INVESTIGATION OF THE USE OF RECOMBINANT BCG, EXPRESSING THE MAJOR CAPSID PROTEIN (LI) OF HUMAN PAPILLOMAVIRUS TYPE 16, AS A CANDIDATE VACCINE FOR CERVICAL CANCER

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CONTENTS

APPENDICES

DECLARATION

The work described in this thesis was done at the Division of Medical Virology, Department of Clinical Laboratory Science and Institute of Infectious Diseases and Molecular Medicine, University of Cape Town, under the supervision of Professor Anna-Lise Williamson. The work is my own. Where use has been made of others, their contribution has been acknowledged.

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ABSTRACT

The identification of a causal link between infection with specific types of human papillomavirus (HPV) and the development of cervical cancer has stimulated considerable interest in the development of HPV vaccines, especially for use in developing countries, where the disease burden is high and resources for widespread screening and follow-up procedures are limited.

As the major structural protein of the HPV capsid, L1 is the antigen of choice for the development of prophylactic vaccines for HPV. L1 has the ability to self-assemble into virus-like particles (VLPs) which are highly immunogenic, and can induce protection from papillomavirus challenge in various animal models. Human phase 2 clinical trials with HPV-16 VLPs have produced encouraging results. The production expense of VLPs, however, makes them unlikely candidates for large-scale use in developing countries. Because affordability is a key factor for vaccine production in South Africa, this study assessed the potential of *Mycobacterium bovis* bacille Calmette-Guérin (BCG) as a HPV vaccine vehicle. BCG is the live, attenuated *M. bovis* strain, which is routinely used to immunise against infection with *M. tuberculosis*. A number of factors contribute to the cost-effectiveness of recombinant BCG (rBCG) vaccines: complicated purification procedures are not required, its potent adjuvant properties prevent the need for additional adjuvants, an extensive cold chain for maintenance of efficacy is not required, and distribution networks already exist. A variety of viral, bacterial and parasitic antigens have been successfully expressed in BCG, inducing both humoral and cell-mediated responses. In experimental models rBCG has proven to elicit protective immunity against Lyme disease, pneumococcal infection, and cutaneous leishmaniasis. rBCG expressing cotton tail rabbit papillomavirus L1 has been shown to partially protect rabbits from CRPV challenge, indicating that rBCG has potential as a vector for a prophylactic HPV vaccine.

In this study, rBCG expressing the L1 capsid protein of the HPV-16 was evaluated as a candidate prophylactic HPV vaccine. The expression and immunogenicity of three HPV-16 L1 gene variants, namely $L1_R$ (full-length gene), $L1_{NLS}$- (a 66 base-pair 3' truncation of $L1_R$ to remove the nuclear localisation signal [NLS]), and $L1_{BCG}$ (a synthetic gene with codons optimised for expression in BCG) were assessed in rBCG. The $L1_R$, $L1_{NLS}$-, and $L1_{BCG}$ genes were cloned into a panel of 8 *E. coli* - mycobacterial expression vectors, resulting in 20 clones which were introduced into BCG (Tokyo and Pasteur substrains) by

electroporation. The expression vectors differed with respect to promoters utilised for foreign gene expression, foreign protein export, and were either integrative or extrachromosomal.

A high frequency of genetic instability was observed in many of the BCG recombinants immediately after electroporation, which was evident by L1 gene excisions; and as a result, only 12 of the 20 L1-expression vectors could be introduced into BCG in their intact form. The 8 unstable plasmids were clones of pMV261NT, p2619, pAB26, and pSMT3, which have two common features: they are extrachromosomal (copy number of 5) and they drive foreign gene expression with the *hsp60* promoter. The integration-proficient plasmids (pMV361 and pNIV192), which also drive foreign gene expression with *hsp60*, were however stably maintained. Clones derived from the extrachromosomal vectors, pCB112 and pCB119, which drive foreign gene expression with the *M. leprae 18kDa* and *mtrA* promoters, respectively, were more stable in BCG than their *hsp60*-driven counterparts. This superior *in vitro* stability may be a consequence of the weak *in vitro* activity of the *M. leprae 18kDa* and *mtrA* promoters. Restriction enzyme analysis and sequencing of the unstable expression plasmids revealed that the gene deletions present in these constructs ranged from deletions spanning the entire L1 gene and promoter region, to smaller deletions within the L1 gene. These gene excisions were never found to occur at exactly the same sites. Stable constructs were sequenced, and surprisingly, no small gene deletions or significant point mutations were detected.

Recombinants that appeared stable after transformation were assessed by PCR or restriction enzyme mapping after a further 16 and 24 generations of growth in liquid medium containing antibiotics. The BCG recombinants containing integration-proficient vectors were genetically stable (100% stable after 16 generations), despite having the strong *hsp60* promoter. The BCG constructs derived from the pCB112 extrachromosomal vector were relatively stable (4 out of 5 cultures were 100% stable after 16 generations). BCG constructs derived from the pCB119 vector were the least stable (3 out of 5 cultures were unstable after 16 generations; between 12% and 90.1% these cultures had lost of the L1 gene). When grown without antibiotic selection, the integrated vectors were more stably maintained than the extrachromosomal constructs. On average, 85% of the bacteria in the cultures containing integrating vectors were still antibiotic resistant after 26 generations of selection-free growth, but only 25% of the bacteria in the cultures containing extrachromosomal constructs remained antibiotic resistant.

It was assumed that the observed plasmid instability was a consequence of high levels of L1 expression, however, the level L1 protein production by rBCG was usually too low to be detected (<0.3% of total rBCG protein). mRNA quantification studies deemed L1 transcription to be efficiently taking place in all rBCG-L1 constructs tested. rBCG containing the extrachromosomal vectors, p112L1$_e$ and p119L1$_e$, produced higher levels of L1 mRNA than p361L1$_e$; which is likely due to the multiple copy nature of the former plasmids. p119L1$_e$ produced more L1 mRNA than p112L1$_e$, implying that the *mtrA* promoter (in p119L1$_e$) is more active than the *M. leprae 18kDa* promoter (in p112L1$_e$) *in vitro*. Codon optimisation of L1, to reflect commonly utilised BCG codons, did not increase L1 production to detectable levels, suggesting that the apparent low expression of L1 is also not due to inefficient translation. A high rate of degradation by proteases is suggested for L1's apparent low expression.

Immune responses elicited by rBCG-L1 were investigated in guinea pigs and mice. Although both humoral and cell-mediated immunity were assessed in guinea pigs, the focus was on the ability of rBCG-L1 to generate antibodies specific to HPV-16 L1, as the aim of this research was to investigate the possibility of utilising rBCG-L1 as a prophylactic vaccine for HPV. The mouse studies focused on the *in vivo* CTL responses elicited to rBCG-L1, by measuring the level of protection in a recombinant vaccinia virus-HPV-16 L1 (rVV-L1$_R$) challenge model. The combined results from the guinea pig and mouse studies demonstrated that immunisation with rBCG-L1 successfully elicited VLP-specific antibody and cell-mediated immune responses that were dependent on expression vector, dose, route of immunisation and L1 variant. Three or more rBCG-L1 immunisations were required for the induction of a strong antibody response. On the other hand, a single inoculum of purified HPV-16 L1 VLP elicited high levels of antibodies to L1. In guinea pigs the rBCG-361L1$_e$ and rBCG-119L1$_e$ constructs induced the highest serum antibody levels, while the rBCG constructs utilising the pCB112 expression vector consistently elicited the weakest antibody responses. When administered at the $2x10^6$ cfu dose, all rBCG-L1 constructs successfully induced L1-specific DTH reactions, which is evidence of BCG's potent cellular immunity adjuvant properties. The observation that rBCG-112L1$_{BCGe}$ (codon optimised L1) elicited higher levels of antibodies than rBCG-112L1$_e$ (native L1) suggests that codon optimisation increased the expression of L1, or that CpG-islets on the optimised gene increased this recombinant's immunogenicity. The observation that the anti-L1 antibody levels elicited by rBCG-361L1$_e$ and rBCG-119L1$_e$ were of similar magnitude, and that rBCG-119L1$_{BCGe}$ did not elicit significantly more antibodies than rBCG-119L1$_e$, suggests an upper threshold level of L1 expression in BCG. The removal of the L1 NLS enhanced the immune response over

full-length L1; this was apparent by the enhanced antibody response induced to L1 in guinea pigs by rBCG-112L1$_{NLS}$-, and by the superior protective ability of rBCG-361L1$_{NLS}$- in the rVV-L1 mouse challenge model. VLP-specific antibodies were detected in the vaginal secretions of a number of animals immunised with rBCG-361L1$_e$, rBCG-119L1$_e$ and rBCG-119L1$_{BCGe}$. Wild-type BCG-primed guinea pigs elicited enhanced L1-specific antibody responses, when compared with non-primed animals, easing concerns that prior vaccination with wild-type BCG could adversely affect the immunity induced to recombinant BCG vaccines. rBCG-L1 did not elicit detectable neutralising antibodies in guinea pigs, which implies that VLPs or capsomeres were not formed within BCG.

Immunisation of mice with rBCG-L1 elicited antigen-specific $CD8^+$ responses, and antigen-specific cytotoxicity, and L1-specific antibody responses. Furthermore, these mice were afforded L1-specific protection of up to 98.89% from challenge with rVV-L1$_R$, confirming the induction of strong $CD8^+$ CTL responses.

In conclusion, rBCG-L1 has produced good preliminary immunogenicity data in small animals. However, the L1 production and stability of these vaccine candidates would need to be improved before these rBCG-L1 would be of clinical use.

Publication

A large portion of Chapter 1 was included in the following publication:
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ABBREVIATIONS

A	adenine
aa	amino acid(s)
ab(s)	antibodie(s)
ADC	adeno-and adenosquamous-carcinoma
AIDS	acquired immune deficiency syndrome
AP	alkaline phosphatase
APS	ammonium persulphate
ASR	age-standardised rate
BCG	*M. bovis* bacille Calmette-Guérin
bp	base pair(s)
BPV	bovine papillomavirus
BSA	bovine serum albumin
C	cytosine
cfu.	colony-forming units
CIN	cervical intraepethelial neoplasia
cm	centimetre
COPV	canine oral papillomavirus
CRPV	cottontail rabbit papillomavirus
CSP	circumsporozoite protein of *Plasmodium facciparum*
CTL	cytotoxic T lymphocyte
DEPC	Diethyl pyrocarbonate
ddNTP	dideoxynucleoside triphosphates
Dig	digoxigenin
DMSO	dimethyl sulphoxide
DNA	deoxyribonucleic acid
dNTPs	deoxynucleoside triphosphates
DTH	delayed-type hypersensitivity
E. coli	*Escherichia coli*
EDTA	ethylenediamine tetra-acetic acid
ELISA	enzyme-linked immunosorbent assay
F	Farad
FCS	foetal calf serum
FITC	fluorescein isothyocyanate
G	guanine
g	grams
β-gal	beta-D-galactosidase
HCl	hydrochloric acid
HIV	human immunodeficiency virus
HLA	human leukocyte antigen
HPV	human papillomavirus
hr(s)	hour(s)
HRP	horseradish peroxidase
hsp	heat-shock protein
HSV	herpes simplex virus
Hyg	hygromycin
I.d.	intradermal
I.n.	intranasal
I.p.	intraperitoneal
i.v.	intravenous
IFN	interferon I
gA,G,M	immunoglobulin A, G, M
IL	interleukin
kbp	kilo base pairs
kD	kiloDalton
L1*	$L1_R$, $L1_{NLS^-}$, or $L1_{BCG}$
LCR	long control region
LNC	lymph node cells
LTR	long terminal repeat
M	*Mycobacterium*
M	molar (moles per litre)
mA	milliamps
Mab	monoclonal antibody
MCS	multiple cloning site
mg	milligram
MHC	major histocompatibility complex
min	minute(s)
ml	millilitre
mm	millimetre
mM	millimolar
mRNA	messenger ribonucleic acid
msec	milliseconds
N	normal
NaCl	sodium chloride
NaOH	sodium hydroxide
NCR	non-coding region
ng	nanogram
NIH	National Institutes of Health
NLS	nuclear localisation signal
OD	optical density
OPD	1, 2 phenylenediamine dihydrochloride
OR	odds ratio
ORF	open reading frame
PAGE	polyacrylamide gel electrophoresis
PBMCs	peripheral blood mononuclear cells
PBS	phosphate-buffered saline
PCR	polymerase chain reaction
PEG	polyethylene glycol
pfu	plaque forming unit
pmol	picomole
PPD	purified protein derivative of tuberculin
PV	papillomavirus
Rb	retinoblastoma
rBCG	recombinant BCG
RBS	ribosome-binding site
RNA	ribonucleic acid
RNase	ribonuclease
rpm	revolutions per minute
s.c.	subcutaneous
S1-TTC	S1 subunit of pertussis toxin fused to fragment C of tetanus toxin
SA	South Africa
SDS	sodium dodecyl sulfate
sec	second(s)
SEM	standard error of mean
T	thymidine
TAE	tris-acetate EDTA
TB	tuberculosis
Tc1/2	cytotoxic T cells type 1/type 2
Th1/2	helper cells type 1/type 2
TNF	tumour necrosis factor
U	unit
µg	microgram
µl	microlitre
µM	micromolar
V	volts
v/v	volume per volume
VLP	virus-like particles
w/v	weight per volume
WHO	World Health Organisation
X-gal	5-bromo-4-chloro-3-indolyl-β-D-galactosidase
ZN	Ziehl-Neelson

LIST OF TABLES

LIST OF FIGURES

CHAPTER 6

CHAPTER 1: INTRODUCTION TO HUMAN PAPILLOMAVIRUS

1.1. HISTORICAL OVERVIEW

The infectious nature of warts has been suspected for many centuries. In 1907, Ciuffo suggested the viral aetiology of human skin warts by experiments demonstrating the transmission of warts from man to man by the inoculation of a cell-free extract of wart tissue (reviewed in Burns, 1992). In 1933 the first papillomavirus (PV) was described by Richard Shope. Shope recognised the cottontail rabbit papillomavirus (CRPV) as the aetiological agent responsible for cutaneous papillomatosis in the cottontail rabbit (Shope, 1933). The first electronmicroscopy identification of papillomavirus particles occurred in 1949; and the structure of the papillomavirus genome was elucidated in 1963, however, further characterisation was slow, since a tissue culture system for the propagation of PVs did not exist (reviewed in zur Hausen, 1996).

One of the main reasons for the current interest in human papillomaviruses was the discovery of their relationship to carcinoma of the cervix. Although a cytologic feature of cervical HPV infection, called "koilocytotic atypia", was observed and named in a study by Koss and Durfee in 1956, the association with HPV infection was not yet recognised. It was only suggested in 1975 by zur Hausen, that PVs may play a role in the induction of cervical cancer (zur Hausen, 1975).

1.2. CLASSIFICATION, NOMENCLATURE AND TAXONOMY

Historically, papillomaviruses and polyomaviruses were grouped together as papovaviruses; however, papillomaviruses are now seen as a family of their own. PVs are widespread in nature and have been isolated from humans, cattle, rabbits, horses, dogs, non-human primates, mice, sheep, birds and deer (reviewed in Howley and Lowy, 2001). Traditionally, each virus was named firstly after its natural host, as the host range of single PVs is usually very restricted and they rarely or never cross the species barrier. PVs from one species were further classified into supergroups, subgroups, and types according to the sequence homologies of the E6, E7, and L1 open reading frames (ORFs). The PV taxonomic classification has recently been revised, introducing the terms "genus" and "species". The supergroup is now referred to as a genus (different genera share less than 60% nucleotide sequence homology of the L1 gene), subgroups are now called species (species within a genus have 60-70% homology), the traditional classification into "types" has persisted (types

within a species have 71-89% L1 nucleotide sequence homology). So far over 100 HPV types have been identified, with 80 types fully characterised (de Villiers *et al.*, 1997, 2004).

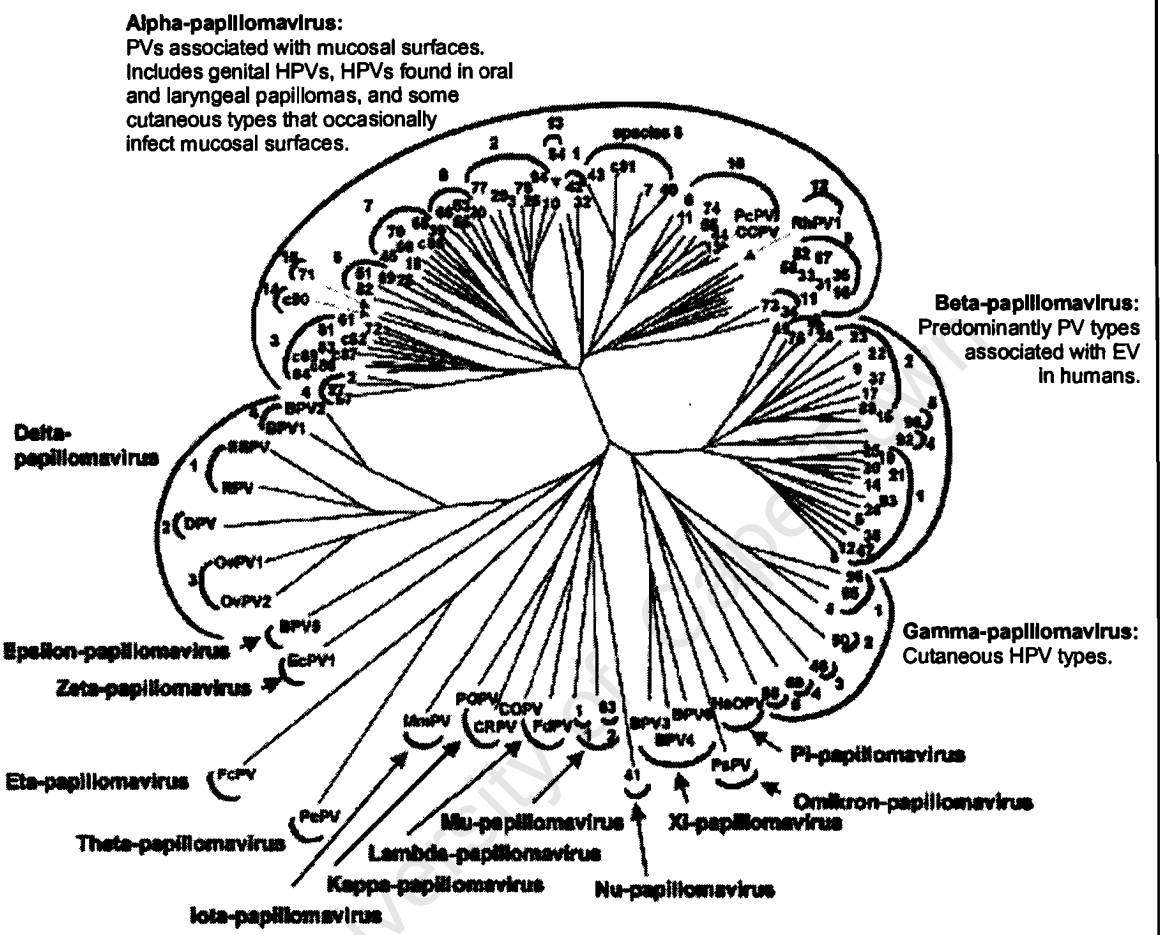

Figure 1.1. The papillomavirus phylogenetic tree. This phylogenetic tree is based on the L1 gene sequences from 118 PV types. The numbers at the end of each branch identify HPV types, while the animal PVs are abbreviated. The numbers at the inner semicircles represent the papillomavirus species, while the outermost semicircles represent the genera. Modified from de Villiers *et al.* (2004).

HPVs can be subdivided according to their tendency to affect either cutaneous or mucosal epithelia, and then further grouped according to their propensities to cause malignancy. Lesions induced by different types of anogenital HPVs show great variation in their risk for malignant progression; anogenital HPVs are therefore broadly classified into high-risk HPVs,

such as types 16, 18, 31, and 33; and low-risk HPVs, including types 6 and 11 (which are very rarely found in malignant tumours) (zur Hausen and de Villiers, 1994).

1.3. PV STRUCTURE AND NUCLEIC ACIDS

1.3.1. Virion structure

PVs are small (~55nm in diameter) non-enveloped viruses with icosahedral symmetry; consisting of a protein capsid which encapsulates a double-stranded, circular DNA genome. The capsid consists of 72 pentameric capsomeres that are arranged on a *T*=7 icosahedral lattice, with all except the cottontail rabbit papillomavirus displaying a *d* (right) hand skew lattice (Baker *et al.*, 1991; Pfister and Fuchs, 1994)).

Figure 1.2. A three-dimensional image reconstruction from cryoelectron microscopic analysis of a papillomavirus particle. From Baker *et al.* (1991).

1.3.2. Genome Organisation and Function

The PV genome consists of between 7500 and 8000 base pairs, and forms a chromatin-like complex by associating with cellular histones. All protein-encoding sequences are located on one of the DNA strands, which contains at least 8 open reading frames (ORFs). All PVs share a similar genetic organisation (see Figure 1.3), which can be divided into three basic regions: (1) an early (E) region, encoding proteins involved in DNA replication, transcription and cell transformation; (2) the late region, encoding the capsid proteins; and (3) the non-coding region, the long control region (LCR), which contains control elements for transcription and replication (Howley and Lowy, 2001; Pater *et al.*, 1994).

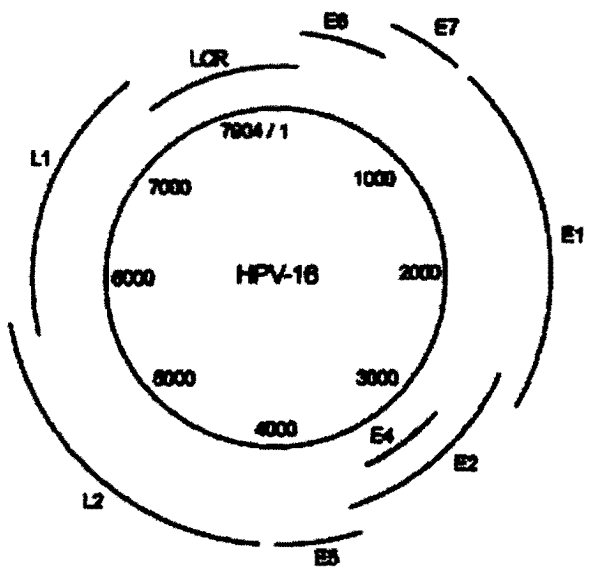

Figure 1.3. Organisation of a papillomavirus genome. The bars represent the open reading frames of the early (E) genes, the late (L) genes, and the long control region (LCR). Modified from Lowy and Howley (2001).

Table 1.1. Summary of the functions of the ORFs of HPV.

ORFs	Function
L1	Major capsid protein
L2	Minor capsid protein
E1	Initiation of viral DNA replication (Chow and Broker, 1994).
E2	Transcriptional regulator with replication functions (zur Hausen and de Villiers, 1994).
E4	HPV-16 E4 causes the collapse of the cytokeratin network, possibly to aid the release of virions (Roberts *et al.*, 1993). E4 can cause the arrest of the G2 phase of the cell cycle (Nakahara *et al.*, 2002)
E5	E5 has weak transforming activity and is able to interact with growth factor receptors (Howley and Lowy, 2001).
E6	Transforming activity. Binds and promotes degradation of the cellular tumour suppressor protein, p53 (Schmitt *et al.*, 1994).
E7	Transforming activity. Binds to the retinoblastoma protein, a tumour suppressor protein (Schmitt *et al.*, 1994).

Genes encoding the capsid proteins, L1 and L2

The late region of the PV genome contains two ORFs, encoding the major capsid protein (L1) and minor capsid protein (L2). Capsid synthesis and virus assembly occurs only in terminally differentiated keratinocytes (Ullman and Emery, 1996). The L1 protein represents about 80-90% of the viral protein, it has an average molecular weight of about 55 kDa (504 amino acids [aa]) and is the most highly conserved of all the PV proteins. In a number of systems, both eukaryotic (Kirnbauer *et al.*, 1992; Rose *et al.*, 1993; Hagensee *et al.*, 1993; Sasagawa *et al.*, 1995) and bacterial (Nardelli-Haefliger *et al.*, 1997), L1 proteins alone have been shown to self-assemble into non-infectious, empty capsid-like structures that closely resemble native virions.

L2 proteins have a molecular weight of approximately 76kDa (473 aa) and are not as well conserved as L1. Although L2 is not required for VLP assembly, the co-expression of L1 and L2 increases the VLP yield by about four-fold in the baculovirus system (Kirnbauer *et al.*, 1993), and by about 100-fold in mammalian cells (Hagensee *et al.*, 1993). L2 is able to bind to DNA, indicating a possible role in binding viral DNA during viral particle assembly (Zhou *et al.*, 1994). It is suggested that the HPV capsid consists of a ratio of 12 L2 molecules to 360 (72 pentamers) molecules of L1 (Kirnbauer *et al.* 1993).

PV virions infect cells via a clathrin-dependent receptor-mediated endocytic pathway (Day *et al.*, 2003). Although VLPs containing only L1 are sufficient for enabling infection, L2 can enhance infection by facilitating virion entry, and plays a role in determining HPV tropism (Kawana *et al.*, 2001; Yang *et al.*, 2003). The finding that neutralising antibodies can be generated to L2 following immunisation with capsids, suggests that the L2 protein is positioned at the surface of the virion (Heino *et al.*, 1995; Roden *et al.*, 1994).

Both the L1 and L2 proteins have nuclear localisation signals (NLS) to facilitate the translocation of these proteins to the nucleus. The NLS of HPV-16 L1 consists of clusters of basic amino acids (KRKKRK, aa 499 to 504; KRK, aa 484-486; and KR, aa 499 and 500), located at the carboxy terminus (Zhou *et al.*, 1991c). The HPV-16 L2 NLS (RKRRKR) is situated at aa 456 to 461 (Zhou *et al.*, 1991c). Nelson *et al.* (2000, 2003) found that HPV capsomeres are recognised by the karyopherin (Kap) α2 adapter, and are then translocated from the cytoplasm to the nucleus via the Kap α2β1-mediated pathway. They established that L1 binds to the Kap β3 and Kap β2 import receptors via interaction with its NLS.

1.4. LIFE CYCLE

Transmission of HPVs is facilitated by the presence of abraded epithelia, allowing the infection of the basal keratinocytes of squamous epithelia. The basal cells are the only cells in the squamous epithelium capable of dividing; the virus must therefore infect this layer in order to induce a lesion which can persist. This is followed by infection which can be latent, subclinical or clinical (Howley and Lowy, 2001).

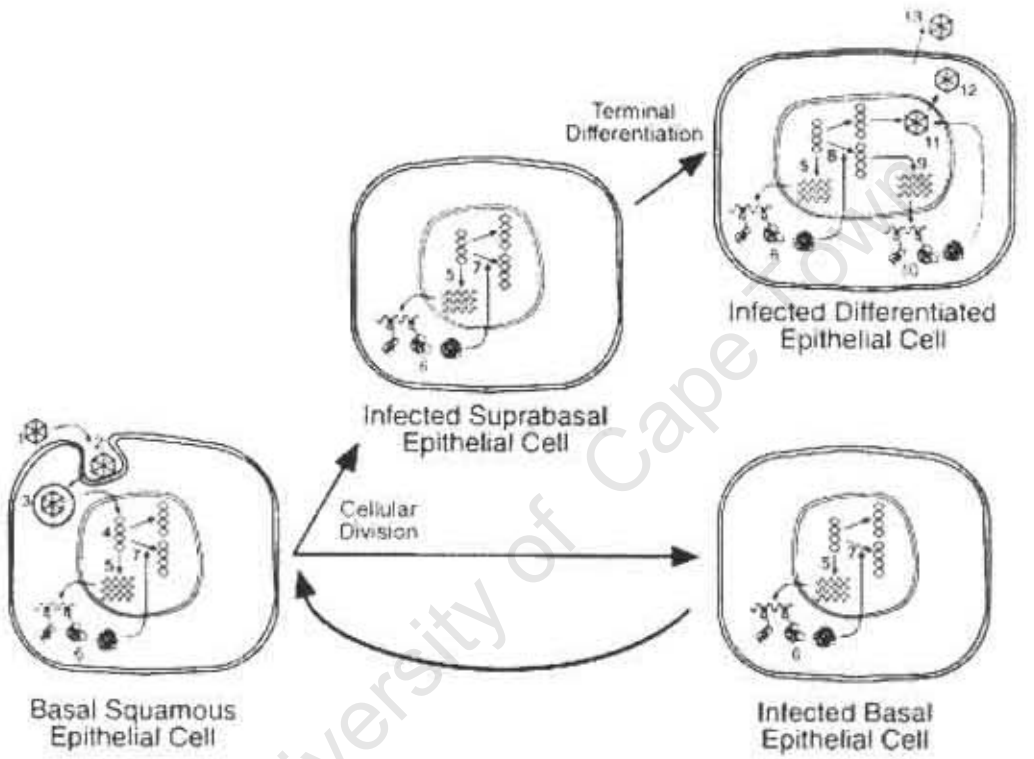

Figure 1.4. The life cycle of papillomavirus. Infection of a basal epithelial cell (1, 2), followed by endocytosis, viral DNA uncoating (3), and transport to the nucleus (4). Transcription of early genes (5), translation (6) and viral replication (7) in basal and suprabasal epithelial cells. Vegetative viral DNA replication (8), late gene transcription (9), production of capsid proteins (10) and assembly of virions (11) take place in differentiated keratinocytes. Nuclear breakdown (12) and release of virus then occurs (13). From Howley and Lowy (2001).

The viral transcriptional activity is regulated at a low level and viral DNA replication only occurs when these basal and parabasal cells enter the S phase of the cell cycle. The viral genome is maintained at low copy numbers, less than 20-50 per cell. After the cells ascend,

they leave the cell cycle and undergo progressive differentiation. In infected cells, the progression of the differentiation program of the epithelium becomes retarded, resulting in an increased thickness of the spinous cells (reviewed in Chow and Broker, 1994). This leads to various grades of papillomatosis and epithelial hyperplasia. During the proliferative phase, viral DNA replication and synthesis of structural proteins remains restricted. This restriction is released upon entry into the differentiating cells layers, where vegetative viral DNA replication, structural protein synthesis, and virion particle assembly take place. Malignant conversion of benign lesions leads to the termination of vegetative PV replication (Taichman and LaPorta, 1987).

Since lytic replication does not take place in the basal keratinocytes, the germinative cells of the epithelium are protected from the cytopathic effects caused by late gene products. Virions are only produced in the terminally differentiated cells, which are later shed into the environment, ensuring the spread of the virus, and the continued growth of the host keratinocytes (Taichman and LaPorta, 1987).

1.5. EPIDEMIOLOGY AND DISEASE

1.5.1. HPV associated diseases

Human papillomaviruses are extremely widespread, and due to the numerous different types almost everybody will come into contact with HPV during their lifetime. The spectrum of clinical manifestations associated with HPV ranges from asymptomatic to benign and malignant lesions, the most common being the production of warts, which are benign tumours. Clinical manifestations associated with the different types can be divided into cutaneous (common wart, plantar wart, flat wart etc.) and mucosal manifestations (genital warts, cervical cancer, respiratory papillomas etc.) (reviewed in Howley and Lowy, 2001).

There is a strong association between the HPV type and clinical manifestation (see Table 1.2). A number of HPV types induce benign self-limiting proliferations of the skin and mucosa. These lesions usually regress, either spontaneously or after treatment. They are classed as low-risk viruses as they are never detected in invasive carcinomas. HPV types 6 and 11 are low-risk viruses that cause genital warts; however, they are occasionally associated with low-grade cervical intraepithelial neoplasia (CIN) (Greer *et al.*, 1995). Some

HPV types are associated with the development of invasive cancers; these are known as high-risk viruses.

Table 1.2. HPV types and major clinical manifestation. From Lowy and Howely (2001).

Clinical type	Associated HPV(s)
Skin warts	
Deep plantar wart	HPV-1
Common wart	HPV-2, -4
Mosaic wart	HPV-2
Flat wart	HPV-3, -10, -28, -41
Reddish-brown plaques of epidermodysplasia verruciformis (EV)	HPV-5, -8, -9, -12, -14, -15, -17, -19, -20, -21, -22, -23, -24, -25, -36, -37, -38, -47, -49
Butcher's warts	HPV-7
Genital tract	
Subclinical infection	All genital HPVs
Exophytic condyloma (any site)	HPV-6, -11
Flat condyloma (especially cervix)	HPV-6, -11, -16, -18, -31, and others
Bowenoid papulosis	HPV-16
Giant condyloma (Bushke-Lowenstein tumour)	HPV-6, -11
Cervical cancer	
Strong association	HPV-16,-18,-31,-45
Moderate association	HPV-33,-35,-39,-51,-52,-56,-58,-59,-68
Weak or no association	HPV-6,-11,-26,-42,-43,-44,-53,-54,-55,-62,-66
Vulvar cancer	HPV-16
Penile cancer	HPV-16
Respiratory papillomas	HPV-6,-11
Conjunctival papillomas	HPV-6,-11
Oral cavity	
Focal epithelial hyperplasia	HPV-13,-32
Infection with genital tract HPVs	HPV-6,-11,-16
Lesions on lip	HPV-2

1.5.2. Cancer of the cervix

In 1995 the World Health Organisation (WHO) declared HPV a human carcinogen (International Agency for Research on Cancer [IARC]. It is now accepted that certain HPV types, such as HPV-16 and HPV-18, play an important role in anogenital carcinogenesis, especially in carcinoma of the cervix (Clifford *et al.*, 2003; Bosch *et al.*, 1995). Cervical cancer is the second most prevalent cancer worldwide and the most common cancer in developing countries (International Agency for Research on Cancer [IARC] 1999). Annually, approximately 500 000 women are diagnosed with cervical cancer and there are over 200,000 deaths from this disease each year, of which, 80% occur in developing countries. Sub-Saharan Africa is the area of highest cervical cancer mortality rates, with age-standardised rates (ASRs) ranging between 10.7 (Western Africa), 24.3 (Eastern Africa) and 17.1 (South Africa) (Pisani *et al.*, 1993). More recently, the risk in South African women was found to be even higher, with an ASR of 30.5 per 100 000 (Sitas *et al.*, 1998).

In an attempt to identify the most prevalent HPV types associated with invasive cervical cancer, Clifford *et al.* (2003) collated published data from 85 studies, with a total of 10 058 cases (8550 squamous cell carcinoma (SCC), 1508 adeno-and adenosquamous-carcinoma (ADC). The overall detection of HPV DNA in the cancer samples was 83-89%. The most prevalent HPV types, in order of decreasing prevalence, were HPV-16, -18, -45, -31, -33, -58, -52, -35, -59, -56, -6, -51, -68, -39, -82, -73, -66 and -70. In SCC the HPV prevalence was 87.3%; HPV-16 was the most common type (46-63%) followed by HPV-18 (10-14%), -45 (2-8%), -31 (2-7%) and -33 (3-5%). In Asia, however, HPV-58 (6%) and HPV-52 (4%) were identified frequently. The HPV prevalence was lower in ADC (76.4%), where HPV-18 (37-41%) was the most common type, followed by HPV-16 (26-36%) and -45 (5-7%). In South Africa, HPV-16 was the most frequently detected type in cervical cancer biopsies (82%), followed by HPV-18 (10%), and HPV 33 (10%) (Kay *et al.*, 2003).

1.5.2.1. Progression of infection to cervical cancer

The first step in the development of cervical cancer is appearance of abnormal cells (non-differentiating basal-like cells) which replaces the normal cervical epithelium. This may regress, remain unchanged or progress to high-grade squamous intraepithelial lesions (HSILs) and invasive cervical cancer. The process from the appearance of the first abnormalities to the development of cervical cancer can take years, and it is during this interval where these abnormalities can be detected by Pap smear and treated (reviewed in Lowy and Howley, 2001). Approximately 75% of detected SIL will not progress (41% will

persist and 34% will regress); while of the 25% that does progress, 10% will progress to carcinoma *in situ* and 1% to invasive cancer (Braly *et al.*, 1997).

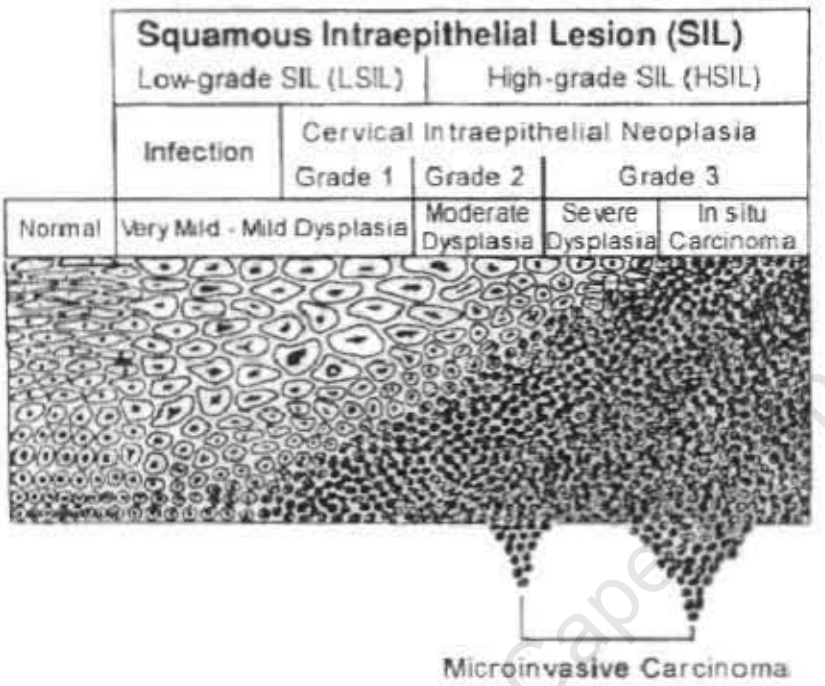

Figure 1.5. A schematic representation of cervical cancer precursors and the various terminologies that are used to refer to them. Modified from Lowy and Howley (2001).

1.5.2.2. Evidence of a HPV-cervical cancer association

Several findings point to an HPV-cervical cancer association:

- HPV DNA is consistently detected in cervical cancer biopsies. Depending on technique used, this ranges from 42 to 100%. Studies coordinated by the International Agency for Research on Cancer, of cervical cancer in 22 countries, identified HPV DNA in 99.7% of these cancers (Clifford *et al.*, 2003). Besides, cervical cancer, 50% of the biopsies from cancers of the vulva and penis are HPV-positive; and about 85% of anal cancers are HPV-positive (reviewed in zur Hausen, 1996; IARC, 1999). The HPV types found in cancers are usually the high-risk types;

with HPV types 16 and 18 detected in the vast majority of cancer samples. The mere presence of HPV DNA, however, does not prove an etiological involvement.

- HPV DNA is maintained as an episome in benign productive lesions, however, integrated HPV DNA is identified in immortalised human keratinocytes, in cells lines isolated from cervical neoplasms, and in 100% of squamous cell cancer biopsies of the cervix (Cooper *et al.*, 1991; Cullen *et al.*, 1991).
- The E2 ORF is usually disrupted during integration leading to disruption of the E2 repressor, which results in over-expression of the E6 and E7 oncoproteins (Cullen *et al.*, 1991).
- E6 and E7 are consistently expressed in cancer cells. These proteins interact with tumour suppressor proteins and are able to immortalise human cells. When E6 and E7 expression is blocked in cervical carcinoma cell lines, using complementary RNA, cell growth is reduced and there is a reversion of the malignant phenotype (von Knebel *et al.*, 1988).

1.5.3. Other cancers

When all the global cancer incidences that have HPV associations are added up, including, cervical cancer, causing approximately 5.8% of worldwide cancer, 50% of cancers of the penis, vagina and vulva, 85% of anal cancers, and 50% of oesophageal cancers; approximately 6-10% of the estimated 9 million cases of cancer worldwide (excluding non-melanoma skin cancers) can be linked to HPV infection (IARC, 1999). Women from developing regions, like Latin America, Southwest Asia, and Sub-Saharan Africa, have the highest prevalences of HPV-induced cancers, with 20-24% of cancers attributable to HPV (IARC, 1999).

Oesophageal cancer accounts for the sixth highest number of cancer deaths worldwide. Southern Africa has the highest age-standardised rate of oesophageal cancer mortality worldwide in the male population (ASR = 29.5) and the second highest in the female population (ASR = 7.9) (Pisani *et al.*, 1993). Recent PCR data suggests an association between HPV and oesophageal cancer as most authors have detected HPV in approximately 50% of these tumours (Matsha *et al.*, 2002; Ringstrom *et al.*, 2002). More studies have to be performed as data are limited and results vary greatly between studies. A study on oesophageal carcinoma samples from Chinese patients detected HPV-16 DNA in

up to 72% of the tumours and HPV-18 in up to 17%, both often in the same tumour (Li *et al.*, 2001). Cooper *et al.* (1995) detected HPV DNA in 52% of South African oesophageal tumours, with HPV-16 in 84% of the HPV-positive cancers. Ritchie and colleagues (Ritchie *et al.*, 2003) established that the prevalence of HPV DNA in carcinomas of the oral cavity and oropharynx was 21%, of which 86% was HPV-16. They found a higher risk of HPV infection in men, and in patients with a history of oral-genital sex.

The earliest evidence for the link of HPV and skin cancer is from studies on patients with EV. There are about 20 HPV-types that are frequently detected in about 90% of EV cancers (zur Hausen, 1996) (see Table 1.2.). HPV may be an etiologic agent in non-melanoma skin cancer and keratoacanthomas as it is detected in about 50% of these lesions, with a higher prevalence in immunosuppressed patients (Lloveras *et al.*, 2002; Forslund *et al.*, 2002). More studies must be done in this field to confirm these results.

It is estimated that HPV (mainly HPV-16) is associated with 85% of the 44 000 cases of anal cancer worldwide (IARC, 1999). Danish and Swedish studies detected high-risk HPV types in 100% of anal squamous carcinoma samples from homosexual men, while 58% and 90% of samples were HPV positive from heterosexual men and women, respectively (Frisch, 2002)

About 50% of cancers of the penis, vagina and vulva are associated with HPV infection (IARC, 1999). HPV DNA is detected in 42% of cases of penile carcinoma, 90% of dysplasia, and 100% of cases of condyloma. The prevalence of HPV is different in different histological cancer subtypes; HPV is detected in 34.9% of squamous cell carcinoma, 33.3% of verrucous carcinoma, 80% of basaloid and 100% of warty tumour subtypes (Rubin *et al.*, 2001).

1.6. IMMUNOLOGICAL ASPECTS OF HPV INFECTION

1.6.1. Natural immunity to HPV

Although immune responses elicited to HPV infections are usually weak, due to limited exposure of viral antigens to the immune system, the immune response does play an important role in the course of PV infection, as not all HPV infections lead to disease and some HPV lesions regress spontaneously.

1.6.1.1. Cell-mediated immunity

Cell-mediated immune responses are thought to play an important role in HPV disease progression as individuals with cell-mediated immune deficiencies, such as AIDS patients and transplant recipients, have an increased risk of infection with HPV (reviewed in Koutsky 1997), and of developing cervical cancer (Petry *et al.*, 1994).

Studies have shown that regressing genital warts are characterised by an active cell-mediated immune response in which regressing tumours are infiltrated with macrophages and T lymphocytes (Heller *et al.*, 2003; Coleman *et al.*, 1994). In the CRPV model, Selvakumar and colleagues (1997) detected the infiltration of mainly $CD8^+$ T cells into the basal and suprabasal layers of the epidermis during wart regression. The human or genital situation may be slightly different; as mainly $CD4^+$ T cells, were found both within the stroma and on the surface epithelium of regressing genital warts. There is also a significant induction of immune accessory molecules, such as the major histocompatibility complex (MHC) class II molecules and the intercellular adhesion molecules (ICAM1) on keratinocytes; and E-selectin and VCAM1 on the endothelial cells in these regressing warts (Coleman *et al.*, 1994).

A number of human T cell epitopes have been mapped to the HPV-16 L1, E6 and E7 proteins (Strang *et al.*, 1990; Stauss *et al.*, 1992). T-cell responses to HPV E2, E6, E7 and L1 have been identified in humans with cervical dysplasia (Bontkes *et al.*, 1999a; Bontkes *et al.*, 2000; Shepherd *et al.*, 1996), but also in a large number women who have cleared infection (de Gruijl *et al.*, 1999). HPV-16 E6-specific (Welters *et al.*, 2003), L1-specific (Shepherd *et al.*, 1996) and E2-specific (de Jong *et al.*, 2002) interferon gamma (IFN)-γ-producing circulating T cells have been detected in up to 60% of healthy individuals, suggesting that circulating T cells may play a role in protection against persistent HPV infection and disease progression. The cervical cytokine patterns detected in women preceding clearance of infection consisted of the expression of IFN-γ and interleukin-4 (IL-4), which are indicative of a T-helper (Th1) type response (Scott *et al.*, 1999). The precise role of naturally occurring HPV-specific cytotoxic T lymphocyte (CTL) immunity must still be established as some studies show high HPV-CTL activity in patients with advanced cervical disease, but not in women who have cleared HPV infection (Bontkes *et al.*, 2000; Welters *et al.*, 2003; Shepherd *et al.*, 1996).

1.6.1.2. Humoral immunity

Numerous studies have looked into the use of HPV-specific antibody detection for diagnostic, prognostic and epidemiological purposes. Serological assays have focused on responses to HPV L1, but many studies have also detected antibodies to L2, E2, E4, E6, E7 (discussed below).

Serum IgG-positivity to HPV L1 increases with increasing severity of cervical disease and with disease persistence (Wideroff *et al.*, 1995; Carter *et al.*, 1996). Natural IgG antibodies to L1 therefore do not seem to have an influence on existing cervical disease; suggesting that other mechanisms are involved cervical lesion clearance. In a study by Wideroff *et al.* (1995), anti-L1 antibodies were detected in 16.6% of HPV-16 positive but normal-cytology controls, compared to 30.8% and 52.4% of cases with low- and high-grade cervical lesions, respectively. They also found that HPV-16 VLP seropositivity increased from 22% in subjects who were HPV-16 DNA-positive once, to 83.3% in those with persistent infections. Other studies have reported HPV-16 VLP seropositivities in up to 81% of women with high grade SIL (Kirnbauer *et al.*, 1994; Nonnenmacher *et al.*, 1995). Seropositivity appears to be lower in patients with invasive cancer, perhaps due to the cessation of productive HPV infection in invasive cancer cells (Nonnenmacher *et al.*, 1995).

Cervical mucosal IgA antibodies to HPV VLPs of the oncogenic HPV types, 16, 18, 31 and 45, are also associated with HPV infection. Mucosal IgA antibodies to the oncogenic HPV VLPs have been detected in up to 72% of women with oncogenic HPV infections (Sasagawa *et al.*, 2003; Wang *et al.*, 1996). As with IgG seropositivity, the mucosal IgA-positivity decreases with progression to CIN II and III and cervical cancer. Mucosal IgA antibody responses are, therefore also, not likely to be involved with disease clearance. In a longitudinal study, Bontkes and colleagues (1999b) investigated the association between local and systemic IgG and IgA immunity to HPV-16 VLPs in 125 patients. Their data showed that the cervical IgG and IgA, and systemic IgG antibodies to VLPs did not correlate with virus clearance, but that systemic IgA responses were associated with clearance. Since the systemic IgA responses were not accompanied by cervical IgA responses, they suggest that the systemic response is a by-product of a successful cell-mediated immune response induced at the cervical lymph nodes, and is mediated by cytokines. However, humoral responses will likely be important to prevent new infection. In a study of female university students, Ho *et al.* (2002) found that the presence of both serum IgA and IgG antibodies to HPV-16 VLPs was associated with a decreased risk for subsequent infection, and that this association decreased as the genetic relatedness to HPV-16 decreased.

The highest anti-E6 and -E7 antibody prevalences are found at in patients with invasive cervical cancer; and do not correlate with the detection of anti-L1 antibodies (de Sanjose *et al.*, 1996; Gaarenstroom *et al.*, 1994; Nonnenmacher *et al.*, 1995). This is perhaps because, although E6 and E7 are early proteins, the normal expression of these proteins is disrupted in high-grade cervical intraepithelial neoplasia (CIN), causing them to be expressed consistently throughout the lesion (reviewed in zur Hausen 1987). HPV-16 E6 seropositivity ranges from 2-6% in CIN patients to 54.1-56% in cancer patients (Nonnenmacher *et al.*, 1995; Sun *et al.*, 1994). Antibody prevalences to HPV-16 E7 range from 4.6-13% in control subjects, to 6-31% in CIN patients, to 43-63% in cancer patients (de Sanjose *et al.*, 1996; Gaarenstroom *et al.*, 1994; Nonnenmacher *et al.*, 1995; Sun *et al.*, 1994). In women with CIN, Marais *et al.* (1997) found that antibody prevalences to HPV-16 E2 increased with age, while seropositivity to HPV-16 VLPs decreased with age. In an interesting study, a cohort of women with mild to moderate cervical dyskaryosis were tested for serum antibodies to HPV-16 E7 for a period of up to 27 months (de Gruijl *et al.*, 1996). The highest proportion of E7 seropositive responders were those who had cleared infection, while the lowest responders had persistent infection. IgG_2 was predominant in the women who had cleared the infection. Patients with cervical carcinoma had equal amounts of IgG_1 and IgG_2, suggesting that clearance was associated with a cell-mediated Th1 response.

1.6.2. Immune evasion

The exposure of HPV viral antigens to the host's immune system is restricted since the majority of HPV protein expression occurs in the upper layers of the epithelium, and lysis of infected cells does not occur, but rather a shedding of virus into the environment (Taichman and LaPorta, 1987). This antigen restriction is the reason why antibodies to the HPV capsid proteins, which are very immunogenic (Kirnbauer *et al.*, 1992), are often only detected in patients with progressed cervical lesions, where the L1 protein is likely expressed in the differentiated epithelium (Wikstrom *et al.*, 1995), and also why it takes so long for women to seroconvert (8.3 months) after infection is detected (Carter *et al.*, 1996).

There is also evidence that cervical carcinomas can limit the presentation of certain HPV epitopes, thereby decreasing recognition by CTLs. This is made possible by the down-regulation of MHC class I expression (Hilders *et al.*, 1994), and the down-regulation of the transporter proteins, TAP1 and TAP2, in cervical carcinoma cells (Evans *et al.*, 2001a). This will likely place constraints on the use of certain HPV CTL epitopes for the immunotherapy of

HPV-associated disease. Cervical cancer cells also promote the production of IL-4 and down-regulate the production of IFN-γ in cancer-encountered T cells, by producing IL-10 and TGF-β (Sheu *et al.*, 2001).

1.7. VACCINES FOR HPV

In 1993 the World Bank released a World Development Report addressing disease burden and cost-utility analysis of a variety of interventions to deal with health problems. The report evaluated 52 interventions and found that basic immunisations (BCG, DTP, OPV, and measles) were amongst the best health investments to make (Miller and Hinman, 1999). The cost of cervical cancer screening, follow-up, and treatment in the USA has been estimated at US $5 billion per year (IARC, 1999). In a study to determine the cost-effectiveness of a prophylactic vaccine to high-risk HPV, Sanders and Taira (2003) calculated that a vaccine with a 75% efficacy, administered to all 12-year-old girls in the USA, would result in a life expectancy gain of 4.0 quality-adjusted life days per individual, and would prevent more than 1,300 deaths from cervical cancer in their lifetimes. Although the life expectancy gain seems small at the individual level, population benefits would be considerable. The calculated cost savings was US$ 22,755 per quality-adjusted life year (QALY).

1.7.1. PV vaccine types and immunogenicity studies

Vaccines, traditionally, have been regarded as prophylactic, preventing new infection. In viral infections this is usually accomplished by the induction of neutralising antibody responses to surface epitopes of the virus. This has usually been achieved by the use of inactivated or attenuated virus; however, this is not possible with PV due to the absence of a permissive culture system, and hence the inability to propagate large amounts of virus (Lowy *et al.*, 1994). PV prophylactic vaccines therefore focus on the use of the virus-like particles (VLPs), which are similar in structure to native virions, and can elicit neutralising antibodies and PV protection in various animal models and humans (described below).

An estimated 300 million women, worldwide, are presently infected with HPV (IARC, 1999); and if left untreated, a percentage of these women will develop HPV-associated cancers. Many HPV vaccine strategies therefore focus on eliminating existing HPV infection and HPV-

associated tumours. These vaccines are predominantly aimed at inducing cell-mediated immunity to the early PV proteins. The early proteins are good targets for therapeutic vaccines as they are produced in the basal layers of HPV-associated lesions (Durst *et al.*, 1992), which are most likely to be infiltrated by lymphocytes. E6 and E7 are especially attractive targets as their expression is maintained during malignant progression (zur Hausen, 1987).

1.7.1.1. VLPs and other peptides

The most successful HPV prophylactic vaccine candidates, to date, are based on L1 VLPs produced by recombinant baculovirus or yeast. In animal and human studies, VLP vaccines have been well tolerated and have induced high titres of neutralising antibodies (Brown *et al.*, 2001; Christensen *et al.*, 1994; Harro *et al.*, 2001; Rose *et al.*, 1994b; Tobery *et al.*, 2003). Studies in the cow, rabbit and dog, have also shown that immunisation with homologous VLPs can protect against viral challenge (Breitburd *et al.*, 1995; Christensen *et al.*, 1996b; Kirnbauer *et al.*, 1996; Suzich *et al.*, 1995). It has been observed that only intact VLPs present the epitopes required to induce protective immunity, as studies using disrupted or denatured VLPs have induced low neutralising antibody responses, and have afforded little protection from challenge in animal models (Breitburd *et al.*, 1995; Suzich *et al.*, 1995; Kirnbauer *et al.*, 1992).

Ohlschlager *et al.* (2003) demonstrated that HPV-16 pentameric capsomeres (10 amino acid truncation at the L1 amino-terminal) were able to elicit CTL responses in mice, which were similar to that produced by intact VLPs, and that this immunity was capable of inducing regression of L1-expressing tumours. Capsomeres were found to induce high-titre, type-specific neutralising antibodies in mice; with neutralisation titres similar (HPV-11 capsomeres) or 10- to 20-fold lower (HPV-33) than those induced by intact VLPs (Rose *et al.*, 1998; Fligge *et al.*, 2001).

In cows, prophylactic and therapeutic immunisation against mucosal bovine papillomavirus (BPV) has been achieved. Immunisation with BPV-4 L2, BPV-4 E7 (Campo *et al.*, 1993), BPV-4 L1/L2 VLPs or BPV-4 L1 VLPs (Kirnbauer *et al.*, 1996) achieved almost complete protection against BPV-4 infection; and also induced a therapeutic response, evident by the faster regression of lesions that had appeared. Immunisation of dogs or rabbits with L1 VLPs or L1/L2 VLPs has successfully induced neutralising antibodies and protection against challenge with canine oral papillomavirus (COPV) (Suzich *et al.*, 1995) or CRPV (Breitburd *et al.*, 1995; Christensen *et al.*, 1996b), respectively. Selvakumar *et al.* (1995) stimulated

regression of papillomas in the CRPV model after immunisation with E1 or E2 protein. They found that the best predictor of wart regression was a T cell proliferative response to E2.

In rhesus macaques, vaccination with HPV-16 L1 VLPs generated a strong neutralising antibody response and a strong L1-specific Th2 response, measured by IL-4 production by $CD4^+$ T cells (Tobery *et al.*, 2003). Systemic immunisation of African green monkeys with HPV-11 VLP in an alum adjuvant induced strong VLP-specific neutralising serum antibodies and significant neutralising antibodies in cervicovaginal secretions; suggesting that protection at the cervix may be possible by systemic VLP immunisation (Lowe *et al.*, 1997).

The mucosal administration of VLP has been investigated to specifically induce mucosal immunity. Oral immunisation of mice, by gavage, with HPV-11 VLPs elicited anti-L1 serum IgG and IgA neutralising antibodies (Rose *et al.*, 1999). Two groups showed that mucosal adjuvants could enhance VLP immunogenicity when administered mucosally. Gerber *et al.* (2001) demonstrated that anti-VLP humoral responses in peripheral blood and in genital mucosal secretions were significantly improved when HPV-16 and HPV-18 VLPs were administered orally with the co-administration of a *Escherichia coli* heat-labile enterotoxin mutant or CpG DNA. Balmelli *et al.* (1998) noted that systemic immunisation of mice with HPV-16 VLPs generated low titres of IgG in genital secretions, but no genital IgA could be detected. However, when VLPs, together with cholera toxin, were administered intranasally, they detected high titres of anti-VLP IgA and IgG in saliva and genital secretions. Serum antibodies were also enhanced 10-fold by the use of cholera toxin. Antibodies in serum, saliva and genital secretions were strongly neutralising. They, however, found that oral immunisation of VLPs, even with the cholera toxin adjuvant, was inefficient at generating antibodies.

The first VLP "proof of principle" phase 2 vaccine trial in humans showed very promising results. In a trial group consisting of about 2400 young women (aged 16-23), Koutsky and colleagues (2002) administered 3 doses of HPV-16 VLPs or placebo. The trial participants were followed for an average of 17.4 months, during which genital samples were analysed every six months. The incidence of persistent HPV-16 infection was 3.8 per 100 woman-years at risk in the placebo group and 0 per 100 woman-years at risk in the vaccine group. The efficacy of the vaccine regimen was therefore 100% (95% CI = 90-100). HPV-16 VLP administration therefore reduced the incidence of HPV-16 infection, but it also appeared to reduce the development of HPV-16-related CIN. In January 2000, a GlaxoSmithKline-coordinated phase 2 trial was started in which a bivalent HPV-16/HPV-18 VLP vaccine was

tested in humans (Harper *et al.*, 2004; Billich, 2003). They reported a vaccine efficacy of 91.6% (95% CI = 64.5-98) against incident infection, and 100% efficacy against persistent infection with HPV-16 and HPV-18. In addition, a vaccine efficacy of 92.9% (95% CI = 70.0-98.3) was achieved against HPV-16/HPV-18-associated cytological abnormalities. In a phase 1 study in humans, HPV-11 VLPs were well tolerated and induced high levels of neutralising antibodies, as well as increased lymphoproliferation to HPV-11 L1. Interestingly, increased lymphoproliferation was also generated to L1 from HPV-6, -16, and -18; which demonstrates that some T cell helper epitopes are conserved across HPV types (Evans *et al.*, 2001a). Other phase 1 human trials have proven the safety and immunogenicity of HPV-18 VLP (Ault *et al.*, 2004), HPV-11 or HPV-16 VLPs (Fife *et al.*, 2004), and HPV-6/11/16/18 quadrivalent VLP (Villa *et al.*, 2002) candidate vaccines. In a small study of HPV-6b VLP vaccination in human subjects, Zhang *et al.* (2000) observed complete regression of genital warts in 25 of the 33 subjects, suggesting the therapeutic use of VLP for this and other genital HPVs.

The minor capsid protein, L2, has been used successfully in vaccine studies, either alone or in combination with L1, in L1/L2 VLPs. L1/L2 VLPs, produced in baculovirus, form VLPs more efficiently than when L1 is used alone (Kimbauer *et al.*, 1993), however, L1 VLPs are preferred as they are sufficient at inducing strong neutralising responses, without making the production process more complicated. Furthermore, since L2 is able to bind DNA non-specifically (Zhou *et al.*, 1994), it may introduce DNA into the VLP preparations. Vaccination of rabbits and cows with L2 protein is able to protect against challenge, indicating that L2 is able to elicit neutralising antibodies (Lin *et al.*, 1992; Gaukroger *et al.*, 1996).

It is possible to fuse small peptides onto VLPs, to produce chimaeric VLPs (cVLPs) that are similar to normal VLPs in structure and antigenicity. Two groups have shown that chimaeric HPV-16 L1/L2 VLPs, to which E7 was fused, were able to protect mice against HPV-16 E7-positive tumour challenge; they showed that this protection was mediated by MHC class I-restricted CTLs (Greenstone *et al.*, 1998; Wakabayashi *et al.*, 2002). Varsani *et al.* (2003a) demonstrated that HPV-16 cVLPs presenting an L2 epitope were able to elicit antibodies to both L1 and L2 in mice.

1.7.1.2. Live viral vectors

VLP preparations are expensive to manufacture, and will likely not be utilised for widespread vaccination in developing countries. Numerous groups have therefore investigated the use of live vaccines as cheaper alternatives to VLPs.

Vaccinia virus is an attractive poxvirus vector due to its extensive use and relatively large capacity for foreign DNA. In 1991, Zhou and colleagues (1991a) demonstrated the generation of VLPs by recombinant vaccinia virus expressing HPV-16 L1 and L2. Vaccinia virus expressing HPV-16 L1 was then shown to elicit L1 specific CTL immunity in mice (Zhou *et al.*, 1991b). Protection against CRPV challenge was observed in rabbits immunised with recombinant vaccinia virus expressing CRPV L1 (Lin *et al.*, 1992). Vaccinia virus expressing the HPV-16 E6 protein has been used successfully to elicited E6-specific antibody, lymphoproliferative and CTL responses in mice (Gao *et al.*, 1994); and immunity elicited by a HPV-16 E7 vaccinia virus recombinant in mice was shown to induced regression of HPV-16 immortalised tumours (Lamikanra *et al.*, 2001).

There are health concerns about the use of vaccinia virus as there is a possibility of dissemination in immunocompromised individuals. The use of modified vaccinia Ankara (MVA), a replication deficient poxvirus vector, may overcome this problem (Baxby, 1993). An MVA-E2 recombinant was successfully used to treat E2-expressing tumours in rabbits (Rosales *et al.*, 2000) and mice (Valdez *et al.*, 2000). Interestingly, antibody responses, but not CTL responses were detectable in the rabbits, which suggested that antibody-dependent macrophage cytotoxicity played a dominant role in the regression of the tumours.

In rhesus macaques, a replication-defective adenovirus vector expressing codon-optimised HPV-16 L1 elicited strong Tc1 (cytotoxic T cells type 1) responses, as measured by production of IFN-γ by $CD8^+$ T cells and a lack of IL-4 production (Tobery *et al.*, 2003). Although a humoral response was elicited, the titre of neutralising antibodies was very low. This is in contrast to vaccination with VLPs in the same animal study, which generated a strong neutralising antibody response but failed to induce a $CD8^+$ T cell-mediated IFN-γ response.

1.7.1.3. Live bacterial vectors

Attenuated avirulent, but invasive *Salmonella* strains have been investigated as delivery vehicles for a number of bacterial, viral, and mammalian antigens (Nardelli-Haefliger *et al.*, 1996; Newton *et al.*, 1989; Srinivasan *et al.*, 1995; Vancott *et al.*, 1996). Live *Salmonella* are effective at delivering heterologous antigens to both the mucosal and systemic immune systems, and can induce cell-mediated, humoral and secretory IgA antibody responses (Vancott *et al.*, 1996). Nardelli-Haefliger *et al.* (1997) demonstrated by electron microscopy

that HPV-16 L1 expressed in recombinant attenuated *S. typhimurium* assembled into VLPs. This was the first demonstration that VLPs can self assemble in prokaryotes. They showed that nasal immunisation of mice was effective at inducing systemic neutralising antibodies, and also oral and vaginal anti-VLP IgA and IgG, indicating possible protection at mucosal sites. Revaz *et al.* (2001) demonstrated that mucosal vaccination of mice with recombinant attenuated *S. typhimurium* expressing HPV-16 L1 induced anti-tumour immunity, which was prophylactic and therapeutic. Immunisation of mice with recombinant *Salmonella* expressing HPV-16 E6 and E7 have also induced antibodies to these HPV proteins (Krul *et al.*, 1996). Although researchers have experienced instability problems with recombinant *Salmonella* expressing L1, more stable recombinants have been developed by using lethal vector systems and by codon-optimisation of L1 (Baud *et al.*, 2002).

The *Mycobacterium tuberculosis* vaccine, *Mycobacterium bovis* bacille Calmette-Guérin (BCG), has attractive properties as a vaccine vector (see Chapter 2). A variety of viral, bacterial and parasitic antigens have successfully been expressed in BCG. In experimental models recombinant BCG (rBCG) has been shown to elicit protective immunity against Lyme disease, pneumococcal infection, and cutaneous leishmaniasis (Abdelhak *et al.*, 1995; Langermann *et al.*, 1994a; Stover *et al.*, 1993). It has also been demonstrated that oral or intranasal immunisation with rBCG induces sustained systemic and mucosal specific immune responses (Langermann *et al.*, 1994a). A study by Jabbar *et al.* (2000) reported that rBCG expressing HPV-6b L1 or HPV-16 E7 antigens were only weakly immunogenic when compared with protein vaccines, and immunisation with rBCG expressing HPV-16 E7 was not able to protect against challenge with an E7-expressing tumour.

Listeria monocytogenes has also been investigated as a live bacterial vector for the expression of HPV genes. Lin *et al.* (2002) demonstrated the induction of an E7-specific CTL response in mice after oral administration with a *L. monocytogenes* recombinant expressing HPV-16 E7. Vaccination with this recombinant was able to prevent tumour growth in mice, as well as cause regression of tumours.

1.7.1.4. DNA

DNA vaccines usually involve the intramuscular injection of plasmid DNA, or delivery of DNA into the epidermis using a gene gun. A DNA vaccine plasmid contains a gene encoding the antigen of interest, the expression of which is controlled by a mammalian promoter. This method of vaccination has successfully elicited antibody and cell-mediated responses in a number of preclinical studies, however, DNA vaccine potency in humans has been low

(reviewed in Liu, 2003). The cost of producing DNA vaccines is also higher than originally thought – it costs approximately US$ 0.6 million for the manufacture of a DNA vaccine for a phase 1 trial (Anna-Lise Williamson Department of Clinical Laboratory Science, University of Cape Town; personal communication).

Rocha-Zavaleta and colleagues (2002) demonstrated the efficacy of DNA vaccines expressing HPV-16 L1 in mice. They showed that intramuscular, subcutaneous and oral vaccination elicited systemic anti-L1 IgG and vaginal IgA neutralising antibodies. The vaccine also elicited L1-specific CTL responses, and slowed tumour growth after challenge with an L1-expressing melanoma cell line.

Rabbits immunised intramuscularly with plasmid carrying the CRPV L1 gene generated high titres of neutralising antibodies, and were protected from CRPV challenge; indicating that L1 was presented in the correct conformational form to generate a protective response (Donnelly *et al.*, 1996). In rhesus macaques, a HPV-16 L1 DNA vaccine generated a primarily Th1/cytotoxic T cell type 1 (Tc1) response, which was measured by the production of IFN-γ by mainly $CD8^+$ T cells (Tobery *et al.*, 2003). Although a humoral response was elicited, the titre of neutralising antibodies was very low.

Immunogenicity of DNA vaccines can be enhanced by codon modification. Liu *et al.* (2002) showed that a DNA vaccine containing a synthetic codon optimised HPV-16 E7 gene expressed higher levels of E7 in mammalian cells than non-optimised E7; and that mice immunised with the optimised vaccine produced significantly higher antibody and CTL responses, and showed enhanced tumour protection. When utilising a codon-optimised COPV E1 sequence in a DNA vaccine, Moore *et al.* (2002) could completely protect dogs against mucosal COPV challenge, however, all animals vaccinated with the wild-type E1 developed papillomas.

An novel way of delivering DNA is by encapsulation in biodegradable polymeric microparticles made of poly-D,L-lactide-coglycolide. Plasmid DNA encapsulated in this manner, encoding HPV E6 and E7 epitopes, has been tested in mice and humans. Mice vaccinated with these microparticles were protected from E7-tumour challenge, and in a phase II human trial, 43% of women showed lesion regression, compared to 27% in the placebo group (Habeck, 2003).

