, CA; Mr K. van Heerden, University of Stellenbosch).

Phylogenetic analysis

The DNA sequences were assembled and translated using Genepro version 6.1(Riverside Scientific, MN, USA) to identify ORFs. The *gag* DNA sequences were compared phylogenetically with a subtype C consensus sequence (http://hiv-web.lanl.gov), Indian subtype C sequences (http://hiv-web.lanl.gov), Malawian subtype C sequences (R. Swanstrom; UNC Centre for AIDS Research, University of North Carolina, USA; personal communication) and reference *gag* sequences for the non-subtype C subtypes (http://hiv-web.lanl.gov). The *gag* DNA sequences were also compared phylogenetically with Southern

African sequences from Johannesburg, Durban, Zambia, Zimbabwe and Malawi (the HIVNET028 study; H. Bredell, unpublished); subtype C sequences from the Los Alamos HIV sequence database (http://hiv-web.lanl.gov) and the derived ancestral sequence for subtype C (http://hiv-web.lanl.gov).

Sequence alignment was performed using ClustalX (Thompson *et al.*, 1997) or BioEdit Sequence Alignment Editor (version 7.0; Isis Pharmaceuticals Inc; Hall, 1999) with final manual adjustment. A neighbour-joining phylogenetic tree was generated with the Kimura two-parameter distance correction (Kimura, 1980) with 100 bootstrap replicates using TreeconW software (van de Peer and de Wachter, 1994). DNA and amino acid distance matrices were calculated with ClustalX (Thompson *et al.*, 1997) or Mega2 (version 2.1, Molecular Evolutionary Genetic Analysis, Kumar *et* al., 2001). An entropy plot was also performed using BioEdit Sequence Alignment Editor (version 7.0, Isis Pharmaceuticals Inc; Hall, 1999).

A South African consensus sequence was generated using DNAMAN (version 4.0, Lynnon Biosoft, Vandreuil-Dorion, Canada) and defined the most common amino acid at each position. The consensus sequence (ZA_CON) was included in the analysis.

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2.3. RESULTS

2.3.1. PHYLOGENETIC RELATIONSHIPS BETWEEN THE *GAG* SEQUENCES

A total of 32 partial *gag* sequences (939bp) were generated; 14 from recently infected individuals from the Durban cohort (Du), 2 from outpatient clinics in Cape Town (CTSC); 7 from women attending antenatal clinics (GG) and 9 from men attending an STD clinic (RB).

All the sequences were classified as subtype C (Fig 2.1). There was limited internal structure in the tree with South African sequences grouping within four subclusters supported by

significant bootstrap values ($\geq$ 70). The sequences in the first subcluster RB14 and 18, were closely related and most likely share a common origin, reflecting either direct transmission or infection from common partner. The linkage between these sequences was maintained in other genes (*pol, env*) confirming that it was not a contamination (Williamson *et al.*, 2003). The second subcluster consisted of sequences from different geographical origin, Du174, GG5 and RB13; the third subcluster was made up of sequences from the Du cohort, Du151 and 422, while the fourth subcluster comprised of two Malawian sequences and the South African GG10 and RB22 sequences. The Ethiopian and Brazilian reference sequences grouped as a subtype C outlier to the southern African and Indian subtype C.

The average nucleotide distance between the *gag* gene sequences of the South African subtype C viruses was 5.9%. The greatest nucleotide distance between sequences was 9.5% (between CTSC1 and Du204) and the closest distance was 1% (between RB14 and 18).

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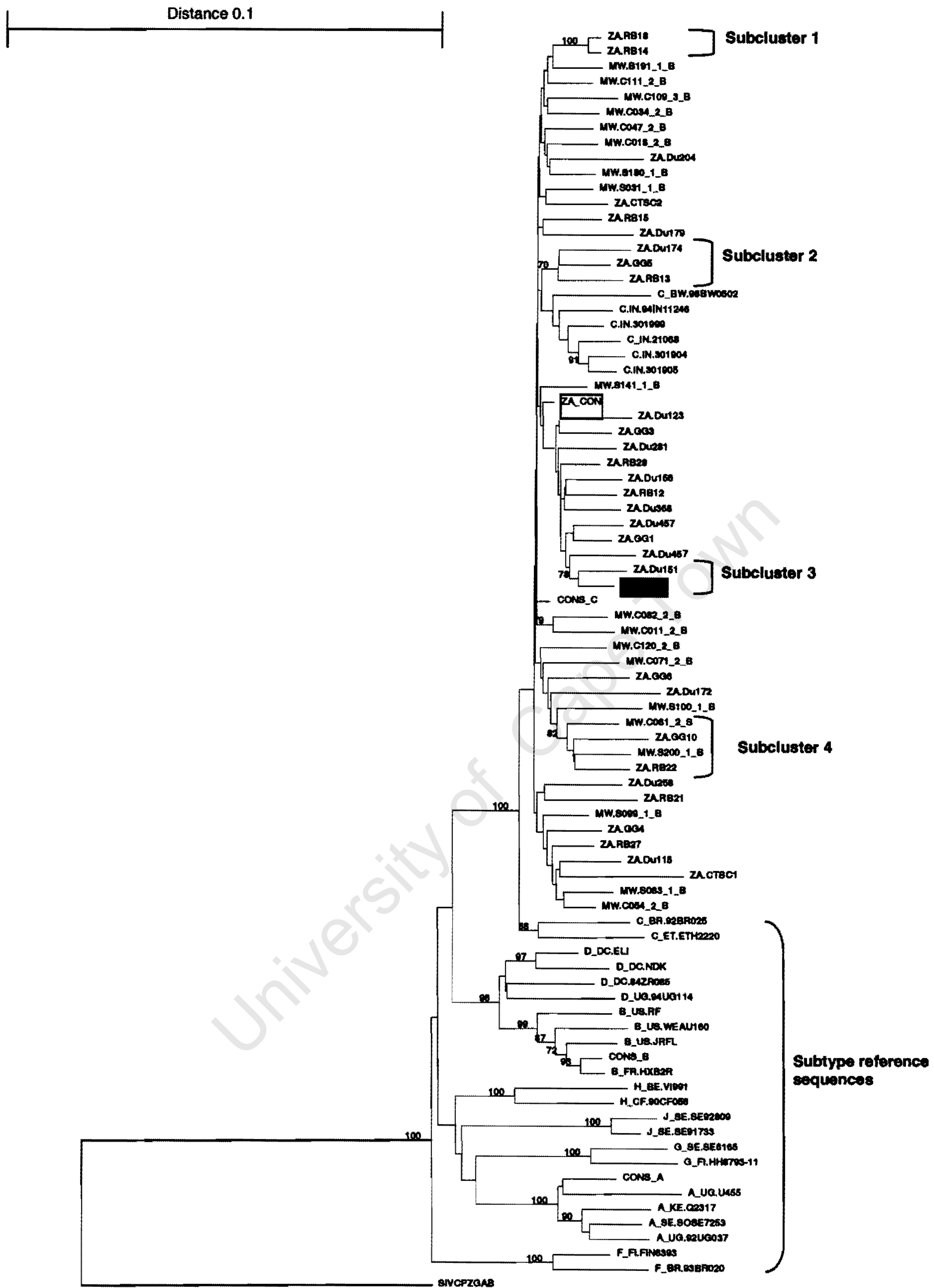

Fig 2.1. Neighbour-joining phylogenetic tree generated to subtype the South Africa HIV-1 Gag sequences (939bp)

100 bootstrap replicates were performed; **Distance bar** = 10%; Red box indicates Du422, the isolate selected for the vaccine (see below)

Key: BE, Belgium; **BR**, Brazil; **BW**, Botswana; **CF**, Central African Republic; **DC**, Democratic Republic of the Congo; **ET**, Ethiopia; **FI**, Finland; **FR**, France; **IN**, India; **KE**, Kenya; **MW**, Malawi; **SE**, Sweden; **UG**, Uganda; **US**, United States; **ZA**, South Africa; **CPZ**, chimpanzee; **ZA_CON**, South African Consensus; **CONS_A/B/C**, Subtype consensus

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2.3.2. SELECTION OF A *GAG* GENE FOR A CANDIDATE VACCINE

In order to identify a representative *gag* gene for inclusion in the vaccine, a South African subtype C consensus sequence was derived by defining the most common amino acid at each position for the South African sequences (n=32). The mean diversity between the South African amino acid sequences was 6.6% with a distance range of 1.4% to 12.5%. The South African sequences were between 91.4 and 98.3% identical to the South African consensus, with a mean identity of 93.6% (Fig.2.2). The Du422 sequence was closest to the consensus (98.3% amino acid identity). This sequence was generated from an individual who had been infected for 2 months and the virus was shown to be viable in tissue culture (performed by Lynn Morris, National Institute of Communicable Diseases, South Africa, Williamson *et al.*, 2003). The mean distance of all the South African sequences from Du422 was 4.7%, with Du281, RB27 and GG1 being most closely related at 2.5% distance and Du174 and Du204 most distant at 7.8%.

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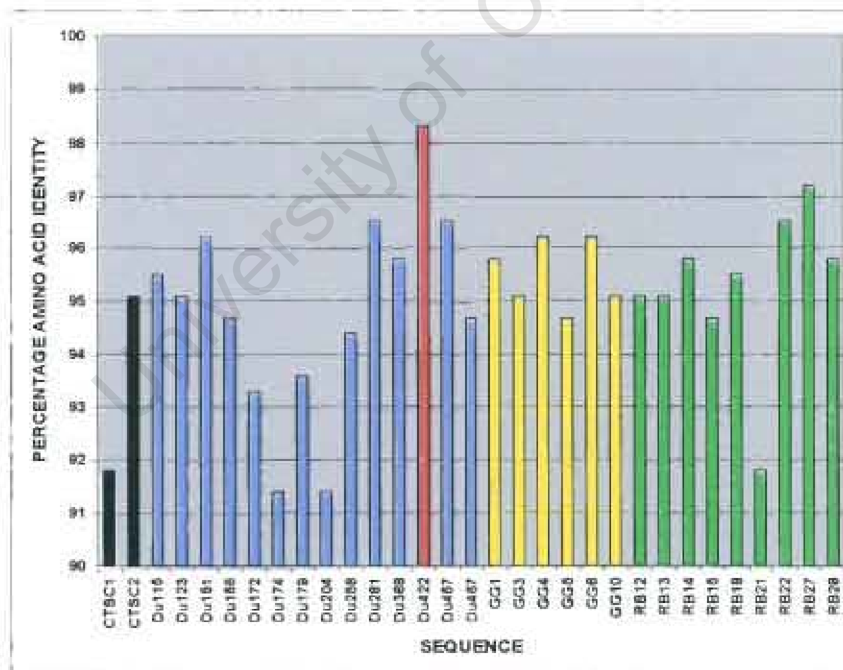

Fig 2.2. Amino acid distances between South African Gag sequences compared to the South African Gag consensus sequence. Black bars indicate the CT sequences; Blue bars indicate the Du sequences (except Du422); Yellow bars indicate the GG sequences; Green bars indicate the RB sequences; Du422, the isolate closest to the consensus is indicated with a red bar

2.3.3. EFFECT OF INCLUDING ADDITIONAL SEQUENCES ON SELECTION OF Du422 AS THE GAG VACCINE STRAIN

The selection of the Du422 strain was based on the analysis of samples collected in 1999. Since then, sequences have become available within the laboratory generated from South Africa, Zambia, Zimbabwe and Malawi (the HIVNET028 study; H. Bredell, unpublished) as well as more recently published sequences from the Los Alamos HIV sequence database (http://hiv-web.lanl.gov). The relatedness of the vaccine strain to southern African sequences, as well as to the derived ancestral subtype C sequence (http://hiv-web.lanl.gov), and to the latest global subtype C consensus (http://hiv-web.lanl.gov) was determined. This was done to determine the current relationship of the vaccine strain to South African and southern African sequences and, in addition, to determine whether any advantage would have been gained from the use of a subtype C consensus or ancestral sequence instead of Du422.

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Du422 remained the closest amino acid sequence to the updated South African consensus (98.2% amino acid identity). The updated South African consensus sequence based on 80 sequences was 99.8% similar to the consensus sequence derived from sequences collected in 1999. In addition, Du422 remained the most closely related South African sequence to both the ancestral and the global subtype C consensus (95.9% and 97.3% amino acid identity).

In this larger database, the mean amino acid distance of all southern African sequences (n = 202) was 5.5% (0.6% to 13%), which is similar to the mean amino acid distance between the 80 South African sequences of 5.9% (range 0.8% to 11.5%), and slightly lower than the mean amino acid distance between the 32 South African sequences in the original 1999 database of 6.6% (range 1.2% to 12.5%) (Fig. 2.3.).

The mean amino acid distance of Du422 to southern African sequences was 4.2% compared to 3.9% distant from South Africa sequences (fig 2.3.). These distances compare well with the earlier analysis (see above) where the distance of the 32 South African sequences from the

Du422 sequence was 4.7%. When the distance of the mean of all the southern African sequences was measured from the updated South African consensus, the global subtype C consensus and ancestral subtype C sequences, it was found to be 3.3%, 3.3% and 4.1%, respectively (fig 2.3.).

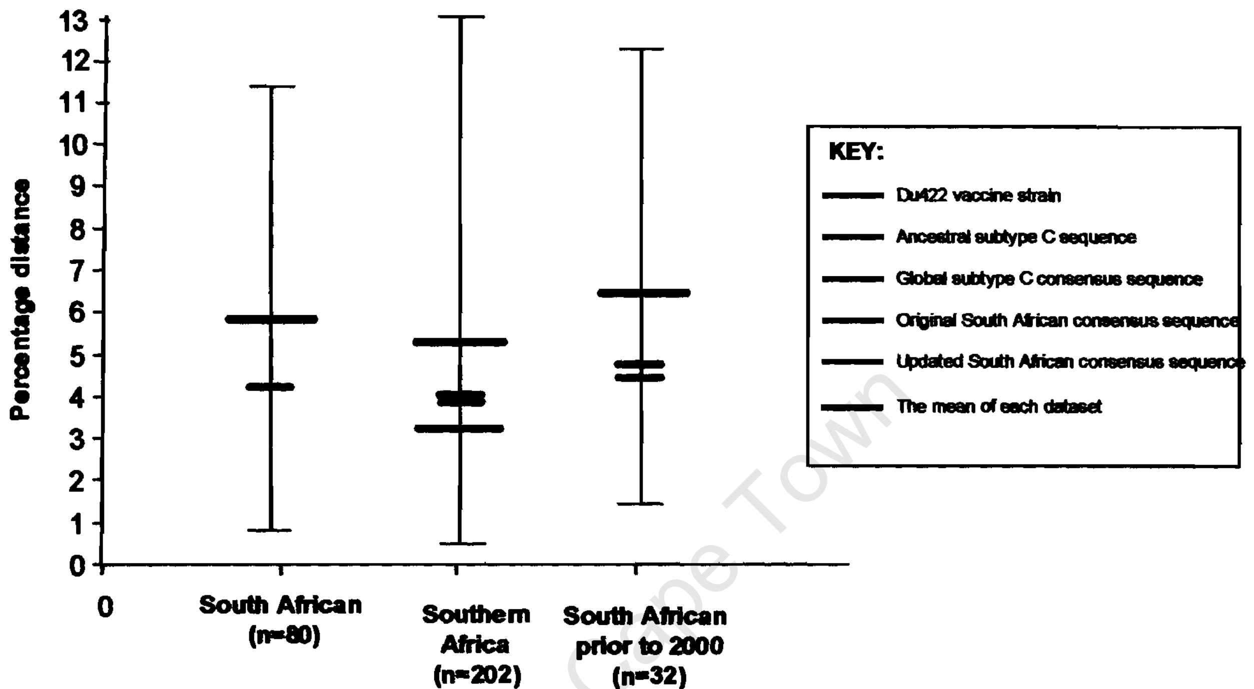

Fig. 2.3. Graph showing the amino acid distance range of the different data sets including South African (n=80); Southern African (n=202) and South Africa prior to 2000. Datasets were compared to Du422 vaccine strain, ancestral subtype C sequence, the global subtype C consensus sequence and the original and updated South African consensus sequences.

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A phylogenetic comparison between Du422 and this larger dataset did not change the initial relationships identified on the smaller subset although there is evidence of a South African cluster evolving (fig 2.4). Du422 falls within a cluster of South African sequences with slightly shorter branch length than the more recent sequences. Similarly, limited geographic clustering of sequences was observed, with Indian, Botswana and Malawian subclusters identified.

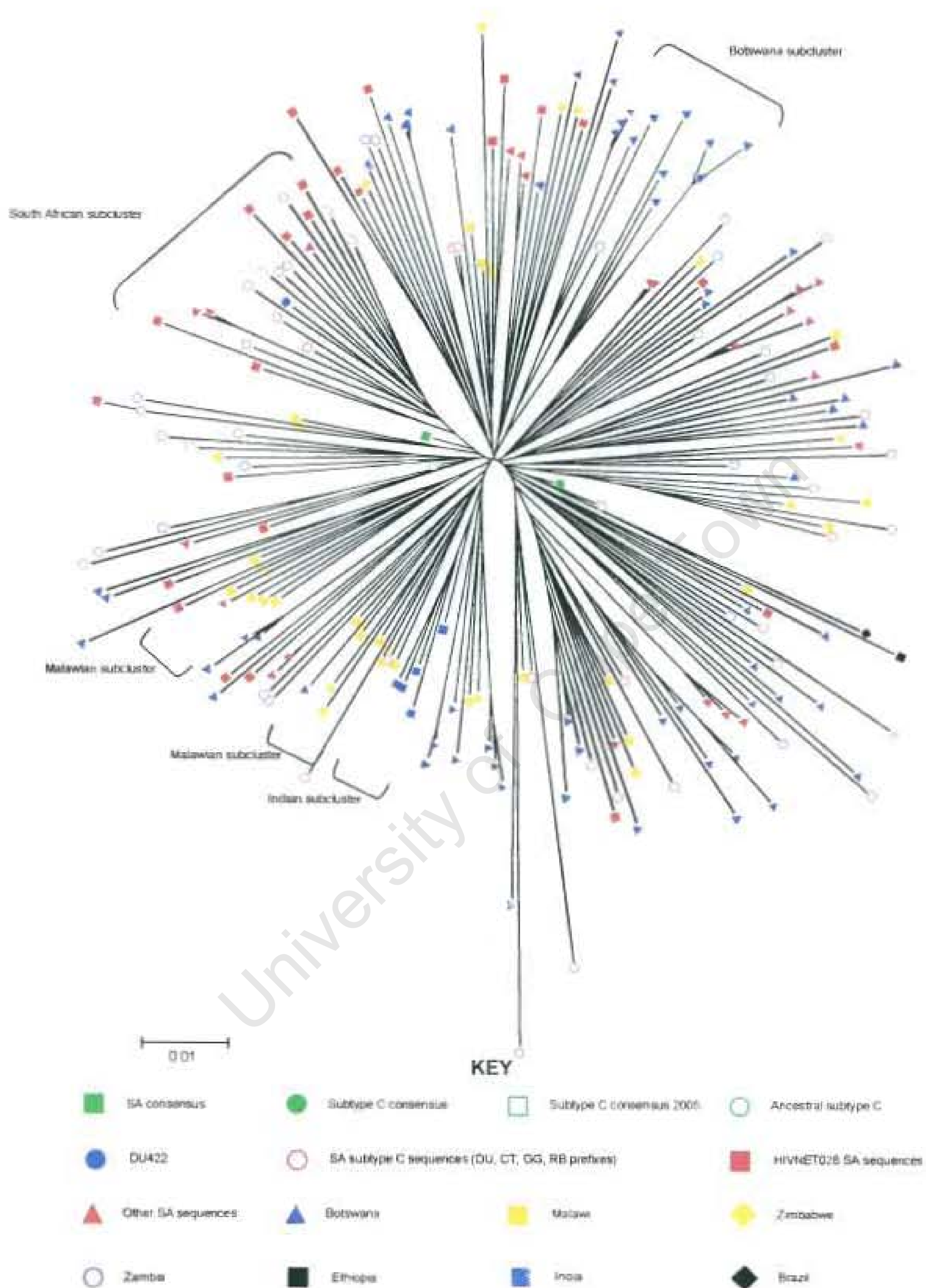

Fig. 2.4. Neighbour-joining phylogenetic tree generated to determine the current relationship of the South Africa HIV-1 Gag sequences (939bp) to other subtype C sequences from Africa and the rest of the world
100 bootstrap replicates were performed; Distance bar = 1%

2.3.4. IMMUNOLOGICAL RELEVANCE OF Du422

CTL epitopes in Gag, which are targeted by southern African individuals infected with subtype C, were described by Masemola *et al.* (2004b). Using an IFN-γ ELISPOT assay, they identified four peptide stretches containing CTL epitopes in p17 and four in p24. These peptides were recognised at different frequencies in the individuals studied (fig 2.5). The p17 peptides are recognised at frequencies ranging from 7.7% to 34.6% while the p24 peptides were recognised at frequencies from 7.7% to 19.2%. This study utilized peptides based on consensus subtype C sequence which is 98% homologous to Du422. We were interested in determining if Du422 contained immunologically reactive peptides identified in this study and to determine the variability of sequences within these epitopes.

The variability of residues within the subtype C *gag* genes from the South African cohorts was assessed by generating an entropy plot on the aligned amino acid sequences (Fig 2.5.). The p17 region was highly variable while the p24 region contained more conserved residues. Of the eight peptides recognised, two were identical to Du422; four had a single amino acid change and two contained 2 amino acid changes (Fig 2.6). One region of p24 which was frequently recognised also had low genetic variability (Fig 2.5). As this peptide is highly conserved it may be important to include in a Gag-based subtype C vaccine although it is not known if responses to this peptide are associated with control of viral replication. Despite high variability in p17, the peptide stretch TGTEELRSLYNTVATLYCVHAGIEV was recognised at the highest frequency (35%). It should be noted that the ELISPOT analysis was based on 15-mers which encompass the 9-mer HLA epitope. Not all amino acid changes will affect recognition and the HLA anchor binding site and T-cell recognition sites are most important.

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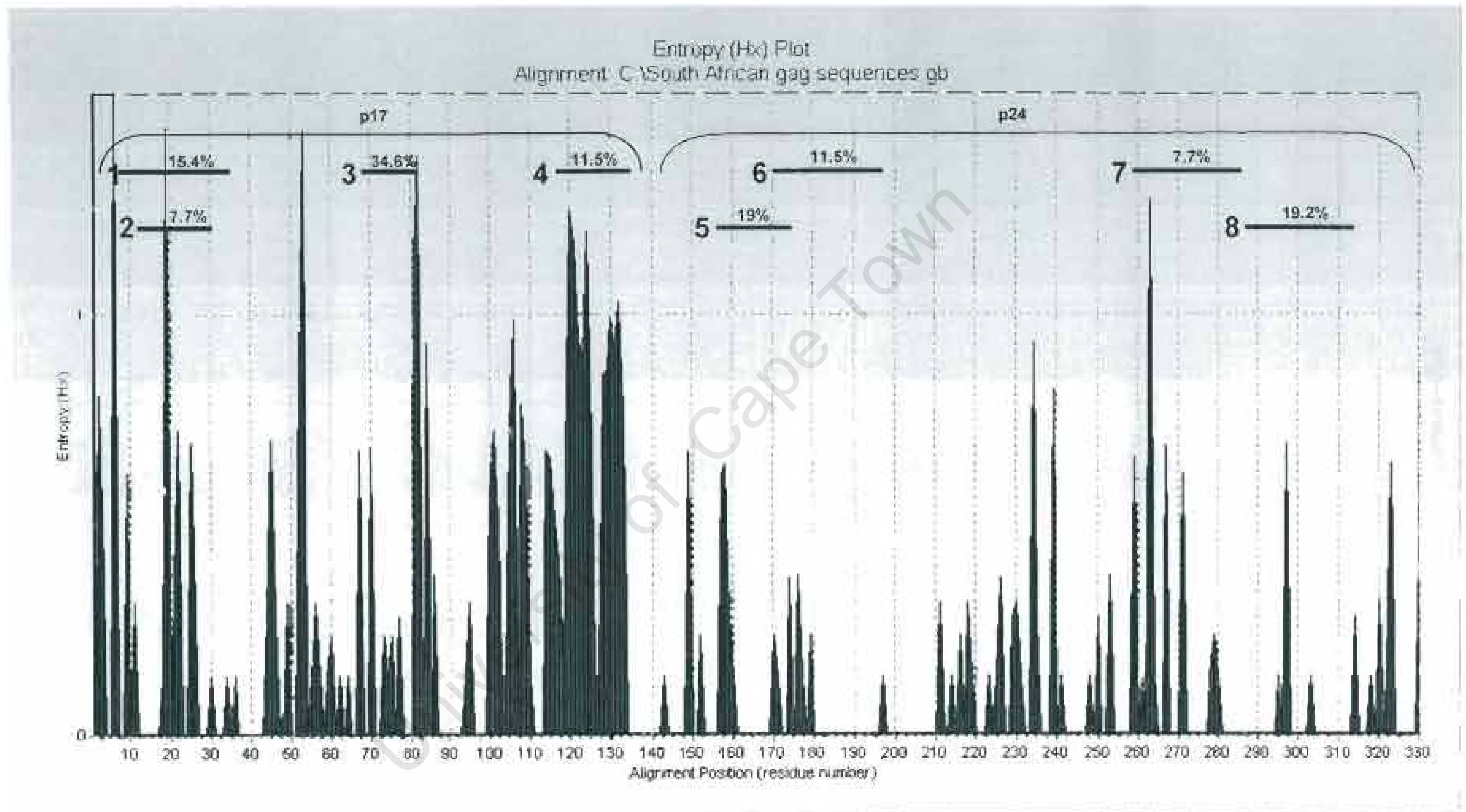

Fig 2.5. Entropy plot illustrating variability across the *gag* gene (p17 and p24) for the South African sequences in this study (Du, CT, GG and RB prefixes). The immunologically reactive peptide stretches identified by Masemola *et al.* (2004b) using IFN-γ ELISPOT are indicated and numbered in bold, along with the frequencies with which they are recognized in the studied population (n=38).

