PRE-CLINICAL ASSESSMENT OF NOVEL CANDIDATE HIV-1 VACCINES USING THE CHACMA BABOON

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DECLARATION

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

Signed by candidate ....

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June 2006

ABSTRACT

Evaluation of HIV vaccines in animal models is an essential part of vaccine development because it generates an important part of the preclinical data upon which applications for approval for human trials of new vaccines are based. The chacma baboon (*Papio ursinus*), which is an abundant species in the wild in southern Africa, could be developed into a realistic and inexpensive animal model for pre-clinical testing of HIV vaccines. This thesis describes immunogenicity evaluation of novel vaccines which are based on HIV-1 subtype C using the chacma baboon model.

This project was undertaken as part of the efforts of the South African AIDS Vaccine Initiative (SAAVI)-funded HIV vaccine development group at the University of Cape Town to develop locally relevant HIV-1 vaccines for southern Africa. A number of 1st generation HIV-1 *gag* vaccines and one multigene vaccine had shown potent immunogenicity in the mouse model and thus, they were selected for further testing in the baboon. These vaccines were derived from the *gag* gene of ZDU422, a HIV-1 subtype C primary isolate that shows the closest amino acid sequence to the consensus sequence of recently transmitted HIV-1 subtype C viruses from southern Africa. One *gag* DNA (pTH-GagC), two rBCG-HIV-1 *gag* (rBCG:RT106 and rBCG:RT108), a PR55^{gag} virus-like particle (GagC VLP) and a multigene rMVA (SAAVI MVA-C) vaccines were used in prime-boost vaccination protocols in the baboon.

This project started by developing immunological methods. To achieve this and gather preliminary immunogenicity data, two baboons were intramuscularly inoculated six times with pTH-GagC (5mg per animal), followed by a single inoculation with 24 μg of GagC VLP (DNA Exp1). Several reagents were identified and verified as useful for development of HIV-1-specific antibody ELISA to detect humoral responses, and IFN-γ ELISPOT and intracellular IFN-γ assays to detect cellular responses, thereby justifying the use of chacma baboon for pre-clinical development of HIV-1 vaccines.

This was followed by a second experiment (DNA Exp2), whose objective was to gather more immunogenicity data on these vaccines. Five baboons were vaccinated with pTH-GagC, three times, four weeks apart to reflect an immunisation regimen commonly used in clinical trials which utilise DNA vaccines to prime the immune system. A dose of 5 mg per animal was given bilaterally by intramuscular injection. This group was boosted twice with 29 μg of GagC VLP. The development of humoral and cellular immune responses at several time points were monitored using the newly established methods.

The immunogenicity data generated from these two experiments revealed a close similarity in the development of immune responses. In both, immunisation with pTH-GagC induced weak HIV-1 Gag-specific antibody and IFN-γ ELISPOT responses, which were efficiently boosted by a single GagC VLP immunisation. The range of peak cumulative response in the IFN-γ ELISPOT assay after pTH-GagC prime was 130-280 and 217-515 SFU/10^6 PBMC for DNA Exp1 and DNA Exp2 respectively indicating that increasing the number of DNA inoculations does not influence the extent of the immune response. A boost with a single GagC VLP inoculation increased the response of baboons in DNA Exp1 and DNA Exp2 to Gag to 1262-2254 and 775-3583 SFU/10^6 PBMC respectively. The response in the IFN-γ ELISPOT assay after the GagC VLP vaccination was to an average of 9 Gag peptides located across all 3 regions of Gag protein. Several of the peptides recognised by baboon PBMC have been reported to be targeted by PBMC from patients with early HIV-1 subtype C infection, indicating the relevance of ZDU422 isolate as a vaccine strain. The phenotype of the cells participating in this response was demonstrated in one GagC VLP-immunised baboon and shown to be predominantly of the CD8+ T cell type. In addition, an array of cytokines investigated for baboons in DNA Exp2 using a CBA kit showed that IL-2, IFN-γ and TNFα were the major cytokines produced by PBMC upon stimulation with specific Gag peptides, indicating that the immune responses to these vaccinations are Th1-biased, which is desirable for T-cell vaccines. These two experiments therefore showed that GagC VLP is efficient in boosting and broadening the immune response induced by a matched DNA vaccine. Additional long-term observation in two of the baboons showed that these responses were detected up to 89 weeks after GagC VLP inoculation. The IFN-γ ELISPOT responses to Gag in the present study are comparable to other vaccine strategies which have entered phase 1 human clinical trials, suggesting that our vaccines pTH-GagC together with GagC VLP in a prime boost regimen may be very successful if tested in similar trials.

The next phase of this project assessed the immunogenicity of rBCG vaccines that were developed in our laboratory. Because of lack of information in the literature in respect to BCG-specific cellular and humoral responses of chacma baboons, a study, whose objective was to investigate the components of the immune response of chacma baboons following vaccination with *Tokyo* and *Pasteur* strains of BCG, was conducted. Two baboons per group were inoculated twice intradermally with either BCG-*Pasteur* or varying doses of BCG-*Tokyo*. Immunisations with BCG resulted in development of local cutaneous ulcerations which healed without medication. Data obtained from this study showed that BCG-specific cell-mediated responses were characterised by a strong PPD-specific DTH, BCG-specific cellular proliferation and IFN-γ production. The magnitudes of these responses appeared to be dose-dependent. An IFN-γ ELISPOT assay detected PPD-specific IFN-γ producing cells beyond 2 years of the last BCG inoculation and flow cytometric investigation of the phenotype of PBMC

producing this cytokine in one of the immunised baboons revealed that it was CD4 cell-mediated. These data support the use of chacma baboons for testing rBCG-based vaccines.

The potential of rBCG-HIV-1 *gag* vaccines to prime the immune system was investigated in a series of two experiments. In the 1st experiment (BCG Exp1), baboons were inoculated four times intradermally with either rBCG:RT106 or rBCG:RT108 and finally boosted twice with GagC VLP one year after the last rBCG inoculation. Gag-specific humoral responses were largely undetectable following rBCG immunisations but were consistently detected after a boost with GagC VLP. An IFN-γ ELISPOT response to Gag was detected for only one baboon (peak response: 157 SFU/10^6 PBMC) after rBCG:RT106 inoculation. However, after the booster immunisation with GagC VLP, strong to moderate responses were detected for all the baboons that were primed with rBCG:RT106 (range: 542-812 SFU/10^6 PBMC) and rBCG:RT108 (range: 156-391 SFU/10^6 PBMC). Results from this study indicated that rBCG:RT106 was more efficient than rBCG:RT108 in priming the immune response for a GagC VLP booster immunisation. To take advantage of the better immunogenicity exhibited by rBCG:RT106, a second experiment (BCG Exp2) was conducted using an inoculation regimen that would be more representative of a regimen that would be used in human clinical trials. Six baboons were primed with two rBCG:RT106 inoculations then boosted with a single inoculation of SAAVI MVA-C (10^8 PFU) followed by a further boost with GagC VLP in a triple vector vaccination protocol. SAAVI MVA-C is a multigene HIV-1 subtype C vaccine expressing a matched HIV-1 Gag and was incorporated into this vaccination regimen as it is soon to be used in human clinical trials in South Africa and the USA. This vaccine has already been shown by our group in a separate study from this one, to be extremely effective in boosting immune responses induced by a matching multigene DNA vaccine in the chacma baboon.

In BCG Exp2, cellular responses to Gag were negative after the rBCG:RT106 inoculation and generally of low magnitude in the majority of baboons after immunisation with SAAVI MVA-C but the subsequent immunisation with GagC VLP was able to boost these responses (range: 86 to 3852 SFU/10^6 PBMC). There was no statistical difference in the mean cumulative IFN-γ ELISPOT response between rBCG:RT106-primed and wt-BCG mock-primed baboons, indicating that two primary inoculations with rBCG:RT106 was not sufficient to prime the immune system.

In BCG Exp1, an IFN-γ ELISPOT response to Gag was detected in four baboons after 37-50 weeks after the GagC VLP boost, demonstrating the longevity of these responses. Also, peptide mapping of IFN-γ responses showed that 5-8 peptides contained in 3 or 4 peptide pools were recognised by baboon PBMC. All the peptides that were recognised belong to the p24 and p2p7p1p6 regions but not p17 Gag protein. Several of these peptides have been reported to be recognised by PBMC from

patients with early HIV-1 subtype C infection, similar to what was observed for baboons immunised with pTH-GagC and GagC VLP vaccines in DNA expl and DNA exp2. For both BCG Expl and Exp2, flow cytometric analysis revealed that these IFN-γ responses were mediated predominantly by the CD8+ T cells. In addition, an investigation of spectrum of cytokines produced by PBMC for baboons in BCG Exp2 showed that IFN-γ, IL-2 and TNFα were the major cytokines produced upon stimulation of PBMC with specific Gag peptides, pointing to Th1-biased immune response. Overall, it was shown that GagC VLP was effective in boosting the immune responses that were induced by rBCG:RT106, rBCG:RT108 or SAAVI MVA-C. While these findings support the advancement of GagC VLP to clinical evaluation, they also suggested that both rBCG:RT106 and rBCG:RT108 are not sufficiently immunogenic to warrant further evaluation.

In conclusion, the present studies succeeded in developing the chacma baboon as an animal model for testing the immunogenicity of HIV-1 vaccines. The immunogenicity data generated using this model showed that GagC VLP is efficient in boosting immune responses that are primed with pTH-GagC. These responses were strong and broadly directed and CD8+ T cell-mediated and with a bias towards a Th1 type cytokine response. As these responses are comparable with those generated by other vaccines already in phase 1 clinical trials, the present studies strongly recommend the advancement of GagC VLP to evaluation in clinical trial. In addition, the potential use of GagC VLP as a booster vaccine in a prime-boost combination with either rBCG:RT106, rBCG:RT108, SAAVI MVA-C or both rBCG:RT106 and SAAVI MVA-C (triple vector) was demonstrated, further supporting the movement of GagC VLP to clinical evaluation. Data from the present study further showed that both rBCG:RT106 and rBCG:RT108 were not sufficiently immunogenic to warrant consideration for clinical evaluation at this stage but their potential use in priming the immune system needs more investigation after further development to improve their immunogenicity.

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ABBREVIATIONS

aa	amino acid(s)
AB	antibody (ies)
AGM	African green monkey
AIDS	acquired immunodeficiency syndrome
AP	alkaline phosphate
APC	antigen presenting cell(s)
-APC	allophycocyanin conjugate
ART	antiretroviral therapy
BCG	*Mycobacterium bovis* bacilli Calmette-Guerin
BSA	bovine serum albumin
CAF	CD8 cell antiviral factor
CBA	cytometric bead array
CCR5	C-C chemokine receptor 5
CD	cluster of differentiation
CFU	colony-forming units
cm	centimetres
CO_2	carbon dioxide
CTL	cytotoxic T lymphocytes
cpz	chimpanzee
CXCR4	C-X-C chemokine receptor 4
dH_2O	de-ionised water
DMSO	dimethyl sulphoxide
DNA	deoxyribonucleic acid
DTH	delayed-type hypersensitivity
E. coli	*Escherichia coli*
EDTA	ethyllendiamine tetra-acetic acid
ELISA	enzyme-linked immunosorbent assay
ELISPOT	enzyme-linked immunospot
env	envelope
FACS	fluorochrome activated cell sorting
FBS	foetal bovine serum
FCS	foetal calf serum
-FITC	fluorescein isothyocyanate
g	grams
Gag	structural protein of lentiviruses
HCl	hydrochloric acid
HIV	human immunodeficiency virus
HLA	human leukocyte antigen
HPV	human papillomavirus
h	hour(s)
HRP	horseradish peroxidase
hsp	heat-shock protein
IAVI	International AIDS Vaccine Initiative
i.d.	intradermal
i.m.	intramuscular
i.n.	intranasal
i.p.	intraperitoneal
i.v.	intravenous
ICS	intracellular cytokine staining
IFN	interferon
Ig	immunoglobulin
IL	interleukin
IPR	Institute of Primate Research
IU	international unit(s)
kD	kiloDaltons
L1	major capsid protein of HPV
LTNP	long-term non-progressor(s)
LTR	long terminal repeat
M	*Mycobacterium*
M	molar (moles per litre)
mac	macaque
mAB	monoclonal antibody(ies)
mg	milligram(s)
MHC	major histocompatibility complex
min	minute(s)
MIP	macrophage inflammatory protein
mL	millilitre(s)
mm	millimetre(s)
mM	millimolar(s)
mRNA	messenger ribonucleic acid
MVA	modified vaccinia virus Ankara
N	normal
NaCl	sodium chloride
NaOH	sodium hydroxide
ng	nanogram(s)
NHP	nonhuman primates
NK	natural killer cells
NKT	natural killer T cells
NMK	National Museums of Kenya
°C	degrees Celsius
OD	optical density
p	protein
PBMC	peripheral blood mononuclear cell(s)
PBS	phosphate-buffered saline
PCR	polymerase chain reaction
-PE	phycoerythrin conjugate
-PerCP	peridinin chlorophyll conjugate
PFU	plaque-forming units
pg	picogram(s)
pH	hydrogen potential
PPD	purified protein derivative of tuberculin
pol	polymerase
RANTES	regulated upon activation, normal T-cell expressed and secreted
rBCG	recombinant BCG
rMVA	recombinant MVA
RNA	ribonucleic acid
RNAse	ribonuclease
rcf	relative centrifugation force (*xg*)
rpm	revolutions per minute
SAAVI	South African AIDS Vaccine Initiative
s.c.	subcutaneous
SDS	sodium dodecyl sulphate
sec	second(s)
SHIV	simian-human immunodeficiency virus
SIV	simian immunodeficiency virus

sm	sooty mangabey
TB	tuberculosis
Th1/2	helper cell type 1/type 2
TMB	tetramethyl benzidine
TNF	tumour necrosis factor
U	unit(s)
UCT	University of Cape Town
UNAIDS	Joint United Nations Programme on HIV/AIDS
µg	microgram(s)
µL	microlitre(s)
µM	micromolar
v/v	volume per volume
VLP	virus-like particles
vpu	viral protein u
vpr	viral protein r
vpx	viral protein x
w/v	weight per volume
WHO	World Health Organisation
wk	week(s)
yr	year(s)
ZN	Ziehl-Neelson

CHAPTER ONE

OVERVIEW AND LITERATURE REVIEW

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1.1. HIV INFECTIONS IN HUMANS

1.1.1. HIV/AIDS pandemic

Since the first case of human immunodeficiency virus/acquired immunodeficiency syndrome (HIV/AIDS) was reported in 1981, HIV has infected more than 60 million people and over 25 million of them have died of HIV/AIDS related infections (UNAIDS, 2005). Currently, an estimated 40.3 million people have HIV infection (UNAIDS, 2005). The biggest burden of HIV infections is in sub-Saharan Africa where 10% of world's population reside but carries >60% of global HIV infections. Although the number of people living with HIV/AIDS has continued to increase with close to 5 million people getting newly infected in 2005 alone, the overall trends seem to be stabilising (UNAIDS, 2005). The development of effective anti-retroviral drugs have had a major impact in controlling the viraemia and effectively delaying the onset of AIDS in the industrialised countries but the same has not been realised in developing countries due to poverty. In these resource-poor countries, there have been concerted efforts in provision of condoms and community education on prevention. Also, provision of anti-retroviral therapy (ART) has increased in the last 5 years, both in scope and scale (UNAIDS, 2004) but data show that these efforts have not resulted in slowing of the AIDS pandemic in the majority of these regions. The stabilisation in adult HIV prevalence that has been seen in recent years is thought to be a sign for the worsening situation of the epidemic, when the number of people getting newly infected with HIV roughly equals the number of people that are dying of AIDS-related causes (UNAIDS, 2004). Development of preventive HIV vaccine(s) is considered the only long-term hope to stop and control the HIV/AIDS epidemic (Esparza & Bhamarapravati, 2000).

1.1.2. Classification of HIV

HIV, the aetiological cause of AIDS, is categorised into HIV-1 and HIV-2. Both have similar biological properties though they differ significantly in some regions of their genomes and serological reactivity. HIV-1 is by far more widely distributed and more pathogenic than HIV-2 and nearly all cases of AIDS in human are caused by HIV-1.

HIV-1 is classified into three groups, namely group M (main), group O (outlier), and group N (non-M, non-O). Greater than 99% of HIV infections are caused by Group M viruses, which are further divided into 9 pure subtypes or clades, which are designated subtype A, B, C, D, F, G, H, J and K. Group M viruses also include 16 circulating recombinant forms (CRF) and several unique recombinant forms (McCutchan, 2006; Robertson, 2000). Subtypes E and I, which were originally classified, as pure subtypes are now known to be recombinants. Thus, subtype E is now designated CRF01_AE and subtype I is CRF04_cpx. Subtypes are unevenly distributed in Africa. Subtypes A and CRF02_AG (and also group N and O viruses) are found in West Africa, subtypes A, D and C in

eastern Africa, subtype C in southern Africa (also in Ethiopia) and subtypes A, F, G, H, J, K and a host of CRFs in central Africa (McCutchan, 2000; Simon *et al*, 1998; Thomson *et al*, 2002). Subtype C accounts for about 56% of HIV-1 infections globally (Esparza & Bhamarapravati, 2000) and >90% of infections in South Africa. Other areas outside Africa with distinct clusters of subtype C include India, Brazil and China.