Chen *et al.* (2000) investigated prime-boost strategies involving a DNA vaccine and vaccinia virus, both expressing the chimera, Sig/E7/Lamp-1, consisting of HPV-16 E7 linked to the sorting signals of the lysosome-associated membrane protein-1 (LAMP-1). Although both the DNA and vaccinia vaccines could induce anti-tumour immunity individually, in mice, they found that sequential vaccination with the DNA vaccine and then recombinant vaccinia generated a very strong E7-specific $CD8^+$ T cell response. Kowalczyk *et al.* (2001) showed that a prime-boost strategy with a DNA vaccine and replication-defective adenovirus, both expressing HPV-16 L1, successfully induced high antibody titres to intact VLPs. Although these vectors only produced marginal antibody titres on their own, when mice were primed i.m. with the DNA-L1 vaccine and boosted i.n. with the replication-defective adenovirus-L1 recombinant, the mice produced antibodies similar to that elicited by two doses of VLP. IgA antibodies specific to HPV-16 VLPs were also induced in the vaginal cavities of these mice.

1.7.1.5. HPV protein production in plants

Another low-cost vaccine strategy is the oral or parenteral delivery of HPV antigens that have been produced in plants. Bacterial and viral antigens have successfully been produced in plants, and have been shown to generate humoral and mucosal immune responses in animals after oral administration (Haq *et al.*, 1995; Richter *et al.*, 1996; Thanavala *et al.*, 1995). Franconi and colleagues (2002) expressed the HPV-16 E7 protein in the *Nicotiana benthamiana* plant using a potato virus X-derived vector. Mice immunised with the E7-containing crude foliar extracts developed both humoral and cell-mediated responses and were protected from E7-expressing tumour challenge.

Three groups have recently demonstrated that VLPs of HPV-16 and HPV-11 can be produced in transgenic plants. Warzecha *et al.* (2003) demonstrated that HPV-11 VLPs made in transgenic potato tubers were weakly immunogenic when fed to mice. Biemelt *et al.* (2003) and Varsani *et al.* (2003b) demonstrated efficacy of producing HPV-16 L1 VLPs in transgenic tobacco (*Nicotiana tabacum*), that were antigenically identical to baculovirus-synthesised VLPs as assayed by conformation-specific MAb binding, and were immunogenic if given to animals via gavage (Biemelt *et al.*, 2003) or by injection (Varsani *et al.*, 2003b). The feasibility of using a recombinant tobamovirus vector to make HPV-16 VLPs in *N. benthamiana* at yields ten times greater than in tobacco transgenic for the same gene, was also demonstrated (Varsani *et al.*, 2001; Varsani, 2003c)

1.7.2. Immunity summary and considerations for HPV vaccine strategies

Due to the nature of HPV infection, with the absence of a systemic stage, mucosal immunity will intuitively be important to prevent and clear infection. There are various methods to stimulate immunity at mucosal surfaces, but generally, mucosal immunisation successfully induces mucosal immunity. Numerous mucosal vaccination strategies, including the use of mucosal adjuvants, have successfully been used to induce papillomavirus-specific genital immunity in animal models (some of these are described in section 1.7.1).

A prophylactic vaccine would aim to provide neutralising antibody protection at genital mucosal surfaces. This protection is provided mainly by local secretory IgA antibodies (McGhee *et al.*, 1992), but these antibody responses are short-lived. Elfgren *et al.* (1996) demonstrated that local IgA antibodies to HPV VLP disappeared soon (months) after successful conisation treatment, whereas systemic IgA persisted for longer (years). Mucosal IgA and IgG antibodies are frequently detected in women with persistent infection, and are not correlated with virus clearance (Sasagawa *et al.*, 2003; Bontkes *et al.*, 1999b), which suggests that mucosal antibodies are not effective at inducing regression of established lesions. These mucosal antibodies are possibly only present due to continuous stimulation by the persistent infection. Although seropositivity to HPV L1 increases with increasing severity of cervical disease, if present before challenge, systemic antibodies can provide protection from mucosal PV challenge, as observed in experimental animal models (Kirnbauer *et al.*, 1996; Suzich *et al.*, 1995) and in natural infection in women (Ho *et al.*, 2002). The observed protection may be a result of sustained transudation of serum antibodies to the mucosa.

Although much emphasis is placed on the development of neutralising antibodies at the site of infection, these antibody responses are usually not long-lasting, and sterilising immunity rarely occurs. Local cell-mediated responses will therefore be vital for elimination of infected cells (Ada, 1999). Therapeutic vaccines aim to eliminate these virus infected cells by generating CTL responses to antigens derived from endogenously processed viral proteins, which have been presented by MHC class I molecules. Therapeutic vaccines for HPV are at a much earlier stage of development than their prophylactic counterparts. Several studies substantiate the role of the cell-mediated response in the clearance of HPV disease (these are described in detail in section 1.6.1.1): Individuals with cell-mediated immune deficiencies have an increased risk of infection with HPV (Koutsky, 1997), regressing HPV-tumours are infiltrated with macrophages and T lymphocytes (Coleman *et al.*, 1994; Heller *et al.*, 2003),

HPV-specific circulating T cells are often detected in healthy individuals (Shepherd *et al.*, 1996; Welters *et al.*, 2003), and anti-HPV IgG_1 and IgG_2 (de Gruijl *et al.*, 1996), and IFN-γ and IL-4 are detected frequently in individuals who have cleared infection, suggesting the role of a cell-mediated Th1 response. Therapeutic vaccines would focus on developing cellular immunity to HPV early proteins; however, the observation by Zhang *et al.* (2000), that immunisation with HPV-6 VLP may have caused regression of genital warts in human subjects, provided evidence the therapeutic potential of HPV L1 should also be evaluated. The presence of natural antibodies to HPV early proteins can be detected a subset of women before the onset of invasive cervical disease, but these responses are generally low and do not provide protection from disease. In cancer patients, however, immunity to E6 and E7 increases, which provides evidence that these proteins are being exposed to the immune system; and suggests the potential of directing vaccines to these proteins. There is also increasing interest in E2. More work must be done to determine the best antigen targets, vaccine types, and routes of immunisation.

Studies in human volunteers show that VLP-based vaccines, of types 6, 11, 16 and 18 (Fife *et al.*, 2004; Evans *et al.*, 2001b; Koutsky *et al.*, 2002; Villa *et al.*, 2002, Harper *et al.*, 2004), are safe and produce high antibody titres in almost all vaccine recipients. The human HPV-16 HPV (Koutsky *et al.*, 2002) and HPV-16/18 (Harper *et al.*, 2004) efficacy studies provided good evidence that VLPs will at least be partially effective in the prevention of HPV infection in humans (described above in section 1.7.1.1).

HPV VLP vaccines will likely have to be multivalent, as virions from different HPV types are antigenically distinct from one another (Rose *et al.*, 1994a), and although, there does appear to be some cross-protection between similar HPV types, these reactions are weak (Combita *et al.*, 2002). In women, the presence of natural IgA and IgG antibodies to HPV-16 VLPs was found to be associated with a decreased risk for subsequent infection; and this association decreased as the genetic relatedness to HPV-16 decreased (Ho *et al.*, 2002). Vaccination of mice with HPV-16, -31, -33, or -45 VLPs was shown to induce weak cross-neutralising reactivity to pseudovirions of the closely related phylogenetic types (Bousarghin *et al.*, 2002a; Combita *et al.*, 2002). Cross-neutralising reactivity was observed mainly between HPV types 16, 31, 33, and 58; however, it is not known whether this cross-reactivity is sufficient to offer cross-protection *in vivo*. Bousarghin *et al.* (2002b) showed that cross-neutralising could also be induced in humans after immunisation with VLP. This group investigated neutralising antibody responses against HPV-16, -18, -31, -33, and -58 after the administration of both HPV-16 and HPV-18 VLPs. They showed that 40% of the vaccine

recipients developed neutralising antibodies against types other than those included in the vaccine. Evans *et al.* (2001b) observed that the immunisation of humans with HPV-11 VLPs generated lymphoproliferation to L1 from HPV-6, -16, and -18; demonstrating the conservation of some T cell helper epitopes across HPV types. Bivalent and quadrivalent HPV VLP-based vaccine studies in women (Harper *et al.*, 2004; Villa *et al.*, 2002) have demonstrated that these candidate vaccines have the ability to produce antibodies to all the included HPV types.

The distribution of HPV types compiled by Clifford *et al.* (2003) suggests that vaccinating against HPV-16 and HPV-18 should prevent over 70% of cervical cancer cases worldwide. In countries where types 16 and 18 are not as prevalent, other types could be included depending on prevalence in that country.

The main target population for HPV vaccination would be adolescent girls, with an aim to induce prophylactic immunity just before they become sexually active. However, due to the risk of acquiring HPV at birth or early in life (Cason *et al.*, 1995), it may be advantageous to deliver HPV vaccines at birth, with a possible booster dose just before the sexually active stage. Since men can act as reservoir for HPV infection (Bosch *et al.*, 1996), both men and women would be vaccinated. Using mathematical models, Hughes *et al.* (2002) predicted the affect of a HPV vaccine program. Under a particular set of assumptions, they found that vaccinating men and women would result in a 44% decrease in prevalence of that specific HPV type; and that vaccinating only women would result in a reduction of 30%.

An ideal HPV vaccine would be affordable, would protect from the most prevalent high-risk HPV types and/or clear the lesions caused by them, and would have a low incidence of side-effects. VLPs, produced in yeast or baculovirus, meet most of these criteria; however, affordability will be a problem in developing countries. Further testing of alternate delivery systems, such as viral, bacterial, or DNA, or plant produced antigens, is therefore warranted. However, vaccines based on these systems may take longer than VLPs to become available for human use, as many are not currently utilised in large-scale vaccination strategies, and would require more thorough testing.

1.8. PROJECT MOTIVATION

Our aim was to develop a prophylactic vaccine for HPV that would be affordable in South Africa and other developing countries, where the burden of HPV disease is at its highest. Due to the production expense of purified protein vaccines, such as VLPs, and therefore the unlikelihood of large-scale use of such vaccines in developing countries, we investigated the potential of recombinant BCG (rBCG) expressing an HPV antigen as a HPV vaccine.

As the major structural protein of the HPV capsid, L1 was the antigen of choice for the development of our HPV prophylactic vaccine candidates. The L1 protein of HPV type 16 was selected for this study, because HPV-16 is the most prevalent HPV type detected in cervical cancer biopsies in South Africa and worldwide (Clifford *et al.*, 2003). A large body of work is available on the immunity elicited by L1 VLPs, and many of these have been reviewed above. Several studies have demonstrated that L1 VLP vaccines can induce high titres of neutralising antibodies in animals and humans (Brown *et al.*, 2001; Christensen *et al.*, 1994; Harro *et al.*, 2001; Rose *et al.*, 1994b; Tobery *et al.*, 2003). Many studies in animal models have also shown that immunisation with VLPs can protect against PV challenge (Campo *et al.*, 1993; Christensen *et al.*, 1996b; Kirnbauer *et al.*, 1996; Suzich *et al.*, 1995). More recently, phase 2 trials in humans have reported reduced incidences of HPV infection and HPV-related CIN in VLP vaccinated individuals (Koutsky *et al.*, 2002; Harper *et al.*, 2004). It has also been demonstrated that immunisation with VLPs can induce CTL responses that are capable of inducing regression of L1-expressing tumours in mice (Ohlschlager *et al.*, 2003), BPV lesions in cows (Kirnbauer *et al.*, 1996); and genital warts in humans (Zhang *et al.*, 2000), which suggests that L1 could be used as a therapeutic vaccine for genital HPVs.

BCG, the live, attenuated *M. bovis* strain, which is routinely used to immunise against infection with *M. tuberculosis*, has many features that make it an attractive vaccine vehicle. These include, a low incidence of serious side effects, the ability to confer long-lasting immunity following a single immunisation, potent adjuvant properties, and a low production cost (Langermann *et al.*, 1994; Stover *et al.*, 1991). The motivation for the use of BCG will be discussed further in Chapter 2.

CHAPTER 2: INTRODUCTION TO BCG

2.1. BCG THE TB VACCINE - HISTORY AND GENERAL FEATURES

Mycobacterium bovis bacille Calmette-Guérin (BCG) is the live, attenuated bovine tubercle bacillus, which is routinely used to immunise against infection with *Mycobacterium tuberculosis*. Since the 1920's, over 4 billion people have been immunised with this vaccine, in almost every country in the world (Plotkin and Plotkin, 1999). The BCG vaccine has also been proven to be protective against infection with *Mycobacterium leprae* (Fine and Rodrigues, 1990; Karonga Prevention Trial Group, 1996); and has been used for immunoprophylaxis and immunotherapeutic treatment of cancer in humans (Fuerst *et al.*, 1992b).

BCG was made by Albert Calmette and Camille Guérin, who sub-cultured virulent bovine tubercule bacillus for 13 years in medium containing glycerol, potato slices and ox bile (Plotkin and Plotkin, 1999). After 231 passages, this strain had lost its virulence by the deletion of large chromosomal regions, and it thus became the BCG strain (Smith and Starke, 1999; Mahairas *et al.*, 1996). Live attenuated BCG was first given orally to infants in Paris in 1921, and was declared safe by the League of Nations in 1928. The use of BCG increased with the introduction of new methods of administration, such as intradermal in 1927, multiple puncture in 1939, and scarification in 1947. Since 1974 the World Health Organisation (WHO) has included BCG in its Expanded Programme on Immunisation to combat disease, especially in children in developing countries. Only two countries, the United States of America and the Netherlands, have never vaccinated with BCG on a national scale (Smith and Starke, 1999).

Despite widespread BCG vaccination, *M. tuberculosis* remains one of the pathogens responsible for the most death and disease in the world. BCG efficacy studies have produced vastly divergent results, with protection ranging from -57% to 75% (Bloom and Fine, 1994). Based on the meta-analysis of 14 prospective efficacy trials and 12 case-control studies, Colditz and colleagues (1994) concluded that BCG vaccination significantly reduces the number of tuberculosis cases and deaths. This study showed that BCG-vaccinated individuals were afforded enhanced protection from *M. tuberculosis* infection (50% protection), pulmonary and disseminated tuberculosis (78% protection), tuberculous meningitis (64% protection), and death (71% protection). Factors contributing to the large variation between the BCG trials include: (1) genetic diversity and different ages of vaccinated populations, (2) prior exposure to environmental mycobacteria, (3) the use of

different BCG substrains, (4) different vaccine preparation methods, and (5) different immunisation schedules, routes and doses (Hess and Kaufmann, 1999).

2.2. BCG SUBSTRAINS

The original BCG strain was distributed to labs throughout the world, at which culturing on different media and under different conditions resulted in the formation of variant substrains. These substrains differ in growth characteristics, colony morphology, biochemical activity, virulence in animals, and the ability to elicit delayed-type hypersensitivity responses. The Brazilian, Tokyo, Swedish, and Russian substrains have two large genomic deletions when compared to *M. bovis,* and they have secreted antigens and methoxymycolates; whereas, the Danish, Dutch, Glaxo, and Pasteur substrains have three large genomic deletions when compared to *M. bovis*, and have no secreted antigens or methoxymycolates (Mahairas *et al.*, 1996; Smith and Starke, 1999).

BCG substrains can also be loosely grouped into "weak" (Glaxo, Tokyo) and "strong" (Pasteur, Danish) substrains. The strong substrains are more immunogenic in various animal models, inducing a greater degree of cutaneous hypersensitivity, better protection from tuberculosis and greater granuloma formation. The "strong" substrains appear to elicit a higher degree of protection from tuberculosis; however, the "weak" substrains have a lower incidence of side effects, such as lymphadenitis and osteitis (reviewed in Smith and Starke, 1999).

2.3. FEATURES MAKING BCG A GOOD VACCINE VEHICLE FOR THE DELIVERY OF HETEROLOGOUS ANTIGENS

Numerous features make BCG an attractive delivery vehicle for heterologous antigens:

- BCG has a proven safety record
  BCG is currently the most widely used vaccine and has been given to billions of people worldwide with a very low incidence of serious complications (Hanson *et al.*, 1995).

- BCG is a potent adjuvant

  The adjuvant properties of BCG and its cell wall components have often been exploited in experimental vaccines in animals and man, being able to induce both humoral and cell-mediated immune responses.

- BCG is inexpensive to produce

  BCG is inexpensive to produce as no complicated purification procedures are required. A dose of the BCG vaccine costs approximately ZAR 0.44 in South Africa (personal communication, Maureen Dennehy, State Vaccine Institute, South Africa); and about US$ 0.06 per dose in America (Labidi *et al.*, 2001). Vaccine cost is particularly relevant when considering large-scale vaccination in developing countries.

- BCG is heat-stable

  BCG is one of the most heat-stable of the vaccines in use, and does not require an extensive cold chain for maintenance of efficacy (Cirillo *et al.*, 1995). This also helps to keep costs at a minimum.

- BCG can be administered at birth

  BCG can be administered at or any time after birth and is unaffected by maternal antibodies (Hanson *et al.*, 1995). BCG is one of the six vaccines recommended by the World Health Organisation to administer to children (Labidi *et al.*, 2001).

- A single dose of BCG can confer long-lasting immunity

  A single BCG inoculum can sensitise for 5 to 50 years to tuberculoproteins (Hanson *et al.*, 1995).

- BCG can be administered orally

  Oral or intranasal administration is of particular importance to confer protective immunity at mucosal sites to prevent entry of pathogens through the mucosa or to prevent colonisation of the mucosa (McNeela and Mills, 2001). Another advantage of these routes of vaccination, over the intradermal or multiple puncture methods, is that they do not require highly-trained personnel for vaccine administration, of whom there may be a shortage in developing countries.

- BCG is a live vaccine

  Recombinant BCG can be used simultaneously as an adjuvant and a vehicle to deliver antigens to the immune system. BCG replicates *in vivo* and thus continually delivers

antigens, which may elicit a more long-lived immune response than that of a simple mixture of adjuvant and antigen (Aldovini and Young, 1991). No protein purification or deactivation procedures are necessary.

- BCG is susceptible to antibiotics
  Unlike live viral vaccine systems, BCG is susceptible to antibiotics. Infection can thus be controlled should any serious complications arise.

- Distribution network
  Due to its widespread use, a worldwide distribution network exists, with medical officers that have BCG vaccine delivery experience (Labidi *et al.*, 2001).

2.4. POTENTIAL LIMITATIONS OF BCG AS A DELIVERY VEHICLE

- Large variations exist in the BCG vaccine efficacy against tuberculosis (mentioned above). If similar efficacy variations exist to a recombinant BCG (rBCG) vaccine, then these immune variations may be carried through to the foreign antigen expressed by rBCG. Worldwide rBCG vaccine production and delivery would have to be carefully monitored to prevent such efficacy variations.

- Serious complications to BCG vaccines are very rare in immunocompetent individuals; however, the number of immunocompromised patients has increased dramatically over the last two decades, mainly due to the HIV pandemic (Smith and Starke, 1999). Although the safety implications of vaccinating HIV-infected patients with BCG are not fully known, there could be a risk of disseminated BCG infections in these individuals (Dietrich *et al.*, 2003). Live vaccines also have the potential of activating the immune system, which could consequently increase HIV replication (Ljungman, 1999). The World Health Organisation currently recommends BCG immunisation in countries with a high risk of tuberculosis infection, including HIV-infected children. Unfortunately, no data are available on the protective efficacy of BCG in HIV-infected patients (Ljungman, 1999). A possible solution to this concern is the use of *in vitro* replication-defective BCG auxotrophs; however, further BCG attenuation may decrease its ability to protect against tuberculosis (Chambers *et al.*, 2000).

- A major technical problem is the observation of genetic instability of rBCG. Unstable recombinants will prove a large problem during large scale production. This is discussed further in Chapter 4.

2.5. INTRACELLULAR MYCOBACTERIAL ANTIGEN PROCESSING AND IMMUNITY

Mycobacteria are facultative intracellular bacteria that are able to survive and replicate in the phagosomes of antigen-presenting cells (APCs). Both macrophages and dendritic cells (considered the most potent APCs) are able to endocytose mycobacteria (Jiao *et al.*, 2002; Neyrolles *et al.*, 2001; Thurnher *et al.*, 1997).

Live mycobacteria are able to evade the immune response by inhibiting acidification and phagosome-lysosome fusion. Mycobacteria limit the trafficking of protein antigens from the phagosome to the cytosol, which prevents MHC class I presentation and recognition by $CD8^+$ T cells. The delay of phagosome-lysosome fusion, also prevents the processing of mycobacterial antigens by MHC class II molecules, and thus delays the $CD4^+$ T cell response to live mycobacteria (Neyrolles *et al.*, 2001; Ramachandra *et al.*, 2001). The mycobacterial phagosome, however, is a dynamic compartment, that is accessible to the recycling endosomal pathway, that allows entry of transferring receptor, glycolipids (Neyrolles *et al.*, 2001; Rhoades *et al.*, 2003), and MHC class II molecules (Ramachandra *et al.*, 2001) into the phogosome. Trafficking of a limited number of mycobacterial glycolipids and lipoproteins from the phagosome to the cytosol is also known to occur (Neyrolles *et al.*, 2001).

Once macrophages and dendritic cells are activated by T cells and IFN-γ, phagosomal maturation occurs, and the mycobacteria are eliminated. Generally, the mycobacterial antigens are processed by the MHC class II molecules, and hence stimulate strong $CD4^+$ T cell responses (Cheadle *et al.*, 2003; Kaufmann and Hess, 1999). BCG is therefore a potent activator of a $CD4^+$ T helper 1 (Th1)-type immune response that is associated with secretion of interleukin-12 (IL-12) by macrophages and dendritic cells, and IFN-γ and TNF-α secretion by $CD4^+$ T helper cells (Esser *et al.*, 2003; Mutis *et al.*, 1993).

One of the suggested reasons for BCG's inadequate ability to control *M. tuberculosis* infection, is its limited activation of $CD8^+$ CTL immune responses, however, *M. tuberculosis*

effectively stimulates $CD8^+$ T cells by MHC class I antigen presentation (Hess and Kaufmann, 1999). The exact mechanisms that make *M. tuberculosis* more successful than BCG at making antigens accessible for MHC class I antigen presentation have not yet been fully elucidated. *M. tuberculosis* and BCG are able to perforate the phagosomal membrane allowing antigens to enter the cytosol (Kaufmann and Hess, 1999), however, this occurs several-fold more readily in *M. tuberculosis*-containing phagosomes (Teitelbaum *et al.*, 1999). Another possible mechanism is due to the observation that *M. tuberculosis*, but not BCG, is able to escape from the phagosome (McDonough *et al.*, 1993); however this finding remains controversial. Recently, human group 1 CD1 molecules (CD1a, -d, -c) were identified that mediate MHC-independent presentation of mycobacteria-derived lipid and glycolipid antigens to $CD8^+$ T cells (Kawashima *et al.*, 2003). These molecules are expressed in intracellular endocytic compartments, where mycobacteria lipid antigens are known to traffic, and are expressed almost exclusively on dendritic cells.

2.6. EXPRESSION OF HETEROLOGOUS PROTEINS IN BCG

2.6.1. Expression vectors

The development of reliable methods for genetic manipulation of mycobacteria has been hindered by the slow growth of these organisms and their complex cell walls. BCG has a doubling time of 24 hours or longer, and can take 3-6 weeks to form a visible colony on solid medium (Ohara and Yamada, 2001). Their thick lipid-rich cells walls and their tendency to clump can make transformation with DNA problematic. In liquid culture, even in the presence of detergents, considerable clumping can occur, and colonies are often grown from a clump, rather than a single bacterium.

In the late 1980's, first generation *E. coli*-mycobacterial shuttle vectors (shuttle phasmids) were developed. These consisted of mycobacteriophage and *E. coli* cosmid DNA, and were able to replicate in *E. coli* as plasmids, and insert foreign DNA into mycobacteria by infection (Jacobs *et al.*, 1987). The system was improved by Snapper *et al.* (1988), who developed a plasmid transformation system, by combining the mycobacterial replicon from the *M. fortuitum* pAL5000 plasmid with an *E. coli* replicon and a kanamycin-resistance gene. Although mycobacterial cells can be somewhat resistant to genetic manipulation, electroporation has overcome this problem and can efficiently allow entry of DNA into the cells by the formation of transient pores (Falkinham and Crawford, 1994; Jacobs *et al.*,

1991). Since the efficacy of this system was established, a number pAL5000-based shuttle vectors have been developed to improve stability, ease cloning, and to remove non-essential sequences (Ranes *et al.*, 1990; Stover *et al.*, 1991). Stover *et al.* (1991) developed the pAL5000-derived multiple-copy number *E. coli*-mycobacterial shuttle vector, pMV261, which contains an *E. coli* plasmid origin of replication (*OriE*), a mycobacterial plasmid origin of replication (*OriM*), a kanamycin resistance gene, a multiple cloning site, and the *hsp60* mycobacterial promoter for expression of heterologous genes. Numerous studies have utilised this vector and its derivatives, including this study.

Due to *in vivo* plasmid stability concerns, integrative shuttle vectors were developed that stably insert heterologous DNA into the mycobacterial chromosome by either, transposition (Martin *et al.*, 1990), gene replacement by homologous recombination (Aldovini *et al.*, 1993; Husson *et al.*, 1990), or by utilising the site-specific integration system of a mycobacteriophage (Haeseleer *et al.*, 1992; Lee *et al.*, 1991). Stover and colleagues (1991) developed an integration-proficient vector, pMV361, by replacing the *oriM* region of pMV261 with the integrase (*int*) gene and attachment site (*attP*) of the mycobacteriophage L5. While site-specific integrative vectors only exist as a single copy per cell, homologous recombination vectors can be present in several copies (Dellagostin *et al.*, 1993), however the latter method has the potential of undesirable gene replacement events. It is possible to introduce both integration-proficient and extrachromosomal vectors into the same cell, providing additional options for antigen expression.

2.6.2. Factors affecting transcription

One of the most important mechanisms regulating the expression of protein is the rate of transcription initiation, which is determined by the strength of the promoter (Mulder *et al.*, 1997). A good understanding of the strength and expression characteristics of the promoters is thus important for the regulation of expression of heterologous antigens in mycobacteria.

Numerous promoters have been used successfully to drive heterologous gene expression in mycobacteria. These include: *hsp60* and *hsp70* from BCG (Stover *et al.*, 1991), the *M. leprae* 18kDa antigen promoter (Dellagostin *et al.*, 1993), the *M. tuberculosis* 19kDa antigen promoter (Stover *et al.*, 1993), *mtrA* from *M. tuberculosis*, the P_{AN} promoter from *M. paratuberculosis* (Murray *et al.*, 1992), the α antigen promoter from *M. kansasil* (Matsuo *et al.*, 1990), the *M. fortuitum* β-lactamase promoter (P*blaF*) (Himmelrich *et al.*, 2000), the *M.*

tuberculosis Erp antigen promoter (P*erp*) (Himmelrich *et al.*, 2000), and the *groES/groEL1* promoter from *Streptomyces albus* (Winter *et al.*, 1991).

The promoters that are used most frequently, *hsp60* and *hsp70*, are those of the 60kDa and 70kDa heat-shock proteins (Hsps), respectively. Hsps belong to a family of stress proteins that are essential for normal mycobacterial growth, but are up-regulated during conditions of stress, such as those encountered in the intracellular environment (Bukau and Horwich, 1998). These promoters have, however, been found to express heterologous antigens constitutively, driving foreign protein expression up to 10% of total BCG protein (Dellagostin *et al.*, 1995; Stover *et al.*, 1991). The *M. leprae* 18kDa antigen and *M. tuberculosis mtrA* promoters express foreign antigens relatively weakly *in vitro*, but are strongly induced *in vivo* to levels almost as high as those by *hsp60* (Dellagostin *et al.*, 1995; Via *et al.*, 1996; Zahrt and Deretic 2000). The 18kDa, *mtrA*, and *hps60* promoters have been used in this study, and have been discussed in more detail in Chapter 3.

2.6.3. Factors affecting translation

Factors likely to influence the translation efficiency of heterologous proteins in mycobacteria include the sequence of the ribosome-binding site, the structure at the 5' end of the mRNA, the sequence surrounding the initiation codon, and the codon bias of the foreign gene (Burlein *et al.*, 1994). Mycobacteria utilise the initiation codon GUG as frequently as they do AUG; the consequences of initiation codon choice are as yet unknown. Mycobacterial translation also utilises a Shine-Dalgarno sequence similar to that of prokaryote organisms, which is situated upstream of the initiation codon. To avoid potential translation initiation problems, researchers have commonly expressed heterologous proteins as fusions with a short portion of the 5' end of the protein which is naturally expressed by the promoter (Burlein *et al.*, 1994). Little is known about mycobacterial expression regulation sequences situated downstream of the initiation codon, which could have possible implications when expressing foreign proteins (Mulder *et al.*, 1997).

Codon optimisation has been utilised to increase foreign gene expression in a viral (Tobery *et al.*, 2003) and DNA-based (Liu *et al.*, 2002) vaccine systems. A few of studies of bacterial vaccine systems, including salmonella (Baud *et al.*, 2002) and BCG (Chujoh *et al.*, 2001; da Cruz *et al.*, 2001), have also utilised codon optimised genes to obtain efficient expression. Due to the high G+C content of the BCG sequence (approx. 65% G+C; Andersson and Sharp, 1996), codon bias will likely play a role in translation efficiency, however it does not

appear to be a key role as many genes with low G+C content are expressed at high levels in BCG, such as *ospA* of *Borrelia burgdorferi* (30% G+C; (Stover *et al.*, 1993) and *Streptococcus pneumoniae* PspA (38% G+C; Langermann *et al.*, 1994; McCool *et al.*, 2002).

2.6.4. Post-translational modification

The rate of translation can also be influenced by the accumulation of protein, which in turn is dependent on the balance between post-translational modification and protein folding, and degradation by endogenous proteases (Burlein *et al.*, 1994). Incorrect folding or incorrect post-translational modification of foreign proteins can decrease protein stability by enhancing protease degradation, and consequently decrease apparent expression levels. It is plausible that these post-translational processes are responsible for the large differences observed in the expression levels of foreign proteins in BCG. The majority of the rBCG studies that have recorded a high production of foreign protein have expressed bacterial proteins, such as *E. coli* β-gal (10% of total BCG protein; Stover *et al.*, 1991), pneumococcal PspA (15% of total BCG protein; Langermann *et al.*, 1994), and *Borrelia burgdorferi* OspA (10% of total BCG protein; Fuerst *et al.*, 1992b; Stover *et al.*, 1993). The expression viral antigens in BCG, however, rarely reaches the level of 1% of total BCG protein (Aldovini and Young 1991; Mederle *et al.*, 2002; Winter *et al.*, 1991). Fuerst *et al.* (1992a) observed that β-gal was expressed to about 15% of total BCG protein, but HIV-1 gp120 was expressed at levels that were 200 times lower, even though the same BCG expression vector was utilised.

The production of heterologous proteins into the cytoplasm of BCG restricts the availability of these proteins to the immune system, and can also limit protein production due to the accumulation and the possible toxicity of these proteins. Researchers have therefore produced fusion proteins consisting of a foreign antigen and a leader peptide which directs newly made proteins to the surface of the mycobacterial membrane, or out of the bacterium into the phagosome. The signal sequences of the BCG-derived α antigen (Stover *et al.*, 1993), the α antigen of *M. kanasii* (Matsuo *et al.*, 1990), the 85A antigen of *M. tuberculosis* (Abomoelak *et al.*, 1999; Supply *et al.*, 1999), and the *M. tuberculosis* Erp antigen have been used to facilitate foreign antigen secretion. The signal sequence of the *M. tuberculosis* 19kDa lipoprotein has been used extensively to direct foreign antigen to the surface of the mycobacterial membrane (Himmelrich *et al.*, 2000; Stover *et al.*, 1993). In general, short peptides (Honda *et al.*, 1995; Uno-Furuta *et al.*, 2003); proteins that are naturally secreted such as *Toxoplasma gondii* GRA1 (Supply *et al.*, 1999), *E. coli* MalE (Himmelrich *et al.*,

2000), and *L. monocytogenes* p60 (Grode *et al.*, 2002); and naturally occurring membrane-anchored proteins, such as *Borrelia burgdorferi* OspA (Stover *et al.*, 1993) and *Streptococcus pneumoniae* PspA (Langermann *et al.*, 1994) have successfully been processed by these two alternative antigen display systems. Some large proteins, and proteins that are naturally cytoplasmic in location, however, have failed to be exported (Abomoelak *et al.*, 1999; Bastos *et al.*, 2002), presumably due to being trapped on the inside of the cell wall.

Targeting of foreign protein for export, or to the membrane of BCG generally has little effect on levels of protein production or leads to a decrease in expression when compared with cytoplasmic expression (Al Zarouni and Dale, 2002; Himmelrich *et al.*, 2000; Langermann *et al.*, 1994). A few studies, however, have reported substantial increases in heterologous protein production when secreting foreign protein from BCG (Al Zarouni and Dale, 2002; Himmelrich *et al.*, 2000). This increase in expression could be due to the reduced accumulation and hence the stimulation of translation, enhancer-like stimulation of transcription by the signal peptide, or decreased protease degradation due to efficient export or due to enhanced protein stability by insertion into the cytoplasmic membrane as an intermediate step in the export of the protein (Al Zarouni and Dale, 2002).

Both the secretory and membrane-bound forms of heterologous antigen display have led to enhanced immunogenicity when compared with cytoplasmically located proteins (Bastos *et al.*, 2002; Langermann *et al.*, 1994; Stover *et al.*, 1993). These superior immune responses are most likely attributable to an improved antigen availability to both MHC I and II molecules, and possibly to the immunogenicity of the signal peptides themselves (Grode *et al.*, 2002; Horwitz *et al.*, 2000; Stover *et al.*, 1993). The α antigen and 19kDa peptides have been discussed in detail in Chapter 3.

2.7. MOTIVATION FOR INVESTIGATING A RECOMBINANT BCG-HPV-16 L1 VACCINE

A variety of viral, bacterial, parasitic and human antigens have been successfully expressed in BCG; and in experimental models rBCG has elicited protective immunity against malaria parasites, Lyme disease, pneumococcal infection, measles, tetanus, cutaneous leishmaniasis, and listeriosis (Matsumoto *et al.*, 2000; Langermann *et al.*, 1994; Stover *et al.*, 1993; Fennelly *et al.*, 1995; Fuerst *et al.*, 1992; Abdelhak *et al.*, 1995; Grode *et al.*, 2002).

The precise correlates of HPV protection and lesion regression may not be fully understood, but it is generally accepted that a prophylactic vaccine for HPV would aim to provide neutralising antibody protection in the genital tract. Studies have demonstrated that rBCG is able to elicit neutralising antibodies to a number of viral proteins (Bastos *et al.*, 2002; Fennelly *et al.*, 1995; Hiroi *et al.*, 2001; Chujoh *et al.*, 2001). It has also been shown that mucosal administration of BCG (such as oral or intranasal) effectively induces sustained specific mucosal immunity (Lagranderie *et al.*, 1998; Langermann *et al.*, 1994b; Biet *et al.*, 2003). Nardelli-Haefliger *et al.* (1997) demonstrated that HPV-16 L1 expressed in recombinant attenuated *S. typhimurium* assembled into VLPs, suggesting the possibility that VLP or capsomere assembly could also take place in other bacterial systems, such as BCG, and thus display the necessary conformational epitopes for the induction of neutralising antibodies. Jabbar *et al.* (2000) reported that HPV-6b L1 and HPV-16 E7 BCG recombinants elicited antigen-specific DTH, T-proliferation and antibody responses, however, these immune responses were weak when compared with protein vaccines; and immunisation with rBCG expressing HPV-16 E7 was not able to protect against challenge with an E7-expressing tumour. They did not report on the ability of the HPV-6b antibodies to neutralise virus. rBCG expressing cotton tail rabbit papillomavirus (CRPV) L1 has been shown to partially protect rabbits from CRPV challenge (Govan *et al.*, 2004), indicating that rBCG has potential as a vector for a prophylactic HPV vaccine.

If a neutralising antibody response fails to provide sterilising immunity, a cell-mediated response will be necessary for the elimination of infected cells. Selvakumar and colleagues (1997) detected the infiltration of mainly $CD8^+$ T cells into the basal and suprabasal layers of the epidermis during CRPV wart regression in rabbits; in contrast, mainly $CD4^+$ T cells were found both within the stroma and on the surface epithelium of regressing human genital warts (Coleman *et al.*, 1994). Mycobacterial antigens are predominantly processed by MHC class II molecules, and thus BCG is a potent activator of a $CD4^+$ Th1-type cellular immune response (Esser *et al.*, 2003; Mutis *et al.*, 1993); however, numerous BCG recombinants have also elicited strong $CD8^+$ CTL responses to heterologous antigens (Mederle *et al.*, 2003; Honda *et al.*, 1995; Uno-Furuta *et al.*, 2003; Lim *et al.*, 1997). The rBCG dose, route of immunisation, and antigen localisation, are factors which influence the immune response to rBCG. These factors are reviewed in Chapters 3 and 5.

Recombinant BCG vaccines are good candidates for vaccines intended for use in developing countries, because BCG is inexpensive to produce, and distribution networks already exist. The main HPV vaccination target population would be adolescent girls, however, it may be

advantageous to deliver HPV vaccines at birth, with a possible booster dose just before the sexually active stage, due to the risk of acquiring HPV at birth or early in life (Cason et al., 1995). BCG would be perfectly suited to a vaccination schedule such as this, as it is routinely administered at birth, and is unaffected by maternal antibodies (Hanson *et al.*, 1995).

The following sections of this report will describe the development of the BCG-L1 recombinants (Chapter 3), the stability of rBCG-L1 (Chapter 4), the immunogenicity elicited by the rBCG-L1 constructs (Chapter 5), and a vaccinia virus-L1 murine challenge model (Chapter 6). Each chapter will contain a short review of the relevant literature for the work presented in that section. Finally, a general conclusion will be presented in Chapter 7.

CHAPTER 3: DEVELOPMENT OF BCG RECOMBINANTS EXPRESSING HPV-16 L1

3.1. INTRODUCTION

Numerous *E. coli*-mycobacterial shuttle vectors have been developed to optimise the expression of heterologous antigens in mycobacteria, as discussed in Chapter 2. A good knowledge of the expression characteristics of the mycobacterial promoters and the features of the signal sequences present in the shuttle vectors is of importance as these will have a direct bearing on the regulation of expression of the foreign protein, and on the immune responses elicited. The promoters, namely, BCG *hsp60*, *M. leprae* 18kDa and *M. tuberculosis mtrA*, and the signal peptides, namely, *M. tuberculosis*-derived 19kDa and BCG-derived α antigen, were utilised in this study and are therefore briefly reviewed below. Furthermore, studies reporting the level and localisation of heterologous protein expression have been reviewed.

3.1.2. PROMOTERS

3.1.2.1. *Hsp60*

Hsp60 belongs to a family of stress response proteins (heat-shock proteins) which are essential for normal bacterial growth, and are up-regulated during conditions of stress (Bukau and Horwich, 1998). The heat-shock proteins themselves are highly immunogenic and can stimulate both humoral and cellular responses (reviewed by Pockley, 2003). In particular, researchers have induced strong MHC class I-restricted $CD8^+$ CTL responses to proteins that have been fused to heat-shock proteins (Cho *et al.*, 2000; Suzue *et al.*, 1997; Suzue and Young, 1996).

The *hsp60* promoter is a very strong promoter; approximately five times stronger than the *hsp70*, *PAN* and *18kDa* promoters. In *M. smegmatis* the *hsp60*, *hsp70* and *18kDa* promoters show similar levels of activity, which is about four times lower than that of the *hsp60* promoter in BCG (Medeiros *et al.*, 2002). Batoni *et al.* (1998) investigated the activity of the *hsp60* promoter in *M. avium* by using a β-gal reporter system. They reported low *hsp60* activity levels during the early growth phases of *in vitro* culture, slightly induced levels during late log phase and maximal activity levels during stationary phase, suggesting that it is responsive to starvation stimuli. Batoni *et al.* (1998) showed that *Hsp60* activity could be induced in liquid culture by a temperature shift from 37 to 45°C (four-fold increase in activity),

but not by a shift from 37-42°C. Stover and colleagues (1991) detected high constitutive expression of *lacZ* under the control of *hsp60* in BCG, and found *hsp60* to be inducible *in vitro* by heat (42°C) and acid (addition of H_2PO_4 to pH 5). Curcic *et al.* (1994), however, could not significantly increase *hsp60* activity by temperature increases. *Hsp60* does not appear to be inducible by hydrogen peroxide, when it is expressed on a plasmid vector (Batoni *et al.*, 1998; Stover *et al.*, 1991), however, when expressed from an integrative vector, it is inducible with heat, acid and peroxide (Stover *et al.*, 1991). It was suggested that these differences in expression can be explained by the differences in the superhelical state of the DNA, which may play a direct role in the regulation of gene expression (Mulder *et al.*, 1997).

A number of studies have described a high, constitutive expression of heterologous antigens by the *hsp60* promoter, with no upregulation upon entry into macrophages (Dellagostin *et al.*, 1995; Stover *et al.*, 1991; Via *et al.*, 1996). Batoni *et al.* (1998), however, detected strong *hsp60* induction after infection of macrophages, which was maximal after 3 hours and persisted for 7 days after infection.

3.1.2.2. *M. Leprae 18kDa*

The *M. Leprae* 18kDa protein is related to the α crystalline family of low-molecular-weight heat-shock proteins (Dellagostin *et al.*, 1993). The *M. leprae* 18kDa antigen promoter expresses foreign antigens relatively weakly in liquid culture, but is strongly induced in macrophages to levels that are almost as high as those of *hps60* (Dellagostin *et al.*, 1995; Zahrt and Deretic, 2000). Contrary to the above, Medeiros *et al.* (2002) found that during liquid culturing of BCG, the *18kDa* promoter had a relatively high level of activity; however, the authors noted that the promoter region in their constructs differed slightly to those used in the studies mentioned above. The *18kDa* promoter functions relatively well in *E. coli* (Al Zarouni and Dale, 2002; Medeiros *et al.*, 2002).

3.1.2.3. *MtrA*

The *M. tuberculosis mtrA* promoter is a response regulator that belongs to a two-component signal transduction system (mtrA-mtrB) (Curcic *et al.*, 1994). Two-component signal systems are the major mechanisms used by bacteria for recognition of environmental conditions, and hence influence the expression of virulence factors and other factors pertinent to adaptation in host cells (Curcic *et al.*, 1994).

Zahrt and Deretic (2000) found the activity of the *mtrA* promoter to be different in BCG and *M. tuberculosis*. They noted a constitutive expression of *mtrA* in *M. tuberculosis*, with similar levels of activity in liquid culture and during expression in macrophages. When expressed in BCG, however, *mtrA* activity was very low in liquid culture, and was induced to a high level in macrophages, which was greater than the *mtrA* activity level in *M. tuberculosis* and similar to that of the *hsp60* promoter. Via *et al.* (1996) also established the induction of *mtrA* in BCG during infection of macrophages. Curcic *et al.* (1994) found that, when expressed in *M. Smegmatis* or BCG in liquid culture, *mtrA* expression was as strong or stronger than that of *hsp60*. They unfortunately did not describe the growth phase at which they harvested the cells. Curcic and colleagues (1994) also noted that *mtrA* was more active when the cells were grown on solid medium than when in liquid medium.

3.1.3. SIGNAL PEPTIDES

3.1.3.1. Alpha (α) antigen

The α antigen forms part of a family of three proteins that show a high degree of homology at both amino acid and DNA level. The α antigen is also referred to as the 30kDa protein or antigen 85B. The other two proteins of this family have molecular masses of approximately 32kDa, and are known as antigen 85A (32A protein) and antigen 85C (32B protein). This group of three proteins, referred to as the 30/32kDa complex or the antigen 85 complex (Harth *et al.*, 1996), are among the most abundantly exported mycobacterial proteins (Wiker and Harboe, 1992). Approximately 45% of extracellular mycobacterial protein belongs to this complex, with the 30kDa protein comprising the bulk (22%) of this. These exported proteins are found at the constant ratio of 3:2:1 (30kDa/32A/32B) (Harth *et al.*, 1996).

All three of the 30/32kDa complex proteins are fibronectin-binding proteins (Wiker and Harboe, 1992), and exhibit mycolic acid transferase activity (Belisle *et al.*, 1997). The disruption of each individual gene in this complex causes impaired bacterial cells wall growth, but not cell death; while disruption of all the genes at once severely inhibits *M. tuberculosis* culture growth, and nearly achieves bacteriostatis (Harth *et al.*, 2002). All three of these proteins are highly immunogenic, with the ability to induce both humoral and T cell responses (Wiker and Harboe, 1992).

The α antigen ORF encodes a polypeptide of 323 amino acids, consisting of a 40 amino acid signal sequence at the amino-terminal, followed by a 283 amino acid mature protein. During the secretion process the signal sequence is cleaved off and the mature protein (30kDa) is released from the cell. The ORF has a G+C content of 64%, with a high G+C preference (86%) in the third codon position (Matsuo *et al.*, 1988). The α antigen exists as a monomer and is not modified post-translationally by either glycosylation or lipidation. It is also highly resistant to degradation by site-specific proteases (Harth *et al.*, 1996).

Investigation of the localisation of the proteins of the 30/32kDa complex in *M. tuberculosis*-infected monocytes revealed that these proteins were mainly present on the bacterial cells wall and in the phagosomal space, but there were also significant amounts in cytoplasmic vacuoles (Harth *et al.*, 1996). Ramachandra *et al.* (2001) demonstrated that MHC class II molecules formed complexes with α antigen within the phagosomes (20 minutes after *M. tuberculosis* infection) and not in endocytic compartments after export of the α antigen from phagosomes. After 20 minutes these complexes could be detected on the plasma membrane. This system, however, is not present to increase antigen presentation, but rather to inhibit MHC antigen processing as a mechanism for intracellular survival, as heat-killed *M. tuberculosis* were processed more rapidly by macrophages than live bacteria, and the phagosomes containing live *M. tuberculosis* contained fewer α antigen-MHC class II complexes.

3.1.3.2. 19kDa lipoprotein

Analysis of the 19kDa lipoprotein shows no obvious homology with other known proteins, and its biological function is yet to be determined. It is primarily associated with the mycobacterial cell wall (Neyrolles *et al.*, 2001). The 19kDA antigen undergoes post-translational modification consisting of cleavage of the signal peptide, glycosylation and acylation (Garbe *et al.*, 1993). The 19kDa lipoprotein is very immunogenic, and can stimulate the innate immune response, including the generation of nitric oxide and IL-12, via Toll-like receptors (Brightbill *et al.*, 1999).

After phagocytosis of live mycobacteria the 19kDa lipoprotein is produced and processed quickly; with the protein being exported from the phagosome and found throughout the infected cell in less than one hour after phagocytosis (Neyrolles *et al.*,

2001). It is generally accepted that "phagosomal" bacteria, such as BCG, primarily stimulate $CD4^+$ T cells via MHC class II antigen presentation; in contrast, antigens from "cytoplasmic" pathogens are available to MHC class I molecules and hence stimulate $CD8^+$ T cells (Kaufmann and Hess, 1999). By and large, antigens present in the cytoplasm are directed by the TAP transporter to the endoplasmic reticulum, where they associate with MHC class I molecules; however, a TAP-independent pathway exists, during which antigens associate with class I molecules within the cell or on the cell surface following endocytosis. After the export of the 19kDa lipoprotein from the phagosome, it appears to be processed by this alternative mechanism, which appears to be dependent on its acylation (Neyrolles *et al.*, 2001).

Recently, human group 1 CD1 molecules (CD1a, -d, -c) were identified that mediate MHC-independent presentation of mycobacterial-derived lipid and glycolipid antigens to $CD8^+$ T cells (Kawashima *et al.*, 2003). These molecules are expressed in intracellular endocytic compartments where mycobacterial lipid antigens are known to traffic, and are expressed almost exclusively on dendritic cells. The fusion of a peptide epitope from the influenza virus NP with the 19kDa lipoprotein expressed by *M. vaccae*, rapidly delivered the influenza epitope for recognition by MHC class I-restricted $CD8^+$ T cells. This MHC class I recognition was determined by the 19kDa lipoprotein, because antigen presentation was blocked with the removal of the lipid tail (Neyrolles *et al.*, 2001)

Although the 19kDa lipoprotein activates innate and specific immunity during early infection, it has been found to inhibit macrophage MHC class II expression and antigen processing during the later stages of infection, which effectively decreases T cells recognition. This MHC class II inhibition is dependent on Toll-like receptor 2 and independent of Toll-like receptor 4 (Noss *et al.*, 2001). Some mycobacterial glycolipids are actively exported from the immature mycobacterial vacuole, and are transferred out of the macrophage in extracellular vesicles, by a process called exocytosis. These vesicles are phagocytosed by adjacent un-infected cells. This cell-to-cell spread of mycobacterial components may explain the continuous immune response modulation at the infection foci, seen with *M. tuberculosis* infection (Beatty *et al.*, 2000; Neyrolles *et al.*, 2001).

3.1.4. LEVEL AND LOCALISATION OF HETEROLOGOUS PROTEIN PRODUCTION

As mentioned in Chapter 2, the level of heterologous protein production in recombinant BCG depends on a number of factors including, promoter strength, signal peptides, and protein stability.

Extrachromosomal expression vectors utilising the *hsp60* promoter have produced the highest levels of cytoplasmically located foreign proteins in rBCG, however, these vectors are also the most unstable (Al Zarouni and Dale, 2002; Medeiros *et al.*, 2002). When under the control of *hsp60* on extrachromosomal vectors, the *E. coli* β-gal (Stover *et al.*, 1991) and *Borrelia burgdorferi* OspA proteins (Fuerst *et al.*, 1992; Stover *et al.*, 1993) accounted for over 10% (20ng/10^6 rBCG) of total BCG protein. Expression of β-gal on an integrative vector utilising the same promoter was lower (Stover *et al.*, 1991), but this was not unexpected as only one copy of the integrative vector is present per cell. Substantially lower levels of OspA (1-5ng/10^6 rBCG) were produced when expressed as fusions with the α antigen or 19kDa lipoprotein (Stover *et al.*, 1993). When OspA was expressed without a signal peptide it was detected exclusively in the aqueous cytoplasmic fraction, while chimaeric OspA-19kDa was located almost exclusively in the detergent-soluble fractions, indicative of a membrane association. Chimaeric OspA-α antigen was detected in the insoluble cell-wall fraction, and in the extracellular medium, but not in the detergent-soluble membrane fraction (Stover *et al.*, 1993). Langermann *et al.* (1994) reported the production of pneumococcal PspA at 15% (100ng/10^6 rBCG) of total BCG protein (*hsp60*, extrachromosomal vector). The levels of PspA were two- to five-fold lower when fused to membrane-directed signal sequences. The 19kDa lipoprotein successfully directed PspA to the surface of the BCG cell wall; however, the PspA's native signal could not facilitate export. Abomoelak *et al.* (1999) found that the *hsp60* promoter could drive expression of a pertussis toxin-tetanus toxin hybrid protein (S1-TTC) to levels 10 times that of the same protein under the control of the antigen 85A promoter and signal peptide. The latter system was not able to export this protein, presumably due to its large size (75kDa).

The addition of signal sequences (membrane or secretory) usually has little effect on protein production levels or leads to a decrease in expression when compared with cytoplasmic expression. A few studies, however, have detected substantial increases in protein production when using secretory signals. When expressing *E. coli* MalE protein in BCG

(extrachromosomal vector, P*blaF* promoter), Himmerlrich *et al.* (2000) found that the highest MalE producers by far were the BCG recombinants that secreted MalE. The highest MalE producer had two contiguous secretion signal sequences, while the lowest producer was the 19kDa lipoprotein (membrane-directed) construct. The highest producer made 9 and 450 times more MalE than the cytoplasmic and lipoprotein constructs, respectively. Al Zarouni and Dale (2002) reported that the expression of β-gal in BCG was relatively low when using the *18kDa* promoter by itself or with the 19kDa lipoprotein signal peptide, however, the addition of the antigen 85A secretion signal increased the expression 7-fold.

The expression of viral antigens in BCG rarely exceeds 1% of total BCG protein. Production of HIV-1 p24 accounted for 0.1% of total BCG protein (extrachromosomal, *hsp70*, cytoplasmic) (Aldovini and Young, 1991), and HIV-1 Nef accounted for 1% of total BCG protein (extrachromosomal, *groES/groEL1* promoter, cytoplasmic) (Winter *et al.*, 1991). rBCG expressing a synthetic SIV *nef-gag* operon on an extrachromosomal vector (P*blaF* promoter, cytoplasmic) expressed high amounts of Gag (approximately 1.11% of total BCG protein), but this construct was unstable. When the same operon was expressed on an integrative vector it was stable, but it produced 8-fold less Gag (Mederle *et al.*, 2002).

BCG does not only have difficulty with the secretion of large proteins, some small proteins are also not able to cross the cell wall; however, at appears that the smaller the protein, the more likely it is to be secreted. Short peptides from the V3 region of HIV-1, which were 19 and 15 amino acids (aa) in length, were not secreted when fused with the α antigen secretory signal, but 12 and 11 aa peptides of the same region were secreted successfully (8.37-17.5μg/ml in culture filtrate) (Chujoh *et al.*, 2001). Uno-Furuta *et al.* (2003) reported that a 12 aa epitope of the hepatitis C virus that fused to the α antigen signal was secreted more efficiently than a similar 16 aa long peptide.

3.2. STUDY OBJECTIVES

The objective of this chapter was to produce a panel of BCG recombinants expressing the L1 protein of HPV-16, with the intention of comparing the L1 expression levels *in vitro*. The L1 gene was cloned into a number of *E. coli* - mycobacterial shuttle vectors, and these clones were then introduced into BCG. Care was taken to confirm BCG transformants, because plasmid instability was often detected in the BCG recombinants. BCG-HPV-16 L1 recombinants were assessed for their production of HPV-16 L1 protein and mRNA. As the study evolved, more *E. coli* - mycobacterial shuttle vectors became available, and additional HPV-16 L1 gene variants were investigated, which lead to the production and assessment of a large array of BCG recombinants.

3.3. MATERIALS AND METHODS

3.3.1. *E. COLI* - MYCOBACTERIAL SHUTTLE VECTORS

A number of *E. coli* - mycobacterial shuttle vectors were utilised in this study, with the intention of comparing foreign protein expression levels and immunogenicity. The main features of these vectors are tabulated below (Table 3.1). Maps of the vectors are available in Appendix C. The origins of these vectors are indicated in the footnotes of table 3.1.

3.3.2. MYCOBACTERIAL SUBSTRAINS

Two *M. bovis* BCG substrains were utilised, namely Tokyo and Pasteur. Freeze-dried ampoules of the BCG Tokyo substrain were obtained from the State Vaccine Institute, Pinelands, Cape Town, South Africa. BCG Pasteur 1172 P2 was obtained from the Statens Seruminstitut, Denmark.

Table 3.1. A summary of the general features of the *E. coli* - mycobacterial shuttle vectors that were utilised in the present study.

Vector	Promoter	Fusion/signal sequence	Other features	Protein expression	References
Extrachromosomal vectors					
pMV261NT[b]	BCG *hsp60*	RBS and 1st 6 codons of Hsp60 protein followed by a multiple cloning site (MCS)	Tn*903*-derived aminoglycoside phosphotransferase (*aph*) gene conferring kanamycin resistance (Kan^R) as a selectable marker, *OriM* from the pAL5000 plasmid, *OriE* from pUC19, MCS followed by a transcription terminator (*rrnABt1*), copy number of 5.	cytoplasmic	Stover *et al.*, 1991
p2619[b]	BCG *hsp60*	*M. tuberculosis*-derived 19kDa lipoprotein RBS and 5' signal peptide sequence (28 codons from the 5' end of the gene)	as above (pMV261 derivative)	export to bacterial surface	Stover *et al.*, 1993
pAB26[b]	BCG *hsp60*	BCG-derived α antigen RBS, signal sequence and structural gene (323 codons)	as above (pMV261 derivative)	export into medium	Stover *et al.*, 1993
pCB119[d]	*M. tuberculosis mtrA*	19kDa lipoprotein (45 codons from the 5' end)	*aph* Kan^R gene, *OriM* from pAL5000, *OriE*, transcription terminator, copy number of 5. Obtained as a clone containing the *Plasmodium falciparum* MSP-1 gene. A lysA gene is also present, which can be used as a non-antibiotic *in vivo* selection system when used in a lysine auxotrophic BCG mutant. The latter system was not utilised in the present study.	export to bacterial surface	W. R. Jacobs (personal communication)
pCB112[d]	*M. leprae 18kDa*	α antigen (41 codons from the 5' end)	as above	export into medium	W. R. Jacobs (personal communication)
pSMT3[a]	BCG *hsp60*	1st 6 aa of Hsp60 protein	Hygromycin resistance gene (Hyg^R) from *Streptomyces hygroscopicus*, *OriM* from the pAL5000 plasmid, *OriE*, transcription terminator, copy number of 5.	cytoplasmic	Garbe *et al.*, 1994
Integration-proficient vectors					
pNIV192[c]	BCG *hsp60*	None	Mycobacteriophage *FRAT 1* integrase gene and attachment site, *aph* Kan^R gene, copy number of 1, *OriE*.	cytoplasmic	Haeseleer *et al.*, 1992; Haeseleer *et al.*, 1993
pMV361[b]	BCG *hsp60*	1st 6 aa of Hsp60 protein	pMV261 derivative with *OriM* removed, *attP* attachment site and integrase gene from mycobacteriophage L5, *aph* Kan^R gene, *OriE*, copy number of 1. Integration occurs at the 3' end of a tRNA^{Gly} gene, and does not disrupt the expression of this gene.	cytoplasmic	Lee *et al.*, 1991; Stover *et al.*, 1991

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[b] MedImmune, Inc.
[c] P. Jacobs, Dept. of Medical Microbiology, University of Brussels
[d] W. R. Jacobs, Dept. of Microbiology and Immunology, Albert Einstein College of Medicine

3.3.3. SOURCE OF HPV-16 L1 GENES

3.3.3.1. Rochester HPV-16 L1 ($L1_R$)

An L1 gene, designated $L1_R$ (aa sequence identical to GenBank AF134175, Appendix E), was kindly supplied by Robert Rose (University of Rochester, Rochester, New York, USA) on the pVL plasmid (Rose *et al.*, 1994).

3.3.3.2. Synthetic L1 with optimised codons for expression in BCG ($L1_{BCG}$)

An important factor influencing protein expression is the compatibility of the codons of the foreign gene with the codon usage of the expression system (as described in Chapter 2). Many HPV-16 L1 codons are used infrequently by BCG, partly due to the high G+C% of the BCG sequence (approx. 65% G+C), and the low G+C% of HPV sequence (L1 is approx. 42% G+C) (Andersson and Sharp, 1996; Howley and Lowy, 2001). To explore the influence of codon usage on the expression of L1 in this system, the HPV-16 L1 gene was resynthesised (Geneart, Germany) to reflect commonly used BCG codons. The L1 amino acid sequence utilised for the codon-optimised gene ($L1_{BCG}$; Appendix E) was based on the L1 Phil sequence (aa sequence identical to Genbank NC001526), which Touze *et al.* (1998) has showed to produce high VLP yields in the baculovirus expression system. This sequence differs by 8 amino acids when compared with the $L1_R$ sequence.

3.3.4. L1 PCR AND CLONING

3.3.4.1. $L1_R$ (Full-length Rochester HPV-16 L1)

The HPV-16 $L1_R$ ORF (1.5 kb) was amplified by PCR from pVL using primers to facilitate directional insertion into the *E. coli* - mycobacterial shuttle vectors pMV261, pMV361, p2619, and pAB26 (Table 3.1). The upstream primer, 5'-GGA**AGATCT**GATGTCTCTTTGGCTG-3' (JM9), contained a *Bgl* II restriction enzyme recognition site (bold) and a start codon (underlined). The downstream primer, 5'-GAGC**AAGCTT**CTCGAGTTACAGCTTA-3' (JM7), contained a *Hind* III restriction enzyme site (bold) and a termination codon (underlined). The primers were produced by the DNA Synthesis Unit (Dept. of Molecular and Cell Biology, University of Cape Town). PCR reactions were prepared and performed as described in Appendix A9. The PCR amplification product was cloned into the pMOS-blue vector using

the pMOS-blue TA cloning kit (Amersham, Buckinghamshire, UK), as described in Appendix A8.3. The resultant clone was called pM-$L1_R$. After restriction enzyme digestion of the pM-L1 clone with *Bgl* II and *Hind* III, the L1 fragment was cloned into the *Bam* HI and *Hind* III sites of pUC19 (*Bam* HI and *Bgl* II form compatible ends), forming pUC-$L1_R$. Refer to Appendix D, Figure D1 for an illustration of the cloning strategy.

The HPV-16 $L1_R$ gene was amplified by PCR using primers to facilitate directional insertion into the *E.coli*-mycobacterial shuttle vectors, pCB112 and pCB119. The upstream primer, 5'-GAC**GGGCCC**ATGTCTCTTTGGCTGC-3' (CBF), contained an *Apa* I restriction enzyme recognition site (bold) and a start codon (underlined). The downstream primer, 5'-CC **ATCGAT**CTAGACTTACAGCTTAC-3' (CBR), contained a *Cla* I restriction enzyme site (bold) and a termination codon (underlined). The $L1_R$ gene was amplified from the pM-$L1_R$ plasmid, and was cloned into pGEM-T Easy vector (Promega, Madison WI, USA) as described in Appendix A8.3, to form pG-$CBL1_R$. Refer to Appendix D, Figure D2 for an illustration of the cloning strategy.

3.3.4.2. $L1_{NLS}$- (Rochester HPV-16 L1 without a nuclear localisation signal)

The $L1_{NLS}$- gene was formed by a 66 base-pair (22 amino acid) truncation at the C-terminal of the HPV-16 gene to remove the nuclear localisation signal (NLS). The truncation was formed by designing a downstream PCR primer to terminate translation just before the NLS.

The $L1_{NLS}$- gene was amplified by PCR from pVL using primers to facilitate directional insertion into the *E. coli* - mycobacterial shuttle vectors, pMV261, pMV361, p2619, and pAB26 (Table 3.1). The upstream primer, 5'-GGA**AGATCT**GATGTCTCTTTGGCTG-3' (JM9), contained a *Bgl* II restriction enzyme recognition site (bold) and a start codon (underlined). The downstream primer, 5'-GAGC**AAGCTT**TTATCCTAATGTAAATTTTGG-3' (JM10), contained a *Hind* III restriction enzyme site (bold) and a termination codon (underlined). The $L1_{NLS}$- gene was amplified from the pVL by PCR, and was cloned into the pGEM-T Easy vector, forming pG-$L1_{NLS}$-. Refer to Appendix D, Figure D3 for an illustration of the cloning strategy.

The HPV-16 L1 gene was amplified by PCR using primers to facilitate directional insertion into the *E. coli* - mycobacterial shuttle vectors, pCB112 and pCB119. The upstream primer, 5'-GAC**GGGCCC**ATGTCTCTTTGGCTGC-3' (CBF), contained an *Apa* I restriction enzyme recognition site (bold) and a start codon (underlined). The downstream primer, 5'-

CC**ATCGAT**TTATCCTAATGTAAATTTTG-3' (CBRT), was used to truncate the L1 gene just before the NLS. The downstream primer contained a *Cla* I restriction enzyme site (bold) and a termination codon (underlined). The $L1_{NLS^-}$ gene was amplified from pM-$L1_R$, and was cloned into pGEM-T Easy, forming pG-CB$L1_{NLS^-}$. Refer to Appendix D, Figure D2 for an illustration of the cloning strategy.

3.3.4.3. $L1_e$ (Rochester HPV-16 L1 with the downstream EpiA tag)

A DNA tag of 75 base pairs, named EpiA, was inserted downstream of the L1 gene in some of the latterly-constructed vectors. EpiA contains a 9 amino acid epitope (IPNPLLGLD) from paramyxovirus SV5, which is recognised by the monoclonal antibody SV5-P-k, and a 10 amino acid BALB/c mouse CTL epitope (RGPGRAFVTI) which originated from the V3 region of the HIV-1 envelope protein.

The presence of EpiA has multiple applications when expressed as a fusion with L1 in rBCG: The SV5-P-k monoclonal antibody epitope gives us an alternative method for evaluating translation of the L1-EpiA transcript. Due to the situation of EpiA, downstream to L1, expression of EpiA is indicative of read-through of the L1 ORF. In mouse immunogenicity studies the CTL tag can be used as an alternative method to establish *in vivo* expression of the recombinant protein. The tag can also be used for the comparison of protein expression between vaccines which contain different foreign genes but the same epitope tag. Dr Will Boum (Department of Clinical Laboratory Science, University of Cape Town) is acknowledged for the construction of the EpiA tag.


```
      R   L   R   G   P   G   R   A   F   V   T   I
5'-CGA TTG CGC GGC CCG GGC CGC GCC TTC GTC ACC ATC

 T   F   K   Q   I   P   N   P   L   L   G   L   D
ACG TTT AAA CAG ATC CCG AAC CCG CTG CTG GGC CTG GAT-3'
```

Figure 3.1. The EpiA DNA sequence (only sense strand shown) and corresponding amino acid sequence (above). To facilitate cloning, *Cla* I sites (bold) were placed on either end of the tag. The BALB/c HIV CTL epitope is underlined with the solid line. The SV5-P-k monoclonal antibody epitope is underlined with the dotted line.

The L1 termination codon was removed to facilitate read-through into the EpiA tag. This was achieved by PCR amplification of the L1 gene using a downstream primer that did not contain a termination codon. The primers were also designed to facilitate cloning into the *E. coli* - mycobacterial shuttle vectors. The HPV-16 L1 gene was amplified from pVL using the upstream primer, 5'-**GGGCCCA**<u>**CAGCTG**</u>GTCGACA<u>TATG</u>TCTCTTTGGCTGC-3' (L1SF), which contained an *Apa* I enzyme site (bold), a *Pvu* II enzyme site (bold and underlined) and a start codon (underlined); and the downstream primer, 5'-**ATCGAT**ACAGCTTACGTTTTT TGCG-3' (L1SR), which contained a *Cla* I restriction enzyme site (bold). The PCR amplification product was cloned into the pGEM-T Easy vector forming pG-L1S. Refer to Appendix D, Figure D4 for an illustration of the cloning strategy. Refer to section 3.3.5.1 for cloning of EpiA into the *E. coli* - mycobacterial shuttle vectors.

3.3.4.4. $L1_{BCGe}$ (BCG codon-optimised HPV-16 L1, with the downstream EpiA tag)

During the synthesis of $L1_{BCG}$, restriction enzyme recognition sequences were added at the 5' and 3' ends to facilitate cloning. *Apa* I and *Pvu* II enzyme sequences were added to the 5' end of the gene, while a *Cla* I sequence was added to the 3' end. $L1_{BCG}$ was designed without a stop codon to enable read-though into the EpiA tag.

3.3.5. CLONING INTO *E. COLI* - MYCOBACTERIAL SHUTTLE VECTORS

3.3.5.1. Cloning into pCB112 and pCB119

- The full-length L1 gene ($L1_R$) and the truncated version ($L1_{NLS}$-) were obtained by *Apa* I and *Cla* I digestion of pG-CBL1_R and pG-CBL1_{NLS}-, respectively, and were then cloned into pCB112 and pCB119, after these vectors had also been digested with *Apa* I and *Cla* I. The resultant clones were p112L1_R, p112L1_{NLS}-, p119L1_R, and p119L1_{NLS}-. Refer to Appendix D, Figure D5 for an illustration of the cloning strategies.