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```
Du422        ^9RGEKLDKWEKIRLRPGGKKHYMLKHI^34
               **-**********************-
Peptide 1    ^9RGGKLDKWEKIRLRPGGKKHYMLKHL^34

Du422        ^16KHIVWASRELERFAL^30
                **-************
Peptide 2    ^16KHLVWASRELERFAL^30

Du422        ^70TGTEELKSLYNTVATLY^86
                ******-**********
Peptide 3    ^70TGTEELRSLYNTVATLY^86

Du422        ^121KAA.D.GKVSQNYPIV^134
                 ***-*-**********
Peptide 4    ^119KAAADKGKVSQNYPIV^134

Du422        ^157WVKVIEEKAFSPEVIPMF^174
                 ******************
Peptide 5    ^157WVKVIEEKAFSPEVIPMF^174

Du422        ^170PMFTALSEGATPQDLNTMLNTVGGH^196
                 ***************************
Peptide 6    ^170PMFTALSEGATPQDLNTMLNTVGGH^196

Du422        ^259PVGDIY.KRWIILGLNKIVRMYSPVSI^284
                 ******-********************
Peptide 7    ^259PVGDIYWKRWIILGLNKIVRMYSPVSI^285

Du422        ^289GPKEPFRDYVDRFFKTLRAEQATQEV^314
                 ************************-*
Peptide 8    ^289GPKEPFRDYVDRFFKTLRAEQATQDV^314
```

Fig.2.6. Comparison of Du422 amino acid sequence with immunologically reactive peptide stretches identified by (Masemola *et al.* (2004b) using IFN-γ ELISPOT
Peptides are numbered to match fig 2.4.
* indicates homologous bases; - indicates base differences; . indicates insertion or deletion
Residue numbers are indicated in superscript

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2.4. DISCUSSION

Gag was selected as a model immunogen in first generation vaccines as it is rich in CTL epitopes and responses to Gag have been associated with control of viral replication. In addition, it is a frequently recognised protein with up to 74% of infected individuals responding to this protein (Masemola *et al.*, 2004a). In this study, we aimed to minimise the potential impact of diversity on vaccine protection by selecting a sequence that was most closely matched the consensus sequence generated from circulating strains in South Africa. The sequence was chosen from a group of strains that were recently transmitted since such strains would have properties close to that of transmitted virus. Derdeyn *et al.* (2004) have recently shown that the envelope glycoproteins of transmitted viruses have characteristics

that influence transmission and that these characteristics are lost during chronic infection due to immune escape. The transmitted viruses were more sensitive to neutralization and thus the selection of recently transmitted variants used in this study may have more relevance for *env* gene-based vaccines.

Based on genetic analyses, Du422 was selected since it was the most closely related sequence to the derived South African consensus sequence for the *gag* gene. This sequence was cloned directly from plasma and was not a derived, artificial gene sequence thus avoiding the need for resynthesis. Although an artificial consensus sequence approach could have been used, further analysis indicated that not much would have been gained from using such a strategy. In addition to being 98.2% related to the South African consensus sequence, Du422 was found to be 95.9% similar to the derived ancestral sequence and 97.3% similar to the derived global consensus sequence for subtype C. The results of this study indicate that Du422 remains well-suited as a regional Gag vaccine strain for southern Africa since a larger database of sequences and an updated consensus sequence did not alter the close relationship it has to the consensus.

Du422 amino acid sequence contains eight epitopes recognized by southern African individuals infected with subtype C HIV-1 (Masemola *et al.*, 2004b), of which two were identical and six had one or more changes. This provides further support for the relevance of Du422. Some of these peptides occur in regions of the *gag* gene that have a limited ability to change, possibly due to functional constraints, and thus may be important for inclusion in a vaccine to produce cross-subtype responses. Since these peptides are recognised by the target population for the vaccine, Du422 *gag* has been incorporated into a number other HIV-1 vaccine candidates. These include vaccines being developed within the South African AIDS Vaccine Initiative (SAAVI) group working at the University of Cape Town DNA vaccine such as recombinant *Salmonella* vaccines, DNA vaccines (van Harmelen *et al.*, 2003) and protein subunit vaccines (Jaffray *et al.*, 2004). In addition, the *gag* gene from Du422 has been included in vaccines which are currently in clinical trial including: Venezuelan equine encephalitis virus replicon particle (VRP) vaccine (http://www.hvtn.org/trials/) and an adeno-

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associated virus (AAV) vaccine (http://www.iavirep.org/trialsbd/). The VRP_{Du422} vaccine candidate was the first vaccine to enter phase I clinical trials in South Africa and the AAV candidate is currently being tested in Germany and is due to enter phase I trials in South Africa in 2005.

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CHAPTER 3

THE CONSTRUCTION OF *E.COLI*/MYCOBACTERIAL SHUTTLE VECTORS EXPRESSING HIV-1 SUBTYPE C GAG: STABILITY AND EXPRESSION STUDIES

3.1. INTRODUCTION

In this chapter the construction of recombinant *M.smegmatis* and *M.bovis* BCG expressing HIV-1 subtype C Gag is described. The *gag* gene from isolate Du422 (see chapter 2) was amplified and cloned in 8 different *E.coli/Mycobacterial* shuttle vectors. These *gag*-shuttle vectors were introduced into *M.smegmatis* where the genetic stability of the plasmids and expression of *gag* were studied. The shuttle vectors were then introduced into *M.bovis* BCG in preparation for immunogenicity studies (Chapter 4). The expression of *gag* in rBCG was also investigated in bacteria and infected macrophage cultures.

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3.1.1. PROMOTERS AND FOREIGN PROTEIN EXPRESSION IN rBCG

A number of different promoters have been used to drive expression of the foreign antigen in the rBCG system; eg. the BCG *hsp60* (Stover *et al.* 1991), the *M. fortuitum pBlaF** (Nascimento *et al.*, 2000), the *Streptomyces albus groES/EL* (Winter *et al.*, 1991) and the *M.kansasii* α-antigen (Matsuo *et al.*, 1990) promoters. The promoters pertinent to this study and the effects they may have on the production of foreign antigens are reviewed briefly below.

The *hsp60* promoter

The highly conserved *hsp60* protein is a major antigen of mycobacteria (Young *et al.*, 1988). It is expressed constitutively under all growth conditions but is expressed in higher amounts in response to stress (Buchmeier and Heffron, 1990). Two transcription start sites that were

responsive to stress with heat and acid have been identified (Stover *et al.*, 1991). The *hsp60* promoter is active in *E.coli* and, compared to the *M.leprae* 18kDa and 28kDa protein promoters, it gave the highest level of expression of β-galactosidase in *M.smegmatis* and BCG (Dellagostin *et al.*, 1995). Al-Zarouni and Dale (2002) also showed activity for the *hsp60* promoter in *E.coli,* however, they found a surprisingly low level of expression of the *dtxB* gene from *hsp60* in BCG Pasteur. Further investigation revealed that this observed level was due to 95% of the plasmid containing deletions that affected or abolished expression. Batoni *et al.* (1998) demonstrated low levels of *hsp60* promoter activity in exponentially growing *M.avium* but activity increased in late exponential phase and was maximal during late stationary phase. This suggested that the *hsp60* promoter is responsive to starvation stimuli. Significant induction in exponentially growing cells was demonstrated by shifting the incubation temperature from 37°C to 45°C but no induction was seen when hydrogen peroxide was added to the cultures (Batoni *et al.*, 1998).

The 18kDa promoter

The *M.leprae* 18kDa protein induces both cellular and humoral responses in tuberculoid leprosy patients (Dellagostin *et al.*, 1993). It is related to the alpha crystallin family of eukaryotic heat shock proteins (Nerland *et al.*, 1988). Initial sequencing of the gene encoding the 18kDa protein did not identify the promoter sequence (Booth *et al.*, 1988) but examination of two fragments upstream of the start codon identified a transcriptional start site as well as the –10 and –35 sequences (Dellagostin *et al.*, 1995). In addition there is an undefined element upstream of the transcription start site that appears to be involved in regulation of expression. During *in vitro* growth in *E.coli* the 18kDa promoter was relatively strong; however, the expression in *M.smegmatis* and BCG was very low compared to expression from the *hsp60* and 28kDa promoters (Dellagostin *et al.*, 1995). The 18kDa promoter appeared to be specifically activated *in vivo* when expression was tested in macrophages (a ten-fold higher expression than the *hsp60* promoter). Al-Zarouni and Dale (2002) found the highest activity from the 18kDa promoter in *E.coli* but the expression in BCG Pasteur was relatively low.

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The *mtrA* promoter

Via *et al.* (1996) isolated a two-component regulatory system, *mtrA-mtrB*, from *M.tuberculosis*. These systems are associated with environmental signal recognition and induction of adaptive responses. The first gene in the system, *mtrA*, is a response regulator. The transcription and translation initiation sites for the *mtrA* promoter were identified (Zahrt and Deretic, 2000) and it has been demonstrated that expression of the gene is induced in macrophages (Via *et al*, 1996). The response regulator is essential for *M.tuberculosis* viability; however, it is not known whether it is also essential for the growth of BCG (Zahrt and Deretic, 2000). In *M.tuberculosis* the *mtrA* gene was expressed constitutively and expression was thus unchanged upon macrophage entry but, in BCG, it was expressed only upon infection of macrophages (Zahrt and Deretic, 2000).

The *katG* promoter

The *M.tuberculosis katG* gene encodes the catalase-peroxidase enzyme that contributes to survival in the macrophage by protecting the bacterium against excess hydrogen peroxidase (Heym *et al.*, 1997). Mulder *et al.* (1999) identified a 559bp region that incorporated the *M.tuberculosis katG* promoter. They mapped two transcription start sites upstream of the *katG* GTG initiation codon and identified putative –10 and –35 sites for each transcription start site. Deletion studies in the promoter fragment revealed a region that was essential for promoter activity in *E.coli* and required for optimal activity in *M.smegmatis*. This upstream activator region was able to increase expression from the *M.paratuberculosis* P_{AN} promoter in both *E.coli* and *M.smegmatis*. The *katG* promoter was induced by ascorbic acid but repressed by oxygen limitation and elevated temperatures. Expression peaked during late exponential growth but declined during stationary phase. The promoter had similar activities in BCG and *M.smegmatis*.

Targeting of the foreign antigen

Efforts to enhance the immunogenicity of the target antigens lead to the use of signal peptides derived from lipoproteins to direct export or membrane-associated surface expression of the antigens (Stover *et al.*, 1993). Numerous antigens were expressed in the cytosol in rBCG but the

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expression levels were highly variable from one antigen to the next and weak humoral responses were elicited to certain antigens even when they were highly expressed (Stover *et al.*, 1993). The signal peptide strategy had the advantages of the potential for improved antigen presentation by surface expression and enhanced immunogenicity – lipid acylation increases immunogenicity of synthetic peptides and lipoproteins from bacterial pathogens are highly immunogenic. rBCG vaccines expressing outer surface protein A (OspA) from *Borrelia burgdorferi* as a membrane-associated lipoprotein (using the *M.tuberculosis* 19kDa gene secretion signal) resulted in protective antibody responses that were 100-1000-fold higher than responses from rBCG expressing OspA in the cytosol or as a secreted fusion with the mycobaterial alpha antigen (Stover *et al.*, 1993). Others have followed a similar strategy with similar results (eg. Langermann *et al.*, 1994b; Bastos *et al.* 2002; Grode *et al.*, 2002; Bao *et al.*, 2003). For more detail and further examples, see various reports described in section 1.B.5.

The level of production of foreign protein

The level of production of foreign antigens in the rBCG system varies widely from one study to the next. Reports range from 15% of the total BCG protein (Fuerst *et al.*, 1991; Stover *et al.*, 1991) to 0.1% (Aldovini and Young, 1991). Bacterial antigens, particularly *Mycobacterial* antigens, tend to yield the highest expression levels irrespective of the promoter/signal sequence combination. However, different promoter/signal sequence combinations have been shown to express the same foreign antigen to different levels. A brief review of reported expression levels follows.

Fuerst *et al.* (1991) expressed β-gal to 15% of the total BCG protein when the *lacZ* gene expression was *hsp60*-driven on an episomal vector. However, HIV-1 *gp120* in the same system could only reach levels that were 200-fold lower than the β-gal expression (Fuerst *et al.*, 1992). When *Streptococcus pneumoniae* PspA protein was expressed under the control of *hsp60* in cytosolic, membrane-associated and secreted form, the cytosolic form accounted ~15% of the total BCG protein (Langermann *et al.*, 1994a). This was 2- to 5-fold higher than the membrane-

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associated or secreted expression. Comparing expression levels of hybrid pertussis toxin-tetanus toxin in rBCG showed that expression under the control of the *hsp60* promoter was approximately 10-fold more than expression from the Ag85A promoter (5ng of total protein vs 0.4ng of total protein; Abomoelak *et al.*, 1999). The production of another bacterial protein, *E.coli* MalE, was optimal in secreting rBCG strains (Himmelrich *et al.*, 2000). The total amount of MalE was 3.3-8.6 times higher when secreted. However, the percentage of MalE in the total BCG protein in each fraction ranged from as low as 0.003% to 2.149%.

The parasite antigen CSP1 from *Plasmodium falciparum* was expressed in rBCG to a level of 1% of the total BCG protein, under the control of the 64kDa antigen promoter of BCG (Haeseleer *et al.*, 1993).

Lower production levels of viral antigens are reported generally. Full-length HIV-1 Gag protein was produced to only 0.1% of total BCG protein from the *hsp70* promoter (Aldovini and Young, 1991), while HIV-1 Nef was expressed to 1% of the total protein under the control of the *S.albus groES/groEL1* promoter (Winter *et al.*, 1991). When SIV *nef* and *gag* genes were expressed from the *pBlaF** promoter as an operon, the percentage of Gag in the total protein was 1.11% in an episomal vector compared to 0.14% in an integrative vector (Médérlé *et al.*, 2002). However, another integrative vector was able to express Gag to 1.04% of the total protein, despite there being only a single copy of the *gag* gene present in that recombinant.

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3.1.2. STABILITY OF rBCG

***In vitro* stability of rBCG**

In order to maintain a sufficient level of antigen expression and ensure a strong immune response, it is important to have a genetically stable rBCG. In addition, for future production in sufficient batches for general vaccination, the rBCG needs to be stable during the manufacturing and storage process.

Various groups have reported results from *in vitro* stability experiments. In one of the first experiments to assess *in vitro* stability, it was found that rBCG carrying vectors with only kanamycin resistance markers and no heterolgous genes were genetically unstable (Matsumoto *et al.*, 1996). Only one vector in their series (pSO246) was stable up to 50 generations. Comparing *E.coli lacZ* expression in *M.smegmatis* transformed with episomal and integrative vectors demonstrated that the integrative vectors were more stable by about 120-fold (Kumar *et al.*, 1998). The loss of stability in episomal vectors was also observed in rBCG expressing a SIVmac251 Gag-Nef fusion from the *pBlaF** promoter, while stable maintenance of integrative vectors carrying the same gene fusion was demonstrated (Méderlé *et al.*, 2002). However, an rBCG expressing a *Mycobacterial* protein – the 30kDa major secretory protein from *M.tuberculosis* – was found to be highly stable even though the gene was carried on an episomal shuttle vector (Horwitz *et al.*, 2000).

The genetic stability of vectors expressing β-gal from different promoters in different *Mycobacterial* host strains has also been investigated (Medeiros *et al.*, 2002). The shuttle vector expressing β-gal from the *hsp60* promoter was highly unstable in *M.smegmatis* but was more stable in *M.vaccae.* The *pAN* promoter shuttle vector was more stable than *hsp60* vector in both host strains. When testing the plasmids expressing the *dtxB* gene in different strains of BCG, 0/30 clones carried intact plasmid in BCG Moreau, while 7/30 were intact in BCG Tokyo and BCG Pasteur (Al-Zarouni and Dale, 2002).

Structural modification of the shuttle vector has been identified as the cause of the loss of expression. Episomal vectors with deletions that encompassed the promoter (*hsp60*) with either part of the heterologous gene or the entire gene have been described (Kumar *et al.*, 1998). Al-Zarouni and Dale (2002) reported that 95% of the plasmids recovered from rBCG contained deletions. An insertion in an integrative vector (Kumar *et al.*, 2002) as well as transposition of an insertion sequence (Chawla and Das Gupta, 1999) has been shown. Loss of the shuttle vector has also been demonstrated (Méderlé *et al.*, 2002). In one case, no gross structural modifications

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were identified despite loss of heterologous gene expression (Medeiros *et al.*, 2002) and in another case, sequencing of the shuttle vector revealed the deletion of one nucleotide at the codon preceding the stop codon, resulting in a frame-shift and expression of a larger protein (Bastos *et al.* 2002).

***In vivo* stability of rBCG**

A number of groups have also investigated the *in vivo* stability of the shuttle vectors usually by plating homogenates of organs from inoculated animals on media with or without selection Table 3.1.). Retention of kanamycin resistance is compared and, in some cases, expression of the heterologous gene is determined. Stability levels vary quite considerably from one study to the next. In some studies, the constructs continue to stably express the heterologous gene after immunization eg. 100% expression of β-gal after 2-4 weeks (Stover *et al.*, 1991); 100% expression of gp63 after 10 weeks (Connell *et al.*, 1991). In most studies, however, there is some loss of expression – 26% β-gal expression after 2 months (Murray *et al.*, 1992) – or complete loss of expression – 0% Env expression in one of two macaques (Méderlé, 2003).

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3.1.3. GENERAL CONCLUSIONS

rBCG candidate vaccines for a variety of pathogens have been developed and many induce immune responses in animal models (section 1.B.5). These rBCG vaccines are often unstable (section 3.1.2) and it has been shown that, in general, integrative vectors are more stable than episomal ones. However, integrative constructs have a lower level of expression. The level of expression of the foreign antigen in rBCG is also influenced by the nature of the antigen, the promoter used to drive expression and the use of a localization signal to direct transport of the antigen (section 3.1.1). Bacterial antigens have been expressed to the highest levels but different promoter/localization signal combinations have been shown to improve the expression of antigens. The three promoters that have been used most often to yield good expression of and immune responses to a wide variety of antigens are *hsp60*, P_{AN} *and pBlaF**. In addition, comparative studies have shown that antigens that are fused to a localization signal tend to

express at a higher level and are more stable than their cytosolic counterparts. A number of rBCG vaccines have shown partial protection against infection in animal models (section 1.B.5). The fact that only partial protection has been observed could mean that both a neutralizing antibody response and a CTL response are required to achieve full protection. An rBCG vaccine in

Table 3.1. *In vivo* stability of rBCG expressing various heterologous proteins

Antigen source	Antigen	*Vector features* Promoter	Localization or gene fusion	Animal	In vivo *stability*	Reference
E.coli	β-gal	hsp60	cytosol	BALB/c mice	recovery from spleens; 2-4 weeks post-immunization: all colonies were Km^R and expressed β-gal	Stover *et al.*, 1991
		P_{AN}	cytosol	BALB/c mice	recovery from spleens; 2 months post-immunization: 45% of colonies retained Km^R but only 26% expressed β-gal	Murray *et al.*, 1992
		P_{AN}	cytosol	Guinea pigs	recovery from spleens; 4 weeks post-immunization: 50% of colonies expressed β-gal recovery from draining lymph nodes; 16 weeks post-immunization: 50% of colonies expressed β-gal	Lagranderie *et al.*, 1993
Borrelia burgdorferi	OspA	hsp60	membrane-associated	mice	recovery from spleens; 9 weeks post-immunization: all Km^R colonies were expressing OspA	Edelman *et al.*, 1998
Pertussis toxin	S1PT	pBlaF*	β-lactamase	BALB/c mice	recovery from spleens; 1 or 2 months post-immunization: 86% of colonies were Km^R	Nascimento *et al.*, 2000
Leishmania	gp63	hsp60	cytosol	mice	recovery from spleens; 10 weeks post-immunization: 5/5 colonies expressing gp63	Connell *et al.*, 1993
Measles virus	N protein	hsp60	cytosol	mice	recovery from spleens; 8 weeks post-inoculation: 100% expressing N protein	Fennelly *et al.*, 1995
				macaques	recovery from draining lymph nodes; 6 months post-immunization: expression of N protein in >90% of Km^R colonies	Zhu *et al.*, 1997
Human papillomavirus	L1 protein	hsp70	cytosol	mice	recovery from spleens; 3 colonies were still expressing HPV-L1	Jabbar *et al.*, 2000
SIV	Nef-Gag fusion	pBlaF*	cytosol	mice	recovery from spleens; after 28 days: episomal vector lost from 40% of colonies; after 100 days: episomal vector lost from 75% of colonies but integrative vectors lost from only 2-15% of colonies; after 70 days: 25% of colonies retained Nef and Gag expression for the episomal vector while all colonies retained expression for the integrative vectors	Médérlé *et al.*, 2002
	Nef, Gag and Env	pBlaF* and P_{AN}	cytosol	2 macaques	recovery from exudating lesions; 70% and 40% Km^R colonies; Gag expression in 58% and 90%; Nef expression in 24% and 10%; Env expression in 18% and 0%.	Médérlé *et al.*, 2003

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combination with another vaccine candidate in a prime-boost strategy could provide a more broad response that offers a higher level of protection from infection.

An rBCG vaccine requires a high level expression of the foreign antigen combined with maximum genetic stability: an episomal vector with a strong promoter and an antigen that is localized to the extracellular milieu or the cell surface.

3.2. METHODS

3.2.1. BACTERIAL STRAINS AND GROWTH CONDITIONS

All cloning and DNA manipulation steps were performed in *E.coli* DH5α (Hanahan, 1983) grown in 2X YT broth or on 2X YT agar supplemented with kanamycin (30μg/ml). *Mycobacterium smegmatis* $mc^2$155 (Snapper *et al.*, 1990) and *M.bovis* BCG (Tokyo and Pasteur) were used as mycobacterial host strains. Liquid cultures of Mycobacteria were grown in 100ml Sauton's medium containing tyloxapol detergent to prevent clumping (Appendix A) in 1l glass bottles on a 4 rpm roller at 37°C, while plating was on Middlebrooks 7H10 medium containing O-ADC (Appendix A), both supplemented with kanamycin (15μg/ml) when appropriate.

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3.2.2. RT-PCR AND CLONING OF FULL-LENGTH Du422 *gag* GENE

The Du422 *gag* gene was amplified from serum by RT-PCR and then cloned in the pGEM-T Easy vector.

Reverse transcription

Reverse transcription was performed on RNA obtained from serum sample Du422 as described in section 2.2.2. using the GIBCO-BRL kit (Invitrogen, MD, USA). The primer used for the reverse transcription was o-gag-r (5`-ACC TCC AAT TCC TCC TAT C-3`; HXB2 numbering: 2390-2408; S. Loubser, Division of Medical Virology, University of Cape Town, unpublished).

PCR

Nested PCR to amplify the Du422 *gag* gene was performed using the primers listed in Table 3.2. The outer reaction components consisted of a 50µl amplification mix with 1x buffer (Southern Cross Biotechnology, South Africa) containing 1.5mM $MgCl_2$ (Southern Cross Biotechnology, South Africa), 2.5mM of each dNTP (Roche, Basel, Switzerland), 1.5U Supertherm *Taq* DNA polymerase (Southern Cross Biotechnology, South Africa) and 25pmol of each primer. The cDNA (4µl) used for the template was obtained from the RT reaction described above. The thermocycle profile consisted of an initial denaturation at 94°C for 5 min, followed by 35 cycles of 94°C for 30 sec, 55°C for 30 sec and 72°C for 1 min. The reaction was ended with a final elongation step of 5 min at 72°C.

The inner reaction was performed using 4µl of the outer PCR product and consisted of a 50µl amplification mix containing 1.5mM $MgCl_2$ (Southern Cross Biotechnology, South Africa), 2.5mM of each dNTP (Roche, Basel, Switzerland), 1.5U SuperTherm *Taq* DNA polymerase (Southern Cross Biotechnology, South Africa) and 25pmol of each primer. The thermocycle profile consisted of an initial denaturation at 94°C for 5 min, followed by 5 cycles of 94°C for 30 sec, 55°C for 30 sec and 72°C for 1 min. This was followed by 35 cycles of 94°C for 30 sec and 72°C for 1 min with a final elongation step of 72°C for 5 min.

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Table 3.2. PCR primers used to amplify the Du422 *gag* gene by nested PCR

Primer name	Sequence (5'-3')	HXB2 numbering[a]	Source
Outer primer set:			
o-gag-f	AAA TCT CTA GCA GTG GCG	623-640	S.Loubser[b]
o-gag-r	ACC TCC AAT TCC TCC TAT C	2390-2408	S.Loubser
Inner primer set 1:			
p17apa-F	GGG CCC ATG GGT GCG AGA GCG TCA ATA[c]	790-810	P.Eickhaus[b]
i-gag-cla-R	ATC GAT AAT ACT GTA TCA TCT GCT CCT G	2329-2350	S.Loubser
Inner primer set 2:			
p17eco-F	GAA TTC ATG GGT GCG AGA GCG TCA ATA	790-810	P.Eickhaus
i-gag-sal-R	GTC GAC AAT ACT GTA TCA TCT GCT CCT G	2329-2350	S.Loubser

[a] www://hiv-web.lanl.gov/; [b] Division of Medical Virology, University of Cape Town, unpublished; [c] restriction enzyme sites underlined

Cloning of the Du422 *gag* gene in pGEM-T Easy

PCR products were purified using the QIAquick PCR purification kit (Qiagen Operon, CA USA) following the protocol supplied. DNA concentrations of the purified products were estimated by

electrophoresis on a 1% agarose gel alongside samples of known DNA concentration and comparing the fluorescence emitted by the samples. The amplicons were then ligated to pGEM-T Easy (Appendix D; Promega, WI, USA), a T-tailed vector, following the manufacturer's instructions. Ligation reactions were introduced into competent *E.coli* DH5α cells by the heat shock method (Appendix B). Transformants containing the amplification products were selected by plating transformation mixes on 2X YT agar (Appendix A) containing 50μg/ml of ampicillin (Ranbaxy; South Africa).

Putative transformants were cultured in 5ml 2X YT agar (Appendix A) containing 50μg/ml of ampicillin (Ranbaxy; South Africa). Small-scale plasmid DNA extraction was performed on the cultures using the High Pure plasmid extraction kit (Roche, Germany) following the manufacturer's protocol. The plasmids were digested with *Eco*RI (Appendix B) and electrophoresed on 1% agarose at 80V to identify positive transformants. A positive transformant for each of the inner primer sets was selected and designated pRT001 (inner primer set 1) and pRT002 (inner primer set 2).

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3.2.3. SEQUENCING OF THE CLONED Du422 *gag* GENE

The DU422 *gag* gene inserts from pRT001 and pRT002 were sequenced on both strands. Template for DNA sequencing was prepared using the High pure plasmid extraction kit (Roche, Germany). DNA sequencing was performed on plasmid DNA as described in Appendix B. The primers used for sequencing are listed below.

Sequencing primers

M13 universal forward and reverse primers

HXB2 numbering in brackets (www://hiv-web.lanl.gov/)

**Gag1d*: 5`- ACA TGG GTA TTA GCT CTG GGC T -3` (1282-1303)

**Gag2u*: 5`- CAC CTA GAA CTT TGA ATG CAT G -3` (1232-1253)

**Gag2d*: 5`- TCT AAT GAA GCC CCT GGT CCT AAT -3` (1797-1820)

Gagend: 5`- TTG GAT GAC AGA CAC CTT G -3` (1724-1753)

* = Williamson *et al.*, 2003

3.2.4. SHUTTLE VECTOR SUBCLONING

Properties of *E.coli/Mycobacterium* shuttle vectors

Six different *E.coli/Mycobacterium* shuttle vectors were modified in this study to generate 8 constructs carrying the Du422 *gag* gene (Table 3.3.).

Table 3.3. Properties of the *E.coli/Mycobacterium* shuttle vectors used in this study

VECTOR	PROMOTER	LOCALIZATION SIGNAL	INTEGRATIVE/ EPISOMAL	SOURCE
pMV361	*hsp60*	none	Integrative	MedImmune[a]
pMV33A	*hsp60*	Full alpha antigen	Integrative	M.Dennehy[b]
pMV3319	*hsp60*	19kDa signal sequence	Integrative	M.Dennehy
pMV119	*mtrA*	19kDa signal sequence	Integrative	J.Maclean[c]
pCB112	18kDa	Alpha antigen signal	Episomal	W.R. Jacobs Jr[d]
pCB119	*mtrA*	19kDa signal sequence	Episomal	W.R. Jacobs Jr[d]

[a] = MedImmune Inc.; Maryland, USA; [b] = M. Dennehy, 2003; [c] = J. Maclean, 2004; [d] = C. Berkower and W. Jacobs Jr.; Howard Hughes Medical Institute, Albert Einstein College of Medicine, New York, USA; unpublished

The integrative shuttle vectors carry the origin of replication for *E.coli* (*oriE*) and an *attP* attachment site and an integrase gene *(int)* from mycobacteriophage L5. The episomal vectors, pCB112 and pCB119, carry the origins of replication for *E.coli* (*oriE*) and *Mycobacteria* (*oriM*), the malarial *msp1* gene and a *lysA* gene expressed from an *hsp60* promoter (for use in auxotrophic hosts). Both integrative and episomal vectors have a kanamycin resistance marker (*aph*).

Amplification and cloning of the *katG* promoter

The *katG* promoter was excised from pJCkat06 (Mulder *et al.*, 1999) using *Bam*HI and *Hin*dIII (Appendix B). The *Bam*HI/*Hin*dIII fragment was then used as a template in an amplification reaction in order to change the restriction sites at the ends. The following primer pair was used in the PCR: **katG-f**: 5`-TCT AGA GAT GGG GGA TCG -3` and **katG-r**: 5`- CAT ATG GAT CCT TCC AGG -3`. The *Xba*I and *Nde*I restriction enzyme sites are underlined. The PCR amplification

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was performed in 50μl containing 1x polymerase buffer (manu), 1.5mM $MgCl_2$, 1.25mM of each dNTP, 10pmol of each primer and 1U of *Taq* DNA polymerase. The thermocycle profile was as follows: 94°C for 5 min; 35 cycles of 94°C for 20 sec, 55°C for 20 sec and 72°C for 20 sec; and a final elongation step of 72°C for 5 min.

The *XbaI/NdeI* PCR product was purified using the QIAquick PCR purification kit (Qiagen Operon, CA USA) following the kit protocol. The purified product was then ligated into the pGEM-T Easy vector (Appendix D; Promega, WI, USA) following the manufacturer's instructions. After introduction into *E.coli* DH5α (Appendix B), transformants were screened by PCR using 2μl of culture in the same amplification mix detailed above. Positive transformants were confirmed by sequencing both strands of the insert (Appendix B) using the M13 forward and reverse primers. The clone was designated pRT004.

Subcloning strategies

Large volume (100ml) cultures of the *E.coli* isolates carrying the 8 shuttle vectors as well as pRT001 and pRT002 were grown and large-scale plasmid isolation was performed using the Nucleobond AX-100 kit (Machery-Nagel, Germany). Ligation reactions were performed on DNA inserts and vectors with compatible ends as described in Appendix B.

In preparation for cloning into pMV361, pMV33A and pMV3319, the Du422 *gag* gene from pRT002 was excised as an *Eco*RI/*SalI* fragment. This fragment was cloned into pUC19 (Appendix D) prepared with *Eco*RI/*SalI* to yield pRT003. The *gag* gene was then excised from pRT003 as an *Eco*RI/*Hin*dIII fragment and cloned into pMV361, pMV33A and pMV3319 prepared with the same enzymes; yielding pRT101, pRT102 and pRT103 (Appendix D). Cloning into pMV119 required a different strategy. The *gag* gene from pRT001 was excised as an *ApaI/ClaI* fragment. Digestion of pMV119 with *ApaI/ClaI* yielded two fragments (3557bp and 1315bp) since there is an *ApaI* site outside of the vector multiple cloning site (see Appendix D). The *ApaI/ClaI*

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gag gene fragment was first ligated to the smaller 1315bp *ApaI/ApaI* vector fragment. This fragment was then ligated to the bigger 3557bp *ApaI/ClaI* vector fragment to yield pRT104.

The Du422 *gag* gene from pRT001 was excised as an *ApaI/ClaI* fragment. The malarial *msp1* gene was excised from pCB112 and pCB119 with an *ApaI/ClaI* digest and the *gag* gene was ligated in its place to yield pRT105 and pRT106, respectively (Appendix D). In order to generate pRT107 and pRT108, the 18kDa promoter from pRT105 and the *mtrA* promoter from pRT106 were excised with an *XbaI/NdeI* digest and replaced with the *katG* promoter (*XbaI/NdeI* fragment) from pRT004 (Appendix D).

The 8 shuttle vectors containing *gag* were confirmed by means of restriction mapping.

***Mycobacterial* transformation: preparation of competent cells**

Mycobacterial cells were prepared for electroporation based on the method of Parish and Stoker (1998). Briefly, liquid mycobacterial cultures were grown until the OD_{600} reached 0.8-1.0. The cells were pelleted at 3000rpm for 15min at room temperature in a benchtop centrifuge. The pellets were resuspended in 50ml of 10% glycerol and then centrifuged as before. This process was repeated three times. The cells were then resuspended in 1-4ml of 10% glycerol.

***Mycobacterial* transformation: electroporation**

One hundred microlitres of the competent Mycobacterial cells were aliquoted into 0.1cm gap electroporation cuvettes (BioRad, CA, USA). The shuttle vector DNA was added (500ng) and the cells were electroporated using a GenePulser II electroporator (BioRad, CA, USA). The GenePulser was set at a capacitance of 25µF and a resistance of 1000Ω and a 1.8kV charge was passed through the cuvette. After electroporation, 0.9ml of Sauton's media was added to each cuvette and the transformation mix was removed to a microfuge tube that was placed at 37°C for 2hr (*M.smegmatis*) or 24hr (*M.bovis* BCG). The transformation mix was pelleted gently at 3000rpm in a microfuge for 2 minutes, 500µl of media was removed and the pellet was

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resuspended in the remaining media. The recombinants were selected by plating the resuspended cells on Middlebrooks 7H10 media containing kanamycin (15µg/ml) at 37°C for 2 days (*M.smegmatis*) or 3-4 weeks (*M.bovis* BCG).

Identification of recombinant *Mycobacteria*: integrative shuttle vectors

Colonies from transformation plates were grown in 5ml of Sautons medium at 37°C until mid-logarithmic phase (OD_{600} = 0.6-0.8). Recombinants carrying the integrative shuttle vectors were identified by Southern hybridization of genomic DNA with an appropriate probe. Genomic DNA was isolated from putative recombinants following the method described by Ausubel *et al.* (1987; Appendix B). The genomic DNA was digested with *Xba*I and *Cla*I restriction enzymes and separated on 0.8% agarose at 20V overnight. The DNA from the gel was transferred to Hybond N membrane using standard methods (Appendix B) and cross-linked to the membrane with ultraviolet light. The probe used in the hybridization was a PCR product encompassing the p24 portion of the DU422 *gag* gene. It was amplified using the *gag* B primer set (fwd: 5` CCATATCACCTAGAACTTTGAAT 3`; rev 5` CTCCCTGACATGCTGTCATCAT 3`). The probe was labeled using the ECL direct nucleic acid labeling and detection system (Amersham Pharmacia Biotech) following the kit protocol. The hybridization was performed using the ECL system and following the manufacturer's instructions.

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Identification of recombinant *Mycobacteria*: episomal shuttle vectors

Recombinants carrying episomal shuttle vectors were identified by restriction mapping of plasmid DNA recovered in *E.coli.* After small-scale isolation of plasmid DNA was performed by alkaline lysis following the method of Sambrook *et al.* (1989; Appendix B) from recombinant Mycobacteria, the plasmid DNA was introduced into *E.coli* DH5α cells by means of the heat shock method (Appendix B). *E.coli* transformants were selected on 2x YT agar containing kanamycin (15µg/ml) and plasmid DNA was isolated from 10-15 transformants. Maps of the plasmids generated by restriction enzymes were compared to parental plasmid maps in order to identify recombinants.

3.2.5. RECOMBINANT *M.smegmatis* (rMS) STABILITY AND EXPRESSION

Growth at 37°C and 42°C

After transformation of *M.smegmatis* with the shuttle vectors, 250µl of the transformation mixes were plated in duplicate on Middlebrooks 7H10 media containing kanamycin. One set of plates was incubated at 37°C and the other at 42°C for 2 days. Duplicate plates were then screened and compared for growth and size of colonies.

Protein extraction

Mycobacterial cultures were grown until the OD_{600} was 0.6-0.7. Total protein was extracted by means of SDS-boiling lysis of the bacterial cells. Briefly, 80ml of culture was centrifuged in a pre-cooled rotor at 12000*g* for 4 minutes at 4°C. Pellets were resuspended in 20ml of ice-cold PBS and centrifuged as before. Pellets were then resuspended in 250µl of 1x lysis buffer (3% SDS, 10% glycerol, 0.0625M Tris-Cl pH 6.8) and the tubes were placed in a boiling water bath for 30 minutes. The lysed cells were centrifuged at 27000*g* for 10 minutes at room temperature. The supernatant was further clarified by centrifugation at 15000*g* in a microfuge and was then transferred to a clean tube and stored at –20°C.
Protein concentrations were determined using the DC protein assay kit (Bio-Rad Laboratories, CA, USA) following the manufacturer's instructions.

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Genetic stability and expression of *gag*

Genetic stability and expression of *gag* were studied by subculturing recombinant *M.smegmatis* for 42 generations. Recombinants were grown in liquid culture until the OD_{600} reached 0.6-0.8 (mid-logarithmic growth) before inoculating new cultures at a starting OD_{600} of 0.01 to 0.03. Total protein was extracted after each subculture. In addition, for the recombinants carrying episomal vectors, plasmid DNA was extracted by standard alkaline lysis (Appendix B) after the first culture (subculture 1) and at subcultures 6 and 10. Recombinants carrying integrative vectors had genomic DNA extracted from them after the first and last subculture. The process was repeated

until 40-42 generations had passed. The number of generations was calculated using the formula: (lnX-lnY)/ln2; where X = OD at harvest and Y = OD at inoculation.

The plasmid DNA extracted from the *M.smegmatis* recombinants was introduced into *E.coli* DH5α and plasmid DNA was then extracted from 10-15 individual *E.coli* transformants (Appendix B). The plasmid samples were subjected to restriction enzyme mapping with *Xba*I and *Pst*I. A selection of these samples was also mapped with *EcoRI*, *Ssp*I and *Not*I. The genomic DNA extracted from recombinants was digested with *Xba*I and *Cla*I and Southern hybridization was performed on the DNA after transfer to a nitrocellulose membrane using the *gag* probe described above (section 3.2.4).

The relative expression level of Gag from each recombinant was determined by measuring the level of p24 in the protein samples using an automated capture ELISA. This was performed using the HIV type 1 (groups M and O) p24 Ag test on the Elecsys 2010 immunoassay analyzer (Roche, Basel, Switzerland) following the manufacturer's instructions. Five micrograms of total protein extracted from the recombinant cultures during the 42 generation-long serial passage was subjected to the Ag test.

Expression during different phases of growth

Recombinant *M.smegmatis* carrying the episomal shuttle vectors were grown to early log phase (OD_{600} ~0.4), mid log phase (OD_{600} ~0.7) and late log/stationary phase (OD_{600} >1). Protein was extracted from the cultures at each point and the level of p24 was measured as described above.

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3.2.6. CYTOSOLIC VS MEMBRANE-ASSOCIATED GAG EXPRESSION

rMS and rBCG Tokyo and Pasteur were cultured to mid-log phase and protein from the cytosolic and membrane fractions was extracted. Protein concentrations were determined using the DC protein assay kit (Bio-Rad Laboratories, CA, USA) following the manufacturer's instructions. Expression of p24 was assayed using 5-10μg of each protein fraction in the HIV type 1 (groups M and O) p24 Ag test described above.

Cytosolic protein extraction

Cultures were grown until mid-log phase and harvested at 3000rpm in a benchtop centrifuge for 20 min. The pellet was resuspended in PBS and centrifuged as before. This washing step was repeated and the pellet was resuspended in 4ml PBS. The suspension was then divided into 2 screw-cap tubes (Quality Scientific Plastics, CA, USA) containing 0.1mm zirconia silica beads (Biospec Products, OK, USA) to the 100µl level. The tubes were placed in a Fastprep FP120 bead beater (Bio 101 Systems, CA, USA) and subjected to four 30 sec shakes at speed 6. Tubes were placed on ice for 3 min between each shake. The lysates were clarified by centrifugation at 10000rpm in a microfuge and the cellular debris was retained for extraction of the cell membrane protein (see below #). The supernatant containing the cytosolic protein was removed and the remaining debris was removed by further centrifugation. The lysates were stored at -20°C.

Cell membrane and insoluble protein extraction #

The cellular debris retained from the cytosolic protein extraction was resuspended in PBS containing 1%SDS. The suspension was boiled for 10 minutes to release the insoluble protein and the protein in the BCG membrane fraction. After centrifugation to repellet the debris (10000rpm, 10 min), the supernatant containing the membrane protein fraction was collected and stored at -20°C.

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3.2.7. rBCG:GAG EXPRESSION IN BALB/C PERITONEAL MACROPHAGES

Infection

The peritoneal lavages from 10 BALB/c mice were pooled and the cells were harvested by centrifugation at 1500rpm for 5 min. The pellet was resuspended in 2ml R10 medium (RPMI-1640 medium with Glutamax-1 containing 10% foetal bovine serum, 100units/mL Penicillin-G and 100 µg/mL Streptomycin). After counting, the cell numbers were adjusted to $6X10^5$ cells/ml in R10 medium. Two milliliters of cells were then aliquotted into the wells of a 24-well plate (Nalge-Nunc International, Denmark). The plates were incubated at 37°C/5%CO_2 for 4 hours; the non-adherent

cells were discarded, the medium was changed and the adherent macrophages were incubated overnight. The cells were washed with R10 without penicillin and streptomycin. The rBCG stocks at a concentration of $1.8X10^6$cfu/ml were added to the macrophages (6 wells per rBCG) such that the BCG:macrophage ratio was 10:1. Infection was allowed to continue for 4hours, the plate was washed three times with R10 (without penicillin and streptomycin) and incubation was continued for 24 hours and 48 hours.

Lysis

Each well was drained of its supernatant and washed twice with 1ml ice-cold PBS/2mM PMSF. Two hundred microlitres of RIPA lysis buffer (RIPA buffer containing 0.01%SDS, 1x protease inhibitor) was added and the plates placed at -20°C overnight. After thawing the lysates, the cells were removed from the well bottoms manually with a syringe plunger. The lysates were sonicated (X3) on ice using a Microson Ultrasonic Cell Disruptor XL (Misonix Inc; Farmingdale, New York, USA) set at 2.5 and 0.01W. After sonication, lysates were clarified by centrifugation at 10000rpm at 4°C in an Eppendorf centrifuge. The protein-containing supernatants were transferred to fresh tubes and stored at -80°C.

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Gag expression

Fifty microlitres of each lysate, diluted 1:5 in dH_2O, was subjected to the HIV type 1 (groups M and O) p24 Ag test described above. Protein concentrations were determined using the Pierce BCA Microassay kit following the manufacturer's instructions.

3.3. RESULTS

3.3.1. CLONING THE FULL-LENGTH *gag* GENE

The *gag* gene from isolate DU422 was amplified by RT-PCR using 2 primer sets with different restriction enzyme sites on the ends. The resulting *Apa*I/*Cla*I and *Eco*RI/*Sal*I amplicons were

cloned into pGEM-T Easy to produce pRT001 and pRT002, respectively, and sequenced. The DNA sequences are shown in Appendix C.

The sequences were compared with the parental DU422 *gag* sequence using DNAMAN alignment software. pRT001 had 3 amino acid changes compared to the parental sequence, while pRT002 had 1. The nucleotide changes responsible are all in the third base position of the respective codons. They are thus reflective of the different viral quasispecies transcribed by the reverse transcriptase and amplified by the Taq DNA polymerase. In addition, the changes are all previously described HIV-1 subtype C *gag* sequences. Fig 3.1. shows an alignment of the three sequences.

3.3.2. SHUTTLE VECTOR SUBCLONING

The *gag* gene from pRT002 was subcloned into pUC19 to yield pRT003 (see section 3.2.4) and after an *Eco*RI/*Hin*dIII restriction digest, was cloned into the four integrative shuttle vectors pMV361, pMV33A, pMV3319 and pMV119 to yield pRT101, pRT102, pRT103 and pRT104, respectively. The *gag* gene from pRT001 was cloned into the episomal vectors pCB112 and pCB119 in place of the malarial *msp1* gene to yield pRT105 and pRT106. pRT107 and pRT108 were generated by replacing the 18kDa promoter of pRT105 and the *mtrA* promoter of pRT106 with the *katG* promoter from pRT004, respectively (Table 3.4. and Appendix D). The shuttle vectors were mapped with restriction enzymes to confirm that the *gag* gene had been successfully cloned into them before introduction into *Mycobacteria smegmatis* and BCG (data not shown). The DNA sequence of the promoters and signal sequences for the different shuttle vectors are shown in Appendix C. Shuttle vectors were introduced into *Mycobacterial* cells by electroporation and recombinants were identified as described in section 3.2.4. rMS were identified for each of the 8 shuttle vectors.

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```
DU422       MGARASILRGEKLDTWEKIRLRPGGKKHYMLKHIVWASRE    40
pRT001      ----------------------------------------    40
pRT002      -----------K----------------------------    40

DU422       LERFALNPGLLETSEGCKQIMKQLQPALQTGTEELKSLYN    80
pRT001      --------------------I------------------D    80
pRT002      ----------------------------------------    80

DU422       TVATLYCVHEKIEVRDTKEALDKIEEEQNKCQQKTQQAKA   120
pRT001      ----------------------------------------   120
pRT002      ----------------------------------------   120

DU422       ADGKVSQNYPIVQNLQGQMVHQAISPRTLNAWVKVIEEKA   160
pRT001      ----------------------------------------   160
pRT002      ----------------------------------------   160

DU422       FSPEVIPMFTALSEGATPQDLNTMLNTVGGHQAAMQMLKD   200
pRT001      ----------------------------------------   200
pRT002      ----------------------------------------   200

DU422       TINEEAAEWDRLHPVHAGPIAPGQMREPRGSDIAGTTSTL   240
pRT001      ----------------------------------------   240
pRT002      ----------------------------------------   240

DU422       QEQIAWMTSNPPIPVGDIYKRWIILGLNKIVRMYSPVSIL   280
pRT001      ----------------------------------------   280
pRT002      ----------------------------------------   280

DU422       DIRQGPKEPFRDYVDRFFKTLRAEQATQEVKNWMTDTLLV   320
pRT001      ----------------------------------------   320
pRT002      ----------------------------------------   320

DU422       QNANPDCKTILRALGPGATLEEMMTACQGVGGPGHKARVL   360
pRT001      ----------------------------------------   360
pRT002      ----------------------------------------   360

DU422       AEAMSQTNSGNIMMQRSNFKGPRRIVKCFNCGKEGHIARN   400
pRT001      ----------------------------------------   400
pRT002      ----------------------------------------   400

DU422       CRAPRKKGCWKCGKEGHQMKDCTERQANFLGKIWPSHKGR   440
pRT001      ----------------------------------------   440
pRT002      ----------------------------------------   440

DU422       PGNFLQNRPEPTAPPAESFRFEETTPAPKQEPIEREPLTS   480
pRT001      --------------------L-------------------   480
pRT002      ----------------------------------------   480

DU422       LKSLFGSDPLSQ                               492
pRT001      ------------                               492
pRT002      ------------                               492
```