1.1.3. HIV preventive vaccines

The hallmark of a successful prophylactic vaccination is induction a memory immune response that is effective in preventing the development of an overt disease in the immunised subject. Thus, an effective HIV vaccine should ideally generate immune responses that are sufficient to prevent infection or to completely clear the virus during early infection to provide a sterilising immunity. Studies have shown that neutralising antibodies (humoral) and cell-mediated (cellular) immune responses are important in preventing HIV infection and controlling the virus replication. The neutralising antibodies, which are directed to the outer coat protein of the virus, thus preventing HIV from entering and infecting the cells, are produced by B cells. The cellular response is mediated by CD8+ cytotoxic T lymphocytes (CTL), which have the ability to recognise and destroy HIV-infected cells. These CTL are assisted by CD4+ T helper cells, which provide the growth factors and signals that are essential in generating and maintaining functional CTL.

Currently, no prophylactic vaccine is available for protection against HIV infections. However, efforts to develop safe, effective and affordable vaccines are underway. To date, over 30 candidate HIV vaccines have been evaluated in over 60 phase 1 and 2 clinical trials (IAVI, 2006). Of these, two candidate vaccines (AIDSVAX B/B and AIDSVAX B/E) have completed phase 3 efficacy trials but did not demonstrate protection (Flynn *et al*, 2005) while two vaccines, in combined immunisation modality, are currently undergoing a phase 3 clinical trial (Pitisuttithum, 2005; Trinvuthipong, 2004). Several other vaccines, which appear to be very promising, particularly the live viral vectored ones, are at various levels of development (Spearman, 2006).

A fundamental question that has been asked is: 'Is development of a vaccine against AIDS possible?' In a review, which addresses this question, McMichael & Hanke (2003) gave a chronological progress in HIV vaccine development and noted examples of successful viral vaccines, such as the measles, polio and hepatitis B virus vaccines. Before the development of such vaccines, it was well known that people who survived the acute natural infection with each of these viruses acquired long-lasting protective immunity to re-infection. This is not the case with HIV since no one is known to have recovered and completely cleared acute infection with HIV-1. Additionally, HIV-1 is not known to cause transient infection and nearly all human infections are life-long and proceed to chronic

stages. For these reasons, HIV-1 vaccine development efforts started from scratch as there was no prior experience and no approach seemed to be better than another.

During the two and a half decades since the discovery of HIV-1, evidence has accumulated to show that vaccine-induce immunity to HIV is possible. Numerous studies in animal models (discussed in details in section **1.3**) have demonstrated that vaccine-induced (i) humoral (Shibata *et al*, 1999; Baba *et al*, 2000; Berman *et al*, 1990; Emini *et al*, 1992; Fultz, 1992; Girard *et al*, 1995 & 1997; Lubeck *et al*, 1997; Mascola *et al*, 2000; Parren *et al*, 2001; Putkonen *et al*, 1991; Veazey *et al*, 2003) and (ii) T-cell mediated (Amara *et al*, 2001; Barouch *et al*, 2000; Shiver *et al*, 2002) immunity is effective against HIV-1 and SIV infections. Further evidence to show that it is possible to develop a vaccine against AIDS comes from human studies, which show that control of virus replication is associated with a rise in generation of CTL in recently HIV-1-infected people with acute infection (Borrow *et al*, 1994; Koup *et al*, 1994) and strong CTL responses in long-term survivors of HIV-1 infection (Migueles *et al*, 2000). Further support that HIV-1 vaccination might prevent infection comes from studies of cohorts of highly exposed but uninfected health-care workers (Clerici *et al*, 1994; Pinto *et al*, 1995) and sex workers (Kaul *et al*, 2000; Rowland-Jones *et al*, 1995 & 1998), which showed the presence of CTL responses to HIV-1 in the absence of HIV infection, suggesting that abortive infection may have occurred after being cleared by natural immune defences.

Recent development of new molecular tools in virology and immunology, and continued gains in knowledge of HIV immunopathogenesis, is hoped to lead to new vaccine approaches, with improved immunogenicity in animal models and human trials. However, available data from studies in macaque virus challenge models have repeatedly demonstrated the difficulty to induce complete protection (sterilising immunity) by vaccination (except with live attenuated virus vaccines, which are unlikely to be used for human vaccination) but that it is possible to reduce the viral load and delay or prevent disease progression. It is thus more realistic to expect that the first generation of successful HIV-1 vaccines will be disease-modifying vaccines, causing long-term control of virus replication at a low or undetectable set point rather than clearing the virus completely. The controlled viral replication will, hopefully, slow disease progression or even abrogate its development. By use of mathematical models, it is predicted that a vaccine that reduces the viral load by 1 to 1.5 $\log_{10}$ could provide important benefits at the population level by reducing transmission (Smith & Blower, 2004) and HIV-associated mortality in the first 20 years after the introduction of such vaccination (Davenport *et al*, 2004; Anderson & Hanson, 2005). These findings provide the reasons to hope that developing a safe and effective AIDS vaccine is possible.

1.1.4. Development of HIV vaccines

Development of safe and effective HIV/AIDS vaccines is a lengthy and challenging task. Initial stages, which start with discovery of the vaccine concept, followed by preclinical development in the laboratory and testing in small laboratory animals and non-human primates may take 5-10 years. Upon regulatory approval (1-2 years), successful candidate vaccines proceed to clinical trials in humans. Before application for licensing, the candidate vaccine has to be tested in at least 3 stages, Phase 1 to Phase 3 (NIAID, 2005; HVTN, 2006; CDC, 2006), a process that might take 5-15 years. Phase 1, involves 10-100 healthy, non-infected low-risk volunteers, and tests for safety and preliminary immunogenicity of the vaccine. Phase 2 requires 100-500 non-infected, low-risk volunteers and investigates further safety issues and immunogenicity as well as routes and dosage of vaccine administration and vaccination schedules. Phase 3 is usually a multi-centre vaccine trial requiring 2,000-5,000 healthy, high-risk volunteers. The main objective of this phase is to test for vaccine efficacy. Phase 4 clinical trial is usually a post-licensing monitoring of efficacy and safety of vaccines. As stated previously, only two phase 3 clinical trials have been completed (Flynn *et al*, 2005) and a third one is in progress (McNeil *et al*, 2004; Pitisuttithum, 2005; Trinvuthipong, 2004). None of the completed trials demonstrated substantial efficacy (Cohen, 2003; Flynn *et al*, 2005; Veljkovic *et al*, 2003) to warrant an application for licensing.

This literature review will focus on preclinical development of HIV vaccines using the non-human primate models with emphasis on efficacy testing.

1.2. TESTING OF HIV VACCINES IN ANIMALS

Pre-clinical testing of HIV vaccines in animal models falls into 2 broad categories namely (i) safety and immunogenicity studies and (ii) efficacy or challenge experiments. Initially, safety and immunogenicity studies are conducted using the small laboratory animals (e.g. mice) and investigate if the proposed candidate vaccine causes detrimental local or systemic reactions and the capacity to induce the desirable immune responses. Further testing in non-human primates is desirable if the vaccine is selected to proceed to clinical trials. On the other hand, efficacy testing investigates the ability of the candidate vaccine to protect against infection and/or disease. These studies require suitable virus challenge models. Most efficacy trials include immunogenicity studies as well in a bid to explore the immune responses associated with protection.

1.2.1. Animal models

Due to the urgent need to identify and develop a safe and effective HIV/AIDS vaccine, a large number of vaccine immunogens have been produced but only a limited number of them can be tested in humans. This makes it necessary to screen potential candidate vaccines, initially in small laboratory animals, and then advancing the promising ones for further preclinical testing in nonhuman primate

models. Evaluation in animal models is an important and essential part of vaccine development because it generates the preclinical data upon which applications for approval for human trials of new vaccines are based. A good animal model should be rational, readily available, inexpensive to maintain, allow vaccination regimens that are suitable for use in human, and give readouts that are relevant and predictive of the ultimate outcome in human trials. It is crucial that animal models that are as close to humans as is practical are used in the final pre-clinical studies in order to select the best candidate vaccines for clinical trials. Non-human primate models best fulfil this requirement.

1.2.2. Nonhuman primate models

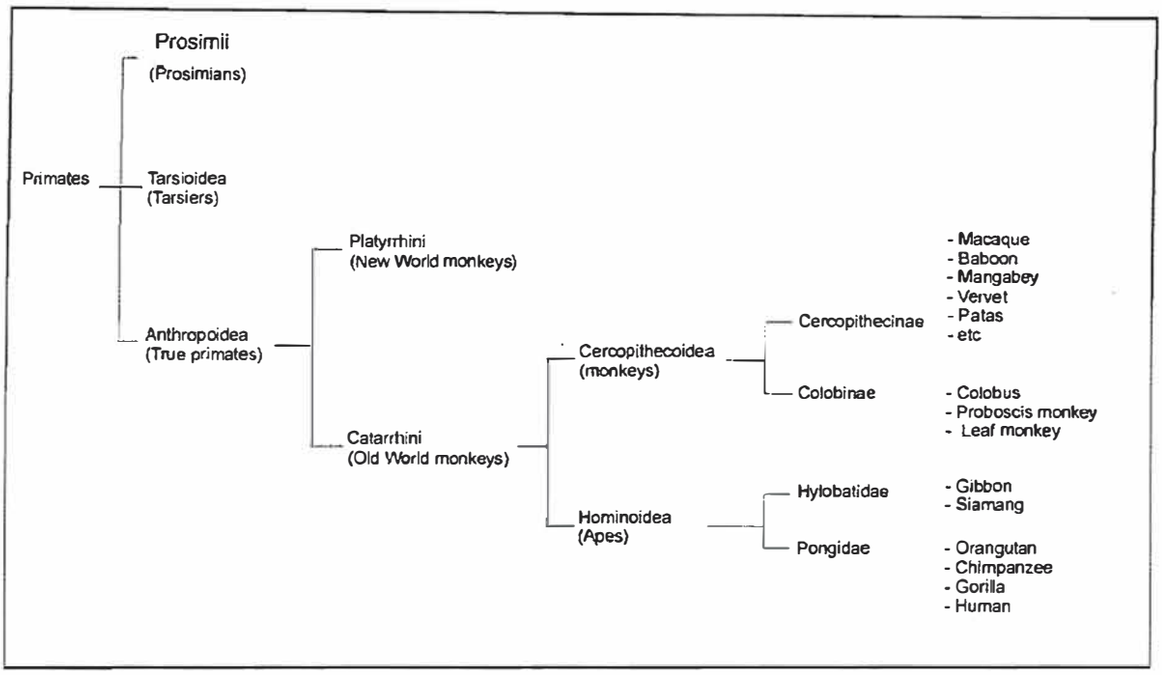
1.2.2.1 Evolutionary aspects of primate species

Primates comprise a diverse group of mammals with the smallest living primate (the pygmy mouse lemur) weighing about 30g and the largest (gorilla) weighing about 175kg (Myers *et al*, 2006). Primates are classified in the Order Primates. The following classification is based on information obtained from Wisconsin Primate Research Center Library (WPRCL; 2006) of the University of Wisconsin-Madison, Emory University (2006), Schwimmer (1996), Kennedy *et al*, (1997) and Disotell *et al*, (1992).

The Order Primates is divided into three sub-orders namely Prosimii, Tarsioidea and Anthropoidea. The members of the sub-order Prosimii are referred to as Pro-simians as they are not true primates and include common species like the lemurs and bush babies (or galagos). The sub-order Anthropoidea represents true primates and is further divided into 2 infra-orders, namely Platyrrhines (or New World monkeys) and Catarrhini (or Old World monkeys). The New World monkeys are found naturally in Central and South America while the Old World monkeys inhabit Africa, Europe and Asia. The New World monkeys are more phylogenetically distant from humans (*Homo sapiens*) than the Old World monkeys. A summary of classification of primates is shown in **Fig 1.1**.

The New World monkeys are further divided into 2 families, the Callitrichidae (e.g. marmosets and tamarins) and Cebidae (e.g. owl, capuchin and squirrel monkeys). It is estimated that New World monkeys diverged over 30 million years ago while the Old World monkeys as a group diverged between 15 and 20 million years ago (Kennedy *et al*, 1997). Of the New World monkeys, only cotton-top tamarins (*Sanguinus oedipus*), common marmoset (*Callithrix jacchus*), owl monkey (*Aotus trivergatus*), capuchin monkeys (*Cebus* spp) and squirrel monkeys (*Saimiri sciureus*) are commonly used in biomedical research (Genain & Hauser, 2001). The majority of the nonhuman primates used in biomedical research are the Old World monkeys.

Fig 1.1: Classification of primates. (Adapted from Kennedy *et al*, 1997)



The Old World monkeys are divided into two main groups (or super-families), the Cercopithecoidea (monkeys, which have tails) and Hominoidae (apes, which lack tails). The Cercopithecoidea comprise of two ecologically and morphologically distinct sub-families, the Cercopithecinae (includes macaques, baboons, mangabeys, vervets and patas monkeys) and Colobinae (includes colobus, proboscis and leaf monkeys). The main difference between the two sub-families is that the former are omnivorous with simple stomachs and cheek pouches while the latter are folivorous with complex stomachs (almost like ruminants') and lack cheek pouches. The sub-family Cercopithecoidea is further divided into 2 tribes namely Papionini and Cercopithecini. The tribe Papionini comprise of macaques (*Macaca* spp), savannah baboons (*Papio* spp), gelada baboons (*Theropithecus*), drills and mandrills (*Mandrillus* spp) and mangabeys (*Cercocebus* and *Lophocebus* spp) (Disotell, 1994).

The super-family Hominoidae is composed of two families, the Hylobatidae (gibbons and siamangs) and the Pongidae (Orang-utans, chimpanzees bonobos, gorillas). Humans (*Homo sapiens*) are classified in the family Pongidae. The genetic similarity between the humans and these apes is >95% (Huh *et al*, 2003; Sakate *et al*, 2003). These nonhuman primates are classified as highly endangered in the wild and are legally protected. Next in evolutionary distance are the monkeys in the Papionini tribe, the majority of which are not endangered in the wild.

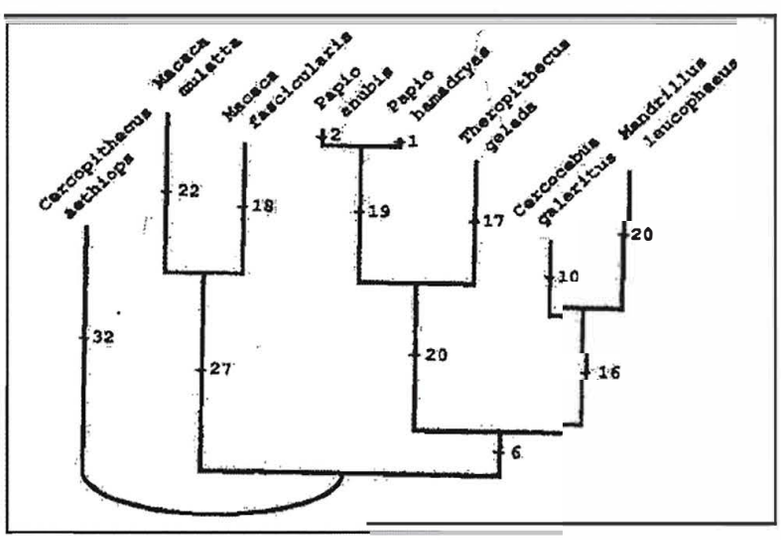
1.2.2.2 The baboon animal model

The baboon belongs to the Papionini tribe in the sub-family Cercopithecoidea. The Papionini is estimated to have radiated during the late Miocene to Plio-Pleistocene period (Kennedy *et al*, 1997)

and therefore is considered to be a sister group of Hominoids (gorilla, chimpanzee, orangutan). Thus, from an evolutionary consideration, members of the Papionini group are the closest non-hominoid Old World monkeys to human in terms of phylogenetical similarity.