- The EpiA epitope tag was cloned into the *Cla* I site of pCB119 vector to form $pCB119_e$ (Appendix D, Figure D6). The L1S gene (with stop codon removed) was cloned from pG-L1S into $pCB119_e$ using *Apa* I and *Cla* I digests, to form p119$L1_e$ (Appendix D, Figure D6). $L1_e$ was then sub-cloned from p119$L1_e$ into pCB112 with *Apa* I/*Hpa* I digests, forming

p112L1$_e$ (Appendix D, Figure D7). This cloning strategy inserted the L1 gene in-frame of the transcription terminator present in the vectors just downstream of the multiple cloning site.

- The L1$_{BCG}$ gene was cloned from the pSK-L1$_{BCG}$ plasmid (Geneart, Germany) into pCB119$_e$ using *Apa* I/*Cla* I digests to form p119L1$_{BCGe}$. The L1$_{BCGe}$ fragment was then cut out of p119L1$_{BCGe}$ with *Apa* I/*Hpa* I and subcloned into pCB112, forming p112L1$_{BCGe}$ (Appendix D, Figure D7).

3.3.5.2. Cloning into pMV361 and p2619

- The L1$_R$ gene was obtained from pUC-L1$_R$ by partial digestion with *Eco* RI (an *Eco* RI site exists elsewhere in the L1 gene) and *Hind* III. The L1$_R$ fragment was then cloned into the *Eco* RI and *Hind* III sites of pMV361 and p2619, forming p361L1$_R$ and p2619L1$_R$, respectively. Refer to Appendix D, Figure D8 for an illustration of the cloning strategy.

- The L1$_{NLS}$- gene was cloned from pUC-L1$_{NLS}$- into pMV361 and p2619, forming p361L1$_{NLS}$- and p2619L1$_{NLS}$-, respectively. The same method used, as described above for L1$_R$ (Appendix D, Figure D9).

- L1$_e$ was sub-cloned cloned from p119L1$_e$ into pMV361 using *Pvu* II/*Hpa* I digests, forming the clone p361L1$_e$ (Appendix D, Figure D10).

3.3.5.3. Cloning into pMV261NT, pAB26 and pSMT3

- The full-length L1 gene was cut out of pM-L1$_R$ by digestion with *Bgl* II and *Hind* III, and the resultant L1 fragment was sub-cloned into the *Bam* HI and *Hind* III sites of pAB26, pMV261 and pSMT3, to form pAB26L1$_R$, p261L1$_R$, and pT3L1$_R$, respectively (Appendix D, Figure D11 and D13).

- The L1$_{NLS}$- gene was cloned from pG-L1$_{NLS}$- into pAB26, pMV261 and pSMT3, using the same methods as described above for the full-length gene (Appendix D, Figure D12 and D13).

3.3.5.4. Cloning into pNIV192

- The cloning of $L1_R$ into pNIV192, forming $pNVL1_R$, was performed by Wendy Burgers (MSc, Dept of Medical Microbiology, University of Cape Town, 1996).

3.3.6. SEQUENCING OF CLONES TO CONFIRM PCR FIDELITY AND CLONING

After the clones were verified by restriction endonuclease digestion (Appendix A6), they were sequenced to confirm PCR fidelity and correct ligation at the cloning sites. In order to obtain clean DNA for sequencing, small scale DNA extractions were performed using the High Pure Plasmid Isolation Kit (Roche Diagnostics, Germany) as described in Appendix A5.5. All primers were made by the DNA Synthesis Unit (Department of Molecular and Cell Biology, University of Cape Town). Unless otherwise stated, all sequencing was performed at the Core Facility (University of Stellenbosch).

- The pM-$L1_R$, pG-$L1_{NLS}$-, pG-CB$L1_R$ and pG-CB$L1_{NLS}$- clones were sequenced by DNA Sequencing Unit (Department of Molecular and Cell Biology, University of Cape Town), using the M13 primers (Roche Diagnostics, Germany). The M13 primers bind on either side of the multiple cloning sites of these vectors.

- pAB26$L1_R$ /$L1_{NLS}$-, p261$L1_R$ /$L1_{NLS}$- and p2619$L1_R$ /$L1_{NLS}$- were sequenced with primers 26F (5'- TGGCGAACTCCGTTGTAGTG -3') and 26R (5'- GTTGGCTAGCTGATCACC -3') to assess the sequences at the cloning sites. Primer 26F binds upstream to the *hsp60* promoter in the vectors and thus sequences the promoter, the 5' cloning site and 5' end of the L1 gene; primer 26R binds downstream to the L1 gene and sequences the 3' cloning site and the 3' end of the L1 gene.

- p361$L1_R$ /$L1_{NLS}$- was sequenced with primers 361F (5'- TCTGTGCGGAGACCTGGGCA -3') and 26R. 361F binds upstream to the *hsp60* promoter and thus sequences the promoter, the cloning site and into the 5' end of the L1 gene. 26R sequences as mentioned above.

- pT3-$L1_R$ was sequenced with primers T3F (5'- GGCAGTGAGCGCAACGCAA -3') and T3R (5'- GATAACGTTCTCGGCTCGATG -3'). Primer T3F binds upstream to the *hsp60* promoter

and thus sequences through the promoter and into the 5' end of the L1 gene; T3R binds downstream to the L1 gene and sequences the 3' cloning site and the 3' end of the L1 gene.

- Eight primers, shown below, were used to sequence the pCB112 (p112L1$_R$ /L1$_{NLS}$-/L1$_e$) and pCB119 clones (p119L1$_R$ /L1$_{NLS}$-/L1$_e$). These primers were designed to sequence the sense and anti-sense strands of approximately four 600bp overlapping regions which included the L1 sequence, its promoter and leader sequence.

CBAF: 5'- AGTGCTTGTGGTGGCATCC -3'
CBAR: 5'- TCAACTTGGCACGTCTGGAG -3'
CBBF: 5'- GTTGTCGGCGCCGATGTTG -3'
CBBR: 5'- CGCTGTGTATCTGGATTG -3'
CBCF: 5'- GTATCAGGATTACAATACAGGG -3'
CBCR: 5'- TTTGCAGTAGCACCAGAGCC -3'
CBDF: 5'- GGGCTGGTGCTGTTGGTG -3'
CBDR: 5'- GGCAGTCGATCGTACGCTAG -3'

- pCB119$_e$ was sequenced with primers CBBF and CBDR to confirm that EpiA was cloned in the correct orientation. When the p119L1$_{BCGe}$ and p112L1$_{BCGe}$ clones were sequenced, the above primers, CBAF, CBAR, CBBF and CBDR were used as these primers bind to sequences in the vectors. The primers below were designed to prime within L1$_{BCG}$.

BCGL1CF: 5'- GTTCTACAACCCGGACACCC -3'
BCGL1BR: 5'- AGATGCCCACGCCCAGCGG -3'
BCGL1DF: 5'- CCACAACAACGGCATCTGCTG -3'
BCGL1CR: 5'- GGCGCACAGCGACATGTTGG -3'

3.3.7. INTRODUCTION OF SHUTTLE VECTORS INTO BCG

3.3.7.1. Electrotransformation of BCG

The mycobacterial expression vectors were introduced into BCG by electroporation, which involves the administration of a high voltage to the cells which induces a transient state of competency, allowing the uptake of DNA (Jacobs *et al.*, 1991).

Large scale plasmid DNA extractions were performed on the L1-mycobacterial expression vector clones (Appendix A5.2). Competent BCG was prepared for electrotransformation (Appendix A2.2) and the vectors were introduced into the competent BCG, as described in Appendix A3.2. Briefly, plasmid DNA was mixed with washed BCG cells and placed in an electrogap cuvette (Bio-Rad Laboratories, Germany). The cell/DNA mixture was then subjected to an electric pulse using a GenePulser (Bio-Rad Laboratories, Germany) to allow uptake of DNA. The cells were incubated with medium to allow for the expression of the antibiotic resistance gene of the newly transferred plasmid, and then plated on solid medium containing the appropriate antibiotic. Single colonies were selected and analysed.

3.3.7.2. Verification of BCG transformants

Due to their thick, complex cell walls, DNA extraction from mycobacteria is not as efficient as from *E. coli*. It was therefore necessary to amplify the mycobacterial plasmid DNA by transforming it into *E. coli*. Briefly, plasmid DNA was extracted from mycobacterial transformants and then transformed into *E. coli*. Plasmid extractions were then performed on the *E. coli* recombinants and the DNA obtained was subjected to endonuclease digestion and agarose gel electrophoresis. To verify whether mycobacterial transformants contained integrated expression vectors, total DNA was extracted from the mycobacteria and Southern blot-hybridisation was performed. These methods are described in detail in Appendix A4.1.

3.3.8. ANALYSIS OF PROTEIN EXPRESSION BY rBCG

3.3.8.1. Total protein extraction from BCG

Mechanical methods have been developed for protein extraction from mycobacteria as these methods are more suitable than lysis buffers for efficient lysis of the thick mycobacterial cells walls. Total protein was extracted from BCG cultures using a bead-beating method described in detail in Appendix A11.1. The protein concentrations of the mycobacterial cell lysates were determined using the Bio-Rad Protein Assay Kit (Bio-Rad Laboratories, Germany; Appendix A11.2).

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

The proteins from the cell lysates were resolved by electrophoresis on a denaturing polyacrylamide gel (Appendix A11.3). Protein gels were typically run in duplicate, after which, one gel was stained with Coomassie blue, while the other was used for western blotting. Coomassie blue staining (Appendix A11.4) is based on non-specific binding of Coomassie blue dye to proteins. After staining, the gel was destained to remove background colour, and the proteins were detected as blue bands on a clear background.

3.3.8.3. Western blot analysis

The duplicate SDS-PAGE gel (section 3.3.8.2) was subjected to western blotting (Appendix A12.1), followed by immunodetection of specific proteins. Immunodetection of L1 protein was carried out using the BM Chemiluminescence Blotting Substrate (POD) kit (Roche Molecular Biochemicals, Germany) according to the manufacturer's specifications (see Appendix A12.2 for a detailed method). Two HPV-16 L1 specific monoclonal antibodies, namely H16.J4 and H16.D9, were used to probe for the L1 protein on the western blots. These antibodies are specific for denatured HPV-16 L1 protein (Christensen *et al.*, 1996a). Purified HPV-16 VLPs were utilised as the positive control.

3.3.9. L1 mRNA QUANTIFICATION

Due to the low production of HPV-16 L1 protein by the BCG-L1 recombinants, the L1 mRNA production by these recombinants was investigated. Briefly, total RNA was extracted from BCG, and reverse transcribed into cDNA using random primers. Specific genes (cDNA) were then quantified by a quantitative PCR technique (LightCycler thermocycler instrument, Roche Molecular Biochemicals, Germany).

3.3.9.1. RNA extraction and reverse transcription

RNA extractions were performed on fresh BCG cultures or on frozen BCG samples (Appendix A14.1). Care was taken to harvest the cultures during the same growth phases. The FastRNA Blue Kit (Bio 101, USA) was used to extract RNA as per the kit's instructions (Appendix A14.1). To remove DNA contamination, the RNA samples were treated with deoxyribonuclease (DNase). The DNase kit, AMP-D1 (Sigma, USA), was used for this purpose, as per the kit's instructions (Appendix A14.2). After DNase treatment, the RNA

samples were tested for the presence of DNA by PCR using the LightCycler (Appendix A9), with primers specific for HPV-16 L1 (947U23 and 1091L21; see below, section 3.3.9.2) and MT10SA, a small stable mycobacterial RNA (Mariani *et al.*, 2000) (primers, 10SAF: 5'-AGGGCCAGGTCGGTGGC -3' and 5'- AGATCCTGGACGATCGGC -3'). DNA-free RNA samples were reverse transcribed into cDNA using random primers. The 1st Strand cDNA Synthesis Kit for RT-PCR (Roche Molecular Biochemicals) was utilised for this purpose, according to the manufacturer's instructions (see Appendix A14.3 for a detailed method).

3.3.9.2. RNA/cDNA quantification

The LightCycler (Roche Molecular Biochemicals) is able to quantify the number of DNA copies of a specific template in an unknown sample, by comparing the PCR efficiency of the template in the unknown sample with that of known standard samples. The PCR reagents were obtained from the LightCycler - FastStart DNA Master SYBR Green I Kit (Roche Molecular Biochemicals). This PCR kit is specifically adapted for use with the LightCycler instrument. This system uses SYBR Green I dye, which specifically binds to double stranded DNA; this binding enhances the dye's fluorescence, which is then measured by the LightCycler. Primers were designed to amplify a small region of the HPV-16 L1 gene, the Tn903 kanamycin resistance gene, and the *M. bovis* 16S rRNA gene.

The following primers were used:
HPV-16 L1 gene (256bp product)
947U23: 5'- ACTGCAAATTTAGCCAGTTCAAA -3'
1091L21: 5'- CATATTCCTCCCCATGTCGTA -3'

Tn903 *kanamycin gene (286bp product)*
245U23 : 5'- CGACCATCAAGCATTTTATCCGT -3'
5110L21: 5'- TGTTCAACAGGCCAGCCATTA -3'

M. bovis 16S rRNA gene (268bp product)
284U23: 5'- AGATACGGCCCAGACTCCTACGG -3'
530L22: 5'- CAACGCGACAAACCACCTACGA -3'

The primers were synthesised by the DNA Synthesis Unit (Dept. of Molecular and Cell Biology, University of Cape Town).

Standard DNA template samples were set up for each gene that was quantified. p119L1$_e$ plasmid was used as a template for the HPV-16 L1 and Tn903 kanamycin genes. Six 10-fold serial dilutions were made with concentrations ranging from 12.5pg/μl (approx. $4.1x10^{10}$ plasmid copies/μl) to $1.25x10^{-4}$ pg/μl (approx. $4.1x10^5$ plasmid copies/μl). The standard DNA template for 16S rRNA was made by PCR amplification (Appendix A9) of DNA from wild-type Tokyo BCG (Appendix A5.4) utilising the 284U23/530L22 primers. The 16S rRNA PCR product was purified by gel electrophoresis (Appendices A7 and A5.6). Six 10-fold serial dilutions were made from the purified 16S rRNA PCR product, with concentrations ranging from 0.4pg/μl (approx. $4.1x10^{10}$ copies/μl) to $0.4x10^{-5}$ pg/μl.

To quantify the number of copies of a specific gene in an unknown cDNA sample, the unknown sample and the known standards (for that gene) were amplified on the LightCycler using the primers for the gene of interest. The PCR reaction mixtures and thermocycler parameters were set up as described in Appendix A14.4. The LightCycler software (Version 3.5; Roche Molecular Biochemicals) was used to calculate the number of copies of the gene of interest in the unknown sample, relative to the number of copies of that in the standard samples. The program uses the standard samples to construct a standard curve, which is the linear regression line through the data points of the crossing points (cycle number) versus the log10 concentration of the standard samples. The crossing point is the cycle number at which the log-linear region of the amplification fluorescence curve intercepts the cycle number axis. The crossing points of the unknowns are plotted against the standard curve and the concentrations are calculated.

16S rRNA was used as the housekeeping gene (endogenous reference gene), according to which the cDNA (reverse transcribed from 16S rRNA) concentrations of the unknown samples were normalised. Once the amounts cDNA were normalised, the amounts of L1 cDNA could be directly compared. The kanamycin gene was used as a second housekeeping gene, to verify the accuracy of the 16S rRNA (cDNA) quantification.

3.4. RESULTS

3.4.1. VECTOR CONSTRUCTION

The $L1_R$, $L1_{NLS}$-, $L1_e$, and $L1_{BCGe}$ genes were successfully cloned into the mycobacterial shuttle vectors, to produce the constructs listed in Table 3.2, and shown in Figure 3.2. Subsequent to every PCR amplification of L1 for cloning purposes, the amplification product was sequenced to verify PCR fidelity. All clones were also sequenced though the ligation sites, to ensure that the appropriate ligation reaction transpired. On the occasions that cloning or PCR errors were detected, the clones were remade or PCR amplification was repeated.

At two occasions, PCR errors in L1 were detected and were not corrected, as these sequence errors did not alter the L1 amino acid sequence. These errors were detected in plasmids pG-L1S (section 3.3.3.3) and pG-$L1_{NLS}$- (section 3.3.3.2). Two point mutations were detected in pG-L1S, resulting in a codon change from CAT to CAC (L1's 47^{th} codon), and a codon change from GAT to GAC (L1's 439^{th} codon). A single point mutation was detected in pG-$L1_{NLS}$-, causing a codon change from TTT to TTA (L1's 420^{th} codon).

3.4.2. INTRODUCTION OF SHUTTLE VECTORS INTO BCG

The mycobacterial shuttle vector-L1 constructs (Table 3.2) were introduced into BCG by electroporation. Recombinants containing extrachromosomal plasmids were verified by endonuclease digestion and agarose gel electrophoresis; and recombinants containing integration-proficient vectors were verified by Southern blot analysis.

Analysis of the DNA migration patterns, of plasmids obtained from BCG-L1 recombinants, indicated that the majority of BCG electroporations resulted in mixed populations of recombinants, some containing the correct plasmid, and others containing genetically unstable plasmids, from which regions of L1 had been excised. In some cases, even though the electroporations were repeated several times, no stable recombinants were detected. The integration-proficient vectors appeared stable by Southern blot analysis (Figure 3.3 shows an example of a Southern blot). Table 3.3 summarises the BCG recombinants that were successfully made, and the recombinants that were always unstable. From the table, it

is clear that the unstable BCG recombinants were those that contained the extrachromosomal vectors which drove the expression of L1 with the *hsp60* promoter. The integration-proficient plasmids which used the *hsp60* promoter appeared stable. The stability of these BCG recombinants will be discussed further in Chapter 4. The BCG recombinants that contained stable plasmids were selected and used to produce stocks for animal immunisations (Chapter 5).

Table 3.2. Mycobacterial shuttle vector – HPV-16 L1 clones constructed during this study.

Shuttle vector	Insert	Clone name
pNIV192	$L1_R$	*$pNVL1_R$
pSMT3	$L1_R$	$pT3L1_R$
	$L1_{NLS^-}$	$pT3L1_{NLS^-}$
pMV261	$L1_R$	$p261L1_R$
	$L1_{NLS^-}$	$p261L1_{NLS^-}$
pAB26	$L1_R$	$pAB26L1_R$
	$L1_{NLS^-}$	$pAB26L1_{NLS^-}$
p2619	$L1_R$	$p2619L1_R$
	$L1_{NLS^-}$	$p2619L1_{NLS^-}$
pMV361	$L1_R$	$p361L1_R$
	$L1_{NLS^-}$	$p361L1_{NLS^-}$
	$L1_e$	$p361L1_e$
pCB119	$L1_R$	$p119L1_R$
	$L1_{NLS^-}$	$p119L1_{NLS^-}$
	$L1_e$	$p119L1_e$
	$L1_{BCGe}$	$p119L1_{BCGe}$
pCB112	$L1_R$	$p112L1_R$
	$L1_{NLS^-}$	$p112L1_{NLS^-}$
	$L1_e$	$p112L1_e$
	$L1_{BCGe}$	$p112L1_{BCGe}$

* Clone made by Wendy Burgers (MSc, Dept of Medical Microbiology, University of Cape Town, 1996).

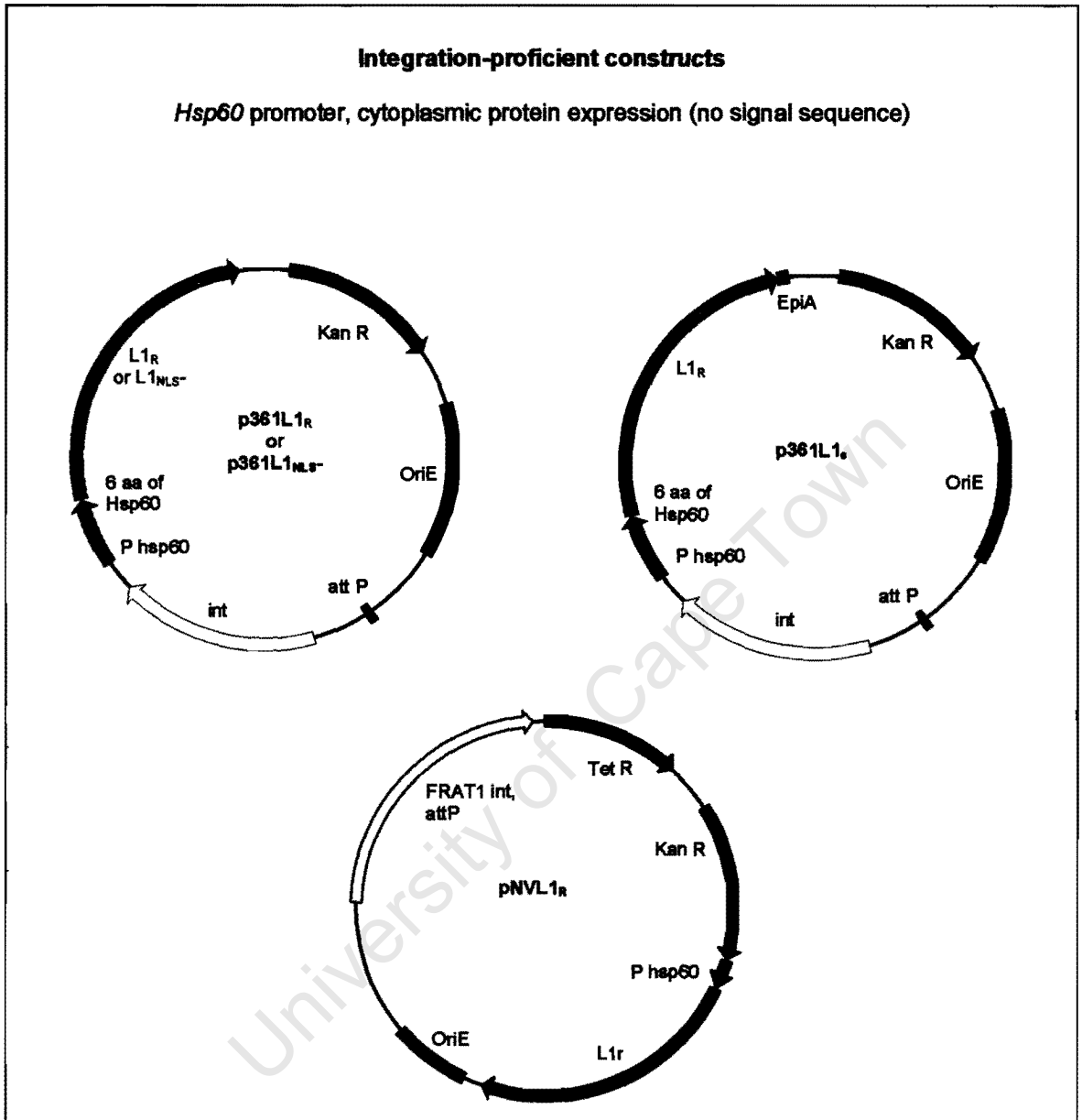


Figure 3.2. Diagrams of the mycobacterial shuttle vector – HPV-16 L1 clones constructed during this study. P hsp60, BCG *hsp60* promoter; P MtrA, *M. tuberculosis mtrA* promoter; P M.lep 18kDa, *M. leprae 18kDa* promoter; Kan R, kanamycin resistance gene; Hyg R, Hygromycin resistance gene; Tet R, tetracycline resistance gene; OriE, *E. coli* origin of replication; OriM, mycobacterial origin of replication; EpiA; epitope tag; int, mycobacteriophage L5 integrase gene; att, mycobacteriophage L5 attachment site; FRAT 1, mycobacteriophage *FRAT 1* integrase gene and attachment site; alpha antigen, BCG alpha antigen signal sequence; 19kDA, *M. tuberculosis* 19kDa lipoprotein signal sequence.

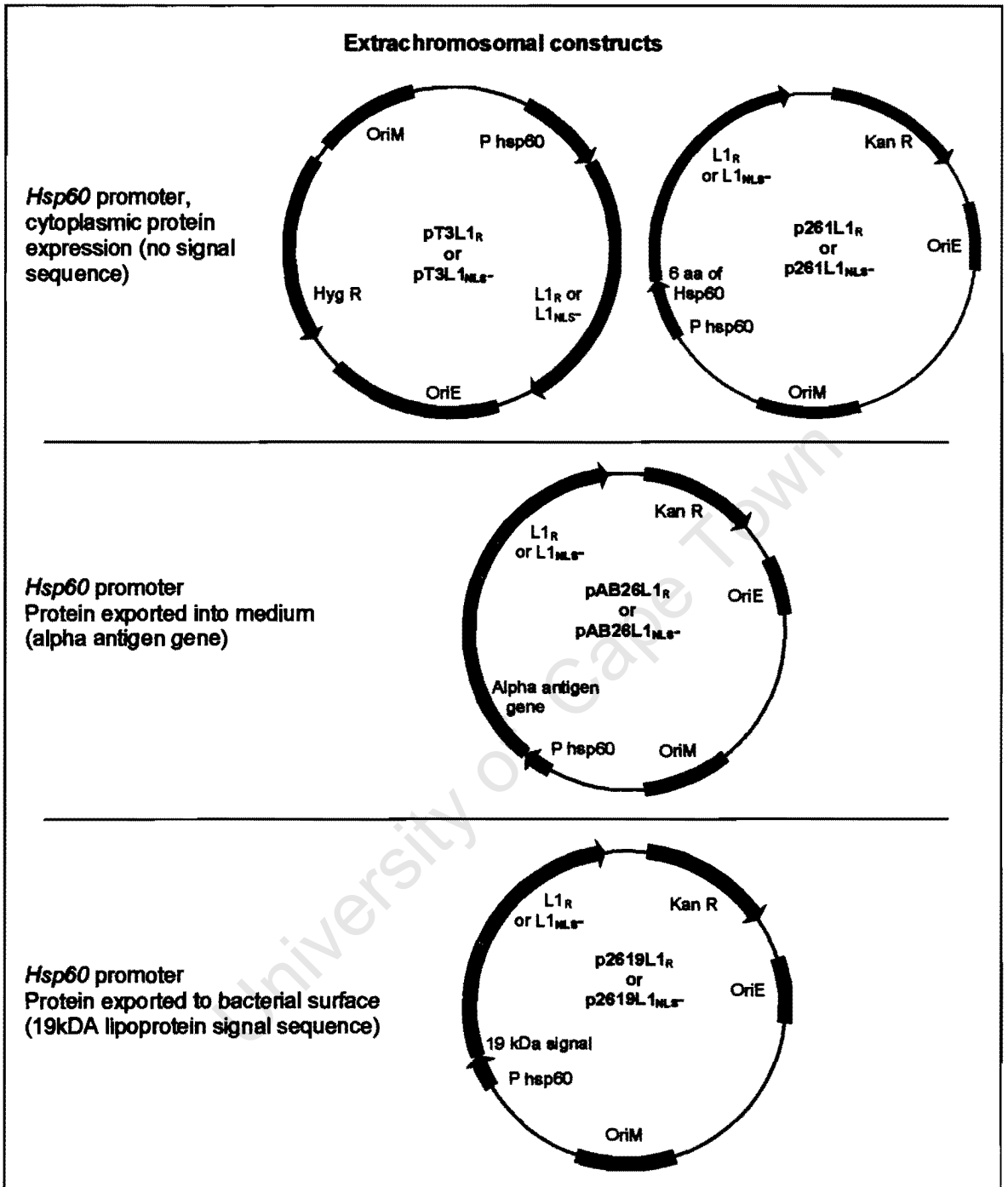
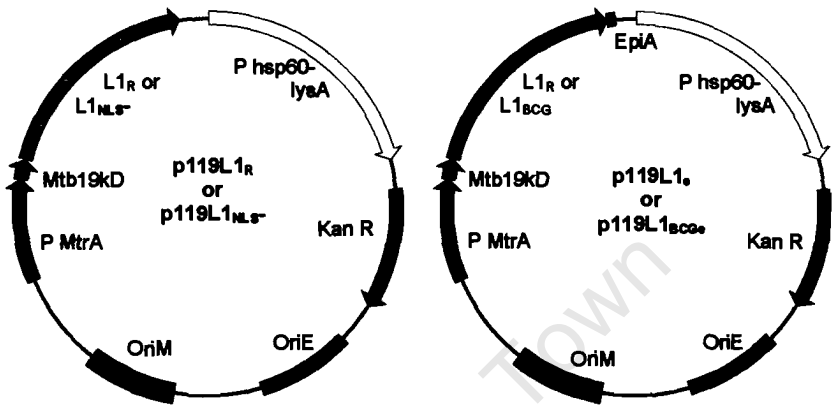


Figure 3.2 (continued). Diagrams of the mycobacterial shuttle vector – HPV-16 L1 clones constructed during this study. P hsp60, BCG *hsp60* promoter; P MtrA, *M. tuberculosis mtrA* promoter; P M.lep 18kDa, *M. leprae 18kDa* promoter; Kan R, kanamycin resistance gene; Hyg R, Hygromycin resistance gene; Tet R, tetracycline resistance gene; OriE, *E. coli* origin of replication; OriM, mycobacterial origin of replication; EpiA; epitope tag; int, mycobacteriophage L5 integrase gene; att, mycobacteriophage L5 attachment site; FRAT 1, mycobacteriophage *FRAT 1* integrase gene and attachment site; alpha antigen, BCG alpha antigen signal sequence; 19kDA, *M. tuberculosis* 19kDa lipoprotein signal sequence.

Extrachromosomal constructs (continued)

MtrA promoter,
19kDa signal



M. leprae 18kDa
promoter,
alpha antigen signal

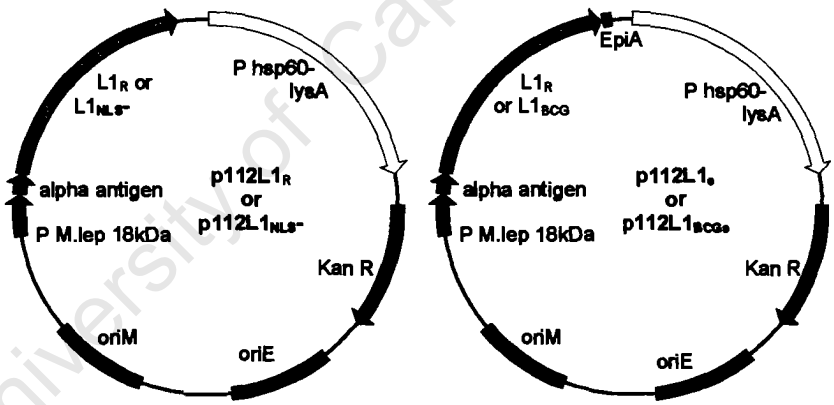


Figure 3.2 (continued). Diagrams of the mycobacterial shuttle vector – HPV-16 L1 clones constructed during this study. P hsp60, BCG *hsp60* promoter; P MtrA, *M. tuberculosis mtrA* promoter; P M.lep 18kDa, *M. leprae 18kDa* promoter; Kan R, kanamycin resistance gene; Hyg R, Hygromycin resistance gene; Tet R, tetracycline resistance gene; OriE, *E. coli* origin of replication; OriM, mycobacterial origin of replication; EpiA; epitope tag; int, mycobacteriophage L5 integrase gene; att, mycobacteriophage L5 attachment site; FRAT 1, mycobacteriophage *FRAT 1* integrase gene and attachment site; alpha antigen, BCG alpha antigen signal sequence; 19kDA, *M. tuberculosis* 19kDa lipoprotein signal sequence.

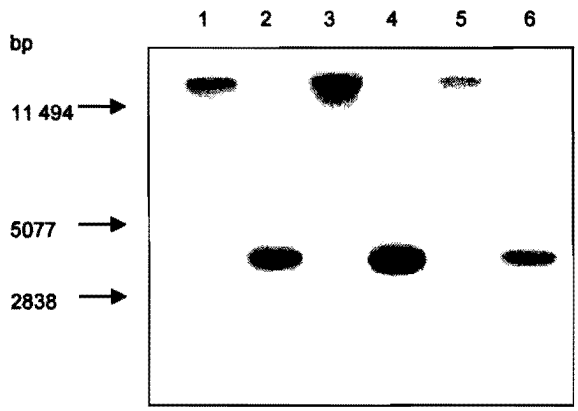


Figure 3.3. Southern blot-hybridisation analysis of chromosomal DNA obtained from Tokyo rBCG-361L1$_{NLS}$-. Three rBCG-361L1$_{NLS}$- recombinants were assessed; lanes 1 and 2, contain DNA from the one recombinant, lanes 3 and 4 contain DNA from the other, and lanes 5 and 6 from a third. The DNA in lanes 1, 3, and 5, was digested with *Hind* III, which cuts the integrated vector once, outside of the L1 gene (it is difficult to predict the size of this band as one does not know where in the chromosome *Hind* III will cut, but it should be larger than the vector, ie. larger than 5919bp). The DNA in lanes 2, 4, and 6, was digested with *Pvu* II and *Not* I, excising a fragment of 3638bp, including the L1 gene. The above image is the autoradiograph of the Southern blot after it was probed with labelled-HPV-16 L1.

3.4.3. ANALYSIS OF PROTEIN EXPRESSION BY rBCG

The expression HPV-16 L1 protein by the BCG-L1 recombinants was investigated by SDS-PAGE, followed by Coomassie blue staining or western blot analysis. Coomassie blue staining failed to detect L1 protein production by the BCG-L1 recombinants (Figure 3.4). Western blot analysis detected L1 protein in rBCG-L1 lysates by the visualisation of faint bands of the expected size (55kDa, Figure 3.5). Due to the low expression of L1 by BCG, this result could not be repeated. To determine the western blot assay sensitivity for HPV-16 L1; HPV-16 VLPs were serially diluted and detected by western blot analysis using the H16.J4 monoclonal antibody. The lowest detectable amount of VLP with this method was 0.03μg. As 10μg of rBCG-L1 lysate was routinely used in these assays, it was established that when L1 was not detectable in rBCG-L1 lysates, the amount of L1 protein was less than 0.3% of the total protein in the samples.

Table 3.3. BCG-HPV-16 L1 recombinants successfully made, including those that were unstable.

Recombinant	Promoter	Signal Sequence	Nature of vector	Successful recombinant or unstable
Tokyo pNVL1$_R$	*hsp60*	None	Integrated	Successful
Tokyo pT3L1$_R$	*hsp60*	None	Extrachromosomal	Unstable
Tokyo pT3L1$_{NLS^-}$	*hsp60*	None	Extrachromosomal	Unstable
Tokyo p261L1$_R$	*hsp60*	None	Extrachromosomal	Unstable
Tokyo p261L1$_{NLS^-}$	*hsp60*	None	Extrachromosomal	Unstable
Tokyo pAB26L1$_R$	*hsp60*	α antigen	Extrachromosomal	Unstable
Tokyo pAB26L1$_{NLS^-}$	*hsp60*	α antigen	Extrachromosomal	Unstable
Tokyo p2619L1$_R$	*hsp60*	19kDa lipoprotein	Extrachromosomal	Unstable
Tokyo p2619L1$_{NLS^-}$	*hsp60*	19kDa lipoprotein	Extrachromosomal	Unstable
Tokyo p361L1$_R$	*hsp60*	None	Integrated	Successful
Tokyo p361L1$_{NLS^-}$	*hsp60*	None	Integrated	Successful
Tokyo p361L1$_e$	*hsp60*	None	Integrated	Successful
Tokyo p119L1$_R$	*mtrA*	19kDa lipoprotein	Extrachromosomal	Successful
Tokyo p119L1$_{NLS^-}$	*mtrA*	19kDa lipoprotein	Extrachromosomal	Successful
Tokyo p119L1$_e$	*mtrA*	19kDa lipoprotein	Extrachromosomal	Successful
Tokyo p119L1$_{BCGe}$	*mtrA*	19kDa lipoprotein	Extrachromosomal	Successful
Tokyo p112L1$_R$	*p18kDa*	α antigen	Extrachromosomal	Successful
Tokyo p112L1$_{NLS^-}$	*p18kDa*	α antigen	Extrachromosomal	Successful
Tokyo p112L1$_e$	*p18kDa*	α antigen	Extrachromosomal	Successful
Tokyo p112L1$_{BCGe}$	*p18kDa*	α antigen	Extrachromosomal	Successful
Pasteur p361L1$_e$	*hsp60*	None	Extrachromosomal	Successful
Pasteur p119L1$_e$	*mtrA*	19kDa lipoprotein	Extrachromosomal	Successful
Pasteur p119L1$_{BCGe}$	*mtrA*	19kDa lipoprotein	Extrachromosomal	Successful
Pasteur p112L1$_e$	*p18kDa*	α antigen	Extrachromosomal	Successful
Pasteur p112L1$_{BCGe}$	*p18kDa*	α antigen	Extrachromosomal	Successful

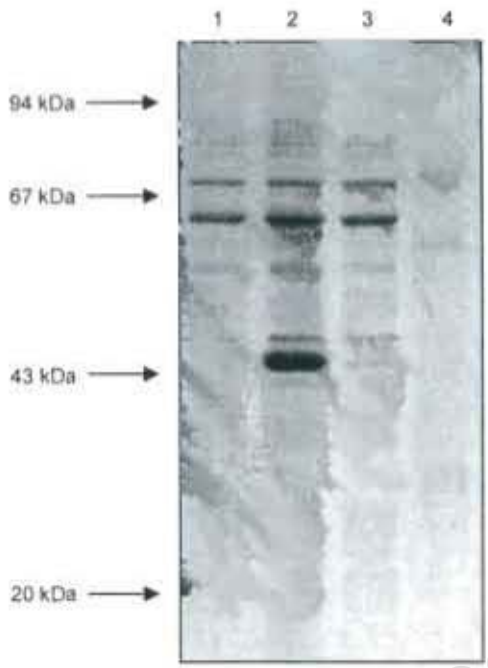


Figure 3.4. Coomassie blue stained SDS-PAGE of rBCG-L1 cell lysates. Lane 1, wild-type BCG; lane 2, rBCG-119L1$_{NLS}$-; lane 3, rBCG-361L1$_{NLS}$-; lane 4, HPV-16 VLP. All the BCG recombinants were of the Tokyo substrain. The expected L1 band (approx. 55kDa) was not apparent in any of the rBCG-L1 lanes. The only band apparent in the rBCG-L1 lysate (lane 2, rBCG-119L1$_{NLS}$-) that was not visible in the wild-type BCG lane, was that of the *lysA* gene product (which is present in the p119L1$_{NLS}$- plasmid, but not in any of the others utilised here).

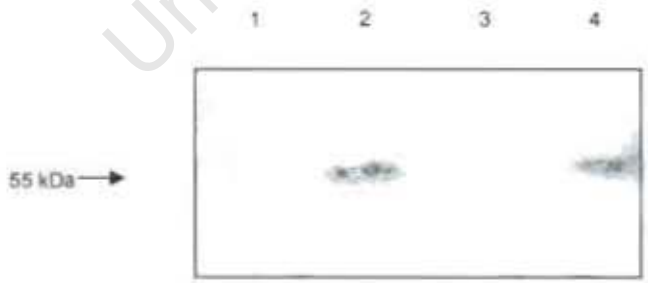


Figure 3.5. Western blot analysis of rBCG-L1 lysates using the H16.J4 monoclonal antibody. Lane 1, wild-type BCG; lane 2, rBCG-361L1$_{NLS}$-; lane 3, rBCG-112L1$_{NLS}$-; lane 4, rBCG-119L1$_{NLS}$-. All the BCG recombinants were of the Tokyo substrain. The arrow indicates the position of L1 monomer, which is approximately 55kDa in size, and which was confirmed by the addition of a HPV-16 VLP positive control (not shown).

Analysis of the western blot (Figure 3.5), on which L1 was successfully detected, shows L1 bands in the rBCG-361L1$_{NLS}$- and rBCG-119L1$_{NLS}$- lysate lanes. L1 was not detected in the rBCG-112L1$_{NLS}$- lane, or in the negative control (wild-type BCG) lane. The position of the L1 bands in the BCG lysate lanes were at the same level as the most prominent band (L1 monomer, 55kDa) occurring in the HPV-16 VLP lane (not shown).

3.4.4. L1 mRNA QUANTIFICATION

Due to the low production of HPV-16 L1 protein by the BCG-L1 recombinants, the L1 mRNA production by these recombinants was investigated. Total RNA was extracted from BCG, and reverse transcribed into cDNA using random primers. The number of copies of specific RNAs were measured by performing quantitative PCR on the cDNA.

16S cDNA, L1 cDNA and kanamycin cDNA quantification was performed on the standard and unknown samples (see Figures 3.6 and 3.7 for examples of 16S cDNA and L1 cDNA quantification, respectively). The LightCycler program compared the PCR efficiencies of the unknown samples to the standard samples (of which the gene copy numbers were known), and determined the concentrations (in cDNA copy number) of the 16S cDNA, L1 cDNA and kanamycin cDNA in each unknown sample. To normalise the amount of RNA in the samples, each unknown sample was divided by a factor to normalise the number of 16S cDNA copies at $1x10^4$. The L1 and kanamycin cDNA copy numbers of the unknown samples were then divided by the same factors, to obtain comparable data.

To verify the accuracy of the 16S cDNA quantification, the kanamycin mRNA in the Tokyo rBCG-112L1$_e$ and Tokyo rBCG-119L1$_e$ samples were quantified and normalised to 10^4 copies of 16S cDNA. The p112L1$_e$ and p119L1$_e$ plasmids utilise an identical kanamycin gene, which is expressed by the same promoter in both vectors; number of kanamycin mRNA copies relative to the 16S cDNA copies produced by these two samples should be the same. The relative number kanamycin mRNA copies were determined at 35.1 for rBCG-112L1$_e$, and 32.7 for rBCG-119L1$_e$ (Table 3.4; margin of error of 2.4 per 10^4 copies of 16S cDNA).

Table 3.4. Quantification of HPV-16 L1 mRNA (cDNA) in BCG-L1 recombinants. The amount of cDNA in each sample was normalised to the number of 16s rRNA-cDNA copies per sample, which enabled the copies of L1 cDNA to be directly compared. RNA was extracted from either freshly-made rBCG cultures or frozen samples.

Sample	L1 cDNA copies per 10^4 16S cDNA copies		Kanamycin cDNA copies per 10^4 16S cDNA copies
	Freshly-made BCG	Frozen BCG	Frozen BCG
Tokyo rBCG-361L1$_e$	15.0 ± 0.5	2.45 ± 0.08	Not tested
Pasteur rBCG-361L1$_e$	12.0 ± 0.12	1.20 ± 0.052	Not tested
Tokyo rBCG-112L1$_e$	30.7 ± 0.02	12.2 ± 0.037	35.1 ± 0.26
Tokyo rBCG-119L1$_e$	63.0 ± 0.18	55.8 ± 2.39	32.7 ± 0.17
*Pasteur rBCG-119L1$_e$	Not tested	51.5 ± 1.5	Not tested

* Only 88% of the frozen rBCG-119L1$_e$ (Pasteur) stock contains the L1 gene, as a result of genetic instability.

When comparing the number of L1 cDNA copies between the freshly-made rBCG-L1 cDNA samples (Table 3.4), it was evident that the integrating construct, p361L1$_e$, produced the least L1 cDNA (and therefore the least L1 mRNA), while the extrachromosomal vectors, p112L1$_e$ and p119L1$_e$, produced approximately 2- and 5-fold more L1 cDNA than p361L1$_e$, respectively. The number of L1 copies in the samples from the frozen BCG-L1 recombinants was reduced when compared with freshly-made BCG-L1 recombinants. This reduction was greater for p361L1$_e$ (6- to 10-fold lower), than it was for p112L1$_e$ and p119L1$_e$, 2.5- and 1.2-fold lower, respectively.

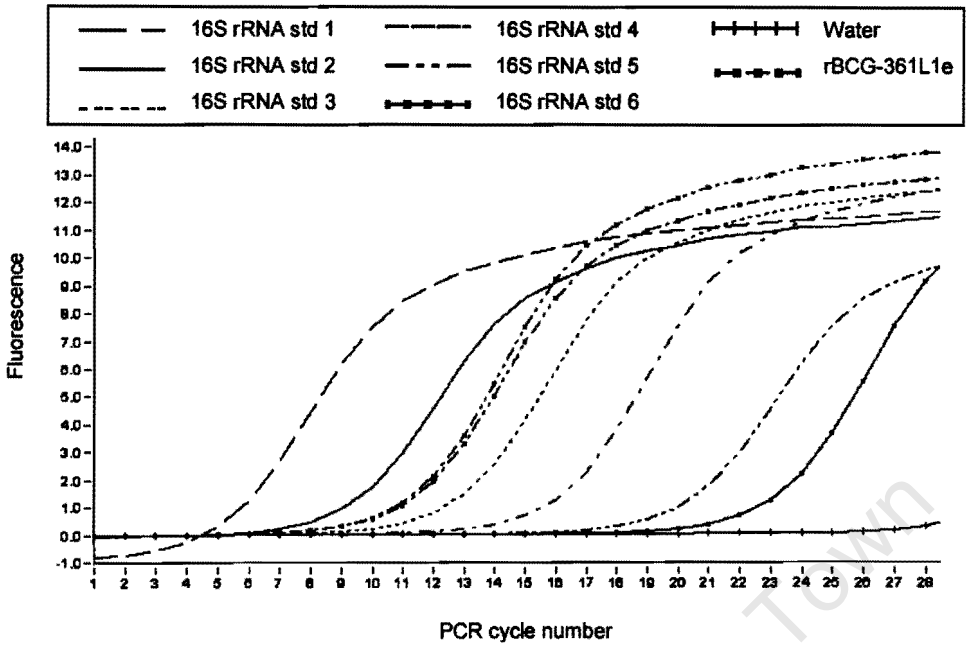


Figure 3.6. Quantification of 16S rRNA-cDNA in a Tokyo rBCG-361L1$_e$ cDNA sample. The 16S rRNA-cDNA was amplified from the rBCG-361L1$_e$ cDNA sample within the range of the standard samples. The amplification was performed in duplicate. The negative control sample (water) did not amplify, indicating that PCR amplification was specific.

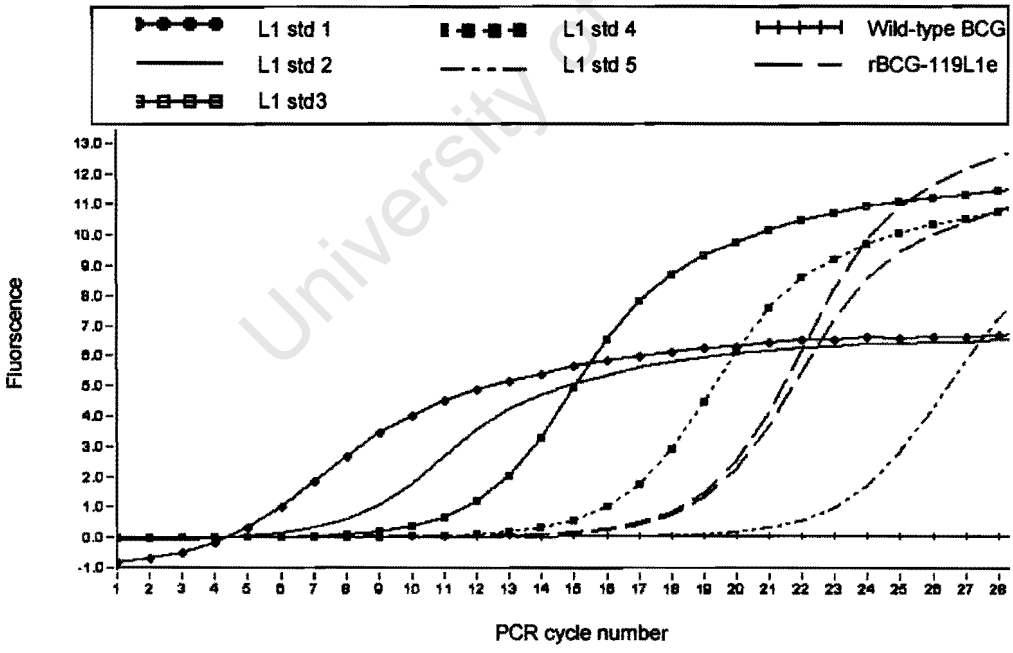


Figure 3.7. Quantification of L1 cDNA (mRNA) in a Tokyo rBCG-119L1$_e$ cDNA sample. The L1 cDNA was amplified within the range of the standard samples. The amplification was performed in duplicate. The negative control sample (wild-type BCG cDNA) did not amplify, indicating that PCR amplification was specific for L1.

3.5. DISCUSSION

Three L1 gene variants were cloned into a panel of *E. coli* - mycobacterial shuttle vectors, producing 20 HPV-16 L1 expression vectors for assessment in BCG. Of these 20 clones, 12 were stably introduced into BCG. Genetic instability, which was frequently detected in rBCG-L1, will be investigated further in Chapter 4. As the study evolved, more *E. coli* - mycobacterial shuttle vectors became available, and additional HPV-16 L1 gene variants were investigated, leading to the production of a large number of BCG recombinants. Initially, the intention was to compare the L1 *in vitro* expression between the various BCG-L1 recombinants; however, the low L1 expression levels prevented this. Finally, in order to compare L1 transcription levels, and also to rule out the vector defects, the BCG-L1 recombinants were assessed for their production HPV-16 L1 mRNA.

3.5.1. SELECTION OF HPV-16 L1 GENES

Three HPV-16 L1 variants namely, $L1_R$, $L1_{NLS}$-, and $L1_{BCG}$ were investigated. The amino acid sequence of $L1_R$ (and $L1_{NLS}$-) differs from the known high VLP-yielding L1 sequences, Phil and Sen32 (Touze *et al.*,1998), by 8 and 4 residues, respectively; however, there is no evidence to substantiate whether it is a high or low VLP-yielding sequence, as the $L1_R$ is dissimilar to all sequences investigated by Touze *et al.* (1998). $L1_{NLS}$- is expected to successfully assemble into stable VLPs (Chen *et al.*, 2001; Paintsil *et al.*, 1996), as only 22 residues were deleted from the C-terminal. Zhou *et al.* (1991c) showed that when cells were infected with vaccinia virus expressing HPV L1, the L1 protein accumulated almost exclusively in the nucleus of the infected cells, however, when the NLS was deleted, L1 was distributed evenly throughout the cell. The NLS was removed to determine whether the cellular localisation of L1 in rBCG-L1 infected cells would have an effect on L1 antigen presentation, and hence on the immune responses elicited by rBCG-L1. To maximise the L1 yield in BCG, the L1 gene was codon optimised to reflect commonly used BCG codons. The amino acid sequence utilised for this gene was based on the L1 Phil sequence (Touze *et al.*, 1998). Although the L1 Phil sequence produces high VLP yields in the baculovirus expression system, it is unknown whether the same will hold true in a bacterial expression system.

Although assembly of L1 into capsomeres and VLPs has been observed in *Salmonella* (Nardelli-Haefliger *et al.*, 1997), *E. coli* does not support *in vivo* VLP formation (Chen *et al.*,

2001; Li *et al.*, 1997; Zhang *et al.*, 1998); and it is therefore unknown whether expression in *Mycobacteria* would facilitate VLP assembly. The only other group (Jabbar *et al.*, 2000) to study the expression of HPV proteins in BCG, established the expression of HPV-6b L1 by western blot analysis but, did not investigate whether HPV-6b L1 assembled into VLPs. The assembly of L1 into capsomeres and VLPs is essential for the induction of conformationally-dependent neutralising antibody responses (Rose *et al.*, 1998; Fligge *et al.*, 2001; Kirnbauer *et al.*, 1992). One of the expression vectors (pCB112) utilised in this study directs heterologous protein out of the bacterium, and another to the bacterial cell wall (pCB119); if L1 export is successful we believe that VLP formation in the mycobacterium would be prevented. If L1 is successfully exported to the BCG cell wall, these monomers will be unable to assemble into VLPs. If L1 is secreted from the bacterium and can accumulate to sufficient quantities in the host cell nucleus, as directed by the nuclear localisation signal, VLP assembly may occur there, but the accumulation of sufficient L1 in the host cell is unlikely. If VLP assembly occurs within the bacterium before export is able to take place, its export would be prevented.

3.5.2. rBCG-L1 INSTABILITY

The above L1 gene variants were cloned into a panel of *E. coli*-mycobacterial shuttle vectors. These L1 expression constructs were introduced in to BCG (Pasteur and Tokyo) with varying rates of success, as genetic instability prevented the stable maintenance of some recombinants. It was clear that the unstable BCG recombinants were those containing extrachromosomal vectors which utilised the *hsp60* promoter to drive L1 expression. This instability, which was presumably a consequence of the overproduction of L1, was most likely due to the accumulative effects of the high *in vitro* activity of *hsp60* (Stover *et al.*, 1991), and the multi-copy nature of the plasmids. The integration-proficient plasmids, which also utilised *hsp60* to drive L1 expression, were more stably maintained; suggesting that chromosomes are less prone to gene deletions, or that the presence of only one copy of the integrating vector per bacterium, compared to five copies of the extrachromosomal vectors, sufficiently decreased L1 expression and hence increased stability. The observation that the *hsp60* promoter causes genetic instability of the vectors from which it expresses is not unique to this study, as this has been observed by other groups (Al Zarouni and Dale, 2002; Medeiros *et al.*, 2002; Stover *et al.*, 1991). In fact, Stover *et al.* (1991) observed that expression of the HIV-1 gp120 gene, under the control of the *hsp60* promoter, was lethal to BCG when expressed on an extrachromosomal vector, but not when expressed on an

integrative vector. The other extrachromosomal constructs (p112-L1* and p119-L1*), although also present in multi-copies, were more stable than the *hsp60*-driven vectors, as stable recombinants were obtained with all these constructs (although more often than not, electroporation produced a mixture of stable and unstable recombinants). The higher *in vitro* stability of the p112-L1* and p119-L1* constructs was presumably due to the weak *in vitro* activity of the *M. leprae 18kDa* and *mtrA* promoters (Dellagostin *et al.*, 1995; Zahrt and Deretic, 2000). The stability of the BCG recombinants will be discussed in more detail in Chapter 4.

3.5.3. EXPRESSION OF HPV-16 L1 BY rBCG-L1

The expression of HPV-16 L1 protein by rBCG-L1 was not apparent by Coomassie staining, and was only detectable by western blot analysis on one occasion, indicating that expression levels were very low. Attempts to up-regulate protein expression by heat-shock, and the investigation of the insoluble protein fractions, also failed to ascertain L1 expression. It was established that L1 was produced at concentrations below the detection threshold (0.03 μg) of the analysis, which was determined using purified HPV-16 VLPs. This equated to an L1 contribution of less than 0.3% of total rBCG protein.

A comparison of the BCG and HPV-16 L1 codon usage revealed that many L1 codons were used extremely infrequently by BCG. In bacteria, transcription-translation coupling is believed to prevent the accumulation of non-functional transcripts, which have the capacity to form R-loop complexes with DNA, thus impeding transcription elongation (Gowrishanka and Harinarayanan, 2004). Bacterial mRNA production and stability are therefore influenced by the efficiency of the transcription-translation coupling, which in turn is influenced by the rate of translation. The importance of efficient translation prompted us to have the HPV-16 L1 ORF re-synthesised to reflect commonly used BCG codons. Unfortunately, codon optimisation did not appear to increase the *in vitro* expression of L1, as L1 production was still at concentrations below the western blot detection threshold. This low production of L1 made it impossible to compare expression levels and localisation between the BCG expression vectors.

3.5.4. L1 mRNA QUANTIFICATION

Other than a slow rate of translation, the poor L1 expression by BCG may be attributed to a low rate of transcription, or a high rate of degradation by proteases; of which the latter is the most plausible. To test the former premise, the amount of L1 mRNA produced by rBCG-L1 was quantified. Although the quantification method utilised can supply absolute values, it does not detect or compensate for PCR inhibitors present in the sample material; and may therefore be inaccurate, unless internal controls are utilised. Quantification relative to a housekeeping gene (which is assumed to be constant) should, however, be accurate without compensating for differences in PCR efficiencies between samples. Even if the absolute values obtained from this study are deemed slightly inaccurate, there is still a large amount of L1 cDNA (and therefore L1 mRNA) present (1.2×10^1 - 6.3×10^1 copies of L1 cDNA per 10^4 copies of 16S cDNA [16S rRNA], which is a very abundant RNA). It is important to bear in mind that the promoters utilised here are strongly up-regulated *in vivo*, or under stress-response conditions; and since these conditions were not employed during this study, relatively low amounts of mRNA were expected. The relative comparison of the L1 mRNA copies between the freshly-made rBCG-L1 samples provided interesting results. The integrating construct, p361L1$_e$, produced the least L1 mRNA, presumably due to the presence of only one L1 gene per BCG chromosome. Although identical L1 mRNA levels were anticipated from the BCG Tokyo p361L1$_e$ and Pasteur p361L1$_e$ samples, a difference of approx. 3 copies (per 10^4 16S cDNA copies) was obtained; which may be due to slight differences in culture growth or RNA extraction conditions, or due to expression differences that exist between BCG substrains (Burlein *et al.*, 1994). However, the accuracy of this assay was determined at 2.4 per 10^4 copies of 16S cDNA, which may prevent the accurate comparisons of samples which have differences similar to this margin of error. The extrachromosomal vectors, p112L1$_e$ and p119L1$_e$, produced higher levels of L1 mRNA than p361L1$_e$; which is likely due to the multiple copy nature of the former plasmids. p119L1$_e$ produced more L1 mRNA than p112L1$_e$, which suggests that the *mtrA* promoter (in p119L1$_e$) is more active than the *M. leprae 18kDa* promoter (in p112L1$_e$) *in vitro*. The relative L1 mRNA copies were also investigated in frozen rBCG-L1 stocks, as these stocks were utilised for animal inoculations, and their genetic stabilities were well characterised. The process of freezing the rBCG-L1 samples reduced the expression of L1 relative to 16S rRNA. This reduction could have been a consequence of RNA degradation during the freezing process; however, the L1 RNA reduction was greater for p361L1$_e$ than it was for the other two vectors. It therefore appears that the drop in temperature during the freezing process achieved a more rapid suppression of *hsp60* promoter activity than it did with *mtrA* or *18kDa*

promoter activity. Temperature reduction is known to cause specific suppression of some heat-shock proteins (Jones and Inouye, 1994), which concurs with the reduction in *hsp60* promoter activity seen here. mRNA was not analysed from the two recombinants (rBCG-361L1$_{NLS}$- and rBCG-119L1$_{NLS}$-) from which L1 protein was initially detected, as these were not considered especially strong L1 producers due to the inability to repeat the detection of L1 from these strains.

The evidence of L1 RNA expression from this study, and the effective use of the same vectors and/or promoters to achieve high foreign antigen expression in other studies (Himmelrich *et al.*, 2000b; Stover *et al.*, 1993), makes it unlikely that the low expression of the L1 protein is a consequence of low L1 transcription. The low expression is also not likely due to inefficient translation, as codon optimisation did not increase L1 production to detectable levels, and the instability is an indication of high expression levels. Degradation by proteases therefore seems to be the most probable cause of the apparent low expression levels. The same conclusion was drawn by Chen *et al.* (2001), who were unable to express the HPV-16 and HPV-11 L1 proteins in *E. coli*, even after rare codons were removed. The rate of degradation by proteases is a property of the antigen itself; which will be different for all proteins, and will be dependent on the system in which it is expressed. It would be interesting to compare wild-type and rBCG-L1 gene regulation by BCG microarray, as this may corroborate protease up-regulation in rBCG. As mentioned in section 3.1.4, bacterial proteins are expressed to higher levels in BCG; this is evident by the Coomassie gel analysis (section 3.4.3) where the *lysA* gene product produced a very prominent band. Here the expression of *lysA* is driven by the *hsp60* promoter, which is also utilised to drive L1 expression in this study, with expression levels nowhere near that of the *lysA* product.

The *in vitro* protein expression levels, however, may not accurately imitate the situation occurring *in vivo*, as the activity of the promoters utilised in this study are known to be induced upon infection of macrophages (Batoni *et al.*, 1998; Zahrt and Deretic 2000). It would be interesting to investigate whether the expression of L1 mRNA or L1 protein levels increase after infection of macrophages with the BCG-L1 recombinants used in this study.

The next chapter provides a short review of *in vitro* and *in vivo* studies, which have reported genetic instability in recombinant mycobacteria. After which, the stability of the recombinants in the present study are assessed further.

CHAPTER 4: GENETIC STABILITY OF BCG RECOMBINANTS

4.1. INTRODUCTION

In the previous chapter, the instability of the BCG-HPV-16 L1 recombinants was reported. *In vitro* genetic stability of recombinant BCG is a necessity for long-term maintenance and large-scale production. *In vivo* stability is also required for the persistent delivery heterologous antigens to the immune system. This chapter provides a short review of the studies which have assessed *in vitro* and *in vivo* genetic stability of recombinant mycobacteria. Thereafter, the *in vitro* stabilities of the recombinants produced in the present study are investigated further.

4.1.1. STABILITY OF HETEROLOGOUS GENES AND EXPRESSED PROTEINS

When heterologous genes are expressed in bacteria, the bacteria are subjected to new conditions that are possibly suboptimal for normal bacterial growth. Depending on the type of protein or amount produced, the "health" of the recombinant could suffer from effects ranging from slower growth (due to the metabolic burden of protein manufacture) to cell death (from protein toxicity). Selective pressures therefore exist for the removal of such heterologous genes. The instability of foreign genes in mycobacteria has been reported frequently; some of these reports are described below.

The *hsp60* promoter appears to beget the majority of the foreign gene instability reports in mycobacteria, presumably a consequence of its high activity. Stover *et al.* (1991) noted that the expression of the HIV-1 gp120 gene under the control of the *hsp60* promoter, from an extrachromosomal vector, was lethal to BCG, and that it was only possible to express this gene on an integrative vector. Al-Zarouni and Dale (2002) reported instability in 95% of plasmids, in the form of deletions in the *hsp60* promoter region, when expressing the *lacZ* gene. No instability was detected when utilising the *18kDa*, *85A*, and *19kDa* promoters. When expressing β-gal in *M. smegmatis*, Medeiros *et al.* (2002) observed gross modifications of the constructs that utilised the *hsp60* promoter, but not in those that used the *18kDa* promoter. They showed that within two subcultures of the cells containing the *hsp60* construct, in liquid medium, 90% of the cells had lost their ability to express β-gal. Kumar *et al.* (1998) studied the genetic rearrangements of the *lacZ* gene under the control of the *hsp60* promoter in *M. smegmatis*. They detected the disruption of the *lacZ* gene by insertion sequences in integrating vectors, and by deletions in the *lacZ* gene and the *hsp60* promoter in extrachromosomal vectors, they however found these rearrangements to be rare

events. The frequency of the loss of *lacZ* phenotype was $1.7x10^{-5}$ for integrative vectors and $2x10^{-3}$ for extrachromosomal vectors.

Al-Zarouni and Dale (2002) observed that deletions occurred during or soon after transformation, but not during subsequent growth. They therefore suggest that there is a transient lethal induction of the *hsp60* promoter during electroporation. Another possible explanation for the high rate of rearrangements seen when the *hsp60* promoter is utilised, is due its natural function of regulating Hsp60 production. Hsp60 is a chaperone protein that is up-regulated during conditions such as heat-shock due to the presence of non-native proteins, and is involved in assisting and directing the folding of unfolded or incorrectly folded proteins (Bukau and Horwich, 1998). In recombinant BCG the presence of unfolded heterologous proteins could up-regulate the *hsp60* promoter, causing further production of the heterologous protein, and thus causing overt stress on the BCG recombinant.

The attenuation of the growth of recombinant BCG relative to wild-type BCG has been described *in vivo* and *in vitro*. The ability of rBCG expressing the *Borrelia burgdorferi* OspA protein (extrachromosomal vector, *hsp60* promoter, 18kDa lipoprotein signal) to replicate and persist *In vivo* was attenuated in comparison to wild-type BCG (Stover *et al.*, 1993). BCG expressing the measles virus nucleoprotein on an extrachromosomal vector (copy number of 5, *hsp60* promoter) had a slower growth rate than wild-type BCG when grown in liquid culture, however, expression the same protein on an integrated vector (one copy per cell) did not alter the growth rate (Zhu *et al.*, 1997).

A few researchers have reported varying rBCG stabilities when expressing different portions of the same genes, suggesting a possible size determinant, or that some areas of a protein are more "toxic" than others. Bastos *et al.* (2002) found that BCG could not successfully express the full-length GP5 protein of porcine reproductive respiratory syndrome virus, due to the development of deletions and frame-shift mutations; however, a truncated form of the gene (lacking the first 90 5' base pairs) was stably expressed. Similarly, Lim *et al.* (1997) were not able to generate BCG recombinants that expressed aa 1-512 or aa 215-521 of the SIV gp110 protein, but aa 1-245 of the same protein was expressed well.

Mederle and colleagues (2002) noted that when the HIV Gag and Nef proteins were co-expressed in BCG from synthetic *gag-nef* operon, Gag was expressed efficiently, while the production of Nef was very low, suggesting differences in protein stability. A number of

studies have reported the presence of possible heterologous protein degradation on western blots (Fennelly *et al.*, 1995; Miyaji *et al.*, 2001; Supply *et al.*, 1999).

4.1.2. VECTOR STABILITY (*IN VIVO* AND *IN VITRO*)

In addition to the stability of the heterologous gene in the expression construct, the retention of the whole expression construct by the BCG recombinant in the absence of antibiotic selection warrants assessment. This is particularly important in the *in vivo* situation, as persistent expression of heterologous protein is desired to allow for continual immune stimulation. Studies of *in vitro* and *in vivo* stability, and comparisons between extrachromosomal and integrating vector stability are reviewed below.

Haeseleer *et al.* (1993) reported that BCG and *M. smegmatis* recombinants expressing the circumsporozoite protein of *Plasmodium facciparum* (CSP) from an integrating vector (*hsp60* promoter) maintained stable expression for at least 10 weeks in the absence of selective pressure. Almost 100% of the colonies retained kanamycin resistance after 400 generations of growth without antibiotic selection in the case of *M. smegmatis*, and 50 generations in the case of BCG. Lee *et al.* (1991) found that after culturing *M. smegmatis* for 30 generation without antibiotic selection, there was no loss of the integrative vector, but 65% of the colonies containing an extrachromosomal vector were no longer kanamycin resistant.

28 and 100 days after immunising mice with rBCG containing an extrachromosomal vector expressing HIV Gag and Nef, Mederle *et al.* (2002) 40 and 75% of colonies isolated from mouse spleens, respectively, had lost their resistance to kanamycin. However, when integrated vectors were utilised less than 15% of the colonies had lost their resistance to kanamycin after 100 days. 2 and 16 weeks after immunising guinea pigs with a BCG-*lacZ* recombinant (extrachromosomal, P_{AN} promoter), 54 and 60% of BCG harvested from spleen and lymph nodes, respectively, had lost the *lacZ* phenotype (Lagranderie *et al.*, 1993). Four weeks after immunising macaques with rBCG expressing SIV Nef, Gag or Env (extrachromosomal vectors), Mederle *et al.* (2003) tested the genetic stability of the BCG isolated from draining lesions. They found that, of the total number of BCG colonies isolated, only 56% were still kanamycin resistant, and of these colonies 58% contained the *gag* gene, 17% contained the *nef* gene, and 9% contained the *env* gene, indicating differing gene stabilities or different levels of rBCG growth attenuation. In human trial patients rBCG-OspA (extrachromosomal vector, *hsp60* promoter) was cultured from the vaccination sites 14-24

days after vaccination; of the BCG colonies isolated, only 67-80% were still resistant to kanamycin (Edelman *et al.*, 1999).