Fig. 3.1. Amino acid sequence alignment of Du422 *gag* gene and the *gag* gene in pRT001 and pRT002. The amino acid differences are indicated

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Table 3.4. List of vectors generated in this study. The parental vector used to generate them and pertinent properties of the vectors are also listed.

VECTOR	PARENTAL VECTOR	PROPERTIES
pRT001	pGEM-T Easy[a]	*gag* with *ApaI/ClaI* ends cloned into pGEM-T Easy
pRT002	pGEM-T Easy[a]	*gag* with *EcoRI/SalI* ends cloned into pGEM-T Easy
pRT003	pUC19[b]	*gag* from pRT002 cloned into pUC19 at *EcoRI/SalI* sites
pRT004	pGEM-T Easy[a]	*katG* promoter cloned into pUC19
pRT101	pMV361[c]	*gag, hsp60* promoter, no localization signal, integrative
pRT102	pMV33A[c]	*gag, hsp60* promoter, α-antigen signal, integrative
pRT103	pMV3319[c]	*gag, hsp60* promoter, 19kDa signal, integrative
pRT104	pMV119[c]	*gag, mtrA* promoter, 19kDa signal, integrative
pRT105	pCB112[c]	*gag,* 18kDa promoter, α-antigen signal, episomal
pRT106	pCB119[c]	*gag, mtrA* promoter, 19kDa signal, episomal
pRT107	pRT105	*gag, katG* promoter, α-antigen signal, episomal
pRT108	pRT106	*gag, katG* promoter, 19kDa signal, episomal

[a,b] Appendix D;
[c] Table 3.2

3.3.3. AN INVESTIGATION OF STABILITY AND EXPRESSION OF SHUTTLE VECTORS IN RECOMBINANT *M.smegmatis*

Genetic stability: Integrative shuttle vectors

Recombinant *M.smegmatis* carrying the four integrative shuttle vectors (pRT101, pRT102, pRT103 and pRT104) were subcultured for about 40 generations. At subcultures 1 and 6 (corresponding to generations 16 and 33, respectively) genomic DNA was extracted from each culture. The genomic DNA was subjected to restriction enzyme digestion with *Xba*I and *Cla*I and Southern hybridization using a *gag* gene probe. Fig.3.2 is a photograph of the agarose gel and a scan of the resulting autorad. The signals obtained for each vector was at the expected molecular weight for both subcultures (2kb for pRT101; 2.8kb for pRT102; 1.97kb for pRT103 and 2.4kb for pRT104). This implies that gross genetic stability has been maintained over the generations assayed.

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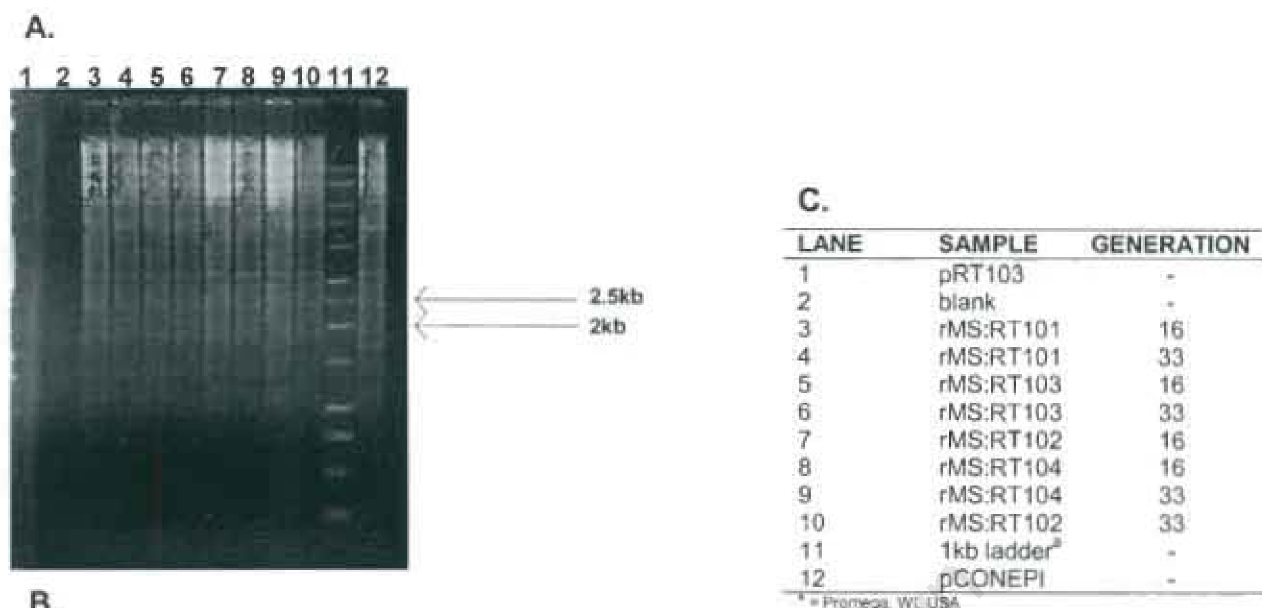
A.



C.

LANE	SAMPLE	GENERATION
1	pRT103	-
2	blank	-
3	rMS:RT101	16
4	rMS:RT101	33
5	rMS:RT103	16
6	rMS:RT103	33
7	rMS:RT102	16
8	rMS:RT104	16
9	rMS:RT104	33
10	rMS:RT102	33
11	1kb ladder[a]	-
12	pCONEPI	-

[a] = Promega, WI, USA

B.

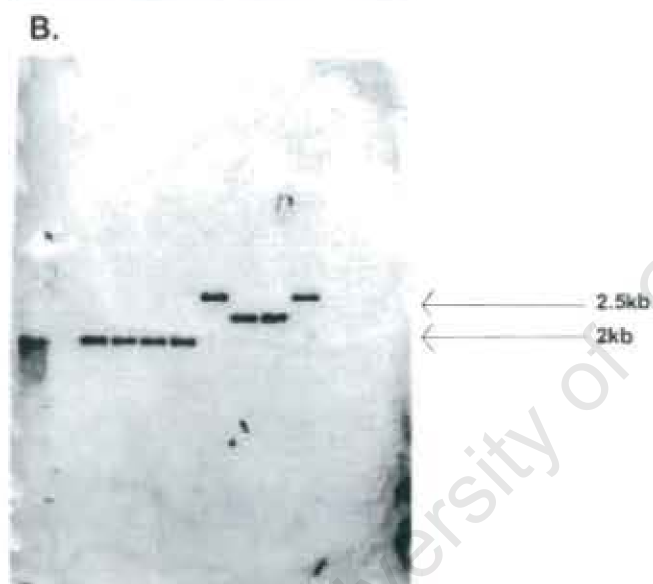


Fig.3.2. Southern blot of gDNA taken from rMS cultures of the integrative vectors.
A. Photograph of agarose gel used to separate restriction digested gDNA. gDNA was digested with *XbaI* and *ClaI* and electrophoresed on a 1% agarose gel at 80V. The DNA was transferred to a nitrocellulose membrane and a Southern blot was performed used a *gag* gene probe (**B**). Samples are loaded as listed in **C.**
B. Scan of the Southern blot autoradiograph showing the *gag*-positive signals.
C. Table listing the order in which the samples were loaded onto the gel.

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Genetic stability: episomal shuttle vectors

Recombinant *M.smegmatis* (rMS) carrying the four episomal gag shuttle vectors (pRT105, pRT106, pRT107 and pRT108) were subcultured for approximately 42 generations. At subcultures 1, 6 and 10 (corresponding to generations 5-6, 20-25 and 42, respectively) plasmid DNA was extracted from each culture that was then introduced into *E.coli* DH5α cells. Introduction into *E.coli* allowed for the separation of individual plasmids (1 per cell) and provided the opportunity to get an overview of the genetic status of the vectors during subculture.

Restriction fragment analysis performed with *Xba*I and *Pst*I indicated that pRT108 was the most genetically stable vector, followed by pRT106. After ~20 generations, 90% and 60% of the plasmids appeared to have no gross deletions or rearrangements, respectively. pRT107 was less stable, as only 40% of the plasmids were identical to the parent after ~20 generations, while pRT105 was completely unstable (Table 3.5.). The shuttle vectors with altered restriction profiles all had deletions of various sizes. When a selection of the plasmids were mapped more precisely with *Eco*RI, *Ssp*I and *Not*I enzymes (fig.3.3.), it was found that they consisted of deletions encompassing the promoter and localization signal with fragments of the gag gene varying in size.

Table 3.5. Summary of the restriction fragment analysis performed on shuttle vectors from recombinant *M.smegmatis* recovered in *E.coli* using *Xba*I and *Pst*I. The number of recovered plasmids with the correct profile for subcultures 1, 6 and 10 are indicated

SHUTTLE VECTOR	SUBCULTURE	GENERATION	CORRECT RESTRICTION PROFILE
pRT105	1	8	10/15
	6	21	0/10
	10	42	0/10
pRT106	1	6	15/15
	6	22	6/10
	10	42	3/10
pRT107	1	5	15/15
	6	21	4/10
	10	42	2/10
pRT108	1	6	15/15
	6	22	9/10
	10	42	4/10

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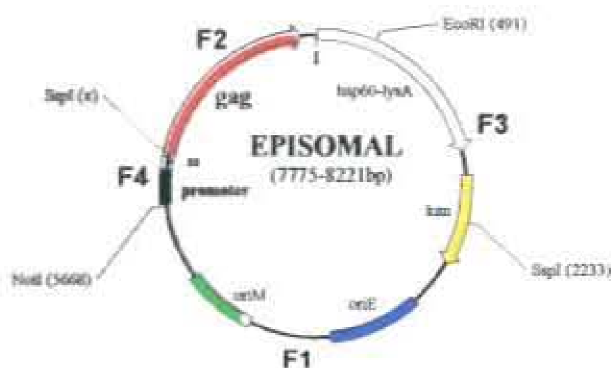
A.

VECTOR	SspI (x)	F 1 (bp)	F 2 (bp)	F 3 (bp)	F 4 (bp)	TOTAL SIZE (bp)
pRT105	6129	3435	2137	1742	461	7775
pRT106	6575	3435	2137	1742	907	8221
pRT107	6416	3435	2137	1742	748	8062
pRT108	6425	3435	2137	1742	757	8071

B.



C.

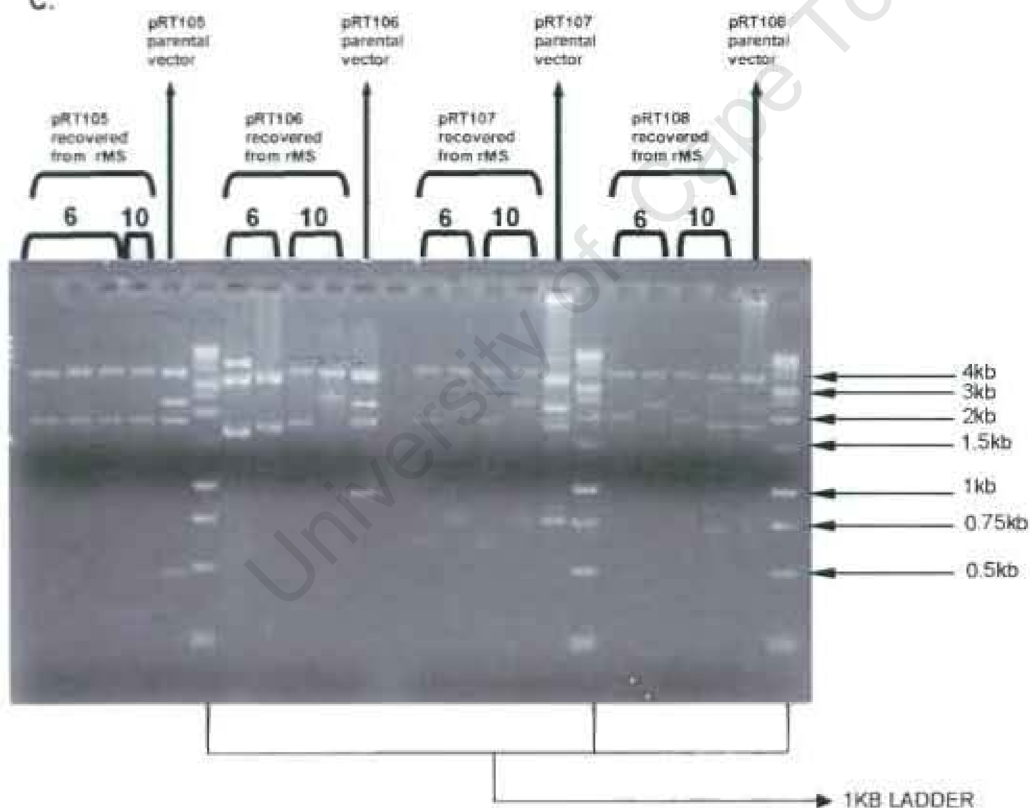


Fig.3.3. Restriction fragment analysis performed on shuttle vectors recovered from recombinant *M.smegmatis* clonally expanded in *E.coli* showing the deletions in fragments F2 and F4. A. Table listing the predicted sizes of the four restriction fragments (F1-F4) for each shuttle vector. The bp position for the *SspI* restriction site (x) is also indicated for each shuttle vector. B. Diagram of a shuttle vector representative of the four vectors used in the restriction fragment analysis. The four restriction fragments (F1-F4) are indicated. C. Photograph of the agarose gel used to separate the digested shuttle vector DNA. Parental vectors were electrophoresed alongside the shuttle vectors removed from the rMS for comparison. Restriction digestion was performed using *EcoRI*, *SapI* and *NotI* enzymes. Digested vector DNA was separated on a 1% agarose gel at 80V. 6 = subculture 6; 10 = subculture 10; 1kb ladder (Promega, WI, USA)

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A comparison of the Gag expression level in the integrative and episomal shuttle vectors

In order to determine how the genetic stability of the recombinant *M.smegmatis* carrying the shuttle vectors impacts on the expression of Gag from these vectors, expression from these cultures was monitored over a number of generations. Total protein was extracted from the recombinant *M.smegmatis* cultures after every subculture as described in section 3.2.5. and the relative level of p24 was measured using the HIV type 1 (groups M and O) p24 Ag test.