Baboons comprise a series of closely related species that are recognised as olive baboon (*Papio anubis*), yellow baboon (*P. cynocephalus*), sacred or hamadryas baboon (*P. hamadryas*), chacma baboon (*P. ursinus*), guinea baboon (*P. papio*), gelada (*Theropithecus gelada*) and Drills and Mandrills (*Mandrillus* spp). Phylogeny studies based on mitochondrial DNA suggest that these baboon series should be defined either as 'phylogenetic' species or 'biological' sub-species (Disotell, 1994; Disotell *et al*, 1992; Newman *et al*, 2004), indicating that these 'species' can cross-breed naturally. All the baboon species are found naturally in Africa and the Arabian Peninsula. Drills and mandrills inhabit West Africa, sacred baboons in the north-eastern Africa and Arabian Peninsula, olive and yellow baboons in eastern Africa, chacma baboons in southern Africa and the guinea baboons in the west central Africa (Cawthon, 2006). The gelada baboons are restricted to the Ethiopian highlands. With the exception of drills and mandrills, all the other baboon species live in open grasslands (savannah) and a few are adapted to desert conditions (eg chacma and hamadryas). Both drills and mandrills live in equatorial forests and are classified as endangered species due to loss of their habitat and hunting for meat. The conservation status of all the other baboon species, except sacred baboons, is listed as lower risk by Convention on International Trade in Endangered Species of Wild Fauna and Flora (CITES). Sacred baboons are classified as vulnerable. **Fig 1.2** shows the phylogenetic relationship between baboon species and other members of the Papionini tribe.

Fig 1.2: Phylogenetic relationship between baboons species and other members of the Papionini tribe. The tree was constructed using total nucleotide substitutions based on mitochondrial DNA sequence data on the cytochrome oxidase subunit II gene (Taken from Disotell *et al*, 1992).



The role of the baboon in medical research was first recognised internationally in 1963, when the 1st International symposium on the Baboon and its use as an Experimental Animal was held in San Antonio, Texas (Vagtborg, 1965). Since then, several studies in biomedical research have utilised the baboon in a diverse fields of research (see review by Murthy *et al*, 2006). In the field of HIV vaccine research, only a few published studies have reported the use of yellow and hamadryas baboons for evaluation of immunogenicity of HIV vaccines. The studies reported in this thesis are the first to report the use of chacma baboon for immunogenicity studies. However, a major limitation of the baboon model is the inability to use it for HIV vaccine efficacy tests as baboons have not been shown to be infected with HIV-1.

1.2.2.3 Considerations for employing nonhuman primates in research

Initial stages of pre-clinical testing should utilise small laboratory animals like mice, rats, rabbits and guinea pigs as these models provide large numbers at reasonable cost (Kennedy *et al*, 1997). However, these animals may not predict what might occur in humans as they differ with humans in fundamental ways. Thus, their major role in preclinical testing is to screen for immunogens that are promising for success in human trials. Prior to clinical trials, nonhuman primates should be considered for evaluation of selected candidate vaccines that have the best chance for future studies in humans.

The best nonhuman primate models would be those closest to human based on phylogeny. The species in the hominoid group – chimpanzees, gorillas and orangutans – would best fit this description. However, due to their endangered status, they are scarce in the wild and their acquisition and transportation from their native habitats is highly restricted. Their large body sizes make their maintenance in captivity cumbersome and costly. Euthanasia after experimentation is not an ethical consideration and these animals must live out their entire lives in captivity (30-40 years), making the maintenance costs extremely high. These issues preclude large studies using these animals.

On the other hand, the majority of Old World monkey species, which are phylogenetically acceptable are not endangered or threatened in the wild. These non-hominoid primate species are readily available for research and euthanasia after experimentation may be considered. Thus, based on maintenance costs and availability, these species offer the best alternative to hominoid primate models for pre-clinical studies of vaccines.

Other considerations include the costs of housing for the nonhuman primates. Performing studies that require biosafety level 3 facilities (when an infectious agent that represents a human pathogen is involved e.g. SIV/SHIV challenge experiments) will inflate the costs of housing, as specialised

facilities that are expensive to maintain will be needed. Studies that can be conducted in animals in group cages represent a cheaper alternative to individual caging.

1.2.2.4 Comparative immunology between nonhuman and human primates

The major histocompatibility complex (MHC) loci encode for cell surface molecules that play a crucial role in the adaptive arm of the immune system. MHC class 1 molecules are involved in antigen presentation to CD8+ cytotoxic T lymphocytes (CTL) whereas MHC class 2 molecules present to CD4+ T helper (Th) cells. The MHC system in nonhuman hominoid and Old World monkeys resemble that of humans (Adams & Parham, 2001; de Groot *et al*, 2000; Bontrop, 1995 & 1999; Boyson *et al*, 1996; Doxiadis *et al*, 2001; Bontrop & Watkins, 2005). For example, the HLA-A-related and HLA-B-related loci have been identified in all apes species, macaques and baboons (Adams & Parham, 2001; Doxiadis *et al*, 2001). HLA-A alleles partition into 2 lineages, A1 and A2, both of which are represented in macaques and baboons as *mamu*-A1, *mamu*-A2 and *paan*-A1 respectively. Like in human, both the HLA-A-related and HLA-B-related loci in these species display a high degree of polymorphism. New World monkeys show dissimilar MHC, which is smaller and condensed compared to that of humans and both MHC class 1 and class 2 molecules in New World monkeys are less polymorphic. In both human and chimpanzee, some specific orthologous alleles in MHC class 1 have been shown to influence resistance to HIV/SIV infection and disease (Carrington & Bontrop, 2002; Bontrop & Watkins, 2005).

The orthologues of HLA-DR, -DQ and -DR (coding for MHC class 2) are present in apes, rhesus macaques and baboons (Doxiadis *et al*, 2001) and are comparable to human analogues in terms of organisation and polymorphism.

The immunoglobulins (Ig) in humans, apes and Old World monkeys are similar. Apes, baboons and humans have four IgG classes (Damian *et al*, 1971) whereas macaques have been reported to have three IgG classes (Martin, 1982). In addition, many human reagents e.g. cluster of differentiation (CD) antigens, immunoglobulins and cytokines cross-react with the nonhuman primate analogues, making it feasible to utilise the same immunologic techniques in pre-clinical studies as in human studies.

1.2.3. Nonhuman primate animal models of AIDS

Lentiviruses, cause a prolonged course of infection in their hosts, which leads to a slowly progressive and inevitably fatal disease in the majority of these species, including humans who are infected with HIV-1. These lentiviruses, which include Maedi Visna virus of sheep, equine infectious anaemia virus of horses, feline immunodeficiency virus (FIV) of cats and simian immunodeficiency viruses (SIV) of non-human primates (NHP) have similar morphological, biological and molecular features (Fukasawa

et al, 1988; Bibollet-Ruche *et al*, 2004). However, due to the phylogenetical closeness between humans and NHP and among their respective lentiviruses (see **Fig 1.4**), the SIV infections of NHP, rather than that of cats and ungulates, is a better model for human AIDS. Simian AIDS in macaques may also be caused by simian retrovirus type D (Lackner *et al*, 1988) but there are significant biological differences between lentiviruses and type D retroviruses. Other models e.g. SCID mice (mice with severe combined immune deficiency), which can be infected with HIV-1 have been reported (Bonyhadi & Kaneshima, 1997; Kitchen & Zack, 1998). SCID mice lack an adaptive immune system. When reconstituted with human tissues such as thymus, lymphocytes etc, they become permissive to HIV-1 infection. This review will focus on NHP utilising SIV, HIV and SIV/HIV infections. The major characteristics of an ideal NHP model of AIDS have been reviewed by Joag (2000) as shown in **Table 1.1**.

The NHP models of AIDS have been used extensively to study AIDS pathogenesis, virus-host cell interactions, virus transmission especially mucosal transmission, immune mechanisms, drug studies and vaccine studies. A vast majority of these studies involve vaccine studies and this is the focus of this review.

Table 1.1: Characteristics of major NHP models of AIDS (adapted from Joag, 2000).

Parameter	HIV-1 in chimpanzees	SIV_{mac}/HIV-2 in macaques	Pathogenic SHIV in macaques
Virus has HIV-1 *env*	Yes	No	Yes
Virus has HIV-1 *gag/pol*	Yes	No	No
Virus has HIV-1 accessory genes	Yes	No	*tat, rev, vpu*
Animals develop CD4+ T-cell loss	No	Yes	Yes
Time for CD4+ T cell count <500/μL*	NA	months to years[1]	weeks to months[2]
Immunodeficiency in animals	No	Yes	Yes
Encephalitis	No	Yes	Yes
Other organ-specific disease	No	Yes	Yes

* Time required for CD4+ T cell count to fall below 500/μL varies with the strain of virus; [1]: for SIV_{mac239} and [2]: for $SHIV_{KU-1}$; NA: not applicable.

1.2.3.1 HIV-1 nonhuman primate models

The chimpanzee is the only NHP, which can be consistently infected with HIV-1 (Fultz, 1993; Heeney JL, 1996). Following experimental inoculation with HIV-1, the chimpanzees sero-convert in the same way as humans and virus can be isolated from blood cells during infection. However, the pathological features associated with HIV-1 infection in chimpanzees are quite different from that seen in HIV-1 infected persons. Chimpanzees do not develop high level viraemia or loss of CD4+ T cells and the development of disease is extremely rare although it has been reported (Novembre *et al*, 1997 & 2001). The chimpanzee model for HIV-1 infection is valuable and several studies have been done using the chimpanzee. However, there are serious limitations to the use of this model due to its endangered status.

HIV-1 has been reported to infect pig-tailed macaques (Agy *et al*, 1992; Bosch *et al*, 1997; Frumkin, 1993). However, efforts to adapt HIV-1 in these macaques, including serial *in vivo* passage (Agy *et al*, 1997), were largely unsuccessful in reproducing a persistent infection of pig-tailed macaques with HIV-1 strains.

1.2.3.2 HIV-2 nonhuman primate models

Certain strains of HIV-2 have been reported to experimentally infect baboons (Barnett *et al*, 1994; Locher *et al*, 1997, 1998a & 1998b) and macaques (Castro *et al*, 1991; Putkonen *et al*, 1989) with development of AIDS-like symptoms in some baboons (Barnett *et al*, 1994; Locher *et al*, 1998b). The baboon/HIV-2 model has been useful, to some extent, for studying HIV pathogenesis (Blackbourn *et al*, 1997; Locher *et al*, 1997, 1998b; 2001 & 2003) but there are limitations associated with the fact that baboons are not readily infected with HIV-2 (Locher *et al*, 1998a) and only a few specific isolates of HIV-2 are infectious.

1.2.3.3 SIV and SHIV nonhuman primate models

The discovery of SIV, shortly after the discovery of HIV-1, provided an important model for AIDS. SIV was discovered coincidentally in two separate primate centres in the USA, New England and California Regional Primate Research Centres, where macaques were dying of an AIDS-like illness (Mansfield *et al*, 1995; Gardner, 2003) in the absence of simian type D retrovirus infection. It was shown that SIV had spread to captive macaques from the captive sooty mangabeys, which are the natural hosts of SIV (Chakrabarti *et al*, 1987; Daniel *et al*, 1985).

SIV infect a wide variety of NHP species in Africa. To date SIV cross-reactive antibodies have been detected in 36 African Old World monkeys and SIV infection confirmed by sequence analysis in 29 of them (Bibollet-Ruche *et al*, 2004). Surveys have revealed cases of cross-species transmission in patas monkeys (Bibollet-Ruche *et al*, 1996), olive baboons (Jin *et al*, 1994) and chacma baboons (van Rensburg *et al*, 1998) from the local species of African green monkeys. The infection in these species is life-long and characterised by high viral loads without progression to disease except for two reported cases (Ling *et al*, 2004; Apetrei *et al*, 2004). In contrast, natural infection in the wild of Asian Old World monkeys with SIV has not been seen and experimental infection of these species with SIV nearly always result to development of AIDS-like symptoms with many parallels to human AIDS. Some common chimpanzees have been reported to be naturally infected with an SIV strain, designated SIVcpz (Gao *et al*, 1999; Santiago *et al*, 2002). The infections in these chimpanzees are apparently non-pathogenic.

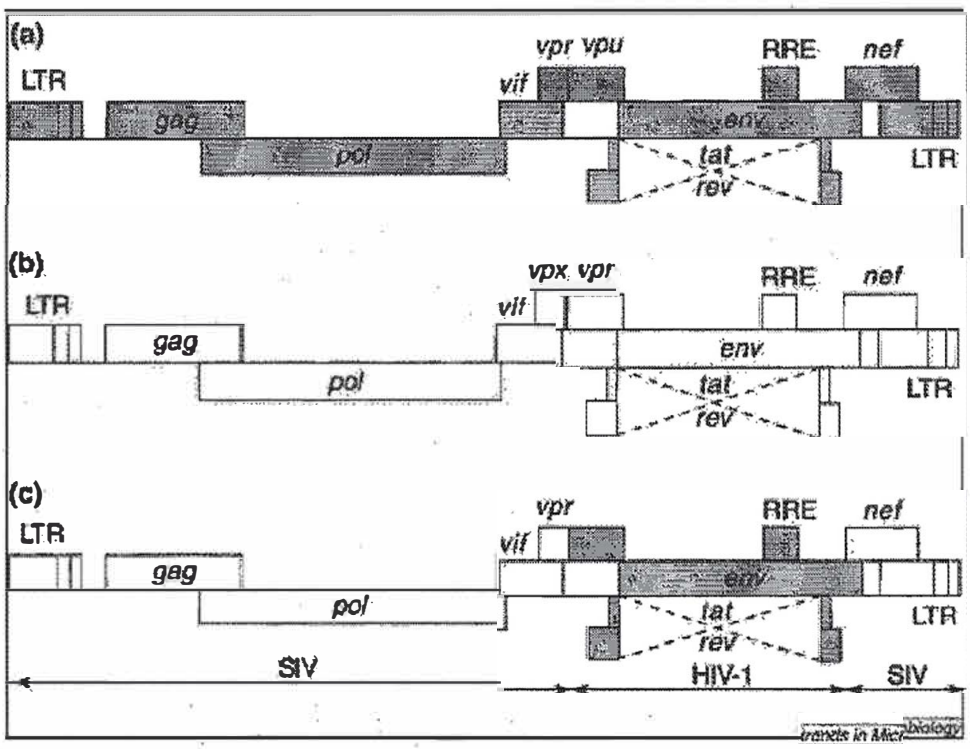
Chimeric HIV-SIV viruses (SHIV) are manufactured lentiviruses. SHIV contain the HIV-1 *env* and associated *tat*, *vpu* and *rev* genes along with the SIV backbone (*gag*, *pol*, *vif*, *vpr* and LTR). Some

SHIV are both infectious and pathogenic in macaques while others are infectious but non-pathogenic (see **Table 1.2**). Baboons can also be infected with SHIV (Allan *et al*, 1995; Klinger *et al*, 1998).

1.2.3.4 Similarity between SIV and HIV

The simian immunodeficiency viruses (SIV) share many features with HIV. The genomic structure and organisation is similar with a minor difference in one of the accessory genes (**Fig 1.3**). While HIV-1 has *vpu*, SIV and HIV-2 have *vpx*. Phylogenetic analysis has shown SIV_{cpz} to be closely related to HIV-1 whereas SIV_{smm} and SIV_{mac} are closely related to HIV-2 (**Fig 1.4**).