Some studies, on the other hand, have reported high stabilities of extrachromosomal vectors. Stover *et al.* (1991) noted that 2-4 weeks after immunisation of mice with BCG expressing β-gal from an extrachromosomal vector, all BCG colonies removed from the spleens expressed β-gal and were resistant to kanamycin. 55 days after inoculating mice with BCG expressing HPV-6 L1 (extrachromosomal vector, *hsp70* promoter), all 3 randomly picked colonies (harvested from spleens) were positive for expression of the L1 protein (Jabbar *et al.*, 2000). 10 weeks after inoculation of mice with rBCG-gp63 (extrachromosomal vector, *hsp60* promoter), the BCG harvested from the spleens produced equal numbers of colonies on plates with or without kanamycin, confirming plasmid maintenance (Connell *et al.*, 1993).

4.2. STUDY OBJECTIVES

The aim of this chapter was to assess the *in vitro* genetic stability of the BCG-HPV-16 L1 recombinants generated in this study, and to characterise the plasmid rearrangements. Culturing rBCG-L1 with antibiotic selection will provide us with an idea of the recombinant's potential for long-term maintenance; while culturing rBCG-L1 without the presence of antibiotics will provide insights into the length of *in vivo* foreign antigen expression.

4.3. METHODS

4.3.1. *IN VITRO* PLASMID STABILITY WITH ANTIBIOTIC SELECTION

To determine the frequency of plasmid rearrangements in originally stable BCG recombinants, the recombinants were grown in liquid culture, and tested for the presence of the L1 gene at various stages.

Directly after electroporation, BCG colonies were selected and grown in 10ml of medium to an OD_{600} of between 0.5 and 0.8 (approximately 10 generations after picking the colony), as described in Appendix A3.2. Each culture was then halved; the one half was frozen, while the other half was used to verify whether a successful transformation event had transpired

(Chapter 3, section 3.3.7). Stable recombinants were thawed and grown in 100ml of Sauton's medium, containing kanamycin, until an OD_{600} of between 0.5 and 0.7 had been attained (Appendix A1.2). The recombinant had now been though approximately 16 generations since the colony had been picked. The cells were prepared for animal immunisations and then frozen (Appendix A15). Before the immunogenicity of the BCG-L1 recombinants were investigated, the stocks were tested for plasmid stability. An aliquot of frozen rBCG-L1 (inoculation stock) was allowed to thaw, and the cells were harvested by centrifugation. Plasmid DNA was extracted from the cells and was then transformed into *E. coli*, as described in Appendix A 5.3. This method was effective at separating a possible mixed population of stable and rearranged plasmids that may have developed in the rBCG-L1 culture. 20 *E. coli* colonies were selected and grown in 5ml of liquid medium. Plasmid DNA was extracted from these cultures and then digested with restriction endonucleases to detect the presence of the L1 gene and its promoter. PCR was used to verify the presence of the L1 gene in the BCG-L1 recombinants containing integrated vectors. After the frozen BCG stocks were allowed to thaw, they were serially diluted in Sauton's medium, and plated to obtain single colonies (Appendix A1.2). PCR reaction mixtures were set up as described in Appendix A9, using the CBF and CBR primers to amplify the L1 gene. Colonies were picked and added directly to the PCR reaction mixtures. A typical thermocycler reaction profile was utilised (Appendix A9), except for an extended initial denaturing step (10min), which was used to promote lysis of the bacteria.

The effect of further culturing was investigated. Frozen recombinant BCG stocks (inoculation stocks) were grown in 100ml of Sauton's medium, and the presence of L1 was again investigated, as described above. The recombinants had now been though approximately 24 generations in liquid culture since the colony picked after electroporation; and approximately 44 generations in total, as it was estimated that a BCG colony forms in 20 generations of growth.

4.3.2. SEQUENCING OF rBCG-L1 STOCKS

Although restriction endonuclease analysis of the BCG recombinants can detect large gene alterations, small changes may possibly go undetected. The recombinants were therefore sequenced to determine whether point mutations or small deletions had occurred.

Stable BCG-L1 recombinants were either sequenced directly after electroporation (Table 3.3, Chapter 3) or after they had been prepared as inoculation stocks. Plasmid DNA was isolated from the BCG recombinants and introduced into *E. coli* (Appendix A5.3). The plasmid DNA was then purified from the *E. coli* recombinants using the High Pure Plasmid Isolation Kit (Roche Diagnostics, Germany; Appendix A5.5). These DNA samples were then sequenced, as described in Chapter 3, section 3.3.6, to obtain sequence of the promoter and L1 gene region.

In order to sequence L1 from BCG containing integrated vectors, the chromosomal BCG DNA was extracted (Appendix A5.4), and the *hsp60*/L1 region was amplified by PCR with the 361F and 26R primers (as described in section 3.3.6). The *hsp60*/L1 PCR amplification product was then cloned into the pGEM-T vector, using the pGEM-T Easy kit (Appendix A8.3). The cloned *hsp60*/L1 regions were then sequenced using the M13 primers, and the CBCR, CBDR and CBDF primers (section 3.3.6).

4.3.3. SEQUENCING OF PLASMID REARRANGEMENTS

As noted in Chapter 3, transformation of certain constructs resulted in stable recombinants, however, transformation of other constructs resulted in mixed populations of stable and unstable recombinants, or in transformants that all contained plasmid rearrangements. A number of unstable recombinants (detected directly after transformation) were sequenced to further characterise the excisions.

Plasmid DNA was prepared as described above (section 4.3.2). The BCG Tokyo recombinants containing unstable pAB26L1$_R$, p261L1$_R$, and p261L1$_{NLS}$- were sequenced with primers 26F and 26R. Pasteur rBCG-112L1$_e$ was sequenced with the CBAF primer. Two unstable Pasteur rBCG-119L1$_{BCGe}$ recombinants were sequenced with primers CBAF, CBBF and CBCF. Unstable Tokyo rBCG-119L1$_e$ was sequenced with primers CBAF, CBBF and BCGL1CF. The primers are described in section 3.3.6. The sequencing was performed at the Core Facility (University of Stellenbosch).

4.3.4. *IN VITRO* rBCG-L1 STABILITY WITHOUT ANTIBIOTIC SELECTION

It is widely accepted that recombinant bacteria eventually loose their plasmid DNA when the selective pressure to keep it is removed; and that recombinant bacteria containing integrated heterologous DNA are often more stable than those containing extrachromosomal plasmids (Haeseleer *et al.*, 1993; Matsumoto *et al.*, 1996). To provide an indication of the stability and length of expression of the BCG-L1 recombinants in animals (i.e. in the absence if antibiotic selection), the BCG-L1 recombinants were cultured in liquid medium without the addition of antibiotics.

50ml of Sauton's broth containing kanamycin (16.7μg/ml) was inoculated with rBCG-L1 and grown on a roller at 37°C until an OD_{600} of between 0.5 and 0.8 was attained. The kanamycin was removed from the cells by centrifugation (3000rpm for 10min) and re-suspension in 50ml of Sauton's medium. The washing step was repeated twice. After the washes, the cell pellet was resuspended in 50ml Sauton's medium and the OD_{600} was measured. 0.5ml of this culture was used to inoculate 50ml of Sauton's medium, without kanamycin. The culture was grown on a roller at 37°C until an OD_{600} of 0.5 to 0.8 was reached. Again, 0.5ml of this culture was used to inoculate 50ml of medium, which was grown as above. This was repeated 4 times until approximately 26 generations were grown without antibiotic selection. A generation was measured as a doubling of the OD_{600}. After 26 generations of growth without antibiotics the culture was serially diluted and spread on 7H10 agar plates, either with or without antibiotics. The number of colonies on the antibiotic-containing and non-antibiotic-containing plates was compared; and the percentage of bacteria retaining antibiotic resistance was calculated.

4.4. RESULTS

4.4.1. *IN VITRO* PLASMID STABILITY WITH ANTIBIOTIC SELECTION

The stability of the BCG-L1 recombinants, that appeared to be stable directly after electroporation, was tested after further culturing in liquid medium containing antibiotics, by assessing for the presence of the L1 gene and promoter. The table below (Table 4.1) summarises the percentage of stable recombinants after 16 and 24 generations of growth in liquid medium with antibiotics. While some recombinant BCG-L1 cultures stably maintained

the L1 gene, other cultures lost the L1 gene at varying rates. BCG containing the integration-proficient construct, p361L1$_e$, and the extrachromosomal p112L1$_R$/L1$_e$/L1$_{NLS^-}$/L1$_{BCGe}$ (L1*) constructs (except for Pasteur rBCG-112-L1$_e$) were 100% stable. Overall, recombinants containing the p119L1* constructs were less stable than the p361L1$_e$ and p112L1* recombinants. The Pasteur recombinants (excluding the integrated recombinants) appeared to be less stable than their Tokyo counterparts.

Table 4.1. Percentage of recombinant BCG retaining the L1 gene after 16 and 24 generations of growth in liquid medium with antibiotics.

Substrain and plasmid	Percentage bacteria containing the promoter and L1 gene	
	After 16 generations	After 24 generations
Integrated plasmids:		
Tokyo rBCG-361L1$_e$	100 (assayed for L1 only)	nd
Pasteur rBCG-361L1$_e$	100 (assayed for L1 only)	nd
Extrachromosomal plasmids:		
Tokyo rBCG-112L1$_R$	100	nd
Tokyo rBCG-112L1$_{NLS^-}$	100	nd
Tokyo rBCG-112L1$_e$	100	100
Tokyo rBCG-112L1$_{BCGe}$	100	100
Pasteur rBCG-112L1$_e$	0	nd
Tokyo rBCG-119L1$_R$	15.8	nd
Tokyo rBCG-119L1$_e$	100	87
Tokyo rBCG-119L1$_{BCGe}$	100	nd
Pasteur rBCG-119L1$_e$	88	nd
Pasteur rBCG-119L1$_{BCGe}$	9.1	nd

nd, not determined

4.4.2. SEQUENCING OF rBCG-L1 STOCKS

BCG-L1 recombinants that appeared stable by restriction enzyme mapping were sequenced to confirm their sequence fidelity. No extensive changes were observed, however, point mutations were detected within the L1 gene of the Tokyo rBCG-112L1$_e$ and Tokyo rBCG-361L1$_e$ recombinants. No amino acid changes resulted from these mutations. The mutations were as follows:

- Tokyo rBCG-112L1$_e$ had two point mutations: At L1 nucleotide 141 there was a change from T to C (codon change from CAT to CAC), and at L1 nucleotide 1317 there was a change from T to C (codon change from GAT to GAC).
- Tokyo rBCG-361L1$_e$ had two point mutations: At L1 nucleotide 90 a change from C to G was detected (codon change from CGC to CGG); and at L1 nucleotide 114 a change from A to G was found (codon change from GGA to GGG).

4.4.3. SEQUENCING OF PLASMID REARRANGEMENTS

Sequencing of the genetically altered plasmids confirmed the data obtained by restriction endonuclease analysis; that deletions had occurred in the L1 gene and its promoter. As seen in Figure 4.1, the genetic rearrangements ranged from deletions spanning the entire L1 gene and promoter region, to smaller deletions within the L1 gene. Often, these deletions occurred in non-essential sequences adjacent to the L1 gene, such as the *lys A* gene. The deletions did not occur at sequence-specific sites.

4.4.4. *IN VITRO* rBCG-L1 STABILITY WITHOUT ANTIBIOTIC SELECTION

After 26 generations of growth in liquid medium without antibiotic selection, the rBCG-L1 cultures were tested for plasmid stability. The cultures were spread onto plates, either with or without antibiotics, and the number of colonies was compared to determine the percentage of bacteria that had retained their antibiotic resistance (Table 4.2). It was assumed that the loss of antibiotic resistance was due to the loss of the entire expression vector.

Figure 4.1. Examples of the gene deletions that were detected in L1 expression vectors obtained from BCG recombinants. The figures show the original plasmid and the deletions which are represented by the ▨ bar.

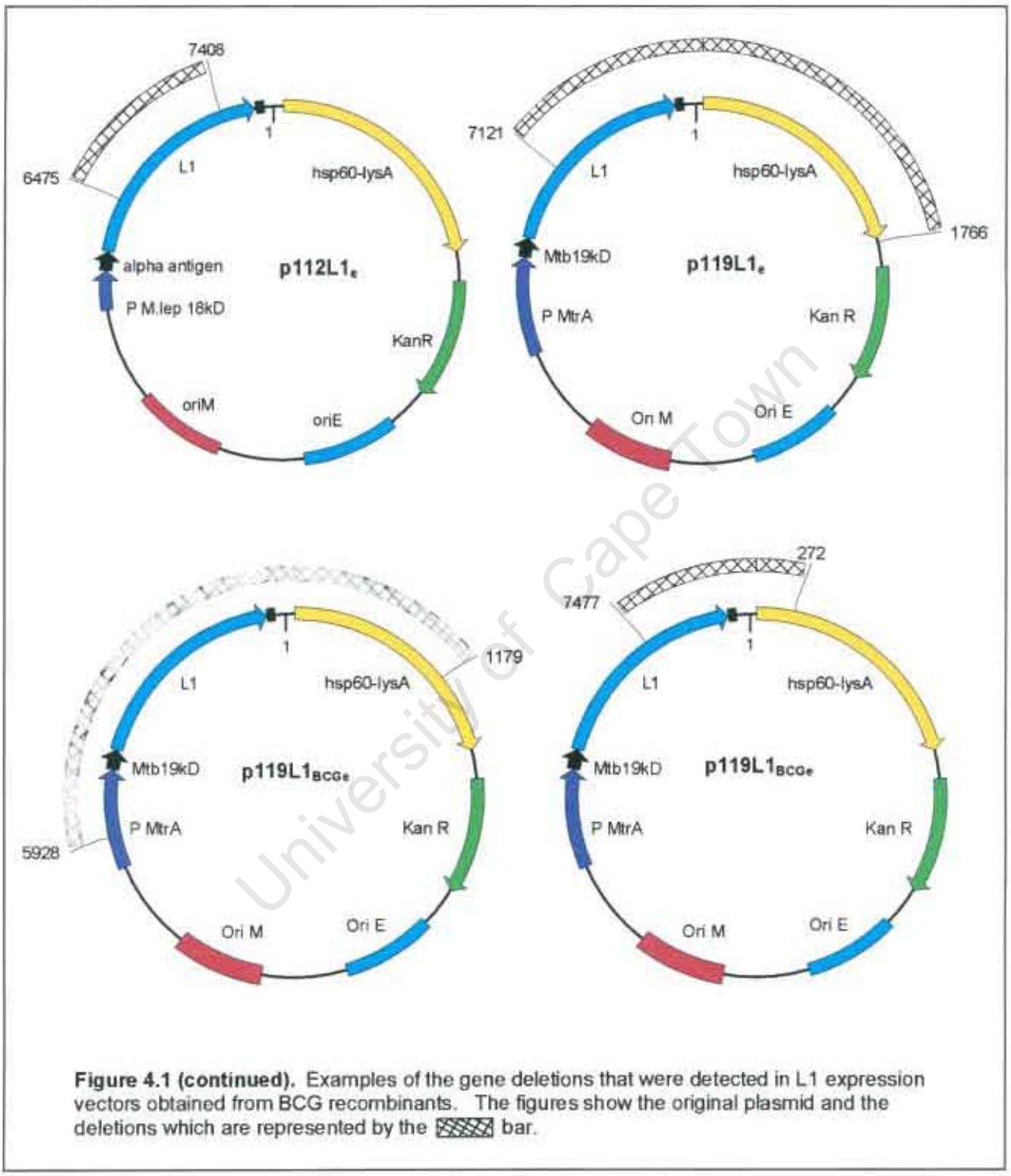


Figure 4.1 (continued). Examples of the gene deletions that were detected in L1 expression vectors obtained from BCG recombinants. The figures show the original plasmid and the deletions which are represented by the ▨ bar.

(BCG Tokyo and BCG Pasteur) containing the integration-proficient vector, p361L1$_e$, were 100% stable after 16 generations. Although the stability of the integrated recombinants was not determined after 24 generations, we believe that the integrated recombinants were more stable than the extrachromosomal recombinants, because in contrast to the extrachromosomal recombinants, both BCG Tokyo and BCG Pasteur integrated recombinants were 100% stable after 16 generations, and enhanced stability of integrated BCG recombinants has been observed in other studies (Stover *et al.*, 1991; Kumar *et al.*, 1998).

The BCG Tokyo recombinants containing the p112L1* constructs were 100% stable at 24 generations; showing a higher stability than the Tokyo recombinants containing the p119L1* constructs, which is possibly due to the stronger promoter of p119L1* (see discussion Chapter 3). It is possible that the Pasteur p112L1$_e$ construct was 0% stable after 16 generations if a deletion mutant appeared early in the culture, which then outgrew the slower-growing stable recombinants. The BCG Pasteur recombinants (excluding the integrated recombinants) were less stable than their Tokyo counter parts. A study by Al-Zarouni and Dale (2002) also detected varying degrees of instability between different BCG substrains, however, they detected gene deletions in BCG Tokyo and BCG Moreau more frequently than in BCG Pasteur. There was a large difference between the stabilities of Tokyo rBCG-119L1$_R$ (15.8% stable) and rBCG-119L1$_e$ (100% stable at 16 generations), but due to the similarity of these constructs it is unlikely that they assert significantly different stresses on BCG. In this case is likely that an early deletion mutant appeared in the Tokyo rBCG-119L1$_R$ culture.

Sequencing of the deletion mutants confirmed that deletions had occurred in the L1 gene and its promoter. None of the excisions occurred at exactly the same sites, indicating that there were no sequence-specific areas that were prone to recombination. Many deletions occurred in non-essential sequences adjacent to L1, such as the *lys A* gene, as the expression of this gene also places a metabolic load onto the bacteria. The *lys A* gene is only necessary for bacterial survival in lysine auxotrophic BCG mutants (W. Jacobs, Dept. of Microbiology and Immunology, Albert Einstein College of Medicine, unpublished),

Screening of recombinants by restriction enzyme digestion, Southern blot analysis, or PCR has limitations as point mutations or small deletions would go undetected. The BCG-L1 recombinants that appeared stable were therefore sequenced to verify the sequence fidelity of the L1 gene and promoter. Very few point mutations, and no significant changes, were

observed in the L1 sequences of the BCG-L1 recombinants. Point mutations were only detected in two recombinants, which did not result in amino acid sequence changes. This was surprising as although many large genetic alterations occurred to reduce the expression of L1, no significant point mutations or small deletions took place.

4.5.2. *IN VITRO* rBCG-L1 STABILITY WITHOUT ANTIBIOTIC SELECTION

The BCG-L1 recombinants containing integrated vectors were more stable than the extrachromosomal constructs when grown without antibiotic selection. On average, 85% of the bacteria in the cultures containing integrating vectors were still antibiotic resistant after 26 generations of selection-free growth, but only 25% of the bacteria in the cultures containing extrachromosomal constructs remained antibiotic resistant. These results concur with other studies (Lee *et al.*, 1991; Mederle *et al.*, 2002). The study by Mederle *et al.* (2002), in which rBCG were isolated from mouse spleens 100 days post immunisation, found that 75% of the rBCG containing an extrachromosomal construct had lost their antibiotic resistance, while over 85% of the rBCG containing an integrative vector retained antibiotic resistance. Measurement of the loss/retention of antibiotic resistance is a relatively crude method of determining the loss of the entire construct, as it does not account for possible deletion mutants that have lost the antibiotic resistance gene, but have maintained the remainder of the construct. It is thought that deletion mutations will be minimal compared with the loss of the entire construct, especially with respect to extrachromosomal constructs. It is noteworthy to point out that although these *in vitro* studies can determine stability trends, they may not accurately reflect the stability situation *in vivo*. Assuming that stability is tied to expression levels, these constructs may be less stable *in vivo*, because of promoter induction.

Although stability is an important issue with all BCG recombinants, little literature is available on methods to increase plasmid stability in BCG. Baud *et al.* (2002) established that codon optimisation of the HPV-16 L1 gene, for expression in *Salmonella*, increased plasmid stability *in vivo*; however, in the present study codon optimisation may have decreased plasmid stability, due to a possible increase of L1 expression levels. Auxotrophic mutants could be exploited to increase plasmid maintenance; for example, mutants lacking the ability to synthesis diaminopimelae (DAP), due to the disruption of the *asd* (aspartate-semialdehyde dehydrogenase) gene, are unable to complete cell wall synthesis and therefore lyse. For *asd*$^-$ mutants to survive they would be forced to maintain a plasmid carrying the wild-type *asd* gene (Cirillo *et al.*, 1995). The pCB112 and pCB119 vectors utilised in this study contain

the *lys A* gene, which is necessary for bacterial survival in lysine auxotrophic BCG mutants (W. Jacobs, Dept. of Microbiology and Immunology, Albert Einstein College of Medicine, unpublished). Although these methods will ensure plasmid maintenance in the absence of antibiotic selection, gene deletions will not be prevented.

The use of inducible promoters may help with respect to the prevention of gene deletions *in vitro*; however, for these promoters to be effective their *in vitro* activity will have to be negligible, with strongly inducible activity *in vivo*. Even low expression can cause instability, as noted in this study with the p119L1* and p112L1* constructs, even though L1 expression is driven by promoters with weak *in vitro* activity (*mtrA* and *M. leprae 18kDa*, respectively). The most promising inducible promoters to date are those of the *M. smegmatis* acetamidase enzyme. Acetamidase is induced in the presence of amides, such as acetamide, butyramide and formamide, enabling the organism to utilise these amides as their sole sources of carbon (Roberts *et al.*, 2003; Parish *et al.*, 1997). Under induced conditions acetamidase levels can increase to 100-fold that of the un-induced state, comprising up to 10% of total cell protein. The functionality of this system has been demonstrated by the successful expression of luciferase (Gordon *et al.*, 1994) and the *M. leprae* 35kDa protein (Triccas *et al.*, 1998) in *M. smegmatis*. The system is unfortunately not perfect as even in the absence of an inducer, a low basal level of acetamidase expression exists (Roberts *et al.*, 2003). More work will have to be done to gain a better understanding of this system.

CHAPTER 5: IMMUNOGENICITY OF RECOMBINANT BCG EXPRESSING HPV-16 L1

5.1. INTRODUCTION

A wide range of immune responses have been detected to foreign antigens expressed by BCG. In the introduction to this chapter these animal studies will be summarised, and factors which influence the immunity generated to recombinant BCG, including route of administration, dose, prior exposure to BCG, and foreign protein localisation, will be reviewed. The introduction is followed by the evaluation of the immune responses elicited by the BCG-HPV-16 L1 recombinants that were constructed during this project.

5.1.1. IMMUNE RESPONSES TO HETERLOGOUS PROTEINS

A multitude of BCG recombinants have been produced that effectively express and generate immune responses to proteins of viral, bacterial, parasitic, and mammalian origin (these are summarised in Table 5.1). Similar to the immune responses induced following wild-type BCG vaccination (Chapter 2, section 2.5), these BCG recombinants most often elicit strong Th1 cellular responses (mainly $CD4^+$ T cell activation, with IL-2, IL-12 and IFN-γ production) to the heterologous antigens that they express; however, some BCG recombinants elicit strong $CD8^+$ T cell and antibody responses to these proteins. Not only have these recombinants been found to be immunogenic in murine, guinea pig, and primate models, but they have been shown to provide protection in murine models of measles (Fennelly *et al.*, 1995), *Streptococcus pneumoniae* infection (Langermann *et al.*, 1994a), Lyme disease (Stover *et al.*, 1993), tetanus (Fuerst *et al.*, 1992), listeriosis (Grode *et al.*, 2002), leishmanisasis (Connell *et al.*, 1993; Streit *et al.*, 2000), and malaria (Matsumoto *et al.*, 2000).

The immune responses generated to the these foreign antigens are dependent on a number of factors (discussed below) which include the nature of the antigen itself, dose, route of inoculation, amount of protein expressed, and the cell compartments into which the protein is directed.

5.1.2. FACTORS AFFECTING IMMUNE RESPONSES TO HETERLOGOUS PROTEINS

5.1.2.1. BCG substrain

As described in section 2.2, BCG substrains differ somewhat in their immunogenicities, this therefore has a direct effect on the immune responses elicited to foreign antigens expressed by these substrains. Also, the ability of the BCG substrain to persist and multiply *in vivo* will contribute to the sustained expression of its foreign antigen, and thus to the continued stimulation of the immune system. In studies comparing the immunogenicity of live versus heat-killed rBCG expressing the fragment C (ToxC) of the tetanus toxin, the heat-killed recombinant produced insignificant levels of serum antibodies to ToxC, and could not protect against challenge with tetanus toxin; while the live rBCG produced high antibody titres, and completely or partially protected mice from challenge (Fuerst *et al.*, 1992; Stover *et al.*, 1991). These results, together the observation that one immunisation with rBCG can lead to an immune response that develops slowly, but that increases over many weeks (Fuerst *et al.*, 1992; Hayward *et al.*, 1999; Leung *et al.*, 2000); point to the importance of the persistence of rBCG.

A comparison of five commonly used BCG substrains (Glaxo, Tokyo, Pasteur, Prague and Russian) by Lagranderie *et al.* (1996), revealed that these substrains differ in their abilities to persist and to elicit protective immunity in mice. Of all the substrains, BCG Prague and Tokyo were least able to multiply and persist in target organs after immunisation. These results correlated well with their ability to protect against challenge with rBCG-*lacZ*: while immunisation with the Pasteur, Glaxo or Russian substrains caused the efficient elimination of rBCG-*LacZ*, the Tokyo and Prague substrains did not show significant protection. When the immunity to these substrains was investigated, they found that the all had similar capacities to induce proliferative responses and cytokine production; however, BCG Prague and Glaxo induced significantly weaker CTL responses than the Pasteur and Russian substrains; and the Tokyo substrain was totally ineffective at inducing CTLs. Of the substrains, BCG Tokyo elicited the lowest levels of anti-PPD antibody. Burlein *et al.* (1994) showed that expression of foreign genes could differ between substrains. They found that while some substrains (Danish

and Pasteur) readily expressed *B. burgdorferi* OspA and HIV-1 pg120, the Tokyo substrain was unable to produce these antigens, even though identical vectors were utilised. In contrast, Al-Zarouni and Dale (2002) found no clear difference in expression of the foreign gene between the three BCG substrains used (Pasteur, Tokyo and Moreau). They did, however, find differences in vector stabilities: BCG Tokyo being the most stable and BCG Moreau the least stable. They went on to suggest that the differences in expression of foreign proteins by different BCG substrains observed by other researchers could be due to undetected vector instabilities.

5.1.2.2. Dose and route of administration

The recommended human BCG dose varies with BCG substrain, method of administration and age of recipient. The Pasteur substrain is administered at $4x10^4$ to $5x10^5$ colony forming units (cfu) per dose, Glaxo at $2x10^5$ to $1x10^6$ cfu per dose, while Tokyo is administered at $3x10^6$ cfu per dose (Smith and Starke, 1999). Presently, the majority of countries administer BCG via the intradermal (i.d.) route. South Africa has recently changed the BCG inoculation regimen from percutaneous (multiple puncture) to the i.d. route (personal communication, Maureen Dennehy, State Vaccine Institute, South Africa; Kibel *et al.*, 1998), as this method administers a more accurate dose. Considerable differences also exist in vaccination schedules between countries. The WHO recommends a single dose at birth; however, many countries give a dose at birth, with repeated doses during childhood (Smith and Starke, 1999).

The BCG dose plays a key role in shaping the Th1/Th2 nature of the immune response. Low BCG doses elicit a predominantly Th1 (cell-mediated) immune response, and is independent of the route of inoculation (Power *et al.*, 1998). Power *et al.* (1998) observed that immunising mice with very low numbers of BCG (40 cfu i.v., 400 cfu s.c., or 2000 cfu i.d.) were able to induce detectable cell-mediated responses to BCG. For the induction of antibodies to BCG high dosed were more effective and were dependent on route; an i.v. dose of $4x10^5$ cfu was effective, but a dose of $4x10^6$ cfu given s.c. induced almost no detectable antibodies to BCG. The high doses induced mixed Th1/Th2 immune responses. Hayward *et al.* (1999) found that immunisation of mice with *E. coli* heat labile enterotoxin (LT-Bh) alone induced a Th2-dominated (antibody) response, however, when LT-Bh was expressed by rBCG, the response to LT-Bh was shifted to a predominantly Th1 response. An i.p. immunisation of mice with 10^6 cfu of rBCG-LT-Bh did not elicit LT-Bh-specific antibodies, but larger doses of 10^7 and 10^8 cfu lead to progressively greater serum IgA and IgG responses.

Kremer *et al.* (1996) compared a panel of routes (i.v., i.p., s.c., or i.n) when they immunised mice with a single dose ($8x10^6$ cfu for all routes) of rBCG expressing glutathione S-transferase of *Schistosoma mansoni* (Sm28GST). Serum antibody responses to Sm28GST were generated by all immunisation routes, the strongest being elicited by the i.p. and i.v. routes (IgG, not IgA). Antibody responses were enhanced by a booster dose. Analysis of the antibody isotypes indicated that i.v. and i.n. immunisation elicited a dominant Th1 response, s.c. immunisation produced a Th1 response that gradually became a mixed Th/Th2 profile, while i.p. gave a mixed profile directly after immunisation. Lagranderie *et al.* (1998) investigated mucosal routes of administering a mixture of rBCG expressing SIV antigens (Env, GAG, Nef) in mice. The oral and rectal doses were $1.5x10^9$ cfu of each rBCG over 5 days; the i.n. dose was 10^7 cfu of each rBCG, and the respiratory dose was about $5x10^6$ cfu of each rBCG. All routes induced similar CTL responses. The highest HIV-specific IgA antibodies were detected in the faeces, moderate levels were found in bronchoalveolar lavage samples, and vaginal secretions contained negligible levels. High titres of HIV-specific serum IgG were obtained by all vaccination routes, with a preferential IgG_1 isotype response (Th2). It therefore appears that dominant Th1 (IgG_{2a} isotype) responses are obtained following parenteral immunisation (Gheorghiu *et al.*, 1994; Kremer *et al.*, 1996), while mucosal immunisation preferentially stimulates Th2 responses.

Langermann *et al.* (1994b) observed a dose response when mice were immunised i.n. with rBCG-OspA. An i.n. dose of 10^3 cfu was unable to elicit detectable *B. burgdorferi* OspA-specific antibodies, a dose of 10^5 cfu produced an intermediate level, while 10^6 and 10^9 cfu produced high antibody titres. Mucosal administration tends to elicit greater serum and mucosal IgA responses than do the parenteral forms of immunisation. Intraperitoneal immunisation of rBCG-OspA ($2x10^6$ cfu) elicited high titres of serum IgG, but no serum IgA; i.n. immunisation (with $2x10^8$ cfu), on the other hand, produced sustained low titres of serum IgA as well as IgG antibodies. Intranasal but not i.p. immunisation with rBCG-OspA elicited an IgA response in vaginal washes (Langermann *et al.*, 1994b). Kawahara *et al.* (2002b) compared single routes (i.d., i.r., and i.n.) and combinations of routes (i.d./i.r., or s.c./i.r.) in guinea pigs. The combined i.d./i.r route proved best at eliciting the highest titres of specific IgA and IgG antibodies, and cell-mediated immunity to rBCG expressing the HIV-1 V3J1 peptide. In a phase I clinical trial to test the safety and immunogenicity of rBCG-OspA, adult volunteers were immunised i.d. with four escalating doses ranging from $2x10^4$ to $2x10^7$ cfu over 28 weeks. The vaccine was deemed safe, but none of the doses generated detectable antibodies to OspA, even though similar doses successfully induced immunity in mice (Edelman *et al.*, 1999; Stover *et al.*, 1993).

5.1.2.3. Prior exposure to BCG

One of the suggested explanations for the low and varied protective efficacy of the BCG vaccine against *M. tuberculosis* is prior exposure to environmental mycobacteria (Agger and Andersen, 2002; Hess and Kaufmann, 1999). Studies have found that when cows have been sensitised to environmental bacteria, the protective affect of subsequent BCG vaccination is adversely affected (Buddle *et al.*, 2002). Therefore, a major concern surrounding the development of recombinant BCG vaccines is whether prior vaccination with wild-type BCG will adversely affect the immunity induced to recombinant BCG vaccines.

One group has completed a number of studies in which the effect of BCG-priming on the immunity induced by rBCG-LacZ and rBCG-Nef in mice was investigated (Gheorghiu *et al.*, 1994; Lagranderie *et al.*, 1997a; Lagranderie *et al.*, 1996). They showed that mice that had been immunised with wild-type BCG were partially protected from challenge with rBCG-lacZ, as the growth of recombinant BCG in the mouse organs (spleen and lymph nodes) was reduced. Proliferative responses to β-gal and Nef were also reduced by varying degrees (0-40% reduction) in BCG-primed mice, depending on the length of time between priming and rBCG vaccination. Antibody responses to β-gal, however, were dramatically increased in the BCG-primed mice. These increased responses were apparent after one or two rBCG immunisations, and could be detected several months after rBCG immunisation. The antibody stimulatory effect was not due to polyclonal stimulation of B cells or to the non-specific adjuvant effect of BCG.

5.1.2.4. Protein localisation and amount of protein produced

A review of the literature established that, as one would expect, the more foreign protein that is produced by rBCG, the stronger the immune response that develops to that protein. Although the utilisation of secretory or membrane-targeted signal sequences often leads to an overall reduction in the levels of the foreign protein, these sequences generally lead to amplified immune responses by improving antigen delivery.

Abomoelak *et al.* (1999) showed that a BCG recombinant expressing large quantities of pertussis/tetanus hybrid protein (S1-TTC) (*hsp60* promoter) was significantly more immunogenic than the lower S1-TTC expresser (85A promoter, 10-fold lower expression). Only the high expresser was able to elicit anti-S1-TTC antibodies and an IL-2 response. Similarly, β-gal-specific antibody and IFN-γ responses were considerably higher in mice when β-gal was expressed by rBCG under the control of a strong promoter (P*blaF*), as compared to

a weaker promoter (P_{AN}) (Lagranderie *et al.*, 1997b). Langermann *et al.* (1994a) showed that although *S. pneumoniae* PspA production was highest when expressed in rBCG as a cytoplasmic protein, only rBCG producing PspA as a secreted (PspA signal) or membrane-bound protein (19kDa lipoprotein signal) elicited responses capable of protecting mice against *S. pneumoniae* challenge. Himmerlrich *et al.* (2000) compared a panel of vectors expressing the *E. coli* MalE protein in BCG. They observed that the highest MalE producers were the BCG recombinants that secreted MalE, and that these were also the recombinants that elicited the highest MalE-specific immune responses. The two secretory constructs elicited antibody and T cell responses to MalE, while the cytoplasmic and lipoprotein constructs did not. Of the two secretory constructs, the one producing the highest levels of MalE generated a more rapid and a higher level of antibodies; it also produced high IFN-γ and proliferative responses, while the lower producer failed elicit specific proliferative responses and produced only a low level of IFN-γ. Mederle *et al.* (2002) compared three BCG recombinants expressing a synthetic SIV *nef-gag* operon. An unstable extrachromosomal vector with high Gag expression (1% of total BCG protein) and a stable integrated construct with low expression (0.14% of total BCG protein) failed to elicit Gag-specific IFN-γ responses in mice after a single immunisation. The low, but persistent level of Gag production by the integrated construct was, however, sufficient to induce a humoral response. A second integrative vector with high, stable Gag expression (1% of total BCG protein) was able to elicited Gag-specific IFN-γ responses. It therefore appears that a threshold level of heterologous protein expression by rBCG must be reached before specific memory T cells are primed.

A comparison of BCG recombinants expressing the OspA protein of *B. bugdorferi* as a cytoplasmic, secretory (α antigen signal) or membrane-anchored protein (19kDa or OspA lipoprotein signals), revealed that although the highest OspA production was by the cytosolic construct, it produced the lowest levels of OspA-specific antibodies (which were not apparent before boosting). The secretory construct produced intermediate antibody levels, while the membrane constructs produced the highest levels of OspA-specific antibodies (titres 100 to 1000 fold higher than the other constructs (Stover *et al.*, 1993). rBCG expressing *E. coli* LT-Bh as either a cytoplasmic, membrane-associated (19kDa signal), or secreted protein (α antigen), displayed similar levels of LT-Bh production, but only the membrane-associated and secretion constructs elicited significant levels of LT-Bh-specific serum IgA antibodies in mice (Hayward *et al.*, 1999). Grode *et al.* (2002) investigated the immune responses to rBCG expressing the p60 protein of *L. monocytogenes* as either a cytoplasmic, membrane-associated (19kDa lipoprotein signal), or secreted protein (Ag85B secretion signal). While the secreting and membrane constructs evoked good protection from listeriosis, 80% and 100% respectively,

cytosolic expression failed to protect. *In vivo* depletion of the T cell subpopulations revealed that the secretory rBCG construct mediated protection with $CD4^+$ T cells, while a combination of $CD4^+$ and $CD8^+$ T cells were involved in the protection conferred by the membrane construct.

5.1.2.5. Animal Strain

The immune responses to foreign antigens delivered by rBCG are under a complex set of genetic influences, and include susceptibility to BCG and ability to respond to the delivered antigen. The vast majority of rBCG studies have tested immune responses in mice, but guinea pigs, macaques, and human volunteers have also been utilised (see Table 5.1). Guinea pigs are probably the most relevant small animal model for the assessment of immunity to recombinant BCG. They are used routinely for testing tuberculosis infection as they resemble tuberculosis infection in humans clinically, pathologically and immunologically (Horwitz *et al.*, 2000).

Multiplication of BCG in mice is controlled by the *Bcg* locus, which has two allelic forms, susceptible (Bcg^S) and resistant (Bcg^R) (Gros *et al.*, 1981; Lagranderie *et al.*, 1997b). The low immunogenicity of recombinant BCG in certain strains of mice can be explained by the inferior multiplication of BCG in these strains, which in turn leads to the expression of less heterologous protein. Lagranderie *et al.* (1997b) investigated the immunogenicity of rBCG-β-gal in a number of mouse strains. They found that rBCG recovery from C3H/HeJ mice (Bcg^R) was low, which corresponded to a low antibody response to β-gal. Although BALB/c and C57BL/6 mice are both Bcg^S, β-gal-specific antibodies were produced in BALB/c and not in C57BL/6; this occurs since BALB/c and C3H/HeJ mice are able to respond to lower doses of β-gal than C57BL/6 mice. Similarly, Himmelrich *et al.* (2000) found that immunity to MalE varied in different strains of mice when expressed by a weak rBCG-MalE producer, but detected strong responses in all strains when immunised with a high MalE producer. When Stover *et al.* (1993) assessed 24 inbred and outbred mouse strains for relative antibody responsiveness to OspA produced by rBCG, they determined that the antibody responses to OspA did not correspond to the strain's BCG susceptibly/resistance alleles; this was most likely due to the high OspA production by rBCG.

Table 5.1. Immune responses elicited to foreign proteins expressed by recombinant BCG.

Antigen Source	Antigen	Expression system	Animal model	Immune response to foreign antigen	Reference
Viral					
HIV-1	Gag, Pol, Env	Extrachromosomal vector Hsp70 promoter	Mice	Antibody (ab) and CTL responses (CD8+ T cell-mediated). IFNγ and IL-2 production.	Aldovini and Young, 1991
	Nef	Extrachromosomal vector GroES/groEL1 promoter	Mice	Proliferative immune responses.	Winter *et al.*, 1991
	V3 env T cell epitope (19aa)	Extrachromosomal vector α antigen secretion system	Mice	A specific CTL response that was MHC class I restricted.	Kameoka *et al.*, 1994
	V3 env T cell epitope (15aa)	Extrachromosomal vector α antigen secretion system	Guinea pig	Specific antibody, DTH and CTL responses. *In vitro* neutralising abs.	Honda *et al.*, 1995
			Mice	Specific CTL responses (MHC-I restricted) Passive protection against HIV-1 challenge in SCID/hu mice using immune serum.	
			Mice	Prolonged (1 year) high titre V3-specific IgG abs, with *in vitro* neutralising activity after nasal immunisation.	Hiroi *et al.*, 2001
	V3 env T cell epitopes of principle neutralising region (Codon optimised for BCG)	Extrachromosomal vector α antigen promoter and signal sequence	Guinea pigs	After oral immunisation of freeze-dried rBCG proliferative responses were detected when testing PBMCs, splenocytes and intestinal intraepithelial lymphocytes. DTH responses to V3 could be detected 1.5 years after immunisation.	Kawahara *et al.*, 2002a
				Enhanced rBCG immunogenicity by combined intrarectal and intradermal immunisation. High titres of serum IgG and IgA. Production of IFN-γ and IL-2. Antigen specific Th1-type memory cells maintained for more than 2 years.	Kawahara *et al.*, 2002b
				Neutralising abs (*in vitro*).	Chujoh *et al.*, 2001
SIV	Gag epitope	Extrachromosomal Hsp70 promoter	Rhesus monkeys	No Gag-specific abs were detected. Specific CTL responses (CD8+, MHC class 1 restricted). No protection from SIV challenge.	Yasutomi *et al.*, 1993; Yasutomi *et al.*, 1995
	Nef	Extrachromosomal PAN promoter	Mice	Proliferative and CTL responses. CTL activity of intestinal intraepithelial CD8β+ T cells after oral administration.	Winter *et al.*, 1995

Table 5.1 (continued). Immune responses elicited to foreign proteins expressed by recombinant BCG.

Antigen Source	Antigen	Expression system	Animal model	Immune response to foreign antigen	Reference
	Nef-Gag fusion	Extrachromosomal and integrative PblaF promoter	Mice	Anti-Gag IFN-γ-producing CD4+ cells, and anti-Gag abs. No immune response to Nef.	Mederle *et al.*, 2002
	Nef, Env, Gag	Extrachromosomal PAN or PblaF promoters	Mice	Immunisation with a rBCG cocktail elicited systemic IgA and IgG responses (against Env and Gag) and strong CTL responses against all antigens, after mucosal immunisation.	Lagranderie *et al.*, 1998
	Gag, Pol, Env and Nef	Extrachromosomal Hsp70 promoter	Rhesus monkeys	SIV-specific IgA and IgG ab responses. CTL and helper T cell proliferation.	Leung *et al.*, 2000
	N-terminal of env (aa 1-245)	Extrachromosomal PblaF promoter	Mice Guinea pigs	Strong CTL and ab responses after 3 immunisations, *in vitro* neutralising abs. Specific IgA abs in faeces after oral immunisation.	Lim *et al.*, 1997
	Nef, Env, Gag	Extrachromosomal PAN or PblaF promoters	Cynomolgus macaques	A single inoculation (i.d.) of a mixture of 3 BCG recombinants elicited CTL and IFN-γ responses to all 3 antigens (Nef, Env, Gag). Mucosal boosting elicited mucosal IgA, but not serum IgG or lymphoproliferation. Animals were not protected from challenge with pathogenic SIVmac251.	Mederle *et al.*, 2003
Measles virus	Nucleocapsid (N) protein	Extrachromosomal Hsp60 promoter 19 kDa lipoprotein, or no signal	Mice	Specific humoral and proliferative responses. Low titre *in vitro* neutralising abs. Protection from viral challenge.	Fennelly *et al.*, 1995
	Nucleocapsid (N) protein	Extrachromosomal or integrative Hsp60 promoter	Rhesus macaques	The extrachromosomal construct elicited abs after one inoculation, while 2 inoculations of the integrative construct were needed to produce similar ab levels. rBCC-N did not protect from measles virus challenge.	Zhu *et al.*, 1997
Hepatitis C virus	CTL epitope of non-structural protein 5a (NS5a)	Extrachromosomal vector α antigen promoter and signal sequence	Mice	NS5a-specific MHC class 1-restricted CD8+ CTL response. Protection against challenge with vaccinia virus expressing NS5a.	Uno-Furuta *et al.*, 2003

Table 5.1 (continued). Immune responses elicited to foreign proteins expressed by recombinant BCG.

Antigen Source	Antigen	Expression system	Animal model	Immune response to foreign antigen	Reference
HPV	HPV-6b L1 and HPV-16 E7	Extrachromosomal Hsp60 and hsp70 promoters	Mice	L1-specific ab, DTH and proliferative responses. E7-specific ab, DTH, CTL and proliferative responses. No protection from E7-expressing tumour.	Jabbar *et al.*, 2000
Rabies virus	nucleoprotein B-cell and T-cell epitopes	Extrachromosomal Hsp60 or 18kDa promoter	Mice	Long lasting and progressively increasing ab response. Higher ab titres than elicited by commercial rabies vaccine (Rai-SAD).	da Cruz *et al.*, 2001
Porcine reproductive respiratory syndrome virus	GP5 and M proteins	Extrachromosomal Hsp60 promoter 19 kD lipoprotein or no signal	Mice	Neutralising ab and IFN-γ responses by rBCG expressing a truncated GP5.	Bastos *et al.*, 2002
Bacterial					
Streptococcus pneumoniae	Surface protein A (PspA)	Extrachromosomal Hsp60 promoter PspA secretion signal, 19 kDa lipoprotein, or no signal	Mice	High titre ab response. Protection against challenge. Passive protection against challenge in CBA/N mice using immune serum.	Langermann *et al.*, 1994a
E. coli	β-gal	Extrachromosomal Hsp60 promoter	Mice	A single immunisation produced high titres of β-gal-specific abs, CTL responses, and IFN-γ production.	Stover *et al.*, 1991; Fuerst *et al.*, 199
	β-gal	α antigen promoter and signal sequence	Mice	High ab titres against β-gal, which peaked 10 weeks after a single inoculation. Production of Th-1 cytokines (IFN-γ & IL-2). Down regulation of IgE ab response.	Kumar *et al.*, 1999
	β-gal	Extrachromosomal PAN or PblaF promoters	Mice (BALB/c)	High titre ab responses after two inoculations. Proliferative responses (CD4+ and CD8+ T cells) and IFNγ production.	Murray *et al.*, 1992
			Guinea pigs	Prolonged antibody responses, CTL (CD4+ T cells) and DTH response following oral and respiratory immunisation of guinea pigs. Serum and intestinal IgA abs.	Lagranderie *et al.*, 1993
			Mice (BALB/c, CBA, C57BL/6, C3H)	High titre ab responses only in BALB/c mice immunised with the PAN construct. Abs in all strains immunised with PblaF construct (stronger expresser).	Lagranderie *et al.*, 1997b

Table 5.1 (continued). Immune responses elicited to foreign proteins expressed by recombinant BCG.

Antigen Source	Antigen	Expression system	Animal model	Immune response to foreign antigen	Reference
	MalE	Extrachromosomal 19kDa, Perp, or PblaF promoters 19kDa lipoprotein signal, erp signal (secretion), double signal consisting of BlaF and MalE sequences, or no signal sequence	Mice	MalE-specific abs, proliferation and IFN-γ production when utilising the double secretion signal construct. Negligible or low responses with the other constructs.	Himmelrich *et al.*, 2000
	B subunit heat labile enterotoxin	Extrachromosomal Hsp60 or 19kDa promoters α antigen, 19kDa lipoprotein, or no signal	Mice	Serum IgA and IgG abs after oral immunisation. The highest ab titres were elicited with cell wall associated-antigen expression.	Hayward *et al.*, 1999
Clostridium tetani	Protective fragment C of the tetanus toxin (ToxC)	Extrachromosomal Hsp60	Mice	High titre abs to ToxC after a single immunisation. Mice developed complete or partial protection from challenge.	Stover *et al.*, 1991; Fuerst *et al.*, 1992
Corynebacterium diphtheriae	Non-toxic mutant of diphtheria toxin (CRM197) and Tetanus toxin fragment C (FC)	Extrachromosomal PblaF promoter B-lactamase secretion signal	Mice	Anti-CRM197 abs were generated, but were unable to neutralise diphtheria toxin activity in an *in vitro* assay. Diptheria toxin neutralising abs were generated when rBCG-CRM197 and rBCG-FC were immunised together.	Miyaji *et al.*, 2001
			Mice, guinea pigs	I.p. immunisation of mice with a rBCG-CRM197 and rBCG-FC combination produced a Th2 immunoglobulin profile. Antisera from guinea pigs immunised with rBCG-CRM197 and rBCG-FC neutralised both diphtheria and tetanus toxins.	Mazzantini *et al.* 2004
Borrelia burgdorferi	Outer-surface protein A (OspA)	Extrachromosomal Hsp60 promoter α antigen, 19 kDa or OspA lipoprotein signals, or no signal	Mice	High titre ab response, CTL responses, and protection against *in vivo* challenge. Prolonged-protective systemic IgG and IgA immune responses (more than one year).	Stover *et al.*, 1993 Langermann *et al.*, 1994b
	OspA	Extrachromosomal Hsp60 promoter 19 kDa lipoprotein signal	Human volunteers	The rBCG vaccine was safe, but did not elicit anti-OspA abs in any of the volunteers.	Edelman *et al.*, 1999
Vibrio cholerae	Toxin B subunit	Extrachromosomal Hsp60 α antigen signal	Mice	Intranasal immunisation elicited serum and mucosal IgA responses.	Biet *et al.*, 2003

Table 5.1 (continued). Immune responses elicited to foreign proteins expressed by recombinant BCG.

Antigen Source	Antigen	Expression system	Animal model	Immune response to foreign antigen	Reference
Bordetella pertussis	S1-TTC (S1 subunit of pertussis toxin fused to ToxC)	Extrachromosomal Hsp60 promoter (no signal), or 85A promoter with its signal sequence	Mice	Specific neutralising antibodies against TTC, and IL-2 production to both S1 and TTC only after immunisation with the hsp60 construct.	Abomoelak *et al.*, 1999
M. tuberculosis	30kDa major secretory protein	Extrachromosomal M. tuberculosis 30kDa protein promoter	Guinea pigs	Strong cutaneous DTH responses. Enhanced protection against challenge when compared with wild-type BCG.	Horwitz *et al.*, 2000
	ESAT-6	Extrachromosomal Hsp60 promoter	Mice, guinea pigs	Enhanced protection against challenge with *M. tuberculosis*.	Pym *et al.*, 2003
Listeria monocytogenes	p60	Extrachromosomal Hsp60 promoter Ag85B secretion signal, 19kDa lipoprotein signal, or no signal	Mice	Protection against listeriosis after immunisation with the secretory or membrane-anchoring constructs, but not with the cytosolic construct.	Grode *et al.*, 2002
M. leprae	18kDa protein	Extrachromosomal Hsp65 promoter	Mice	Specific humoral, proliferative responses, and DTH skin reactions.	Baumgart *et al.*, 1996
Protozoa					
Schistosoma mansoni	Glutathione S-transferase (Sm28GST)	Extrachromosomal vector Hsp60 promoter	Mice	i.p., i.v., s.c., and i.n. immunisation elicited prolonged (over 1 year) specific ab (mainly IgG2a) responses.	Kremer *et al.*, 1996
Toxoplasma gondii	GRA1	Extrachromosomal 85A antigen promoter and signal sequence	Mice, sheep	Immunisation of mice failed to induce measurable GRA1-specific humoral or cellular responses, and conferred very limited protection against *T. gondii* challenge. Immunisation of sheep failed to elicit GRA1-specific abs, but did produce proliferative and IFN-γ responses.	Supply *et al.*, 1999
Leishmania major	Surface proteinase Gp63	Extrachromosomal hsp60 promoter	Mice	Protection against challenge in resistant CBA mice, but not susceptible BALB/c mice.	Connell *et al.*, 1993
	Surface proteinase Gp63	Extrachromosomal PAN promoter Pblaf promoter and signal sequence for secretion	Mice	BALB/c mice were protected against challenge when immunised with rBCG the Pblaf construct.	Abdelhak *et al.*, 1995

Table 5.1 (continued). Immune responses elicited to foreign proteins expressed by recombinant BCG.

Antigen Source	Antigen	Expression system	Animal model	Immune response to foreign antigen	Reference
Leishmania chagasi	LCR1	Extrachromosomal hsp60 promoter	Mice	Immunisation with rBCG-LCR1 provided better protecting immunity against *L. chagasi* infection than immunisation with LCR1 protein alone.	Streit *et al.*, 2000
Plasmodium falciparum	Circumsporo-zoite protein (CSP)	Extrachromosomal Hsp70	Mice	Specific ab and proliferative responses. Production of IL-2 and IFN-γ.	Zheng *et al.*, 2002
Plasmodium yoelii	15kDa fragment of merozoite surface protein (MSP-1)	Extrachromosomal α antigen promoter and signal sequence	Mice	Immunisation with rBCG induced greater protection against blood-parasite infection than immunisation with MSP1 protein and adjuvant.	Matsumoto *et al.*, 1998; Matsumoto *et al.*, 2000
Human					
	IL-2	Integrative vectors α antigen promoter and signal sequence or hsp60 promoter	Mice	Secretion of active IL-2 which augmented the immune response to BCG, thus enhancing BCG clearance.	Kong and Kunimoto, 1995
	IL-2, IL-18	Extrachromosomal hsp60 promoter α antigen for secretion	Mice	rBCG-IL-2 (but not rBCG-IL-18) improved BCG's ability to induce and maintain a strong type1 immune response. Stronger proliferative, IFN-γ, and IgG2a responses were elicited after rBCG-IL-2 vaccination. rBCG-IL-2 vaccination did not enhance BCG's protective immunity against *M. bovis* challenge.	Young *et al.*, 2002
	IL-18	Extrachromosomal hsp60 promoter α antigen for secretion	Mice	rBCG-IL-18 augmented BCG's ability to induce type1 immune response, with greater production of IFN-γ, IL-10 and granulocyte-macrophage colony-stimulating factor; but with decreases production of serum IgG.	Biet *et al.*, 2002

5.2. STUDY OBJECTIVES

The objective was to evaluate and compare the immunity elicited by the BCG-HPV-16 L1 recombinants constructed during this study (Chapter 3). Since the potential use of rBCG-L1 as a prophylactic vaccine for HPV was under evaluation, the focus of the assays lay in testing the ability of rBCG-L1 to generate antibodies that were specific for HPV-16 L1; however, cell-mediated immunity was also assessed. Guinea pigs were the animals of choice, as they are the most relevant small animal model for the assessment of immunity to mycobacteria.

5.3. MATERIALS AND METHODS

5.3.1. SOURCE OF ANIMALS

Outbred Dunkin-Hartley guinea pigs, weighing 200-400g were obtained from South African Vaccine Producers (South Africa) and were housed in the UCT Animal Unit. All animal protocols were approved by the UCT Animal Research Review Committee. Immunisations, serum and organ collections were performed by trained animal technologists.

5.3.2. PREPARATION OF IMMUNOGENS

rBCG-L1 and wild-type BCG were prepared for animal immunisations by growing the cultures to middle log phase, followed by centrifugation and re-suspension in PBS, as described in Appendix A15. The BCG immunisation samples were either used directly after preparation, or were frozen at -80ºC and thawed just prior to immunisation. The genetic stabilities of the immunisation stocks were tested prior to use (section 4.2.1).

HPV-16 VLPs were produced by the recombinant baculovirus system, and were supplied by Robert Rose (University of Rochester, Rochester, New York, USA) or the Department of Molecular and Cell Biology (UCT, South Africa). Phosphate-buffered saline (PBS) was used as a control immunogen.

5.3.3. ANIMAL IMMUNISATIONS AND SAMPLE COLLECTION

5.3.3.1. Immunisation of guinea pigs with freshly-made rBCG-L1 for comparison of expression vectors

Guinea pigs were anaesthetised by intramuscular (i.m.) administration of ketamine/rompun in the hind leg. Pre-immune blood samples were collected by cardiac puncture, and the guinea pigs were then immunised by i.m. injection in the hind leg (the opposite leg to that used for anaesthesia) with a 100µl dose of BCG, containing $2x10^6$ colony forming units (cfu). Four groups of 4 animals each, received either rBCG-361L1$_R$, rBCG-112L1$_{NLS}$-, rBCG-119L1$_{NLS}$-, or wild-type BCG. All BCG was of the Tokyo substrain. One to 2ml of blood were collected by cardiac puncture every 1 or 2 weeks.

The blood was kept at room temperature for 2 hours to complete the clotting process, and then separated by centrifugation at 3000rpm for 10min. The sera were collected and stored at -80°C. Booster immunisations were administered 4 and 13 weeks after the first immunisation. Antibodies specific for HPV-16 VLPs were measured by enzyme linked immunosorbent assay (ELISA), as described in section 5.3.4.1.

5.3.3.2. Immunisation of guinea pigs for comparison of freshly-made versus frozen/ thawed rBCG-L1, and investigation of priming with wild-type BCG

Guinea pigs were anaesthetised as above. Pre-immune blood samples were collected and the guinea pigs were split into 4 groups. One group, of three animals, was immunised subcutaneously (s.c.) with $2x10^6$ cfu of wild-type Tokyo BCG, and received booster inoculations, of the same dose and route, 4 and 12 weeks after the first dose. Two groups, of 7 animals each, were primed subcutaneously with $2x10^6$ cfu of wild-type BCG, after which they were immunised intramuscularly or subcutaneously with $4.6x10^6$ cfu of rBCG-112L1$_{NLS}$-. Both groups were then boosted with $2x10^6$ cfu of rBCG-112L1$_{NLS}$-, 4 and 12 weeks after the first rBCG-112L1$_{NLS}$- immunisation. Two groups, of 6 guinea pigs each, were immunised s.c. with $4.6x10^6$ cfu of rBCG-112L1$_{NLS}$- and were boosted s.c. with $2x10^6$ cfu of rBCG-112L1$_{NLS}$-, 4 and 12 weeks afterwards; while the one group received freshly-made BCG, the other received frozen/thawed BCG. Another group, of 6 guinea pigs, was immunised i.m. with $4.6x10^6$ cfu of frozen/thawed rBCG-112L1$_{NLS}$-, and was boosted i.m. with $2x10^6$ cfu of frozen/thawed rBCG-112L1$_{NLS}$- at 4 and 12 weeks.

Table 5.2. Guinea pig immunisation schedule utilised for comparison of freshly-made versus frozen/thawed rBCG-L1, and to investigate priming with wild-type BCG

	Group name	Wild-type BCG prime	Immunisations		
		4 weeks prior to week 0	Week 0	Week 4	Week 12
1.	i.m rBCG-112L1$_{NLS}$- only	none	i.m rBCG-112L1$_{NLS}$-	i.m rBCG-112L1$_{NLS}$-	i.m rBCG-112L1$_{NLS}$-
2.	s.c. rBCG-112L1$_{NLS}$- only	none	s.c. rBCG-112L1$_{NLS}$-	s.c. rBCG-112L1$_{NLS}$-	s.c. rBCG-112L1$_{NLS}$-
3.	s.c. wild-type prime + i.m. rBCG-112L1$_{NLS}$- boost	s.c. wild-type	i.m rBCG-112L1$_{NLS}$-	i.m rBCG-112L1$_{NLS}$-	i.m rBCG-112L1$_{NLS}$-
4.	s.c. wild-type prime + s.c. rBCG-112L1$_{NLS}$- boost	s.c. wild-type	s.c. rBCG-112L1$_{NLS}$-	s.c. rBCG-112L1$_{NLS}$-	s.c. rBCG-112L1$_{NLS}$-
5.	s.c. wild-type only	none	s.c. wild-type	s.c. wild-type	s.c. wild-type

One to 2ml of blood were collected every 2 weeks by cardiac puncture. The blood samples were allowed to clot at room temperature for 2 hours, after which they were separated by centrifugation at 3000rpm for 10min. The sera were collected and stored at -80°C. Two weeks after the last immunisation, the guinea pigs were tested for delayed-type hypersensitivity (DTH) responses to PBS, purified protein derivative of tuberculin (PPD, 0.2μg/animal), sodium carbonate buffer (pH 9.6), intact HPV-16 VLPs (5μg/animal) and disrupted HPV-16 VLPs (5μg/animal); as described in section 5.3.4.3. Antibodies specific for HPV-16 VLPs were measured by ELISA, as described in section 5.3.4.1.

5.3.3.3. Immunisation of guinea pigs with frozen/thawed BCG for comparison of expression vectors, doses and routes

Guinea pigs were anaesthetised as above. Pre-immune blood and vaginal wash samples were collected. The guinea pigs were immunised, either, i.m. in the hind leg, or intradermally (i.d.) on the back. Before the i.d. inoculations were performed, the hair on the backs of the guinea pigs was cut with an electric hair clipper. Two negative control groups, of four animals each, were inoculated with 100μl of either PBS or rBCG-361-GAG ($2x10^6$ cfu, a HIV-1 GAG BCG recombinant; obtained from Robin Thomas, Division of Medical Virology, Department of Clinical Laboratory Science, UCT). A positive control group, of 6 guinea pigs, was inoculated with 15μg of HPV-16 VLP. Seven groups, of 6-8 animals, were inoculated

i.d. (unless otherwise indicated) with $2x10^6$ cfu of either, rBCG-361L1$_e$, rBCG-112L1$_{NLS}$-, rBCG-112L1$_{NLS}$- (i.m.), rBCG-112L1$_e$, rBCG-112L1$_{BCGe}$, rBCG-119L1$_e$, or rBCG-119L1$_{BCGe}$. Two groups, of 6 animals, were given a lower dosage ($1x10^4$ cfu) of rBCG-361L1$_e$ or rBCG-119L1$_{BCGe}$. All BCG was of the Tokyo substrain.

Booster immunisations, of the same routes and doses, were administered 4, 8, and 12 weeks after the initial immunisation. One to 2ml of blood were collected by cardiac puncture every 1 or 2 weeks. Blood was kept at room temperature for 2 hours to complete the clotting process, and then separated by centrifugation at 3000rpm for 10min. The serum was collected and stored at -80°C. Vaginal washes were performed on the female guinea pigs (which comprised a half of each group), using 50µl of PBS. The wash samples were collected 2 weeks after each immunisation, and were stored at -80°C. Six weeks after the 3rd booster dose, the last dose was administered, and 3 weeks later the guinea pigs were tested for DTH responses (described in section 5.3.4.3) to PBS, PPD (0.2µg/animal), and HPV-16 VLPs (5µg/animal). Serum and vaginal antibodies, specific for HPV-16 VLPs, were measured by ELISA, as described in section 5.3.4.1.

HPV-16 pseudovirus neutralisation assays (described in 5.3.4.4) were performed using serum from the above mentioned guinea pigs. The prebleed serum and week 20 serum from one animal from each of the following groups were tested: rBCG-361L1$_e$, rBCG-112L1$_{NLS}$-, rBCG-112L1$_e$, rBCG-112L1$_{BCGe}$, rBCG-119L1$_e$, or rBCG-119L1$_{BCGe}$, HPV-16 VLP, rBCG-GAG, and PBS.

5.3.4. IMMUNOLOGICAL ASSAYS

5.3.4.1. Detection of serum and vaginal antibodies by ELISA

Antibodies specific for HPV-16 VLPs were detected by indirect enzyme-linked immunosorbent assays (ELISAs). Serum antibodies were detected by ELISA according to the procedure described in Appendix A16.1. Serum dilutions of 1:40 were utilised. HPV-16 VLP-specific antibodies in guinea pig vaginal wash samples were detected by ELISA using the polyvinyl alcohol and polyvinylpyrrolidone blocking method (Appendix A16.2) to minimise non-specific antibody binding. The vaginal wash samples were diluted 1:5 before use.

5.3.4.2. Endpoint antibody titre determination

The titres of HPV-16 VLP-specific serum antibodies were determined by ELISA, using the polyvinyl alcohol and polyvinylpyrrolidone blocking method (Appendix A16.2). The sera were serially diluted by factors of 3, starting from 1:40 and ending at 1:787320. The antibody titres were calculated as the reciprocal of the highest dilution where the OD was ≥2x that of the 1:300 dilution of the prebleed.

5.3.4.3. DTH skin reaction measurement

The delayed-type hypersensitivity (DTH) skin reaction is an antigen-dependent reaction, which includes erythema, induration and monocytic or neutrophilic infiltration at the site of antigen injection, within 24 to 72 hours. These reactions are dependent on both $CD4^+$ and $CD8^+$ memory T cells. The deposition of fibrinogen from the blood vessels, and to a lesser extent, the influx of T cells and monocytes, causes the tissue become indurated (Black 1999).

Briefly, the hair on the back of each guinea pig was removed, and antigens were administered by intradermal injection into this area. The inoculation sites were observed 24, 48 and 72 hours after inoculation and the induration (swelling) was measured at each site using calipers. This method is described in detail in Appendix A17.

5.3.4.4. Neutralising antibody assay

Due to the difficulty of infecting laboratory animals with HPV, *in vitro* methods have been developed for the investigation of neutralising antibodies to this virus. The neutralisation assays were performed by John Schiller (Laboratory of Cellular Oncology, National Cancer Institute, Bethesda, USA) as reported by Pastrana *et al.* (2004). This assay is based on the ability of pseudoviruses to transfer plasmid DNA, carrying a secreted alkaline phosphatase (SEAP) reporter gene, into cells. Antibody neutralisation of the pseudoviruses prevents the delivery of the plasmid into the cells, and hence prevents SEAP expression. Neutralisation titres were defined as the reciprocal of the highest serum dilution that caused at least a 50% reduction in SEAP activity.

5.4. RESULTS

5.4.1. COMPARISON OF THE IMMUNITY INDUCED IN GUINEA PIGS BY IMMUNISATION WITH FRESHLY-MADE rBCG-L1 CONSTRUCTS

Guinea pigs were immunised three times with freshly-made rBCG-L1 (rBCG-112L1$_{NLS}$-, rBCG-119L1$_{NLS}$-, or rBCG-361L1$_R$) or wild-type BCG. Figure 5.1 (A) shows that antibodies reactive with HPV-16 VLPs were detectable in all rBCG-L1-immunised groups after three immunisations. Figures 5.1 (B-D) depict the same results as Figure 5.1 (A), but here the groups are shown individually, and error bars have been included. The results are displayed as the group mean ratio of the ELISA OD_{492nm} value to the OD value of the prebleed of each animal. This was necessary because the prebleed OD ELISA values varied greatly (from OD_{492nm} of 0.054 to 0.20) between the outbred guinea pigs. When the VLP-specific antibody responses were averaged, all rBCG-L1 inoculated groups showed a significant difference to wild type-inoculated animals after the third inoculation (P<0.05, week 17). After the 3rd inoculation (week 17) the rBCG-112L1$_{NLS}$-, rBCG-119L1$_{NLS}$-, and rBCG-361L1$_R$ groups showed significant differences to the wild-type group (P=0.03, P=0.03, and P=0.04, respectively). No significant differences were observed between the rBCG-L1-immunised groups (P>0.6, week 17).

A subset of the above animals was chosen for the assessment of antibody titres. Sera obtained on the 17th week of the study (Figure 5.2), from the wild-type BCG- and rBCG-361L1$_R$-immunised guinea pigs, were analysed for antibody titres specific for HPV-16 VLPs. High L1-specific antibody titres of 3420-29160 were elicited in the rBCG-361L1$_R$-immunised animals. The titres to L1 were 3- to 81-fold higher than the background levels observed in the animals immunised with wild-type BCG.