When comparing the expression levels from the genetically stable integrative shuttle vectors, it was clear that expression from rMS:RT101 was higher than from the other recombinants. However, all four recombinants expressed at a low level that was maintained (fig 3.4). In other words, the genetic stability of these rMS allowed for a maintenance of expression level but that level was low.

The levels of p24 from recombinants carrying the episomal vectors show that there were 3 distinct patterns of expression. rMS:RT105 expressed at a high level initially but this level dropped rapidly. rMS:RT106 expressed at a more moderate level and was able to maintain this level of expression for about 30 generations before declining. The expression patterns for these recombinants compared well with their genetic stability profile. rMS:RT105 was highly unstable over the period assayed and in keeping with this, lost expression capacity most quickly. rMS:RT106 had a more moderate loss of genetic stability which was reflected in a more moderate drop in Gag expression. The two recombinants expressing Gag from the *katG* promoter, rMS:RT107 and rMS:RT108, did so at a lower level compared to rMS:RT105 and rMS:RT106 and this level remained fairly constant over the growth period assayed (fig 3.4.). Although these *katG* recombinants had low but constant Gag expression levels, they had different patterns of genetic stability. rMS:RT107 lost genetic stability quite soon during the assay period but this was not partnered with a rapid loss of Gag expression. rMS:RT108 had a slower loss of genetic stability

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Table 3.4. List of vectors generated in this study. The parental vector used to generate them and pertinent properties of the vectors are also listed.

VECTOR	PARENTAL VECTOR	PROPERTIES
pRT001	pGEM-T Easy[a]	*gag* with *ApaI/ClaI* ends cloned into pGEM-T Easy
pRT002	pGEM-T Easy[a]	*gag* with *EcoRI/SalI* ends cloned into pGEM-T Easy
pRT003	pUC19[b]	*gag* from pRT002 cloned into pUC19 at *EcoRI/SalI* sites
pRT004	pGEM-T Easy[a]	*katG* promoter cloned into pUC19
pRT101	pMV361[c]	*gag*, *hsp60* promoter, no localization signal, integrative
pRT102	pMV33A[c]	*gag*, *hsp60* promoter, α-antigen signal, integrative
pRT103	pMV3319[c]	*gag*, *hsp60* promoter, 19kDa signal, integrative
pRT104	pMV119[c]	*gag*, *mtrA* promoter, 19kDa signal, integrative
pRT105	pCB112[c]	*gag*, 18kDa promoter, α-antigen signal, episomal
pRT106	pCB119[c]	*gag*, *mtrA* promoter, 19kDa signal, episomal
pRT107	pRT105	*gag*, *katG* promoter, α-antigen signal, episomal
pRT108	pRT106	*gag*, *katG* promoter, 19kDa signal, episomal

[a,b] Appendix D; [c] Table 3.2

3.3.3. AN INVESTIGATION OF STABILITY AND EXPRESSION OF SHUTTLE VECTORS IN RECOMBINANT *M.smegmatis*

Genetic stability: integrative shuttle vectors

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To determine the effect of growth phase on expression of Gag, the expression level in the recombinants carrying the episomal vectors was investigated during different phases of growth (early logarithmic, mid-logarithmic and late-logarithmic/stationary phase). The differences seen were not significant and would not have an effect on the results of the expression level study (data not shown).

Growth at 37°C and 42°C to investigate the stress response of the recombinant *M.smegmatis*

The hypothesis for this experiment was that the bacteria were being stressed by an accumulation of misfolded, denatured Gag protein. This stress results in a heat shock/stringent response that allows the bacteria to grow at 42°C. The implication is that the higher the expression level, the more the bacteria are stressed, leading to smaller colony sizes and survival at 42°C. The constructs in this study varied according to promoter and localization signal. The results from this experiment thus give an indication of the level of expression from each construct. Combined with the expression and stability data detailed above this experiment should give further insight into the differences between the constructs and confirm the results of the expression and stability studies. pCONEPI is an empty vector expressing only kanamycin resistance (W. Bourn, Division of Medical Virology, University of Cape Town). This was used as a control in the experiments and the colony size for *M.smegmatis* transformed with this vector was taken as "normal."

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This experiment was performed three times with the same result each time. Table 3.6 summarizes the colony size differences for the rMS. The results can be summarized as follows:

- recombinants carrying the plasmid, pCONEPI, did not survive heat shock
- only recombinants carrying the episomal constructs survived heat shock, implying that those carrying integrative constructs were not as stressed.

- based on the hypothesis of the experiment and the colony size differences, the comparative Gag expression levels were :
    - episomal vectors: highest RT105>RT106>RT108>=RT107 lowest
    - integrative vectors: highest RT101>RT102 and RT104>RT103 lowest
- the size or growth of the RT105 colonies was limited compared to the other rMS colonies. This implies, based on the experimental hypothesis that rMS:RT105 was producing the largest amount of Gag.

Table 3.6. Colony size differences for recombinant *M.smegmatis* grown under normal (37°C) and heat shock (42°C) conditions.

rMS:	GROWTH at 37°C	GROWTH at 42°C	EXPRESSION LEVELS[a]
RT101	large	none	Low
RT102	large	none	Low
RT103	large	none	Low
RT104	large	none	Low
RT105	minute	minute	High
RT106	small-medium	small	Moderate
RT107	medium-large	small-medium	Low
RT108	medium-large	small-medium	Low
pCONEPI	large	none	n/a

[a] = see Fig.3.4; low = <100 COI; moderate = between 100 and 400 COI; high = > 400 COI

The results seen in this experiment were consistent with the data from the expression study. The integrative constructs expressed Gag at a lower level than the episomal constructs. They were thus less stressed and unable to induce a stringent response that would have allowed survival at 42°C. There was a stringent response in the recombinants carrying the episomal constructs and the colony sizes did correlate with of the expression levels. rMS:RT105 had the smallest colonies and the highest expression levels, rMS:RT106 had small-medium colonies and a more moderate expression level while the rMS:RT107 and RT108 had the largest colonies and the lowest expression levels.

3.3.4. AN INVESTIGATION OF EXPRESSION OF GAG IN CYTOSOLIC VS MEMBRANE-ASSOCIATED/INSOLUBLE PROTEIN FRACTIONS

The data from the stability and expression studies in recombinant *M.smegmatis* are summarized in Table 3.7. As can be seen, the recombinants could be divided into 5 categories:

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- integrative low producers with high stability (RT101, 102, 103 and 104)
- episomal high producer with low stability (RT105)
- episomal moderate producer with moderate stability (RT106)
- episomal low producer with low stability (RT107)
- episomal low producers with high stability (RT108)

Table 3.7. Summary of the recombinant *M.smegmatis* stability and expression data

Shuttle vector	Promoter	Signal	Int/Epi	Genetic stability	p24 levels	Colony size	Stress response
RT101	*hsp60*	None	Int	High	Low	Large	No
RT102	*hsp60*	α-Ag	Int	High	Low	Large	No
RT103	*hsp60*	19kDa	Int	High	Low	Large	No
RT104	*mtrA*	19kDa	Int	High	Low	Large	No
RT105	18kDa	α-Ag	Epi	Unstable	High	Minute	Yes
RT106	*mtrA*	19kDa	Epi	Moderate stability	Moderate	Small-Medium	Yes
RT107	*katG*	α-Ag	Epi	Unstable	Low	Medium-large	Yes
RT108	*katG*	19kDa	Epi	High stability which drops	Low	Medium-large	Yes

The genetically unstable constructs were not selected for further study. The integrative constructs appear to be the most stable but were also the lowest Gag producers. For this reason and because episomal plasmids present more ease-of-use, the integrative constructs were also not used in rBCG studies. The medium stability/moderate producer, RT106 and the high stability/low producer, RT108 were introduced into BCG Tokyo and Pasteur.

Since the 19kDa antigen signal sequence was designed to result in membrane-associated expression of Gag, the expression levels in rMS:RT106 and RT108 and rBCG:RT106 and RT108 (Tokyo and Pasteur) cytosolic and membrane fractions were compared. For each of the strains tested the percentage of p24 protein in the membrane/insoluble fraction of the RT106 *Mycobacteria*, compared to that in the cytosol was the same (Table 3.8). Although the percentage of p24 for rMS:RT108 was the same for both fractions, rBCG:RT108 Tokyo had 4.8x more p24 in the membrane/insoluble fraction than in the cytosolic fraction. rBCG:RT108 Pasteur did not express any p24 in the membrane/insoluble fraction. Overall, the expression of Gag in all cases was a very small percentage of the total protein. The highest level was seen in rBCG:RT106

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Pasteur while the rBCG:RT108 Tokyo had the lowest expression levels, even though it had the biggest difference between p24 in the cytosol versus the cell surface.

Table 3.8. Cytosolic vs membrane-associated expression of Gag in rMS and rBCG (Tokyo and Pasteur)

	RT106 smeg	RT108 smeg	RT106 Tokyo	RT108 Tokyo	RT106 Past	RT108 Past
*COI:**cytosolic**	776.6	178	107	16.5	1278	45.38
total protein in sample: cytosolic (µg)	5	5	10	10	5	5
p24 in cytosolic protein (ng)	1.16	0.27	0.16	0.025	1.9	0.068
p24 in cytosolic protein (%)	0.02	0.005	0.0016	0.00025	0.038	0.0014
COI:**membrane**	832.7	219.7	103	82.81	1171	No p24
total protein in sample: membrane (µg)	5	5	10	10	5	
p24 in membrane protein (ng)	1.24	0.328	0.154	0.124	1.75	
p24 in membrane protein (%)	0.02	0.007	0.0016	0.0012	0.035	

*COI: p24 levels are reported in the form of a CutOff Index that is calculated by the Elecsys 2010 analyser using the readings from the negative and positive calibrators supplied with the HIV type 1 (groups M and O) p24 Ag test kit

3.3.5. GAG EXPRESSION IN BALB/C MOUSE MACROPHAGES

Before preclinical immunological testing of the rBCG vaccines in mice was undertaken, Gag expression in BALB/c mouse macrophages was investigated for the two recombinants, rBCG:RT106 and rBCG:RT108 (Tokyo). Expression of Gag from both recombinants was demonstrated successfully (Table 3.9). Unlike the culture expression work, the level of expression from rBCG:RT106 and rBCG:RT108 was not different after both 24 and 48 hours of infection.

Table 3.9. Gag expression levels (± standard deviation) from rBCG:RT106 and rBCG:RT108 in macrophages.

	Corrected COI[a] 24hr	Corrected COI[a] 48hr
macrophages	0.33 ± 0.109	0.37 ± 0.051
RT106	13.97 ± 0.154	9.49 ± 0.227
RT108	11.88 ± 0.312	6.13 ± 0.222

[a] Corrected COI indicates the Elecsys readout corrected for the amount of protein in each sample. The results represent the average of the lysates from 6 separate infections.

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3.4. DISCUSSION

The approach taken with this project was to use the available resources to produce a suitable rBCG-HIV vaccine candidate. MedImmune and Prof W. Jacobs Jr made *E.coli*/Mycobacterial shuttle vectors available for this purpose. Further shuttle vectors were constructed in our laboratory by modifying the ones already available. The strategy employed was to produce *E.coli*/*Mycobacterium* Gag shuttle vectors and test these in the model *M.smegmatis* system for stability of the constructs and production of Gag before selecting the most appropriate construct(s) for introduction into the BCG system and immunogenicity testing.

3.4.1. FEATURES OF THE VECTORS

Various HIV antigens have been expressed in rBCG previously (Stover *et al.*, 1991; Aldovini and Young, 1991; Winter *et al.*, 1991; Fuerst *et al.*, 1992; Kameoka *et al.*, 1994; Wada *et al.*, 1996; Kawahara *et al.*, 2002 and Chujoh *et al.*, 2002) but this is the first study to report the use of HIV-1 subtype C Gag. Chujoh *et al.* (2002) also used a non-subtype B virus – subtype E HIV-1 epitopes were used to construct a V3-loop rBCG. Of the studies listed above, HIV-1 Gag was one of the heterologous antigens in the following:

- Stover *et al.* (1991) and Fuerst *et al.* (1992) expressed *gag* from the *hsp60* promoter in both an episomal and an integrative vector (subtype B);
- Aldovini and Young (1991) used the *hsp70* promoter and an episomal vector to express the *gag* gene (subtype B); and
- Wada *et al.* (1996) expressed a B-cell epitope of p17 under the control of the *M.kansasii* α-antigen promoter and secretion signal (subtype B).

In this study, in addition to the pMV361 vector used by Stover *et al.* (1991) and Fuerst *et al.* (1992), a number of shuttle vectors not previously used to express HIV-1 Gag were employed. This was the first time the *katG* promoter was used to control expression of any heterlogous gene in rBCG. In addition, the *mtrA* and *M.leprae* 18kDa promoters have not been used in the expression of the HIV-1 *gag* gene before.

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Export or surface expression are desirable since it is thought that these features promote stability of the shuttle vectors by moving the heterologous antigen out of the bacterium and also enhance the immune response to the antigen by making it more readily accessible to presentation by MHC molecules (Stover *et al.*, 1993). Other groups have shown better immune responses to rBCG antigens that are exported or expressed on the surface of the bacterium (Stover *et al.*, 1993; Langermann *et al.*, 1994b; Bastos *et al.* 2002; Grode *et al.*, 2002; Bao *et al.*, 2003). Only Wada *et al.* (1996) expressed a Gag epitope fused to a secretion signal. In this study the fusion of GagC to both the α-antigen secretion signal and the 19kDa lipoprotein signal is described. pRT101, which was derived from pMV361 (Stover *et al.*, 1991), was the only shuttle vector with features similar to any used previously for rBCG:Gag vaccines.

While expression from the *hsp60* promoter is constitutive, expression from the 18kDa promoter and the *mtrA* promoter is induced in macrophages. Expression from the *katG* promoter peaks during late exponential phase. The properties of the vectors were thought to be sufficiently broad to ensure that there would be a suitable candidate identified for further study.

The range of vectors used could be grouped in various ways:

- 3 integrative vectors with the same promoter (*hsp60*) but different localization of the Gag protein product [viz. pRT101 (cytosolic), pRT102 (secreted) and pRT103 (membrane associated)] that were all derived from pMV361.
- 2 sets of 2 episomal vectors that have different promoters (*M.leprae* 18kDa promoter vs *katG* promoter and *mtrA* promoter vs *katG* promoter) but localize the Gag product to the same place [viz. pRT106 and pRT108 (membrane associated) and pRT105 and pRT107 (secreted)]
- 2 episomal vectors with the same promoter (*katG*) but different localization [viz. pRT107 (secreted) and pRT108 (membrane associated)]
- 2 vectors with the same promoter (*mtrA*) and signal sequence (membrane associated) but one was integrative (pRT104) and one was episomal (pRT106).

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3.4.2. STABILITY AND EXPRESSION IN RECOMBINANT *M.smegmatis*

The *in vitro* stability and expression of the shuttle vectors was studied in rMS with a view to using the data to decide which constructs would be suitable for further study in rBCG. Das Gupta *et al.* (1998) reported that fast growing *M.smegmatis* recognizes mycobacterial promoters with efficiency comparable to the slower growing *M.bovis* BCG and *M.tuberculosis*. Their observations suggest that *M. smegmatis* is a suitable surrogate for analysis of gene expression.

The induction of a good immune response against the foreign antigen expressed in rBCG usually goes hand-in-hand with a high expression level (Fuerst *et al.*, 1991 and 1992; Himmelrich *et al.*, 2000). However, as mentioned before (section 3.3.3), high levels of a foreign antigen may result in a metabolic load that is disadvantageous to the bacterium. Partly for this reason, the constructs are often unstable and genetic rearrangements that negate expression of the foreign gene are more common and more necessary in higher expressers. Another consideration is that these vaccines are intended for eventual general distribution to a large population. In order to scale-up the production of the rBCG from a laboratory-level culture to the volumes required for large-scale production of the vaccine, the rBCG would need to be stable over numerous generations and subcultures. A successful rBCG vaccine would balance sufficient expression with good stability. It was important for the stability of the constructs used in this study to be measured in order to assist in deciding which rBCGs were suitable for immunogenicity experiments. The experiments were designed to first investigate the genetic stability of the constructs in recombinant *M.smegmatis*, then to determine what effect the stability had on the expression levels of Gag in these recombinants. The heat shock stress response in the recombinants was also tested to further establish the link between expression levels and stability. The data from these experiments were used to select the constructs that would be introduced into BCG for immunogenicity studies.

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The stability of the constructs was studied by monitoring genetic rearrangement of shuttle vectors in the rMS over time. Recombinant *M.smegmatis* carrying integrative vectors were highly stable for the entire period assayed. This was similar to results in other studies showing increased stability for integrative shuttle vectors when compared to episomal ones (Méderlé *et al.*, 2002). However, the recombinants carrying the episomal vectors were far less stable. The recombinant carrying pRT105 showed instability most rapidly. The two vectors expressing Gag in combination with the 19kDa lipoprotein signal were the most stable episomal vector recombinants. rMS:RT108 was able to retain 90% plasmid integrity after ~20 generations, while rMS:RT106 retained 60% integrity. Loss of stability was shown to result from deletions encompassing the promoter/signal sequence region and the *gag* gene. These results compare favourably with other studies where *in vitro* stability was investigated. Horwitz *et al.* (2000) found their episomal vector expressing a *Mycobacterial* protein to be highly stable, while other groups report more instability and genetic rearrangements in their episomal vectors (Kumar *et al.*, 1998; Medeiros *et al.*, 2002; Al-Zarouni and Dale, 2002 and see section 3.1.4).

One would expect the genetic stability to be mirrored in the expression levels: the higher the stability, the better the expression level will be maintained and the more rapid the accumulation of deletions, the more rapid the drop in expression. For the recombinants carrying the integrative constructs, the results seen in genetic stability experiment were predictive of the results in the experiment tracking the expression level changes over time. The integrative constructs expressed at a lower level compared to the episomal constructs and this level was fairly constant over the assay period. The recombinant carrying pRT101 was the only integrative rMS that expressed at a level approaching the level of any of the episomal vectors. For this reason, no doubt, the integrative recombinants were also more stable. Since the *hsp60*-driven rMS:RT101 differs from rMS:RT102 and rMS:RT103 by not having a signal sequence fused to the *gag* gene, it is possible that expression of Gag from the latter recombinants was lower because of the presence of the signal peptides. Expression from *mtrA*-driven rMS:RT104, which had a 19kDa lipoprotein fusion, was equivalent to the other integrative recombinants carrying fusions.

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Since the integrative constructs were only present at 1 copy per bacterium whereas the episomal constructs were present at 5 copies per cell, one would expect a difference in expression level. rMS:RT105 expressed at a high level initially but rapid accumulation of deletions lead to a rapid drop in expression level. For rMS:RT106, the higher genetic stability yielded a more moderate loss of expression. These recombinants had expression profiles that matched their genetic stability profiles. However, the expression data for the two *katG*-based recombinants did not match the genetic stability data. Although there was an accumulation of deletions (rapid for rMS:RT107 and slow for rMS:RT108), the expression of Gag was at low but consistent levels. Smaller plasmids are taken up more readily by competent cells and are more readily extracted by the alkaline lysis method than larger DNA molecules that are sheared more easily (Maniatis, 1982). Since only 10-15 colonies were selected for plasmid extraction in the genetic stability study, it is conceivable that a higher percentage of the colonies contained plasmids with deletions. The expression levels give a clearer indication of the construct stability because they were performed on the entire culture rather than a sample.

An experiment in rMS was designed to establish the influence the expression level of Gag from the different constructs had on the response to heat stress of the recombinants. Low-level expression would produce a low metabolic load and recombinants would not be under stress, while high-level expression would result in stress allowing for the recombinants to grow at the higher incubation temperature of 42°C. Colony size was also hypothesized to be an indicator of metabolic stress with the hypothesis that a high expression level will result in high metabolic load and smaller colonies and vice versa. It was a crude evaluation of the differences between the constructs but it yielded interesting results.

The *hsp60* promoter in the integrative constructs did not produce Gag at a level high enough to place a metabolic stress on the recombinants and thus no colonies were seen at 42°C. Further evidence for the low expression levels lies in the fact that the *hsp60* integrative constructs

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produced colonies (at 37°C) of a size almost equal to that of the pCONEPI recombinants (control for "normal" size). The same result was obtained for the integrative construct expressing Gag from the *mtrA* promoter. This was expected as the integrative constructs did produce low levels of p24 when assayed over time.

The episomal constructs gave rise to a different scenario. In all cases, production of Gag was at a level sufficient to impart a metabolic load on the recombinants to such an extent that they underwent a stringent response and were able to grow at 42°C. The higher the load, the more the recombinants struggled, ie. the smaller the colonies, implying that the expression of Gag was higher. Based on the sizes of the colonies, the constructs expressing Gag from the *katG* promoter did so at a level almost equivalent to expression in the integrative constructs but still high enough to induce the stringent response. However, the *mtrA* and 18kDa promoters were producing Gag at the highest level yielding the smallest colonies. Again, these results support the experimental hypothesis since the higher expressers (as shown over time in the p24 assay) were also the constructs that induced the most metabolic stress as seen by colony size and heat shock survival.

What could be concluded from the experiments was that the recombinants carrying the episomal shuttle vectors were expressing Gag at the highest level; rMS:RT105 and rMS:RT106 being the best expressers. Although the integrative constructs were stable, they also produced Gag at the lowest level. In addition, episomal plasmids are practically easier to use and and most studies in rBCG utilize episomal vectors (see section 1.B.3). However, Méderlé *et al.* (2002) were able to express an SIV *gag* gene to the same level from an integrative construct as the level from an episomal one. This is not the norm, however, and the results of this study were similar to what is usually seen. The medium stability/moderate producer, RT106 and high stability/low producer, RT108 were thus selected for use in further experiments.

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3.4.3. EXPRESSION OF GAG IN rBCG

pRT106 and pRT108 were introduced into BCG Tokyo and Pasteur. Before commencing immunogenicity testing, the expression of Gag from these rBCG was evaluated in culture and in infected macrophages. The expression in culture was tested in the cytosolic and the membrane fractions because the 19kDa lipoprotein signal is meant to export the Gag antigen to the membrane of the rBCG. The RT106-containing recombinants (*M.smegmatis* and BCG) had the same percentage of p24 protein in both fractions. This indicates that there was some transport to the cell surface but that it was not efficient. The same result was seen for rMS:RT108, however, rBCG:RT108 (Tokyo) had a higher percentage of p24 in the membrane/insoluble fraction implying that transport was more efficient in this recombinant. Gag expression levels only formed a small percentage of the total protein. Other work has also shown that viral antigens are not expressed as well in BCG as bacterial antigens (Aldovini and Young, 1991; Winter *et al.* 1991 and Méderlé *et al.*, 2002); however, the expression levels in those studies were all better than those achieved in this study. In both rBCG Tokyo and Pasteur, RT106 produced more Gag than RT108, as was seen in the rMS experiments.

Expression of Gag from rBCG:RT106 and RT108 Tokyo in macrophages was investigated as a final test before preclinical immunological studies were undertaken. If the rBCG were unable to express Gag in a macrophage infection model, there would be little chance of expression in an *in vivo* mouse system. However, expression was successfully demonstrated for both recombinants. Unlike the other expression studies where rBCG:RT106 was found to express Gag at a higher level, in the macrophages, expression from the *katG* promoter (rBCG:RT108) was the same as expression from the *mtrA* promoter (rBCG:RT106) after both 24 and 48 hours of infection. Based on the results shown in this chapter, further immunological testing of the rBCG:RT106 and rBCG:RT108 vaccines was undertaken.

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CHAPTER 4

IMMUNOGENICITY TESTING OF rBCG:GAG VACCINE CANDIDATES

4.1. INTRODUCTION

In this chapter immunogenicity testing of two different rBCG:Gag is described. Based on the results of the genetic stability and expression studies in culture and in macrophages (Chapter 3), a medium stability/high-moderate producer (rBCG:RT106) and a highly stable/low producer (rBCG:RT108) were selected for this testing. Both vectors express the Gag gene fused to the 19kDa localization signal, while rBCG:RT106 drives expression of Gag from the *mtrA* promoter and rBCG:RT108 from the *katG* promoter.

The BALB/c mouse model is the most frequently used model for initial immunogenicity testing of rBCG vaccines (section 1.B.3). This model was chosen for the immunogenicity studies of rBCG using three different vaccination strategies. rBCG vaccine strategies have made use of multiple inoculations of the rBCG in order to elicit a stronger response (Aldovini and Young, 1991; Stover *et al.*, 1993; Abdelhak *et al.*, 1995; Fennelly *et al.*, 1995; Wada *et al.*, 1996; Kremer *et al.*, 1996, 1998; Abomoelak *et al.*, 1999; Mederle *et al.*, 2002 and Biet *et al.*, 2003). In light of these studies the initial strategy employed in this study was five consecutive rBCG inoculations.

A single rBCG inoculation and, although it would obviously be advantageous to have a single vaccine for HIV, a DNA prime followed by an rBCG boost strategy was also attempted. Prime-boost strategies with different vaccines have been proven to elicit stronger and broader immune responses (eg. Hanke *et al.*, 1999; Allen *et al.* 2000; Casimiro *et al.*, 2002 and Leung *et al.*, 2004). Mederle *et al.* (2002) demonstrated that rBCG expressing SIV Gag and Nef provided an efficient prime for a recombinant SIV Gag protein boost – CD4 IFN-γ production was enhanced

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using this rBCG prime:protein boost regimen. In contrast, Martin *et al.* (2001) performed a DNA vaccine prime followed by a rBCG boost using the *M.avium* 35kDa antigen (and vice versa) and found that there was no increase in IFN-γ production compared to the application of either vaccine alone.

The Tokyo and Pasteur substrains were chosen for the immunogenicity studies since they were used in the majority of rBCG studies in the literature. In addition, the Tokyo substrain was the one in use in South Africa at the time the study was first started and the Pasteur substrain is the standard BCG vaccine strain. The strategy using five rBCG inoculations studied IFN-γ production in the draining lymph node cells and the generation anti-p24 antibodies in the sera of mice inoculated with either rBCG:RT106 or rBCG:RT108 (Tokyo). The single inoculation and prime-boost experiments investigated the production of different cytokines in the splenocytes of mice inoculated with rBCG:RT106 (Pasteur) by means of ELISPOT assays and cytokine cytometric bead arrays. The sera of these mice were also tested for the generation of antibodies against p24.

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4.2. METHODS

4.2.1. PREPARATION OF rBCG VACCINE STOCKS

rBCG:RT106, rBCG:RT108 (Tokyo and Pasteur) and rBCG:119L1e (Pasteur) were grown in 100ml Sauton's medium at 37°C on a roller until mid-logarithmic phase (OD_{600} = 0.6-0.7). The cultures were then harvested by centrifugation at 3000rpm for 20 min in a benchtop centrifuge (Eppendorf Centrifuge 5702, Eppendorf, Hamburg, Germany). The supernatants were removed and the pellets were resuspended in a volume of resuspension medium (Appendix A) to give an OD_{600} = 10. This yielded a concentration of 1×10^9cfu/ml which were checked by plating dilutions on Middlebrooks 7H10 media supplemented with OADC and containing kanamycin (15μg/ml). The genetic stability of the vaccine stocks was determined by restriction enzyme analysis of plasmid DNA extracted from the stocks. Before aliquotting the vaccine stock in 200μl, for storage

Five mice out of each group were sacrificed 8 weeks and 12 weeks after the final inoculation and spleens were removed. Blood was collected before inoculation and at each sacrifice. Serum was stored at –20°C.

4.2.5. PREPARATION OF MOUSE CELLS

A suspension of cells in R10 medium (RPMI-1640 medium with Glutamax-1 containing 10% foetal bovine serum, 100units/mL Penicillin-G and 100 μg/mL Streptomycin) was prepared from the harvested organs (spleens or lymph nodes). The suspension was purified further using a lympholyte M (Cedarlane Laboratories Ltd, Ontario, Canada) gradient after which an enriched T cell suspension was prepared by removing the B cells using B220 magnetic beads (Dynal, Oslo, Norway) according to manufacturer's instructions. T cells were resuspended at $5x10^6$ cells/ml in R10.