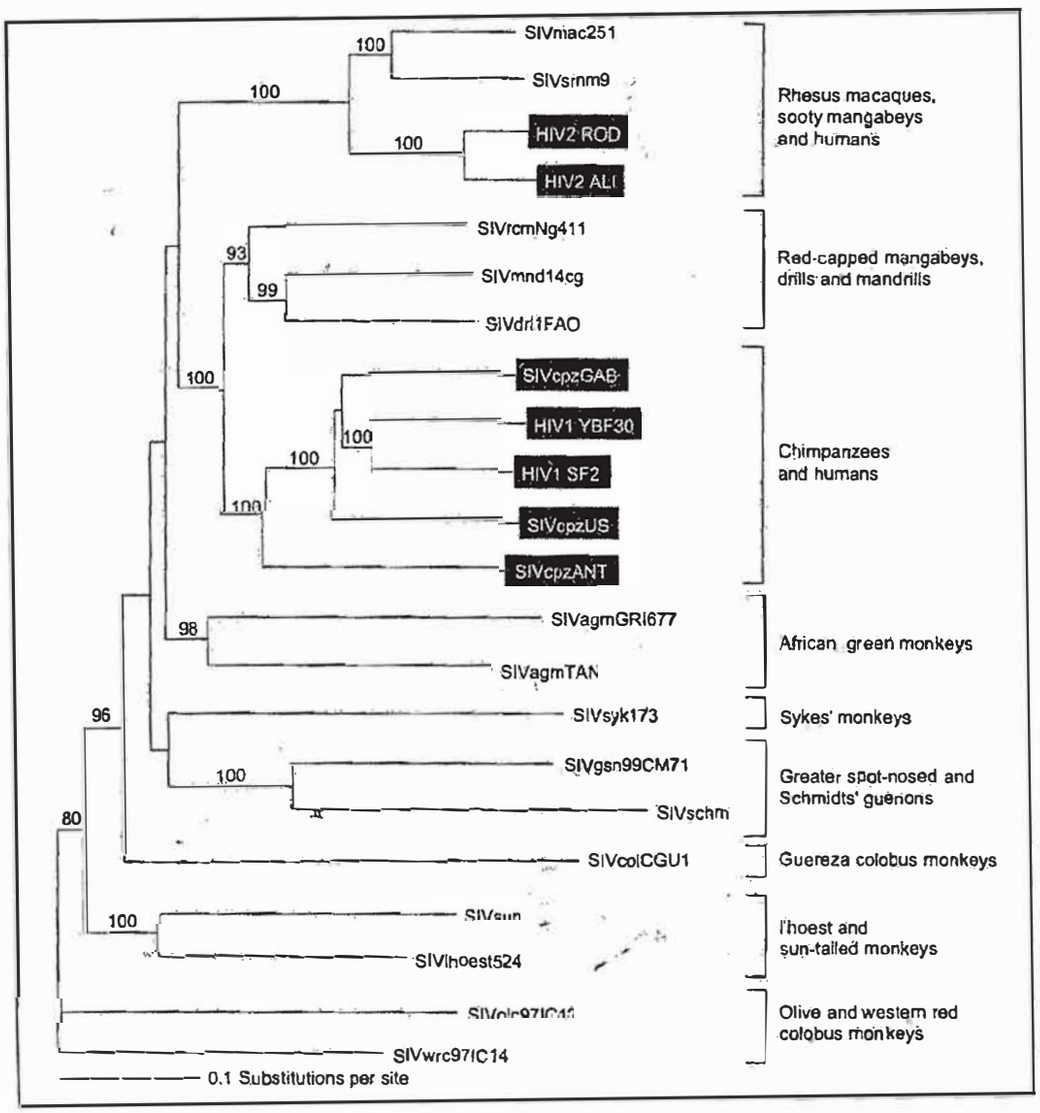
Fig 1.3: The genetic organization of immunodeficiency viruses. **(a)** Shows the HIV genome containing three major genes *env, gag* and *pol*, three regulatory genes *rev, tat* and *nef*, and three accessory genes *vif, vpr* and *vpu*. **(b)** Shows the simian immunodeficiency virus (SIV) genome. SIV differs from HIV as *vpx* is present rather than *vpu*. **(c)** Shows the SHIV genome, which is a combination of HIV *env, rev, tat* and *vpu* sequences with an SIV backbone. Dashed lines show the Rev and Tat proteins, which are derived from multiply spliced sequences. LTR: long terminal repeat; RRE: rev response element. (Taken from Nath *et al*, 2000)



The mechanism of replication and tropism for CD4-bearing cells is similar for both SIV and HIV primary isolates. In conjuction with CD4 receptors, both HIV and SIV use CCR5 (R5) as the main co-receptor for viral entry into cells (Chen *et al*, 1997; Hill *et al*, 1997; Vodros *et al*, 2001). Similarly, SIV, like HIV can be transmitted via intravaginal, intravenous, intrarectal, oral and penile mucosa. The severity of SIV-induced disease and viral loads are similar to those seen in HIV-infected humans (Whetter *et al*, 1999) resulting in loss of CD4+ T cells and immunosuppression and similar AIDS-related symptoms. SHIV utilise R5 and/or CXCR4 (X4) co-receptor depending on the HIV-1 *env*

gene that is inserted into the SIV backbone (Nath *et al*, 2000). Thus, both SIV and SHIV have provided useful surrogate models for HIV-1 (Bogers *et al*, 1997).

Fig 1.4: Phylogenetic analysis of HIV and SIV partial polymerase gene sequences of human, chimpanzee and several Old World monkey species. (Taken from Bontrop & Watkins, 2005)



1.2.3.5 Limitation of SIV and SHIV models

There are important differences between SIV and HIV-1 that may impact on interpretation of results from the monkey models in regard to guiding the human trials. Firstly, the SIV and HIV-1 envelopes are considerably divergent and antibodies directed against the envelope of SIV do not neutralise HIV-1 and vice versa. The use of SHIV may overcome this problem as they contain HIV-1 envelope. Secondly, CTL specific for HIV-1 do not recognise SIV-infected cells and vice versa. Another notable difference between HIV-1 and SIV/SHIV is found in the co-receptor usage. While most HIV-1 primary isolates predominantly use R5 or X4 in very rare cases for cell entry, almost all SIV isolates

use R5 exclusively whereas the majority of pathogenic SHIV utilise X4 exclusively (Chen *et al*, 1997; Hill *et al*, 1997; Vodros *et al*, 2004; Zhang *et al*, 2000; **Table 1.2**) as previously mentioned. In addition, a phenotypic shift from R5 to X4 usage is seen in up to 67% of infected people as the infection progresses from asymptomatic phase to AIDS (Shankarappa *et al*, 1999). This phenotypic switch to X4 usage has not been reported for SIV-infected monkeys. It is now known that X4-utilising viruses massively infect and eliminate both resting naïve CD4+ and memory CD4+ T cells whereas R5-using viruses selectively infect memory CD4+ T cells (Nishimura *et al*, 2004 & 2005). Thus, X4-using viruses (e.g. $SHIV_{89.6P}$, $SHIV_{DH12R}$) cause a rapid, irreversible and complete depletion of CD4+ T cells, inducing simian AIDS within weeks of infection. In contrast, R5-using viruses (e.g. HIV-1, SIV_{mac}) cause a slower loss of CD4+ T cells and hence a slow disease progression within 1-3 years (SIV-infected macaques) or several years (HIV-1-infected humans). This difference in co-receptor usage-associated pathogenicity probably reflects differences in host-virus interactions among the SIV/SHIV-infected macaques and HIV-1-infected humans and may further complicate the interpretation of results from macaque challenge experiments.

1.3. HIV VACCINE STUDIES USING NHP MODELS

1.3.1. Efficacy testing of AIDS vaccine in NHP

It is generally accepted that due to the differences that exist between HIV infection in humans and various NHP models of AIDS, it is extremely difficult to develop an ideal animal model for AIDS (Joag, 2000). However, NHP models provide two main uses namely (i) to understand the mechanisms of pathogenesis and immunity and (ii) to determine the relative efficacy of different vaccine strategies. These two features allow the improvement of current vaccine approaches and most importantly, allow selection of the most promising ones for phase 3 trials in human (Uberla, 2005).

Protection (or efficacy) tests for HIV vaccines can be based on two different levels, induction of sterilising or non-sterilising immunity. Humoral immune responses provided by neutralising antibodies could theoretically mediate sterilising immunity but induction of sufficient neutralising antibodies by the current vaccine approaches has proved to be extremely difficult. Since plasma viral RNA levels have been shown to predict disease progression in HIV-1 infected human and SIV/SHIV-infected NHP, vaccine-induced reduction in viral loads is often used to assess protection in the absence of sterilising immunity. Moreover, it has been predicted by mathematical models that a HIV vaccine leading to a reduction of viral load by 1-1.5 $\log_{10}$ would cause an impact at the population level in reducing the rate of HIV transmission and HIV-associated mortality (Smith & Blower, 2004; Davenport *et al*, 2004; Anderson & Hanson, 2005).

The assessment of vaccine efficacy is directly influenced by the level of stringency of the virus challenge system that is used. Although various factors, such as pathogenicity of challenge virus, the

susceptibility of the challenged monkey species, the route and dose of challenge virus etc, can influence the stringency that is applied in efficacy testing, it is possible to recognise certain important vaccine-induced immunological features that would allow ranking of efficacies of different vaccine strategies. A list of the commonly used challenge viruses in HIV vaccine studies using non-human primate models is shown in **Table 1.2.**

1.3.2. Nonhuman primate HIV/SIV vaccine trials database

A database of published pre-clinical AIDS vaccine studies, which is dedicated to reporting on previously and newly published studies involving HIV/SIV vaccine development using NHP, has been made available at the Los Alamos National Library (LANL) (Warren, 2002). The information is available via a website administered by LANL (http//hiv-vacdb.lanl.gov).

The current database at LANL lists 285 studies that have been conducted between 1987 and 2005 (**Table 1.3a**). Of these studies, 211, 31 and 20 used rhesus, cynomolgus and pig-tailed macaques respectively. Chimpanzees and baboons (yellow baboon) were utilised in 20 and 3 of these studies respectively. Of the 285 studies, 239 and 223 were immunogenicity and challenge experiments respectively, indicating that the majority of them involved both objectives. The majority of the immunogens for these studies were derived from SIV (130 studies) and HIV-1 (70 studies) (**Table 1.3b**). Although the majority of challenge experiments utilised rhesus macaques, it was noteworthy that 1 of 3 baboon experiments was a challenge experiment using HIV-2 as a challenge virus following immunisation with various DNA vaccines (Locher *et al*, 2004). Baboons are endemic in Africa and readily available for HIV vaccine research, unlike the macaques for which a shortage has been reported (Cohen, 2000).

Several vaccine strategies have been developed (McMichael & Hanke, 2003; Nabel, 2002) and assessed in preclinical studies (Smith, 2002). As seen from the LANL database DNA vaccines (62 studies), recombinant live vectors (57 studies; mostly viral vectors) and live attenuated lentiviruses (40 studies) were the leading vaccine formats (**Table 1.3b**). The various vaccine strategies involving studies in NHP will be reviewed briefly in the following sections, with more details on DNA, recombinant BCG and virus-like particles, which are the main subjects of this thesis.

1.3.3. HIV vaccine strategies using NHP models

1.3.3.1 Inactivated whole virus

The Salk form of the polio vaccine is an example of inactivated (killed) vaccine that has been used successfully in protection against a viral pathogen and this may have influenced initial efforts in HIV vaccine development, which focused on this approach. Inactivated SIV was shown to confer protection to vaccinated rhesus macaques against pathogenic SIV challenge (Desrosiers *et al*, 1989;

Murphey-Corb *et al*, 1989). It soon became apparent that whole inactivated SIV grown in human cells gave protection against pathogenic SIV grown in human cells but not SIV grown in macaque cells (Goldstein *et al*, 1994; Le Grand *et al*, 1992) indicating that the protective immune response was directed against human antigens in the vaccine preparation and in the challenge virus (called xeno-immunisation). Arthur *et al* (1995) immunised macaques with human HLA-DR and showed that this protection was associated with antibodies to human HLA-DR, raising concerns about inducing auto-immune processes. Safety is a major concern for this vaccination approach and these may have slowed research in this field. However, new ways of inactivating the whole virus using sub-lethal doses of formalin followed by heat inactivation at 62 °C (Poon *et al*, 2005a) or by alkylation with N-ethyleneamine resulting in complete inactivation and preservation of conformational and functional intregrity of Env glycoproteins (Rossio *et al*, 1998) have re-kindled some interest in this approach leading into more pre-clinical studies in NHP (Lifson *et al*, 2004; Poon *et al*, 2005b).

1.3.3.2 Passive immunisation

Putkonen *et al* (1991) demonstrated that passive transfer of serum containing high titres of SIV antibody protected cynomolgus macaques against SIV and HIV-2. Other studies have shown similar protection against SIV (van Rompay *et al*, 1998) in newborn macaques and SHIV (Shibata *et al*, 1999) in pig-tailed macaques. Administration of monoclonal antibodies that had strong HIV-1 neutralising activity has similarly demonstrated protection against non-pathogenic (Baba *et al*, 2000) and pathogenic (Eda *et al*, 2006; Ferrantelli *et al*, 2004; Mascola *et al*, 2000; Nishimura *et al*, 2003; Parren *et al*, 2001; Veazey *et al*, 2003) SHIV challenge.

This immunisation approach is not practical for long-term vaccination strategy against HIV-1 infections although it demonstrates that neutralising antibodies are capable of protecting against HIV-1 infections. Moreover, passive immunisation required high titres of antibody to be given within a very short time after exposure to confer protection (Nishimura *et al*, 2003) and the development of a vaccine, which is capable of inducing such high titres of antibody, is a serious challenge.

1.3.3.3 Live attenuated lentiviruses

The measles vaccine is an excellent example of a safe and effective live attenuated vaccine. Using the same vaccine strategy, live attenuated SIV has been used experimentally in animal studies. The first live attenuated SIV vaccine, termed SIV1A11, a molecular clone isolated from tissue culture-adapted SIV, was reported to cause low-grade but persistent infection in rhesus macaques. However, upon challenge with wild-type SIV, the vaccinated monkeys were not protected against super-infection but disease progression was delayed (Marthas *et al*, 1990). The attenuation of SIV1A11 was later found to be caused by one premature stop codon in the *vpr* gene and two others in the gp41 transcytoplasmic domain (Luciw *et al*, 1998).

Table 1.2: Commonly used challenge viruses in HIV vaccine studies using NHP models.

Challenge virus	Description	Co-receptor usage	Pathogenic?*	Reference(s)
SIV_{mac251}	SIV isolated from spleen cells of a rhesus macaque with simian AIDS	CCR5	Yes	Kestler *et al*, 1988; Lewis *et al*, 1994
SIV_{mac239}	An infectious molecular clone of SIV isolated from rhesus macaque	CCR5	Yes	Kestler *et al*, 1990
SIV_{smE660}	Uncloned SIV isolated from rhesus macaques experimentally infected with SIV.	NA	Yes	Goldstein *et al*, 1994
$SHIV_{HXBc2}$	Also known as SHIV-4; constructed with *env* gene derived from lab-adapted HIV-1_{HXBc2} (also called HXB2) clone	CXCR4	No	Li *et al*, 1992
$SHIV_{89.6}$	Constructed with *env* gene derived from dual tropic HIV-$1_{89.6}$ primary isolate; constructed in the background of $SHIV_{HXBc2}$	Dual	No	Reimann *et al*, 1996b
$SHIV_{89.6P}$	Generated from $SHIV_{89.6}$ by two sequential *in vivo* passage by intravenous blood to naïve rhesus macaques. Virus isolated from cells.	CXCR4	Yes	Reimann *et al*, 1996a
$SHIV_{89.6PD}$	Generated from same $SHIV_{89.6}$–infected rhesus macaques from which $SHIV_{89.6P}$ was isolated. Virus isolated from plasma	CXCR4	Yes	Lu *et al*, 1998
$SHIV_{KU-1}$	Generated from $SHIV_{HXBc2}$ by *in vivo* serial passage in pig-tailed macaques by bone marrow-to-bone marrow transfusion into naïve monkeys	CXCR4	Yes	Joag *et al*, 1996
$SHIV_{KU-2}$	Generated by further *in vivo* serial passage of $SHIV_{KU-1}$ in rhesus macaques	CXCR4	Yes	Joag *et al*, 1998
$SHIV_{SF33}$	Constructed with *env* gene derived from T-tropic HIV-1_{SF33} primary isolate from patient with terminal AIDS disease (Cheng-Mayer *et al*, 1991)	CXCR4	No	Mandell *et al*, 1999
$SHIV_{SF33A}$	Generated by *in vivo* adaptation of $SHIV_{SF33}$ during chronic infection of a juvenile rhesus macaque	CXCR4	Yes	Luciw *et al*, 1999
$SHIV_{SF162}$	Constructed with *env* gene derived from M-tropic HIV-1_{SF162} primary isolate	CCR5	No	Luciw *et al*, 1995
$SHIV_{SF162P}$	Generated from $SHIV_{SF162}$ by three *in vivo* serial passage in rhesus macaque by bone marrow-to-blood transfusion into naïve monkeys	CCR5	Yes	Harouse *et al*, 1999
$SHIV_{DH12}$	Constructed with *env* derived from dual tropic HIV-1_{DH12} (Shibata *et al*, 1995) primary isolate	Dual	Yes[1]	Shibata *et al*, 1997
$SHIV_{DH12R}$	Generated from $SHIV_{DH12}$ by *in vivo* passage into naïve rhesus macaques accompanied by depletion of CD8+ cells during primary infection	CXCR4	Yes	Igarashi *et al*, 1999
$SHIV_{W6.1D}$	Constructed with *env* derived from dual tropic HIV-$1_{W6.1D}$ (Groenink *et al*, 1992) primary isolate	Dual	No	Ranjbar *et al*, 1997
$SHIV_{CHN19}$	Constructed with *env* derived from HIV-1_{CHN19}, a subtype C from China, macrophage-tropic primary isolate	CCR5	No	Chen *et al*, 2000
$SHIV_{MJ4}$	Constructed with *env* derived from HIV-1, a subtype C from Botswana, macrophage-tropic primary isolate	CCR5	No	Ndung'u *et al*, 2001
HIV-1	Several molecular clones (e.g. IIIB, W6.1D, DH12 etc)	Depending[2]	No[3]	(e.g. ten Haaft *et al*, 2001)
HIV-2_{UC2}	HIV-2 primary isolate from a patient from Cote I'voire (W. Africa)	NA	Yes[4]	Locher *et al*, 2004

*: refers to pathogenicity in macaques; [1]: pathogenic to pig-tailed but not rhesus macaques; [2]: CXCR4/CCR5 co-receptor usage depending on the HIV-1 isolate used; [3]: refers to pathogenicity in chimpanzee model; [4]: refers to pathogenicity in baboon model; NA: information not available.