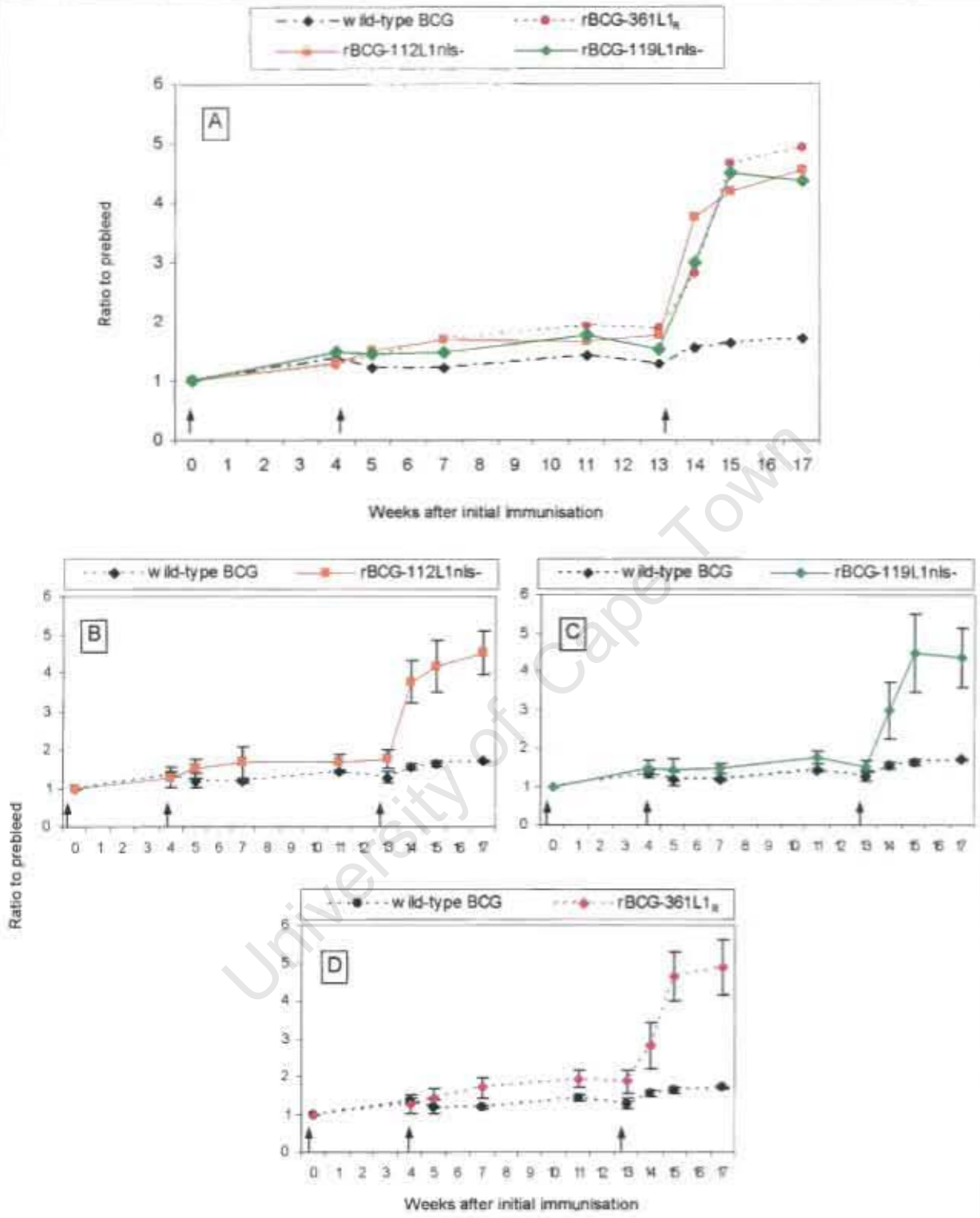


Figure 5.1. Systemic antibody responses specific to HPV-16 VLPs induced following immunisation of guinea pigs with rBCG-L1 (rBCG-112L1$_{NLS^-}$, rBCG-119L1$_{NLS^-}$, and rBCG-361L1$_R$) or wild-type BCG. Guinea pigs were immunised and then boosted at 4 and 13 weeks (represented by arrows). The data are represented as the ratios of the OD_{492nm} value to the OD of the prebleed. A, each data point represents the mean OD ratio per group (of 4 animals). B, C, and D, display the individual groups from graph A, ± standard error of mean (SEM).

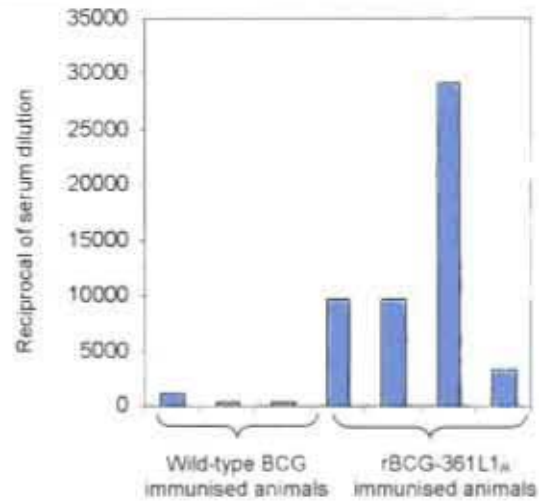


Figure 5.2. Systemic antibody titres induced in guinea pigs specific to HPV-16 L1 VLPs after immunisation with rBCG-361L1$_R$. Sera from week 17 (4 weeks after the 3rd inoculation) were serially diluted and used in an ELISA against intact HPV-16 VLPs. The OD 492nm values were measured and the results recorded as the reciprocal of the highest dilution where the OD is >2x that of the prebleed. Each column represents the serum titre from one animal.

5.4.2. THE EFFECT OF WILD-TYPE BCG PRIMING ON THE IMMUNITY INDUCED IN GUINEA PIGS BY rBCG-L1, AND THE COMPARISON BETWEEN FRESHLY-MADE AND FROZEN/THAWED rBCG-L1

BCG wild-type priming

This animal study was conducted to investigate whether prior immunisation with BCG would prevent subsequent immunity to recombinant BCG. The immune responses elicited in guinea pigs that had been primed with wild-type BCG and then boosted with rBCG-112L1$_{NLS^-}$, were compared to animals that were immunised with rBCG-112L1$_{NLS^-}$, but that had not been primed with wild-type BCG.

Figures 5.3 (A-C) illustrate antibodies reactive with HPV-16 VLPs. Figure 5.3 (A) includes the groups immunised by both the subcutaneous and intramuscular routes. Figures 5.3 (B and C) display the same results as Figure 5.3 (A), but here the groups have been split up according to route of immunisation, and error bars have been included. All groups immunised with rBCG-112L1$_{NLS^-}$, whether with or without wild-type BCG priming, elicited

significant serum antibody levels, when compared to the wild-type BCG negative control group. The animals immunised s.c. with rBCG-L1 (without a wild-type BCG prime) elicited significant levels of VLP-specific antibodies when compared with the wild-type BCG-only-immunised group after the 3rd immunisation (week 14, P=0.057, Figure 5.3 B), but not after the 2nd immunisation (week 6, P=0.54). On the other hand, the animals primed with wild-type BCG and boosted with s.c. rBCG-L1 developed significant specific antibodies after the 2nd and 3rd rBCG-112L1$_{NLS}$- immunisations when compared with the wild-type BCG-only group (weeks 6 and 14, P=0.05 and P=0.011, respectively). The animals that had been primed with a dose of wild-type BCG followed by s.c. rBCG-L1 boosts had generated higher levels of anti-VLP antibodies than the s.c. rBCG-L1-only group (Figure 5.3 B); these responses were significantly different after the 2nd and 3rd rBCG-112L1$_{NLS}$- boosts (weeks 6 and 14, P=0.01 and P=0.022, respectively).

The wild-type BCG prime/i.m. rBCG-L1 boost group elicited significant levels of VLP-specific antibodies when compared with the wild-type BCG-only group at week 6 (P=0.017, Figure 5.3 C) and at week 14 (P=0.001). The i.m. rBCG-L1-only group was not significantly different to the wild-type BCG-only group at week 6 (P=0.28) but was significantly different 2 weeks after the 3rd inoculation (P=0.014, week 14). The wild-type BCG prime/i.m. rBCG-L1 boost inoculation regimen elicited higher levels of anti-VLP antibodies than the i.m. rBCG-L1-only immunisation regimen (week 6, P=0.007; week 14, P=0.04).

The inoculation routes did not appear significant (Figure 5.3 A). The anti-VLP-16 antibodies generated by the wild-type BCG prime/s.c. rBCG-L1 boost and the wild-type BCG prime/i.m. rBCG-L1 boost vaccination regimens were not significantly different (P=0.428 at week 6; P=0.68 at week 14). The anti-VLP-16 antibody responses generated by the s.c. rBCG-L1-only and the i.m. rBCG-L1-only inoculation regimens were also not significantly different to each other (P=0.42 at week 6; P=0.33 at week 14).

Freshly-made versus frozen/thawed rBCG-L1

Freshly-made and frozen/thawed BCG-L1 recombinants were tested in the guinea pig model, to determine whether an immunogenicity difference existed between the two methods of preparation (Figure 5.4). Two weeks after the 2nd rBCG-L1 inoculation (week 6), the freshly-made rBCG-L1 had elicited a significantly greater anti-L1 antibody response than frozen/thawed rBCG-L1 (P=0.016); however, 2 weeks after the 3rd inoculation (week 14), no difference was observed between the 2 groups (P=0.615).

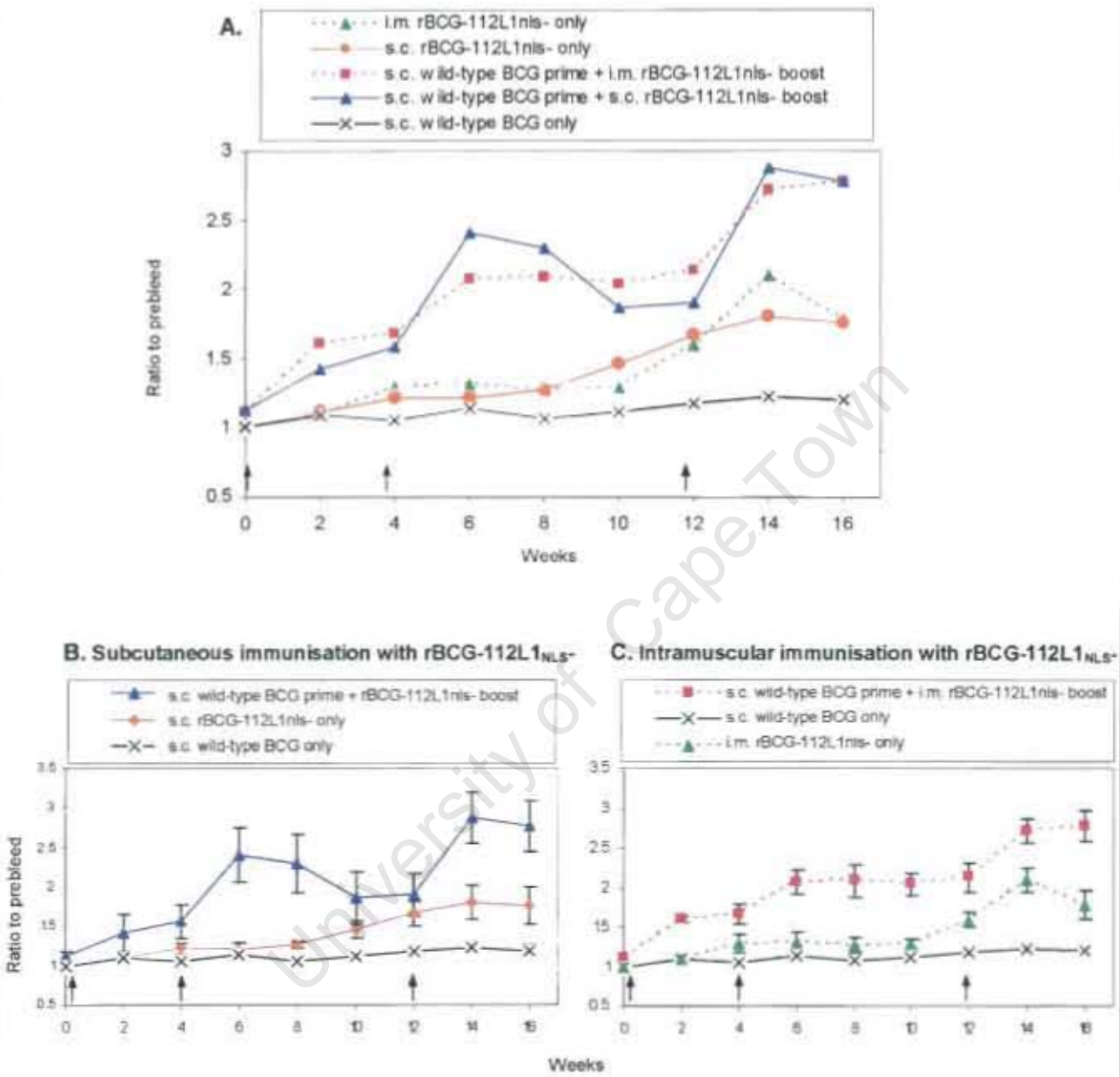


Figure 5.3. Systemic antibody responses specific to HPV-16 L1 VLPs induced following priming of guinea pigs with wild-type BCG and boosting with rBCG-112L1$_{NLS}$-. Guinea pigs were either primed by an immunisation of wild-type Tokyo BCG and then boosted with Tokyo rBCG-112L1$_{NLS}$- 4, 8 and 16 weeks afterwards; or only immunised with Tokyo rBCG-112L1$_{NLS}$- with booster doses at weeks 4 and 12. The graph shows humoral responses after the rBCG-112L1$_{NLS}$- immunisations which occurred at weeks 0, 4, and 12 (represented by arrows). The data are represented as the ratios of the OD_{492nm} value to the OD of the prebleed. A, each data point represents the mean OD ratio per group. The groups from graph A have been separated into graph B (s.c. rBCG-112L1$_{NLS}$- immunisation) and C (i.m. rBCG-112L1$_{NLS}$- immunisation), and error bars (± SEM) have been included.

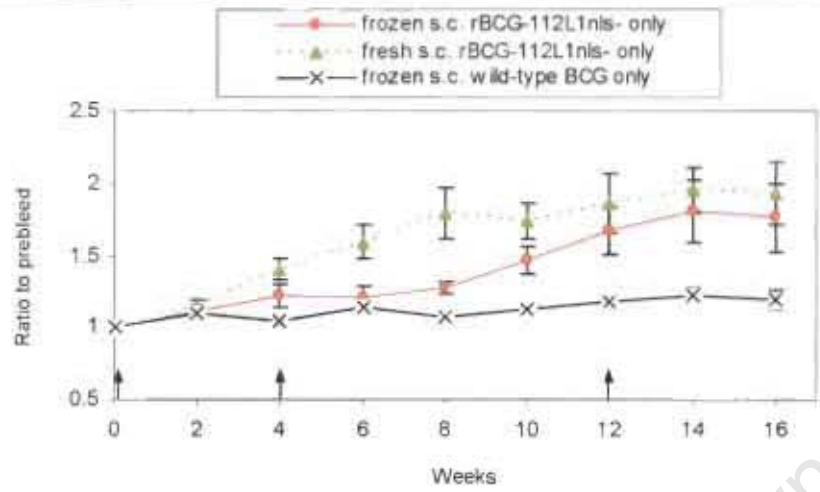


Figure 5.4. Systemic antibody responses specific to HPV-16 L1 VLPs induced following immunisation of guinea pigs with freshly-made or frozen/thawed rBCG-112L1$_{NLS^-}$. Guinea pigs were immunised at week 0, 4 and 12 with Tokyo rBCG-112L1$_{NLS^-}$ (represented by arrows). The data are represented as the mean ratio of the OD_{492nm} value to the OD of the prebleed ± SEM.

The fresh-made rBCG-L1 group was significantly different to the wild-type BCG-only group at week 6 (P=0.036) and at week 14 (P=0.015). The frozen/thawed rBCG-L1 group was significantly different to the wild-type BCG-only group at week 6 (P=0.02) and at week 14 (P=0.02).

DTH skin reactions

To investigate the antigen-specific T-cell immunity elicited in rBCG-L1-immunised guinea pigs, DTH skin tests were performed (Figure 5.5) to PPD, intact HPV-16 VLPs and disrupted HPV-16 VLPs. All groups immunised with rBCG-L1 elicited DTH skin reactions to intact and disrupted HPV-16 L1 VLPs, while the wild-type control animals did not. The reactions were, on average, greatest 48 hours after DTH antigen inoculation, and were often still prominent after 72 hours. No animals developed reactions to PBS or to carbonate buffer (not shown), indicating that the reactions were antigen-specific.

All animals immunised with BCG (wild-type or rBCG-L1) showed positive DTH reactions to PPD. The induration reactions to PPD in the group immunised intramuscularly with rBCG-L1 were significantly smaller than the PPD responses in the subcutaneously-immunised groups (P<0.05). The sizes of the skin reactions to PPD in all the subcutaneously-immunised groups were similar (P>0.05).

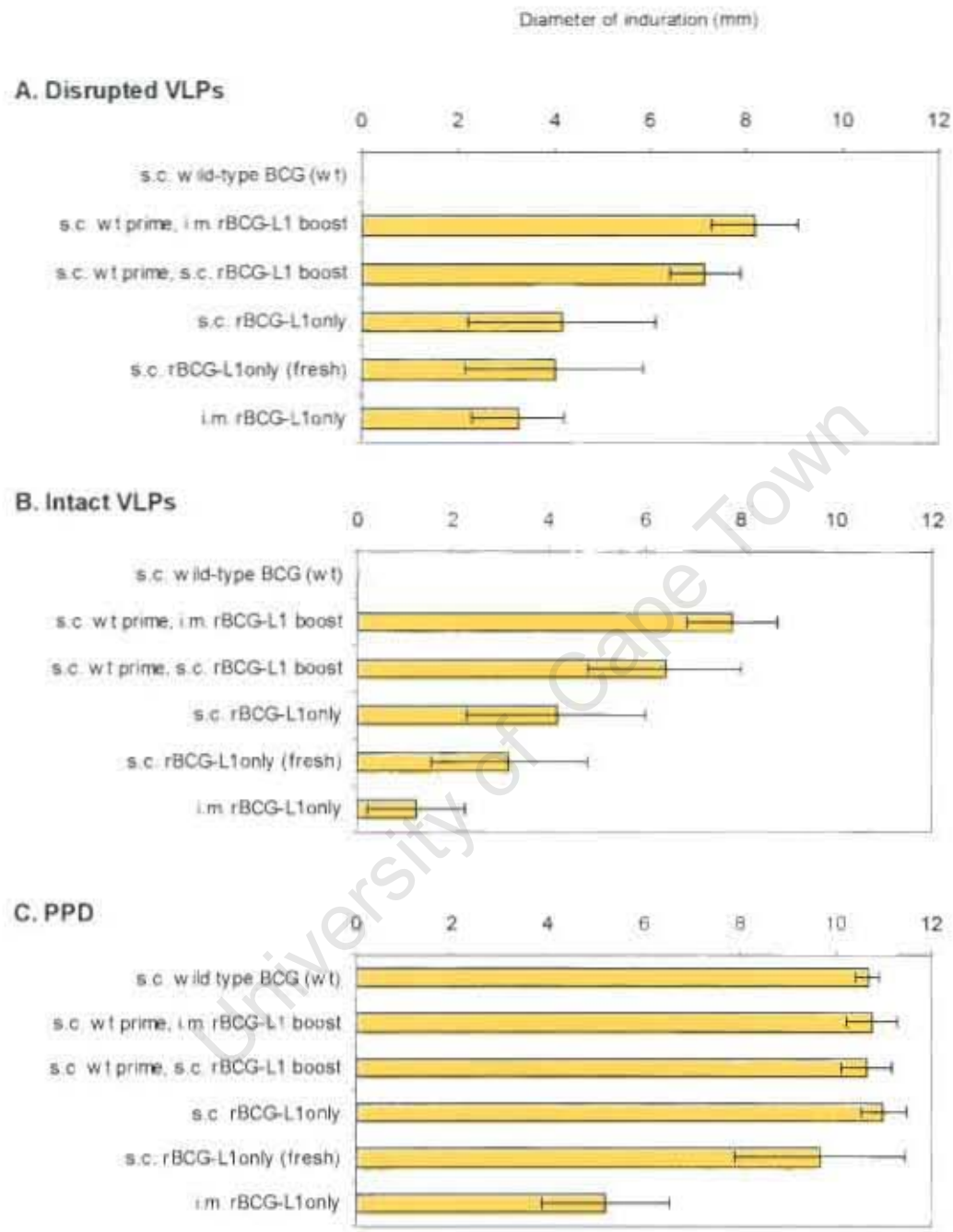


Figure 5.5. Delayed-type hypersensitivity skin reactions displayed by guinea pigs. Guinea pigs were immunised with freshly-made (fresh) or frozen/thawed (no label) Tokyo rBCG-112L1$_{NLS}$- with booster doses at weeks 4 and 12, or they were primed with an immunisation of wild-type Tokyo BCG and then boosted with Tokyo rBCG-112L1$_{NLS}$- 4, 8 and 16 weeks afterwards. DTH reactions were measured 48 hours after i.d. inoculation of disrupted HPV-16 L1 VLPs (A), intact VLPs (B), or PPD (C). The results are expressed as the mean DTH reaction size in mm ± SEM.

The DTH reactions to both intact and disrupted VLPs were significantly greater in the animals that received a priming dose of wild-type BCG prime, followed by a i.m. rBCG-L1 immunisation, than they were in the i.m. rBCG-L1-only group (P=0.014 and P=0.034, respectively). The average induration response displayed by the BCG wild-type prime/s.c. rBCG-L1 animals, to intact and disrupted VLPs, was greater than that of the s.c. rBCG-L1-only animals, however, the difference was not significant (P>0.05). The DTH reactions to VLPs were similar (P>0.05) in the groups immunised with freshly-made rBCG-L1 and with frozen/thawed rBCG-L1.

5.4.3. COMPARISON OF EXPRESSION VECTORS, DOSES, CODON OPTIMISATION AND IMMUNISATION ROUTES ON THE IMMUNITY INDUCED IN GUINEA PIGS BY VACCINATION WITH rBCG-L1

The antibody and DTH responses elicited by guinea pigs following immunisation with a panel of BCG-L1 recombinants were compared. The immune responses induced by different rBCG-L1 doses, routes of inoculation, and effects of L1 codon optimisation and L1 truncation were also compared. Figure 5.6 (A) includes all the BCG-L1 recombinants, routes and doses tested; and provides an overall picture of the systemic antibodies reactive with HPV-16 VLPs. All the groups, with the exception of 3, that were immunised with rBCG-L1, elicited significant levels of antibodies to HPV-16 VLPs (Fig 5.6). Of the 3 groups that had failed to elicit L1-specific immunity, two had been immunised with low doses (10^4 cfu) of rBCG-119L1$_{BCGe}$ or rBCG-361L1$_e$. The third group had been immunised with a high dose ($2x10^6$ cfu) of rBCG-112L1$_e$. Figures 5.6 (B-F) depict the same results as Figure 5.6 (A), but here specific groups are compared and error bars have been included.

Comparison of expression vectors

At the high dose ($2x10^6$ cfu), rBCG-112L1$_e$ was the only construct that failed to elicit significantly higher levels of anti-L1 antibodies than the BCG control (rBCG-GAG) group (P=0.075, week 20, Figure 5.6 B). There does however appear to be a trend of increasing antibodies in the rBCG-112L1$_e$ group, and greater animal numbers may have produced a significant result. The anti-L1 antibodies measured in the rBCG-119L1$_e$ and rBCG-361L1$_e$ groups were higher than those in the rBCG-GAG group (P<0.05, week 20, Figure 5.6 B). Significantly more HPV-16 L1-specific antibodies were induced by rBCG-361L1$_e$ than by rBCG-112L1$_e$ (P=0.04); however, no difference was observed between the rBCG-361L1$_e$ and

rBCG-119L1$_e$ (P=0.31) groups, and no difference between the rBCG-112L1$_e$ and rBCG-119L1$_e$ (P=0.1) groups. The level of L1-specific antibodies induced by rBCG-119L1$_{BCGe}$ was not significantly different to that induced by rBCG-112L1$_{BCGe}$ (P=0.7, week 20, Figure 5.6 C). Although there appears to be a gradual increase in L1-antibody levels in the rBCG-GAG-immunised group, these antibody levels are not significantly higher than those in the PBS-immunised group (P=0.14, week 20). Whereas 3 or 4 immunisations with rBCG-L1 were necessary to elicit significant levels of antibodies to L1, high antibody levels were elicited after the first purified HPV-16 VLP immunisation (ratio of prebleed of 20.25±7.3 at week 4, data not shown).

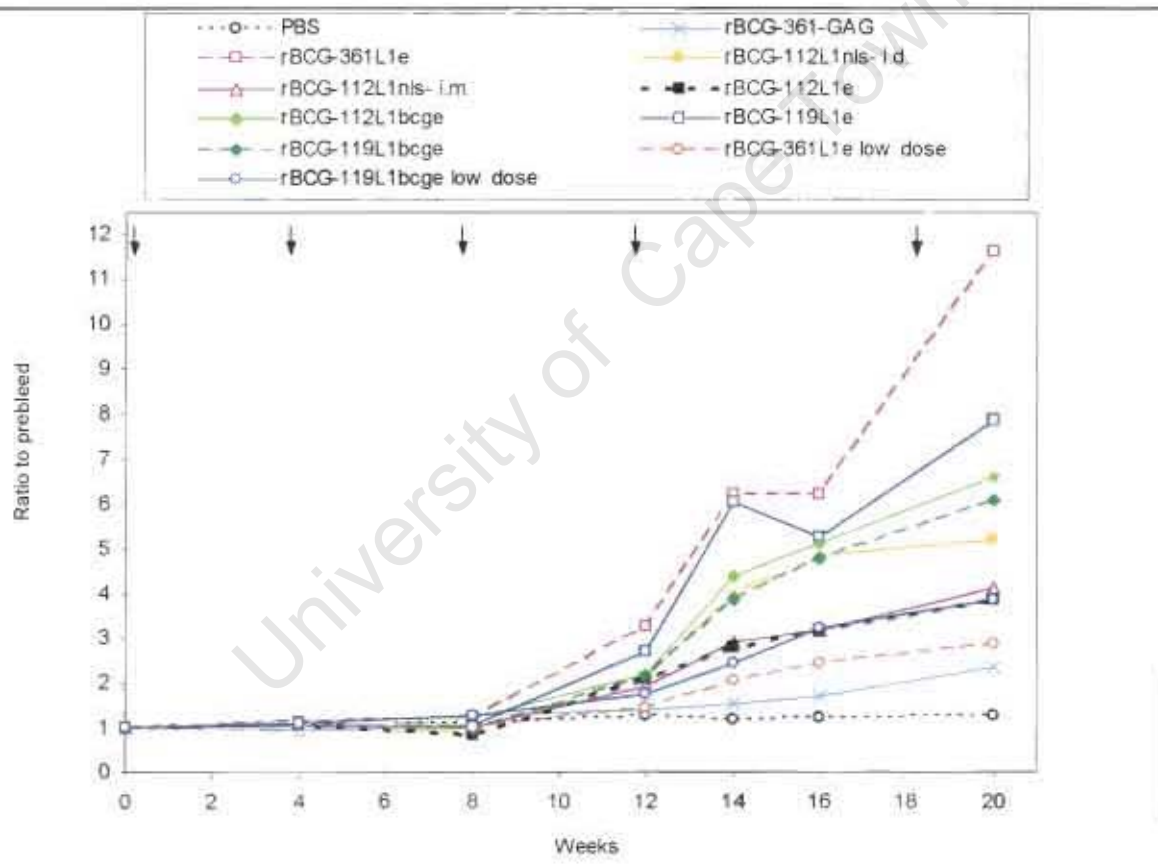
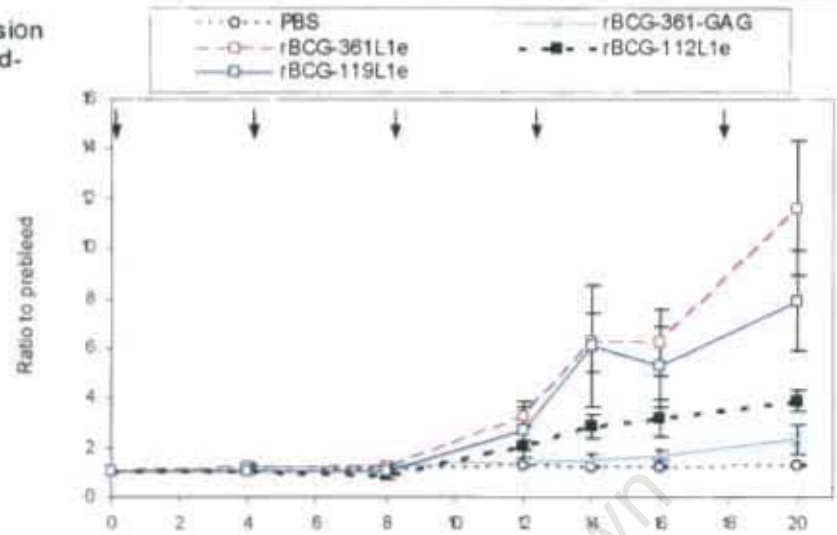


Figure 5.6 (A). HPV-16 L1 VLP-specific systemic antibody responses induced following immunisation of guinea pigs with rBCG-L1 (as listed in the legend). Guinea pigs were immunised i.d. or i.m. (only rBCG-112L1$_{NLS}$-) at weeks 0, 4, 8, 12 and 18 weeks (represented by arrows) with either, PBS, rBCG-GAG, or recombinant BCG expressing HPV-16 L1. All doses were $2x10^6$ cfu, except where indicated by "low dose" (10^4 cfu). The data are represented as the mean ratio of the OD_{492nm} value to the OD value of the prebleed. The average value obtained from the VLP-16 immunised animals was 20.25±7.3 at week 4 and 34.06±15.3 at week 8 (not shown on graph).

B. Comparison of expression vectors expressing wild-type L1



C. Comparison of expression vectors expressing codon optimised L1 ($L1_{BCG}$)

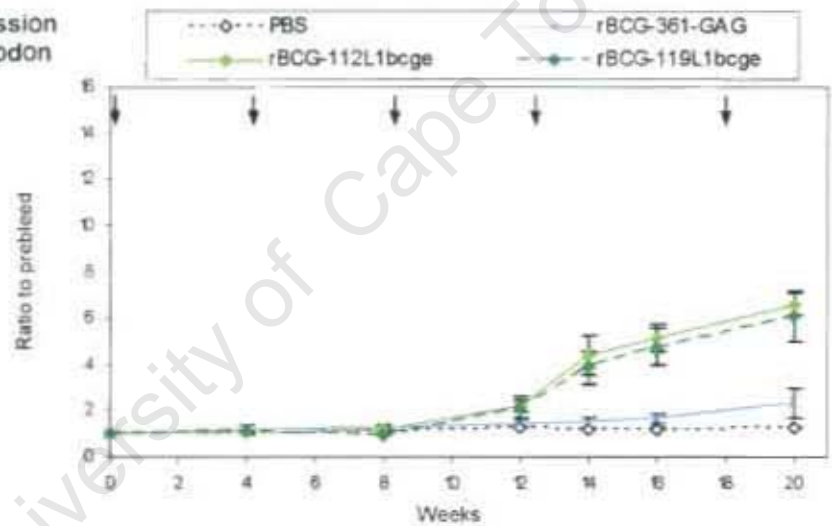


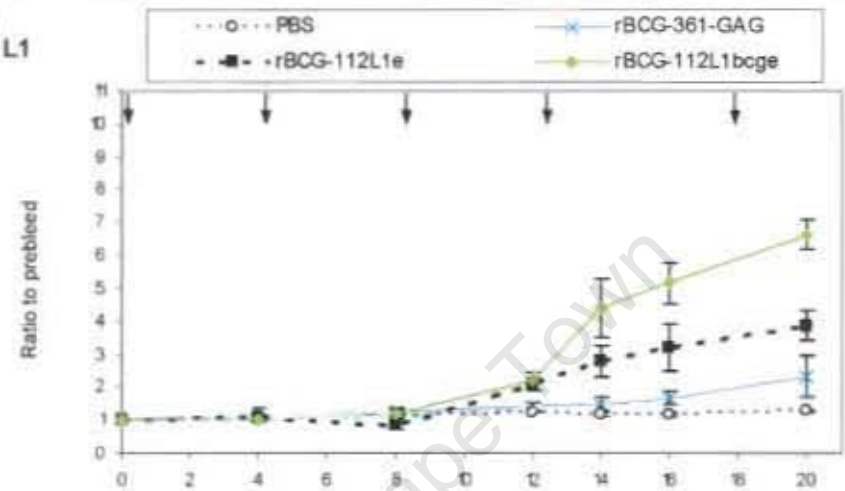
Figure 5.6 (B and C). HPV-16 L1 VLP-specific systemic antibody responses induced following immunisation of guinea pigs with rBCG-L1 (as listed in the legend). Guinea pigs were immunised i.d. or i.m. (only rBCG-112L1$_{NLS}$-) at weeks 0, 4, 8, 12 and 18 weeks (represented by arrows) with either, PBS, rBCG-GAG, or recombinant BCG expressing HPV-16 L1. All doses were $2x10^6$ cfu, unless otherwise indicated. The data are represented as the mean ratio of the OD_{492nm} value to the OD value of the prebleed ± SEM.

Codon optimisation

In contrast to the rBCG-112L1$_e$ construct, immunisation with rBCG-112L1$_{BCGe}$ (expressing codon-optimised L1) elicited significant levels of anti-L1 antibodies (Figure 5.6 D). At week 20, the level of L1-specific antibodies elicited by rBCG-112L1$_{BCGe}$ was higher than that elicited by rBCG-GAG (P=0.0004), and higher than that elicited by rBCG-112L1$_e$ (P=0.002).

Codon optimisation of L1, however, did not always result in an increased immune response to L1, as rBCG-119L1$_{BCGe}$ (codon optimised) and rBCG-119L1$_{e}$ (non-codon optimised) produced similar levels of L1-antibodies (P=0.43, week 20, Figure 5.6 E).

D. **Comparison of rBCG expressing wild-type L1 or L1$_{BCG}$ from the pCB112 vector**



E. **Comparison of rBCG expressing wild-type L1 or L1$_{BCG}$ from the pCB119 vector**

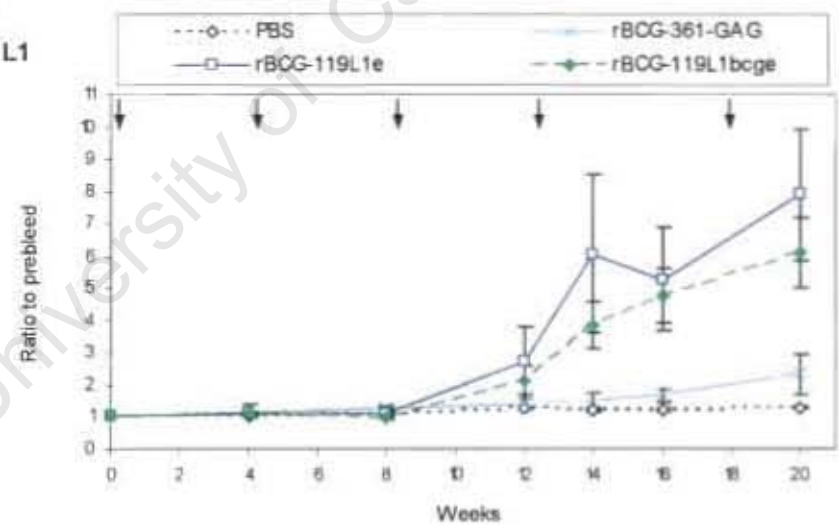


Figure 5.6 (D and E). HPV-16 L1 VLP-specific systemic antibody responses induced following immunisation of guinea pigs with rBCG-L1 (as listed in the legend). Guinea pigs were immunised i.d. or i.m. (only rBCG-112L1$_{NLS}$-) at weeks 0, 4, 8, 12 and 18 weeks (represented by arrows) with either, PBS, rBCG-GAG, or recombinant BCG expressing HPV-16 L1. All doses were 2x10^{6} cfu, unless otherwise indicated. The data are represented as the mean ratio of the OD$_{492nm}$ value to the OD value of the prebleed ± SEM.

Dose comparison

The immune responses elicited by the recombinants, rBCG-119L1$_{BCGe}$ or rBCG-361L1$_e$, were assessed at two doses. When administered in the larger dose (2x10^6 cfu), these recombinants successfully elicited antibody responses to VLPs. Neither recombinant elicited significant anti-L1 antibody levels at the low dose (10^4 cfu), when compared to rBCG-GAG (P>0.05, week 20, Figures 5.6 F and G).

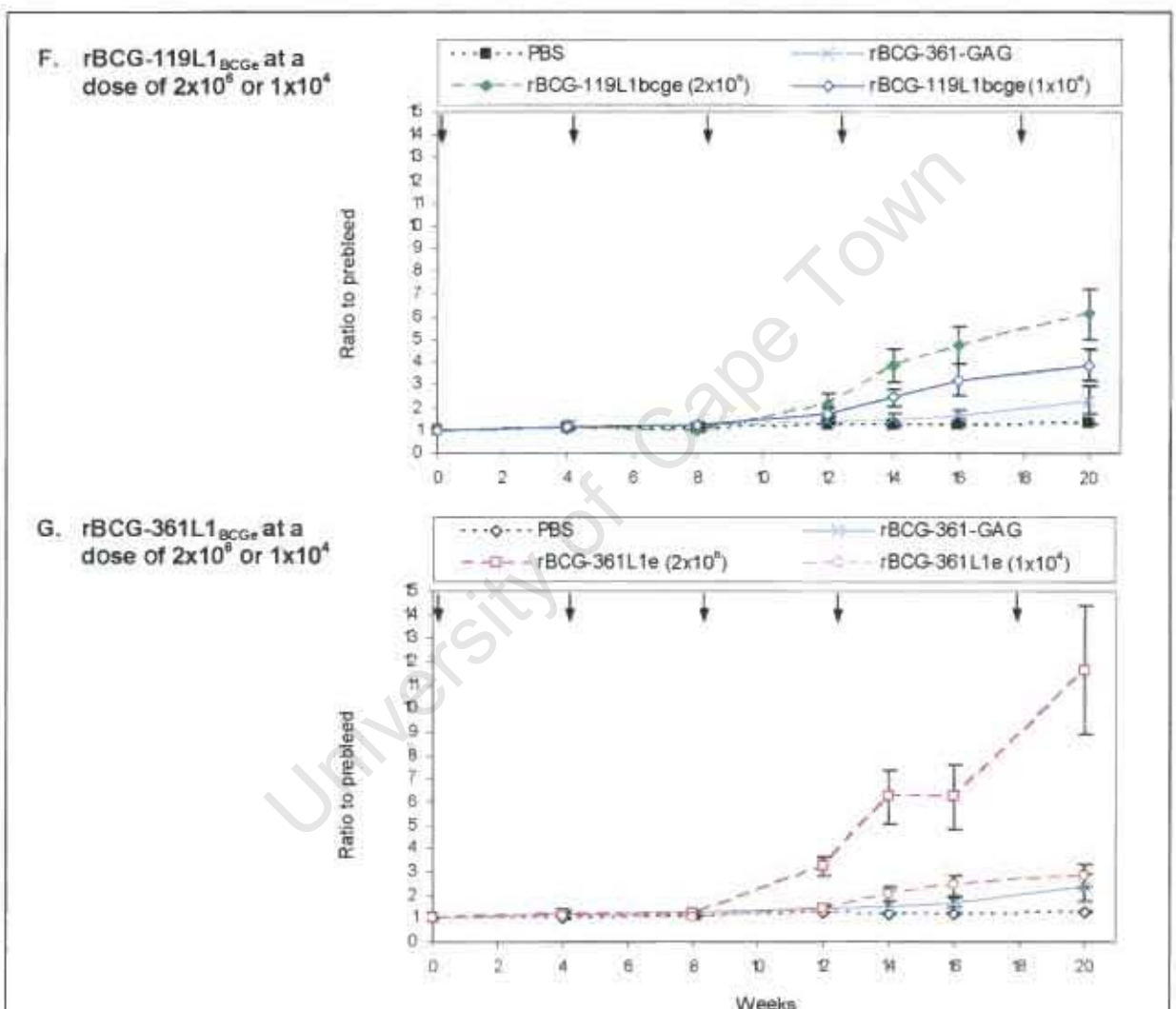


Figure 5.6 (F and G). HPV-16 L1 VLP-specific systemic antibody responses induced following immunisation of guinea pigs with rBCG-L1 (as listed in the legend). Guinea pigs were immunised i.d. or i.m. (only rBCG-112L1$_{NLS}$-) at weeks 0, 4, 8, 12 and 18 weeks (represented by arrows) with either, PBS, rBCG-GAG, or recombinant BCG expressing HPV-16 L1. All doses were 2x10^6 cfu, unless otherwise indicated. The data are represented as the mean ratio of the OD$_{492nm}$ value to the OD value of the prebleed ± SEM.

Route comparison

In previous guinea pigs studies (sections 5.4.1 and 5.4.2) BCG was administered intramuscularly, however, it was decided to change to intradermal administration for the current study. For comparison to these previous studies, in which rBCG-112L1$_{NLS}$- was administered i.m., the present study included i.m. and i.d. immunisations of rBCG-112L1$_{NLS}$-. Intramuscular immunisation with the NLS-truncated L1 recombinant, rBCG-112L1$_{NLS}$-, did not produced significant levels of anti-L1 antibodies when compared to the rBCG-GAG control (P=0.163, week 20; Figure 5.6 H). In contrast, i.d. immunisation with the same recombinant successfully elicited antibodies to L1 (P=0.0084 at week 20).

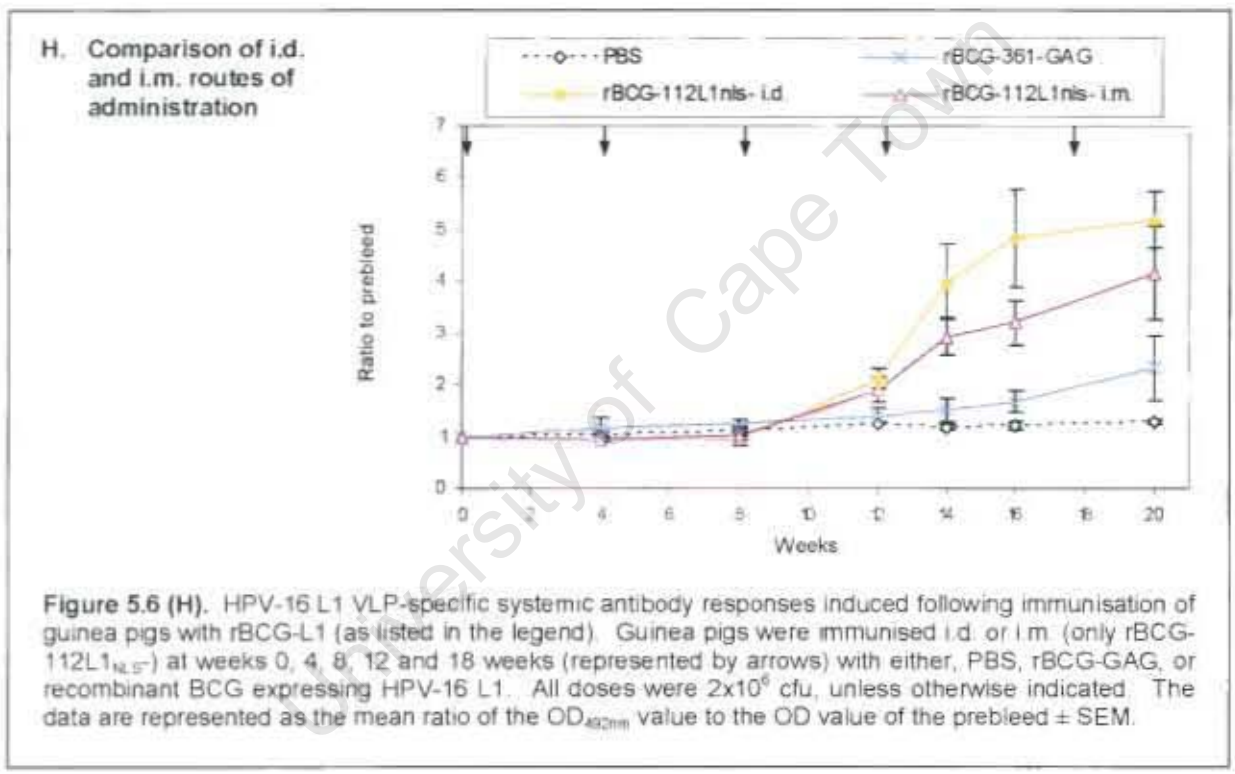


Figure 5.6 (H). HPV-16 L1 VLP-specific systemic antibody responses induced following immunisation of guinea pigs with rBCG-L1 (as listed in the legend). Guinea pigs were immunised i.d. or i.m. (only rBCG-112L1$_{NLS}$-) at weeks 0, 4, 8, 12 and 18 weeks (represented by arrows) with either, PBS, rBCG-GAG, or recombinant BCG expressing HPV-16 L1. All doses were $2x10^6$ cfu, unless otherwise indicated. The data are represented as the mean ratio of the OD_{492nm} value to the OD value of the prebleed ± SEM.

NLS truncation

rBCG-112L1$_e$ and rBCG-112L1$_{NLS}$- were compared to investigate whether the deletion of the L1 nuclear localisation signal (NLS) would affect the immune responses elicited to L1. The construct expressing full-length L1, rBCG-112L1$_e$, failed to elicit significant levels of anti-L1 antibodies in guinea pigs (P=0.075, week 20; Figure 5.6 I), however, immunisation with the NLS-truncated L1 recombinant, rBCG-112L1$_{NLS}$-, successfully did so (P=0.0084, week 20).

I. **Comparison of full-length L1 vs. NLS-truncated L1**

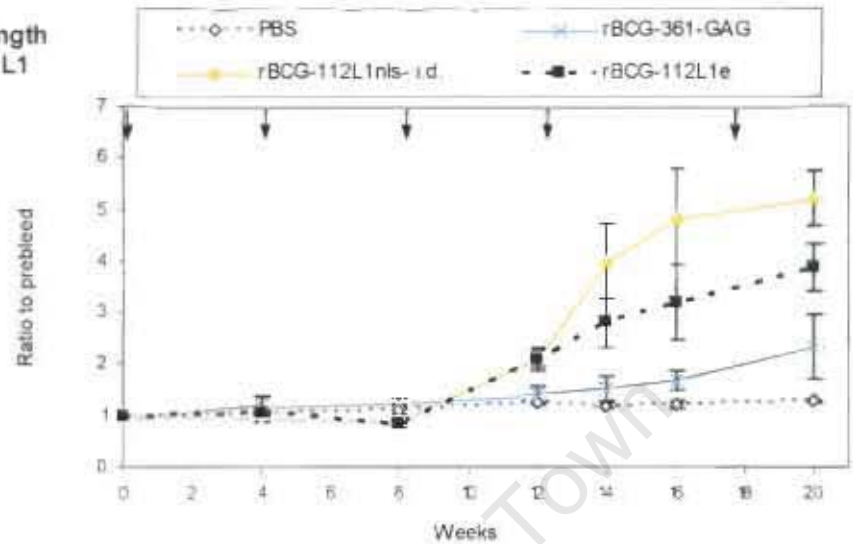


Figure 5.6 (I). HPV-16 L1 VLP-specific systemic antibody responses induced following immunisation of guinea pigs with rBCG-L1 (as listed in the legend). Guinea pigs were immunised i.d. or i.m. (only rBCG-112L1$_{NLS^-}$) at weeks 0, 4, 8, 12 and 18 weeks (represented by arrows) with either PBS, rBCG-GAG, or recombinant BCG expressing HPV-16 L1. All doses were $2x10^6$ cfu, unless otherwise indicated. The data are represented as the mean ratio of the OD_{492nm} value to the OD value of the prebleed ± SEM.

Antibodies in genital secretions

Vaginal wash samples were collected from the guinea pigs 2 weeks after the second and fourth immunisations; and were tested for the presence of antibodies specific for HPV-16 VLPs (Figure 5.7). After the fourth immunisation, strong VLP-specific responses were detected in a number of the rBCG-L1-immunised animals (especially in the rBCG-361L1$_e$, rBCG-119L1$_e$ and rBCG-119L1$_{BCGe}$ groups); however, large variations between animals within the same groups reduced the group averages substantially. Although the antibodies elicited in the rBCG-361L1$_e$, rBCG-119L1$_e$ and rBCG-119L1$_{BCGe}$ groups were >2-fold that observed in their respective pre-wash samples, only the rBCG-119L1$_e$ group elicited a response that was more than twice that of the rBCG-361-GAG control group. The average antibody level in the rBCG-119L1$_e$ group was 3 times that of the rBCG-361-GAG control group (P=0.02, after the fourth immunisation).

High levels of VLP-specific antibodies were detected in the vaginal washes from the VLP-16-immunised animals. After the fourth immunisation the average antibody level in this group was 15.3-fold that of the PBS control group (P=0.003).

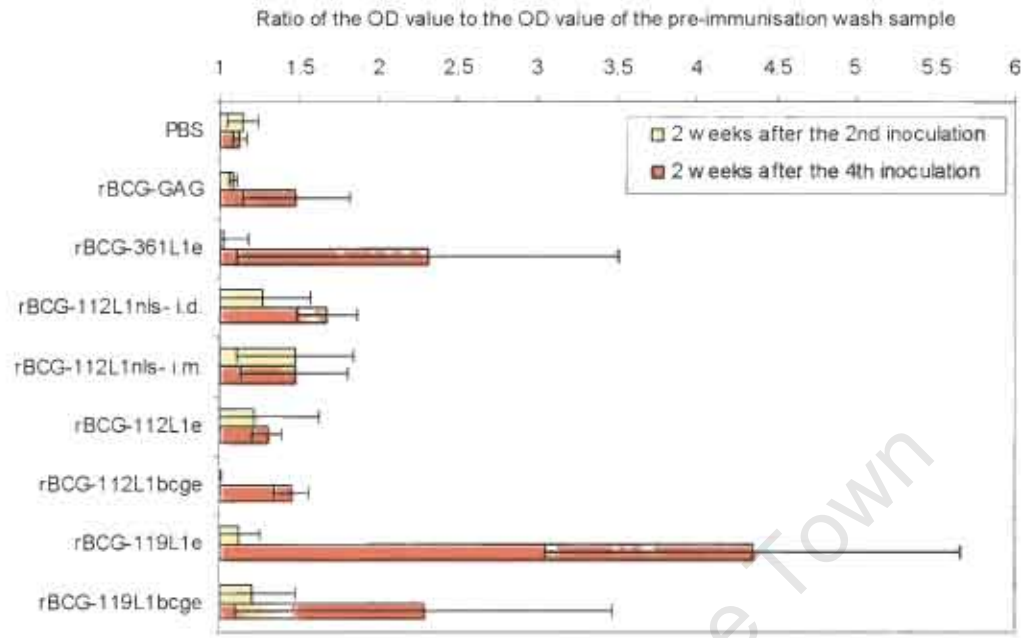


Figure 5.7. Vaginal antibodies specific to HPV-16 L1 VLPs elicited in guinea pigs after immunisation with recombinant BCG expressing HPV-16 L1 (as listed above). Guinea pigs were immunised i.d. or i.m. (only rBCG-112L1$_{NLS}$-) with either, PBS, rBCG-GAG, or recombinant BCG expressing HPV-16 L1. The animals received 4 immunisations, with 4 weeks between each administration. All doses were $2x10^6$ cfu. The data are represented as the group average ratio of the OD_{492nm} value to the OD value of the pre-wash ± standard deviation. VLP immunised animals had values of 20.236 ± 7.27 and 23.482 ± 3.38 after the second and fourth immunisations, respectively.

DTH skin reactions

To investigate the antigen-specific T-cell immunity elicited by rBCG-L1 in these guinea pigs, DTH skin tests were performed (Figure 5.8) with PPD, HPV-16 VLPs, and PBS. On average, the DTH skin reactions displayed by the rBCG-L1-immunised animals peaked 48 hours after the DTH inoculations; the reactions of the VLP-immunised animals, on the other hand, peaked 24 hours after the DTH inoculations, and were declining by 48 hours. In the VLP-immunised animals the induration reactions to VLP were 16.75mm ± 2.1 at 24 hours; but decreased significantly by 48 hours, and were absent by 72 hours, in all but one animal. Animals immunised with low doses (10^4 cfu) of rBCG-119L1$_{BCGe}$ or rBCG-361L1$_e$ did not display significant DTH skin reactions to VLP (P>0.05); however, animals immunised with the same BCG recombinants, in the higher dose ($2x10^6$ cfu) had positive reactions. While

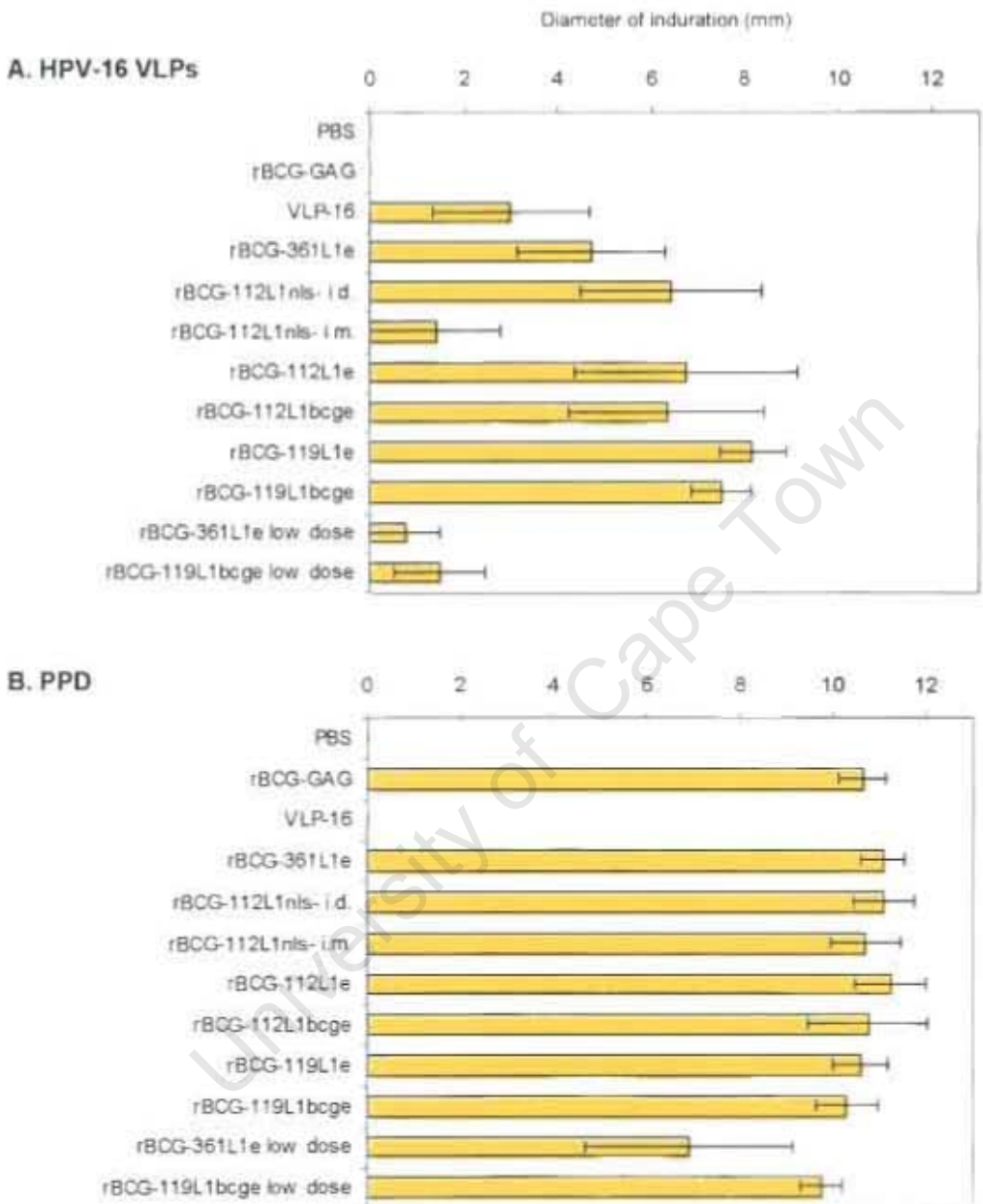


Figure 5.8. Delayed-type hypersensitivity skin reactions displayed by guinea pigs. Guinea pigs were immunised i.d. or i.m. (only rBCG-112L1$_{NLS}$-) with either, PBS, VLP, rBCG-GAG, or recombinant BCG expressing HPV-16 L1 (as listed above). The animals received 5 immunisations, with 4 week intervals between each administration. All doses were $2x10^6$ cfu, unless indicated by "low dose" (10^4 cfu). DTH reactions were measured 48hrs after i.d. inoculation of HPV-16 VLP (A), PPD (B) or PBS (not shown). The results are expressed as the DTH reaction size in mm ± SEM. No DTH reactions developed to PBS in any of the animals (not shown).

the animals that were immunised intradermally with rBCG-112L1$_{NLS}$- displayed significant skin reactions to VLP, the animals immunised intramuscularly with the same recombinant did not (P>0.05). With the exception of the low BCG dose and intramuscularly immunised groups, the DTH reactions to VLPs were similar (P>0.05) in all other rBCG-L1-immunised groups.

No animals developed skin reactions to PBS. No rBCG-361-GAG-immunised animals developed induration reactions to HPV-16 VLP (P>0.05). All groups immunised with BCG (rBCG-GAG or rBCG-L1) displayed positive skin reactions to PPD, which were of similar magnitude (P>0.05).

HPV-16 pseudovirion neutralisation

An *in vitro* neutralisation assay was performed, which is based on the ability of SEAP HPV-16 pseudoviruses to transfer the SEAP reporter gene into cells. Neutralisation titres were defined as the reciprocal of the highest serum dilution that caused at least a 50% reduction in SEAP activity. The prebleed serum and week 20 serum from one animal of each of the above mentioned guinea pig groups were tested for neutralising antibodies (Table 5.3).

Table 5.3. SEAP pseudovirus neutralisation assay to test whether rBCG-L1 elicited HPV-16 specific neutralising antibodies in guinea pigs.

Immunogen	Neutralisation (reciprocal of the highest serum dilution to cause at least a 50% reduction in SEAP activity)	
	HPV-16	HPV-18
PBS	<20	<20
rBCG-GAG	<20	<20
rBCG-361L1$_e$	<20	<20
rBCG-112L1$_{NLS}$-	<20	<20
rBCG-112L1$_e$	<20	<20
rBCG-112L1$_{BCGe}$	<20	<20
rBCG-119L1$_e$	<20	<20
rBCG-119L1$_{BCGe}$	<20	<20
HPV-16 VLP	43 700	<20

Note: Titres below 20 were considered negative.
The sera utilised for the data tabulated above were from week 20 (2 weeks after the 5th immunisation).

The only serum that was shown to cause neutralisation of HPV-16 pseudoviruses was that of the VLP-immunised animal; which produced a 50% neutralisation titre of 43 700 (Table 5.3). All other samples including prebleeds (not shown) and post-immunisation sera of the rBCG-L1, rBCG-GAG, and PBS immunised animals were negative at serum dilutions of 1:20. All samples, including that of the VLP-immunised animal, were negative in the BPV neutralisation control assay, at serum dilutions of 1:20. Serum was considered positive for neutralising antibodies if its HPV-16 neutralisation value was at least 4-fold higher than that of the HPV-18 assay. This assay was repeated and the results confirmed (Debbie Stewart; Department of Clinical Laboratory Science, University of Cape Town).

5.5. DISCUSSION

The aim of this research was to investigate the possibility of utilising rBCG, expressing HPV-16 L1, as a prophylactic vaccine for HPV; I therefore focused on the ability of rBCG-L1 to generate antibodies that were specific for HPV-16 L1 VLPs. HPV-16 VLP-specific cell-mediated immunity was also assessed, as these responses would be valuable for the elimination of infected cells, when sterilising antibody immunity fails to occur. The primary objective of this study was to find a suitable end product vaccine, this influenced the approach to the project; and accordingly it was only of secondary importance to compare results from the BCG-L1 recombinants in a carefully controlled manner.

5.5.1. IMMUNITY INDUCED IN GUINEA PIGS BY IMMUNISATION WITH FRESHLY-MADE rBCG-L1 CONSTRUCTS

In the first animal experiment, guinea pigs were immunised three times with freshly-made rBCG-L1 (grown to the correct OD by the day of immunisation, and harvested just prior to immunisation). Here the ability of rBCG expressing two HPV-16 L1 variants, full-length HPV-16 L1, and L1 without the NLS ($L1_{NLS}$-, 22 aa C-terminal truncation), to elicit antibodies specific for HPV-16 L1 was demonstrated. Guinea pigs were utilised as they share a number of similarities with humans in their response to mycobacterial infection, clinically, pathologically and immunologically (Horwitz *et al.*, 2000; McMurray, 1994). The outbred nature of the guinea pigs, which often resulted in large immune response variations between

individual animals, also more closely resembles the anticipated situation for the human population. Although there were animals in each group that responded very strongly, some animals did not respond, thus creating large standard deviation values and low probability values. For each rBCG-L1 construct, three immunisations were required before a sharp increase in the antibody level was observed; the need for repeated immunisations is most probably a consequence of low L1 expression. The antibody levels elicited by the three different rBCG-L1 constructs were comparable, which suggests that there may be an upper threshold of L1 expression in BCG, however, we cannot make direct comparisons, as the constructs were expressing different L1 variants. High anti-L1 antibody titres (3240-29160; 3- to 81-fold higher than those observed in the wild-type BCG-immunised animals) were observed in the rBCG-361L1$_R$-immunised animals. These titres are comparable with other guinea pig immunogenicity studies, in which viral proteins were delivered by rBCG (Lim *et al.*, 1997; Honda *et al.*, 1995). The moderate antibody response seen in the one wild-type BCG-immunised animal was most likely due to the strong non-specific adjuvant properties of BCG (Fuerst *et al.*, 1992). Gradually increasing non-specific antibody responses have been noted by other groups, after immunisation of guinea pigs with BCG (Lim *et al.*, 1997; Lagranderie *et al.*, 1993).

It should be noted that, although the VLPs against which the sera were routinely tested are referred as "VLPs", one can not assume that all the L1 protein present in these samples is in an assembled form, as breakdown products and linear species can be expected to form during the coating of the ELISA plates. The possibility therefore exists that L1-positive sera from rBCG-L1-immunised animals may bind to linear L1 forms and linear surface epitopes on VLPs/capsomeres or to conformational epitopes on VLPs/capsomeres, or to a mixture of both. Preliminary ELISAs with sera from the above guinea pigs, which were performed in parallel on plates coated with VLPs in PBS or in carbonate buffer (causing breakdown of VLPs) resulted in similar data being obtained from the utilisation of both buffers, and hence only VLPs in PBS were utilised for further serum analysis.

5.5.2. COMPARISON BETWEEN FRESHLY-MADE AND FROZEN/THAWED rBCG-L1

In subsequent experiments, the use of frozen BCG immunisation stocks was preferred, as these could be consistent from one immunisation to the other, and could be tested for genetic stability before they were used. When freshly-made immunisation stocks were compared with frozen/thawed stocks, the freshly-made rBCG-L1 elicited a significantly

greater anti-L1 antibody response when measured two weeks after the 2nd rBCG-L1 inoculation; however, 2 weeks after the 3rd inoculation, no difference was observed between the two groups. After the 2nd freshly-made rBCG-L1 immunisation, a steady increase in anti-VLP antibody levels was elicited; however, the increase in the antibody levels elicited by frozen/thawed rBCG-L1 was preceded by an initial lag phase, suggesting that BCG needed a recovery stage. At the end of the study, the DTH reactions to VLPs were comparable in the groups immunised with freshly-made rBCG-L1 and with frozen/thawed rBCG-L1. Although the antibody levels were comparable after the 3rd immunisation, the antibody levels produced by freshly-made rBCG-L1 in this animal study were lower those elicited in the previous study by the same rBCG-L1 construct (rBCG-112L1$_{NLS}$-); suggesting possible inconsistencies in the preparation of fresh rBCG. A study with larger animal numbers and more rBCG-L1 constructs would need to be performed to confirm the differences between immunogen preparations; however, the impracticality of utilising fresh rBCG preparations for large-scale vaccination roll-out does not warrant such an investigation.

5.5.3. THE EFFECT OF WILD-TYPE BCG PRIMING ON THE IMMUNITY INDUCED IN GUINEA PIGS BY rBCG-L1

A concern surrounding the development of recombinant BCG vaccines is whether prior vaccination with wild-type BCG would adversely affect the immunity induced to recombinant BCG vaccines. This question is often asked because one of the suggested explanations for the poor efficacy of BCG vaccination is that prior exposure to environmental mycobacteria primes the immune response, causing a more rapid control of the BCG vaccination dose, and a consequent reduction in the immune response elicited to it (Agger and Anderson, 2001; Hess and Kaufmann, 1999). This was confirmed by Buddle *et al.* (2002) when they showed that when cows had been sensitised to environmental bacteria, the protective affect of subsequent BCG vaccination was adversely affected. Similar results have been obtained with live recombinant *Salmonella* vaccination, where prior immunisation with the carrier strain and, to a lesser extent with a heterologous strain, reduced the immune responses to foreign proteins delivered by subsequent immunisation with recombinant *Salmonella* (Roberts *et al.*, 1999; Kohler *et al.*, 2000). In contrast to these results, a number of studies established that prior immunological experience with *Salmonella* enhanced the immune responses to heterologous proteins carried by the same strain (Whittle and Verma, 1997; Bao and Clements, 1991). A group investigating the effect of BCG-priming on the immunity induced by rBCG-LacZ in mice (Gheorghiu *et al.*, 1994; Lagranderie *et al.*, 1997a; Lagranderie *et al.*,

1996), showed that antibody responses to β-gal were dramatically increased in the BCG-primed mice, however, proliferative responses were reduced. Also of interest is the observation by Mazzantini *et al.* (2004), of the adjuvant effect of rBCG-CRM_{197} (diphtheria toxin) on the antibody response induced to rBCG-FC (tetanus toxin), when these recombinants were delivered simultaneously. In the current study, I found that wild-type BCG-primed guinea pigs elicited enhanced L1-specific antibody responses, when compared with non-primed animals. The wild-type BCG-primed guinea pigs also showed a trend of increased L1 cell-mediated responses, as these animals displayed larger DTH induration reactions. It is thought that the augmentation of the immune responses observed in BCG-primed animals is, as suggested by Gheorghiu *et al.* (1994), a result of the activation of BCG-specific T cells, leading to enhanced macrophage activation and enhanced antigen presentation of both the mycobacterial and recombinant proteins.

5.5.4. COMPARISON OF EXPRESSION VECTORS, DOSES, CODON OPTIMISATION AND IMMUNISATION ROUTES ON THE IMMUNITY INDUCED IN GUINEA PIGS BY VACCINATION WITH rBCG-L1

In the final guinea pig study, comparisons were drawn between a panel of rBCG-L1 constructs; aspects such as type of expression vector, codon optimisation, dosage, route of immunisation, and NLS deletion were compared. Large differences in the antibody levels were observed between animals within the same groups, as noted before, and hence significance values were often low. Antibody levels were compared with that of the rBCG-GAG control group and not to the PBS group, as the non-specific response observed in the rBCG-GAG group was considerable. As seen previously, at least three immunisations were required before noticeable increases in anti-L1 antibody levels were observed. On the other hand, only one immunisation with purified VLPs was required to induce high levels of antibodies to L1. The rBCG constructs, rBCG-$112L1_e$ and rBCG-$112L1_{NLS}$-, utilising the pCB112 expression vector, consistently elicited the lowest antibody responses; which is possibly a consequence of a weak promoter (*18kDa*) driving L1 expression. In fact, rBCG-$112L1_e$ was the only construct that failed to elicit significant levels of antibodies to L1, when administered at the high dose. Although the average antibody response in the rBCG-$361L1_e$-immunised animals was greater than that in the rBCG-$119L1_e$-immunised animals, the difference between them was not significant. The only clear reason, therefore, for a possible preference of rBCG-$361L1_e$ over rBCG-$119L1_e$ would be due to its superior stability.