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4.2.6. IFN-γ ELISA

Gag T cell stimulation prior to IFN-γ ELISA

T cells ($0.5x10^6$) were cultured (37°C, 5% CO_2) in duplicate in a final volume of 200 μl containing 50 μl mitomycin C-treated naïve splenocytes ($1x10^7$ cells/ml) with the peptides listed in Table 4.1 as stimuli. Culture supernatants were collected at 48h and 72h and stored at –20°C before quantification of IFN-γ as a measure of T cell activation by the stimuli. Unstimulated splenocytes were also cultured as negative controls.

Table 4.1. Peptides used for T cell stimulation prior to the IFN-γ ELISA.

PEPTIDE	DESCRIPTION	FINAL CONC In ASSAY	SEQUENCE
ConA[a]	Non-specific +ve control	1μg/ml	N/A
AMQM[b]	CD8 p24 peptide	2μg/ml	AMQMLK<u>D</u>TI
MRC 2[b]	CD4 p24 peptide	2μg/ml	VHQAISPRTLNAWVKVVEEL
MRC 13[b]	CD4 p24 peptide	2μg/ml	NPPIPVGEIYKRWIILGLNK
MRC 17[b]	CD4 p24 peptide	2μg/ml	FRDYVDRFYKTLRAEQASQD

[a]= Sigma, MO, USA; [b] = Bachem, Bubendorf, Switzerland

The AMQM peptide (Mata *et al.*, 1998) was altered to reflect HIV-1 subtype C sequence (altered bases underlined in the table). The MRC peptides (Mata *et al.*, 2001) were used as published (HIV-1 subtype B sequence).

at -80°C, the resuspension was passed through a syringe needle (21 gauge) 5-10 times to break up any clumps. Prior to inoculation, appropriate dilutions of the rBCG were made in resuspension medium and passed through a syringe needle (21 gauge) 5-10 times to break up any clumps.

rBCG:119L1e is equivalent to rBCG:RT106 except that an HPV-16 L1 gene is expressed from the *mtrA* promoter rather than the *gag* gene. Vaccine stocks of this rBCG were prepared as described for rBCG:RT106 and rBCG:RT108. This rBCG was used a negative control in the ELISPOT and CBA experiments (see section 4.2.4)

4.2.2. HIV ANTIGEN PRODUCTION

Baculovirus-derived recombinant HIV-1 p24 (Quality Biological, Inc; MD, USA) and Gag VLPs produced using the Bac-to-Bac® Expression System (Invitrogen, CA, USA; Jaffray *et al.*, 2004) were used in various assays. The Gag VLPs were kindly provided by Dr. A. Jaffray (Department of Molecular and Cell Biology, University of Cape Town, South Africa).

In addition, subtype C p24 was produced in *E.coli.* The p24 gene from pRT001 (see section 3.3.1.) was cloned into pProEX HTb (GibcoBRL Life Technologies, CA, USA) and expressed with a histidine tag in *E. coli* DH5α cells. The pProEX HTb clone carrying p24 was kindly supplied by Dr. F. Tanzer (Department of Molecular and Cell Biology, University of Cape Town, South Africa). The recombinant p24 protein was column-purified over Nickel-Nitrilotriacetic acid resin (Qiagen, CA, USA), following manufacturer's instructions.

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4.2.3. PREPARATION OF BCG LYSATE

Wild-type BCG Pasteur was grown until late logarithmic phase and harvested at 3000rpm in a benchtop centrifuge for 20 min. The pellet was resuspended in PBS and centrifuged as before. This washing step was repeated and the pellet was resuspended in 4ml PBS. The suspension was then divided into 2 screw-cap tubes containing zirconian silicon beads. The tubes were placed in a FastPrep FP120 bead beater (Bio 101 Systems, CA, USA) and subjected to four 30

sec shakes at speed 6. Tubes were placed on ice for 3 min between each shake. The lysates were clarified by centrifugation at 10000rpm in a microfuge. The supernatant containing the protein was removed and the remaining debris was removed by further centrifugation. The lysates were stored at -20°C.

4.2.4. INOCULATION OF MICE

Mice were housed in the University of Cape Town Animal Unit. All animal inoculations, bleeding and other handling were performed by Mr. R. Lucas, Ms. M. Rheeder and Ms. E. Janse van Rensburg (South African AIDS Vaccine Initiative, University of Cape Town, South Africa). Ethics approval for the experiments was obtained from the University of Cape Town Research Ethics Committee (Approval Number 01/041).

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For the IFN-γ ELISA experiments, female Balb/c mice (5-6 weeks old) in groups of 10 were given 5 subcutaneous inoculations of either rBCG:RT106 or rBCG:RT108 ($1x10^7$ cfu; Tokyo) in the back of the neck. One group of mice was not inoculated and served as a negative control group. The inoculations were at monthly intervals, the mice were sacrificed a month after the final inoculation and draining lymph nodes were removed. Blood was collected 2 weeks after the 3rd and 4th inoculation and at sacrifice. Serum was stored at –20°C.

For further experiments, female Balb/c mice (5-6 weeks old) in groups of 10 were given either:

- one intraperitoneal inoculation of either rBCG:RT106 or rBCG:119L1e (Pasteur) at $1x10^3$cfu per inoculum or $1x10^5$cfu per inoculum or
- one intramuscular inoculation of pTHGagC (100μg) followed a month later by one intraperitoneal inoculation of either rBCG:RT106 or rBCG:119L1e (Pasteur) at $1x10^3$cfu per inoculum or $1x10^5$cfu per inoculum.

IFN-γ ELISA

IFN-γ production during stimulation was determined by an IFN-γ specific ELISA using pairs of specific mAb (Pharmingen, CA, USA) according to the manufacturer's instructions. IFN-γ concentration in individual samples was measured against a supplied rIFN-γ standard.

4.2.7. IFN-γ AND IL-4 ELISPOT ASSAY

The ELISPOT assays were performed on cells from mice that were inoculated with one dose of rBCG Pasteur or mice that were primed with a dose of DNA vaccine (pTHGagC; van Harmelen *et al.*, 2003) and then boosted with one dose of rBCG Pasteur.

T cell plating and stimulation

Ninety-six well ELISPOT plates from the ELISPOT Set kit (BD Biosciences, San Diego, USA) were coated with 100μl of either purified anti-mouse IFN-γ or purified anti-mouse IL-4 capture antibody supplied with the kit. The capture antibodies were diluted 1:200 in PBS. The plates were incubated at 4°C overnight. After washing with 200μl R10 (Appendix A) per well, the plates were then blocked with R10 for 2 hr at room temperature. Peptides were diluted to the required working concentrations and then plated in triplicate (100μl/well). The splenocytes were added at a concentration of 500 000 cells in 100μl per well. For the IFN-γ plates, 1.6μl IL-2 was added to the 100μl of the cells that were incubated with CD8 peptides (see Table 4.1). The plates were covered in foil and incubated at 37°C, 5% CO_2 for 22-24 hr (IFN-γ) or 46-48 hr (IL-4).

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Peptides

Table 4.2. Peptides used in the ELISPOT assay.

PEPTIDE	DESCRIPTION	FINAL CONC in ASSAY	SEQUENCE
ConA[a]	Non-specific +ve control	1μg/ml	N/A
Irrel[b]	-ve control	2μg/ml	TYSTVASSL
AMQM[b]	CD8 p24 peptide	2μg/ml	AMQMLKDTI
Gag CD4 2[b]	CD4 p24 peptide	2μg/ml	VHQAISPRTLNAWVKVIEEL
Gag CD4 13[b]	CD4 p24 peptide	2μg/ml	NPPIPVGRIYKRWIILGLNK
IL-2[c]	Costimulatory signal	0.01μg/ml	N/A
BCG lysate[d]	+ve control	5μg/ml	N/A

[a]= Sigma, MO, USA; [b] = Bachem, Bubendorf, Switzerland; [c] = R&D Systems, MN, USA; [d] = this study

The CD8 and CD4 p24 peptides are recognized by BALB/c mice (Mata *et al.*, 1998 and 2001) and the sequences were altered to reflect HIV-1 subtype C sequence (altered bases underlined in table).

ELISPOT plate development

The plate development was performed using reagents supplied with the ELISPOT Set kit and following the manufacturer's protocol. After discarding the cell/peptide suspension, the wells were washed twice with 200μl of deionized water per well with a soak of 3-5 minutes between washes. The plates were washed thrice with PBS containing 0.3% Tween-20 using a EL_x50 Auto Strip Washer (Bio-Tek Instruments, Inc; VT, USA). The detection antibody was diluted in PBS containing 10% FBS and 100μl were added per well. The plates were incubated at room temperature for 2 hr. After 3 washes with PBS containing 0.3% Tween-20, avidin-horseradish peroxidase was diluted in PBS containing 10% FBS and 100μl were added per well. A 1 hr incubation at room temperature followed. The plates were washed as before and then a further 3 washes were performed using PBS. Nova Red Substrate solution (Vector Laboratories, CA, USA) was used according to the manufacturer's instructions to develop the spots. The spot development was monitored for 5-12 minutes to avoid high background. Washing the wells with deionized water stopped the substrate reaction. The plate was allowed to air-dry at room temperature overnight before counting the spots.

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ELISPOT plate counting

An ImmunoSpot (Cellular Technology Ltd, Cleveland, Ohio) automated plate analyser together with ImmunoSpot Version 3.0 software was used to count the spots in each well. Mean background spots in the absence of peptide were subtracted from the mean spot value in the presence of peptide and data are reported as the net spot forming unit per million cells (sfu/10^6 cells) +/- the standard deviation.

4.2.8. MOUSE Th1/Th 2 CYTOKINE CYTOMETRIC BEAD ARRAY (CBA)

Mouse splenocytes from the 8 and 12 week sacrifice time points were prepared as described in section 4.2.4. The cells were stimulated with the peptides listed in Table 4.1 for 48 hours. Cytokines (IFN-γ, TNF-α, IL-4 and IL-5) released during the stimulation were measured using a Mouse Th1/Th2 cytokine cytometric bead array (CBA; BD Biosciences, San Diego, USA) following the manufacturer's instructions.

4.2.9. p24 ANTIBODY ELISA

Maxisorp C96 plates (Nalge-Nunc International, Denmark) were coated overnight at 4°C with 50μl/well of *Baculovirus* rp24 antigen (5 μg/ml in carbonate bicarbonate buffer pH 9.6; Quality Biological, Inc, MD, USA). Plates were washed 3 times with PBS containing 0.3% Tween-20 and once with 200μl of blocking buffer (PBS containing 0.3% Tween-20, 1% goat serum, 2% fat free milk powder) then blocked in blocking buffer (4°C overnight). Pooled mouse sera was diluted 1:50 in dilution buffer (PBS containing 1% fat free milk powder, 1% goat serum), 100μl was added per well and the plates were incubated at 4°C overnight. Plates were washed 6 times in blocking buffer and then incubated at 37°C for 1.5 h with 100μl biotinylated goat-anti-mouse IgG (ICN Biomedicals, CA, USA; diluted 1:2000 in dilution buffer). Following this plates were washed 5 times with wash buffer (PBS containing 0.3% Tween) and 50 μl horseradish peroxidase-streptavidin conjugate (4μg/ml wash buffer) was added to each well. After incubation for one hour at 37°C, the plates were washed with wash buffer and developed with 50 μl per well para nitro

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phenyl phosphate. The reaction was allowed to continue for 10 minutes before the optical density at 405nm was read.

4.2.10. WESTERN BLOT OF MOUSE SERUM

For a selection of the mouse sera, the presence of gag antibodies was confirmed using the New LAV Blot 1 kit (Bio-Rad Laboratories, CA, USA) following manufacturer's instructions.

4.3. RESULTS

4.3.1. IFN-γ PRODUCTION BY LYMPHOCYTES FROM MICE THAT RECEIVED FIVE rBCG TOKYO INOCULATIONS

The ability of rBCG:RT106 and rBCG:RT108 in the Tokyo strain to induce Gag specific T cells after multiple inoculations was investigated in BALB/c mice. Mice received five subcutaneous inoculations of rBCG:RT106 or rBCG:RT108 at 10^7cfu. Twenty-eight days after the last inoculation with rBCG, T cells harvested from the draining lymph nodes were stimulated with various Gag-specific peptides, rp24 or Gag VLPs for 2-3 days. The Gag CD8 (AMQMLKDTI) and CD4 (MRC 2, 13 and 17) peptides did not stimulate a high level of IFN-γ production (Table 4.3). However, IFN-γ production by T cells from inoculated mice was induced by rp24 and Gag VLP stimulation. In general, IFN-γ production was higher after 3 days of stimulation and production from T cells of mice inoculated with rBCG:RT106 was approximately twice that of T cells from rBCG:RT108 inoculated mice. The production of IFN-γ by T cells from immunized mice or non-immunized mice cultured without Gag protein was minimal (Table 4.3.).

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Table 4.3. Gag-specific IFN-γ production (ng/ml) by T cells from rBCG inoculated mice.

CONSTRUCT	DAY	ANTIGEN	IFN-γ production (ng/ml)	DAY	IFN-γ production (ng/ml)
rBCG:RT106	2	background	0.04 ± 0.03	3	0.36 ± 0.01
		conA	34.29 ± 1.40		44.55 ± 0.86
		p24 (6μg/ml)	1.70 ± 0.64		4.67 ± 0.15
		Gag VLPs (12ng)	24.55 ± 2.07		49.99 ± 13.46
		AMQMLKDTI	0.08 ± 0.06		0.24 ± 0.03
		MRC2	0.10 ± 0.04		0.31 ± 0.03
		MRC13	0.17 ± 0.07		0.43 ± 0.04
		MRC17	0.12 ± 0.08		0.56 ± 0.09
rBCG:RT108	2	background	0.03 ± 0.00	3	0.40 ± 0.00
		conA	31.20 ± 0.70		31.05 ± 2.98
		p24 (6μg/ml)	1.03 ± 0.13		2.25 ± 0.30
		Gag VLPs (12ng)	15.87 ± 1.29		21.57 ± 3.02
		AMQMLKDTI	0.03 ± 0.00		0.20 ± 0.11
		MRC2	0.03 ± 0.00		0.31 ± 0.10
		MRC13	0.03 ± 0.03		0.05 ± 0.00
		MRC17	0.03 ± 0.03		0.12 ± 0.01

The results are the mean ± standard deviation of duplicate determinations of a representative experiment

4.3.2. p24 ANTIBODY ELISA ON SERA FROM MICE THAT RECEIVED FIVE rBCG TOKYO INOCULATIONS

The humoral response to 5 inoculations of rBCG:RT106 or rBCG:RT108 (Tokyo) was also investigated. The levels of anti-p24 antibodies were 14.8 fold or 2.7 fold above the background level of these antibodies in naïve mice, respectively (Fig 4.1.A.). Minimal antibody production to Gag could be detected for either of the two rBCG prior to a 5th inoculation. Western blotting confirmed the presence of p24 antibodies in the sera from mice given 5 inoculations of rBCG:RT106 but was not sensitive enough to confirm the lower level of antibodies in the sera from the mice that received rBCG:RT108 (Fig 4.1.B.).

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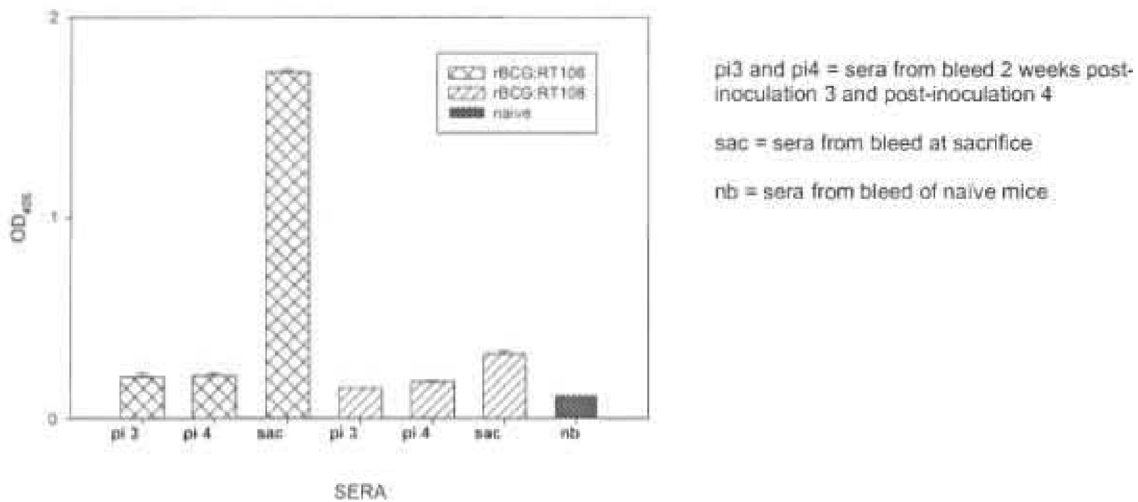

Fig.4.1.A. p24 antibodies determined by ELISA in serum samples diluted 1:50 from mice inoculated with rBCG:RT106 and rBCG:RT108.

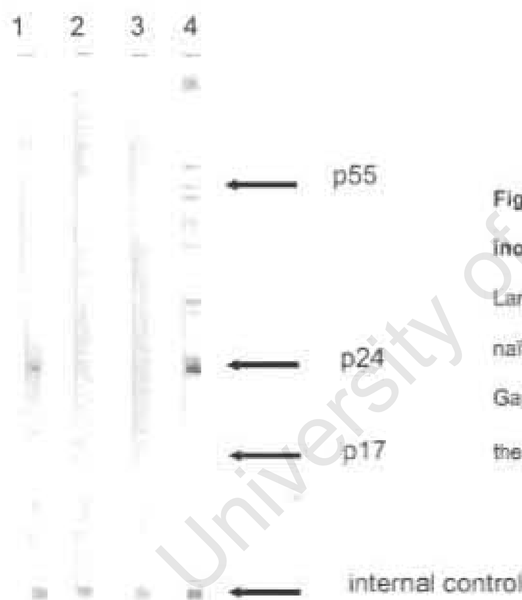

Fig.4.1.B. Western blot of sera taken at sacrifice from mice inoculated with rBCG:RT106 and rBCG:RT108 at a 1:50 dilution. Lane 1 = rBCG:RT106; lane 2 = rBCG:RT108; lane 3 = sera from naïve mice; lane 4 = HIV-1 positive sera control. The positions of the Gag proteins and the internal control for the strips are indicated by the arrows.

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Since rBCG:RT108 produced lower amounts of IFN-γ as measured by the ELISA and also lower levels of anti-p24 antibodies than rBCG:RT106, further immunogenicity testing was performed using only rBCG:RT106. An immunization regimen that involves five consecutive inoculations would not be practically feasible, so a single vaccination approach was attempted initially. The

availability of newer, more sensitive assays (ELISPOT and CBA) also meant that the vaccine-induced immune response could be more accurately tested.

4.3.3. IFN-γ AND IL-4 ELISPOT ON SPLENOCYTES FROM MICE RECEIVING rBCG PASTEUR

The ELISPOT experiment was designed to test whether mice inoculated with the rBCG:RT106 vaccine candidate were able to mount a Gag-specific cell-mediated immune response 8 and 12 weeks after inoculation. The production of IFN-γ and IL-4 by mouse splenocytes in response to stimulation with a Gag CD8 peptide and Gag CD4 peptides was investigated. ConA and BCG lysate were positive controls. The splenocytes were obtained from groups of mice that received either one inoculation of rBCG:RT106 or a priming vaccination with a DNA vaccine (pTHGagC) followed by an rBCG:RT106 boost. Mice inoculated with rBCG expressing the HPV-16 L1 gene were used as a negative control in these experiments.

In all groups of mice for both single inoculation and prime-boost experiments, the background number of spots in response to stimulation with an irrelevant peptide or absence of peptide were equivalent and low and have been subtracted from the spots obtained in response to the specific peptides, BGC lysate and conA to get net spots per million splenocytes.

Response to a single rBCG vaccination

A single inoculation of rBCG (RT106 and 119L1e) at both doses yielded splenocytes that produced IFN-γ and IL-4 in response to the non-specific conA stimulus (Fig 4.2.). Both rBCG:RT106 and rBCG:119L1e were able to induce BCG-specific splenocyte production of IFN-γ and IL-4 (Fig 4.2.). A larger IFN-γ response was generated in response to BCG-lysate stimulation indicating that the response is predominantly Th1 in nature.

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In contrast, the single inoculation of rBCG:RT106 at the doses administered was insufficient to generate a Gag-specific immune response when assayed by ELISPOT (data not shown). There were no IFN-γ or IL-4 producing cells when splenocytes were stimulated with the Gag CD8 (AMQM; Table 4.2) and CD4 peptides (CD 4 2 and 13; Table 4.2) at both 8 and 12 weeks post-inoculation (between 0 and 10 sfu/million splenocytes). For this reason, a pTHGagC DNA vaccine prime followed by an rBCG:RT106 boost was used in an attempt to generate Gag-specific responses in the mice.

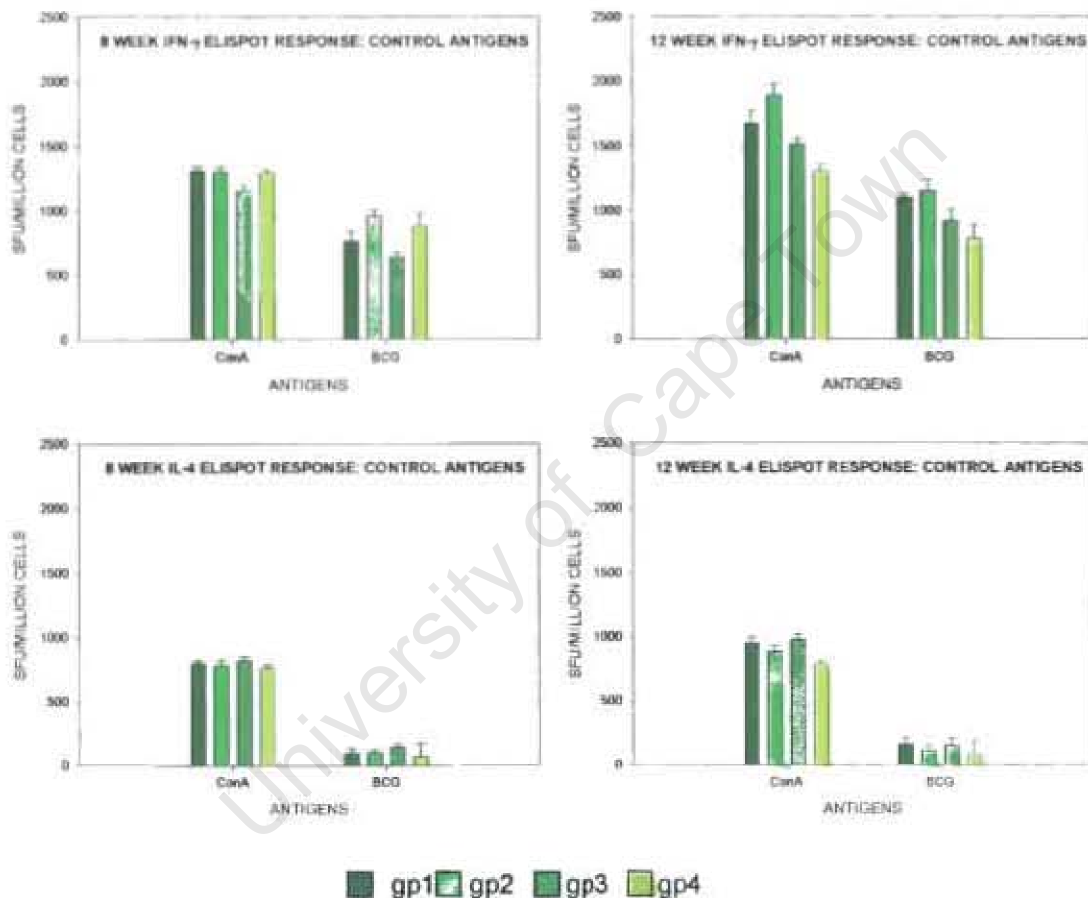

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Fig.4.2. IFN-γ and IL-4 ELISPOT responses to ConA and BCG stimulation 8 and 12 weeks after the single rBCG inoculation.
Responses are shown in spot forming units/million cells. The net sfu/10^6 cells were measured in triplicate and the average response + standard deviation are shown. Inoculations: gp 1 = rBCG:RT106 (10^3cfu); gp 2 = rBCG:RT106 (10^5cfu); gp 3 = rBCG:119L1e (10^3cfu); gp 4 = rBCG:119L1e (10^5cfu)

Response to a pTHGagC DNA vaccine prime followed by a rBCG boost

A DNA prime followed by a single boost with rBCG:RT106 (groups 1 and 2) or rBCG:119L1e (groups 3 and 4) was also able to generate splenocytes producing both IFN-γ and IL-4 in the mice in response to conA and BCG lysate stimulation (Fig 4.3.). This response was also predominantly Th1 in nature since more IFN-γ was produced in response to both stimuli.

Both IFN-γ and IL-4 producing splenocytes were detected in all 5 groups of mice (8 and 12 weeks post-inoculation) after stimulation with the Gag-specific peptides (Fig 4.4.). In general, a larger response was observed after stimulation with the Gag CD8 peptide than after stimulation with the CD4 peptides.

When comparing the Gag-specific production of IFN-γ and IL-4 generated by the splenocytes of rBCG-boosted mice to the responses generated by the splenocytes of positive control mice inoculated with pTHGagC only (group 5), it was clear that the response to the DNA prime had diminished after rBCG boosting (Fig 4.4.). This was particularly evident in the response to CD8 peptide stimulation. The IFN-γ and IL-4 production after the rBCG:RT106 (groups 1 and 2) boosts was not significantly different to the production after the rBCG:119-L1e (groups 3 and 4) boosts (Fig 4.4.).

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4.3.4. CYTOKINE PRODUCTION FROM SPLENOCYTES 8 AND 12 WEEKS AFTER pTHGag PRIME-rBCG BOOST

Production of IFN-γ, TNF-α, IL-4 and IL-5 by the splenocytes of mice primed with a DNA vaccine (pTHGagC) and boosted with rBCG expressing Gag or HPV-16 L1 were investigated using a cytometric bead assay. These mice were the same as evaluated in the ELISPOT assay after receiving a pTHGagC prime followed by a rBCG boost. The production of these cytokines was measured following 48 hour stimulation in culture with a Gag CD8 peptide or two Gag CD4 peptides. The background cytokine production in response to an irrelevant peptide and culture

medium was also determined. These background responses were very low and equivalent and were subtracted from the data presented in fig. 4.5.A-F and fig. 4.6.A-F.

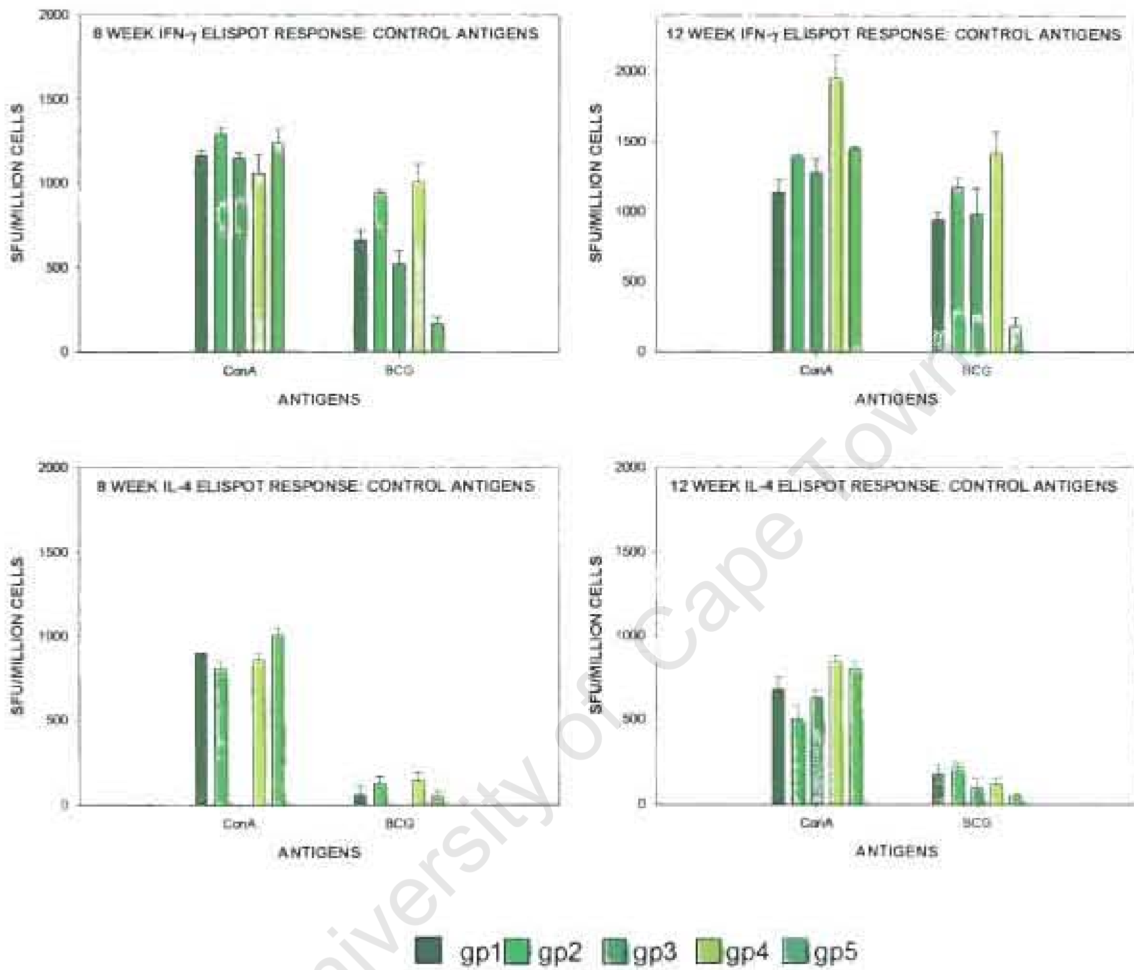

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Fig.4.3. IFN-γ and IL-4 ELISPOT responses to ConA and BCG stimulation 8 and 12 weeks after the rBCG boost. Responses are shown in spot forming units/million cells. The net sfu/10^6 cells were measured in triplicate and the average response + standard deviation are shown. All 5 groups received 100μg of pTHGagC, then 28 days later: gp 1 = rBCG:RT106 (10^3cfu); gp 2 = rBCG:RT106 (10^5cfu); gp 3 = rBCG:119L1e (10^3cfu), rBCG:119L1e (10^5cfu); gp 5 = resuspension medium.