Table 1.3a: Number of HIV/SIV vaccine studies using nonhuman primates from 1987 to 2005. (Compiled from HIV vaccine trials database at http//hiv-vacdb.lanl.gov).

NHP species	DNA	Live attenuated virus	Live virus	Recombinant vector (viral or bacterial)	Whole inactivated virus	Passive antibody	Purified viral products	Recombinant sub-unit proteins	Synthetic protein or peptide	Virus-like particles	Others[a]
Rhesus macaque (Macaca mulatta)	49	32	9	44	15	11	15	19	9	2	6
Cynomolgus macaque (Macaca fascicularis)	6	3	2	8	3	3	3	2	1	0	0
Pig-tailed macaque (Macaca nemestrina)	2	5	0	4	0	4	0	4	0	1	0
Chimpanzee (Pan Troglodytes)	3	0	2	1	1	4	1	7	0	0	1
Baboon (Papio cynocephalus)	2	0	0	0[b]	0	0	0	0	1	0	0
Total	**62**	**40**	**13**	**57**	**19**	**22**	**19**	**32**	**11**	**3**	**7**

[a]: includes cell/tissue culture and live recombinant virus; [b]: study by Casimiro *et al* (2003) not included.

Table 1.3b: Number of HIV/SIV vaccine studies using nonhuman primates from 1987 to 2005 indicating the source of immunogens and the study objectives. (Compiled from HIV vaccine trials database at http//hiv-vacdb.lanl.gov).

NHP species	Source of immunogen				Study objective				
	HIV-1	**HIV-2**	**SHIV**	**SIV**	Immunogenicity	Challenge	Pathogenicity	Passive immunisation	Others[a]
Rhesus macaque	51	2	9	106	174	159	4	12	17
Cynomolgus macaque	3	3	1	16	27	29	1	3	2
Pig-tailed macaque	5	0	0	7	15	15	0	4	2
Chimpanzee	10	0	0	1	21	19	0	3	3
Baboon (P. cynocephalus)	1[b]	1	0	0	2[b]	1	1	0	0
Total	**70**	**6**	**10**	**130**	**239**	**223**	**6**	**22**	**24**

[a]: includes chemotherapy, immunotherapy and passive immunotherapy; [b]: study by Casimiro *et al* (2003) not included.

As the strategy to produce live attenuated vaccines via *in vitro* tissue culture passage or *in vivo* passage through unnatural hosts is unpredictable, this approach has been replaced by targeted gene deletion in HIV vaccine research. Interest in this approach started after Kestler *et al* (1991) reported a strong positive correlation between the presence of a full-length SIV *nef* and development of simian AIDS in macaques. Subsequently, Daniel *et al* (1992) showed that rhesus macaques that were infected with SIV_{mac239} containing a 182 base pair (bp) *nef* deletion (SIVΔnef) maintained low viral loads and stable CD4 counts and were protected from super-infection with SIV_{mac251}. Subsequent studies confirmed that SIV without the *nef* gene conferred protective immunity against closely related pathogenic SIV with intact *nef* gene (Almond *et al*, 1995; Connor *et al*, 1998; Cranage *et al*, 1997; Stahl-Hennig *et al*, 1996; Gauduin *et al*, 1998). Efforts to improve on the safety of live SIV vaccines by making more deletions showed that the degree of protection correlated inversely with the level of attenuation (Wyand *et al*, 1996; Johnson *et al*, 1999). However, a combination of DNA and live attenuated SIVΔnef vaccine strategy has been reported to provide enhanced protection from $SHIV_{89.6P}$-induced disease (Amara *et al*, 2005b).

Multiple deletions of SIV_{mac239} by Gibbs *et al* (1994) produced $SIV_{macΔ3}$, which contained a 101 bp deletion in *vpr*, a 182 bp deletion in *nef* and a second 172 bp deletion in the *nef* region that overlaps with the 3-prime LTR. Infection of macaques with $SIV_{macΔ3}$ induced lower viral loads than infection with wild-type parental virus and protected 50% of macaques against i.v. challenge with wild-type virus (Wyand *et al*, 1996). Both live attenuated SIVΔnef and $SIV_{macΔ3}$ have been shown to confer sterilising immunity against homologous but not heterologous challenge (Lewis *et al*, 1999; Wyand *et al*, 1999). The protection can be mediated in the absence of neutralising antibodies (Langlois *et al*, 1998; Connor *et al*, 1998). Although the protection conferred by live attenuated SIV was associated with development of SIV-specific CTL, other unidentified mechanisms appeared to play some role in protection (Nixon *et al*, 2000) and the mechanisms of protection are not fully understood. A recent study (Schmitz *et al*, 2005) in which macaques were immunised with $SIV_{macΔ3}$ vaccine, followed by CD8+ T cell depletion and intravenous SIV_{mac251} challenge revealed CD8+ T cells playing a central role and neutralising antibodies a supportive role in viral replication control.

While the live attenuated SIV vaccines are very effective in providing protection against homologous virus in NHP models, the use of attenuated HIV-1 is considered too risky for use in human studies (Whitney & Ruprecht, 2004). Consequently, various innovative strategies aimed at modulating SIV virulence while maintaining or increasing the immunogenicity of live attenuated vaccines have been employed (Evans *et al*, 2005; Guan *et al*, 2001; Mori *et al*, 2001; Shacklett *et al*, 2002; von Gegerfelt *et al*, 2002; Stebbings *et al*, 2002; Kuate *et al*, 2003). One of these strategies, termed as 'gain-of-function' approach, involves the use of a 'constitutively-dead, conditionally-live' SIV as the vaccine

virus. This strategy comprises of the inactivation of viral transcriptional activator (Tat) and its responsive (TAR) binding site by mutagenesis and functionally replacing it with a tetracycline-inducible gene expression system so that the replication of the virus is dependent upon the presence of tetracycline or its derivatives (Smith *et al*, 2001; Verhoef *et al*, 2001; Zhou *et al*, 2006). Upon vaccination, the replication of vaccine virus would be temporarily activated by and controlled to the extent needed for induction of immune responses by transient administration of tetracycline. Once the immune responses develop, the tetracycline administration would be discontinued and virus replication would diminish (as replication is dependent upon the presence of the drug).

A similar strategy that aims at reducing the risks associated with live attenuated lentivirus vaccines by controlling vaccine virus replication has explored the use of single-cycle immunodeficiency viruses (SCIV). SCIV have been generated by introducing mutations in the prime binding (Kuate *et al*, 2003) or the *gag-pol* frameshift (Evans *et al*, 2005) sites of the virus genome, resulting into a defect in reverse transcription or a deficient expression of Pol protein respectively. This effectively limits the virus replication to a single cycle. To rescue the replication for vaccine production, an artificial tRNA complementary to the mutated primer binding site or *gag-pol* site is provided and to further enhance the safety of these SCIV, additional deletions were also introduced in the *vif*, *vpr* and *nef* (Kuate *et al*, 2003) or *nef* and downstream to *pol* (Evans *et al*, 2005) genes. Macaques that were immunised with single-cycle SIV were observed to develop SIV-specific humoral and cellular responses (Evans *et al*, 2005; Kuate *et al*, 2003) and exhibited 1-3 $\log_{10}$ reductions in acute-phase plasma viral loads following intravenous challenge with pathogenic SIV_{mac239} (Evans *et al*, 2005).

Other studies investigated the exchange of SIV *nef* with cytokine genes in an attempt to provide genetic adjuvants *in cis*. For example, the expression of the IFN-γ gene in the genome of the attenuated virus was reported to increase virus attenuation and reduce the post challenge viral load in vaccinated rhesus macaques (Giavedoni *et al*, 1997). Other studies have shown that it is possible to increase the immunogenicity of live attenuated vaccines without affecting the viral replication by expressing IFN-γ and IL-4 (Stahl-Hennig *et al*, 2003) or IL-2 (Gundlach *et al*, 1997) in the genome of attenuated virus.

Despite the many approaches being employed to improve the live attenuated vaccines, a major safety concern still remains. The possibility of live attenuated vaccines reverting to virulence through molecular evolution and immune selection has been reported (Alexander *et al*, 2003; Chakrabarti *et al*, 2003; Whitney & Ruprecht, 2004). For this reason, it still remains to be seen whether the above-mentioned efforts will lead to the development of safe and effective candidate HIV vaccines to be considered for future trials in humans. Even if live attenuated lentiviral vaccine approach does not

eventually lead to clinical trials in the near future, these studies utilising macaques immunised with live attenuated viruses have provided invaluable tools for identifying of the immunologic mechanisms of protection against lentivirus infections or disease and understanding the molecular determinants of viral immunopathogenesis (reviewed in Whitney & Rupretch, 2004).

1.3.3.4 Envelope-based sub-unit vaccines

The envelope glycoproteins (gp120 & gp41) provide the main antigens that are responsible for inducing neutralisation epitopes in HIV and SIV infections. Development of vaccine strategies based on HIV-1 envelope has proved to be difficult despite the successful development of a Hepatitis B vaccine, which is based on the surface protein of Hepatitis B virus. Initial reports in this field indicated that gp160 or gp120 was effective in preventing infection of macaques (Ahmad *et al*, 1994; Hu *et al*, 1992) and chimpanzees (Arthur, *et al*, 1989; Berman *et al*, 1990) with SIV and HIV-1 respectively. It was soon realised that these Env glycoproteins did not induce neutralising antibodies (nAB) and protection against pathogenic strains (Haynes, 1996). Most of the neutralisation epitopes are hidden by glycosylated moeities. The 'native' Env complex on the surface of HIV and SIV virions contains gp120 molecules bundled together in groups of three and held together in this trimeric conformation by non-covalent association with a bundle of three transmembrane gp41 molecules (Burton, 1997; Hill *et al*, 1996). Using recombinant technology, soluble multimeric gp140 were made to simulate the natural ("native") glycoproteins by deleting the cleavage site between gp120/41. Immunisation of macaques with the multimeric gp140 induced a different antibody profile from gp120 in addition to low titres of antibodies to V3 loop but failed to elicit neutralising antibodies to heterologous primary isolates (Earl *et al*, 2001). Later analysis revealed that gp140 existed in mixtures of monomers, dimers and tetramers rather that trimers (native).

Attempts to stabilise oligomeric gp140 with intact cleavage sites between gp120 and 41 by introducing disulphide bonds between either gp41 or gp120 and 41 molecules were shown to maintain overall "correct" structure by recognition with conformation-dependent anti-gp120 antibodies and by immunochemical probing (Binley *et al*, 2000). To increase the exposure and immunogenicity of recombinant gp140 (rgp140) by removal of glycosylation sites from envelope on hypervariable loops was done and the rgp140 was evaluated in rhesus monkeys. DNA vectors encoding HIV-1_{SF162} gp140 and SF162ΔV2 were used to prime the immune system and protein boosting (with gp140 cleavage site deleted). Only the SF162ΔV2 induced neutralising antibodies to some heterologous primary isolates (Barnett *et al*, 2001). Further protective potential was assessed after iv challenge with homologous $SHIV_{SF162P}$ in the CD8+T-depleted macaques. Partial protection was achieved and the viral loads in vaccinated monkeys were lower than in unvaccinated controls (Cherpelis *et al*, 2001). Several studies have sought to improve the ability of gp140 proteins to elicit broadly nAB by

increasing the stability of disulphide bonds between gp41 and gp120 (Beddows *et al*, 2005; Center *et al*, 2004; Chakrabarti *et al*, 2002; Sanders *et al*, 2002; Srivastava *et al*, 2003; Yang *et al*, 2002) but generation of nAB that are capable of protecting macaques against heterologous pathogenic challenge has proved difficult to achieve (Xu *et al*, 2006).

Other studies have explored the possibility of inducing effective nAB via immunisation with peptides or polypeptides derived from Env protein. Letvin *et al* (2001) investigated the protective efficacy of Env V3-loop specific antibodies by vaccinating rhesus monkeys with V3 loop peptides. The vaccinated monkeys showed controlled viral loads after challenge with $SHIV_{89.6}$ (non-pathogenic) but not with $SHIV_{89.6P}$ (pathogenic). In another study, a polyvalent envelope glycoprotein vaccine was found to elicit broader neutralising antibodies in pig-tailed monkeys but failed to provide sterilising immunity to a non-pathogenic SHIV challenge (Cho *et al*, 2001). Kumar *et al* (2000) evaluated the immune responses induced by HIV-1 gp120 protein in rhesus monkeys and reported that the vaccine had no effect on infection or viral load after challenging with pathogenic SHIV.

Another strategy with a potential of eliciting antibodies against the native trimeric Env complex has explored the use of pseudovirions, which are produced by means of specific cell-lines after transduction with appropriate plasmid DNA (Hammonds *et al*, 2003 & 2005; Montefiori *et al*, 2001; Radaelli *et al*, 2003). Pseudovirions are non-infectious virus-like particles (VLP), which are capable of exhibiting the native Env trimers on their surface membranes. Both SIV and HIV pseudovirions have shown induction of cellular and humoral responses in animals (Deml *et al*, 1997b; Montefiori *et al*, 2001). Hammonds *et al* (2005) have further shown induction of nAB against HIV-1 primary isolates using the guinea pig model. Prime-boost vaccination strategy using DNA prime, soluble protein boost regimen has also been employed (see **Table 1.4**) in a bid to elicit nAB.

Overall, the HIV envelope approach of developing vaccines have yield disappointing results in inducing protective responses in NHP. Not surprisingly, two monomeric rgp120-based vaccines (designated AIDSVAX B/B and AIDSVAX B/E), have been reported to give no protection when tested in two phase 3 efficacy trials (Cohen *et al*, 2003; Flynn *et al*, 2005; Veljkovic *et al*, 2003).

1.3.3.5 Pseudovirions

Pseudovirions or virus-like particles (VLP) are morphologically similar to immature virions but lacking the viral genetic material. Hence, they are non-infectious. The HIV-1 Gag precursor protein ($Pr55^{gag}$), like those of other retroviruses, is sufficient to generate VLP in the absence of other viral gene products (Freed, 1998; Ono *et al*, 2005). These pseudovirions can be produced *in vitro* in cell cultures via transduction with plasmid DNA or infection with viral vectors that express HIV-1 $Pr55^{gag}$ with an intact myristylation site (Chen *et al*, 2005; Deml *et al*, 2004; Fang *et al*, 1999; Karacostas *et*

al, 1989; Luo *et al*, 2003; Wagner *et al*, 1994). The VLP produced in this manner can be separated and purified into sub-unit vaccines. This vaccine development strategy is discussed in greater details in section **1.5**.