DTH skin reaction tests were performed to measure cell-mediated immunity; these Type IV Hypersensitivity reactions are dependent on both $CD4^+$ and $CD8^+$ memory T cells, but with a predominance of MHC class II-restricted $CD4^+$ T cell activity, and hence an association with a Th1 cytokine profile (Ohtani *et al.*, 2004; Black, 1999). All rBCG-L1 constructs, when administered at the higher dose, successfully induced L1-specific DTH reactions. The potent cellular immunity adjuvant properties of BCG are evident here, because immunisation with rBCG-112L1$_e$, which failed to elicit a significant antibody response to L1, successfully induced a DTH response was comparable to that induced by the other recombinants. The DTH reactions displayed by the rBCG-L1-immunised animals followed the typical Type IV Hypersensitivity reaction profile, with maximum induration appearing 48 hours after the skin test. Interestingly, the DTH reactions displayed by the VLP-immunised animals to the VLP skin tests did not follow the typical Type IV profile, in that these reactions started to decrease after 24 hours. There is a small possibility that these responses were caused by the non-specific activation of the inflammatory response, or by a IgG-mediated-Type III Hypersensitivity response; however these reactions develop 1-2 hours after the skin test (Ponvert and Scheinmann, 2003), and unfortunately the animals were first examined 24 hours post skin test.

rBCG-L1 dosage and route of inoculation both played important roles in the induction of immune responses. The low rBCG-L1 dose (10^4 cfu) failed to elicit significant antibody or cellular responses to L1, but the same recombinants at the high dose ($2x10^6$ cfu) delivered sufficient L1 for the induction of a response. The low dose was not sub-immunogenic, since responses were induced to BCG (PPD), but not to L1 because of its low expression. Intramuscular immunisation of rBCG-112L1$_{NLS}$- was not as efficient as the i.d. route at eliciting antibody or cell-mediated immunity to L1.

rBCG-112L1$_{BCGe}$ elicited more antibodies than rBCG-112L1$_e$, providing evidence that either codon optimisation increased the amount of L1 expressed by BCG, or that the presence of 5 CpG-islets on the optimised gene provided an enhanced adjuvant effect over the native gene (containing one weak CpG-islet at position 1184). However, in the case of rBCG-119L1$_e$, its codon optimised equivalent, rBCG-119L1$_{BCGe}$, did not induce a higher antibody level. This again suggests that there is an upper threshold level of L1 expression in BCG. rBCG-112L1$_{BCGe}$ can express more L1 than rBCG-112L1$_e$ because the threshold has not been reached in rBCG-112L1$_e$, but since L1 expression in rBCG-119L1$_e$ is already at the threshold level, codon optimisation can not enhance expression further. It's difficult to speculate the cause of this apparent threshold, but it may possibly be due to a limited number of

chaperones, which bind to newly produced L1, and in the process of trying to fold it, also protect it from degradation. Since this chaperone-bound L1 is protected from degradation, this may be the L1 that is generating an immune response. When the amount of L1 is in excess of that which can be bound to the chaperones, then the unbound L1 is rapidly degraded, and hence does not mount an immune response (William Boum, personal communication; Department of Clinical Laboratory Science, University of Cape Town).

The removal of the NLS, as in the rBCG-112L1$_{NLS}$- construct, also successfully enhanced the immune response induced to L1. Here the improved immune response could have been a result of superior L1 presentation in the BCG-infected cells, or increased expression of the truncated protein, of which the former is more likely since the truncation was only by 22 amino acids.

5.5.5. HPV-16 PSEUDOVIRION NEUTRALISING ANTIBODY ASSESSMENT

The finding that neutralising antibodies were not detectable in the sera from the rBCG-L1-immunised animals, suggests that, either, L1 could not assemble into VLPs or capsomeres within BCG, or if VLPs did form, the conformational epitopes were destroyed during lysosomal degradation of BCG. The former is more likely, since L1 production in rBCG-L1 was not high enough to be detected, and it is therefore unlikely that the concentration would have been sufficient for VLP formation. This observation is of considerable concern since the proposed use of our rBCG-L1 vaccine was as a prophylactic one, and hence the need for neutralising antibodies.

5.5.6. ASSESSMENT OF ANTIBODIES IN GENITAL SECRETIONS

Since the genital tract is the site of HPV infection, the core objective of a prophylactic HPV vaccine is the generation of antibody and cell-mediated responses at the genital mucosa. In our study, L1-specific antibodies were detected in the vaginal wash samples from a number of the rBCG-L1-immunised animals, especially in the rBCG-361L1$_e$, rBCG-119L1$_e$ and rBCG-119L1$_{BCGe}$ groups. Again, as a result of large differences between animals in the same groups, when averaged, only the rBCG-119L1$_e$ group elicited a L1-specific response that was significantly higher than that seen in the BCG control group. The groups with the highest anti-L1 serum antibody levels (VLP, rBCG-361L1$_e$, and rBCG-119L1$_e$), also boasted

the highest average genital antibody levels; suggesting that the vaginal IgG antibodies originated by transudation from serum. It would be interesting to investigate mucosal routes of delivery, such oral or nasal, as these routes are expected to generate stronger responses at the genital mucosa.

In summary, rBCG-L1 elicited VLP-specific antibody and cell-mediated immune responses that were dependent on expression vector, dose, route of immunisation and L1 variant. rBCG-361L1$_e$, and rBCG-119L1$_e$ induced the highest serum antibody levels, but only rBCG-119L1$_e$ generated significant VLP-specific vaginal antibody responses. The low level of L1 expression by these recombinants required a strategy of multiple immunisations; however, the use of purified VLP necessitated only one immunisation to induce a strong antibody response. While immunisation with VLP generated neutralising antibodies, immunisation with rBCG-L1 failed to do so. Codon optimisation increased immunity to L1, but only to an upper threshold level, and it is unknown whether this is a consequence of increased expression or CpG adjuvant effects of the codon optimised gene. The deletion of the L1 NLS appeared to increase the immune response, possibly by improving its presentation in rBCG-L1-infected cells. Finally, prior immune exposure to wild-type BCG did not prevent the development of an immune response to rBCG-L1, but in fact, increased it.

CHAPTER 6: VACCINIA VIRUS–HPV-16 L1 MURINE CHALLENGE MODEL

6.1. INTRODUCTION

In this chapter I investigated the ability of recombinant BCG, expressing HPV-16 L1, to protect mice against challenge with recombinant vaccinia virus-HPV-16 L1 (rVV-$L1_R$); which we have described previously (Marais *et al.*, 1999). The difficulty experienced with the propagation of HPV in culture, and the lack of animal models for HPV, have hindered the development of animal models to evaluate papillomavirus protection. The rVV-$L1_R$ challenge model is therefore a valuable tool for investigating the efficacy of candidate HPV vaccines. The rVV-$L1_R$ challenge model was first described by Marais *et al.* (1999), in which mice immunised with HPV-16 VLPs elicited a T cell response that was able to provide protection against intraperitoneal (i.p.) challenge with rVV-$L1_R$. The T cell responses were characterised by a Th1 cytokine profile, with some Th2 IL-2 production. Protection was demonstrated by a 4.6 $\log_{10}$ reduction in the ovarian titres of rVV-$L1_R$ in vaccinated BALB/c mice, and a 2.3 $\log_{10}$ reduction in vaccinated C57 BL/6 mice.

Recombinant vaccinia virus (rVV) is widely used as a laboratory expression system due to its broad host range and large capacity for foreign DNA. Vaccinia virus (VV) replicates within the cytoplasm of infected cells, allowing heterologous proteins to enter the MHC class 1 pathway, and thereby inducing $CD8^+$ CTL responses (Moss, 1996). Early, intermediate, and late pox promoters are available for the control of heterologous gene expression by rVV. It has been shown that antigens expressed by rVV from early pox promoters are able to induce CTL responses to these antigens; however, late pox promoters are not able to induce CTL responses to the antigens that they express (Bronte *et al.*, 1997; Zhou *et al.*, 1991b). rVV gene products are not expressed on the VV envelope and are not directly accessible to antibodies (Zinkernagel *et al.*, 1990); rVV challenge therefore allows for evaluation of cell-mediated, rather than neutralising antibody immune mechanisms.

A number of groups have shown that vaccination can successfully control challenge with rVV (Bachmann *et al.*, 1994; Belyakov *et al.*, 1998; Binder and Kundig 1991; Kent *et al.*, 1998; Murata *et al.*, 2003; Pancholi *et al.*, 2003; Uno-Furuta *et al.*, 2003). Binder and Kündig (1991) showed that mice immunised with vesticular stomitis virus (VSV) were protected from challenge with rVV expressing the VSV nucleoprotein, and that the predominant immune response was a CD8-mediated CTL response. They showed that in mice depleted of $CD8^+$ T cells, the rVV virus was able to initiate an infection equivalent to that in non-immunised mice. Depleting the challenged mice of $CD4^+$ T cells had no effect, and the immunised mice were able to clear the rVV infection. Bachmann *et al.* (1994) immunised mice with the

nucleoprotein (NP) of VSV and then challenged intraperitoneally with recombinant VV-VSV-NP. The immunised mice displayed 5.6 log_{10} specific protection from VV-VSV-NP. In another study, mice immunised intrarectally with a HIV-1 peptide vaccine (PCLUS3-18IIIB) showed similar levels of protection to Bachmann's group after intrarectal challenge with recombinant VV expressing the HIV-1 gp160 (Belyakov *et al.*, 1998). Murata *et al.* (2003) showed that immunisation of mice with hepatitis C (HCV) virus-like particles lead to a >5 log_{10} reduction in vaccinia titres after challenge with rVV expressing HCV proteins (vvHCV.S); a DNA vaccine expressing the same HCV proteins, however, only displayed 1 log_{10} protection after challenge with vvHCV.S. Uno-Furuta *et al.* (2003) showed that mice immunised with a BCG recombinant expressing the CTL epitope, NS5a, of HCV demonstrated specific protection of approx. 2.5 log_{10} when challenged with rVV-HCV-NS. They determined that the major immune response present in the mice was of the Th1-type, with production of IFN-γ, IL-2 and IL-12.

6.2. STUDY OBJECTIVES

The primary aim of this study was to determine the *in vivo* CTL responses elicited to BCG-L1 recombinants in mice, by measuring the level protection in a rVV-$L1_R$ challenge model. The humoral responses that were generated to the BCG-L1 recombinants were measured, and compared with the rVV-$L1_R$ protection levels, to determine whether antibodies could be correlated with protection. Finally, the cell-mediated responses generated by rBCG-L1 were investigated further by means of a cytotoxicity assay and by the measurement of $CD8^+$ T cell IFN-γ production.

6.3. MATERIALS AND METHODS

6.3.1. ANIMALS

Inbred BALB/c ($H\text{-}2^b$) mice, 8-12 weeks old, were obtained from South African Vaccine Producers (South Africa) and were housed in the UCT Animal Unit. All animal protocols were approved by the UCT Animal Research Review Committee. Immunisations, serum and organ collections were performed by trained animal technologists.

6.3.2. CLONING INTO THE VACCINIA VIRUS SHUTTLE VECTOR

The HPV-16 L1 gene was cloned into the vaccinia virus shuttle vector, pSC65, forming pSC65-L1, as we have previously described (Marais *et al.*, 1999). This cloning strategy inserted the L1 gene downstream of the constitutive, synthetic early/late promoter, PE/L. The pSC65 shuttle vector also contains an *E. coli* origin of replication (ColE1), an ampicillin resistance gene, and a β-D galactosidase (*LacZ*) gene, which is under the control of the poxvirus early/late p7.5 promoter. The multiple cloning site and *LacZ* gene are flanked by regions of the poxvirus thymidine kinase (*TK*) gene, which facilitates homologous recombination into the non-essential *TK* gene in the wild-type VV genome. The interruption of the *TK* gene by this insertion also provides a selection mechanism for the recombinant viruses. In the presence of the thymidine analogue, 5-bromodeoxyuridine (BUdR), TK competent viruses (TK^+) allow phosphorylation of BUdR, which is lethally incorporated into the virus genome. When the *TK* gene is interrupted in recombinant VVs, BudR is not phosphorylated, and hence has no lethal effect (Chakrabarti *et al.*, 1985).

pSC65-L1 was amplified by large scale plasmid preparation (Appendix A5.2); and was then linearised with *Sca* I, to ensure double crossover recombination (Di Marais, personal communication).

6.3.3. RECOMBINANT VIRUS FORMATION

Recombinant rVV-$L1_R$ was developed, as described in Appendix A22. Briefly, CV-1 cells were infected with vaccinia virus (Western Reserve strain; obtained from Dr John Williamson St Mary's Hospital, London) for 1hr; after which DMEM medium and transfection medium (containing DOTAP and linearised pSC65-L1) were added to the cells. Transfection was allowed to proceed for 15min at room temperature. The infected/transfected cells were then incubated at 37°C for 2-3 days, until cytopathic effect (cpe) was detected. To discern whether the transfection had been successful, the cells were subjected to 5-bromo-4-chloro-3-indoyl-β-D galactosidase (X-gal) staining (see Appendix A23 for protocol). During this staining method, β-galactosidase present in rVV-infected cells hydrolyses X-gal and results in the formation of blue cells. Cells from duplicate wells, that had not been stained, were frozen and thawed to release the newly-formed recombinant virus. The expression of L1 by rVV-$L1_R$ was confirmed by immunofluorescence of infected CV-1 cells (Marais *et al.*, 1999;

Di Marais is acknowledged for the final rVV-$L1_R$ recombinant and the immunofluorescence assay). To reduce contamination by wild-type vaccinia virus, the newly formed rVV-$L1_R$ viral stocks were put through several rounds of plaque purification and serial passage in $HuTK^-$ cells with BUdR selection (Appendix A22), until only blue plaques were detectable by X-gal staining.

6.3.4. LARGE-SCALE VIRUS PROPAGATION IN EGGS

Both rVV-$L1_R$ and rVV-GAG (an HIV-1 GAG VV recombinant, from Therion Biologics Corporation) were grown to high titres on the chorio-allantoic membranes of 11 day old fertilised chicken eggs. The resultant viral stocks were titrated on CV-1 cells. These protocols are described in detail in Appendices A24 and A25.

6.3.5. MOUSE IMMUNISATIONS AND rVV-$L1_R$ CHALLENGE

6.3.5.1. Immunisation with Tokyo rBCG-L1

Groups of three female BALB/c mice were immunised i.p. with either HPV-16 VLP (10 μg/mouse), BCG wild-type, rBCG-361$L1_R$, rBCG-361$L1_{NLS}$-, or rBCG-NV$L1_R$. The BCG doses were $2x10^6$ cfu/mouse and were prepared immediately prior to immunisation (Appendix A15). All the BCG inocula were of the Tokyo substrain. A control group of 3 mice received no immunisations. The mice were given booster inoculations, of the same dose and route, 4 and 8 weeks after the first inoculation. Two weeks after the last boost, the mice were challenged i.p. with 10^8 plaque forming units (pfu) of rVV-$L1_R$ per mouse. Five days post-challenge the ovaries were harvested and homogenised to release the virus (Appendix A26). The virus obtained from group was pooled and the viral loads were measured by titration in triplicate on CV-1 cells (Appendix A25).

Retro-orbital blood samples were collected from each mouse at the beginning of the study, and just prior to challenge. Humoral responses to HPV-16 VLPs were investigated by ELISA (Appendix A16.2). In order to reduce non-specific binding of serum to trace amounts of baculovirus and insect cell proteins present in the VLP preparations, with which the ELISA plates had been coated, the serum samples were incubated for 1.5hrs at room temperature

in the presence of baculovirus/sf9 insect cell extract (kindly supplied by R. Rose, Rochester University, Rochester, USA) before they were added to the ELISA plates. A serum dilution of 1:20 was utilised.

6.3.5.2. Immunisation with Pasteur rBCG-L1

Groups of 8 female BALB/c mice were immunised s.c. with either PBS, HPV-16 VLP (10 μg/mouse), BCG wild-type, rBCG-361L1$_e$ or rBCG-119L1$_e$. The BCG doses were $2x10^6$ cfu/mouse, and were prepared from frozen stocks (Appendix A15). All the BCG inocula were of the Pasteur substrain. The mice were given booster inoculations of the same dose and route, 4 and 8 weeks after the first inoculation. Two weeks after the last boost the mice were challenged i.p. with 10^8 pfu of rVV/mouse. Four mice from each group of 8, were challenged with rVV-L1$_R$, while the other four were challenged with the control virus, rVV-GAG. Five days post-challenge the ovaries were harvested and homogenised to release the virus (Appendix A26). Each mouse was assessed for viral load, which was measured by titration, in triplicate, on CV-1 cells (Appendix A25).

Retro-orbital blood samples were collected from each mouse at the beginning of the study, and just prior to challenge. Humoral responses HPV-16 VLPs were investigated by ELISA (Appendix A16.2). Before the sera were added to the ELISA plate, they were incubated with baculovirus/insect cell extract, as described above. A serum dilution of 1:20 was utilised.

6.3.6. IMMUNISATION OF MICE WITH PASTEUR rBCG-L1 FOR INVESTIGATION OF INTRACELLULAR CYTOKINE AND CTL RESPONSES

As our lab had established immunological assays for the detection of responses to the HIV gp120 CTL epitope, we evaluated the cell-mediated immune responses to this epitope after immunisation with rBCG-119L1$_e$. The HIV gp120 CTL epitope was included in the EpiA tag, which was cloned downstream of the L1 gene. The detection of immune responses to this epitope after rBCG-119L1$_e$ immunisation would provide evidence of successful antigen delivery, and L1 gene read-through.

Twenty BALB/c mice received a single subcutaneous inoculation behind the neck, with $2x10^6$ cfu of Pasteur rBCG-119L1$_e$. Two weeks later inguinal lymph nodes cells were prepared, and were stimulated for 6 days in the presence of gp120 peptide. Intracellular cytokine flow

cytometry with rat monoclonal anti-mouse CD3, CD8 and IFN-γ was used to detect gp120 peptide-specific $CD8^+$ cells producing IFN-γ, after a further 4 hour stimulation in the absence or presence of the peptide (described in detail in Appendix A20).

A CTL assay was also performed to measure ability of the mouse lymphocytes to cause specific lysis of gp120-pulsed p815 cells. Briefly, effector cells were prepared from mouse lymph nodes (above), as described in Appendix A19. The cytotoxic activity of the effector cells was tested in triplicate against P815 (mouse mastocytoma, MHC class I cells) target cells, that had been labelled with ^{51}Chromium (^{51}Cr) and had been pulsed with gp120 peptide (Appendix A21). The ^{51}Cr released from the lysed target cells was measured, and reported as a percentage of specific lysis for each effector:target ratio utilised.

6.4. RESULTS

6.4.1. HUMORAL RESPONSE ELICITED IN MICE AFTER IMMUNISATION WITH TOKYO rBCG-L1

The humoral immunity elicited in BALB/c mice was assessed following 3 immunisations with rBCG expressing HPV-16 L1. Two serum samples were collected: One before the start of the immunisations, and the other two weeks after the third immunisation (just prior to the challenge). The sera were tested for antibody responses specific for HPV-16 VLPs.

The VLP-immunised animals, and the rBCG-NVL1$_R$-immunised group elicited significant levels of antibodies to HPV-16 VLPs, when compared with the relevant controls (Figure 6.1; P<0.04). The VLP-immunised animals elicited the most notable VLP-specific antibody response, which was significantly greater than that generated by the rBCG-L1-immunised animals. The BCG recombinants that failed to elicit significant levels of antibodies (P>0.13) were both based on the pMV361 vector.

6.4.2. PROTECTION FROM rVV-L1$_R$ CHALLENGE AFTER IMMUNISATION WITH TOKYO rBCG-L1

We had already established that immunisation with HPV-16 VLPs was able to protect mice from challenge with rVV-L1$_R$ (Marais *et al.*, 1999). In the current study I investigated whether

rBCG expressing HPV-16 L1 would provide protection from challenge with rVV-$L1_R$, and how this protection would compare with that afforded by immunisation with VLPs.

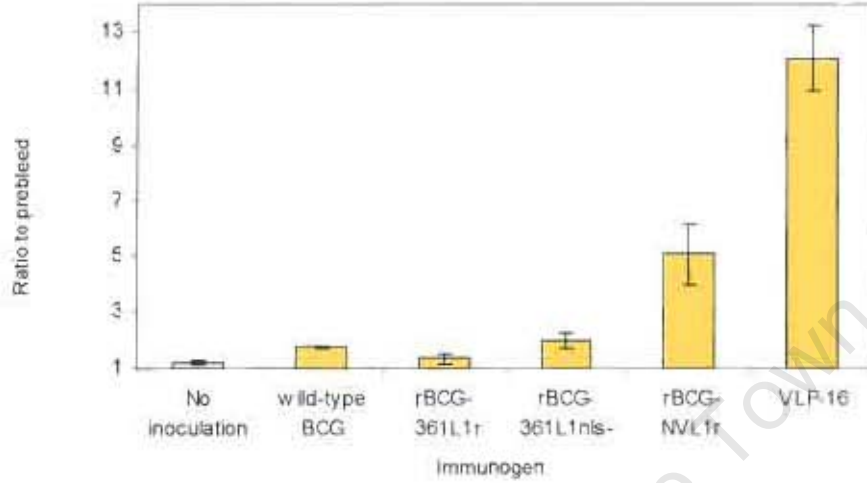


Figure 6.1. Systemic antibody responses specific for HPV-16 L1 VLPs induced in mice following immunisation with Tokyo rBCG-L1 (rBCG-361$L1_R$, rBCG-361$L1_{NLS^-}$, or rBCG-$NVL1_R$). Mice were immunised 3 times at 4 week intervals. Blood samples were collected 2 weeks following the 3rd immunisation. The data are represented as the group mean OD_{492nm} ratios to the group mean prebleed value ± SEM.

BALB/c mice were immunised 3 times with rBCG-L1 (BCG-361$L1_R$, rBCG-361$L1_{NLS^-}$, or rBCG-$NVL1_R$), and then challenged with vaccinia virus expressing L1. Five days after the rVV-$L1_R$ challenge, the vaccinia virus titres in the ovaries were titrated. The vaccinia virus obtained from each group of mice was pooled and the viral load was measured by titration in triplicate. For each group, the average amount of vaccinia virus per mouse was calculated and is presented Table 6.1 and Figure 6.2. The degree of protection is presented as the log_{10} pfu difference between the groups.

Mice immunised with VLPs were protected by 1.42 log_{10} when compared to the un-inoculated control group. Wild-type BCG-immunised animals did not elicit a non-specific protective response, as no reduction in rVV-$L1_R$ titres was observed when comparing this group to the non-immunised group. The amount of rVV-$L1_R$ recovered from mice immunised with wild-type BCG appears greater than that recovered from the non-immunised animals, however, this difference is not significant (P=0.234). Animals immunised with rBCG-$NVL1_R$ displayed low levels of L1-specific protection when compared to the wild-type BCG-

immunised animals (P=0.0043). Animals vaccinated with rBCG-361L1$_R$ and rBCG-361L1$_{NLS}$- were afforded enhanced protection from rVV-L1$_R$ challenge when compared to rBCG-NVL1$_R$. Animals immunised with rBCG-361L1$_{NLS}$- were protected by 0.5 $\log_{10}$ more than those immunised with rBCG-361L1$_R$ (P=0.041).

Table 6.1. Protection from rVV-L1$_R$ challenge in mice immunised with Tokyo rBCG-L1 (BCG-361L1$_R$, rBCG-361L1$_{NLS}$-, or rBCG-NVL1$_R$). Protection afforded by vaccination is represented by the $\log_{10}$ reduction in viral titre compared to the control groups (non-inoculated or BCG wild-type). No mice were afforded 100% protection.

Immunogen	rVV-L1$_R$ pfu / mouse	$\log_{10}$ pfu / mouse	$\log_{10}$ reduction compared with the "no inoculation" group	$\log_{10}$ reduction compared with the wild-type BCG group
No inoculation	1.26×10^8	8.1	–	0.1 (P = 0.234)
BCG wild-type (Tokyo)	1.58×10^8	8.2	– 0.1 (P = 0.234)	–
VLP-16	4.79×10^6	6.68	1.42 (P = 0.0002)	1.52 (P = 0.0001)
rBCG-361L1$_R$ (Tokyo)	2.0×10^7	7.3	0.8 (P = 0.006)	0.9 (P = 0 0038)
rBCG-361L1$_{NLS}$- (Tokyo)	6.31×10^6	6.8	1.3 (P = 0.00009)	1.4 (P = 0.000037)
rBCG-NVL1$_R$ (Tokyo)	5.89×10^7	7.77	0.33 (P = 0.017)	0.42 (P = 0 0043)

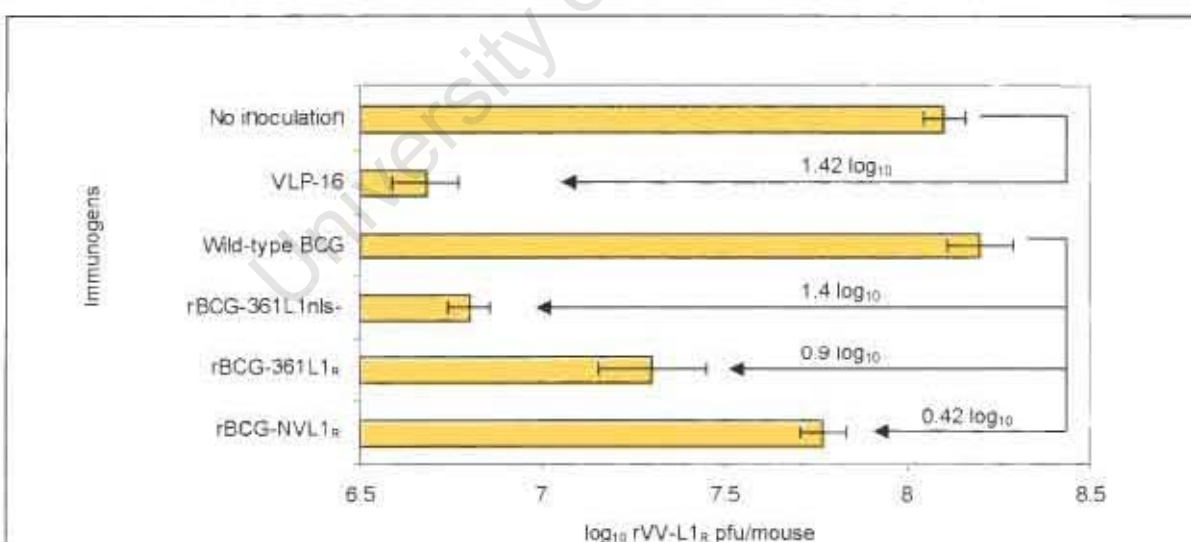


Figure 6.2. Protection from rVV-L1$_R$ challenge in mice immunised with Tokyo rBCG-L1 (BCG-361L1$_R$, rBCG-361L1$_{NLS}$-, or rBCG-NVL1$_R$). Ovarian titres of rVV-L1$_R$ were measured 5 days after the challenge and are expressed as the average $\log_{10}$ rVV-L1$_R$ pfu/mouse ± SEM. The $\log_{10}$ reductions in VV titres are illustrated for selected groups.

6.4.3. CTL RESPONSES AND CD8$^+$ T LYMPHOCYTE IFN-γ PRODUCTION IN MICE IMMUNISED WITH PASTEUR rBCG-L1

In Chapter 2, the existence immunologically "weak" (Glaxo, Tokyo) and "strong" (Pasteur, Danish) substrains, was discussed. A study by Lagranderie *et al.* (1996) also revealed that these substrains differ in their abilities to persist and to elicit protective immunity in mice. Of all the strains, the Tokyo strain was least able to multiply and persist *in vivo*, and was the most ineffective strain at inducing CTLs in mice. This information prompted us to assess rBCG-L1 immunisation in mice using Pasteur recombinants.

The cellular immune responses elicited by Pasteur rBCG-119L1$_e$ were assessed by a peptide-specific cytotoxicity assay and by the investigation of IFN-γ production by CD8$^+$ T cells. These assays were directed at the HIV gp120 CTL epitope, which was cloned downstream, in frame with the L1 gene. After a single s.c. inoculation of rBCG-119L1$_e$, the immune responses that were elicited to the HIV gp120 CTL epitope, present in the EpiA tag, were measured. From Figure 6.3 it is clear that, although the lysis of the control p815 cells was high, a significantly larger percentage of gp120-pulsed p815 target cells was lysed; this was most evident at the 6:1 effector:target ratio, at which there was a specific lysis of 36%. These mice also showed gp120 peptide-specific CD8$^+$ cellular at levels almost 5-fold above background (Figure 6.4), with 8.5% of the CD8$^+$ T cells producing IFN-γ when stimulated with gp120 peptide, as opposed to 1.8%, when not stimulated.

6.4.3. HUMORAL RESPONSE ELICITED IN MICE AFTER IMMUNISATION WITH PASTEUR rBCG-L1

The humoral immunity elicited in BALB/c mice was assessed following 3 immunisations with Pasteur rBCG expressing HPV-16 L1 (rBCG-361L1$_e$, or rBCG-119L1$_e$). Animals immunised with rBCG-361L1$_e$ elicited significant levels of antibodies specific for HPV-16 VLPs (Figure 6.5; P<0.004), but the rBCG-119L1$_e$-immunised animals failed to produce L1 antibody levels greater than that observed in the BCG control group (P=0.74). The highest L1-specific antibody response was elicited in the VLP-immunised group (P<0.0005).

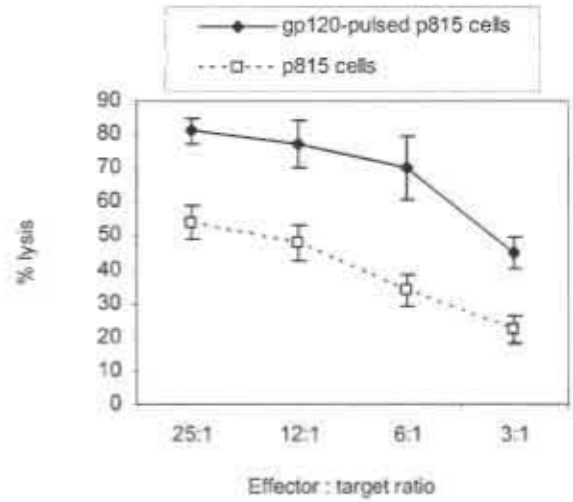


Figure 6.3. gp120-specific cytotoxicity of mouse T lymphocytes after immunisation with Pasteur rBCG-119L1$_e$ as measured by a ^{51}Cr-release assay. Two weeks after a single immunisation with Pasteur rBCG-119L1$_e$, the lytic activity of the mouse T lymphocytes was assessed using gp120-pulsed p815 cells or p815 cells as targets.

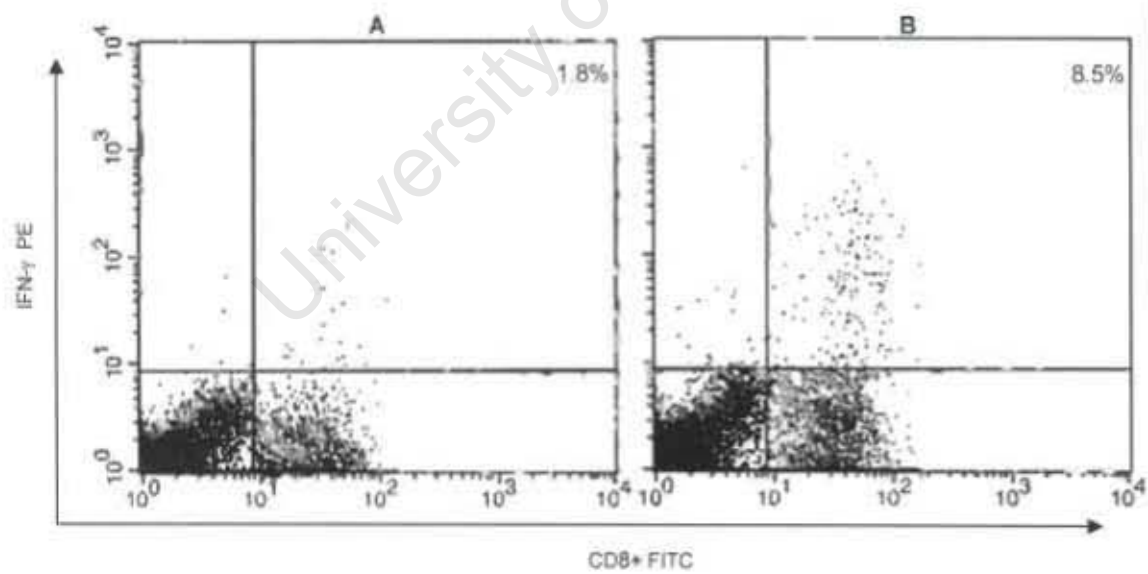


Figure 6.4. gp120-specific IFN-γ production by CD8$^+$ T lymphocytes from mice immunised with a single dose of Pasteur rBCG-119L1$_e$. The lymphocytes were either un-stimulated (A) or stimulated with gp120 peptide (B).

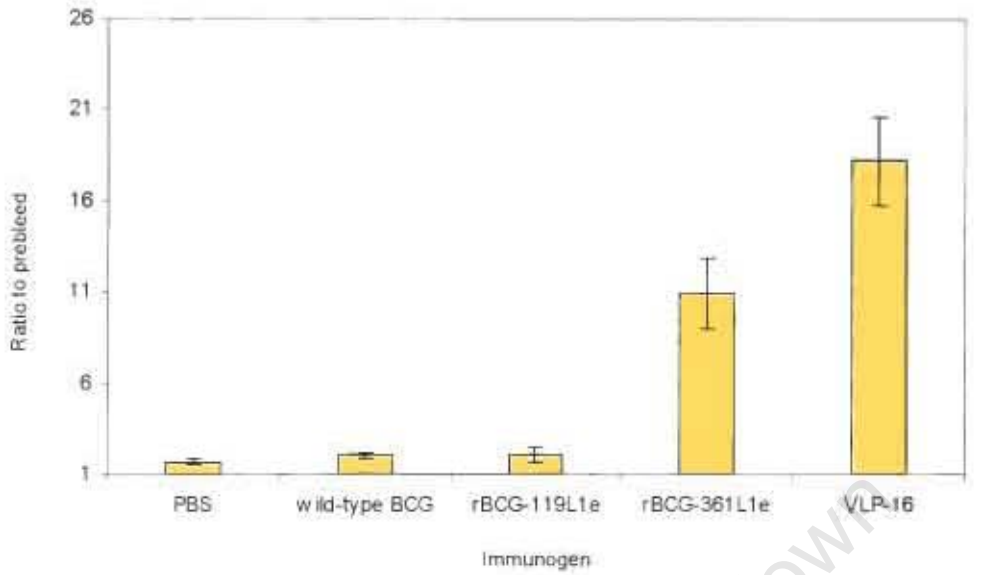


Figure 6.5. Systemic antibody responses specific for HPV-16 L1 VLPs induced in mice following immunisation with Pasteur rBCG-L1 (rBCG-361L1$_e$, or rBCG-119L1$_e$). Mice were immunised 3 times at, 4 week intervals. Blood samples were collected 2 weeks following the 3rd immunisation. The data are represented as the group mean OD_{492nm} ratios to the group mean prebleed value ± SEM.

6.4.4. PROTECTION FROM rVV-L1$_R$ CHALLENGE AFTER IMMUNISATION WITH PASTEUR rBCG-L1

BALB/c mice were immunised 3 times with rBCG-L1 (rBCG-361L1$_e$, or rBCG-119L1$_e$), and then challenged with rVV-L1$_R$ or rVV-GAG. Five days after the challenge, each mouse was assessed for vaccinia viral load. For each group, the average amount of vaccinia virus per mouse was calculated.

In order to investigate non-specific protection of vaccinia virus, mice were challenged with a recombinant vaccinia virus expressing an irrelevant antigen (HIV GAG) (Table 6.2, Figure 6.6). Mice immunised with VLPs were not protected from rVV-GAG challenge. Mice immunised with BCG (wild-type or rBCG-L1) showed non-specific protection, which ranged from 1.25-0.91 log_{10}. Most importantly is that the rVV-GAG protection levels observed in the wild-type- and rBCG-L1-immunised animals were similar (P>0.18, that the protection was dissimilar).

L1-specific protection was investigated by challenge with rVV-L1$_R$ (Table 6.3, Figure 6.7). Mice that were immunised with VLPs were afforded L1-specific protection of 2.94 log_{10} rVV-

$L1_R$ when compared with the PBS-vaccinated animals. Wild-type-immunised mice showed a non-specific protection of 0.7 log_{10}. Mice vaccinated with rBCG-361$L1_e$ or rBCG-119$L1_e$ showed L1-specific protection of 1.95 log_{10} and 1.81 log_{10}, respectively. The protection afforded by the rBCG-119$L1_e$ and rBCG-361$L1_e$ vaccines were not significantly different to each other (P=0.58).

Table 6.2. Protection form rVV-GAG challenge, in mice immunised with Pasteur rBCG-L1 (rBCG-361$L1_e$, or rBCG-119$L1_e$). Protection afforded by vaccination is represented by the log_{10} reduction in viral titre compared to the control immunogens (PBS or wild-type BCG).

Immunogen	rVV-GAG pfu / mouse	Log_{10} pfu / mouse	Log_{10} reduction compared with PBS group	Log_{10} reduction compared with wild-type BCG group
PBS	3.80×10^5	5.58	–	– 1.25 (P = 0.0023)
VLP-16	5.50×10^5	5.74	– 0.16 (P = 0.418)	– 1.41 (P = 0.0007)
BCG wild-type (Pasteur)	2.14×10^4	4.33	1.25 (P = 0.0023)	–
rBCG-119$L1_e$ (Pasteur)	3.63×10^4	4.56	1.02 (P = 0.0023)	– 0.23 (P = 0.34)
rBCG-361$L1_e$ (Pasteur)	4.68×10^4	4.67	0.91 (P = 0.007)	– 0.34 (P = 0.18)

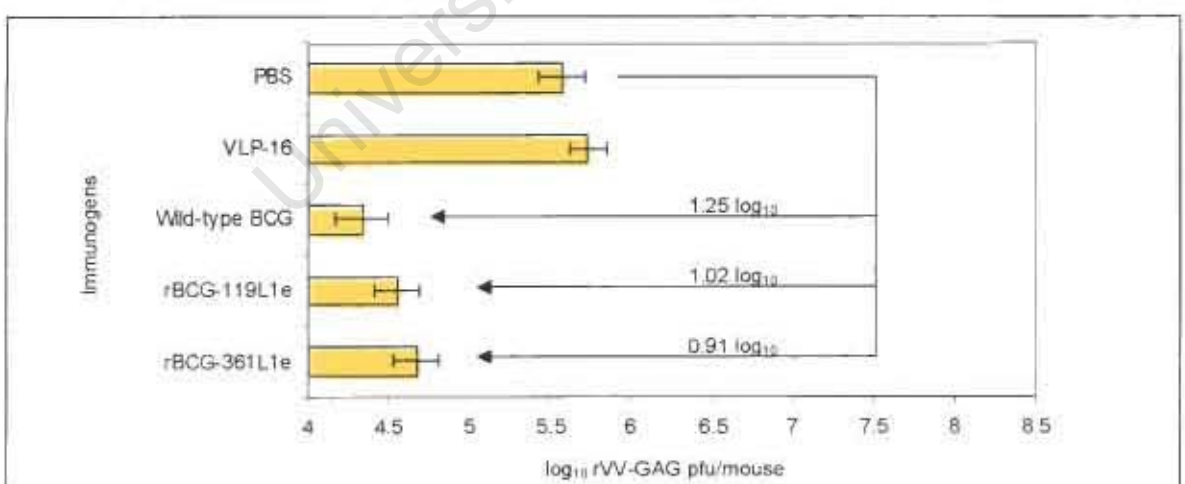


Figure 6.6. Protection from rVV-GAG challenge in mice immunised with Pasteur rBCG-L1 (rBCG-361$L1_e$, or rBCG-119$L1_e$). Ovarian titres of rVV-GAG were measured 5 days after the challenge and are expressed as average log_{10} rVV-GAG pfu/mouse ± SEM. The log_{10} reductions in VV titres are illustrated for selected groups.

Table 6.3. Protection from rVV-L1$_R$ challenge, in mice immunised with Pasteur rBCG-L1 (rBCG-361L1$_e$, or rBCG-119L1$_e$). Protection afforded by vaccination is represented by the log_{10} reduction in viral titre compared with the control immunogens (PBS or wild-type BCG). No mice were afforded 100% protection.

Immunogen	rVV-L1$_R$ pfu / mouse	Log_{10} pfu / mouse	Log_{10} reduction compared with the PBS group	Log_{10} reduction compared with the wild- type BCG group
PBS	1.07×10^8	8.03	–	– 0.7 ($P = 0.06$)
VLP-16	1.23×10^5	5.09	2.94 ($P = 0.000032$)	2.24 ($P = 0.00034$)
BCG wild-type (Pasteur)	2.14×10^7	7.33	0.7 ($P = 0.06$)	–
rBCG-119L1$_e$ (Pasteur)	3.31×10^5	5.52	2.51 ($P = 0.00055$)	1.81 ($P = 0.0048$)
rBCG-361L1$_e$ (Pasteur)	2.40×10^5	5.38	2.65 ($P = 0.00022$)	1.95 ($P = 0.0013$)

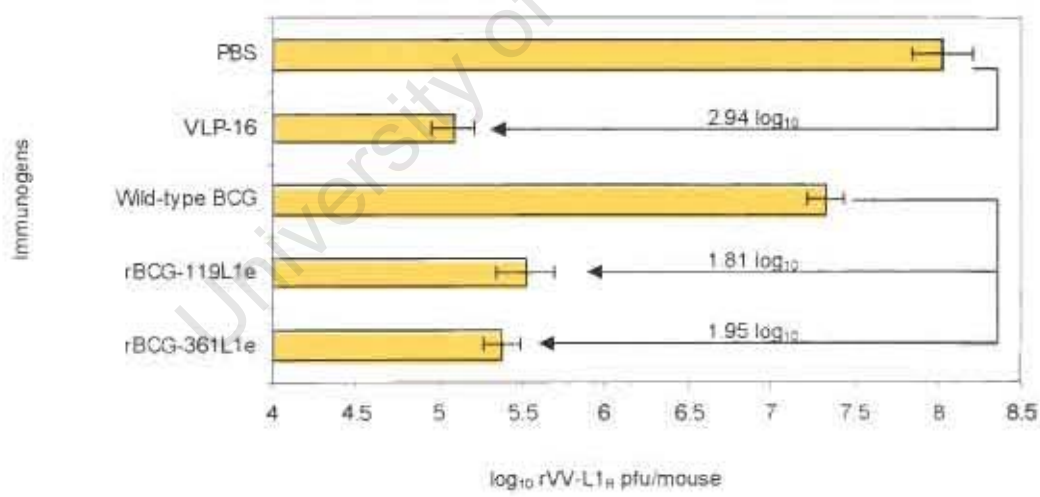


Figure 6.7. Protection from rVV-L1$_R$ challenge, in mice immunised with Pasteur rBCG-L1 (rBCG-361L1$_e$, or rBCG-119L1$_e$). Ovarian titres of rVV-L1$_R$ were measured 5 days after the challenge and are expressed as average log_{10} rVV-L1$_R$ pfu/mouse ± SEM. The log_{10} reductions in VV titres are illustrated for selected groups.

6.5. DISCUSSION

This chapter focused on the ability of recombinant BCG, expressing HPV-16 L1, to protect mice against challenge with recombinant vaccinia virus-HPV-16 L1 (rVV-$L1_R$). The use of mucosal and cutaneous animal PV models (Campo *et al.*, 1993; Suzich *et al.*, 1995b), and the development of *in vitro* pseudovirions systems (Pastrana *et al.*, 2004) have somewhat overcome difficulties experienced with the propagation of HPV in culture and the lack of animal models for HPV. The rVV-$L1_R$ surrogate challenge model was developed as another tool for testing HPV vaccines (Marais *et al.*, 1999). Since heterologous gene products are not expressed on the rVV surface, neutralising antibody immune mechanisms do not play a role in protection; instead, rVV challenge allows for the evaluation of cell-mediated responses by allowing heterologous proteins to enter the MHC class 1 pathway. It is therefore accepted that $CD8^+$ CTL responses are responsible for protection against challenge with rVV (Uno-Furuta *et al.* 2003; Moss, 1996; Marais *et al.*, 1999; Binder and Kündig, 1991). The antibody responses generated to the rBCG-L1 recombinants were assessed, and the antibody levels were compared to the corresponding levels of rVV-$L1_R$ protection. Finally, the cell-mediated responses generated by rBCG-L1 were investigated by means of a cytotoxicity assay and by the measurement of $CD8^+$ T cell IFN-γ production.

6.5.1. PROTECTION FROM rVV-$L1_R$ CHALLENGE AFTER IMMUNISATION WITH TOKYO rBCG-L1

In the first challenge experiment, BALB/c mice were immunised 3 times with Tokyo rBCG-L1 or HPV-16 VLPs, after which they were challenged with rVV-$L1_R$. The VLP-immunised mice were afforded protection of 1.42 $\log_{10}$, this was regarded as a good level of protection because VLP-immunisation was seen as the "gold standard" to which other immunogens would be compared. When calculated as % of virus cleared, the VLP-immunised mice cleared 96.2% of the rVV-$L1_R$. Although animals immunised with the rBCG-NV$L1_R$ construct were afforded a low level of rVV-$L1_R$ protection, immunisation with the rBCG-361L1 constructs (rBCG-361$L1_R$ and rBCG-361$L1_{NLS}$-) afforded high levels of protection, with rBCG-361$L1_{NLS}$- achieving similar protective levels to VLPs (96.0% protection). In Chapter 5, rBCG-L1 expressing NLS-deficient L1 elicited higher antibody responses than full-length L1; here the removal of the NLS enhanced the cell-mediated immunity to L1, as rBCG-361$L1_{NLS}$- induced greater protection than rBCG-361$L1_R$. It is still unclear whether this

enhanced immune response is a result of superior L1 presentation in the BCG-infected cells, or due to increased expression of the truncated protein. The challenge results can not be compared directly with those obtained in the Marais *et al.* (1999) study (4.6 $\log_{10}$ rVV-$L1_R$ protection in BALB/c mice that were immunised once, i.p., with 10μg of HPV-16 VLP), as the dose, route of inoculation and rVV-$L1_R$ stock were dissimilar.

It is interesting to note that the absence of an anti-L1 antibody response to rBCG-L1 did not indicate the failure of rBCG-L1 to elicit anti-L1 immunity, as anti-L1 cellular immunity (rVV-$L1_R$ protection) could occur in the absence of anti-L1 antibodies. Although the rBCG-NVL1_R construct showed the lowest protective ability, it induced the highest antibody levels (possibly a response skewed to Th2), while the rBCG-361L1 constructs, which showed good protective ability, failed to induce antibodies to L1 (response skewed to Th1). This again confirms that rVV-$L1_R$ protection in this model is mediated by cellular rather than antibody responses. It is unclear why the responses generated by the rBCG-361L1 and rBCG-NVL1_R constructs were along different arms of the immune response, since the doses were equivalent, the vectors are both integration proficient, and both express L1 cytoplasmically with the *hsp60* promoter. These vectors do, however, have different integration sites, which may influence L1 transcription.

6.5.2. CTL RESPONSES AND $CD8^+$ T LYMPHOCYTE IFN-γ PRODUCTION IN MICE IMMUNISED WITH PASTEUR rBCG-L1

The knowledge that, of all the BCG substrains, BCG Tokyo induces the weakest immune responses in mice (Lagranderie *et al.*,1996), prompted us to investigate rVV-$L1_R$ protection after immunisation with Pasteur BCG-L1 recombinants. Firstly, the immunity induced by Pasteur rBCG-L1 was assessed by a peptide-specific cytotoxicity assay and by the investigation of IFN-γ production by $CD8^+$ T cells. A single inoculation with rBCG-119L1_e, induced a strong cellular immune response to the HIV CTL tag, that achieved specific lysis of 36% in a cytotoxic assay, and $CD8^+$ T cell IFN-γ production that was almost 5-fold above background. This experiment reassured us that our BCG recombinants could correctly deliver CTL epitopes for association with MHC class I molecules and presentation to CD8+ T cells. Although BCG is known to predominantly activate the $CD4^+$ T Th1-type immune response, the secretion of IFN-γ and IL-2 by Th1 cells (Esser *et al.*, 2003; Mutis *et al.*, 1993), is thought to mediate CTL and DTH activity (Ada, 1999; Uno-Furuta *et al.*, 2003).

6.5.3. PROTECTION FROM rVV-L1$_R$ CHALLENGE AFTER IMMUNISATION WITH PASTEUR rBCG-L1

For the second rVV-L1$_R$ challenge experiment, BALB/c mice were immunised 3 times with Pasteur rBCG-L1 or HPV-16 VLPs, after which they were challenged with rVV-L1$_R$. Unlike in the BCG Tokyo challenge experiment, wild-type BCG Pasteur showed a non-specific protection of 0.7 $\log_{10}$. After taking this into account, the L1-specific protection afforded by rBCG-119L1$_e$ and rBCG-361L1$_e$ was 1.81 $\log_{10}$ and 1.95 $\log_{10}$, respectively (98.5% and 98.89% protection, respectively). VLP immunisation effectively protected from rVV-L1$_R$ challenge, and cleared 99.89% of challenge virus. Unfortunately one can not compare protection levels induced by Tokyo and Pasteur rBCG-L1, as these experiments were performed at different times, and challenge virus and VLP inoculates may not have been identical. Superior immune responses were, however, expected from the BCG Pasteur recombinants, as the Tokyo substrain has an inferior ability to persist and generate immune responses in mice (Lagranderie *et al.*, 1996); and this was apparent in these experiments with regard to both humoral and cell-mediated immune responses.

The above rVV-L1$_R$ challenge experiment was accompanied by a rVV-GAG challenge, to test for the non-specific clearance of vaccinia virus. VLP-immunised mice did not clear rVV-GAG, however, mice immunised with BCG (whether wild-type or rBCG-L1) showed non-specific protection from rVV-GAG challenge. Most importantly, the observation that the levels of rVV-GAG protection were similar in animals immunised with wild-type BCG and rBCG-L1, indicated rBCG-L1 elicited L1 protective immunity during the rVV-L1$_R$ challenge. The level of non-specific rVV-GAG protection was slightly greater than that afforded by the wild-type BCG-immunisation of the rVV-L1$_R$ challenge experiment; however, these values are not directly comparable as the total amount of rVV-L1$_R$ and rVV-GAG obtained from the PBS-immunised mice was significantly different, and this will have had an affect on the protection dynamics.

It was interesting that rBCG-361L1$_e$ elicited antibodies to VLPs, but rBCG-119L1$_e$ failed to do so, since of both these recombinants achieved similar levels of rVV-L1$_R$ protection. Analysis of the initial rVV-L1$_R$ challenge experiment revealed that immunisation with a particular Tokyo BCG-L1 recombinant either elicited an antibody response, or afforded rVV-L1$_R$ protection, but not both; here this is the same for Pasteur rBCG-119L1$_e$, which failed to elicit antibodies, but did protect from rVV-L1$_R$ challenge. In contrast, the Pasteur rBCG-361L1$_e$ recombinant effectively induced both humoral and cellular immunity, which can perhaps be

attributed to its integrative nature, allowing sustained antigen production from increased genetic stability.

CHAPTER 7: SUMMARY AND GENERAL CONCLUSIONS

7.1. HPV VACCINE CONSIDERATIONS AND POTENTIAL USE OF RECOMBINANT BCG

Cervical cancer is the second most prevalent cancer among woman worldwide and the most common cancer among woman in developing countries. South African women are at a particularly high risk of developing cervical cancer, with an ASR of 30.5 per 100 000 (Sitas *et al.*, 1998). There is naturally considerable interest in the development of vaccines for HPV, especially for use in developing countries, where the disease burden is high and resources for widespread screening and follow-up procedures are limited. Recently, phase 2 trials utilising VLPs have produced encouraging results, achieving protection against HPV infection and reducing the development of HPV-related CIN (Koutsky *et al.*, 2002; Harper *et al.*, 2004).

It is generally accepted that a prophylactic vaccine for HPV would aim to provide neutralising antibody protection at the genital mucosa. Several studies have demonstrated that when systemic neutralising antibodies to L1 are present, they provide protection from mucosal PV challenge, as observed in experimental animal models (Kirnbauer *et al.*, 1996; Suzich *et al.*, 1995) and in natural infection in women (Ho *et al.*, 2002). Prophylactic PV vaccines focus on the delivery of L1 VLPs. In animal and human studies, VLP vaccines have been well tolerated and have induced high titres of neutralising antibodies (Brown *et al.*, 2001; Christensen *et al.*, 1994; Harro *et al.*, 2001; Rose *et al.*, 1994b; Tobery *et al.*, 2003, Evans *et al.*, 2001b). Studies in the cow, rabbit and dog, have also shown that immunisation with VLPs can protect against challenge with homologous virus (Breitburd *et al.*, 1995; Christensen *et al.*, 1996b; Kirnbauer *et al.*, 1996; Suzich *et al.*, 1995). In order to elicit a neutralising antibody response it is required that L1 is in the form of capsomeres or intact

VLPs, as the presentation conformational epitopes is essential. Studies using disrupted or denatured VLPs have induced low neutralising antibody responses, and have afforded little protection from challenge in animal models (Breitburd *et al.*, 1995; Suzich *et al.*, 1995; Kirnbauer *et al.*, 1992)

Although much emphasis is placed on the development of neutralising antibodies in the genital tract, these antibody responses are usually not long-lasting, and sterilising immunity rarely occurs (McGhee *et al.*, 1992). Local cell-mediated responses will therefore be vital for elimination of infected cells. Several findings substantiate the role of the cell-mediated response in the clearance of established HPV infection, these include, the detection of mucosal IgA and IgG antibodies in women with persistent infection (Sasagawa *et al.*, 2003; Bontkes *et al.*, 1999b), the observation that individuals with cell-mediated immune deficiencies have an increased risk of infection with HPV (Koutsky, 1997), and the finding that regressing HPV-tumours are infiltrated with macrophages and T lymphocytes (Coleman *et al.*, 1994; Heller *et al.*, 2003). Although therapeutic studies focus on the early proteins of PV, VLPs can induce CTL responses that are capable of inducing regression of L1-expressing tumours in mice (Ohlschlager *et al.*, 2003), BPV lesions in cows (Kirnbauer *et al.*, 1996); and genital warts in humans (Zhang *et al.*, 2000), which suggests that L1 could be used as a therapeutic vaccine for genital HPVs. In the above study by Zhang *et al.* (2000), regression of genital warts was observed in 76% of HPV-6b VLP-immunised subjects, in contrast to regression of 0-29% observed in non-immunised control groups. Although L1 expression is generally thought to be limited to the superficial layers of the epidermis, Zhang *et al.* (2000) attributed the observed wart regression to L1-specific T-cell mediated lysis of HPV infected basal keratinocytes, in which L1 was being expressed, but at levels too low to be detected.

An ideal HPV vaccine would be affordable, would protect from the most prevalent high-risk HPV types and/or clear the lesions caused by them, and would have a low incidence of side-effects. VLPs can meet many of these criteria, but their production expense make them unlikely candidates for large-scale use in developing countries. The cost of vaccinating an individual with HPV VLP, in a 3 injection protocol with a booster required every 10 years, has been estimated at US$ 100 per injection (Sanders and Taira, 2003). Because affordability is a key factor for vaccine production in South Africa, this study investigated the use of live recombinant BCG, as a HPV vaccine vector. A number of factors contribute to the cost-effectiveness of recombinant BCG vaccines: complicated purification procedures are not required, its potent adjuvant properties prevent the need for additional adjuvants, an

extensive cold chain for maintenance of efficacy is not required, and distribution networks already exist. The main HPV vaccination target population would be adolescent girls, however, it may be advantageous to deliver HPV vaccines at birth, with a possible booster dose just before the sexually active stage, due to the risk of acquiring HPV at birth or early in life (Cason *et al.*, 1995). BCG would be perfectly suited to a vaccination schedule such as this, as it is routinely administered at birth, and is unaffected by maternal antibodies (Hanson *et al.*, 1995).

Numerous rBCG studies have established its ability to elicit neutralising antibodies to viral proteins (Bastos *et al.*, 2002; Fennelly *et al.*, 1995; Hiroi *et al.*, 2001; Chujoh *et al.*, 2001), suggesting that rBCG-HPV L1 could possibly induce neutralising antibodies to L1. Nardelli-Haefliger *et al.* (1997) demonstrated that HPV-16 L1 expressed in recombinant attenuated *S. typhimurium* assembled into VLPs, suggesting the possibility that VLP or capsomere assembly could also take place in other bacterial systems, such as BCG, and thus display the necessary conformational epitopes for the induction of neutralising antibodies. rBCG expressing cotton tail rabbit papillomavirus (CRPV) L1 has been shown to partially protect rabbits from CRPV challenge (Govan *et al.*, 2004), indicating that rBCG has potential as a vector for a prophylactic HPV vaccine. Mucosal administration of BCG effectively induces sustained specific mucosal immunity (Lagranderie *et al.*, 1998; Langermann *et al.*, 1994b; Biet *et al.*, 2003), which will be important for the control of mucosal HPV infections.

If a HPV vaccine fails to provide sterilising immunity, a cell-mediated response will necessary for the elimination of infected cells. An rBCG-L1 vaccine should be able to provide the appropriate cell-mediated response. Mycobacterial antigens are predominantly processed by MHC class II molecules, and thus BCG is a potent activator of a $CD4^+$ Th1-type cellular immune response (Esser *et al.*, 2003; Mutis *et al.*, 1993); however, numerous BCG recombinants have also elicited strong $CD8^+$ CTL responses to heterologous antigens (Mederle *et al.*, 2003; Honda *et al.*, 1995; Uno-Furuta *et al.*, 2003; Lim *et al.*, 1997).

The following sections summarise and discuss the development and pre-clinical investigation of candidate rBCG-HPV-16 L1 vaccines. This chapter will conclude by discussing whether these vaccines fulfilled the requirements of a HPV vaccine, and the possible future rBCG-HPV-16 L1 vaccines.

7.2. SUMMARY AND DISCUSSION OF KEY RESULTS

7.2.1. Construction and stability of rBCG-L1

Three HPV-16 L1 gene variants, namely $L1_R$, $L1_{NLS}$-, and $L1_{BCG}$, were acquired or constructed for evaluation in BCG. Because the formation of VLPs or capsomeres is necessary for the presentation of conformational epitopes, it was necessary to express the full-length L1 gene, or short C-terminal truncations thereof. $L1_R$ was supplied by Robert Rose (University of Rochester, Rochester, New York, USA), and was utilised for the majority of the rBCG-L1 constructs. $L1_{NLS}$- was formed by a 66 base-pair (22 amino acids) truncation at the 3' terminal of $L1_R$, in order to remove the L1 nuclear localisation signal. The NLS was removed to investigate whether the cellular localisation of L1 in rBCG-L1 infected cells would have an effect on L1 antigen presentation, and hence on the immune responses elicited by rBCG-L1. $L1_{NLS}$- is expected to be proficient at VLP assembly (Chen *et al.*, 2001; Paintsil *et al.*, 1996). The $L1_{BCG}$ sequence was generated by employing commonly used BCG codons. The $L1_{BCG}$ amino acid sequence was based on the Phil sequence, described by Touze *et al.* (1998) as high VLP-yielding in the baculovirus system. Phil differs from $L1_R$ by 8 amino acids. $L1_R$, $L1_{NLS}$-, and $L1_{BCG}$ were cloned into a panel of *E. coli* - mycobacterial shuttle vectors, producing 20 HPV-16 L1 expression vectors for assessment in BCG.

The introduction the L1-expression vectors into BCG often generated unstable recombinants, which had excisions of the L1 gene. As a consequence of this genetic instability, only 12 of the 20 clones were introduced into BCG in their intact form. The 8 unstable plasmids had two common features: they were all extrachromosomal in nature, and they all drove L1 expression with the *hsp60* promoter. Vector instability in BCG is not unique to this study, and is frequently detected in vectors utilising the *hsp60* promoter to drive heterologous gene expression (Al Zarouni and Dale, 2002; Medeiros *et al.*, 2002; Stover *et al.*, 1991). The integration-proficient plasmids which utilised *hsp60* to drive L1 expression were stably maintained, suggesting that chromosomes are less prone to gene deletions, or that the presence of only one integrated L1 gene copy per bacterium reduced the expression of L1 sufficiently to increase BCG stability. The other extrachromosomal constructs, p112-L1* and p119-L1*, were more stable than the *hsp60*-driven extrachromosomal vectors, as recombinants were obtained with all these constructs. The higher *in vitro* stability of the p112-L1* and p119-L1* constructs was presumably due to the weak *in vitro* activity of the *M. leprae 18kDa* and *mtrA* promoters (Dellagostin *et al.*, 1995; Zahrt and Deretic, 2000). Restriction enzyme analysis and sequencing of the unstable expression plasmids revealed

that the gene deletions present in these constructs ranged from deletions spanning the entire L1 gene and promoter region, to smaller deletions within the L1 gene. These gene excisions were never found to occur at exactly the same sites, indicating that there were no sequence-specific areas that were prone to recombination. Stable constructs were sequenced as point mutations or small deletions would not have been detected by restriction enzyme digestion or PCR. Surprisingly, no small gene deletions or significant point mutations were detected, since large genetic alterations occurred frequently.

It is interesting to note that although I assumed that the observed plasmid instability was a consequence of overproduction of L1, the level L1 protein production by rBCG was typically too low to be detected (<0.3% of the total rBCG protein). The evidence of L1 RNA expression from this study, and the effective use of the same vectors and/or promoters to achieve high foreign antigen expression in other studies (Himmelrich *et al.*, 2000b; Stover *et al.*, 1993), makes it unlikely that the low expression of the L1 protein is a consequence of low L1 transcription. The low expression is also not likely due to inefficient translation, as codon optimisation did not increase L1 production to detectable levels. The observation that the expression of bacterial antigens can account for over 10% of total BCG protein (*lysA* gene product, this study; Fuerst *et al.*, 1992; Stover *et al.*, 1993; Langermann *et al.*, 1994), but that expression viral antigens rarely exceeds 1% of BCG protein (Aldovini and Young, 1991; Winter *et al.*, 1991; Mederle *et al.*, 2002), suggests that the incorrect folding a viral antigens in this bacterial system facilitates their degradation by proteases. This assumption is strengthened by the high rate of instability observed when utilising the *hsp60* promoter. Because *hsp60* is a heat-shock promoter (Bukau and Horwich, 1998), it will be up-regulated in the presence of non-native proteins (in this case L1 protein), which will cause further production of these non-native protein, causing overt stress on the BCG recombinant.

Two categories of rBCG stability are of importance, the first being the stability in the presence of antibiotic selection, which is essential for the long-term maintenance and large-scale production of recombinant BCG, the second being the retention of the expression vector in the absence of antibiotic selection, which provides an indication of the length of *in vivo* foreign antigen expression. BCG-L1 recombinants containing integration-proficient vectors were more stable than the extrachromosomal recombinants, providing an advantage over the extrachromosomal constructs for long-term maintenance and sustained *in vivo* expression. Enhanced stability of integrated BCG recombinants has also been observed in other studies (Mederle *et al.*, 2002; Stover *et al.*, 1991; Kumar *et al.*, 1998).

Extrachromosomal recombinants may have the advantage of a higher copy number with the potential of higher protein expression, but their stability seems to be a limitation.

Although stability will be of concern for all BCG recombinants, limited literature is available on methods for increasing plasmid stability in BCG. Baud *et al.* (2002) found that codon optimisation of the HPV-16 L1 gene, for expression in *Salmonella*, increased plasmid stability *in vivo*; in the present study, however, codon optimisation for expression in BCG may have decreased plasmid stability, due to a possible increase of L1 expression levels. Auxotrophic mutants could be exploited to enhance plasmid maintenance in the absence of antibiotic selection (Cirillo *et al.*, 1995), however this does not diminish the risk of foreign gene deletion events. The use of inducible promoters, like that of *M. smegmatis* acetamidase (Roberts *et al.*, 2003; Parish *et al.*, 1997), seem promising as these may help with respect to the prevention of gene deletions *in vitro*; however, for these promoters to be effective their *in vitro* activity will have to be negligible, with strongly inducible activity *in vivo*, since even low expression may cause instability.

7.2.2. Immunogenicity of rBCG-L1 in small animals

The immune responses elicited by the rBCG-L1 constructs produced during this study were assessed in guinea pigs and mice. The majority of the animal studies were performed with guinea pigs, as they share a number of similarities with humans in their response to mycobacterial infection (Horwitz *et al.*, 2000; McMurray, 1994). Although these studies assessed both humoral and cell-mediated immunity, the focus was on the ability of rBCG-L1 to generate antibodies specific to HPV-16 L1, since the aim of this research was to investigate the possibility of utilising rBCG-L1 as a prophylactic vaccine for HPV. The mouse studies focused on the *in vivo* CTL responses elicited to rBCG-L1, by measuring the level of protection in a rVV-$L1_R$ challenge model. In addition to the rVV-$L1_R$ challenge, humoral responses were assessed, and cell-mediated responses were investigated further by means of a cytotoxicity assay and by the measurement of IFN-γ production by $CD8^+$ T cells. A summary of all the immune responses induced by rBCG-L1 are tabulated in Appendix F.

The combined results from the guinea pig and mouse studies demonstrated that immunisation with rBCG-L1 successfully elicited VLP-specific antibody and cell-mediated immune responses that were dependent on expression vector, dose, route of immunisation and L1 variant. These results therefore also established that, although L1 expression could not be detected by western blot, all the rBCG-L1 constructs were expressing low amounts of

L1. The low expression of L1 necessitated the employment of a vaccination regimen consisting of more than 3 rBCG-L1 immunisations for the induction of a strong antibody response. On the other hand, a single inoculum of purified HPV-16 L1 VLP elicited high levels of antibodies to L1. In guinea pigs the rBCG-361L1$_e$ and rBCG-119L1$_e$ constructs induced the highest serum antibody levels, while the rBCG constructs utilising the pCB112 expression vector consistently elicited the weakest antibody responses; which is possibly a consequence of its weaker promoter (*18kDa*). rBCG-L1 dosage and route of inoculation both played important roles in the induction of immune responses. This was evident when the low dose (10^4 cfu) failed to elicit significant antibody or cellular responses to L1, but the same recombinants at a higher dose ($2x10^6$ cfu) delivered sufficient L1 for the induction of a response. Intramuscular immunisation was not as efficient as the i.d. route at eliciting antibody or cell-mediated immunity to L1. When administered at the $2x10^6$ cfu dose, all rBCG-L1 constructs successfully induced L1-specific DTH reactions, which is evidence of BCG's potent cellular immunity adjuvant properties.