Note that for gp 3 week 8 ELISPOT there were insufficient cells to perform the assay for both IFN-γ and IL-4, so only IFN-γ ELISPOT was performed.

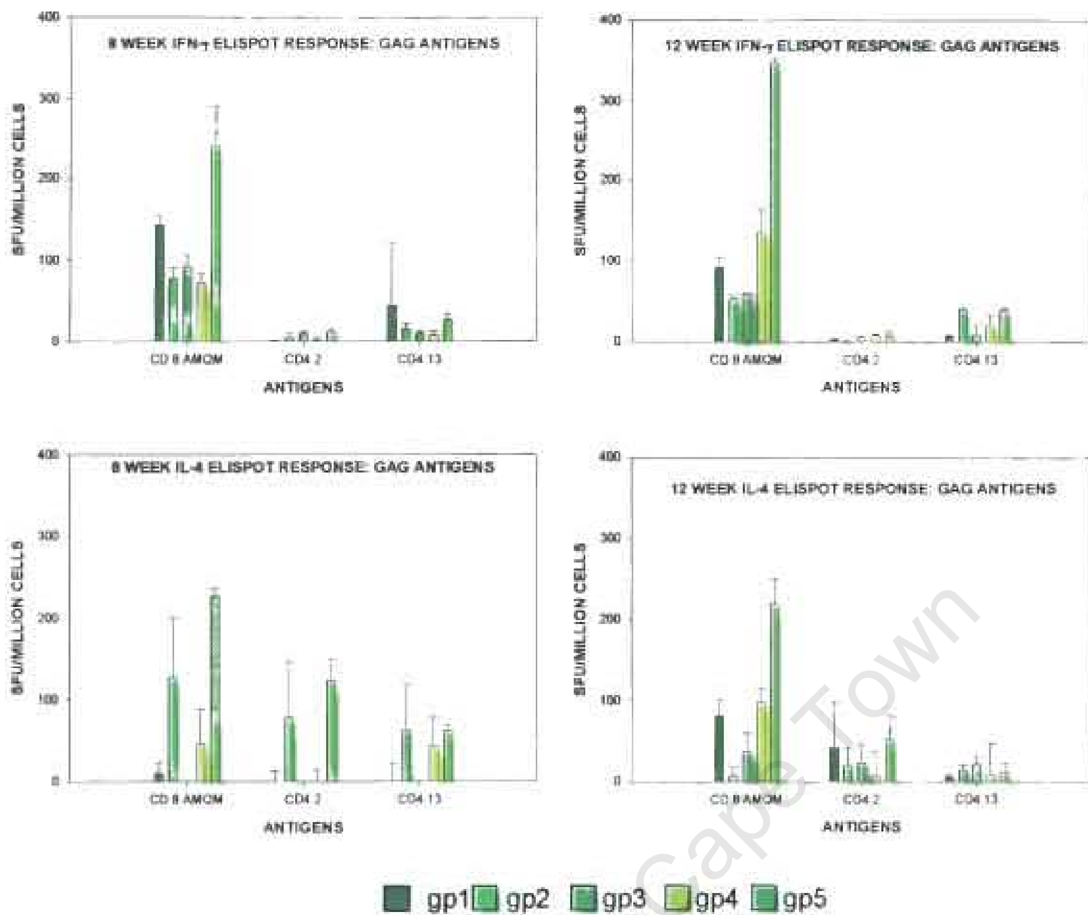

Fig.4.4. IFN-γ and IL-4 ELISPOT responses to Gag CD8 and CD4 antigens 8 and 12 weeks after the rBCG boost. Responses are shown in spot forming units/million cells. The net sfu/10^6 cells were measured in triplicate and the average response + standard deviation are shown. All 5 groups received 100μg of pTHGagC, then 28 days later: gp 1 = rBCG:RT106 (10^3cfu); gp 2 = rBCG:RT106 (10^5cfu); gp 3 = rBCG:119L1e (10^3cfu); rBCG:119L1e (10^5cfu); gp 5 = resuspension medium

Note that for gp 3 week 8 ELISPOT there were insufficient cells to perform the assay for both IFN-γ and IL-4, so only IFN-γ ELISPOT was performed.

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As shown in fig 4.5.A-F, at the 8 weeks time point, the production of IFN-γ, TNF-α, IL-4 and IL-5 by splenocytes from mice inoculated with pTHGagC during stimulation with the Gag CD8, CD4 (MRC-2) and CD4 (MRC-13) peptides was generally increased after a booster inoculation of both doses of rBCG:RT106 when compared to the equivalent doses of rBCG:L1 (Table 4.3). However, at this time point, a dose of 10^3cfu boosted this cytokine production more than a boost with a dose of 10^5cfu. Although there is a clear boost to the response with rBCG:RT106 administration compared to rBCG:L1, rBCG boost appears to suppress the antigen-specific cytokine production from the Gag-specific cells generated in response to a pTHGagC inoculation. This emphasizes the practical necessity to include a negative control vaccination as part of the rBCG testing, since the boost would not have been recognized if it was only compared to the response to the pTHGagC vaccine. This was particularly evident in the CD4 IFN-γ and TNF-α production after stimulation with CD4 2 and CD4 13 (Fig. 4.5. C, D).

As shown in fig 4.6.A-F., at the 12 weeks time point, the production of the cytokines by mouse splenocytes was also increased in general after a rBCG:RT106 booster inoculation when compared to a boost with rBCG:L1 (Table 4.3). The response also appears to be time dependent since the amount of cytokine produced at 12 weeks were lower than at 8 weeks. However, the antigen-specific cytokine response from the Gag-specific cells was again suppressed by administration of rBCG since the production of CD8 IFN-γ and TNF-α as well as CD4 IL-4 and IL-5 was highest in splenocytes from mice that received only pTHGagC (Fig 4.6. A, B, E and F). In general, as was seen at 8 weeks, the cytokine production from the splenocytes of mice that received the 10^3 cfu rBCG:RT106 inoculation was higher than the production from the splenocytes from other groups. However, only IFN-γ production after CD4 2 stimulation was enhanced to a level above the pTHGagC level (Fig 4.6.C) and the production of TNF-α after CD4 13 stimulation was boosted more effectively by the rBCG:RT106 10^5 cfu inoculation (Fig 4.6.D.).

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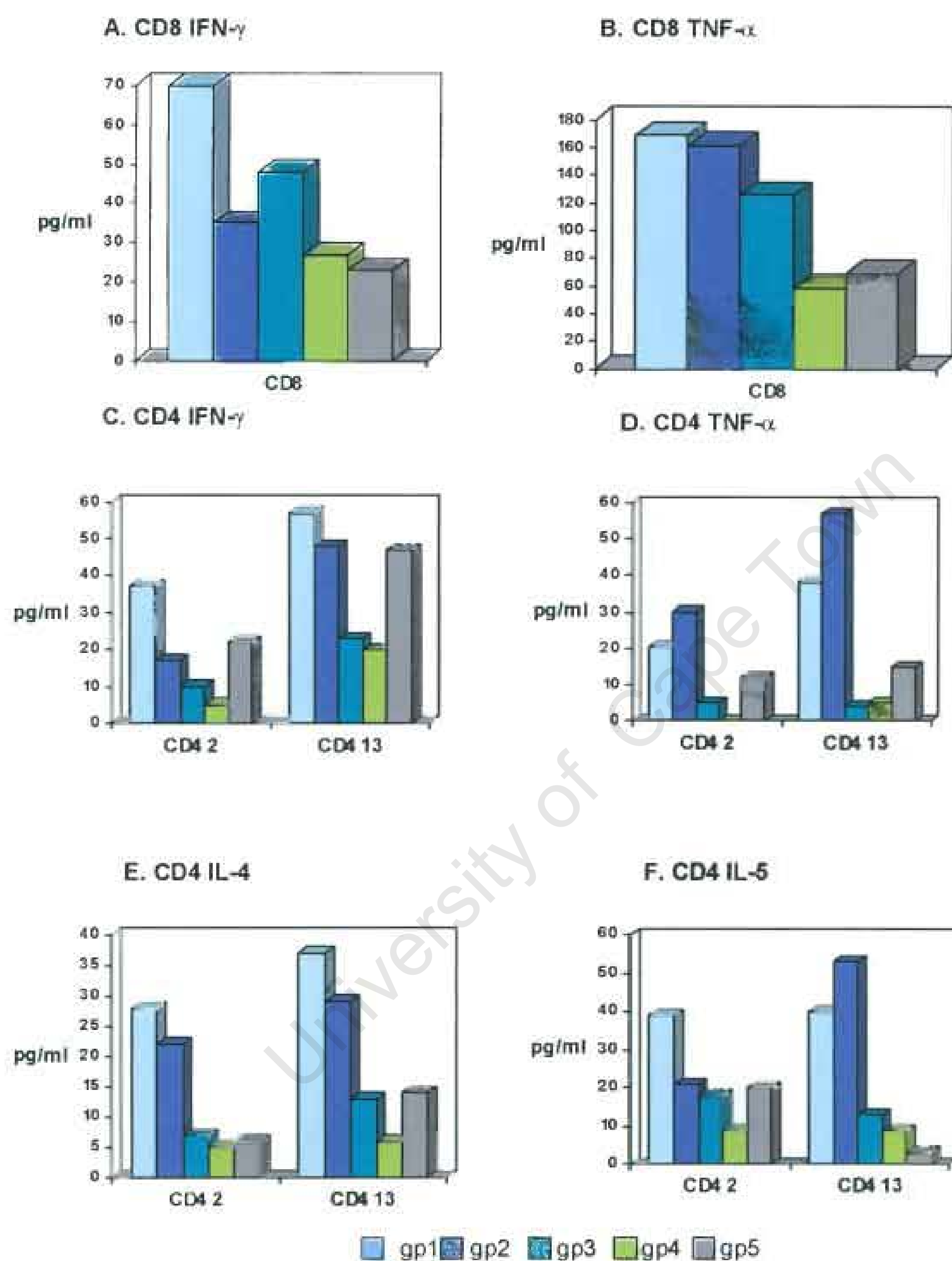

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Fig. 4.5. Cytokine production from mouse splenocytes to Gag CD8 and CD4 antigens measured by cytometric bead assay 8 weeks after the rBCG boost.
All groups of mice received 100μg of pTHGagC followed by: gp1 = rBCG:RT106 (10^3cfu); gp2 = rBCG:RT106 (10^5cfu); gp3 = rBCG:119L1e (10^3cfu); gp4 = rBCG:119L1e (10^5cfu); gp5 = resuspension medium

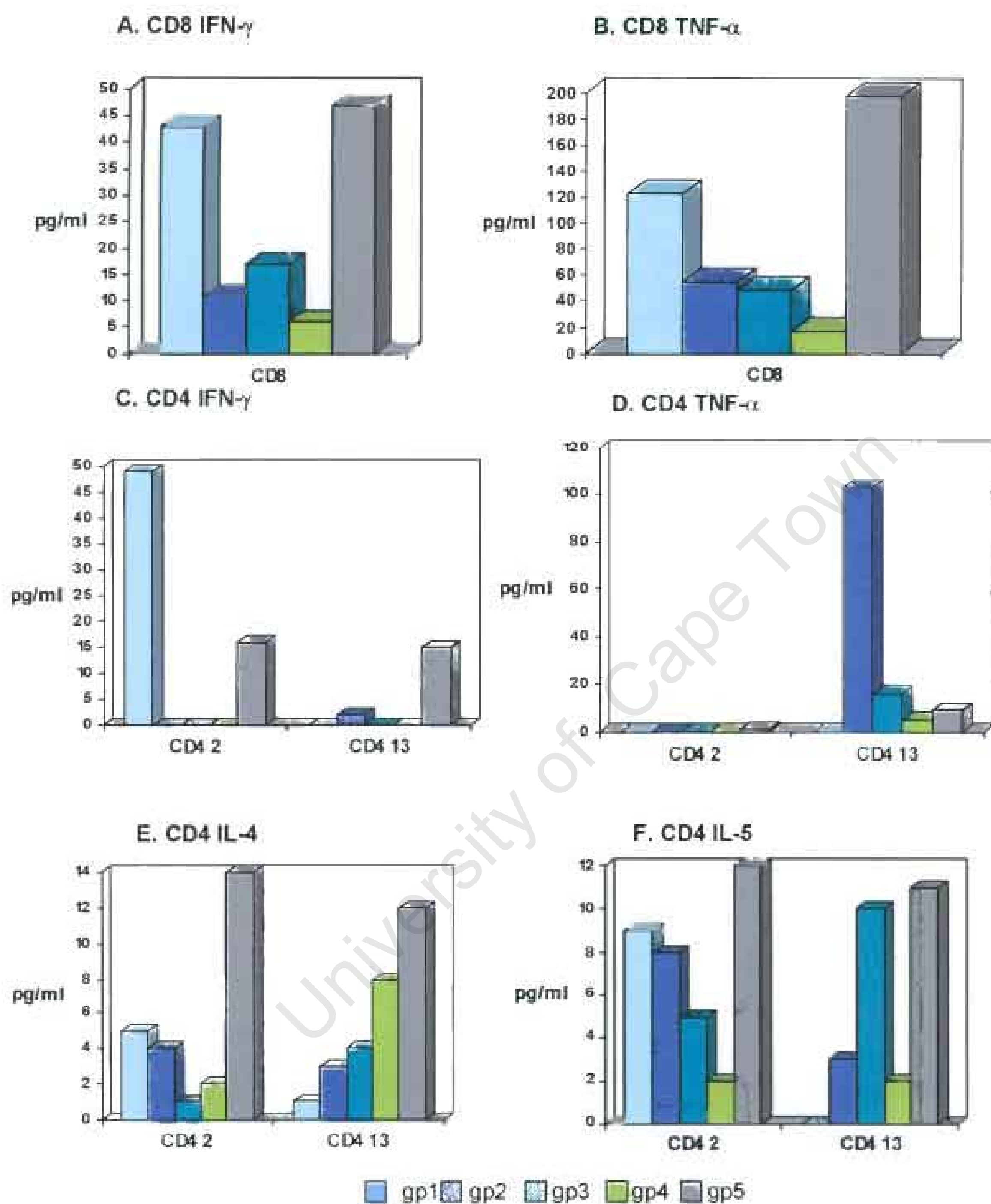

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Fig. 4.6. Cytokine production from mouse splenocytes to Gag CD8 and CD4 antigens measured by cytometric bead assay 12 weeks after the rBCG boost.
All groups of mice received 100μg of pTHGagC followed by: gp1 = rBCG:RT106 (10^{3}cfu); gp2 = rBCG:RT106 (10^{5}cfu); gp3 = rBCG:119L1e (10^{3}cfu); gp4 = rBCG:119L1e (10^{5}cfu); gp5 = resuspension medium

Table 4.4. Summary of the CBA results indicating whether the response elicited by the rBCG:RT106 boost was greater than the response elicited by the rBCG:119L1e boost.

		BOOSTED RESPONSE WHEN COMPARING rBCG:RT106 AND rBCG:119L1e	
	PEPTIDE	**10^7 CFU DOSE**	**10^5 CFU DOSE**
8 weeks	CD8	none	TNF-α
	CD4 2	IFN-γ, TNF-α, IL-4, IL-5	IFN-γ, TNF-α, IL-4, IL-5
	CD4 13	IFN-γ, TNF-α, IL-4, IL-5	IFN-γ, TNF-α, IL-4, IL-5
12 weeks	CD8	IFN-γ, TNF-α	None
	CD4 2	IFN-α	None
	CD4 13	None	TNF-α

These data highlight the fact that the memory response to the Gag peptide stimulation was also predominantly a Th1 response (as measured by CBA) as was seen in the initial response in the *ex vivo* ELISPOT data above.

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4.3.5. p24 ANTIBODY ELISA ON SERA FROM MICE RECEIVING rBCG PASTEUR

Sera taken from mice before inoculation and at each sacrifice were subjected to an ELISA designed to determine the presence of anti-p24 antibodies. The mice receiving only one rBCG inoculation yielded no antibodies to p24 (data not shown). The mice receiving the DNA prime-rBCG boost did produce antibodies to p24 but the differences in level between the groups of boosted mice were low. The antibody production in the boosted mice was similar to the production in mice receiving only the DNA vaccine (Fig 4.7) – even for the mice that received rBCG expressing HPV-16 L1.

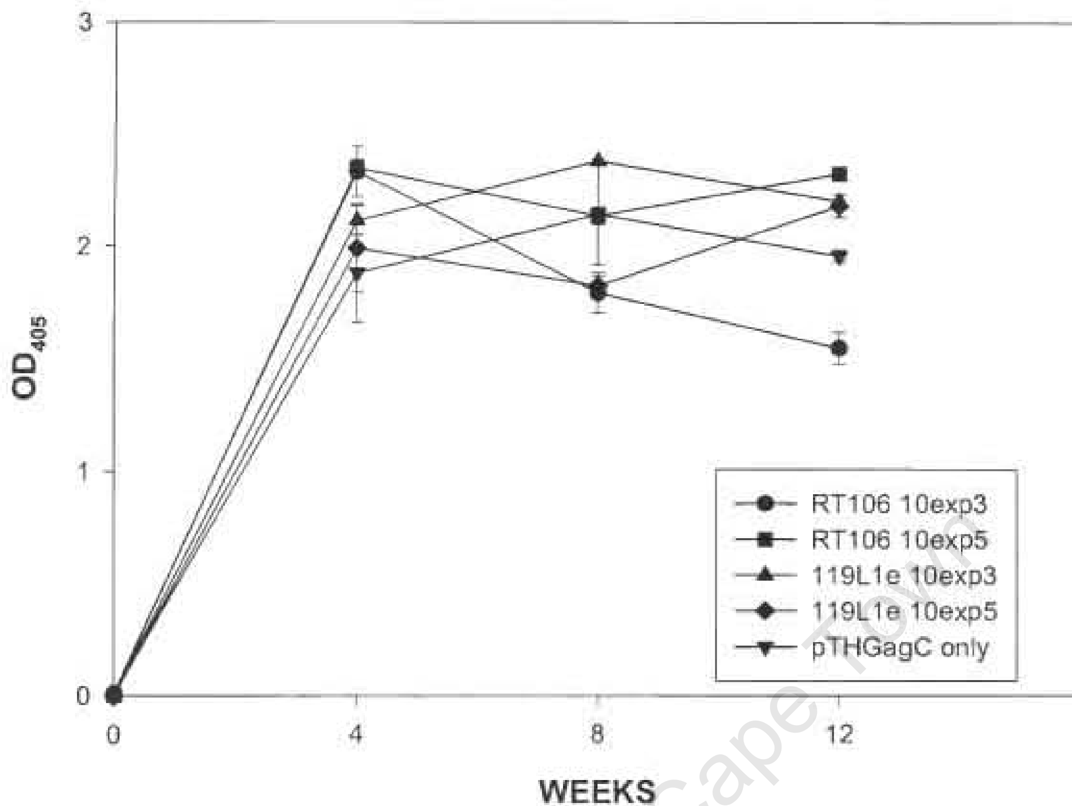

Fig 4.7. p24 antibodies determined by ELISA in serum samples diluted 1:50 from mice inoculated with pTHGagC and boosted with rBCG.

Mice were bled before the pTHGagC vaccination (week 0) and then 4, 8 and 12 weeks after the rBCG vaccination.

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4.4. DISCUSSION

Surprisingly, despite numerous rBCG studies employing HIV-1 antigens (Stover *et al.*, 1991; Aldovini and Young, 1991; Winter *et al.*, 1991; Fuerst *et al.*, 1992; Kameoka *et al.*, 1994; Wada *et al.*, 1996; Kawahara *et al.*, 2002; Chujoh *et al.*, 2002 and Someya *et al.*, 2004), only two of these have investigated the immunogenicity of HIV-1 Gag expressed in rBCG (Aldovini and Young, 1991 and Wada *et al.*, 1996). In the first, the *hsp70* promoter was used to drive expression of a subtype B *gag*, *pol* and *env* genes on an episomal vector in rBCG. Mice inoculated with the Gag and Env expressing rBCG had low but detectable levels of IgG antibodies against p24, p17 and gp160. Splenocytes from mice inoculated with Gag expressing rBCG produced substantial levels

of IFN-γ and IL-2 when stimulated with Gag peptides after 4 day *in vitro* culture and, in addition, were able to lyse P815 cells pulsed with Gag peptides. In the second study, Wada *et al.* (1996) demonstrated that high titres of antibodies to a B-cell epitope of HIV-1 p17 fused to the α-antigen from *M.kansasii* were produced in 3 of 7 mice inoculated with the rBCG. This study demonstrated both cellular and humoral immunogenicity in the mouse model using rBCG:RT106, and rBCG:RT108 (Tokyo), which express Gag at high and low levels, respectively.

Although it has been shown that the BCG Pasteur substrain induces better immune responses than the BCG Tokyo substrain (Lagranderie *et al.*, 1996), numerous studies have been performed successfully using the Tokyo substrain (eg. Kameoka *et al.*, 1994; Honda *et al.*, 1995; Wada *et al.*, 1996; Hiroi *et al.*, 2001; Chujoh *et al.*, 2002 and Kawahara *et al.*, 2002). In addition, the Tokyo substrain was the one used for BCG vaccination in South Africa at the time this study was undertaken. For these reasons, rBCG:RT106 and rBCG:RT108 Tokyo initially were tested for immunogenicity in the mouse model.

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Mice receiving 5 inoculations of either rBCG:RT106 or rBCG:RT108 (Tokyo) at 10^7cfu generated Gag-specific T cells that were able to produce IFN-γ when stimulated with rp24 or Gag VLPs. In addition, anti-p24 antibodies were generated. The other Gag-specific peptides used in this ELISA did not stimulate IFN-γ production. Although the Gag-specific peptides have been shown to be recognized by mice, they represent individual epitopes. The rp24 and Gag VLPs, on the other hand, consist of all the possible Gag-specific epitopes. It is thus more likely that the mouse T cells which were Gag-specific would be more easily stimulated by the wider range of epitopes presented by the recombinant protein or VLPs.

Better IFN-γ production and anti-p24 antibody levels were elicited by rBCG:RT106 vaccination than rBCG:RT108 and as mentioned above, the Pasteur substrain has been shown to induce better immune responses than the Tokyo substrain. rBCG:RT106 (Pasteur) was tested further.

Additionally, newer, more sensitive assays were available for measuring vaccine-induced responses in the mice, and these ELISPOT and CBA assays were used in the testing.

Since a five-inoculation regimen is practically unfeasible, a single inoculation of rBCG:RT106 (Pasteur) at two different doses was attempted. This inoculation induced BCG-specific IFN-γ and IL-4 responses in vaccinated mice. However, no Gag-specific IFN-γ or IL-4 producing cells could be detected in the mouse splenocytes in an ELISPOT assay. In addition, no anti-p24 antibodies were detected by ELISA in the mouse sera. Since prime-boost regimens have been successfully used to broaden and strengthen immune responses, a DNA prime-rBCG boost was attempted next.

A pTHGagC prime followed by an rBCG:RT106 or rBCG:119L1e (Pasteur) boost, at two different doses, was performed. An ELISPOT assay indicated that BCG-specific IFN-γ and IL-4 producing cells could be detected in all groups of mice receiving the rBCG boost. IFN-γ and IL-4 were produced after Gag-specific peptide stimulation (particularly with the Gag CD8 peptide) in all groups of mice. However, there was no significant difference in IFN-γ responses when comparing rBCG:RT106 boosted mice to rBCG:119L1e boosted mice. In general, the cellular response as measured by ELISPOT after rBCG boosting (Gag and L1) was reduced in comparison to the response in mice receiving DNA-Gag only. The cellular response to the DNA Gag vaccine was reduced when mice are inoculated with wild-type BCG. This suppressive effect could also have masked any boosting effect the rBCG vaccination might have had and highlights the importance of negative controls when testing rBCG-generated immune responses in mice. In addition, it has been shown in other studies that administration of BCG can have a suppressive effect on the generation of T cell responses to non-BCG antigens (Dudani *et al.*, 2002 and Smith *et al.*, 2002).

A cytometric bead assay was used to investigate the production of different cytokines in response to Gag CD8 and CD4 peptide stimulation. When the assay was performed 8 weeks after the mice received the rBCG boosters, it was shown that both CD8 and CD4 peptides elicited Gag-specific

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IFN-α and TNF-α production. IL-4 and IL-5 were produced on stimulation with CD4 peptides. Higher levels of CD4 Gag-specific TNF-α, IL-4 and IL-5 were produced in the splenocytes of mice that received both the pTHGagC DNA vaccine prime followed by the rBCG:RT106 when compared to that produced in splenocytes from mice receiving only the pTHGagC DNA vaccine or those mice boosted with rBCG:119L1e. The rBCG:RT106 vaccine was thus shown to be an effective Gag-specific CD8 and CD4 boost for the DNA prime. The cytokine production was boosted more effectively with a 10^3cfu dose of rBCG:RT106 than a 10^5cfu dose. At 12 weeks, the Gag-specific production of cytokines was at a lower level than at 8 weeks. This indicates the importance of determining the timing of the peak response when measuring the vaccine-elicited immunogenicity. The rBCG:RT106 vaccination again boosted the response to the pTHGagC prime compared to the rBCG:119L1e boost.

The ELISPOT data point to a predominantly Th1 response after rBCG:RT106 boosting implying that the *ex vivo* response as seen by ELISPOT assays was a Th1 response. The memory response which is measured by CBA was also shown to be a predominantly Th1 response.

This study was the first demonstration of cellular and humoral immune responses in mice to rBCG expressing a subtype C *gag* gene. The rBCG:RT106 vaccine was able to stimulate better cellular and humoral responses than rBCG:RT108. This implies that an initial high level of Gag expression, which may drop off gradually as the effects of construct instability become apparent (rBCG:RT106) resulted in better immune responses in the mice than a lower level of Gag expression that is maintained (rBCG:RT108). Méderlé *et al.* (2002) also reported that an initial high level of Gag expression in the rBCG was critical for the induction of cellular immune responses.

The immunogenicity of rBCG:RT106 and rBCG:RT108 was also demonstrated in the baboon model in studies performed by Dr G.K. Chege in our laboratory (Appendix E). Dr Chege was able to show clearly that both rBCG:Gag constructs induced Gag-specific cellular immune responses

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in the immunised baboons using an ELISPOT assay with pools of Gag peptides (Fig. E.1.). These responses were weak but they were efficiently boosted by Gag VLPs which were administered one year after the last rBCG immunization. An ELISA performed on the baboon sera showed that weak humoral response was detected in 1 of 4 immunised baboons after immunisation with rBCG:Gag (Table E.1.). However, antibodies against p24 were detected in all the rBCG:Gag immunised animals after the 1st Gag VLP boost, whereas they were only detectable in the rBCG:L1-primed baboons after a Gag 2nd VLP boost. The rBCG:Gag vaccine induced weak humoral and cellular responses in the immunised animals but a single VLP immunisation was enough to give them a strong boost. Masemola *et al.* (2004) have identified and characterized epitopic regions across the Gag protein have in subtype C HIV-1-infected people in Southern Africa. Dr Chege found that several of these regions of Gag were also targeted by PBMC from Gag-immunized baboons.