1.3.3.6 DNA vectors

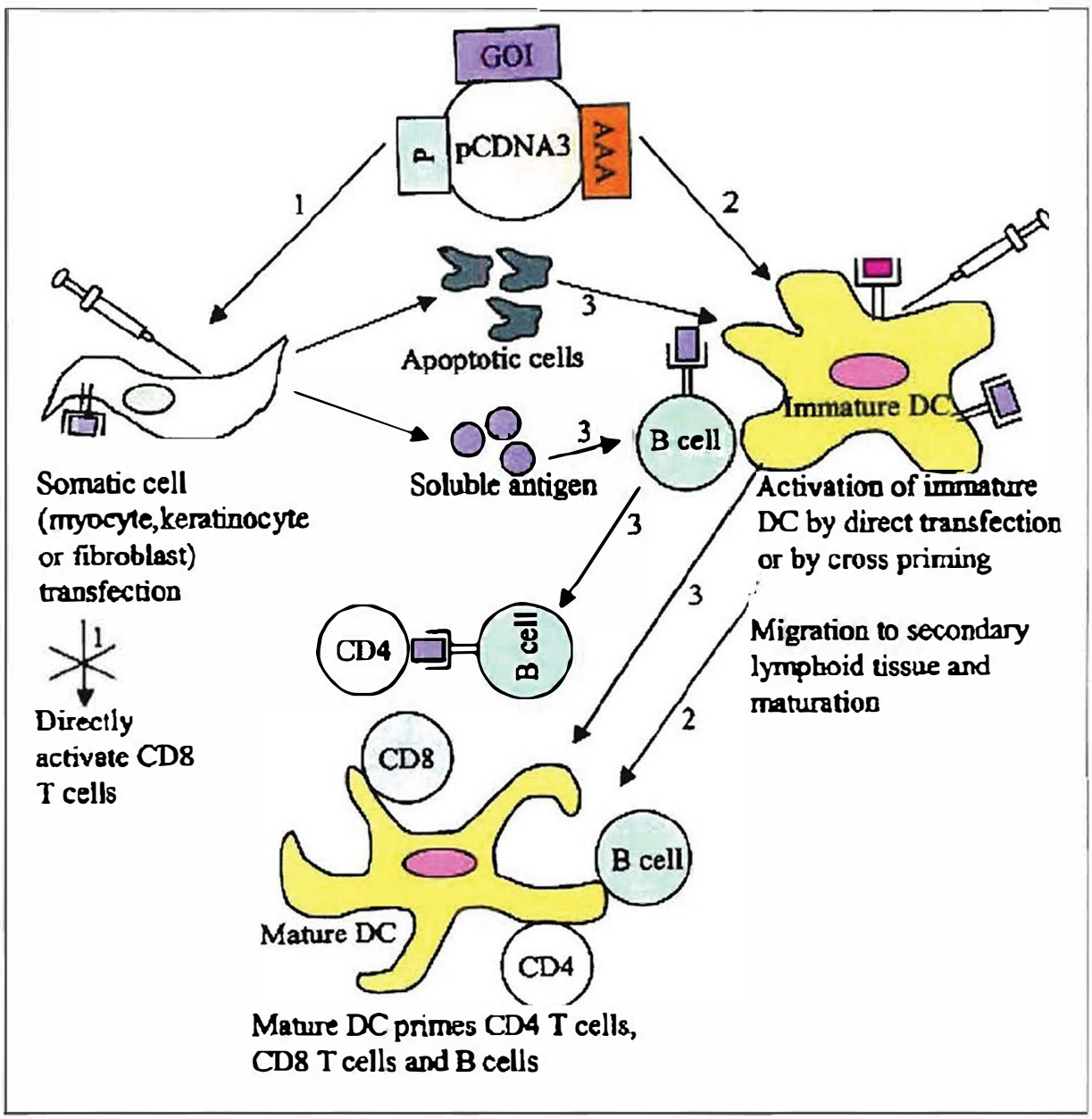
DNA vaccines contain plasmid DNA, which carry portions of pathogens in form of genes coding for specific subunits or immunogens. Following DNA vaccine inoculation, the plasmid DNA is taken up (by transfection) by antigen presenting cells (APC) or somatic cells such as myocytes, keratinocytes and fibroblasts (**Fig. 1.5**). Using the host cell's machinery, the vaccine genes are transcribed and translated into vaccine immunogen proteins, which are then processed for MHC presentation by three possible mechanisms, as shown in **Fig 1.5**. Since non-APC are incapable of antigen presentation to prime the T cells, the vaccine immunogens must be cross-presented to APC (Estcourt *et al*, 2004; Giri *et al*, 2004; Stevenson, 2004). Upon migration to secondary lymphoid tissues, the antigen-activated APC undergo maturity and become capable of priming B and T cells, leading to induction of both humoral (B cells) and cellular (CD4 and CD8 T cells) responses. However, DNA vaccines induce primarily a cellular immune response.

Since cellular immunity was recognised as a key mechanism of controlling HIV-1 replication in early stages of infection, development of DNA vaccines have been attempted to induce cell-mediated responses. The clinical potential of DNA immunisation was first demonstrated by Ulmer *et al* (1993) using the influenza disease model. Boyer *et al* (1997) showed that vaccinating chimpanzees with DNA vaccine protected them from high-dose heterologous HIV-1 challenge. Another study (Mossman *et al*, 1999) compared vaccination of pig-tailed macaques with DNA vectors alone and with protein boosts followed with pathogenic SIV_{mne} challenge. Surprisingly, animals receiving DNA alone were reported to control virus replication better than animals given DNA and protein boosts. Several other studies using NHP models have shown protection by DNA vaccines (Gorelick *et al*, 2000; Wang *et al*, 2000).

A recent strategy of using plasmid DNA to express the full genome of SIV or SHIV is aimed at production of non-infectious virus in the tissues of vaccinated animals. Using this strategy, vaccination of macaques with $SHIV_{KU-2}$ DNA (Liu *et al*, 2006) or $SHIV_{NMr}$ (Horiuchi *et al*, 2006) has demonstrated protection against CD4+ T cell loss and disease to heterologous highly pathogenic viral challenge.

DNA vaccines alone are poorly immunogenic and generate low and transient levels of immune responses in man and nonhuman primates (Boyer *et al*, 1999; Calarota *et al*, 1998, 1999 & 2001; Giri *et al*, 2004; MacGregor *et al*, 1998). These studies show that the potency of DNA vaccines when used

Fig 1.5: Proposed mechanisms of antigen presentation by Giri *et al* (2004). Step 1: shows that expression of the gene of interest (GOI) as a protein antigen can occur following plasmid transfection of the somatic cells. Step 2: shows that immature dendritic cells (DC) can be transfected directly and, on maturation, can activate CD4 T cells, CD8 T cells, and B cells. Step 3: shows that immature DC can acquire antigen following uptake of transfected apoptotic muscle cells, and present the antigen to CD4 T cells and CD8 T cells. Alternatively, immature DC and B cells, following uptake of secreted soluble antigen, can present the antigen to CD4 T cells and CD8 T cells. P: promoter; AAA: polyadenylation signal; pcDNA3: plasmid vector. (Taken from Giri *et al*, 2004).



alone is not sufficient to generate effective and protective immunity. Several approaches of augmenting these responses induced by DNA vaccines have been tried, such as multiple DNA deliveries using biojectors (Haynes *et al*, 1996; Raviprakash *et al*, 2003), use of genetic adjuvants (Barouch & Letvin, 2000; Barouch *et al*, 2000; Bertley *et al*, 2004; Seaman *et al*, 2004; Xin *et al*, 1999), inclusion of immunostimulatory bacterial DNA sequences such as unmethylated cytosine-phosphate-guanosine (CpG) motifs (Davis, 2000; Gursel *et al*, 2002; Hemmi *et al*, 2000; & 2003;

Verthelyi *et al*, 2002); and their use in heterologous prime-boost immunisation regimens. Of these DNA vaccine approaches, the heterologous prime-boost strategy (see section **1.3.11**) has shown great promise.

In general, DNA vaccine strategies have shown great successes (reviewed by Estcourt *et al*, 2004; Giri *et al*, 2004) especially in combination with live attenuated viral vectors such as rMVA and rAd in heterologous prime-boost vaccination modality. In addition, DNA-based vaccines have proved to be safe in human studies (Boyer *et al*, 1999; Calarota *et al*, 1998, 1999 & 2001; MacGregor *et al*, 1998) and have potential for further development. In addition to prime-boost strategies, other feasible approaches to enhance DNA vaccine-induced immune responses include construction of chimeric epitopes derived from different HIV-1 strains to improve on elicitation of cross-reactive T cells; inclusion of multiple HIV-1 genes in the plasmid vaccine to broaden the vaccine immunity (Amara *et al*, 2002; Muthumani *et al*, 2003); co-administration with cytokine genes such as IL-12 and IL-15 to enhance Th1-type responses; and inclusion of CD40L to enhance antigen presentation ability by DC.

1.3.3.7 Live viral vectors

Several live (usually attenuated) viral vectors have been developed for delivery of HIV genes to the immune system. These viral vectors are used as live, live attenuated or replication-defective (or replicon) viruses. Replicons are made by deleting most of viral genes so that the virion "replicons" are completely defective for replication, providing only the structural and/or regulatory proteins and in effect provide just a packaging system. Live vectors (e.g. vaccinia virus) raise considerable safety concerns whereas replicons have excellent safety features but their purification is expensive.

Poxviruses are by far the most used viral vectors especially modified vaccinia virus Ankara (MVA), NYVAC™ (brand name for an attenuated vaccinia virus; Aventis Pasteur), fowlpox virus and ALVAC™ (brand name for a modified canarypox virus; Aventis Pasteur) (Franchini *et al*, 2004). A study by Daniel *et al* (1994) showed that rhesus macaques immunised with recombinant vaccinia virus, which expressed SIV *gag*, *pol* and/or *env* induced strong immune responses in vaccinated macaques but almost all the animals (17 of 18) became infected after low-dose SIV challenge. The disease course was similar in both control and vaccinated groups. Other studies using recombinant vaccinia virus or canarypox (ALVAC) viral vectors showed variable levels of protection of vaccinated macaques to macaque-adapted strain of HIV-2 challenge (Andersson *et al*, 1996; Franchini *et al*, 1995; Myagkikh *et al*, 1996). Recombinant MVA, which expressed SIV antigens were also shown to induce SIV-specific immune responses, including CD8+ T cells (Hirsch *et al*, 1996; Ourmanov *et al*, 2000; Seth *et al*, 2000). The viral loads in vaccinated macaques were 1-2 $\log_{10}$ lower than in unvaccinated controls following SIV challenge. The NYVAC strain of vaccinia, which

expressed SIV env has also been used in NHP studies (Benson *et al*, 1998) to immunise macaques followed by either intra-rectal or intravenous challenge with SIV_{mac251} (highly pathogenic). Suppression of viral replication was seen in vaccinated macaques. Vaccine studies, done in macaques, involving recombinant fowlpox virus as the viral vector have been described (Kent *et al*, 1998 & 2005; Dale *et al*, 2004a & 2004b; De Rose *et al*, 2005; Coupar *et al*, 2006). Most of these studies included a DNA vaccine prime.

In general, poxvirus-based vaccines, when used alone, showed limited protection against pathogenic SIV. However, when used to boost the immune response after DNA priming, excellent boosting effects on the immune responses and protection were obtained (see section **1.3.11**). Repeated vaccination with poxvirus-based vaccines could impact negatively on their immunogenicity due to induction of anti-poxvirus immunity following initial vaccination (Sharpe *et al*, 2001). Because of this concern, efforts have been directed to further improvements of immunogenicity elicited by poxvirus viral vectors. The use of vaccines co-expressing IFN-γ and IL-12 have been shown to increase immunogenicity and protective efficacy of poxvirus-based vaccines (De Rose *et al*, 2005; Dale *et al*, 2004a & 2004b; Coupar *et al*, 2006; Abaitua *et al*, 2006).

Immunization of rhesus with Venezuelan equine encephalitis virus (VEE) replicon particles expressing SIVsm/H4i *gag* and *env* genes induced both humoral and cellular immune responses in vaccinated animals and all 4 vaccinated animals were protected against disease for at least 16 months following iv challenge with a pathogenic SIV_{smE660} (Davis *et al*, 2000). Also, the mean peak viral load reduced by 100-fold in vaccinated macaques. A subsequent vaccination study of macaques with the SIV immunogens delivered by VEE replicon particle vectors followed by a mucosal challenge with SIV_{smE660} was shown to elevate levels of CD4+ T cells and reduce viraemia (Johnston *et al*, 2005). Polio virus replicons expressing HIV-1 and SIV antigens have also demonstrated protection against $SHIV_{89.6P}$ disease in pig-tailed macaques when used in prime-boost vaccination strategy with purified $HIV\text{-}1_{89.6}$ rgp 160 and SIV_{mac239} p55 Gag (Fultz *et al*, 2003). Semliki forest virus (Hanke *et al*, 2003) and Sindbis virus (Vajdy *et al*, 2001) replicon particles expressing HIV-1 and SIV antigens have been reported to be at development stages for use in NHP studies.

Replication-incompetent adenovirus serotype 5 (Ad5) vector has been developed to express SIV or HIV-1 Gag and shown to elicit strong cellular responses in macaques (Shiver *et al*, 2002; Casimiro *et al*, 2003a & 2005; Santra *et al*, 2005) and baboons (Casimiro *et al*, 2003b). The vaccinated macaques had lower viral loads after intravenous challenge with pathogenic $SHIV_{89.6P}$ and suppression of viral replication correlated with pre-challenge cellular responses. The immunogenicity of these vaccines has been further enhanced by heterologous DNA prime-viral vector boost vaccination protocols (see section **1.3.11**). The use of a replication-competent Ad5 vector and DNA plasmid vectors expressing

SIVsmH4 *env* and *rev* genes in a DNA prime/viral vector boost combination has been reported to be effective in inducing strong humoral and cellular immunity to SIV envelope (Malkevitch *et al*, 2004). A major limitation for use of Ad5 vector is the widespread pre-existing immunity in the human population (Kostense *et al*, 2004; Nwanegbo *et al*, 2004). To overcome this problem, adenovirus serotype 35 (Ad35) has been identified for future adenovirus-based vaccines in human (Barouch *et al*, 2004).

Recombinant vesicular stomatitis virus (rVSV), expressing SIV *gag* and HIV *env* have also been evaluated and shown to cause suppression of viral replication in vaccinated macaques after intravenous viral challenge with $SHIV_{89.6P}$ (Rose *et al*, 2001; Egan *et al*, 2004). Egan *et al* (2005) have further shown that priming with plasmid DNA expressing IL-12 and SIV Gag enhanced the immunogenicity and post challenge efficacy of rVSV vaccine. Rhesus macaques receiving this combination had significantly increased SIV Gag-specific cellular and humoral immune responses and significantly lower viral loads after challenge with pathogenic $SHIV_{89.6P}$ relative to macaques receiving only the rVSV immunisation. Sabin vaccine strain of poliovirus (Crotty *et al*, 2001; Crotty & Andino, 2004), Herpes simplex virus (HSV; Murphy *et al*, 2000) and measles virus (Lorin *et al*, 2004) have also been investigated for use as live viral vectors in NHP studies.

Sendai virus, an enveloped RNA virus that causes a fatal pneumonia in mice but non-pathogenic in primates (Kano *et al*, 2000a), has been used as a viral vector. A recombinant Sendai virus expressing SIV Gag was capable of controlling viraemia in cynomolgus macaques challenged with high dose SIV_{mac239} (Kano *et al*, 2000b). When given as a booster vaccine after a matched plasmid DNA prime vaccine, high levels of SIV Gag-specific T cells, control of viremia after pathogenic $SHIV_{89.6P}$ challenge and protection from AIDS progression were demonstrated (Matano *et al*, 2001). In addition, a recombinant replication-defective version of the vaccine given after a single DNA priming was reported to confer protective efficacy against $SHIV_{89.6P}$ challenge (Kawada *et al*, 2006; Takeda *et al*, 2003).

Other novel viral vectors being developed for HIV vaccine delivery include rabies virus (McKenna *et al*, 2004; McGettigan *et al*, 2006; Tan *et al*, 2005), influenza (flu) virus (Gonzalo *et al*, 1999; Gherardi *et al*, 2003), Semliki Forest virus (Berglund *et al*, 1997; Hanke *et al*, 2003) and foot-and-mouth disease virus.

Overall, live viral vectors, especially rMVA and rAd5 or 35, offer great opportunity of developing HIV vaccines that are capable of generating effective T cell responses, particularly when used to boost DNA vaccines.

1.3.3.8 Live bacterial vectors

Bacteria, unlike viruses, lack protein glycosylation machinery, and thus they are unable to express "native" env proteins. However, intracellular bacteria have been found to be most suitable as bacterial vectors. Examples include *Salmonella* spp, *Shigella* spp, *Listeria monocytogenes* and *Mycobacteria bovis* BCG. The use of BCG as live bacterial vector is discussed in greater details in section **1.4**.

A major advantage of using *Salmonella* spp as a bacterial vector is the potential for use as an oral vaccine as *Salmonella* species are common gut pathogens. A few studies using *Salmonella* vectors expressing SIV proteins (Franchini *et al*, 1995; Steger *et al*, 1999) have shown induction of immune responses in macaques and to act as an effective prime for subsequent boosting with rMVA vaccines (Evans *et al*, 2003). However, the macaques that were primed with *Salmonella* vectored vaccine and boosted with a matching rMVA vaccine did not show any improvement in control of virus replication following a rectal challenge with SIV_{mac239} (Evans *et al*, 2003).

Besides having potential for mucosal immunisation, recombinant attenuated *Listeria monocytogenes* infects monocytes, which are key antigen presenting cells. Immunogenicity studies in NHP have been reported (see section **1.3.11**; Boyer *et al*, 2005).

1.3.3.9 Heterologous prime-boost regimens

Heterologous prime-boost vaccination protocols utilise combination vaccines involving immunisation with the same vaccine immunogen(s), which are delivered to the immune system by two different delivery systems. This strategy usually utilises primary immunisation with a live vector or DNA vaccine, followed by booster immunisation with either a live vector or subunit/protein vaccine containing a matched immunogen. This mode of vaccination has been shown to induce strong immune responses compared to vaccination with either vaccine alone. Several prime-boost vaccination modalities have been tested in NHP models. **Table 1.4** gives a list of notable examples.

A substantial boost of the immune response to a DNA vaccine can be achieved with viral or "adjuvanted" protein boosts. DNA vaccines seem to be best at priming the immune system. The efficiency of priming is due to DNA vaccine focussing the immune response generated by the booster vaccine to the vaccine antigen as DNA itself is non-immunogenic (Giri *et al*, 2004). Initial work in this field studied the DNA prime and protein boost strategies, which were found to enhance the antibody responses and little or no increase in CTL response upon protein boosting (Barnett *et al*, 1997; Letvin *et al*, 1997; Mossman *et al*, 1999). The prime- boost immunisations involving DNA prime and recombinant viral vector boost attained excellent CTL boosting effect in many animal models (Hanke *et al*, 1998 & 1999; Allen *et al*, 2000; Robinson *et al*, 1999; Amara *et al*, 2001; Casimiro *et al*, 2003a, 2003b & 2004; Wee *et al*, 2002; Shiver *et al*, 2002).