The L1 variants played a small role with respect to immune responses elicited to them, and/or with respect to the L1 expression levels within BCG. Codon optimisation of L1 for expression in BCG quite likely increased L1 expression levels over native L1 levels, as rBCG-112L1$_{BCGe}$ elicited higher levels of antibodies than rBCG-112L1$_e$. However, in the case of rBCG-119L1$_e$ (native L1), its codon optimised equivalent, rBCG-119L1$_{BCGe}$, failed to induce higher antibody levels, illustrating that numerous factors contribute to the accumulation of L1 in BCG. The observation that the anti-L1 antibody levels elicited by rBCG-361L1$_e$ and rBCG-119L1$_e$ were of similar magnitude, and that rBCG-119L1$_{BCGe}$ did not elicit significantly more antibodies than rBCG-119L1$_e$, suggests an upper threshold level of L1 expression in BCG. This putative threshold is likely facilitated by protease degradation and mediated by chaperones. The removal of the L1 nuclear localisation signal appeared to enhance the immune response over full-length L1; this was apparent by the enhanced antibody response induced to L1 in guinea pigs by rBCG-112L1$_{NLS}$-, and by the superior protective ability of rBCG-361L1$_{NLS}$- in the rVV-L1 mouse challenge model. It is uncertain whether these enhanced immune responses are as a result of improved L1 presentation in the BCG-infected cells, or increased expression of the truncated protein.

In view of the fact that the chief objective of a prophylactic HPV vaccine is the generation of immune responses at the genital mucosa, the detection of L1-specific antibodies in the vaginal secretions of a number of rBCG-L1-immunised animals, especially in those immunised with rBCG-361L1$_e$, rBCG-119L1$_e$ and rBCG-119L1$_{BCGe}$, was an important result.

These vaginal IgG antibodies were thought to have originated by transudation from the serum. It would have been interesting to investigate mucosal routes of delivery, such as oral or nasal, as these routes are expected to generate stronger responses at the genital mucosa.

The question of whether prior vaccination with wild-type BCG could adversely affect the immunity induced to recombinant BCG vaccines is not an unwarranted one, and needed to be investigated. The present study eased these concerns, because prior immune exposure to wild-type BCG increased the immune responses elicited to rBCG-L1. Similar results were obtained by Gheorghiu *et al.* (1994), who investigated the immunity induced by rBCG-LacZ. I concur with this group's suggestion that the enhanced immune responses observed in BCG-primed mice were a result of the activation of BCG-specific T cells, leading to improved macrophage activation and enhanced antigen presentation of both the mycobacterial and recombinant proteins.

The observation that rBCG-L1 did not elicit detectable neutralising antibodies in guinea pigs was perhaps the result that will most limit the use of rBCG-L1 as a prophylactic HPV vaccine. This finding suggests that VLPs or capsomeres were not formed within BCG, and hence the conformational epitopes necessary for the induction of neutralising antibodies were not created. However, as mentioned above (section 7.1), too much emphasis may be placed on the development of neutralising antibodies in the genital tract, as these antibody responses are usually not long-lasting, and sterilising immunity rarely occurs (McGhee *et al.*, 1992). Consequently, local cell-mediated responses will be required for the elimination of infected cells. A rBCG-L1 vaccine should be able to provide the appropriate responses for the elimination of these infected cells as the cell-mediated adjuvant properties of BCG are well documented; with numerous recombinant BCG studies demonstrating strong $CD4^+$ and $CD8^+$ CTL responses to heterologous antigens (Mederle *et al.*, 2002; Murray *et al.*, 1992; Mederle *et al.*, 2003; Honda *et al.*, 1995; Uno-Furuta *et al.*, 2003; Lim *et al.*, 1997). Here I have demonstrated that immunisation of mice with rBCG-L1 elicited antigen-specific $CD8^+$ responses, and antigen-specific cytotoxicity. Furthermore, I showed that mice immunised with rBCG-L1 were afforded L1-specific protection of up to 98.89% from challenge with rVV-$L1_R$, confirming the induction of strong $CD8^+$ CTL responses.

7.3. POTENTIAL OF rBCG-L1 AS A HPV VACCINE

This study demonstrated the ability of recombinant BCG expressing HPV-16 L1 to elicit L1-specific antibody and cell-mediated responses in small animals. Although the cell-mediated immune responses were substantial, the antibody responses were considerably lower that those elicited by purified VLPs, and hence a strategy of multiple immunisations was required. The potential for these vaccine candidates to proceed to human testing is therefore low.

The main obstacle impeding the success of the rBCG-L1 vaccine candidates is poor L1 expression. If L1 expression could be improved, the need for multiple immunisations would be decreased. Higher L1 expression, and thus higher L1 protein monomer concentration, would increase the likelihood VLP formation in BCG, thereby also improving the possibility of neutralising antibody induction. If our premise is correct and the low L1 levels are a consequence of degradation, it will be difficult to increase expression levels. Co-expression of chaperones could possibly be employed to increase native L1 folding and thus decrease degradation.

The second obstacle is the high rate of high genetic instability, which is directly linked to heterologous protein expression. Integrated expression vectors provide enhanced vector stability, and gene stability can be increased with the use of inducible promoters. If the export of L1 from rBCG can be optimised, this could also lead to enhanced stability. The use of short epitopes may increase stability and possibly increase protein expression levels, by decreasing burden, but this would not be optimal for a prophylactic HPV vaccine where full-length L1 protein is needed for the formation of conformational epitopes. rBCG may therefore be better suited as a therapeutic HPV vaccine, where early HPV proteins or epitopes would be expressed, and where antibody responses are not as vital as cell-mediated immunity.

Finally, the cost-effectiveness of recombinant BCG vaccines, especially in a developing country setting, may make it viable to utilise these vaccines in combination with other types of HPV vaccines, where recombinant BCG could prime or boost immunity to HPV antigens.

APPENDIX A: GENERAL TECHNIQUES

A1. BACTERIAL CULTURE PREPARATION

A1.1. *E. coli*

A starter culture, of approximately 5ml of 2x yeast tryptone (YT) broth, was inoculated with a single colony of *E. coli* DHα bacteria, and was grown overnight at 37°C, with vigorous shaking. The following day, the bacterial culture was diluted 1/100 in 50-100ml of 2xYT broth in a flask 5-10 times the culture volume. The culture was incubated at 37°C, with vigorous shaking, until the appropriate culture optical density (at 600nm) was attained. The appropriate antibiotic was added to the medium to prevent the growth of non-recombinant bacteria, and hence select for recombinant bacteria. Kanamycin (Novo Nordisk, SA) was used at a final concentration of 50μg/ml; Ampicillin (Sigma-Aldrich, Germany) was added at a final concentration of 50μg/ml; and Hygromycin B (Roche Diagnostics, Germany) was used at a final concentration of 200μg/ml.

2xYT agar was used as solid medium. After the bacteria were spread on 2xYT agar, they were inverted and incubated overnight at 37°C.

A1.2. Mycobacteria

Mycobacteria were grown in Middlebrook 7H9 medium (Difco, Detroit, USA) or Sauton's liquid medium (Appendix B2). Clumping was reduced by the addition of Tyloxapol (Sigma). Starter cultures of 5-10ml were grown at 37°C as stationary cultures for 7-14 days. The starter culture was then used to inoculate 50-100ml of medium, which was grown at 37°C in rolling bottles for 7-14 days.

7H10 (Difco, Detroit, USA), supplemented with 10% albumin dextrose catalase (State Vaccine Institute, South Africa), was used as solid growth medium. The plates were inverted and incubated at 37°C for 3-5 weeks for BCG. Prior to plating, the bacterial suspension was vigorously passed through a 26 gauge needle, to reduce clumping. The plates were sealed in plastic bags to prevent them from drying out during the long incubation times.

When appropriate, the following antibiotics were utilised: Kanamycin (Novo Nordisk) was used at a final concentration of 16.7μg/ml; and Hygromycin B (Roche Diagnostics) was used at a final concentration of 50μg/ml.

A2. PREPARATION OF COMPETENT CELLS

A2.1. Preparation of competent *E. coli*

The *E coli* strain DH5α was used for manufacture of competent cells according to the method of Chung and Miller (1988). A single colony of bacteria was inoculated into 5ml of 2xYT broth and was grown overnight at 37°C with shaking. 0.5ml of this culture was used to inoculate 50ml of 2xYT, which

was grown to early log phase (OD_{600} 0.2-0.4), and harvested by centrifugation at 5 000rpm for 5min at 4°C (Beckman J2-21 centrifuge). The supernatant was discarded and the cell pellet was re-suspended in 1/10th the culture volume of ice cold TSB solution (Appendix B3). Glycerol was added to a final concentration of 10% (v/v), the cells were aliquoted and frozen at -80°C.

A2.2. Preparation of competent BCG

Competent mycobacteria were prepared for electrotransformation according to the procedure of Jacobs *et al.* (1991). 50-100ml of liquid medium were inoculated with BCG, as described in Appendix A1, and grown at 37°C until an OD_{600} between 0.4 and 0.7 was attained. Once the purity of the culture was verified by Ziehl-Neelson stain (Appendix A13), the cells were collected by centrifugation at 4000rpm for 10min at 25°C (JA-21 rotor, Beckman J2-21 centrifuge). The cells were washed by re-suspending the cell pellet in 40ml of distilled water and collecting as above. The washing step was repeated with water and then again with 10% (v/v) glycerol. The cells pellet was finally re-suspended in 3-6ml of 10% glycerol. Electrocompetent BCG preparations were kept at room temperature, and were used within 1hr of preparation.

A3. TRANSFORMATIONS

A3.1. Transformation of competent *E. coli*

Frozen aliquots of competent cells were allowed to thaw on ice. One to 100ng of DNA were mixed with 100µl of competent cells, and left on ice for 30min; after which, the cell/DNA mixture was placed at 42°C for 2min. After the addition of 900µl of 2xYT broth the cells were incubated at 37°C for 30min, to allow expression of the antibiotic resistance gene of the newly transferred plasmid. The transformed bacteria were plated on 2xYT agar containing the appropriate antibiotic.

A3.2. Electrotransformation of competent BCG

A 100µl volume of competent cells was mixed with 0.1-1µg of plasmid DNA, and incubated at 37°C for 5min in a 0.1cm electrogap cuvette (Bio-Rad Laboratories, Germany). It was important that neither the cells nor the DNA contained high concentrations of salts, which can result in arcing when a voltage is applied to the cuvette. The cuvette was placed in a GenePulser set at 1.8kV and 25µF, with the Pulse Controller (Bio-Rad Laboratories, Germany) set at 1000 Ohms. A single pulse was sent through the cuvette. 900µl of Middlebrook 7H9 or Sauton's medium were added to the cells, which were then incubated at 37°C for 4hr, to allow expression of the antibiotic resistance gene of the newly transferred plasmid. 100µl of the expression mixtures were spread onto Middlebrook agar 7H10 (Difco, Detroit, MI, USA) plates, supplemented with 10% albumin dextrose catalase, containing the appropriate antibiotic, and incubated at 37°C for 3-5 weeks. Single colonies were picked and grown to log phase in 10ml cultures. 5ml of each culture was used to analyse for successful transformation. Glycerol was

added to the remainder of the culture, at a final concentration of 10% (v/v), and the cells were frozen at -80°C.

A4. SELECTION AND VERIFICATION OF TRANSFORMANTS

A4.1. Verification of *E. coli* transformants

Transformation mixtures were spread onto plates (containing the appropriate antibiotics), and were incubated overnight. Single colonies were chosen, and small scale plasmid extractions were performed (Appendix A5.1). The DNA obtained was subjected to endonuclease digestion and gel electrophoresis (Appendices A6 and A7) to determine whether the cells contained the correct DNA.

A4.1. Verification of BCG transformants

Due to their thick, complex cell walls, DNA extraction from mycobacteria is not as efficient as from *E. coli*; it was therefore necessary to amplify mycobacterial DNA by transforming it into *E. coli*. This was done by first extracting plasmid DNA from mycobacterial transformants (Appendix A5.3), and then transforming these plasmids into *E. coli*. Small scale plasmid extractions were performed on the *E. coli* recombinants (3-5 *E. coli* recombinants tested per BCG colony) (Appendix A5.1), and the DNA obtained was subjected to endonuclease digestion and gel electrophoresis (Appendices A6 and A7). If it was determined that an *E. coli* recombinant contained the vector of interest, it was assumed that the corresponding mycobacterial transformant contained the same.

To verify mycobacterial transformants containing expression vectors that had integrated into the chromosome, total DNA was extracted (Appendix A5.4) and Southern blot-hybridisation analysis was performed (Appendix A10) to verify recombinants.

A5. PREPARATION OF DNA

A5.1. Small scale plasmid extraction from *E. coli*

This protocol was based on the method described by Ish-Horowicz and Burke (1981), employing an alkaline/sodium dodecyl sulphate (SDS) lysis method. A single colony of recombinant bacteria was picked and inoculated into 5ml of 2xYT broth containing the appropriate antibiotic and grown overnight at 37°C with shaking. Two millilitres of culture were transferred to a 2ml tube (Eppendorf), and the cells were harvested by centrifugation at 14 000rpm for 2min in a microfuge. The supernatant was discarded and solutions 1, 2 and 3 (Appendix B4) were added sequentially. The cells were first re-suspended in 0.2ml of solution 1, after which, 0.4ml of solution 2 was added to facilitate cells lysis and degrade chromosomal DNA. After 5min at room temperature, 0.3ml of solution 3 was added. This mixture was left on ice for 5min to neutralise the mixture and to precipitate the cellular protein and

membrane material. The flocculant was removed by centrifugation at 14 000 rpm for 15min in a micro-centrifuge, and the clear supernatant was transferred to a new 1.5ml tube. The plasmid DNA was precipitated by adding 0.6 volumes of isopropanol, and collected by centrifugation at 14 000 rpm for 10min. The supernatant was decanted and the DNA pellet was washed with 300µl 70% (v/v) ethanol. The ethanol was removed and the DNA was allowed to dry before dissolving it in an appropriate amount of sterile, distilled water.

A5.2. Large scale plasmid extraction from *E. coli*

For preparation of up to 100µg of plasmid, the Nucleobond® AX PC-Kit 100 (Machery-Nagel, Germany) was used according to the manufacturer's instructions. This method is also based on an alkaline/sodium dodecyl sulphate (SDS) lysis method, with further DNA purification in the AX100 column (a silica-based, anion-exchange column). Recombinant bacteria were grown in 50-100ml of 2xYT broth, overnight at 37°C with shaking. The bacterial cells were then harvested by centrifugation at 5 000rpm for 5min at 4°C (Beckmann J2-21 centrifuge, JA-20 rotor). The supernatant was discarded and the bacterial pellet was re-suspended in 4ml of buffer S1 (50mM Tris/HCl, 10mM EDTA, 100µg Rnase A/ml). The re-suspended bacteria were mixed with 4ml of buffer S2 (200mM NaOH, 1%SDS) and incubated at room temperature for 5min. 4ml of buffer S3 (2.8M KAc) were added to the suspension which was then incubated for 5min on ice. The cellular debris were separated by centrifugation at 12 000rpm for 30min at 4°C (Beckmann J2-21 centrifuge). The supernatant was loaded onto the AX 100 column and allowed to flow through. The column was then washed with 8ml of buffer N3 (100mM Tris/H_3PO_4, 15% ethanol, 1150mM KCl). The purified plasmid DNA was eluted from the column with 2ml of buffer N5 (100mM Tris/H_3PO_4, 15% ethanol, 1000mM KCl). The eluted plasmid DNA was precipitated with 0.7 volumes of isopropanol and collected by centrifugation at 14 000rpm in a microfuge for 10min. The pellet was washed with 70% ethanol and dissolved in an appropriate volume of distilled water.

A5.3. Plasmid extraction from BCG

10ml of Middlebrook 7H9 or Sauton's medium, containing the appropriate antibiotic, were inoculated with a single colony and then incubated at 37°C for 10-15 days, with occasional shaking. The Alkaline/SDS lysis method (described in Appendix A5.1) was utilised for the DNA extraction, except for a modification of the initial cell re-suspension step, during which lysozyme (final concentration of 10mg/ml; Roche Diagnostics, Germany) was added to the S1 buffer, and incubated overnight at 37°C, to aid cell lysis. The mycobacterial plasmid DNA was transformed into competent *E. coli* DHα cells (Appendix A3.1) and plated to form single colonies. Small scale plasmid extractions (Appendix A5.1) were performed on the *E. coli* colonies and the DNA was subjected to restriction enzyme analysis and agarose gel electrophoresis (Appendices A6 and A7) to verify the presence of the correct clone.

A5.4. Chromosomal DNA extraction from BCG

Chromosomal DNA was extracted from BCG by a method adapted from Ausubel *et al.* (1987). A BCG

colony was inoculated into 10ml Middlebrook 7H9 or Sauton's medium containing antibiotics, and incubated at 37°C with occasional shaking for 10-15 days. 4ml of cells were collected by centrifugation, and the cell pellet was re-suspended in 500µl of Tris-EDTA (TE) buffer. 50µl of lysozyme (10mg/ml) were added, and the mixture was incubated at 37°C for 1hr. 70µl of 10% sodium dodecyl sulphate (SDS) and 3µl of Proteinase K (20mg/ml) were added, and the mixture was incubated at 65°C for 10min. 100µl of 5M NaCl were added, and the solution was mixed well. The solution was mixed well with 80µl of CTAB/NaCl solution (Appendix B5), and was incubated at 65°C for 10min. CTAB precipitates the cell wall debris, denatured protein and polysaccharides; while nucleic acids stay in solution. To remove the CTAB-protein/polysaccharide complexes, an equal volume (800µl) of chloroform:isoamyl alcohol (24:1) was added, the solution was vortexed, and then separated at 14000rpm for 5min in a desktop centrifuge. The aqueous supernatant was transferred to a 1.5ml tube, 0.6 volumes of isopropanol were added and the sample was placed at -80°C for 30min. The DNA was then collected by centrifugation at 14 000rmp for 10min in a desktop centrifuge. The supernatant was discarded and the DNA pellet was washed with 300µl 70% (v/v) ethanol. The ethanol was removed and the DNA pellet re-suspended in 50µl of distilled water.

A5.5. Preparation of DNA for sequencing

The High Pure Plasmid Isolation Kit (Roche Diagnostics, Germany) was utilised as per the kit's instructions. Briefly, 2ml of an overnight DH5α *E. coli* culture (Appendix A1.1) was collected by centrifugation. The cells were resuspended in 250µl of the kit's Suspension Buffer (50mM Tris-HCl, 10mM EDTA, 100µl RNase A/ml), and then lysed by the addition of 250µl of Lysis Buffer (0.2M NaOH, 1% SDS). 350µl of Binding Buffer (4M guanidine hydrochloride, 0.5M potassium acetate) were added to precipitate the cell debris and chromosomal DNA. The precipitate was separated by centrifugation. The supernatant was loaded into a High Pure filter tube and was transferred through the filter column by centrifugation. The filter was washed with 700µl of Wash Buffer II (20mM NaCl, 2mM Tris-HCl, 80% ethanol) by a centrifugation step. 50µl of Elution Buffer (10mM Tris-HCl) were added to the filter tube, and the plasmid DNA was eluted into a microfuge tube by centrifugation.

A5.6. DNA purification from agarose gel

DNA was purified from agarose gel with the QiAquick gel extraction kit (Qiagen, Germany), according to manufacturer's instructions. After restriction enzyme digestion and agarose gel electrophoresis (Appendices A6 and A7) the relevant DNA fragments were excised from the agarose gel with a scalpel. The gel fragments were add to a microfuge tube and weighed. 3 volumes of the kit's Buffer QG (300µl per 100µg of gel) were added, and the gel was allowed to dissolve at 50°C for about 10min. The gel mixture was transferred through a MiniElute filter tube by centrifugation. After the filter was washed with the kit's wash buffer, the DNA was eluted with 50µl of water.

A6. RESTRICTION ENDONUCLEASE DIGESTION

Restriction endonucleases and incubation buffers were supplied by Roche Diagnostics (Germany). Unless otherwise specified, restriction endonuclease digestions were carried out according to the method described by Sambrook *et al.* (1989); in a 10-20µl volume, including between 0.5 and 10µg of DNA, 1-2µl 10X incubation buffer, and between 3 and 10 units of enzyme. Enzymatic digestion was stopped by the addition of 6X loading buffer. If enzyme buffers were compatible, multiple restriction enzymes were combined in a single digest, if their. If not, the digestion was done sequentially and the DNA was purified to remove the first buffer using the QiAquick gel extraction kit (Appendix A5.6).

A7. AGAROSE GEL ELECTROPHORESIS

Agarose gel electrophoresis was performed using the submerged horizontal gel slab method, as described by Sambrook *et al.* (1989). Agarose was prepared at concentrations ranging from 0.8 to 2% (w/v) in 1X Tris-acetate buffer. Ethidium bromide was added at a final concentration of 0.1µg/ml. DNA samples were mixed with 6X loading buffer, which were then added to the wells. Electrophoresis was performed at 60-100 volts for 1-3hrs, using a PS500X DC Power Supply (Hoefer Scientific Instruments, San Francisco, USA). Standard DNA size markers were electrophoresed alongside samples to determine the DNA fragment sizes. After electrophoresis, the DNA fragments were visualised on an ultraviolet (UV) light box (UVP Incorporated, USA). The gel was photographed with a Kodak DC120 digital camera, and analysed with Kodak Digital Science ID (version 2.0.3) software.

A8. CLONING

A8.1. Preparation of vector and Insert DNA

Vector and insert DNA were prepared by digestion with the appropriate restriction endonuclease/s thereby creating compatible termini for cloning. The digested DNA samples were electrophoresed, and the relevant fragments were excised and purified from the gel (Appendix A5.6).

A8.2. Ligations

Ligations were carried out in a volume of 10µl; containing vector and insert DNA (which were generally mixed together in a ratio of 1:3), 1µl of T4 DNA ligase (1U/µl, Roche Diagnostics), and 1µl of ligation buffer (Roche Diagnostics). The ligations were carried out overnight at 16°C, after which the ligation mixtures were used to transform competent *E. coli*, as described above (Appendix A3.1).

A8.3. Cloning of PCR products

pMOS-blue TA cloning kit (Amersham, Buckinghamshire, UK)

Ligation was performed as per manufacturers' instructions.

The ligation mixture contained:

1µl 10X DNA ligase buffer

0.5µl 100mM DTT

0.5µl 10mM ATP

1µl pMOS vector (50ng)

2-3µl PCR product

0.5µl T4 DNA ligase

The ligation mixture was made up to 10µl with water and then incubated at 16°C for 2hrs. 1µl was transformed into 100µl of competent DH5α cells.

pGEM-T Easy cloning kit (Promega, Madison, WI, USA)

Ligation was performed as per manufacturers' instructions.

The ligation mixture contained:

5µl 2X DNA ligase buffer

1µl pGEM-T Easy vector

2-3µl PCR product

1µl T4 DNA ligase

The volume was made up to 10µl with water. The ligation mix was incubated at 25°C for 1hr, or at 4°C for 16hrs; after which 5µl was transformed into competent *E. coli* DHα cells.

A8.4. Verification of clones

Transformants were selected as described in Appendix A4.1. The DNA obtained from the transformants was subjected to endonuclease digestion and gel electrophoresis to determine whether the desired recombination event had transpired. When the desired recombinant was found, a large-scale plasmid preparation was performed.

A9. POLYMERASE CHAIN REACTION

Primers were designed according to the guidelines described by Rolfs *et al.* (1992), and evaluated on Primer Designer for Windows Version 2.0 (Scientific and Educational Software, Pasadena, USA).

All reagents were supplied by Takara Bio (Japan) or Roche Molecular Biochemicals (Germany). Reaction volumes were typically 50µl and contained the following:

10x buffer	5ul
(10mM Tris-HCl, 1.5mM $MgCl_2$, 50mM KCl, pH 8.3)	
Taq DNA polymerase	1 unit (U)

$MgCl_2$ (25mM)	3-5µl
dNTPs	each at a final concentration of 200uM
Primers	10-50 pmol of each primer
Distilled water	to make the reaction volume to 49µl

1µl of template DNA or water (no DNA control) was added to each tube.

The amplification reactions were performed in a GeneAmp PCR System 9700 (Perkin Elmer, USA) thermocycler. The PCR reaction parameters were as follows:

- Initial Denaturation at 94°C for 3min
- Denaturation at 94°C for 45sec
- Primer annealing at 40-43°C for 30sec } 5 cycles
- DNA synthesis at 72°C for 2min
- Denaturation at 94°C for 45sec
- Primer annealing at 55-65°C for 30sec } 30 cycles
- DNA synthesis at 72°C for 2min
- Final DNA synthesis at 72°C for 4min

When the LightCycler thermocycler (Roche Molecular Biochemicals, Germany) was utilised, the reactions contained the following:

10x buffer (10mM Tris-HCl, 1.5mM $MgCl_2$, 50mM KCl, pH 8.3)	2ul
Taq DNA polymerase	1 unit (U)
$MgCl_2$ (25mM)	4µl
dNTPs (2.5mM)	1.6µl
Bovine serum albumin (20mg/ml)	2µl
SYBR Green I dye (1/1000 dilution, Roche)	1µl
Primers	5 pmol of each primer
Distilled water	to make the reaction volume to 18µl

2µl of template DNA

The PCR reaction parameters on the LightCycler thermocycler were as follows:

Initial Denaturation	95°C	30 sec	
Denaturation	95°C	0 sec	
Primer annealing	57°C	10 sec	30 cycles
DNA synthesis	72°C	10 sec	

Melting curve	40°C	5 sec
	95°C	0 sec (temperature transition of 0.20°C/sec)
	40°C	0 sec

A10. SOUTHERN TRANSFER OF DNA ONTO A MEMBRANE AND HYBRIDISATION WITH A LABELLED OLIGONUCLEOTIDE PROBE

BCG chromosomal DNA, containing integrated expression vector DNA, was digested and transferred onto a nylon membrane. The membrane was then probed with a labelled L1-specific oligonucleotide to verify the presence of the L1 gene. The method utilised was a modification of that described by Smith and Summers (1980).

A10.1. Southern transfer

DNA was isolated from BCG using the CTAB method (Appendix 5.4) and subjected to restriction endonuclease digestion and agarose gel electrophoresis. The agarose gel was soaked in 0.25M HCl for 10min to introduce nicks in the DNA by depurination, which assists in the transfer of large DNA fragments to the nitrocellulose membrane. The DNA was then denatured by soaking the gel in denaturing solution (Appendix B6) for 30min with gentle shaking (Red Rotor PR70 shaker, Hoefer Scientific Instruments, San Francisco, USA). After which, the gel was neutralised by soaking it in neutralising solution (Appendix B6) for 40min.

A capillary transfer apparatus was set up. The gel was placed upside down a glass support that was covered by a wick of Whatman 3MM chromatography paper that extended into a reservoir of transfer solution (10X sodium citrate, SSC). A piece of Hybond-N nylon hybridisation membrane (Amersham PLC, UK) was cut to fit exactly over the gel. The membrane was soaked in the transfer solution for 5min and placed on the gel. Three pieces of Whatman 3MM paper were soaked in transfer solution and placed on the membrane, followed by a 5cm-thick layer of absorbent paper towelling and a weight of approximately 1kg. The flow of liquid by capillary action from the bottom reservoir, through the gel and membrane into the absorbent towels results in the transfer of DNA from the gel onto the membrane. Transfer was allowed to take place overnight. The apparatus was disassembled, and the DNA was cross-linked onto the membrane in a UV cross-linker (Hoefer Scientific Instruments, San Francisco, USA).

A10.2. Labelling of the probe and hybridisation

Once cross-linked, the membrane was soaked in pre-hybridisation solution (Appendix B7). This took place at 68°C, with shaking, for 2-4hrs (Hybaid incubator, South African Scientific Products, SA). The HPV-16 L1 gene was labelled and used to probe for L1 DNA on the Southern blot. HPV-16 L1 was

isolated from pUC-L1 by restriction enzyme digestion and agarose gel electrophoresis. The L1 band was purified from the agarose gel, as described in Appendix A5.6.

The probe was labelled with digoxigenin (DIG)-dUTP according to the instructions of the DIG High Prime DNA Labelling and Detection Starter Kit 2 (Roche Molecular Biochemicals). Briefly, approximately 1μg of DNA was denatured at 100°C, chilled quickly in an ice/ethanol bath, mixed with DIG-High Prime, and incubated at 37°C for 20hrs. DIG-High Prime generates labelled probe according to the random primed labelling technique.

When the pre-hybridisation step was completed, the labelled probe was placed in a boiling water bath for 10min, to denature the DNA. The probe solution was then added to the pre-hybridisation solution, in which the membrane had been incubating. Hybridisation was allowed to proceed from 4hrs to overnight at 68°C, with gentle shaking.

A10.3. Washing and developing

The membrane was washed to remove un-hybridised probe. The washes were first performed with a 2X SSC, 0.1% SDS (w/v) solution at room temperature; and then with a 0.5X SSC, 0.1% SDS (w/v) solution at 68°C.

A10.4. Detection and photographic development

The membrane was incubated for 30min at room temperature in blocking solution, consisting of 0.1M Maleic acid buffer (Appendix B7) and 1X blocking reagent (Roche Molecular Biochemicals). Anti-Digoxigenin-AP antibody was added to the blocking solution, in which the membrane had been incubating. Antibody binding was allowed to proceed for 30min at room temperature, with gentle shaking. Unbound antibody was washed off with large volumes of washing buffer (Appendix B7); after which the membrane was equilibrated in detection buffer (Appendix B7) for 5min. The membrane was then covered with the kit's CSPD Ready-to-use solution (chemiluminescence substrate). The membrane was sealed in plastic and exposed to Agfa Curix RP1 X-ray film. The X-ray was developed as described in Appendix A12.3.

A11. ANALYSIS OF PROTEIN EXPRESSION BY BCG

A11.1. Total protein extraction from BCG

Due to the thick mycobacterial cell wall, protein extraction methods that make use of lysis buffers are often not suitable for the extraction of protein from mycobacteria. A mechanical bead-beating method was therefore utilised to extract protein from BCG.

BCG was grown to mid-log phase (OD_{600} 0.5-0.7) in 100ml of Sauton's broth, and the cells were harvested by centrifugation at 5000rpm for 10min (Beckman J2-21 centrifuge, JA-10 rotor). To investigate up-regulation of protein expression by the heat-shock response, some cultures were incubated at 45°C for 30min before collecting the cells. The cell pellet was re-suspended in phosphate-buffered saline (PBS), and centrifuged as above to wash the cells. The cell pellet was then re-suspended in 5ml PBS, and was placed into 2ml screwcap tubes (Quality Scientific, USA). 100-200µl of 0.1mm-diameter zirconia spheres (Biospec Products, USA) were placed into the tubes. The tubes were placed into a FastPrep FP120 machine (Bio 101, USA) and processed for four 30 second periods at speed 6. The tubes were cooled in ice between each processing period. The FastPrep machine shakes the tubes at very high speeds, which in the presence of the spheres, effectively lyses most bacteria. The tubes were centrifuged at 10 000rpm for 10min (Eppendorf Desktop centrifuge, Germany) to sediment the cellular debris and silica/ceramic spheres. The supernatant fluid (containing the soluble protein fraction) was collected. The bead/cell debris pellets were resuspended in 10% SDS and boiled for 30min. The supernatant, containing the insoluble protein fraction, was removed after centrifugation as above.

A11.2. Protein assay

The Bio-Rad Protein Assay kit (Bio-Rad Laboratories, Germany) was used as specified in the kit's protocol. This assay is based on the observation that the optical density of Coomassie Brilliant Blue G-250 shifts from 465nm to 595nm when protein binding occurs. The kit was used to determine the concentration of protein extracted from BCG cultures, and hence, the amount of protein to use for SDS-PAGE analysis. Several dilutions of bovine serum albumin (BSA; Roche Diagnostics, Germany) were prepared, with concentrations ranging from 1 to 25µg/ml. These were used to plot a standard curve. 0.8ml of the BSA standards, and appropriately diluted BCG cell lysates, were placed into clean 2ml tubes. 0.2ml of the Dye Reagent Concentrate was added to each tube, and mixed well. The OD_{595} of each sample was measured and the concentrations of the unknown samples were determined by plotting their ODs against the standard curve.

A11.3. Sodium dodecyl sulphate - polyacrylamide gel electrophoresis (SDS-PAGE)

A discontinuous system was used in which a large-pore gel (stacking gel) was layered above a separating (running) gel. A gel mould was made by "sandwiching" one alumina and one glass plate (10cmX10cm) together, with 0.75mm spacers between the plates on either side. A 12.5% acrylamide running gel (Appendix B8) was poured into the gel mould and was overlaid with water-saturated n-butanol to exclude oxygen (to aid polymerization) and to create a flat gel surface. The gel was left at 4°C overnight to polymerise completely. A 2-3cm deep 4% acrylamide stacking gel (Appendix B8) was poured above the running gel and a comb was inserted. Polymerisation was left to proceed for about 1hr. The comb was removed and the gel "sandwich" was clamped into the electrophoresis apparatus (Hoefer Mighty Small SE250/260, San Francisco, USA). The buffer chambers were filled with tank buffer (Appendix B8).

The BCG protein samples were diluted to 1µg/µl in PBS, and then combined with the same volume of 2X treatment buffer (Appendix B8). The proteins were denatured in a boiling water bath for 5min, and then cooled on ice. 20µl (10µg) of the treated protein sample were loaded per well. The gel was electrophoresed at 20mA, until the tracking dye reached the bottom of the gel. A molecular weight marker (LMW Electrophoresis Calibration Kit, Pharmacia Biotech, USA) was electrophoresed alongside the protein samples, for estimation of protein sizes. The gel apparatus was disassembled, and the gel was carefully removed from the glass plates. Protein gels were generally run in duplicate, and either stained with Coomassie blue or used for western blotting.

A11.4. Coomassie blue staining

Coomassie blue staining is based on non-specific binding of Coomassie blue dye to proteins. After electrophoresis, the gel mould was disassembled carefully and the gel was placed in Coomassie blue staining solution (Appendix B9), and was left to shake gently on a rotary shaker (Red Rotor PR70, Hoefer Scientific Instruments, San Francisco, USA) for 1.5hrs. The methanol-acetic acid solution effectively fixes the proteins on the gel. The staining solution was poured off and the gel was washed in destaining solution I (Appendix B9) for 30min to remove the majority of the background stain. The gel was then washed in destaining solution II (Appendix B9) until it was sufficiently destained. Once destained, the gel was placed on a light box and photographed (Kodak DC120 digital camera).

A12. WESTERN BLOT PROTEIN ANALYSIS

A12.1. Western blot protein transfer

After electrophoresis, the SDS-PAGE gel was equilibrated in Towbin transfer buffer for 15 min. A Hybond-P membrane (polyvinylidene difluoride [PVDF] membrane; Amersham Pharmacia Biotech, UK) was cut to the size of the gel, and prepared by wetting it first in methanol, and then in Towbin transfer buffer. Two 1cm high stacks of Whatman 3MM chromatography paper were soaked in Towbin transfer buffer. The one paper stack was placed on the anode of the western blot transfer apparatus (Transblot Semi-Dry Transfer Cell, Bio-Rad Laboratories, Germany), followed by the membrane and the gel. The other stack of saturated paper was placed on the gel. Transfer was allowed to proceed at 200 mA for 2hrs.

A12.2. Immunodetection of proteins on western blot

Immunodetection of specific proteins was carried out using the BM Chemiluminescence Blotting Substrate (POD) kit (Roche Molecular Biochemicals, Germany) according to the manufacturers' specifications. After western blotting was completed, the PVDF membrane was blocked for 1hr at room temperature in 1% blocking solution, which consisted of the kit's blocking solution diluted in tris-buffered saline (TBS). The primary antibody (HPV-16 L1-specific) was diluted in 0.5% blocking

solution; and the membrane was incubated in this solution for 16hrs at 4°C. The membrane was washed in 3 large volumes of TBST (TBS with 0.1% Tween 20), and then once in 0.5% blocking solution, to remove unbound antibody. The secondary antibody (a horseradish peroxidase [HRP]-labelled antibody, specific for the species of the primary antibody) was diluted in 0.5% blocking solution, and incubated with the membrane for 30-60min at room temperature. The membrane was then washed 4x with TBST, and sealed between two pieces of transparent plastic. The kit's detection reagent was prepared, and added to the membrane for 1min. The detection substrate (luminol, a cyclic diacylhydrazide) is oxidised by horseradish peroxidase (HRP, POD) in the presence of hydrogen peroxide, and as a result, emits light. The membrane was exposed to X-ray film for 1-5min, and then developed.

A12.3. X-ray development

Autoradiographs were developed by immersing in developer for 3min, stopper solution for 1min, and fixer solution for 4min. They were and washed in water, dried and viewed on a light box.

A13. ZIEHL-NEELSON STAIN OF BCG

The purity of BCG cultures was verified by means of the Ziehl-Neelson stain. Cells were heat-fixed onto a glass slide, and were then stained with carbol fuchsin, with intermittent heating for 5min. The slide was washed with water and decolourised with a 3% acid-alcohol solution. The cells were counter-stained with methylene blue for 30sec, and were then washed with water. Mycobacteria appear as red rods as they are acid-fast, and hence are not decolourised by the acid-alcohol wash. Other bacteria are decolourised by the acid-alcohol wash, and therefore take up the blue stain (Prescott *et al.*, 1993).

A14. QUANTIFICATION OF BCG RNA

A14.1. RNA Extraction

RNA extractions were performed on freshly-made BCG cultures or on frozen BCG samples. rBCG cultures were grown to middle log phase (OD_{600} 0.5-0.7) in 50ml of Sauton's medium (Appendix A1.2). The cells were then collected by centrifugation, at 4000rpm for 10min at 4°C, and re-suspended in 200µl of water. RNA was also extracted from frozen BCG inoculation stocks (Appendix A15).

The FastRNA Kit, Blue (Bio 101, USA) was utilised for the RNA extractions, as per the kit's instructions. Briefly, 500µl of CRSR-Blue (chaotropic RNA stabilising reagent), 500µl PAR (phenol acid reagent), 100µl CIA (chloroform isoamyl alcohol), and 200µl of the BCG re-suspension were added to a FastPrep Blue tube (containing lysing matrix). The tube was placed in a FastPrep FP120 machine (Bio 101, USA), at processed at speed 6, for three 30sec periods. The tube was cooled on

ice between each processing period. The FastPrep instrument shakes samples at very high speeds, allowing mechanical disruption of cells by the lysing matrix. The tube was spun at 14000rpm for 15min in a microcentrifuge to separate the phases, and pellet cell debris. The top phase was removed and placed in a clean tube. 500µl CIA was added and mixed well. The phases were separated again, and the top phase was removed and placed in a new tube. 500µl of DIPS (DEPC-treated isopropanol solution) was added, and the RNA was allowed to precipitate at room temperature for 1min. The RNA was collected by centrifugation at 14000rpm for 5min. The RNA pellet was washed twice with 250µl of SEWS (ethanol washing solution), allowed to dry, and then re-suspended in 100µl of SAFE (RNase-free water). The RNA sample concentration was measured and calculated as described in Appendix A5.7. DEPC-treated water (Appendix B10) was utilised for all RNA dilutions.

A14.2. DNase Treatment of RNA

RNA samples were treated with deoxyribonuclease (DNase) to remove all DNA contamination. The DNase kit, AMP-D1 (Sigma, USA), was utilised for this purpose, as per the kit's instructions. This process involved the addition of 1 unit of DNase and a 10x reaction buffer to 1µg of RNA, and a 15min incubation step at room temperature. The reaction was stopped with the addition of the kit's stop solution (50mM EDTA) and with a 10min heating step at 70°C.

A14.3. cDNA synthesis / Reverse transcription of RNA

The 1st Strand cDNA Synthesis Kit for RT-PCR (Roche Diagnostics, Germany) was utilised to synthesise cDNA, according to the manufacturer's instructions. 1µg of RNA (extracted from BCG was added to a mixture of reaction buffer, $MgCl_2$, dNTPs, random $p(dN)_6$ primers, RNase inhibitor, and AMV reverse transcriptase. The reaction mixture was incubated at 25°C for 10min to allow primer annealing to take place, 42°C for 60min for cDNA synthesis, 99°C for 5min to deactivate the reverse transcriptase, and then at 4°C for 5min to cool. The cDNA concentration was measured (Appendix A5.7) and then stored at -20°C.

A14.4. cDNA amplification and quantification using the LightCycler

The LightCycler - FastStart DNA Master SYBR Green I Kit (Roche Molecular Biochemicals, Germany) was used for the quantification of the cDNA. This PCR kit is specifically adapted for use with the LightCycler instrument. All PCR components, excluding the template DNA and primers were supplied with the kit, and were mixed according to the kit's instructions. The kit contains the FastStart Taq DNA polymerase, which has been shown to reduce non-specific amplification. SYBR Green I dye specifically binds to double stranded DNA, this binding enhances its fluorescence, which is then measured by the LightCycler instrument.

A typical reaction mixture contained the following reagents per reaction:

FastStart Mastermix	2µl
(containing *Taq* DNA polymerase, dNTPs, SYBR Green dye, 10x reaction buffer)	
$MgCl_2$ (25mM)	2.4µl
Forward primer (5pmol/µl)	2µl
Reverse primer (5pmol/µl)	2µl
H_2O	9.6µl
DNA / cDNA template (50-100ng)	2µl

The PCR reaction parameters were as follows:

- Initial Denaturation at 95°C for 10 min
- Denaturation at 95°C for 0 sec
- Primer annealing at 57°C for 10 sec
- DNA synthesis at 72°C for 10 sec

} 30 cycles

A melting curve was then obtained by running the following DNA Denaturation step:

- 40°C for 5 sec
- 95°C for 0 sec (with a temperature transition of 0.20°C/sec)
- 40°C for 0 sec

A15. PREPARATION OF BCG FOR IMMUNISATIONS

100ml of BCG was grown in Sauton's medium, in rolling bottles, to middle log phase (OD_{600} 0.5-0.7). The cell concentration (in cfu/ml) was calculated using the equation that a BCG culture OD_{600} measurement of 1.2 is approximately $2x10^8$ cfu/ml (Stover *et al.*, 1993). The culture was either used immediately, or was frozen at -80°C. When used freshly, the cells were pelleted by centrifugation at 3000rpm for 10min (Beckman J2-21 centrifuge, JA-20 rotor), and then re-suspended PBS. The cells were used for immunisation within an hour preparation. The concentration of the inoculation sample was then more accurately determine by serial dilution in Sauton's medium and plating on 7H10 medium (6 plates per dilution). In order to prepare the frozen inoculation stocks, the culture was sedimented as described above, and the pellet was re-suspended in PBS (with 10% v/v glycerol) to an approximate concentration of $4x10^8$ cfu/ml. The cell suspension was aliquoted and then frozen at -80°C. Once the inoculation stocks had been frozen for approximately 7 days, an aliquot was thawed and plated to accurately determine the BCG concentration (cfu/ml). Just prior to inoculation, an aliquot was thawed and diluted in PBS to an appropriate concentration.

A16. DETECTION OF ANTI-L1 ANTIBODIES BY ENZYME LINKED IMMUNOSORBENT ASSAY (ELISA)

A16.1. Non-fat milk blocking method

HPV-16 VLPs were diluted in PBS, to a concentration 2µg/ml, and were added to the wells (100µl/well) of a 96-well curved-bottomed microtitre plate (Nunc-Immuno™, Denmark). The plate was placed at 4°C overnight to allow the VLPs to coat the wells. The wells were washed three times with PBS using a LP55 Sanofi plate washer (Pasteur Diagnostics, Paris, France), to remove unattached antigen. The wells were then blocked with 200µl of 1% milk solution (Elite® milk powder in PBS) for 2hrs at room temperature. The plate was washed three times, as above. The sera were diluted 1:40 in 1% milk solution, and 100µl were added in duplicate wells. The plate was incubated at 37°C for one hour, and then washed three times with PBS. The peroxidase-conjugated secondary antibody, rabbit anti-guinea pig-HRP immunoglobulins (DAKO, Denmark) was diluted 1:2000 in 1% milk solution, and was added to the wells. The plate was incubated at 37°C for 1hr, and then washed as before. The substrate, 1,2-phenylenediamine dihydrochloride (OPD; DAKO, Denmark) was made according to the manufacture's instructions; using 12ml distilled water, 4 OPD tablets and 5µl H_2O_2. OPD is a chromogenic substrate for horseradish peroxidase, which develops a yellow colour upon hydrolysis. The substrate solution was added, and the colour reaction was allowed to proceed for 5 to 10min, after which, the reaction was stopped with the addition of 0.5M H_2SO_4. The absorbance was read using the Anthos ELISA plate reader (Labtech Instruments) at 492nm, with a reference filter of 620nm.

A16.2. Polyvinyl alcohol and polyvinylpyrrolidone blocking method

A modified polyvinyl alcohol (PVA)-blocking ELISA (Studentsov *et al.*, 2002) was utilised to minimize non-specific binding. A 96-well curved-bottomed microtitre plate was coated with HPV-16 L1 VLPs overnight, as described above (A16.1). The wells were then blocked for 3hrs at room temperature with 300µl of PVA blocking solution (0.5% PVA in PBS, pH 7.4) per well. The plate was washed six times with PBS. Sera were diluted with PVA blocking solution, and 100µl were loaded onto the plate in duplicate. The plate was incubated at 37°C for 1hr, and then washed six times with PBS. The peroxidase-conjugated secondary antibody was diluted 1:2000 in a 0.5% PVA and 0.8% polyvinylpyrrolidone (PVP) solution in PBS, and was then added to the plate. The plate was incubated at 37°C for 30min, after which it was washed six times with PBS. Detection was performed with OPD substrate, as described above.

A17. DELAYED-TYPE HYPERSENSITIVITY SKIN REACTIONS

The hair on the back of each guinea pig was cut with an electric clipper, and was further removed with a depilatory cream (No Hair, Veet®). Each animal received a number of 50µl intradermal injections in the hairless region of the back. The inocula included PBS (pH 7.4), purified protein derivative of

tuberculin (PPD, MT702.703, 0.2μg/animal), 0.1M sodium carbonate buffer (pH 9.6), intact HPV-16 VLPs (5μg/animal) and denatured HPV-16 VLPs in carbonate buffer (5μg/animal). The denatured VLPs were made by leaving them in 0.1M sodium carbonate buffer for 2 hours before inoculation. The inoculation sites were observed 24, 48 and 72 hours after inoculation and the diameter of erythema and induration was measured at each site using calipers.

A18. MAMMALIAN CELL CULTURE CONDITIONS AND MEDIUM

The African green monkey kidney (CV-1) and Human TK$^-$ 143B (HuTK$^-$) cell lines were obtained from the American Type Tissue Culture Collection (Rockville, MA, USA). These cells were grown as monolayers in 50-200ml tissue culture flasks (Greiner, Germany), in Dulbecco's Modified Eagle's Medium (DMEM; Gibco, UK), containing 10% fetal calf serum (FCS; Sigma-Aldrich, Germany), 0.15mg/ml penicillin (P; Gibco), 0.25mg/ml streptomycin (S; Gibco), and 1% Fungizone (F; Gibco). The cultures were incubated at 37°C in a humidified 5% CO_2 incubator. Confluent cell layers were split by adding trypsin-EDTA solution (Gibco, UK) for 5-10min to lift the cells, and then by diluting the cells1/5 to 1/10 in fresh DMEM with 10% FCS and PSF.

Mouse lymphocytes and P815 (mouse mastocytoma, MHC class I) cells were cultured in suspension in RPMI medium (Gibco, Paisely, UK), at 37°C in a humidified 5% CO_2 incubator. RPMI complete medium was prepared by the addition of 10% FCS, 0.15mg/ml P, 0.25mg/ml S, 1% F, and 2μM β-mercaptoethanol (Gibco).

A19. PREPARATION OF EFFECTOR CELLS

The inguinal lymph nodes from a group of mice were collected aseptically, and were pooled in 10ml of RPMI, with 10% FCS (Appendix A18). A single cell suspension was prepared by pushing the lymph nodes through a sterile metal grid (pore size 0.5mm, Sigma) with the plunger of a 2ml syringe. 10ml of RPMI were poured through the grid to collect residual cells. The cell suspension was placed into a 50ml conical centrifuge tube, into which a further 20ml of RPMI were added. The cell suspension was centrifuged at 1500rpm for 10min (Jouan CR412 centrifuge) to pellet the cells. The supernatant was decanted and the cells were washed by re-suspending the cell pellet in 40ml of RPMI. The centrifugation and washing steps were repeated. The cells were collected again by centrifugation and the cell pellet was re-suspended in 50ml of RPMI complete medium (Appendix A18).

10μl of the cell suspension were diluted with Trypan Blue, and counted in a Neubauer counting chamber. The cell concentration was adjusted to $10x10^6$ cells/ml with RPMI complete medium, in a 200ml tissue culture flask (Greiner, Germany). Gp120 peptide (obtained from the National Institute for

Biological Standards and Control, Hertfordshire, UK) was added to the cells, at a final concentration of 4μg/ml, and the cells were incubated at 37°C in a humidified 5% CO_2 incubator (Forma Scientific) for 6 days. After day six, the cells were transferred to a 50ml tube and collected by centrifugation at 1500rpm for 5min. The supernatant was decanted and the cells were re-suspended in 30ml of RPMI. The cell concentration (both live and dead cells) was adjusted to 15-30x10^6/ml with RPMI. The cell suspension was transferred to 10ml conical-based tubes, and the cells were carefully underlaid with 5ml of Lympholyte-M (Cedarlane). This gradient was spun at 2200rpm (Jouan CR412 centrifuge) for 20min at room temperature, to separate the cells. After centrifugation, the cells at the interface were removed and added to a conical-based tube. The cells were collected by centrifugation at 1500rpm and washed by re-suspension in 10ml RPMI. The washing step was repeated 3 times. The cells were finally re-suspended in 1-5ml of RPMI complete medium and counted. These effector cells were used in immunogenicity studies as required.

A20. DETECTION OF CD8-SPECIFIC INTRACELLULAR IFN-γ BY FLOW CYTOMETRY

For each group of mice, a 200ml tissue culture flask (Greiner) was seeded with 50ml of P815 cells, at a concentration of 0.2x10^6 cells/ml of RPMI complete medium (Appendix A18). The cells were incubated overnight, and then collected by centrifugation at 1500rpm for 5min. The supernatant was discarded; the cells were re-suspended in 5ml of RPMI complete medium, and then counted.

P815 cells, gp120 peptide, effector cells (Appendix A19), and Brefeldin A were added to 4 FACS tubes as follows:

	Tubes			
	CD3/FITC/PE+pep	CD3/CD8/IFN+pep	CD3/FITC/PE-pep	CD3/CD8/IFN-pep
P815 cells	3x10^6	3x10^6	3x10^6	3x10^6
Gp120 peptide (4mg/ml)	0.6μl	0.6μl	-	-
Effector cells	3x10^6	3x10^6	3x10^6	3x10^6
Brefeldin A	0.6μl	0.6μl	0.6μl	0.6μl

RPMI complete medium was added to each tube to a total volume of 600μl. The cells were mixed and incubated for 3 hours. After incubation, the content of each tube was mixed with 1ml of FACS buffer (PBS, 1% FCS, 0.02% sodium azide). The cells were collected by centrifugation at 1500rpm for 5min, and the supernatant was discarded. The cells pellets were re-suspended in 75μl of blocking buffer (141μl Facs Buffer, 1μl CD16/32 (Scientific Group), 3.75μl normal mouse serum, and 3.75μl normal rat

serum), and left on ice for 20min. The cells were washed with 5ml of FACS buffer, and then collected by centrifugation, as before.

75µl of diluted antibody were to the tubes as follows, and then left on ice for 30min: rat monoclonal anti-mouse CD3 APC (1:25 dilution) to all of the tubes, rat monoclonal anti-mouse IgG2a FITC (1:50) to the CD3/FITC/PE tubes, and rat monoclonal anti-mouse CD8 FITC (1:50) to the CD3/CD8/IFN tubes. All antibodies were from BD-Pharmingen. The cells were washed twice in FACS buffer (as above) and the cell pellets were each re-suspended in 3ml of 1x FACSLyse (Scientific Group) for 10min at room temperature. The cells were washed twice in FACS buffer, as before, and then re-suspended in 1ml of FACS buffer overnight, at 4°C. The cells were collected by centrifugation, and the supernatant was discarded. 1ml of 1x PermWash solution (Scientific Group) was added, and the cells were left on ice for 15min. The cells were collected by centrifugation and the supernatants were discarded.

Rat IgG1 PE isotype and IFN-γ PE antibody were diluted 1:50 with PermWash solution. 150µl of isotype or antibody were added to the appropriate tubes, which were then left on ice for 30min. The cells were washed twice with PermWash solution, and re-suspended in 900µl of FACS buffer. A FACS Caliber flow cytometer (Becton-Dickinson, San Jose, CA) was used to perform the immunophenotyping and intracellular cytokine analysis.

The number of gated events for IFN-γ and CD8 in the CD3/FITC/PE and CD3/CD8/IFN tubes were calculated as follows:

For IFN-γ: $$UR \times \frac{\text{gated events of CD3/CD8/IFN}}{\text{gated events of CD3/FITC/PE}} = A$$

For CD8: $$(UR + LR) \times \frac{\text{gated events of CD3/CD8/IFN}}{\text{gated events of CD3/FITC/PE}} = B$$

The percentage of IFN-γ-producing cells, as a percentage of CD8 cells, was calculated as follows:
IFN-γ positive events: UR of CD3/CD8/IFN tube minus A
CD8 positive events: (UR + LR) of CD3/CD8/IFN tube minus B

$$\% \text{ IFN-}\gamma\text{-producing cells as a \% of CD8} = \frac{\text{IFN-}\gamma \text{ positive events} \times 100}{\text{CD8 positive events}}$$

A21. ^{51}CHROMIUM-RELEASE CYTOTOXIC KILL ASSAY

Preparation of target cells

P815 cells were labelled with ^{51}Chromium (^{51}Cr; Amersham). 5ml of healthily growing cells were collected by centrifugation and re-suspended in 200µl of ^{51}Cr (2mCi/ml). The cells were incubated at 37°C with 5% CO_2 for 1.5hrs, with gentle shaking every 15min. The cells were washed 3 times in 10ml RPMI, counted, and then diluted in RPMI complete medium to $1x10^5$ cells/ml. The cells were split into 2 tubes. The cells in one tube were pulsed with gp120 peptide, by the addition of peptide at a final concentration of 2µg/ml.

Preparation of effector cells

Effector cells were prepared (Appendix A19), and were adjusted to a concentration of $10x10^6$ cells/ml.

Kill assay

50µl of the p815 target cells, unpulsed and gp120-pulsed, were added in triplicate to a round-bottomed, sterile 96-well microtitre plate (Nunc, Denmark). The effector cells were serially diluted and added to the same wells at varying concentrations to achieve final effector:target ratios of 200:1, 100:1, 50:1, 25:1, 12:1, 6:1, 3:1 and 1:1. To determine spontaneous ^{51}Cr release, effectors were not added to the target cells, instead, medium was used. To determine maximum ^{51}Cr release, 5% Triton X-100 (Sigma-Aldrich) was added to the target cells.

The plates were incubated for 4hrs at 37°C in a 5% CO_2 incubator. 100µl of supernatant was removed from each well and added into Durham counting vials. The gamma radiation in each tube, measured as counts per minute (cpm), was determined using a Packard gamma counter.

The % of specific sample lysis for the triplicate wells of each effector:target ratio was calculated as follows:

$$\% \text{ specific lysis} = \frac{\text{mean sample cpm} - \text{mean spontaneous cpm}}{\text{mean maximum cpm} - \text{mean spontaneous cpm}} \times 100$$

A22. RECOMBINANT VACCINIA VIRUS FORMATION

African green monkey kidney (CV-1) cells were grown in a six well plate (Nunc, Denmark) until 60-80% confluency. Wild-type vaccinia virus was diluted to $3x10^5$ viruses/ml in virus diluent (DMEM with 1% 1M Hepes-buffered-saline, Gibco), and was added to each well (0.5ml/well). Cell infection was allowed to proceed for 1hr, with occasional agitation. The transfection mixture was set up by incubating 10µg of linearised pSC65-L1 and 60µl of DOTAP (Boehringer Mannhein, Germany) at room temperature for 10-15min. The virus inoculum was removed from the cells, and 2ml of fresh DMEM,

containing 4% FCS and PSF, and the transfection medium were mixed, and added to the cells. The infected/transfected cells were incubated for 2-3 days, until cytopathic effect (cpe) was detected. The cells were then stained with X-gal (Appendix A23) to discern whether the transfection had been successful. Cells from duplicate wells, that had not been stained, were re-suspended in 200µl of virus diluent; and freeze/thawed repeatedly to lyse the cells and release the virus.

To eliminate contamination by wild-type vaccinia virus, the newly formed rVV-$L1_R$ viral stocks were put through several rounds of plaque purification and serial passage in $HuTK^-$ cells with BUdR selection. $HuTK^-$ cells were grown in 6-well plates until 80% confluent. These cells were then infected with the newly formed rVV-$L1_R$ for 1hr. The virus inoculum was removed from the cells and replaced with 2ml of selection medium, consisting of DMEM with 10% FCS and 25µg/ml BUdR (Sigma, St Louis, USA). After a 3-day incubation, the medium was removed and the infected cells were overlayed with a 1:1 mixture of DMEM and 2% low melting point agarose (SeaPlaque, FMC Bio Products), containing 25µg/ml BudR and 250µg/ml X-gal, for detection of recombinant plaques. The agarose mixture was added at 42°C, and allowed set at room temperature for 10min. The cells were incubated overnight at 37°C with 5% CO_2. The incorporation of the *LacZ* gene into the recombinant virus allows for the visual distinction between non-recombinant and recombinant virus plaques. Blue plaques, indicative of rVV-$L1_R$ infection, were picked using a Pasteur pipette and added to 200µl of virus diluent. The infected cells were stored at -20°C or freeze/thawed 3 times, and used for the next round of purification.

A23. X-GAL STAIN

CV-1 cells were grown in a 6-well plate to 80% confluency. The cells were infected with rVV-$L1_R$ for 2 days, after which they were washed with PBS and fixed with 1ml of 4% para-formaldehyde, for 10 minutes at room temperature. The cells were washed with PBS, and then covered with X-gal stain solution (in 10mls; 9.2ml PBS, 0.2ml of 50mg/ml X-gal, 0.2ml of 250mM ferricyanide, 0.2ml of 250mM ferrocyanide and 0.2ml of 100mM $MgCl_2$). The cells were incubated at 37°C until blue plaques were visible (2-16hrs).

A24. VACCINIA VIRUS PROPAGATION IN EGGS

Infection of eggs with VV

Vaccinia virus was grown to high titres on the chorio-allantoic membranes (CAMs) of 11-day-old fertilised chicken eggs. To view the structures within an egg, a light beam was shone through the egg, in a dark room. The eggs were placed on their sides and marked on the round side (at the air sac), and on the flatter side (top most side) near a vein. Small holes were made in the shell near the marks (mentioned above), taking care not to break the membranes. The hole at the air sac was slightly enlarged with a needle. A drop of sterile PBS was placed at the top hole, and the top membrane was

loosened with a needle, which let the PBS run in, onto the top amniotic membrane. A drop of melted wax was placed next to this hole. The air was then sucked out of the air sac hole with a rubber bulb, causing the top membrane to drop. The eggs were incubated for 1-2hrs, to let the chicks settle. 100µl of virus (10^3-10^5 pfu) were injected into the top hole of each egg, without puncturing the chorio-allantoic membrane. The hole was sealed with the wax, and the eggs were incubated at 37°C for 3 days.

Extraction of virus

The eggs were held on their sides, with the inoculation hole at the top. Starting from air sac hole, the shell of each egg was cut in half with a pair of scissors. The top half of the shell, containing the infected membrane, was retained, while the rest of the egg was discarded. Poxes could be seen as white dots on the membrane. The egg shell and uninfected membrane were cut away and discarded. The infected membranes were rinsed in 200ml of PBS. Three or 4 membranes were placed in a cooled McCartney bottle, containing approximately 1.5-2 cm of glass beads (2-4 cm in diameter). Three ml of McIlvaine's buffer (Appendix B11) and 1ml of Arklone® (1,1,2-trichloro-1,2,2-trifluoroethane; AECI, South Africa), an organic solvent, were added to each bottle; and the bottles were shaken vigorously for two minutes, to disrupt the membranes and release the virus particles.

The virus suspension was subjected to centrifugation at 1 000rpm (approximately 600g) for 10mins at 4°C, to sediment the membrane debris. The supernatants were transferred to new McCartney bottles and left on ice. Three ml of McIlvaine's buffer were added to the glass beads/membrane debris and the bottles were shaken again for 2min. The bottles were centrifuged as before. The supernatants were transferred to new McCartney bottles, and were left on ice for 1.5hrs. The McCartney bottles were spun at 2000rpm for 15min, to remove remaining membrane debris from the supernatant. The supernatants were transferred to a Beckman® centrifuge tube, and underlain with 500µl of sterile 36% (w/v) sucrose solution (in PBS). The tubes were centrifuged at 11 000rpm for 1hr (Beckman J2-21 swing-out rotor). The supernatant was discarded and the virus pellet was re-suspended gently overnight in 1ml McIlvaine's buffer at 4°C.

A25. VACCINIA VIRUS TITRATION AND ZIEHL NEELSON CARBOL FUCHSIN STAINING

CV-1 cells were grown in a 24-well plate (Nunc, Denmark) until approximately 80% confluent. Vaccinia virus was serially diluted in virus diluent. 200µl of the 10^{-3} to 10^{-9} dilutions were utilised to infect cells, in triplicate. The infection was allowed to proceed for 2hrs at 37°C, with occasional shaking. The virus inoculum was removed and 1ml of fresh DMEM with 10% FCS and PSF was added. The infection was allowed to progress until cpe was evident, this usually occurred 2-3 days after infection. The medium was removed from the cells, and then approximately 0.5ml Ziehl Neelson

Carbol Fuchsin was added to each well. The wells were washed carefully with water, and allowed to dry. The plaques were counted and the number of pfu per ml was calculated for the undiluted virus.

A26. EXTRACTION OF VACCINIA VIRUS FROM MOUSE OVARIES

The ovaries of each mouse were finely chopped with a sterile scalpel. The chopped ovaries and 1ml of McIlvaine's buffer were transferred to a Ten Broeck grinder and then homogenised with 30 grinding strokes. The ovary homogenate was transferred to a 2ml tube, and subjected to 3 freeze/thawed cycles. Cell debris was removed from the ovary preparation by centrifugation at 2000rpm for 10min. The virus in the supernatant was titrated (Appendix A26), and the number of vaccinia virus pfus/mouse was calculated.

A27. STATISTICAL TESTS

Data were compared using the Student *t*-test. Data were considered significantly different at $P \leq 0.05$.

APPENDIX B: SOLUTIONS, BUFFERS AND MEDIA

B1. Albumin-dextrose complex (ADC)

Bovine albumin factor V	5g
Glucose	2ml
Distilled water	to 100ml

The solution was filter-sterilised and stored at 4 °C.

B2. Sauton's broth

KH_2PO_4	0.5g
$MgSO_4.7H_2O$	1.05g
L-asparagine monohydrate	4.0g
Glycerol	60ml
Ferric ammonium citrate	0.05g
Citric acid	2g
$ZnSO_4$	0.1ml
Tyloxapol	10% (w/v)
Distilled water	800ml

The pH was adjusted to 7.4 with 0.1M KCl. The volume was adjusted to 1 litre, and the solution was autoclaved.

B3. TSB solution

Peptone	1.6g
Yeast extract	1.0g
NaCl	0.5g
PEG (polyethylene glycol)	10.0g
$MgCl_2$ (1M)	1ml
$MgSO_4$ (1M)	1ml
DMSO	5.0ml
Distilled water	to 100ml

B4. Plasmid preparation solutions:

Solution 1

1M Tris-HCl	5ml
0.5M EDTA	2ml
Rnase	10mg
Distilled water	to 100ml

Adjust pH to 8.0 with HCl.

Solution 2

NaOH	0.8g
10% SDS	10ml
Distilled water	to 100ml

Solution 3

Potassium acetate	27.5g
Distilled water	to 100ml

B5. CTAB/NaCl solution

NaCl	4.1g
Distilled water	80ml
CTAB	10g

The solution was placed on a stirrer to dissolve the NaCl, CTAB (n-acetyl-N, N, N,-trimethyl ammonium bromide) was added and heated to 65 °C if necessary. The volume was adjusted to 100 ml and the solution was stored at room temperature for no longer than 6 months.

B6. Southern transfer solutions:

Denaturing solution

NaOH	20g
NaCl	87.68g
Distilled water	to 1000ml

Neutralising solution

NaOH	0.8g
Ammonium acetate	192g
Distilled water	to 1000ml

SSC (20X)

sodium citrate (0.3M)	88.2g
NaCl (3M)	175g
Distilled water	800ml

The pH was adjusted to 7.0 with a few drops of 10N NaOH and the volume made up to 1 litre. The solution was autoclaved and stored at room temperature.

B7. DIG hybridisation solutions:

Pre-hybridisation solution
5X SSC
1X blocking reagent (Roche Molecular Biochemicals, Germany)
0.02% SDS
0.1% N-lauroylsarcosine

Maleic acid buffer
0.1M Maleic acid
0.15M NaCl
NaOH to pH 7.5

Washing buffer
0.1M Maleic acid buffer
0.3% (v/v) Tween 20

Detection buffer
0.1M Tris-HCl
0.1M NaCl

B8. SDS-PAGE gel solutions and buffers:

Monomer solution

Acrylamide	60g
Bisacrylamide	1.6g
Distilled water	to 200ml

Acrylamide is a neurotoxin and was weighed out wearing gloves and a gas mask. The solution was warmed at 55 °C to dissolve the components, it was filter sterilised and stored in the dark at 4 °C.

4X Running gel buffer

Tris	36.3g
Distilled water	150ml

The pH was adjusted to 8.8 with HCl and made to 200ml with distilled water.

4X Stacking gel buffer

Tris	3g
Distilled water	40ml

The pH was adjusted to 6.8 with HCl and made to 50ml with distilled water.

Separating (running) gel (12.5% acrylamide)

Monomer solution	6.3ml
4X running gel buffer	3.8ml
10% SDS	0.15ml
Distilled water	4.8ml
10% ammonium persulphate	75µl
TEMED (N,N,N',N' tetramethyl-ethylenediamine)	5µl

Stacking gel (4% acrylamide)

Monomer solution	0.44ml
4X stacking gel buffer	0.83ml
10% SDS	33µl
Distilled water	2.03ml
10% APS	16.7µl
TEMED	1.7µl

Tank buffer

Tris	30.28g
Glycine	144.13g
SDS	10g
Distilled water	to 10 litres

2X Treatment buffer

4X stacking gel buffer	2.5ml
10% SDS	4ml
Glycerol	2ml
Bromophenol blue	2mg
Dithiothreitol	0.31g
Distilled water	to 10ml

B9. Coomassie blue stain solutions:

Staining solution	
Coomassie Brilliant blue R	0.5g
Methanol	800ml
Acetic acid	140ml
Distilled water	to 2 litres

Destaining solution I	
Methanol	400ml
Acetic acid	70ml
Distilled water	to 1000ml

Destaining solution II	
Methanol	50ml
Acetic acid	70ml
Distilled water	to 1000ml

B10. DEPC-treated water

Diethyl pyrocarbonate (DEPC)	200µl
Distilled water	100ml

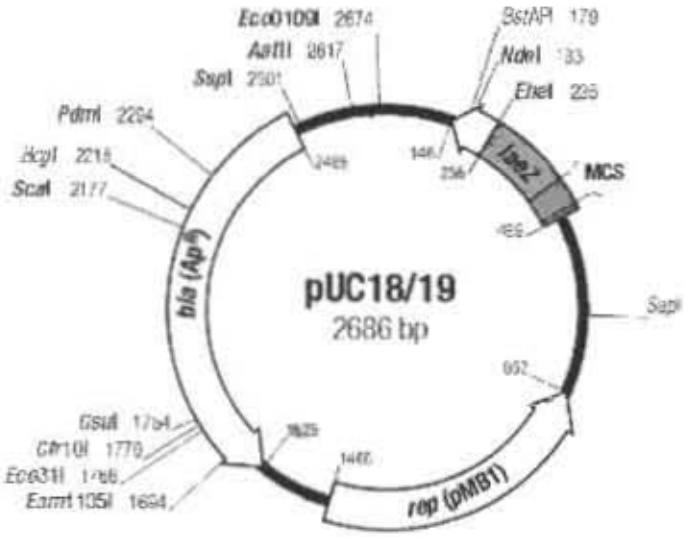
The solution was left at room temperature for 10min, and then autoclaved to inactivate the DEPC.