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CHAPTER 5: CONCLUSIONS

This project is part of the efforts of the South African AIDS Vaccine Initiative group working at the University of Cape Town to develop an affordable and effective HIV-1 subtype C vaccine for South Africa. The group has a number of candidate vaccines in various stages of development and the work presented here is the initial foray into the use of recombinant BCG technology as a likely candidate.

Since this was a first generation HIV-1 vaccine, the gene chosen for the rBCG vaccine was the *gag* gene. The Gag protein is one of the more conserved HIV-1 proteins and many reports have documented Gag-specific CTL responses in small animals, non-human primates and humans. Also, many other first generation CTL vaccines have been Gag-based.

The selection of the isolate for use in the Gag vaccine was made from a group of recently transmitted viruses so that it would be as close to the currently circulating strains as possible. Subtype C HIV-1 predominates in South Africa and all the viruses in the study were found to be subtype C. A Gag C consensus sequence was derived from all the sequences in the study and the vaccine strain isolate was chosen on the basis of its close relation to the consensus sequence. Du422 was selected since it was 98.7% similar to the consensus. Updated analyses performed at the time of writing, indicated that Du422 was a good choice as a regional Gag vaccine strain because it has not lost relevance since the original selection process – using a larger database of sequences and a current consensus sequence did not alter the fact that it is most closely related to the consensus. It was also shown to be 96% similar to the derived ancestral sequence and 97% similar to the derived consensus sequences for the whole of subtype C. Further, it was shown that Du422 was an appropriate choice for immunological reasons since it contains peptides recognised by southern African individuals that are infected with subtype C HIV-1.

Since vaccine projects are long term efforts, the final outcome of which are only seen many years after the project commences, it is of great importance that proper care be taken when selecting the gene that will be used in the candidate. The fact that the samples used to make the selection at the start of this project were taken in 1999 meant that there was a possibility that the phylogeny of the South

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African epidemic would evolve to make the choice of Du422 less optimal and the vaccine candidates less useful. However, analysis using updated datasets proved that Du422 has not lost relevance and that it was a good choice of Gag vaccine strain for the southern African region. The Du422 *gag* gene has been used in other SAAVI HIV-1 vaccine candidates including recombinant *Salmonella* vaccines, DNA vaccines (van Harmelen *et al.*, 2003) and protein subunit vaccines (Jaffray *et al.*, 2004). The gene has also been incorporated in a Venezuelan equine encephalitis VRP vaccine and an AAV vaccine that are currently being evaluated in clinical trials (http://www.hvtn.org/trials and http://www.iavirep.org/trialsdb). The VRP_{Du422} candidate was the first HIV-1 vaccine to enter phase I trials in South Africa and the AAV candidate will be entering trials here in 2005.

BCG, the tuberculosis vaccine, has a number of advantages associated with its use, including the fact that it is already in widespread use globally, it is safe, oral administration is possible, it has proven adjuvant potential and the capacity exists to manufacture the vaccine locally and inexpensively. However, there are practical limitations involved in working with BCG: it is a slow-growing organism that takes up to 2 weeks to grow in liquid culture and 3-4 weeks on solid media; the shuttle vectors used in rBCG technology are often genetically unstable and expression levels of the heterologous protein vary widely; monitoring the immune response to the heterologous protein is often difficult.

In this study, the Du422 *gag* gene was cloned in 8 *E. coli/Mycobacterium* shuttle vectors that either integrated into the *Mycobacterium* genome or were maintained episomally. These vectors also differed with respect to the promoters used to drive expression of Gag (*hsp60*, *M. leprae* 18kDa, *mtrA* or *katG*) and the localization signals (α-antigen or 19kDa signals) fused to the *gag* gene. In order to determine which of the 8 shuttle vectors were the best candidates for use in an rBCG-HIV vaccine, the genetic stability and Gag expression levels were compared *in vitro.* A stable vaccine with high expression levels is important not only to ensure a good immune response in vaccinees but also for future scale-up of the vaccine candidate from laboratory to population level doses.

The genetic stability of the constructs was studied in recombinant *M. smegmatis* (rMS) by monitoring genetic rearrangement over time. rMS integrative vectors were found to be highly stable while the recombinants carrying the episomal vectors were less stable. rMS:RT105 (18kDa, α-antigen)

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demonstrated rapid instability, Gag expression in combination with the 19kDa lipoprotein signal (rMS:RT106 and RT108) was more stable. Deletions that encompassed the promoter/signal sequence region and the *gag* gene were the source of the genetic instability.

Comparison of the expression levels was also performed in rMS by measuring the p24 levels in cultures over time. The integrative constructs did not produce Gag at a high level and higher expression levels were observed for the episomal constructs. The *katG* promoter induced the lower expression levels for the episomal constructs than the *mtrA* and 18kDa promoters which were producing Gag at the highest level. The stability of the constructs appeared to be linked to the expression levels with the higher expressing episomal constructs being more unstable than the integrative ones.

The use of colony-size comparisons on solid media and the indirect metabolic load measurement using differential growth at 42°C and 37°C was also used to compare the 8 recombinants. Importantly, results from this experiment were shown to support the data from the more accurate measurements of genetic stability and p24 levels in rMS cultures. Larger colony sizes were seen in the lower expressing integrative constructs, with colony size decreasing as expression levels increased. Accumulation of misfolded or denatured Gag was hypothesized to create a metabolic load on the bacteria that would trigger the stress response and allow growth at 42°C. Again, the lower expressing integrative constructs did not experience a metabolic load high enough to trigger the heat shock response, while the higher expressing episomals were able to grow at 42°C. This provides a useful initial screening method for testing the utility of candidate shuttle vectors that is less labour-intensive and quicker than the conventional method of measuring expression levels directly.

pRT106 (episomal, moderate expression, medium stability) and pRT108 (episomal, low expression, high stability) were selected for further investigations in rBCG since they were thought to combine the best mix of stability and expression level as well as practical ease-of-use (episomal). The expression of Gag from rBCG:RT106 and rBCG:RT108 (Tokyo and Pasteur) was evaluated in culture and in infected macrophages. Export of the Gag antigen to the membrane of the rBCG by the 19kDa

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lipoprotein signal was not efficient and Gag was only expressed to a small percentage of the total BCG protein. As was seen in the rMS experiments, RT106 produced more Gag than RT108 in both rBCG Tokyo and Pasteur. Expression was successfully demonstrated for both recombinants in infected macrophages but in this case expression from the *katG* promoter (rBCG:RT108) was at the same level as expression from the *mtrA* promoter (rBCG:RT106) after both 24 and 48 hours of infection. Immunological testing of the rBCG:RT106 and rBCG:RT108 vaccines was undertaken in the BALB/c mouse model.

Mice receiving 5 inoculations of either rBCG:RT106 or rBCG:RT108 (Tokyo) at 10^7cfu generated Gag-specific T cells that were able to produce IFN-γ when stimulated with rp24 or Gag VLPs. These mice were also able to generate anti-p24 antibodies.

rBCG:RT106 (Pasteur) was tested at two different doses as a single inoculation or as a booster inoculation following a DNA_{Gag} prime. After a single inoculation, BCG-specific but not Gag-specific IFN-γ and IL-4 responses were induced in vaccinated mice as measured by ELISPOT. In addition, no anti-p24 antibodies were detected by ELISA in the mouse sera. The DNA_{Gag} prime was able to elicit Gag-specific IFN-γ and IL-4 responses (ELISPOT) and anti-p24 antibodies but these responses were not enhanced by the rBCG:RT106 boost even when compared to rBCG:119L1e boosted mice. As has been shown in other studies (Dudani *et al.*, 2002 and Smith *et al.*, 2002), the cellular response boosting with rBCG was suppressed in comparison to the response in mice receiving DNA-Gag only making it difficult to conclude whether there was no boosting effect or if this was masked.

This *ex vivo* response was not mirrored in the memory or recall response seen in a CBA measuring CD8 and CD4 cytokine production in the mice than had received the DNA_{Gag} prime and rBCG:RT106 boost. The memory response demonstrated that rBCG:RT106 was an effective Gag-specific boost for the DNA prime. Gag-specific IFN-α, TNF-α, IL-4 and IL-5 were produced in the splenocytes of the vaccinated mice after 2 days of stimulation. There was a clear boosting effect seen particularly in the production of CD4 Gag-specific TNF-α, IL-4 and IL-5 in the splenocytes of mice that received both the DNA_{Gag} prime followed by the rBCG:RT106 in comparison to mice receiving only the DNA vaccine or

mice boosted with rBCG:119L1e. This boost was shown to be time specific and dose dependent. However, the assay again demonstrated that the rBCG vaccination had a suppressive effect on the overall response. Another finding was that both the *ex vivo* response and the memory response were predominantly Th1 responses. Since the CBA indicates the type of response (memory) one would expect in vaccinees upon challenge, it is a more informative result.

These data were the first demonstration of cellular and humoral immune responses in mice to rBCG expressing a subtype C *gag* gene. rBCG:RT106 yielded better cellular and humoral responses than rBCG:RT108. This implies that a higher initial level of Gag expression which waned as the effects of genetic instability became more pronounced was more effective than a lower level of expression that is maintained. These data also provide insight into the usefulness of the mouse model when studying responses to rBCG vaccine candidates. Since BCG tends to suppress the responses to non-BCG antigens in the mouse model, it is not well-suited to immunogenicity testing of rBCG candidates. As mentioned in section 4.4, work in our laboratory by Dr G.K. Chege has demonstrated that despite the difficulty in measuring the responses elicited by rBCG:RT106 and rBCG:RT108 in mice, good Gag-specific cellular immune responses could be induced in the baboon model.

In conclusion, Du422, a Gag vaccine strain highly suited to the southern African region was selected. The *gag* gene from this strain was cloned in *E. coli/Mycobacterium* shuttle vectors. The genetic stability and Gag expression levels of these shuttle vectors were evaluated *in vitro* in *M. smegmatis* and BCG. Cellular and humoral immune responses were demonstrated in the mouse model using rBCG:Gag subtype C vaccines. This study provides a good basis for the HIV-1 subtype C rBCG vaccine candidate being developed by the UCT SAAVI group. It demonstrates that it is possible to generate an immune response to HIV-1 subtype C antigens expressed in rBCG. These vaccines may be useful in prime-boost modalities as shown in recent work by Dr Chege (section 4.4). Improvements in the expression level and construct stability are being conducted by Drs Bourn and Stutz and may further enhance the immune responses generated by the recombinants.

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APPENDIX A: MEDIA, BUFFERS AND SOLUTIONS

A.1. MEDIA

Note: volumes were adjusted with distilled water unless otherwise indicated.

2X YT Medium

Broth (1l): 10g Yeast extract
16g Tryptone
5g NaCl
Autoclave

Agar: 100ml 2X YT broth
1.5g Agarose
Autoclave

Middlebrook's Medium

Broth (1l): 4.7g Middlebrook's 7H9
450µl Tween-80
2.5ml 80% glycerol
dH_2O to 900ml
Autoclave
Add 100ml O-ADC

Agar (1l): 19g of Middlebrook's 7H10
5ml glycerol
dH_2O to 900ml
Mix thoroughly; boil for 1 min to dissolve fully; autoclave
Add 100ml of O-ADC (State Vaccine Institute, SA)

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Sauton's Medium

1l of broth: 0.5g KH_2PO_4
1.05g $MgSO_4(7H_2O)$
4.0g L-asparagine
60ml glycerol
0.05g ferric ammonium citrate
2.0g citric acid
0.1ml 1% $ZnSO_4$
2.5ml 10% (w/v) tyloxapol
pH to 7 with 2N KOH
Autoclave.

Resuspension medium for rBCG vaccine stocks

100ml: 0.85% NaCl
10% glycerol
0.025% tyloxapol
Autoclave

R10 cell culture medium

RPMI-1640 medium with Glutamax-1 containing 10% foetal bovine serum (FBS), 100units/mL Penicillin-G and 100 µg/mL Streptomycin

A.2. BUFFERS AND SOLUTIONS

Note: volumes were adjusted with distilled water unless otherwise indicated and all buffers/solutions were made up volumetrically.

1M $CaCl_2$
14.7g $CaCl_2.2H_2O$
Autoclave

Chloroform-Isoamylalcoholo
Mix in a ratio of 24:1

CTAB/NaCl
Dissolve 4.1g NaCl in 80ml dH_2O and slowly add 10g CTAB.
Heat at 65°C to dissolve and adjust the volume to 100ml.
Store at RT for no longer than 6 months.

0.5M EDTA (500ml)
Dissolve 93.95g EDTA in 400ml dH_2O by stirring vigorously.
Adjust the pH to 8.0 with NaOH pellets (~10g).
Autoclave.

1M HCl (1l)
86.2ml HCl

5M Potassium acetate (100ml)
49.1g Potassium acetate
Store at 4°C.

10% SDS
10g SDS
Heat to about 80°C to dissolve.
Add a few drops of HCl to adjust pH to 7.2

5M Sodium chloride/NaCl (100ml)
29.22g NaCl
Autoclave.

10N Sodium hydroxide/NaOH (100ml)
40g NaOH
Store in a plastic container

1M Tris-Cl (100ml)
121.1g Tris
Adjust pH to require level with concentrated HCl.
Autoclave.

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Tris-EDTA/TE buffer
100mM Tris-Cl (pH 8.0)
1mM EDTA (pH 8.0)
Autoclave

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APPENDIX B: GENERAL MOLECULAR BIOLOGY PROTOCOLS

B.1. TRANSFORMATION STUDIES

Preparation of competent E.coli cells

E.coli cells were made competent by a $CaCl_2$-shock procedure based on the method described by Dagert and Ehrlich (1979). A single colony of the bacterium was inoculated into 5ml of 2x YT broth (Appendix A) and grown at 37°C overnight with shaking. This starter culture was diluted 1/100 in 100ml of 2x YT broth and cultured to early logarithmic phase (OD_{600} 0.2 to 0.4). The cells were harvested by centrifugation at 5000rpm for 5 min at 4°C in a Beckman Model J2-21 centrifuge. Cells were kept at 4°C during all subsequent procedures. Cells were resuspended in 50ml ice-cold 0.1M $CaCl_2$ (Appendix A) and kept on ice for 1 hr. They were harvested as before and resuspended in 10ml ice-cold 0.1M $CaCl_2$, thereby inducing the transient state of competence. Ice-cold, sterile glycerol was added to the cells to a final concentration of 10% (v/v), mixed and held on ice for 30 min. Aliquots of the competent cells were stored at -80°C.

Transformation

Aliquots of competent cells were thawed on ice before addition of DNA. The tube was then held on ice for a further 20-30 min before heat shocking at 42°C for 2 min. 0.9ml of 2x YT broth was added to the tube and the cells incubated at 37°C for 1 hr. Transformation mixes were plated onto appropriate selective media and incubated at 37°C overnight.

B.2. RESTRICTION ENDONUCLEASE DIGESTION

DNA was digested with enzymes and buffers supplied by Roche (Basel, Switzerland). The appropriate buffers were used at 1x concentration with 0.5-1U of enzyme in a total volume of 20μl (made up with deionized water). All digests were performed at 37°C for 1-2 hr, except for genomic DNA digests, which were allowed to continue overnight. Digests with multiple enzymes were performed simultaneously using compatible buffers.

B.3. DNA SEQUENCING

All DNA sequencing was performed using a capillary system and Big Dye terminators on an automated DNA sequencer (ABIPRISM 3100 Sequencer; Applied Biosystems; Perkin Elmer; CA, USA). The sequencing was perfomed at the University of Stellenbosch (RSA).

B.4. LIGATION

Vector and insert DNA with compatible ends were ligated in volumes of 20μl. The insert:vector ratio in the ligation mix was 3:1 by weight. T4 DNA ligase (Roche, Basel, Switzerland) was used (2.5U/reaction) in 1x ligation buffer (Roche, Basel, Switzerland) and deionized water. Ligations

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were performed at 16°C overnight. Control ligations testing ligase activity and levels of undigested vector and insert DNA were performed in parallel.

B.5. GENOMIC DNA EXTRACTION FROM MYCOBACTERIA

Genomic DNA was extracted following the method of Ausubel *et al.* (1987). Briefly, 5ml Mycobacterial cultures were harvested in a microfuge at 12000*g* for 2 min. The supernatant was discarded and the pellet resuspended in 500µl TE buffer (Appendix A). The cells were lysed by the addition of 50µl of lysozyme (10mg/ml) and incubation at 37°C for 1 hr. Further lysis and digestion of cellular proteins were achieved by the addition of 70µl of 10% SDS (Appendix A) and 3µl of proteinase K (20mg/ml). The suspension was mixed and incubated at 65°C for 10 min. To precipitate the cellular debris, 100µl of 5M NaCl (Appendix A) and 80µl of CTAB/NaCl (Appendix A) were added, mixed in thoroughly and incubated at 65°C for a further 10 min. An equal volume of chloroform/isoamyl alcohol (Appendix A) was added, the tubes were vortexed and centrifuged for 5 min at 12000*g*. The aqueous phase was transferred to a new tube and the DNA was precipitated with 0.6x volume of isopropanol at -80°C for 1 hr. The DNA was collected by centrifugation at 12000*g* for 15 min. The DNA pellets were washed with 70% ethanol, air-dried and resuspended in 20µl of TE buffer.

B.6. SOUTHERN TRANSFER

DNA was transferred from agarose gels to Hybond™-N membranes (Amersham International, UK) using a mono-directional transfer based on the method of Smith and Summers (1980). Before the transfer was performed, the gel was soaked twice in 2-3 gel volumes of 0.25M HCl (Appendix A) for 15 min at room temperature. It was then soaked twice in 2-3 gel volumes of 1.5M NaCl, 0.5M NaOH at room temperature for 15 min. Finally, it was neutralized by soaking it once in 2-3 gel volumes of 0.5M Tris pH7.2, 1.5M NaCl, 1mM EDTA at room temperature for 30 min. Between each step and after neutralization, the gel was rinsed with water. On completion of the pretransfer treatment, DNA was transferred from the gel to the membrane. The gel was placed face down on a flat surface covered in a sheet of foil. A piece of Hybond™-N membrane was cut to size and layered onto the gel, ensuring that no air bubbles were trapped beneath it. Three pieces of Whatmans No.1 filter paper were soaked in neutralization solution and layered on the Hybond™-N. About 50mm of dry paper towel was placed on the filter paper, followed by a piece of Perspex and brick to compress the sandwich. Transfer was allowed to continue for 3 hr. The different materials were removed and the membrane was peeled off. The DNA was then fixed to the the membrane by UV cross-linking at 254nm in a UV Cross-linker (Hoefer Scientific Instruments, CA, USA) for 30 secs.

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B.7. PLASMID EXTRACTION

Small-scale plasmid extraction was performed on 5ml cultures of putative recombinants. Two milliliters of each culture was pelleted at 14000rpm in a microfuge. Pellets were resuspended in 0.2ml of Solution I (25mM Tris-HCl pH8.0; 50mM EDTA, 1% w/v glucose). After resuspension, 0.4ml of Solution II (0.2N NaOH; 1% w/v SDS) was added; the tubes inverted to mix thoroughly and held on ice for 5 min. Pre-cooled Solution III (5M potassium acetate; 3M glacial acetic acid) – 03ml – was added and the tubes were kept on ice for a further 5 min. The precipitates were centrifuged at 14000rpm for 10 min; the supernatants were removed and centrifuged as before to further clarify them. The plasmid DNA was precipitated from the supernatants with 100% ethanol, washed with 70% ethanol, air-dried and resuspended in 20-50µl of TE buffer.

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APPENDIX C: DNA SEQUENCES

pRT001 *gag* gene

GGGCCC**ATG**GGTGCGAGAGCGTCAATATTAAGAGGGGAAAAATTAGATACATGGGAAAAAATTAGGTTAAGGCCAGGGGGAAAGAAACA
TTATATGTTAAAACACATAGTATGGGCAAGCAGGGAGCTGGAAAGATTTGCACTTAACCCTGGCCTTTTAGAAACATCAGAAGGATGTA
AACAAATAATAAAACAGCTACAACCAGCTCTCCAGACAGGAACAGAGGAACTTAAATCATTATACGACACAGTAGCAACTCTCTATTGT
GTACATGAAAAGATAGAAGTACGAGACACCAAGGAAGCCTTAGATAAGATAGAGGAAGAACAAAACAAATGTCAGCAAAAAACGCAGCA
GGCAAAAGCGGCTGACGGAAAGTCAGTCAAAATTATCCTATAGTGCAGAATCTCCAAGGGCAAATGGTACATCAAGCCATATCACCTA
GAACCTTGAATGCATGGGTAAAAGTAATAGAGGAAAAGGCTTTTAGCCCAGAGGTAATACCCATGTTTACAGCATTATCAGAAGGAGCC
ACCCCACAAGATTTAAACACCATGTTAAATACGGTGGGGGACATCAAGCAGCCATGCAAATGTTAAAAGATACCATCAATGAAGAGGC
TGCAGAATGGGATAGATTACATCCAGTACATGCGGGGCCTATTGCACCAGGCCAGATGAGAGAACCAAGGGGAAGTGACATAGCAGGAA
CTACTAGTACCCTTCAGGAACAAATAGCATGGATGACAAGTAACCCACCTATTCCAGTGGGAGACATCTATAAAAGATGGATAATTCTG
GGGTTAAATAAAATAGTGAGAATGTATAGCCCGGTCAGCATTTTGGACATAAGACAAGGGCCAAAGGAACCCTTTCGAGACTATGTAGA
TCGGTTCTTTAAAACTTTAAGAGCTGAACAAGCTACACAAGAAGTAAAAAATTGGATGACAGACACCTTGTTAGTCCAAAATGCGAACC
CAGATTGTAAGACCATTTTGAGAGCATTGGGACCAGGGGCTACATTAGAAGAAATGATGACAGCATGTCAAGGGGTGGGAGGACCTGGC
CACAAAGCAAGAGTATTGGCTGAGGCAATGAGTCAAACAAACAGTGGAAACATAATGATGCAGAGAAGCAATTTTAAAGGCCCTAGAAG
AATTGTTAAATGTTTTAACTGTGGCAAGGAAGGGCACATAGCCAGAAATTGCAGAGCCCCTAGGAAAAAAGGCTGTTGGAAATGTGGAA
AAGAAGGACACCAAATGAAAGACTGCACTGAGAGGCAGGCTAATTTTTTAGGGAAAATTTGGCCTTCCCACAAGGGGAGGCCAGGGAAT
TTCCTTCAGAACAGACCAGAGCCAACAGCCCCACCAGCAGAGAGCTTCAGGCTCGAAGAGACAACCCCCGCTCCGAAACAGGAGCCGAT
AGAAAGGGAACCCTTAACTTCCCTCAAATCACTCTTTGGCAGCGACCCCTTGTCTCAA**TAA**AAGTAGGGGGCCAGACAAGGGAGGCTCT
CTTAGACACAGGAGCAGATGATACAGTATTATCGAT

pRT002 *gag* gene

GAATTC**ATG**GGTGCGAGAGCGTCAATATTAAGAGGGGAAAAATTAGATAAATGGGAAAAGATTAGGTTAAGGCCAGGGGGAAAGAAACA
TTATATGTTAAAACACATAGTATGGGCGAGCAGGGAGCTGGAAAGATTTGCACTTAACCCTGGCCTTTTAGAAACATCAGAAGGATGTA
AACAAATAATGAAACAGCTACAACCAGCTCTCCAGACAGGAACAGAGGAACTTAAATCATTATACAACACAGTAGCAACTCTCTATTGT
GTACATGAAAAGATAGAAGTACGAGACACCAAGGAAGCCTTAGATAAGATAGAGGAAGAACAAAACAAATGTCAGCAAAAAACGCAGCA
GGCAAAAGCGGCTGACGGAAAGTCAGTCAAAATTATCCTATAGTGCAGAATCTCCAAGGGCAAATGGTACATCAAGCCATATCACCTA
GAACCTTGAATGCATGGGTAAAAGTAATAGAAGAAAAGGCTTTTAGCCCAGAGGTAATACCCATGTTTACAGCATTATCAGAAGGAGCC
ACCCCACAAGATTTAAACACCATGTTAAATACAGTGGGGGACACCAAGCAGCCATGCAAATGTTAAAAGATACTATTAATGAAGAGGC
TGCAGAATGGGATAGATTACATCCAGTCCATGCGGGGCCTATTGCACCAGGCCAGATGAGAGAACCAAGGGGAAGTGACATAGCAGGAA
CTACTAGTACCCTTCAGGAACAAATAGCATGGATGACAAGTAACCCACCTATTCCAGTGGGAGACATCTATAAAAGATGGATAATTCTG
GGGTTAAATAAAATAGTGAGAATGTATAGCCCGGTCAGCATTTTGGACATAAGACAAGGGCCAAAGGAACCCTTTCGAGACTATGTAGA
TCGGTTCTTTAAAACTTTAAGAGCTGAACAAGCTACACAAGAAGTAAAAAATTGGATGACAGACACCTTGTTAGTCCAAAATGCGAACC
CAGATTGTAAGACCATTTTGAGAGCATTAGGACCAGGGGCTACATTAGAAGAAATGATGACAGCATGTCAAGGGGTGGGAGGACCTGGC
CACAAAGCAAGAGTATTGGCTGAGGCAATGAGTCAAACAAACAGTGGAAACATAATGATGCAGAGAAGCAATTTTAAAGGCCCTAGAAG
AATTGTTAAATGTTTTAACTGTGGCAAGGAAGGGCACATAGCCAGAAATTGCAGAGCCCCTAGGAAAAAAGGCTGTTGGAAATGTGGAA
AAGAAGGACACCAAATGAAAGACTGCACTGAGAGGCAGGCTAATTTTTTAGGGAAAATTTGGCCTTCCCACAAGGGGAGGCCAGGGAAT
TTCCTTCAGAACAGACCAGAGCCAACAGCCCCACCAGCAGAGAGCTTCAGGTTCGAAGAGACAACCCCCGCTCCGAAACAGGAGCCGAT
AGAAAGGGAACCCTTAACTTCCCTCAAATCACTCTTTGGCAGCGACCCCTTGTCTCAA**TAA**AAGTAGGGGGCCAGACAAGGGAGGCTCT
CTTAGACACAGGAGCAGATGATACAGTATTGTCGAC

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Fig.C.1. Sequences of the *gag* gene cloned into pRT001 and pRT002

The restriction enzyme sites at the ends are underlined. The start and stop codons are indicated in bold.

pRT101: *hsp60* promoter + *gag* gene

XbaI
TCTAGACGGTGACCACAACGACGCGCCCGCTTTGATCGGGGACGTCTGCGGCCGACCATTTACGGGTCTTGTTGTCGTTGGCGGTCATGGGCCGAACATACTCACCCGGA

-35 -10 tss
TCGGAGGGCCGAGGACAAGGTCGAACGAGGGGCATGACCCGGTGCGGGGCTTCTTGCACTCGGCATAGGCGAGTGCTAAGAATAACGTTGGCACTCGCGACCGGTGAGTC

GTAGGTCGGGACGGTGAGGCCAGGCCCGTCGTCGCAGCGAGTGGCAGCGAGGACAACTTGAGCCGTCCGTCGCGGGCACTGCGCCCGGCCAGCGTAAGTAGCGGGGTTGC

RBS TSC EcoRI
CGTCACCCGGTGACCCCCGGTTTCATCCCCGATCCGGAGGAATCACTTCGCAATGGCCAAGACAATTGCGGATCCAGCTGCAGAATTCATGGGTGCGAGGCGTCAATATT

gag
AAGAGGGGAAAAATTAGATA//GAACCCTTAACTTCCCTCAAATCACTCTTTGGCAGCGACCCCTTGTCTCAATAAAAGTAGGGGGCCAGACAAGGGAGGCTCTCTTAGA

SalI HindIII
CACAGGAGCAGATGATACAGTATTGTCGACCTGCAGGCATGCAAGCTT

pRT102: *hsp60* promoter + α-antigen signal + *gag* gene

XbaI -35 -10 tss
TCTAGACAAGGTCGAACGAGGGGCATGACCCGGTGCGGGGCTTCTTGCACTCGGCATAGGCGAGTGCTAAGAATAACGTTGGCACTCGCGACCGGTGAGTCGTAGGTCGG

GACGGTGAGGCCAGGCCCGTCGTCGCAGCGAGTGGCAGCGAGGACAACTTGAGCCGTCCGTCGCGGGCACTGCGCCCGGCCAGCGTAAGTAGCGGGGTTGCCGTCACCCG

α-SD TSC
GTGACCCCCGGTTTCATCCCCGATCGCTAGATATCCATGGATCTGAGCACACGACGACATACAGGACAAAGGGGCACAGGTATGACAGACGTGAGCCGAAAGATTCGAGC

Whole alpha antigen gene
TTGGGGACGCCGATTGATGATCGGCACGGCAGCGGCTGTAGTCCTTCCGG//CGCGGGCGGGCACAACGCCGTGTTCAACTTCCCGCCCAACGGCACGCACAGCTGGGAG