Table 1.4: Examples of heterologous prime-boost vaccination modalities that have been used in HIV vaccine studies utilising NHP animal models.

Prime-boost protocol	Reference(s)
DNA prime - Sub-unit/protein boost	
▪ DNA + Env protein	Barnett *et al*, 2001; Cherpelis *et al*, 2001; Mossman *et al*, 1999; Rasmussen *et al*, 2002; Letvin *et al*, 1997; Putkonen *et al*, 1998; Mossman *et al*, 2004; Pal *et al*, 2006a
▪ DNA + virus-like particles	Leung *et al*, 2004.
DNA prime – Poxviral vectors boost	
▪ DNA + vaccinia virus	Someya *et al*, 2006.
▪ DNA + MVA	Allen *et al*, 2000; Amara *et al*, 2001, 2002 & 2005b; Hanke *et al*, 1999; Heeney *et al*, 2000; Puaux *et al*, 2005.
▪ DNA + NYVAC	Hel *et al*, 2001.
▪ DNA + fowlpox	Coupar *et al*, 2006; Kent *et al*, 2005; Robinson *et al*, 1999; Stratov *et al*, 2005.
DNA prime – Other viral vectors boost	
▪ DNA + adenovirus	Casimiro *et al*, 2003a, 2003b, 2004 & 2005; Malkevitch *et al*, 2004; Suh *et al*, 2006.
▪ DNA + alphavirus (e.g. SFV)	Michelini *et al*, 2004; Koopman *et al*, 2004; Negri *et al*, 2004.
▪ DNA + rhabdovirus (e.g. VSV)	Egan *et al*, 2004 & 2005.
▪ DNA + paramyxovirus (e.g. Sendai virus)	Matano *et al*, 2001; Takeda *et al*, 2003.
DNA prime – bacterial vectors boost	
▪ DNA + salmonella	Franchini *et al*, 1995; Steger *et al*, 1999.
▪ DNA + listeria	Boyer *et al*, 2005 & 2006.
Viral vector prime – Sub-unit/protein boost	
▪ Canarypox (ALVAC) + Env protein	Andersson *et al*, 1996; Pal *et al*, 2002 & 2006b; Wright *et al*, 2004.
▪ Vaccinia (MVA) + Env protein	Nilsson *et al*, 2002.
▪ Picornavirus (poliovirus) + Env protein	Fultz *et al*, 2003.
Viral vector prime – viral vector boost	
▪ Adenovirus type 5 + adenovirus type 35	Barratt-Boyes *et al*, 2006.
▪ Alphavirus (SFV) + poxvirus (MVA)	Nilsson *et al*, 2001.
▪ Rhabdovirus (VSV) + poxvirus (MVA)	Ramsburg *et al*, 2004
Bacterial vector prime – viral vector boost	
▪ BCG + vaccinia virus	Ami *et al*, 2005.
▪ Salmonella + MVA	Evans *et al*, 2003

MVA: modified vaccinia virus Ankara; NYVAC: brand name (Aventis Pasteur) for an attenuated vaccinia virus strain; ALVAC: brand name (Aventis Pasteur) for a modified canarypox virus strain; SFV: Semliki forest virus; VSV: vesicular stomatis virus; BCG: *Mycobacterium bovis* Calmette-Guerin bacillus.

In a classical study involving a prime-boost vaccination modality, Hanke *et al* (1999) showed that, by using a multicytotoxic CTL epitope gene in a DNA prime-MVA boost vaccination regimen, high levels of CTL specific for a single SIV *gag*-derived epitope were elicited in vaccinated rhesus macaques. These CTL were capable of killing SIV-infected cells *in vitro*. FACS analysis using

soluble tetrameric MHC-peptide complexes showed that the vaccinated animals had 1 to 5% circulating $CD8^+$ lymphocytes specific for the vaccine epitope, frequencies, which were comparable to those obtained in SIV-infected monkeys. However, upon intra-rectal challenge with pathogenic SIV_{mac251}, no protection against infection was observed in at least 2 of 3 vaccinated macaques.

Replication-defective recombinant human adenovirus serotype 5 (rAd5) vaccine vector expressing a codon-optimized HIV-1 *gag* gene has demonstrated effective boosting potentials. Rhesus macaques vaccinated with a DNA and rAd5 vectors expressing SIV_{mac} gag were protected against disease progression after intravenous SHIV (Casimiro *et al*, 2003a) and intrarectal SIV_{mac239} (Casimiro *et al*, 2005) challenge. Also, the ability of multigenic SIV DNA prime-rAd5/SIV boost to induce SIV-specific immune responses and protection against intrarectal challenge with SIV_{mac251} has been reported and shown to be promising (Suh *et al*, 2006). Baboons vaccinated with plasmid DNA and rAd5 vector expressing a codon-optimized HIV-1 *gag* gene exhibited consistently strong and long-lived CD8-biased T-cell responses and *in vitro* cytotoxic activities in vaccinated baboons. The DNA vaccine alone elicited weak immune responses but formulation with chemical adjuvants led to moderate increases in the levels of Gag-specific T cells (Casimiro *et al*, 2003b). A recent study by Casimiro *et al* (2004) has further shown that induction of higher cellular responses was achieved in vaccinated macaques when rAd5 vaccine was used to prime for rMVA and not vice versa.

As noted earlier, high prevalence of pre-existing immunity to Ad5 would adversely affect the use of rAd5-based vaccine in clinical settings. Based on low prevalence of Ad35 neutralising antibodies in the general human population, rAd35 has been found to be suitable for vaccine delivery. A recent study by Barratt-Boyes *et al* (2006) investigated the immunogenicity of a first generation replication-competent Ad35-based vaccine using the rhesus macaque model. rAd35 expressing SIV_{mac239} gag gene effectively boosted T cell responses that were induced by a matched rAd5 replication-defective vaccine. Also, Ad5-specific neutralising antibodies did not neutralise rAd35, indicating that pre-existing immunity to Ad5 is not likely to reduce the potency of Ad35-based vaccination in clinical settings.

Using rBCG expressing SIV Gag to prime and a replication-deficient vaccinia virus expressing SIV Gag, Ami *et al* (2005) demonstrated induction of high levels of IFN-γ spot forming cells and effective immunity against mucosal challenge with pathogenic SHIV in rBCG-primed cynomolgus monkeys but not in animals receiving opposite combination or single-modality vaccines.

Boyer *et al* (2005) used plasmid DNA and *L. monocytogenes* that expressed and secreted SIV Gag and Env antigens in a prime-boost vaccination protocol. The recombinant *L. monocytogenes* (rLm-

Gag/Env) was delivered orally. Rhesus macaques receiving DNA SIV Gag/Env prime and rLm-Gag/Env boost developed significantly increased SIV-specific CD4+ and CD8+ T cell responses than those receiving single modality vaccinations. Also, prime-boost vaccination afforded some limited suppression of SIV_{mac239} viral replication after challenge. The level of vaccine-induced IFN-γ-producing cells at the time of challenge were further investigated and found to correlate more strongly with viral loads at set-point than peak viral loads (Boyer *et al*, 2006).

Other efforts directed at improving the current DNA vaccination approaches are aimed at stimulating the induction of effective CD4+ T cell response, in addition to induction of potent CD8+ T cell responses. HIV-1 $Pr55^{gag}$ virus-like particles in prime-boost modalities is particularly attractive because of their demonstrated potential in introducing antigens in both MHC class 1 and class 2 processing and presentation pathways, leading to induction of both CD4+ and CD8+ T-cell mediated immune responses (Sester *et al* 2000; Heintel *et al* 2002).

Overall, heterologous prime-boost vaccination strategy is a very promising one, with various DNA vaccines prime-boost combinations already in clinical trials. A phase 3 efficacy clinical trial, the third phase 3 trial so far, and currently in progress in Thailand, utilises a prime-boost vaccination protocol. This trial, code-named RV144, uses a canarypox viral vector (ALVAC-HIV or vcP1521) as a prime vaccine and a monomeric rgp120 vaccine (AIDSVAX B/E) as a booster vaccine (McNeil *et al*, 2004; Pitisuttithum, 2005; Trinvuthipong, 2004). As mentioned earlier, AIDSVAX B/E, a mixture of subtype B- and subtype E-derived rgp120, failed to protect vaccinated individuals when given alone (Cohen, 2003; Flynn *et a*l, 2005).

1.4 RECOMBINANT BCG VACCINES

1.4.1 A histological background of BCG vaccination

The Bacille Calmette-Guerin (BCG) is an attenuated *Mycobacterium bovis* that was developed by Albert Calmette and Camille Guerin between 1904 and 1908 at the Institute Pasteur de Lille in France (Hoft and Georghiu, 1996). They achieved this attenuation by doing repeated passage of a virulent strain of *M. bovis* on potato-glycerine medium supplemented with ox bile. Extensive testing *in vitro* and in animals was carried out between 1908 and 1921 which confirmed attenuation was non-reversible and vaccinated animals developed resistance to challenge with virulent tubercle bacilli (Hawgood, 1999). The first human vaccination was done in 1921 and by 1926, over 50,000 children had been vaccinated against tuberculosis (TB) and a mortality of only 1.8% compared with 25-32% among unvaccinated children was demonstrated by Calmette himself (Hoft and Georghiu, 1996). In 1928, the League of Nations recommended the use of BCG for protection against TB. It was put on the WHO's expanded programme on immunisation in 1974 (WHO, 2001) and has been adopted by

many countries worldwide, especially in developing countries, in childhood immunization programmes.

The original vaccine strain has developed into several different substrains, which have been used for production of BCG vaccines since 1921. Between 1924 and 1926, at least 34 countries received BCG cultures from Calmette and more than 26 others are reported to have obtained BCG cultures from Paris in 1927 (Oettinger *et al*, 1999). These cultures were propagated in the various countries in different ways until 1950s when the freeze-drying method was developed and seed lots were established. By this time, more than 50 BCG substrains had emerged. Many of these are no longer in use. Today, only 7 sub-strains (Danish-1331, Glaxo-1077, Pasteur-1173, Tokyo-172, Sofia-SL 222, D2PB302 and Russian) are extensively used for vaccine production (WHO, 2001). A number of genetic and antigenic variations among these BCG vaccine sub-strains have been reported and morphological, biochemical and immunological differences among them have been observed (Oettinger *et al*, 1999).

1.4.2 Advantages of BCG as a vaccine vector

BCG is a non-pathogenic intracellular bacillus that infects and colonises the macrophages and dendritic cells where it can survive and replicate for long periods of time. In granulomas, BCG form foci, remaining alive for many years, and generating strong and long-lived immune responses (Langermann, 1996). The cell wall of BCG confer strong non-specific immunomodulating effects, hence making it unnecessary (and cheaper) to administer it together with an adjuvant. Since 1948, over 3 billion people have been vaccinated with BCG with very few complications (Lotte *et al*, 1984), giving BCG vaccine an outstanding safety profile. Oral (or mucosa) administration is possible; indeed, BCG was first administered by oral route until around 1948 when most countries changed to intradermal administration (Langermann, 1996; Hoft & Georghiu, 1996). The vaccine is heat stable making it easy to preserve and store. It is inexpensive to produce and the infrastructure exists to manufacture the vaccine in some developing countries, including South Africa. These features make BCG an attractive vector for the delivery of vaccine immunogens. There are, however, a number of challenges involved in manufacturing recombinant BCG (rBCG). These include the slow growth rate of BCG, instability of the rBCG and low expression of the heterologous protein by the rBCG.

1.4.3 Immune responses to BCG

Data from animal models, BCG-vaccinated persons and TB patients indicate that the immune responses to BCG and other mycobacteria are mainly cell-mediated, which involve the T-cells, macrophages and Th-1 cytokines secreted by activated T cells and macrophages. Macrophages (and dendritic cells) take up the bacteria through normal phagocytosis (Inaba *et al*, 1993), after which, the phagosomes are formed in their cytoplasm. These are the major sites where the bacteria reside and

multiply in the host macrophage. The bacilli can colonize the macrophages for long periods of time. Phagocytosed bacterial proteins are secreted from the phagosomes and taken up for processing via the endosomal pathway and presented to CD4+ T cells in the context of MHC class 2 molecules leading to priming and activation of these cells. Cross-presentation to CD8+ T cells in the context of MHC class 1 molecules can also occur especially in conditions of chronic infection or re-infection. In addition to this, the process of phagocytosis induces IL-12 release from macrophages (Ladel *et al*, 1997; Henderson *et al*, 1997). IL-12 helps in the development of T-helper 1 (Th-1) cells (Sano *et al*, 1999). Activated Th-1 cells in turn produce Th-1 cytokines such as IFN-γ that triggers the anti-mycobacterial activity of macrophages. Several studies have demonstrated the importance of IL-12 in mycobacterial immunity. Exogenous administration of IL-12 to BALB/c mice has shown improvement of survival in *M. tuberculosis* infection (Flynn *et al*, 1995a) while IL-12 double knockout mice are quite susceptible to infection (Cooper *et al*, 1997). Humans with defective IL-12p40 or IL-12 receptor genes were found to have reduced IFN-γ production from T cells (Ottenhof *et al*, 1998).

The important role of IFN-γ was demonstrated by studies that showed that IFN-γ knockout mice rapidly succumbed to *M. tuberculosis* infection and showed defective macrophage activation (Cooper *et al*, 1993; Flynn *et al*, 1993). Similarly, humans with defective genes for IFN-γ receptor are unusually susceptible to TB (Ottenhof *et al*, 1998). Tumour necrosis factor-alpha (TNF-α), another Th-1 cytokine, is produced by T cells, macrophages and dendritic cells during *M. tuberculosis* infection (Henderson *et al*, 1997; Ladel *et al*, 1997; Serbina & Flynn, 1999) and has been reported to play multiple roles in immune and pathologic responses in mycobacterial immunity. It acts in synergy with IFN-γ in macrophage activation (Flesch & Kaufmann, 1990; Chan *et al*, 1992), although it is ineffective on its own. This cytokine also plays a central role in granuloma formation in BCG (Kindler *et al*, 1989) and other mycobacterial diseases (Flynn *et al*, 1995b; Bean *et al*, 1999; Mohan *et al*, 2001; Garcia *et al*, 1997). The main effector mechanism of anti-mycobacterial activity that is triggered by IFN-γ and TNF-α is via the activation of macrophages, leading to increased activity of nitric oxide synthase and release of nitric oxide and related reactive nitrogen intermediates by the macrophages. Nitric oxide and the reactive intermediates cause acidification of phogosomes and killing of mycobacteria.

The primary role of CD4+ T cells in mycobacterial immunity is the production of IFN-γ, which plays a major role in activation of macrophages, help for CD8+ cytotoxic cells and B cells, conditioning of antigen-presenting cells and induction of apoptosis. Apoptosis has been reported to play a role in the control of mycobacterial infections. There may be other cytokines produced by CD4+ T lymphocytes and participating in mycobacterial immunity. The induction of CD4+ T cell proliferative responses

and production of IFN-γ in humans and nonhuman primates vaccinated with BCG has been documented in a number of studies (Kemp *et al*, 1996; Ravn *et al*, 1997; Hoft *et al*, 1998 & 1999; Abolhassani *et al*, 2000; Shen *et al*, 2002; Lai X *et al*, 2003)

Initially, the major role of combating a mycobacterial infection was attributed exclusively to CD4+ T lymphocytes and hence the focus was on CD4+ T-cell research. It later emerged that mice deficient in MHC class I-restricted lymphocytes succumbed rapidly to *M. tuberculosis* infection (Flynn *et al*, 1992). The role of CD8+ T cells in mycobacterial immunity has now become the focus of intense investigations. Studies in human and various mouse models have indicated that CD8+ T cells are necessary for the control of TB infections (Behar *et al*, 1999; Flynn *et al*, 1992; Lalvani et el 1998; Rolph *et al*, 2001; Serbina & Flynn 2001; Sousa *et al*, 2000; van Pinxteren *et al*, 2000). It has now been shown that CD8+ T cells mediate direct cytolytic activity by lysing mycobacteria-infected macrophages, directly killing the intracellular bacteria by production of granulysin (Stenger *et al*, 1998) and by producing IFN-γ for activation of macrophages. Earlier reports had indicated that CD8+ T cells produce no or negligible amounts of IFN-γ, this being the exclusive role of CD4+ T cells (Ravn *et al*, 1997). Later studies demonstrated that CD8+ T cells from BCG-vaccinated humans (Davids *et al*, 2006; Hoft *et al*, 1999) and macaques (Lai X *et al*, 2003) produce IFN-γ in addition to the amounts produced by the CD4+ T cells. Other studies have suggested that the CD8+ T cells play only a modest role during the first phase of infection (Feng & Britton, 2000)

Unconventional T cell subsets, the gamma-delta T-cells (γδ T), and CD4-/8- (double negative) αβ T cells have been reported to play a role in mycobacterial immunity, including BCG (Shen *et al*, 2002; Lai *et al*, 2003; Hoft *et al*, 1998). These subsets recognize mycobacterial non-protein antigens, e.g. glycolipids, in the context of CD1 molecules.