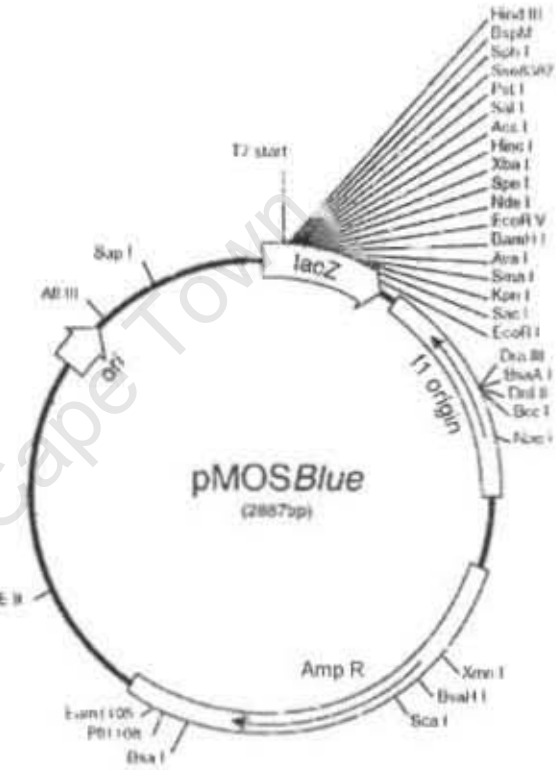
B11. McIlvaine's buffer

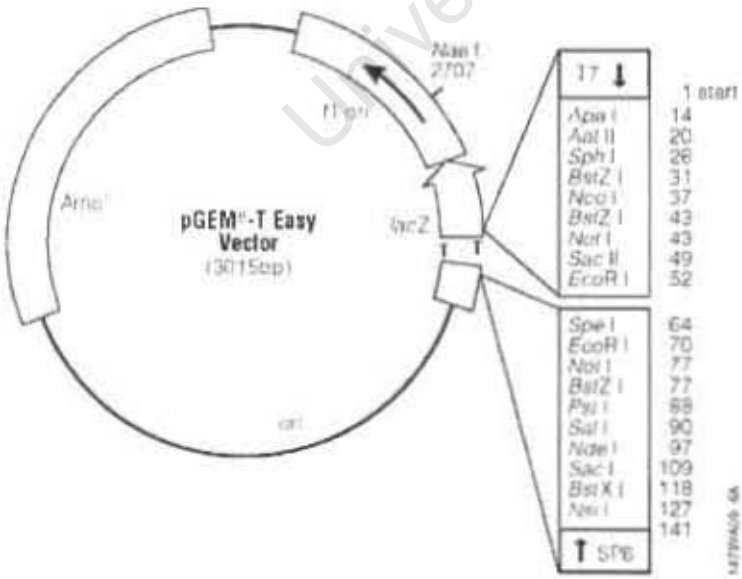
0.1M citric acid	1.83ml
0.2M $Na_2HPO_4.12H_2O$	218.7ml
Distilled water	800ml

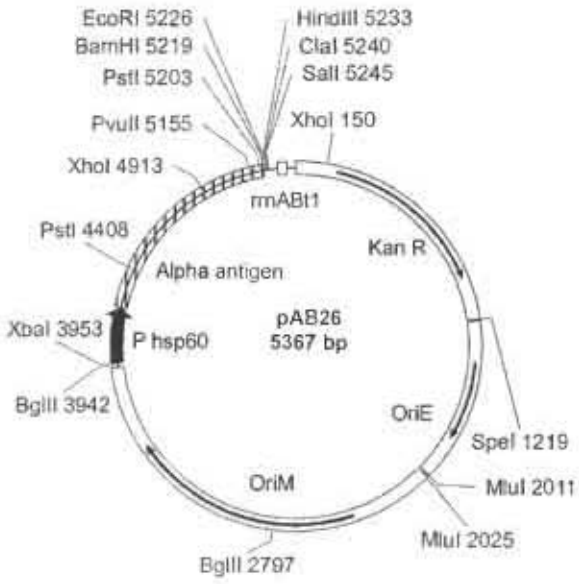
The pH was adjusted to 7.4, and made up to 1000ml with distilled water. The solution was autoclaved, and stored at 4°C.

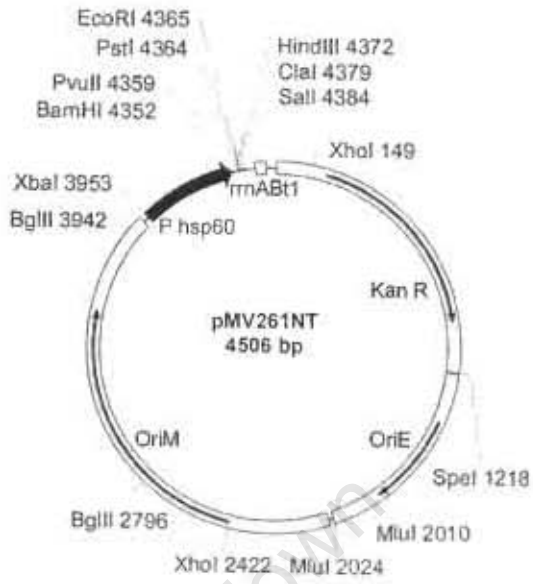
APPENDIX C: VECTOR MAPS

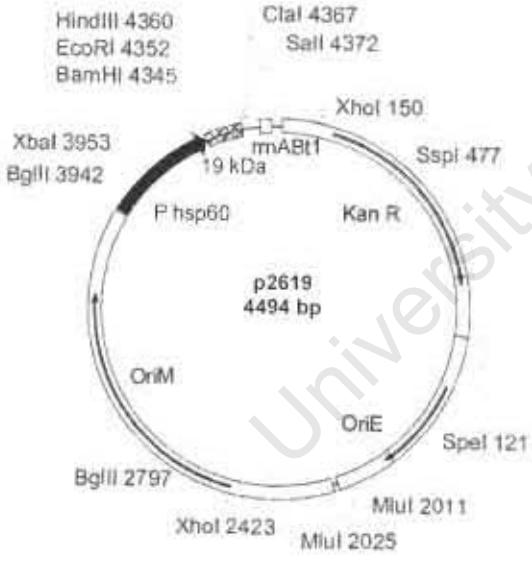


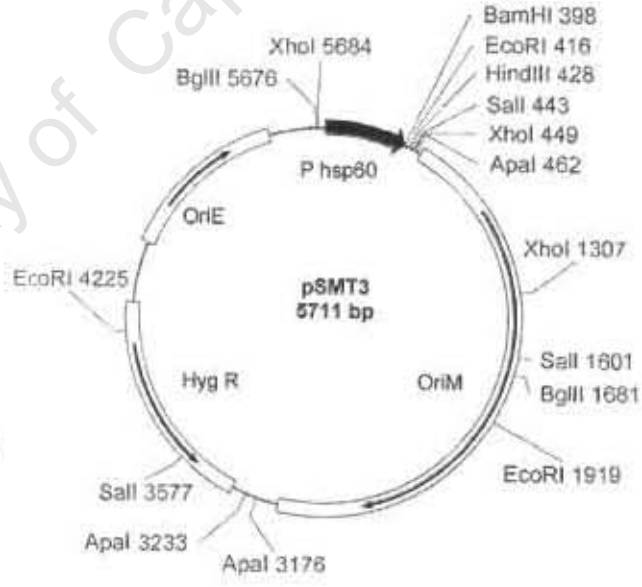












APPENDIX D: CLONE CONSTRUCTION

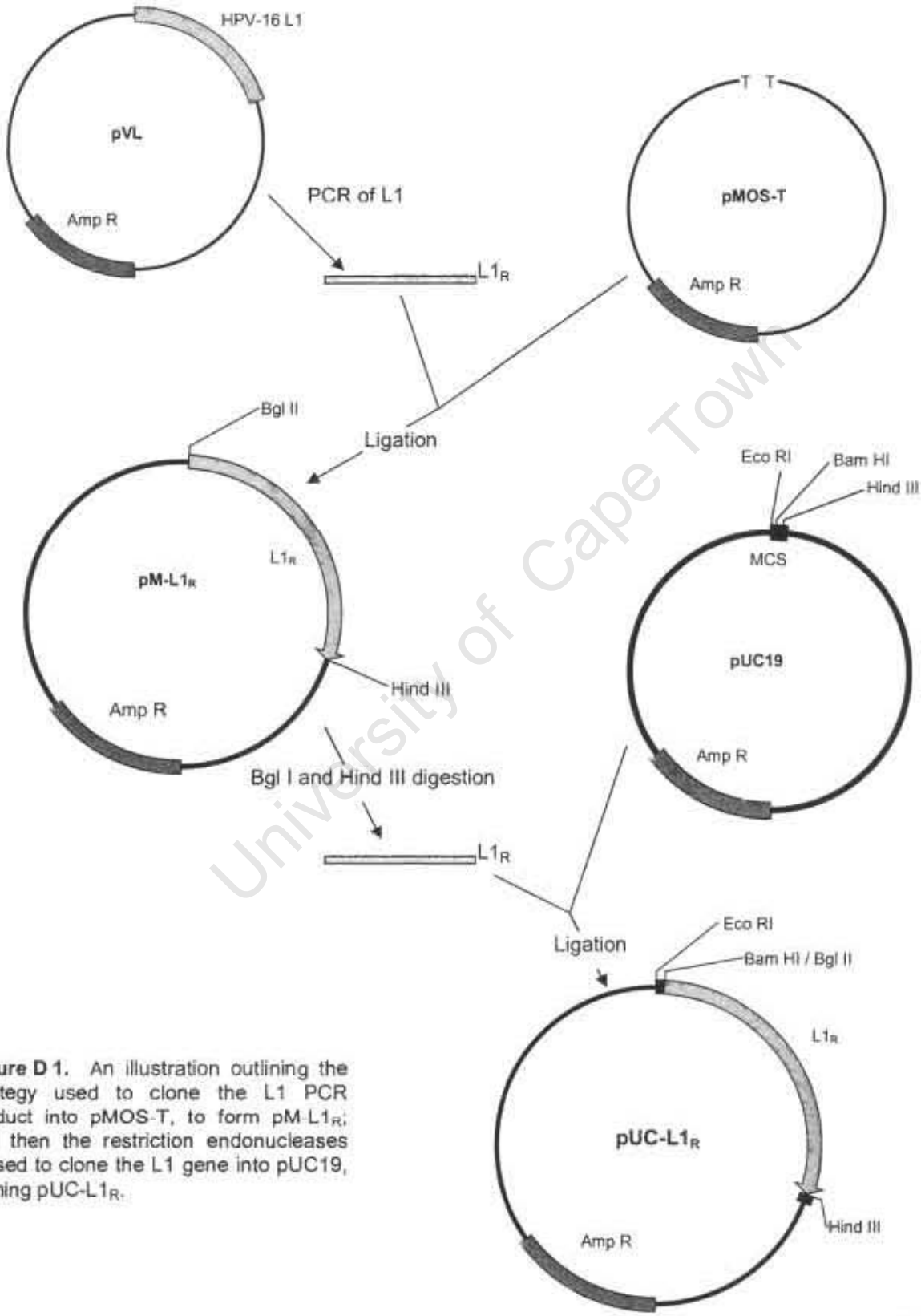


Figure D 1. An illustration outlining the strategy used to clone the L1 PCR product into pMOS-T, to form pM-$L1_R$; and then the restriction endonucleases utilised to clone the L1 gene into pUC19, forming pUC-$L1_R$.

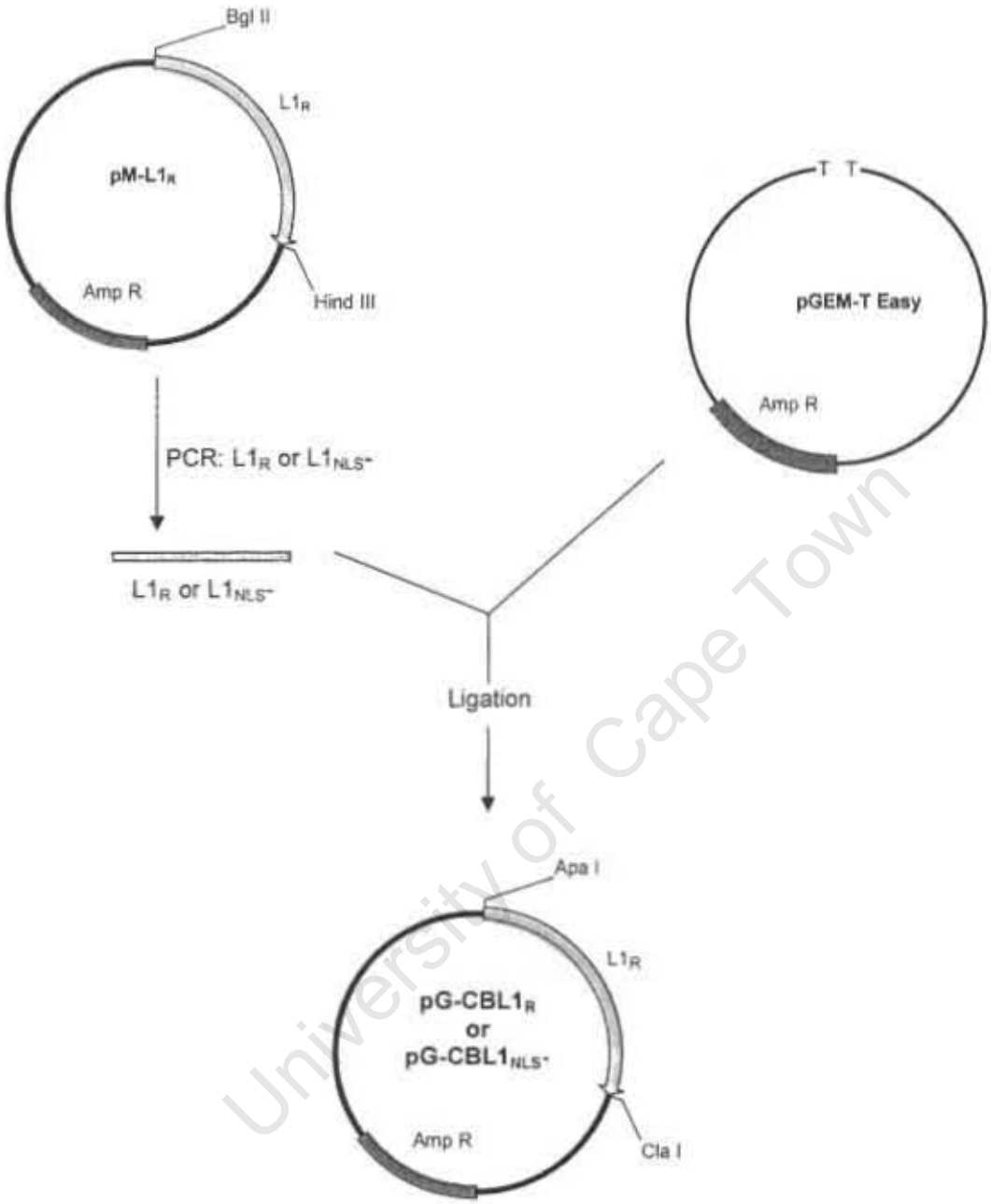


Figure D2. An illustration outlining the strategy used to the clone $L1_R$ and $L1_{NLS^-}$ PCR products into pGEM-T Easy, to form the clones pG-$CBL1_R$ and pG-$CBL1_{NLS^-}$, respectively.

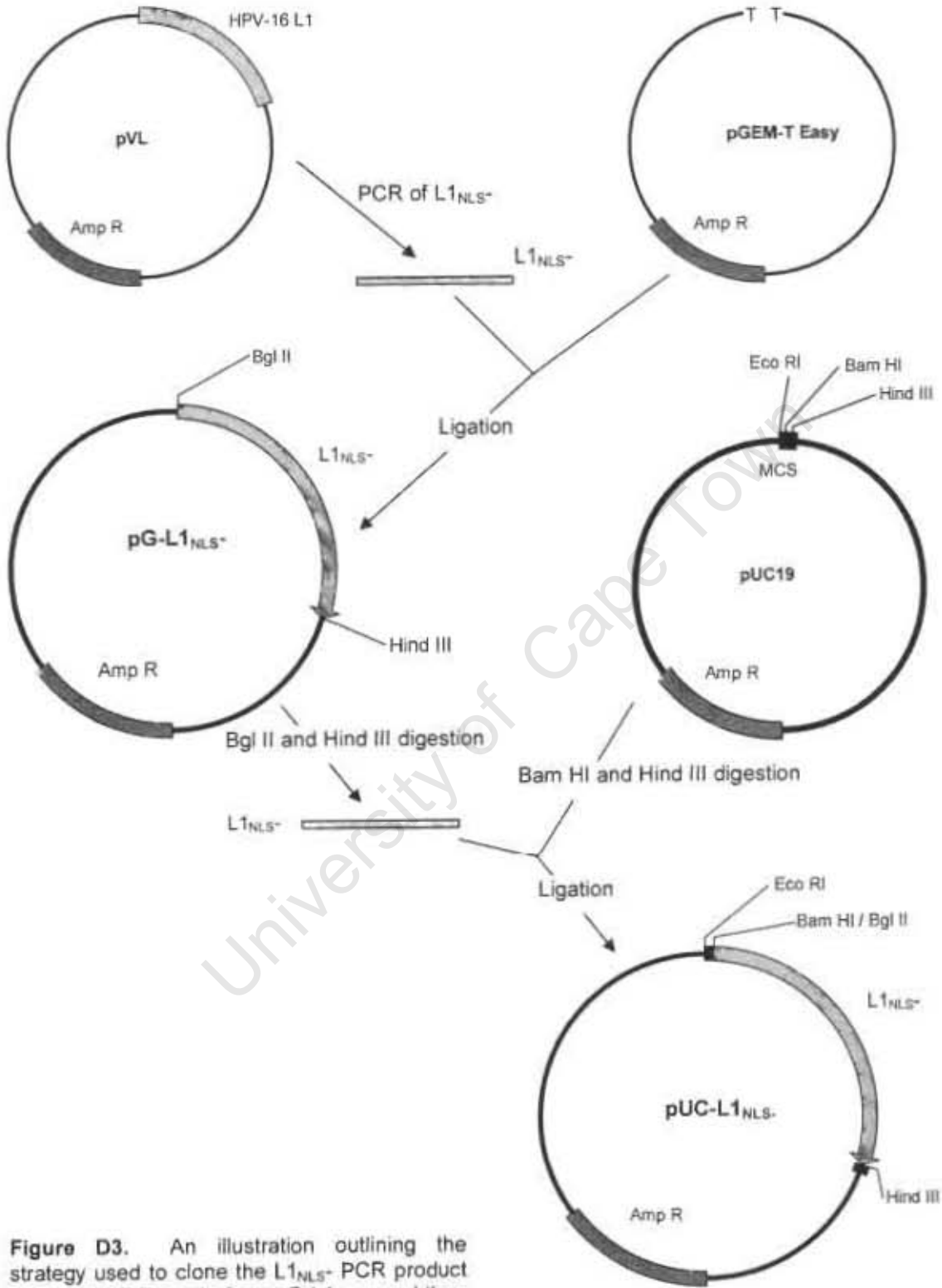


Figure D3. An illustration outlining the strategy used to clone the $L1_{NLS-}$ PCR product into pGEM-T Easy, to form pG-$L1_{NLS-}$; and then the restriction endonucleases utilised to clone the L1 gene into pUC19, forming pUC-$L1_{NLS-}$.

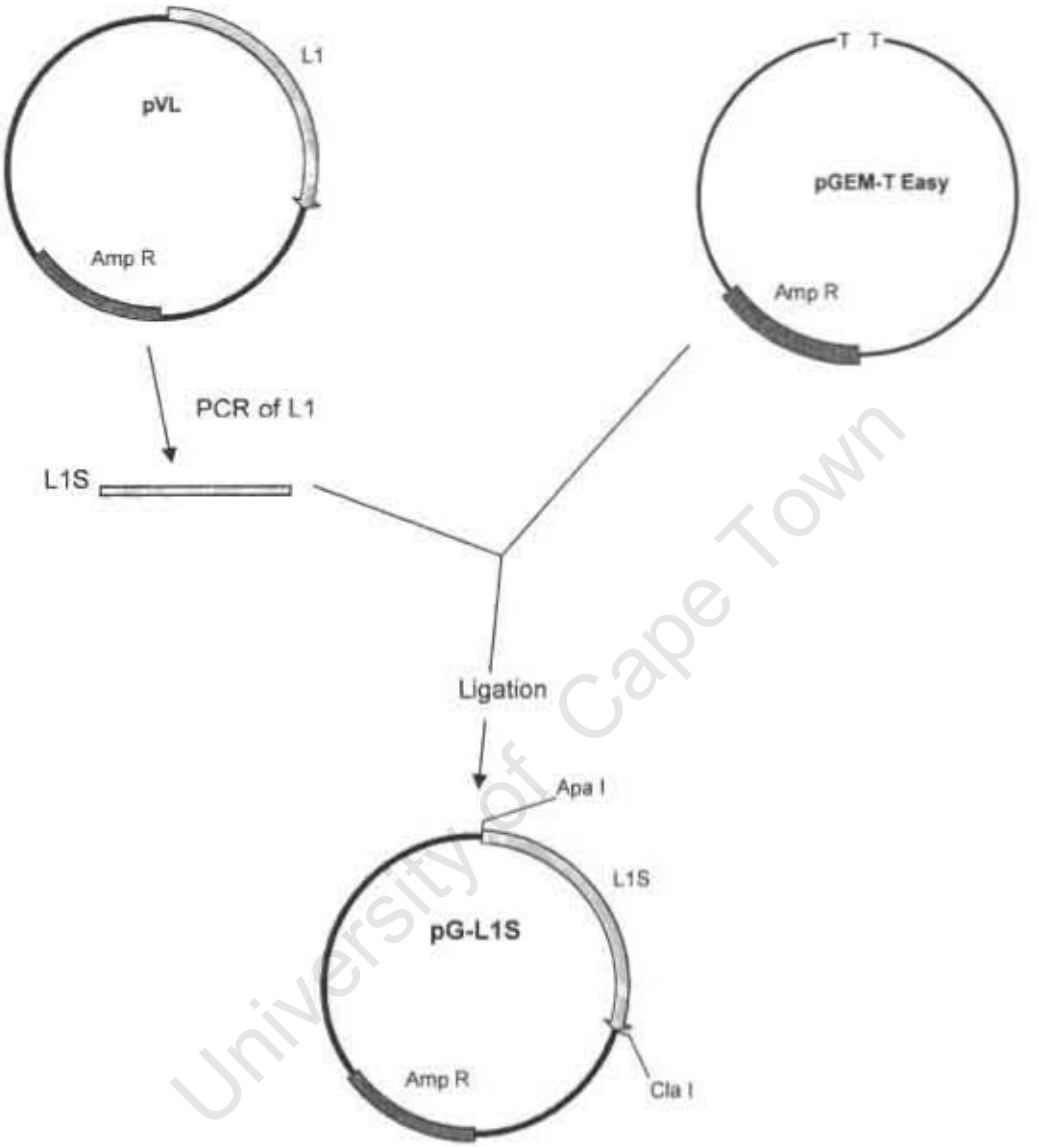


Figure D4. An illustration outlining the PCR and cloning of the L1S gene, from which the termination codon had been removed. The L1 gene was cloned into pGEM-T, forming pG-L1S.

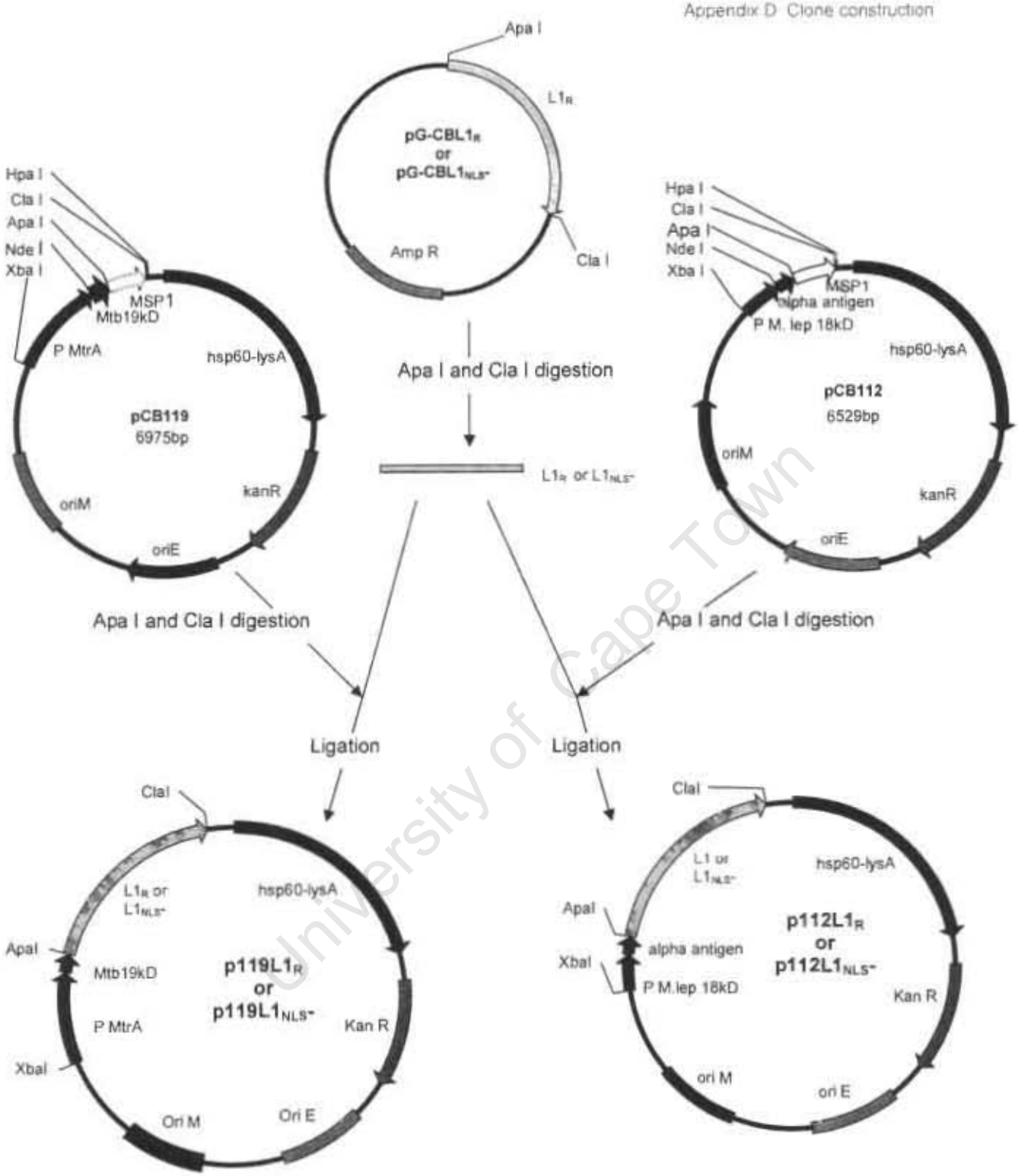


Figure D5. An illustration outlining the strategy used to clone $L1_R$ and $L1_{NLS^-}$, into pCB119 and pCB112.

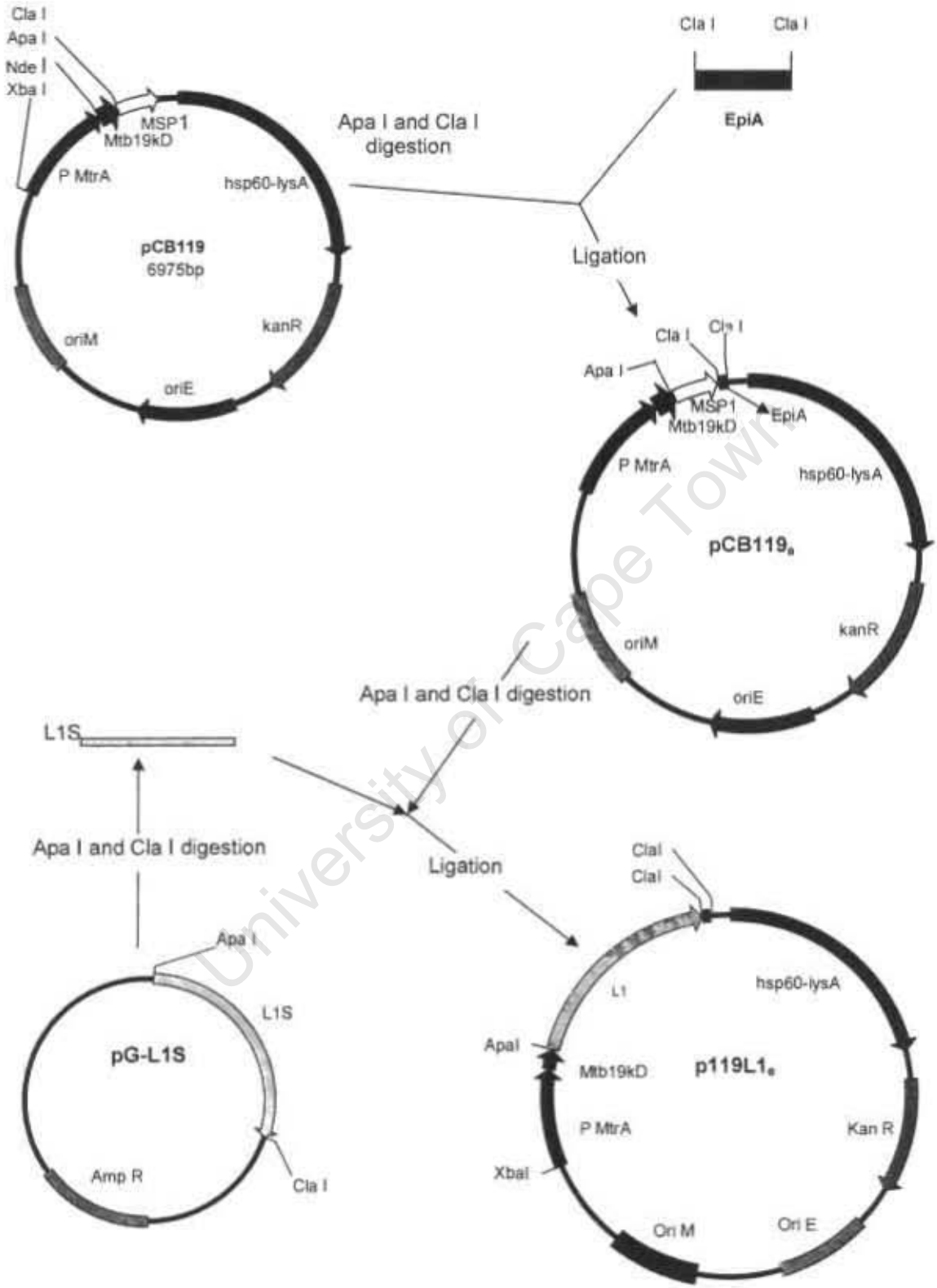


Figure D6. An illustration outlining the cloning of the L1S gene into pCB119$_e$ (containing the EpiA tag), forming p119L1$_e$.

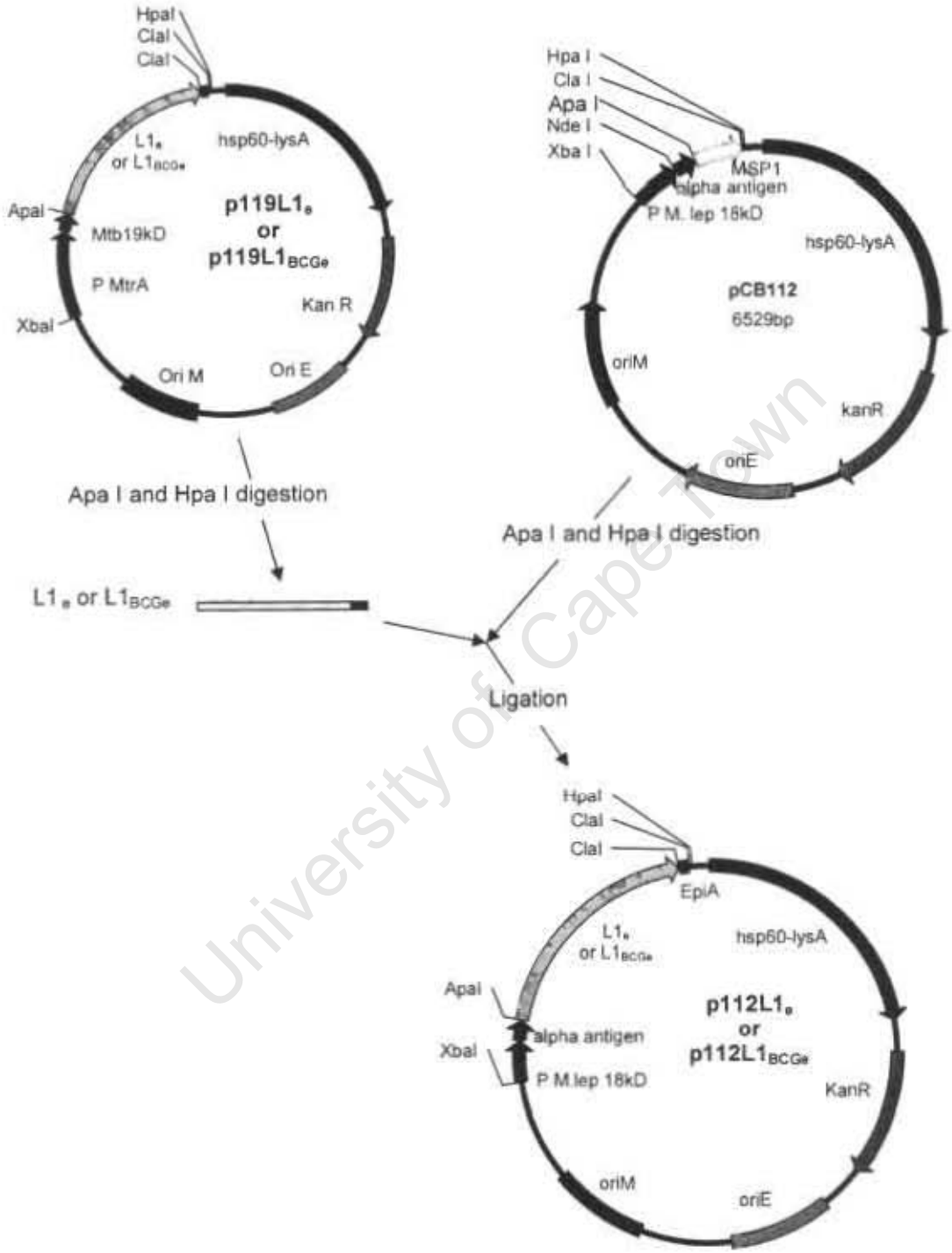


Figure D7. An illustration outlining the cloning of $L1_e$ or $L1_{BCGe}$ into pCB112; forming $p112L1_e$ or $p112L1_{BCGe}$, respectively.

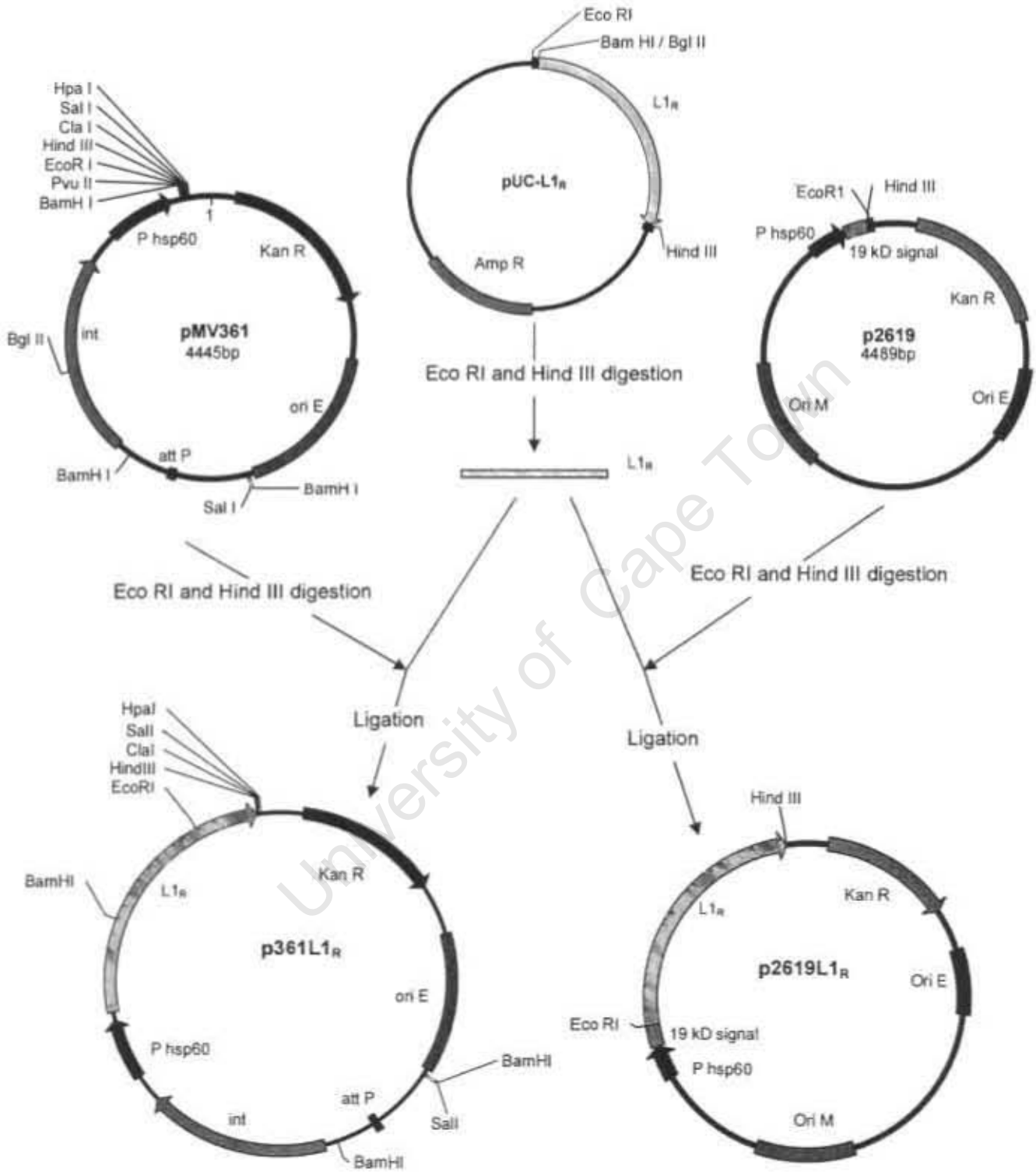


Figure D8. An illustration outlining the strategy used to clone $L1_R$, from pUC-$L1_R$ into pMV361 and p2619, to form p361$L1_R$ and p2619$L1_R$, respectively.

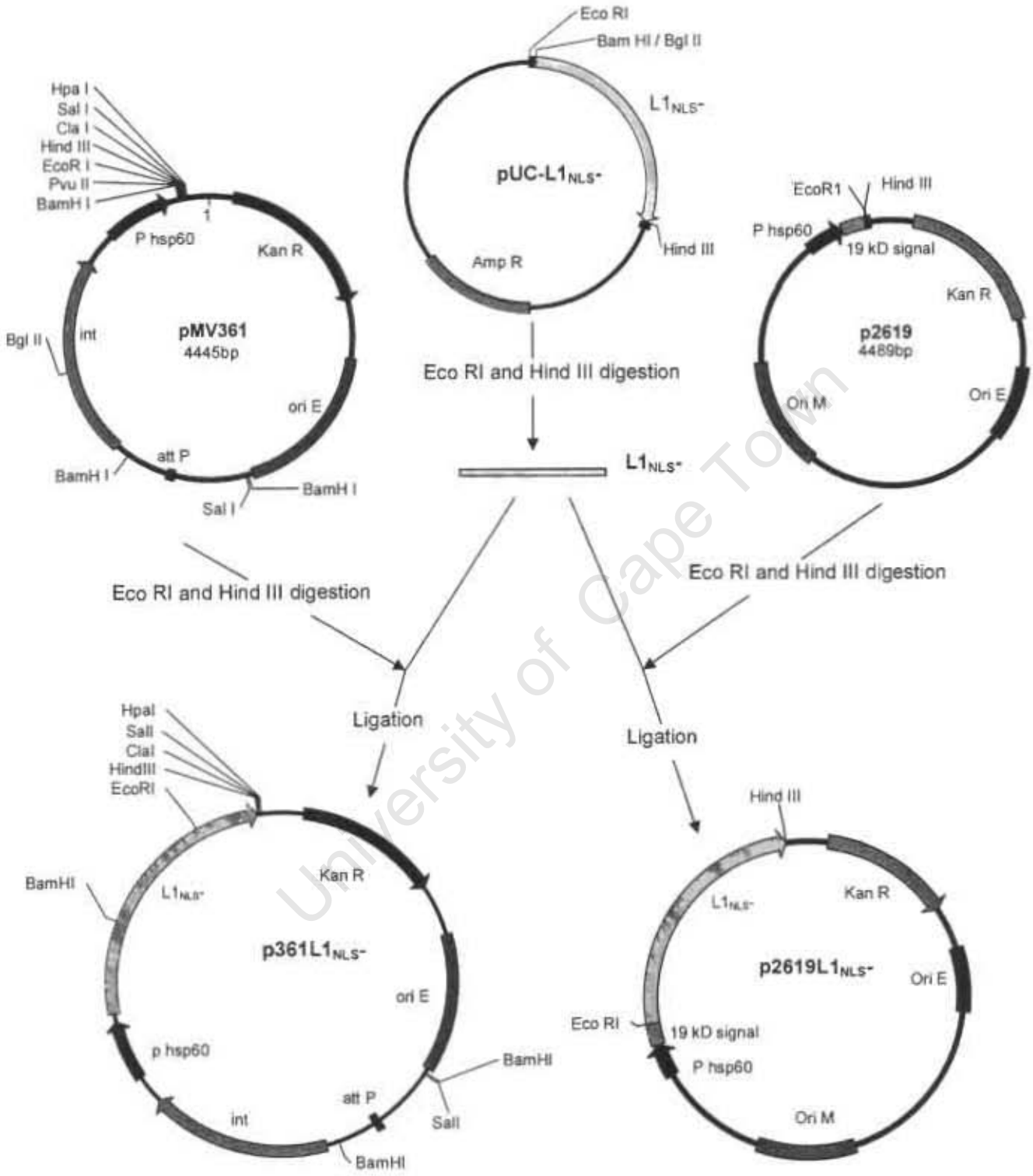


Figure D9. An illustration outlining the strategy used to clone $L1_{NLS^-}$, from pUC-$L1_{NLS^-}$ into pMV361 and p2619, to form p361$L1_{NLS^-}$ and p2619$L1_{NLS^-}$, respectively.

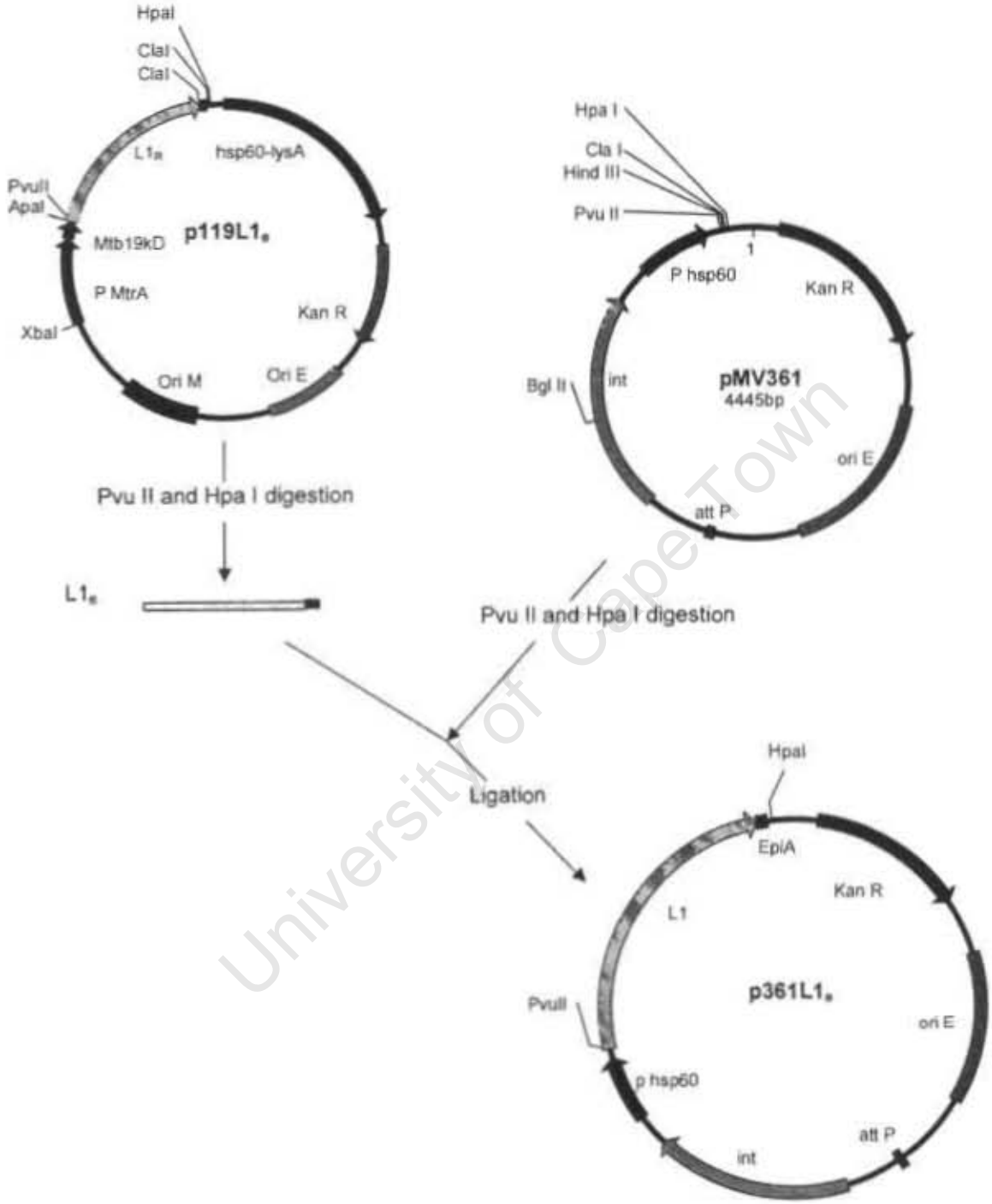


Figure D10. An illustration outlining the cloning of $L1_e$ or $L1_{BCGe}$ into pMV361; forming $p361L1_e$ or $p361L1_{BCGe}$, respectively.

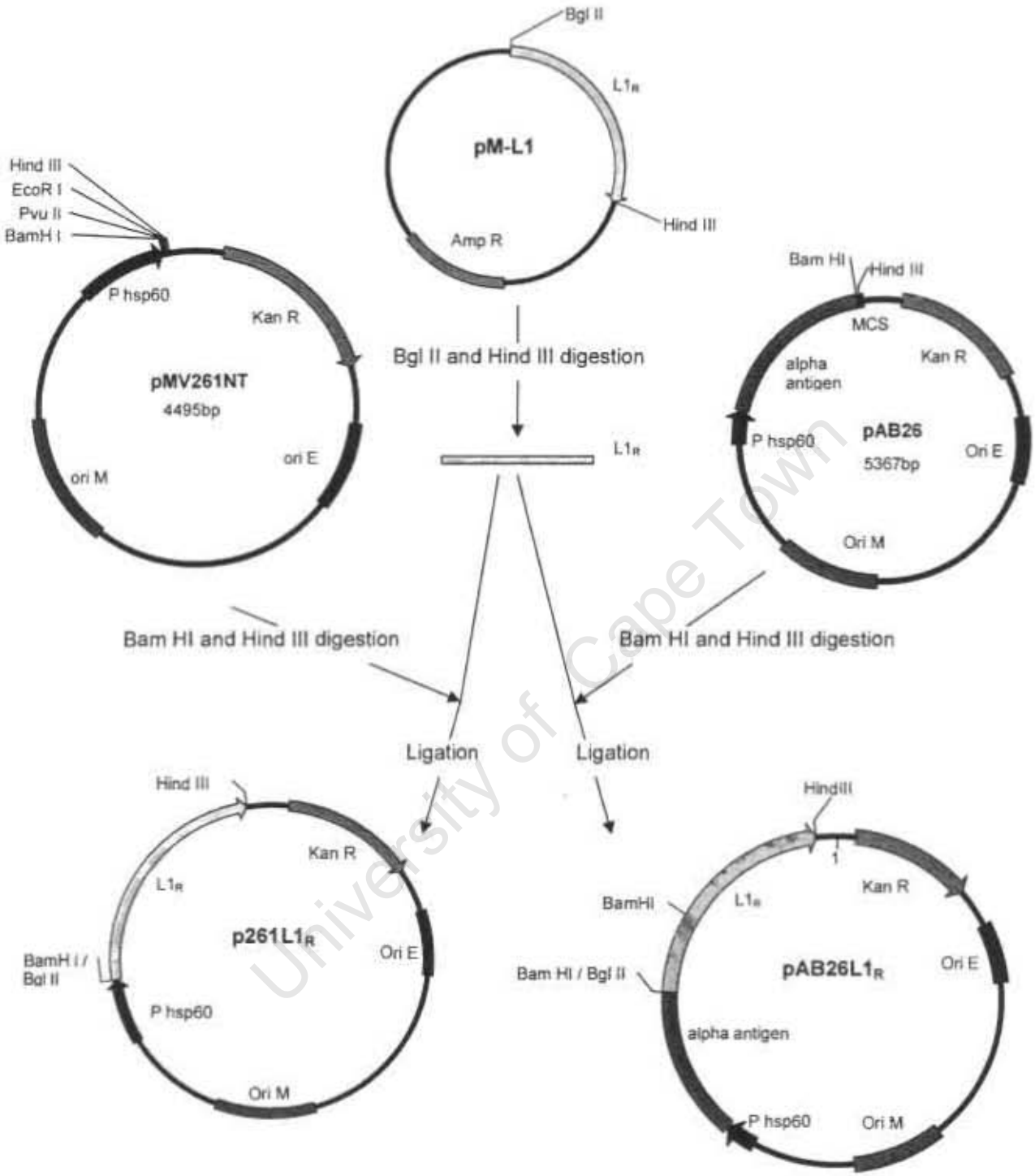


Figure D11. An illustration outlining the strategy used to clone $L1_R$, from pM-L1, into pAB26 and pMV261NT, to form the clones pAB26L1 and p261L1, respectively.

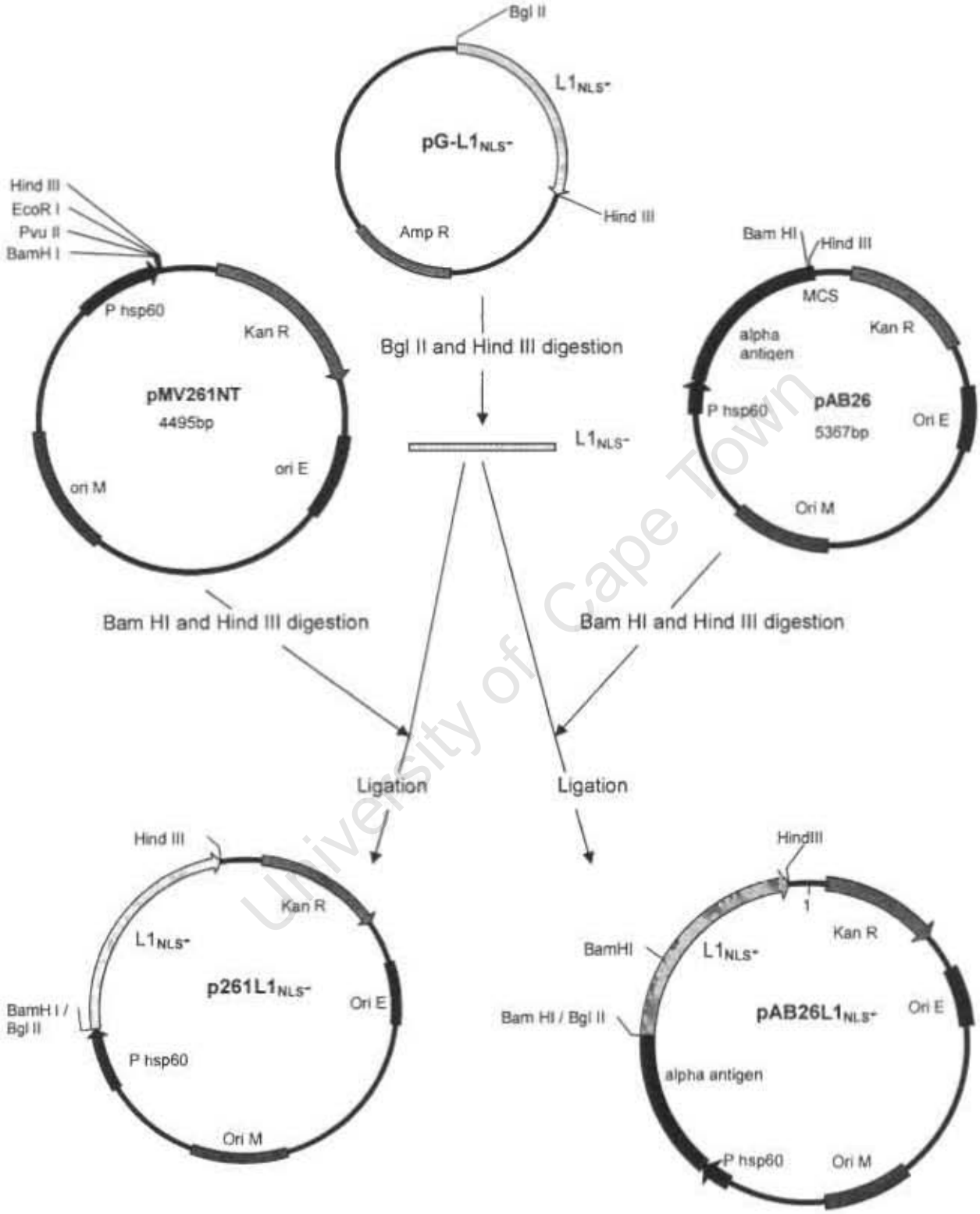


Figure D12. An illustration outlining the strategy used to clone $L1_{NLS}$- from pG-$L1_{NLS}$-, into pAB26 and pMV261NT, to form the clones pAB26$L1_{NLS}$- and p261$L1_{NLS}$-, respectively.

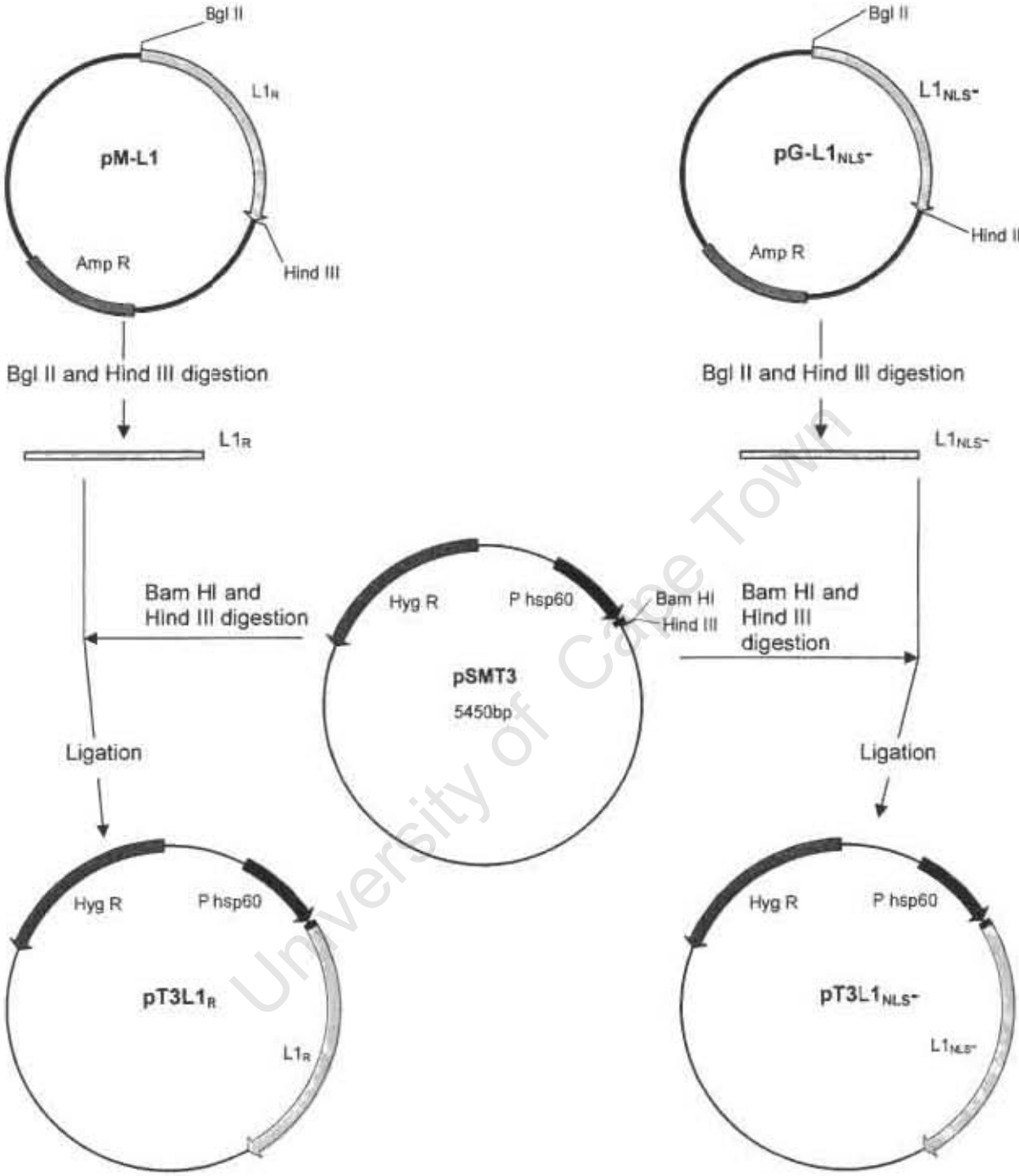


Figure D13. An illustration outlining the strategy used to clone the $L1_R$ and $L1_{NLS^-}$, into pSMT3, forming $pT3L1_R$ and $pT3L1_{NLS^-}$, respectively.

APPENDIX E: HPV-16 L1 SEQUENCES

HPV-16 L1 amino acid sequence alignment:

```
Phil        MSLWLPSEATVYLPPVPVSKVVSTDEYVARTNIYYHAGTSRLLAVGHPYFPIKKPNNNKI   60
Sen32       ------------------------------------------------------------   60
L1R         ------------------------------------------------------------   60
L1nls-      ------------------------------------------------------------   60
L1bcg       ------------------------------------------------------------   60

Phil        LVPKVSGLQYRVFRIHLPDPNKFGFPDTSFYNPDTQRLVWACVGVEVGRGQPLGVGISGH  120
Sen32       --------------y-----------------------------------------------  120
L1R         --------------y-----------------------------------------------  120
L1nls-      --------------y-----------------------------------------------  120
L1bcg       ------------------------------------------------------------  120

Phil        PLLNKLDDTENASAYAANAGVDNRECISMDYKQTQLCLIGCKPPIGEHWGKGSPCTNVAV  180
Sen32       ----------------------------------------------------n-------  180
L1R         ----------------------------------------------------n-------  180
L1nls-      ----------------------------------------------------n-------  180
L1bcg       ------------------------------------------------------------  180

Phil        NPGDCPPLELINTVIQDGDMVDTGFGAMDFTTLQANKSEVPLDICTSICKYPDYIKMVSE  240
Sen32       ------------------------------------------------------------  240
L1R         t----------------------s-------------------------------------  240
L1nls-      t----------------------s-------------------------------------  240
L1bcge      ------------------------------------------------------------  240

Phil        PYGDSLFFYLRREQMFVRHLFNRAGTVGENVPDDLYIKGSGSTANLASSNYFPTPSGSMV  300
Sen32       -------------------------a----------------------------------  300
L1R         -------------------------a----------------------------------  300
L1nls-      -------------------------a----------------------------------  300
L1bcg       ------------------------------------------------------------  300

Phil        TSDAQIFNKPYWLQRAQGHNNGICWGNQLFVTVVDTTRSTNMSLCAAISTSETTYKNTNF  360
Sen32       ------------------------------------------------------------  360
L1R         ---------------------------------------------------p--------  360
L1nls-      ---------------------------------------------------p--------  360
L1bcg       ------------------------------------------------------------  360

Phil        KEYLRHGEEYDLQFIFQLCKITLTADVMTYIHSMNSTILEDWNFGLQPPPGGTLEDTYRF  420
Sen32       ------------------------------------------------------------  420
L1R         --------------------------s---------------------------------  420
L1nls-      --------------------------s---------------------------------  420
L1bcg       ------------------------------------------------------------  420

Phil        VTSQAIACQKHTPPAPKEDPLKKYTFWEVNLKEKFSADLDQFPLGRKFLLQAGLKAKPKF  480
Sen32       ------------------------------------------------------f-----  480
L1R         ------------------------------------------------------f-----  480
L1nls-      ------------------------------------------------------f-----  480
L1bcg       ------------------------------------------------------------  480

Phil        TLGKRKATPTTSSTSTTAKRKKRKL*                                    505
Sen32       -------------------------*                                    505
L1R         -------------------------*                                    505
L1nls-      ---*                                                          483
L1bcg       -------------------------                                     505
```

Nucleotide sequence of BCG-codon-optimised HPV-16 L1 ($L1_{BCG}$):

```
      HindIII      ApaI PvuII  L1 start codon
        |            |   |        |
1     AAGCTTTCGG GCCCGCAGCT GACATGTCGC TGTGGCTGCC GTCGGAGGCC ACCGTGTACC
61    TGCCGCCGGT GCCGGTGTCG AAGGTGGTGT CGACCGACGA GTACGTGGCC CGCACCAACA
121   TCTACTACCA CGCCGGCACC TCGCGCCTGC TGGCCGTGGG CCACCCGTAC TTCCCGATCA
181   AGAAGCCGAA CAACAACAAG ATCCTGGTGC CGAAGGTGTC GGGCCTGCAG TACCGCGTGT
241   TCCGCATCCA CCTGCCGGAC CCGAACAAGT TCGGCTTCCC GGACACCTCG TTCTACAACC
301   CGGACACCCA GCGCCTGGTG TGGGCCTGCG TGGGCGTGGA GGTGGGCCGC GGCCAGCCGC
361   TGGGCGTGGG CATCTCGGGC CACCCGCTGC TGAACAAGCT GGACGACACC GAGAACGCCT
421   CGGCCTACGC CGCCAACGCC GGCGTGGACA ACCGCGAGTG CATCTCGATG GACTACAAGC
481   AGACCCAGCT CTGCCTGATC GGCTGCAAGC CGCCGATCGG CGAGCACTGG GGCAAGGGCT
541   CGCCGTGCAC CAACGTGGCC GTGAACCCGG GCGACTGCCC GCCGCTGGAG CTGATCAACA
601   CCGTGATCCA GGACGGCGAC ATGGTGGACA CCGGCTTCGG CGCCATGGAC TTCACCACCC
661   TGCAGGCCAA CAAGTCGGAG GTGCCGCTGG ACATCTGCAC CTCGATCTGC AAGTACCCGG
721   ACTACATCAA GATGGTGTCG GAGCCGTACG GCGACTCGCT GTTCTTCTAC CTGCGCCGCG
781   AGCAGATGTT CGTGCGCCAC CTGTTCAACC GCGCCGGCAC CGTGGGCGAG AACGTGCCGG
841   ACGACCTGTA CATCAAGGGC TCGGGCTCGA CCGCCAACCT GGCCTCGTCG AACTACTTCC
901   CGACCCCGTC GGGCTCGATG GTGACCTCGG ACGCCCAGAT CTTCAACAAG CCGTACTGGC
961   TGCAGCGCGC CCAGGGCCAC AACAACGGCA TCTGCTGGGG CAACCAGTTG TTCGTGACCG
1021  TGGTGGACAC CACCCGCTCG ACCAACATGT CGCTGTGCGC CGCCATCTCG ACCTCGGAGA
1081  CCACCTACAA GAACACCAAC TTCAAGGAGT ACCTGCGCCA CGGCGAGGAG TACGACCTGC
1141  AGTTCATCTT CCAGCTCTGC AAGATCACCC TGACCGCCGA CGTGATGACC TACATCCACT
1201  CGATGAACTC GACCATCCTG GAGGACTGGA ACTTCGGCCT GCAGCCGCCG CCGGGCGGCA
1261  CCCTGGAGGA CACCTACCGC TTCGTGACCT CGCAGGCCAT CGCCTGCCAG AAGCACACCC
1321  CGCCGGCCCC GAAGGAGGAC CCGCTGAAGA AGTACACCTT CTGGGAGGTG AACCTGAAGG
1381  AGAAGTTCTC GGCCGACCTG GACCAGTTCC CGCTGGGCCG CAAGTTCCTG CTGCAGGCCG
1441  GCCTGAAGGC CAAGCCGAAG TTCACCCTGG GCAAGCGCAA GGCCACCCCG ACCACCTCGT
1501  CGACCTCGAC CACCGCCAAG CGCAAGAAGC GCAAGCTGCA TCGAT
                                          |
                                         ClaI
```

APPENDIX F: SUMMARY OF rBCG-L1 EXPRESSION AND IMMUNITY

Table F1. Summary of rBCG-L1 expression and immunity in guinea pigs

Recombinant	L1 mRNA expression	Anti-L1 antibody response in serum	DTH skin reaction to L1	Anti-L1 antibody response in genital secretions
Tokyo p361L1$_R$	nd	++	nd	nd
Tokyo p361L1$_e$	+	+++	+++	+
Tokyo p119L1$_{NLS^-}$	nd	++	nd	-
Tokyo p119L1$_e$	+++	+++	+++	++
Tokyo p119L1$_{BCGe}$	nd	++	+++	+
Tokyo p112L1$_{NLS^-}$	nd	++	+++	-
Tokyo p112L1$_e$	++	-	+++	-
Tokyo p112L1$_{BCGe}$	nd	++	+++	-

- , not significantly positive; +, low; ++, moderate; +++, high; nd, not determined

Table F2. Summary of rBCG-L1 expression and immunity in mice

Recombinant	L1 mRNA expression	Anti-L1 antibody response in serum	Level of protection from rVV-L1 challenge
Tokyo p361L1$_R$	nd	-	++
Tokyo p361L1$_{NLS^-}$	nd	-	+++
Tokyo pNVL1$_R$	nd	++	+
Pasteur p361L1$_e$	+	+++	+++
Pasteur p119L1$_e$	+++	-	+++

- , not significantly positive; +, low; ++, moderate; +++, high; nd, not determined

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