EcoRI *gag*
TACTGGGGCGCTCAGCTCAACGCCATGAAGGGTGACCTGCAGAGTTCGTTAGGCGCGGATCCGGAATTCATGGGTGCGAGGCGTCAATATTAAGAGGGGAAAAATTAGAT

A//GAACCCTTAACTTCCCTCAAATCACTCTTTGGCAGCGACCCCTTGTCTCAATAAAAGTAGGGGGCCAGACAAGGGAGGCTCTCTTAGACACAGGAGCAGATGATACA

SalI HindIII
GTATTGTCGACCTGCAGGCATGCAAGCTT

Fig.C.2. DNA sequences of the promoters, signal sequences and the *gag* gene for the different shuttle vectors

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pRT103: *hsp60* promoter + 19kDa signal sequence + *gag* gene

XbaI -35 -10 tss
TCTAGACAAGGTCGAACGAGGGGCATGACCCGGTGCGGGGCTTCTTGCACTCGGCATAGGCGAGTGCTAAGAATAACGTTGGCACTCGCGACCGGTGAGTCGTAGGTCGG

GACGGTGAGGCCAGGCCCGTCGTCGCAGCGAGTGGCAGCGAGGACAACTTGAGCCGTCCGTCGCGGGCACTGCGCCCGGCCAGCGTAAGTAGCGGGGTTGCCGTCACCCG

TSC
GTGACCCCCGGTTTCATCCCCGATCGCTAGATATCCATGGATCTGTCCTCAATGCCGATGGACCGCTACGACAGGCAAAGGAGCACAGGGTGAAGCGTGGACTGACGGTC

19kDa signal EcoRI *gag*
GCGGTAGCCGGAGCCGCCATTCTGGTCGCAGGTCTTTCCGGATGTTCAAGCAACAAGTCCACGGATCCTGAATTCATGGGTGCGAGGCGTCAATATTAAGAGGGGAAAAA

TTAGATA//GAACCCTTAACTTCCCTCAAATCACTCTTTGGCAGCGACCCCTTGTCTCAATAAAAGTAGGGGGCCAGACAAGGGAGGCTCTCTTAGACACAGGAGCAGAT

SalI HindIII
GATACAGTATTGTCGACCTGCAGGCATGCAAGCTT

pRT104: *mtrA* promoter + 19kDa signal sequence + *gag* gene

XbaI
TCTAGAGCGATTGAGGGCGTTGACGGCGCTGGCAAGCGGACGTTGGTGGAAAAGCTGTCCGGGGCCTTTCGAGCAGCCGGGAGATCGGTGGCCACACTGGCGTTCCCGCG

CTACGGACAGTCGGTGGCCGCCGACATCGCAGCGGAGGCGCTGCACGGCGAGCACGGTGACCTCGCATCGTCGGTGTATGCGATGGCGACGCTGTTCGCGCTCGACCGCG

CTGGCGCGGTCCACACGATCCAGGGGCTGTGTCGCGGCTACGACGTGGTGATCCTGGATCGCTACGTCGCCTCCAACGCGGCCTACAGCGCGGCGCGCCTACATGAAAAC

GCGGCCGGGAAGGCAGCGGCCTGGGTTCAGCGGATCGAATTTGCAAGACTCGGGTTGCCCAAGCCCGACTGGCAGGTGCTCCTTGCGGTCTCTGCCGAGCTCGCCGGGGA

ACGATCCCGCGGCCGTGCCCAGCGTGACCCCGGTCGGGCGCGCGACAATTACGAACGCGACGCTGAACTTCAGCAGCGCACCGGTGCGGTCTACGCCGAGTTGGCGGCCC

AAGGGTGGGGCGGCCGGTGGCTGGTTGTCGGCGCCGATGTTGATCCGGGCCGACTAGCGGCGACTTTGGCGCCTCCAGACGTGCCAAGTTGAGGGTATTTGCCCTAATCA

RBS NdeI TSC
AGCAAGAAACGCCCGTGTGGTGCGCCAGTTTTGTCCCGATGTGGTGACACCATGGACCATATGAAGCGTGGACTGACGGTCGCGGTAGCCGGAGCCGCCATTCTGGTCGC

19kDa signal ApaI
AGGTCTTTCCGGATGTTCAAGCAACAAGTCGACTACAGGAAGCGGTGAGACCACGACCGCGGCAGGCACGACGGCAAGCCCCGGCGGTGGGCCCATGGGTGCGAGGCGTC

gag
AATATTAAGAGGGGAAAAATTAGATA//GAACCCTTAACTTCCCTCAAATCACTCTTTGGCAGCGACCCCTTGTCTCAATAAAAGTAGGGGGCCAGACAAGGGAGGCTCT

SalI HindIII
CTTAGACACAGGAGCAGATGATACAGTATTGTCGACCTGCAGGCATGCAAGCTT

Fig.C.2. DNA sequences of the promoters, signal sequences and the *gag* gene for the different shuttle vectors, continued

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pRT105: *M.leprae* 18kDa promoter + α-antigen signal sequence + *gag* gene

```
XbaI
TCTAGAATAGCCCGCAGCGTTCATAGAGCCGCAGCGACGGCACCGGGAACGCCGGAAAGTTCGGCCGCTACCGATGATGTCGTATACGCTGCGTTGCAGTGCCGACGTAC

                                                                       -35                  -10             tss
CCGTGCCGGCACTACAATCGGTCATGAGCAATCTCCTCAGCTGTTCAGACAGAAAACTTGTCTATCACAACTTGCATCAATATATCGACCAGTGCTATATCAAATCTATG

                                                    RBS     NdeI TSC
TAGTCAGGAACAGCTATATAGTTATAGTTTGTCACAACAGATTGGAGTGCGAGGTGACCACATATGGCCACAGACGTGAGCCGAAAGATTCGAGCTTGGGGACGCCGATT

                              alpha antigen                                              ApaI
GATGATCGGCACGGCAGCGGCTGTAGTCCTTCCGGGCCTGGTGGGGCTTGCCGGCGGAGCGGCAACCGCGGGCGCGTTCGGTGGGCCCATGGGTGCGAGAGCGTCAATAT

             gag
TAAGAGGGGAAAAATTAGATA//GAACCCTTAACTTCCCTCAAATCACTCTTTGGCAGCGACCCCTTGTCTCAATAAAAGTAGGGGGCCAGACAAGGGAGGCTCTCTTAG

                       ClaI
ACACAGGAGCAGATGATACAGTATTATCGAT
```

pRT106: *mtrA* promoter + 19kDa signal sequence + *gag* gene

```
XbaI
TCTAGAGCGATTGAGGGCGTTGACGGCGCTGGCAAGCGGACGTTGGTGGAAAAGCTGTCCGGGGCCTTTCGAGCAGCCGGGAGATCGGTGGCCACACTGGCGTTCCCGCG

CTACGGACAGTCGGTGGCCGCCGACATCGCAGCGGAGGCGCTGCACGGCGAGCACGGTGACCTCGCATCGTCGGTGTATGCGATGGCGACGCTGTTCGCGCTCGACCGCG

CTGGCGCGGTCCACACGATCCAGGGGCTGTGTCGCGGCTACGACGTGGTGATCCTGGATCGCTACGTCGCCTCCAACGCGGCCTACAGCGCGGCGCGCCTACATGAAAAC

GCGGCCGGGAAGGCAGCGGCCTGGGTTCAGCGGATCGAATTTGCAAGACTCGGGTTGCCCAAGCCCGACTGGCAGGTGCTCCTTGCGGTCTCTGCCGAGCTCGCCGGGGA

ACGATCCCGCGGCCGTGCCCAGCGTGACCCCGGTCGGGCGCGCGACAATTACGAACGCGACGCTGAACTTCAGCAGCGCACCGGTGCGGTCTACGCCGAGTTGGCGGCCC

AAGGGTGGGGCGGCCGGTGGCTGGTTGTCGGCGCCGATGTTGATCCGGGCCGACTAGCGGCGACTTTGGCGCCTCCAGACGTGCCAAGTTGAGGGTATTTGCCCTAATCA

                                                      RBS  NdeI TSC                          19kDa signal
AGCAAGAAACGCCCGTGTGGTGCGCCAGTTTTGTCCCGATGTGGTGACACCATGGACCATATGAAGCGTGGACTGACGGTCGCGGTAGCCGGAGCCGCCATTCTGGTCGC

                                                                                                ApaI
AGGTCTTTCCGGATGTTCAAGCAACAAGTCGACTACAGGAAGCGGTGAGACCACGACCGCGGCAGGCACGACGGCAAGCCCCGGCGGTGGGCCCATGGGTGCGAGAGCGT

             gag
CAATATTAAGAGGGGAAAAATTAGATA//GAACCCTTAACTTCCCTCAAATCACTCTTTGGCAGCGACCCCTTGTCTCAATAAAAGTAGGGGGCCAGACAAGGGAGGCTC

                                ClaI
TCTTAGACACAGGAGCAGATGATACAGTATTATCGAT
```

Fig.C.2. DNA sequences of the promoters, signal sequences and the *gag* gene for the different shuttle vectors, continued

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pRT107: *katG* promoter + α-antigen signal sequence + *gag* gene

XbaI
TCTAGAGATGGGCGATCGGGTCCTAGCAGACGCCTGTCACGCTAGCCAAAGTCTTGACTGATTCCAGAAAAGGGAGTCATATTGTCTAGTGTGTCCTCTATACCGGACTA

CGCCGAACAGCTCCGGACGGCCGACCTGCGCGTGACCCGACCGCGCGTCGCCGTCCTGGAAGCAGTGAATGCGCATCCACACGCCGACACGGAAACGATTTTCGGTGCCG

TGCGTTTTGCGCTGCCCGACGTATCCGGCAAGCCGTGTACGACGTGCTGCATGCCCTGACCGCCGCGGGCTTGGTGCGAAAGATCCAACCCTCGGGCTCCGTCGCGCGCT

ACGAGTCCAGGGTCGGCGACAACCACCATCACATCGTCTGCCGGTCTTGCGGGGTTATCGCCGATGTCGACTGTGCTGTTGGCGAGGCACCCTGTCTGACGGCCTCGGAC

-35 -10 tss
CATAACGGCTTCCTGTTGGACGAGGCGGAGGTCATCTACTGGGGTCTATGTCCTGATTGTTCGATATCCGACACTTCGCGATCACATCCGTGATCACAGCCCGATAACAC

RBS NdeI TSC alpha antigen
CAACTCCTGGAAGGATCCATATGGCCACAGACGTGAGCCGAAAGATTCGAGCTTGGGGACGCCGATTGATGATCGGCACGGCAGCGGCTGTAGTCCTTCCGGGCCTGGTG

ApaI *gag*
GGGCTTGCCGGCGGAGCGGCAACCGCGGGCGCGTTCGGTGGGCCCATGGGTGCGAGAGCGTCAATATTAAGAGGGGAAAAATTAGATA//GAACCCTTAACTTCCCTCAA

ClaI
ATCACTCTTTGGCAGCGACCCCTTGTCTCAATAAAAGTAGGGGGCCAGACAAGGGAGGCTCTCTTAGACACAGGAGCAGATGATACAGTATTATCGAT

pRT108: *katG* 18kDa promoter + 19kDa signal sequence + *gag* gene

XbaI
TCTAGAGATGGGCGATCGGGTCCTAGCAGACGCCTGTCACGCTAGCCAAAGTCTTGACTGATTCCAGAAAAGGGAGTCATATTGTCTAGTGTGTCCTCTATACCGGACTA

CGCCGAACAGCTCCGGACGGCCGACCTGCGCGTGACCCGACCGCGCGTCGCCGTCCTGGAAGCAGTGAATGCGCATCCACACGCCGACACGGAAACGATTTTCGGTGCCG

TGCGTTTTGCGCTGCCCGACGTATCCGGCAAGCCGTGTACGACGTGCTGCATGCCCTGACCGCCGCGGGCTTGGTGCGAAAGATCCAACCCTCGGGCTCCGTCGCGCGCT

ACGAGTCCAGGGTCGGCGACAACCACCATCACATCGTCTGCCGGTCTTGCGGGGTTATCGCCGATGTCGACTGTGCTGTTGGCGAGGCACCCTGTCTGACGGCCTCGGAC

-35 -10 tss
CATAACGGCTTCCTGTTGGACGAGGCGGAGGTCATCTACTGGGGTCTATGTCCTGATTGTTCGATATCCGACACTTCGCGATCACATCCGTGATCACAGCCCGATAACAC

RBS NdeI TSC 19kDa signal
CAACTCCTGGAAGGATCCATATGAAGCGTGGACTGACGGTCGCGGTAGCCGGAGCCGCCATTCTGGTCGCAGGTCTTTCCGGATGTTCAAGCAACAAGTCGACTACAGGA

ApaI *gag*
AGCGGTGAGACCACGACCGCGGCAGGCACGACGGCAAGCCCCGGCGGTGGGCCCATGGGTGCGAGAGCGTCAATATTAAGAGGGGAAAAATTAGATA//GAACCCTTAAC

ClaI
TTCCCTCAAATCACTCTTTGGCAGCGACCCCTTGTCTCAATAAAAGTAGGGGGCCAGACAAGGGAGGCTCTCTTAGACACAGGAGCAGATGATACAGTATTATCGAT

Fig.C.2. DNA sequences of the promoters, signal sequences and the *gag* gene for the different shuttle vectors, continued

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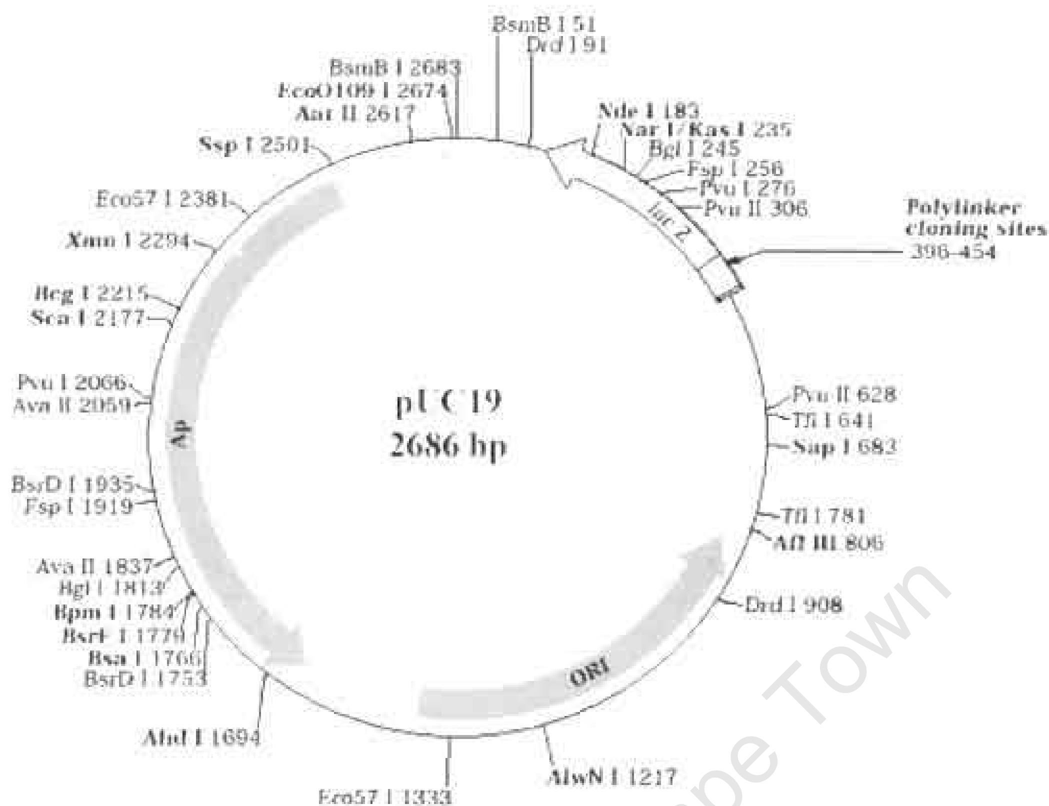

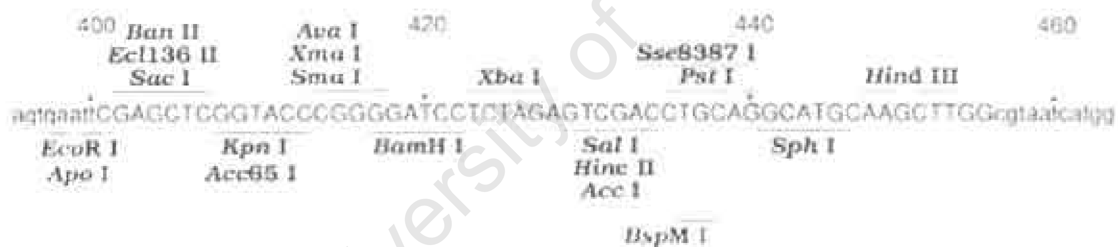

Fig. D.2. Schematic diagram of the plasmid, pUC19 with the multiple cloning site/polylinker region

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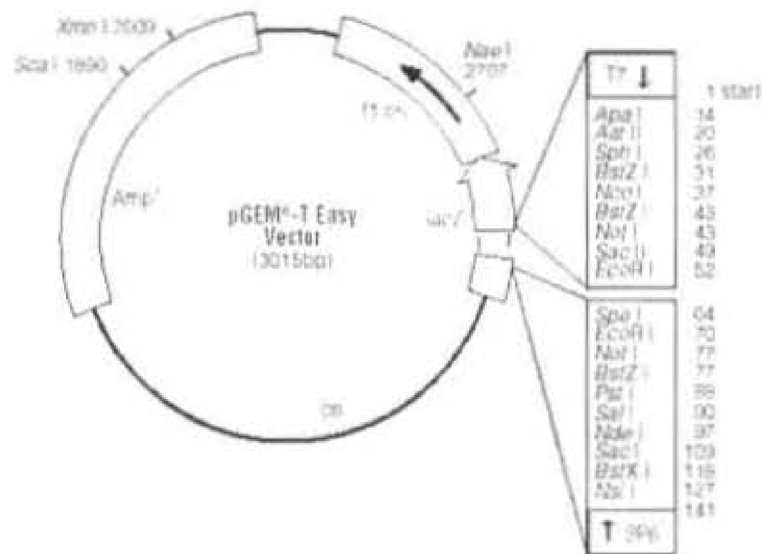

pGEM®-T Easy Vector Sequence reference points:

T7 RNA Polymerase transcription initiation site	1
SP6 RNA Polymerase transcription initiation site	141
T7 RNA Polymerase promoter (−17 to +3)	2999–3
SP6 RNA Polymerase promoter (−17 to +3)	139–158
multiple cloning region	10–128
lacZ start codon	180
lac operon sequences	2836–2996, 166–395
lac operator	200–216
β-lactamase coding region	1337–2197
phage f1 region	2380–2835
binding site of pUC/M13 Forward Sequencing Primer	2956–2972
binding site of pUC/M13 Reverse Sequencing Primer	176–192

Fig D.3. Schematic diagram of the plasmid, pGEM-T Easy with the multiple cloning site and a list of the vector sequence reference points

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APPENDIX E
RESULTS FROM BABOON EXPERIMENT
(Dr G.K. Chege, personal communication)

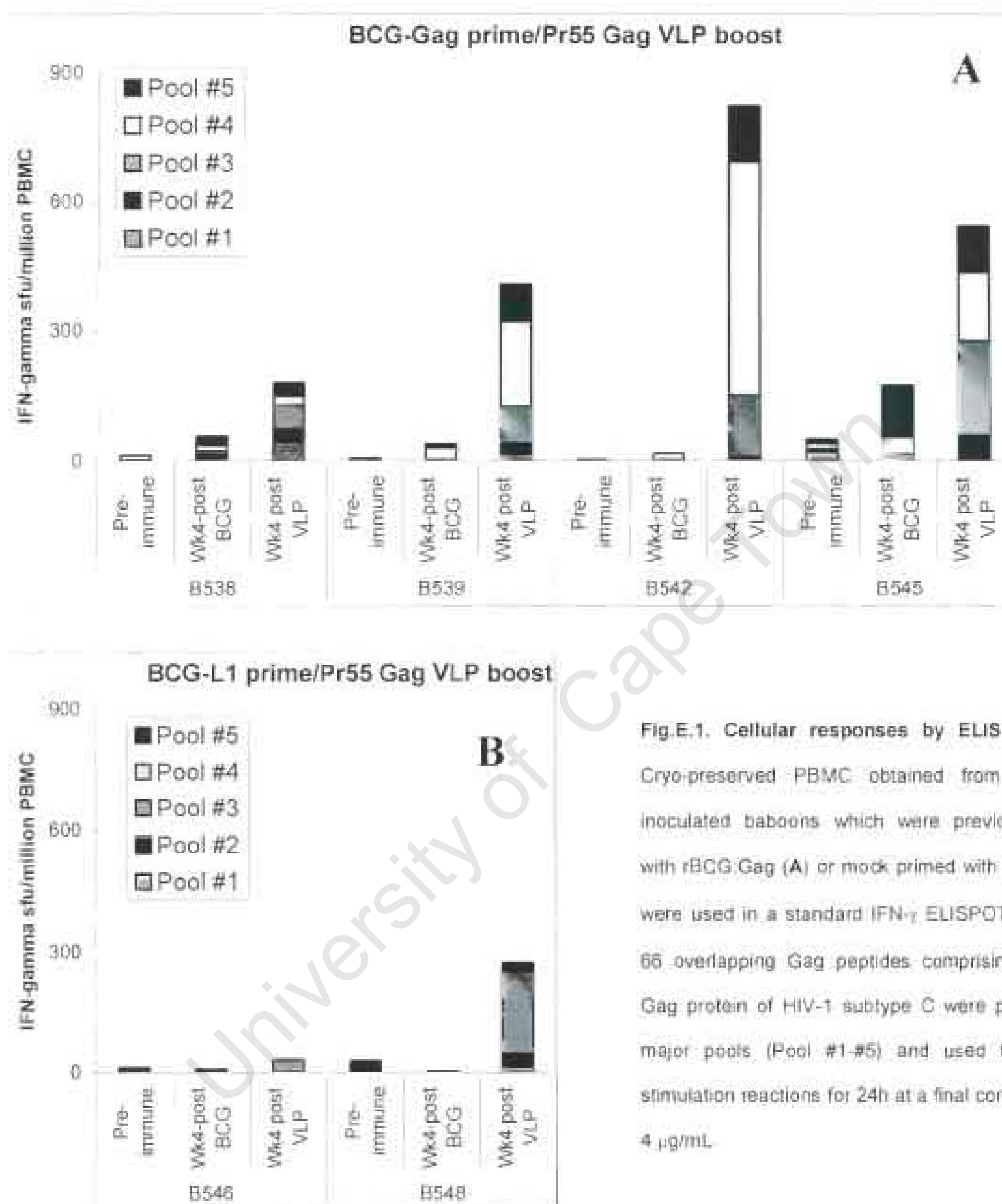

Fig.E.1. Cellular responses by ELISPOT assay. Cryo-preserved PBMC obtained from Gag VLP-inoculated baboons which were previously primed with rBCG:Gag (**A**) or mock primed with rBCG:L1 (**B**) were used in a standard IFN-γ ELISPOT assay. The 66 overlapping Gag peptides comprising the entire Gag protein of HIV-1 subtype C were pooled into 5 major pools (Pool #1-#5) and used for antigenic stimulation reactions for 24h at a final concentration of 4 μg/mL.

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Table E.1. HIV-1 p24 immunoglobulin G antibody titres in the serum of immunised baboons obtained at pre- and post-immunization period

rBCG	Baboon Number	HIV-1 p24 IgG Antibody Titres in the Serum								
		Pre-	*BCG-2*	*BCG-3*	*BCG-4*	*Pre-VLP*	*VLP-1*	*Pre-VLP-2*	*VLP-2*[a]	*VLP-2*[b]
RT106	**B542**[Gag]	<40	<40	<40	<40	<40	<40	60	1280	160
	B545[Gag]	80	80	80	80	160	320	160	15360	2560
RT108	**B538**[Gag]	40	<40	<40	40	40	80	120	10240	1280
	B539[Gag]	<40	<40	40	40	40	60	240	10240	640
119L1e	**B546**[Mock]	<40	<40	<40	<40	<40	<40	<40	640	160
	B548[Mock]	<40	<40	<40	<40	<40	<40	<40	3840	320

Pre-, Pre-immunization; ***BCG-2***, 6 weeks post 2nd BCG-VLP immunization; ***BCG-3***, 4 weeks post 3rd BCG-VLP immunization; ***BCG-4***, 4 weeks post 4th BCG-VLP immunization; ***Pre-VLP***, 0 weeks pre-Gag-VLP immunization; ***VLP-1***, 4 weeks post 1st VLP-Gag immunization; ***Pre-VLP-2***, 0 weeks pre-2nd Gag-VLP immunization; ***VLP-2***[a], 4 weeks post 2nd VLP-Gag immunization; ***VLP-2***[b], 8 weeks post 2nd VLP-Gag immunization; [Gag], BCG-Gag primed; [Mock], BCG-L1 mock primed.

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APPENDIX D: VECTOR MAPS

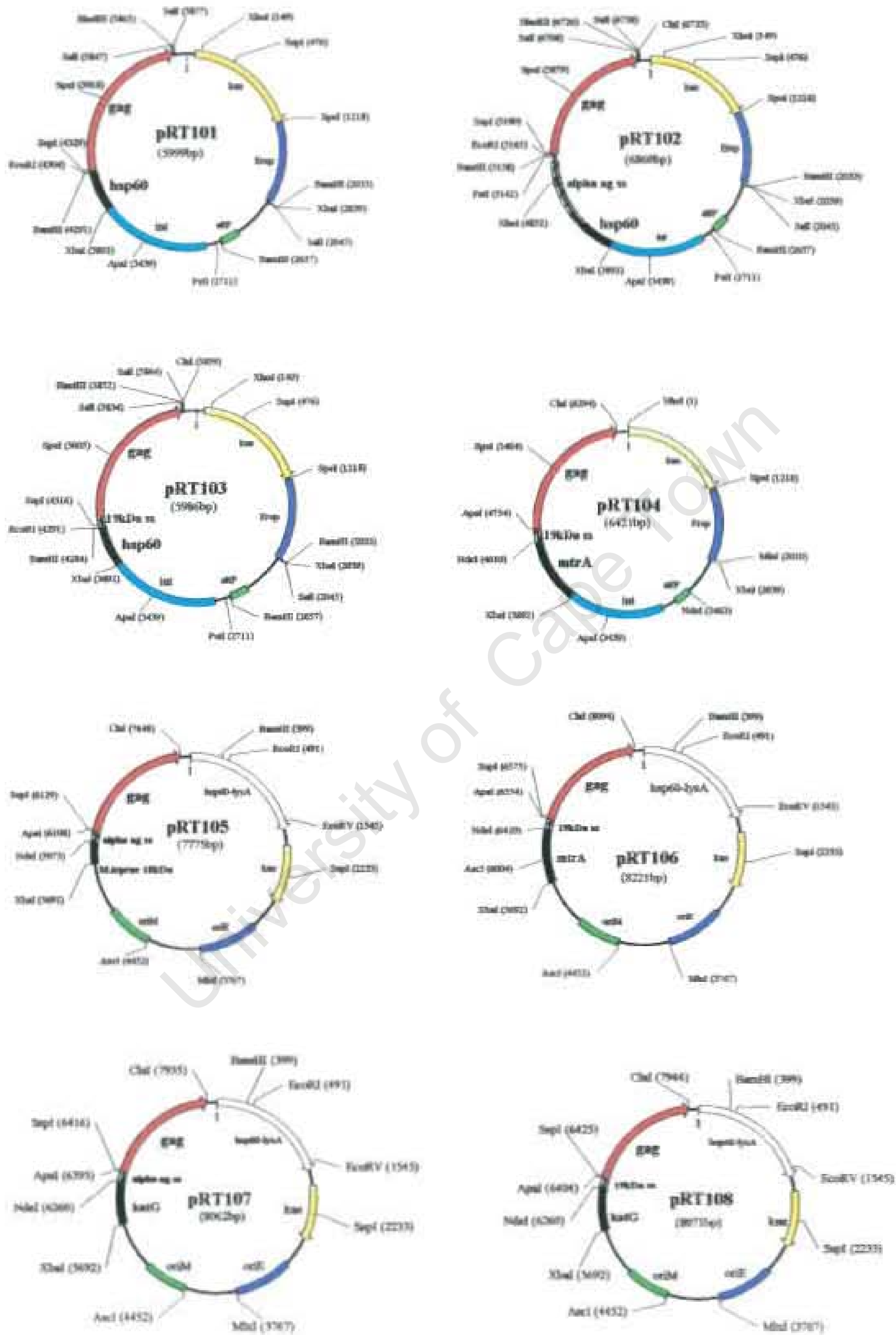

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Fig D.1.Schematic diagrams of the *E.coli/Mycobacterium* shuttle vectors carrying the Du422 *gag* gene