Delayed-type hypersensitivity (DTH) reaction is a cell-mediated response that is induced by several bacterial and viral antigens, including purified protein derivative (PPD). The reaction has been shown to be a CD4+ and CD8+ T cell-mediated memory effector mechanism (Black, 1999) in which the IFN-γ plays a major role (Seabrook *et al*, 2005). The DTH skin test is therefore an important *in vivo* test in screening for TB infection (in both humans and animals) as well as a research tool for measuring induction of cellular immune response to BCG vaccination. When a small amount of PPD is administered intradermally to mycobacteria-sensitised humans or animals, a cascade of inflammatory reactions is triggered, which includes monocytic infiltration and release of cytokines and pro-adhensive molecules into the site of injection. This culminates into the appearance of erythrema (reddening) and an induration (swelling), both of which are measurable between 24 and 72h after injection. The PPD responses induced by BCG vaccination have been reported to persist for

>10 years (Comstock *et al*, 1971; Menzies & Vissandjee, 1992) and boosting can occur after BCG vaccination (Hoft & Tennant, 1999).

Th-2 cytokines, which are associated with humoral responses, are induced only at low levels in most animal models and they have not been shown to play any role in protective immunity in mycobacterial infections.

1.4.4 Development of rBCG HIV vaccines

The discovery of *E. coli/Mycobacterium* shuttle vectors made it possible to express foreign proteins in BCG. The application of this technology led to development of many recombinant BCG (rBCG) vaccine constructs, which express various foreign proteins (Abomoelak *et al*, 1999; Ami *et al*, 2005; Connell *et al*, 1993; Kawahara *et al*, 2006; Leung *et al*, 2000; Matsuo *et al*, 1990; Stover *et al*,, 1991 & 1993; Yasutomi *et al*, 1993), including those of HIV-1 (Aldovini & Young, 1991; Chujoh *et al*, 2001; Gheorghiu *et al*, 1994; Hiroi *et al*, 2001; Honda *et al*, 1995; Kameoka *et al*, 1994; Kawahara *et al*, 2002a & 2002b; Someya *et al*, 2005; Winter *et al*, 1991). These rBCG vaccines have been shown to elicit both humoral and cellular responses in various animal models, including the macaques (Ami *et al* 2005; Leung *et al*, 2000; Someya *et al*, 2005). It is believed that the success of recombinant BCG vaccines is dependent on successful colonisation of the immune system by the rBCG bacteria (Labidi *et al*, 2001) but pre-existing immunity to BCG is thought to be an obstacle in achieving such a success (Brandt *et al*, 2002; Buddle *et al*, 2002; Palmer & Long, 1966).

1.4.5 Nonhuman primate models of rBCG

The mouse and guinea pig are simple, cost-effective models and they have been used for extensive studies in BCG and TB research. The pathogenesis and induction of immunity to mycobacterial infections, including BCG, are very well characterized in these models. Thus, almost all initial preclinical screening of potential rBCG vaccines is carried out in these animals to select the vaccines with the greatest potential for further evaluation in nonhuman primates.

When testing a vaccine for human use it is important that the immune system of the animal model chosen is as close to that of the human if possible. In this respect, non-human primates have unique advantages over the mouse and guinea pig models. As noted earlier, there is high sequence homology between components of the human immune system and that of nonhuman primates (Adams & Parham, 2001; Bontrop, 1995 & 1999; Bontrop & Watkins, 2005; Boyson *et al*, 1996; Damian *et al*, 1971; de Groot *et al*, 2000; Doxiadis *et al*, 2001; Hainsey *et al*, 1993; Rogers & Hixson, 1997). In regard to rBCG vaccines, the nonhuman primates may be more suitable models than the murine and guinea pig models because they are susceptibility to infection with *M. tuberculosis* by natural route (respiratory) and develop a human-like disease (McMurray, 2000). In addition, it has been reported

that several host molecules implicated in mycobacterial infections are present and comparable in human and nonhuman primates but absent or differ fundamentally in the mouse and guinea pig (Geluk *et al*, 1993; Porcelli *et al*, 1992; Shinkai and Locksley 2000; Behar *et al*, 1999). Examples of these similarities between human and nonhuman primates are found in the MHC-DR, -DQ and –DP regions (Bontrop *et al*, 1999; Doxiadis *et al*, 2001), and these products are reactive with some specific mycobacterial peptides in both species (Geluk *et al*, 1993). Also, group 1 CD1 molecules, which are involved in presentation of nonpeptide mycobacterial products to T cells, are present in both human and primates (Porcelli *et al*, 1992; Shinkai & Locksley 2000). A related CD1 molecule in the mouse is reported not to participate in resistance to *M. tuberculosis* infection (Behar *et al*, 1999). In addition, the human T cell antigen receptor repertoire, including γδ T cells, which recognize several mycobacterial ligands (Contant *et al*, 1994; Carding & Egan, 2000) are fundamentally different from those of small laboratory animals but are comparable to those of macaques (Poccia *et al*, 1999; Bontrop *et al*, 1995). These and the fact that nonhuman primates are essential for safety testing of new live and attenuated vaccines make them attractive models for the critical and final stages of pre-clinical evaluation of the rBCG vaccines.

1.4.6 The baboon model for rBCG

Baboons like other non-human primates, are susceptible to BCG and other mycobacterial infections (Theon *et al*, 1977; Fourie & Odendall 1983; Tarara *et al*, 1985; Saposky & Else 1987; Keet *et al*, 2000; Thorel *et al*, 1998; McMurray 2000; Langermans *et al*, 2001) and will develop human-like TB disease when infected with *M. tuberculosis* and *M. bovis*. Although a few studies investigating immune responses to BCG in the macaque (Abolhassani *et al*, 2000; Langermans *et al*, 2001; Shen *et al*, 2002; Lai *et al*, 2003) have been reported, there is virtually no information on the immune response of baboons, and in particular, immune responses of the chacma baboon to BCG.

Chacma baboons are readily available in South Africa and could provide the model required for rBCG testing using vaccination regimens reflecting those to be used in human trials. This baboon species has been reported to be infected with *M. tuberculosis* and *M. bovis* (Fourie & Odendall 1983; Keet *et al*, 2000; Thorel *et al*, 1998) but there are no published attempts to study immune responses to BCG and other mycobacterial infections in this species or any attempts to develop this model for evaluation of rBCG vaccines.

There are a number of advantages of using baboons in vaccine research. Baboons like other nonhuman primates are phylogenetically related to humans (Rogers and Hixson, 1997) and have similar anatomy and physiology to humans (Hainsey *et al*, 1993). In addition, baboons are abundant in the wild and adapt well in captivity. Unlike macaques, baboons are not naturally infected with herpes B virus (Kennedy *et al*, 1997) providing safer handling of animals and blood products for

animal caretakers and laboratory workers. Herpes B infection is a potential fatal infection in humans. Immunologically, baboons like humans, have four immunoglobulin G subclasses (Damian *et al*,, 1971), while rhesus macaques have only three (Martin, 1982). These, and the reported shortage of rhesus macaques for use in research (Cohen, 2000) has led to increasingly more use of baboons as alternative models in HIV vaccine evaluations (Locher *et al*, 2004, Casimiro *et al*, 2003b; Leung *et al*, 2004). Moreover, the baboon can be infected experimentally with SHIV (Allan *et al*, 1995; Klinger *et al*, 1998) and certain isolates of HIV-2. Some HIV-2-infected baboons develop a slow clinical AIDS-like disease, which is similar to that observed in HIV-1-infected humans (Locher *et al*, 1997; 1998; 2001). Locher and co-workers have further characterized the pathological lesions after infection of baboons with a pathogenic isolate of HIV-2 (Locher *et al*, 2003b) and showed for the first time the possibility of using the HIV-2/baboon model for challenge experiments in HIV vaccine protective efficacy trials (Locher *et al*, 2004).

In conclusion, BCG has been shown to generate long-lived immune responses due to its ability to persist for long periods of time after vaccination and its ability to induce strong adjuvant properties. It is also inexpensive to produce and has a good safety profile as a vaccine against TB. The technology to express foreign genes in BCG has been developed and such rBCG have been found to induce both cellular and humoral immune responses to the heterologous proteins. On these considerations, BCG can be viewed as a highly suitable live bacterial vector for development of effective, safe and affordable HIV-1 vaccines. Pre-clinical evaluation of potential rBCG-based candidate vaccines would be crucial and the baboon promises to be a suitable and valuable non-human primate model.

1.5 SUB-UNIT VACCINES

1.5.1 Safety and immunogenicity of sub-unit vaccines

Non-replicating viral proteins or sub-unit vaccines are considered to fulfil the safety demands of HIV vaccines. On one hand, the soluble viral proteins such as peptides and viral polypetides frequently induce poor immunogenicity and are unable to stimulate cellular responses unless they are co-administered with adjuvants. On the other hand, particulate antigens such as virosomes (Gluck & Metcalfe, 2000; Zurbriggen, 2003), immunostimulating complexes, ISCOMS (Takahashi *et al*,1990), poly (lactide-co-glycolide) [PLG] microparticles (Vajdy & O'Hagan, 2001) and virus-like particles (Schirmbeck *et al*, 1994 & 1996; Wagner *et al*, 1996a) have been found to induce more effective humoral response than their soluble counterparts and potent cellular immune response. These particulate antigens possess the efficiency of vaccines that utilise the live or live attenuated vectors but they are safer than live attenuated vaccines.

1.5.2 Virus-like particles

Among the particulate sub-unit vaccines, VLP are particularly attractive for use as sub-unit vaccines because they include the relevant immunogenic structures of viruses and are safe since they lack the viral regulatory proteins and infectious genetic material. Capsid and envelope proteins from numerous viruses have the ability to self-assemble into highly organised particulate structures. Examples are: hepatitis B virus core and surface antigen (Deml *et al,* 1999; von Brunn *et al,* 1991), papilloma virus L1 or L1/L2 (Breitburd *et al,* 1995; Kirnbauer, 1996; Lin *et al,* 1993), parvovirus VP2 (Rueda *et al,* 1999; Sedlik *et al* 1997), SIV, HIV and SHIV Gag and Env proteins (Buonaguro *et al* 2001; Delchambre *et al,* 1989; Deml *et al,* 1997a & 1997b; Gheysen *et al,* 1989; Griffiths *et al,* 1991; Harris *et al,* 1992; Sakuragi *et al*, 2002; Wagner *et al,* 1992; Yamshchikov *et al,* 1995).

VLP possess numerous advantages over the conventional protein immunogens. Firstly, most VLP can be easily produced in large quantities using heterologous expression systems and purified by well-established protocols (Doan *et al*, 2005). Secondly, the VLP-forming core structures can be adapted by recombinant technology to contain and display one or more antigens, including chimeric antigens. Thirdly, less amount of vaccine can be used without the need to co-administer with adjuvants, Finally, the immune system responds well to particulate antigens that are the size of viruses (Harris *et al,* 1992) and VLP have potential in stimulating strong Th-1 CD4+ T and cytotoxic T lymphocyte responses in addition to stimulating B-cell mediated humoral immunity (Deml *et al*, 2004; Wagner *et al*, 1996b; Kang *et al*, 2004; Yao *et al*, 2004).

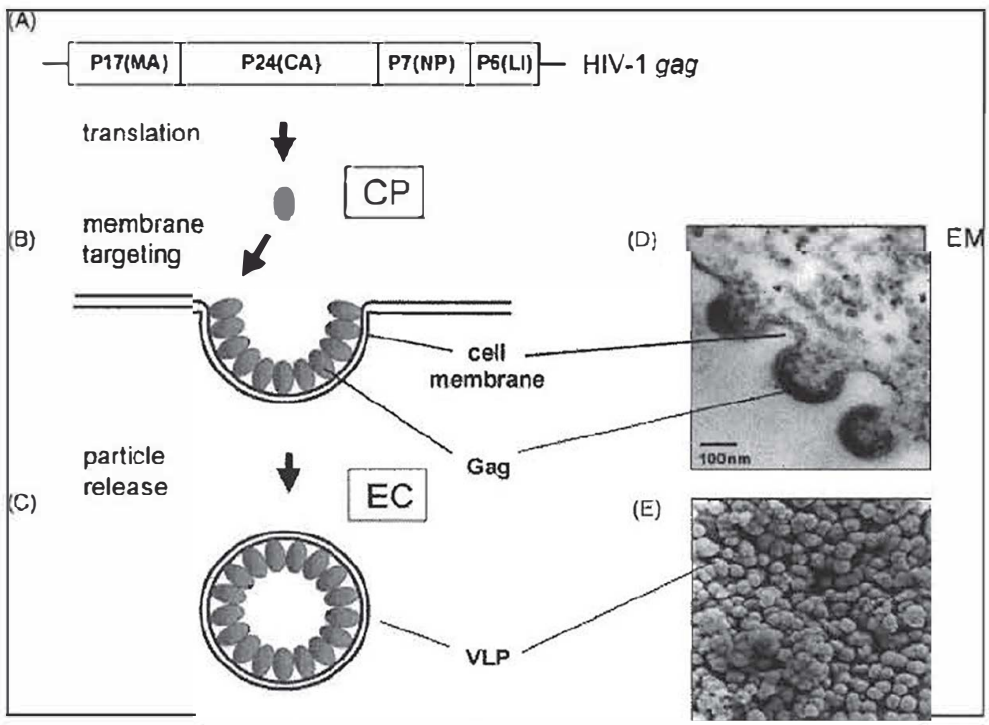
1.5.3 Expression and production of HIV-1 $Pr55^{gag}$ VLP

The HIV-1 *gag* gene encodes a highly conserved structural polyprotein, $Pr55^{gag}$, which forms the viral capsid and viral matrix. $Pr55^{gag}$ comprises of 4 major structural proteins and 2 minor spacer peptides, p1 and p2. The major structural proteins are; p17 (matrix protein), p24 (capsid protein), p7 (nucleocapsid) and p6 (linker protein). The p1 and p2 form the spacer peptides between p7 and p6, and p24 and p7 respectively, altogether making up the 3 main regions of the unprocessed Gag precursor namely, p17, p24 and p2p7p1p6 regions. The unprocessed Gag precursor in the absence of other viral proteins and viral RNA possess an ability to catalyse the formation and release of non-infectious VLP (reviewed in Deml *et al*, 2005 and Doan *et al*, 2005; **Fig 1.6**) when expressed in a suitable eukaryotic cell system (Mergener *et al,* 1992; Sakuragi *et al,* 2002; Wagner *et al,* 1996a).

The process of $Pr55^{gag}$ VLP formation involves the expression of HIV-1 *gag* gene in appropriate eukaryotic cell, synthesis of $Pr55^{gag}$ polyprotein, which is then directed to the plasma membrane, where it acquires a lipid envelope from the plasma membrane of host cell and buds in form of VLP. The host cells' single stranded RNA aids this process, which are recruited by the Gag polyprotein. Several expression systems have been developed. The most common ones are (i) baculovirus (Deml

et al 2004; Gheysen *et al*, 1989), and (ii) yeast (Sakuragi *et al*, 2002) expression systems, which use insect cells and yeast spheroplasts or retrotransposon respectively for VLP expression. HIV VLP have also been produced using vaccinia (Haffar *et al*, 1990; Vzorov *et al*, 1991; Wagner *et al*, 1994) and adenovirus (Luo *et al*, 2003) expression systems, both of which use mammalian cells, Hep2 and human embryonic kidney cells (293 cells) respectively for VLP expression. The baculovirus system is probably the most commonly used system because a large amount of protein can be produced readily (milligram quantities) and the insect cells are easy to handle. In addition, baculovirus does not infected mammalian cells, thus posing no safety threat to vaccinated individuals. Purification of VLP from the culture supernatant is achieved by ultracentrifugation through sucrose gradients to yield particles of >80% purity with a size of 100-120nm and made up of 1500 to 1800 Gag monomers. These particles resemble the virions both morphologically and antigenically (Haffar *et al*, 1990; Wagner *et al*, 1992).

Fig. 1.6: Schematic representation of the HIV Gag polyprotein and particle formation. (A) Shows the HIV $Pr55^{gag}$ polyprotein consisting of the p17 matrix (MA), the p24 capsid (CA), the p7 nucleocapsid (NC) and the p6 linker protein (LI). (B) Shows $Pr55^{gag}$ polyprotein targeting to the inner leaflet of the membrane of the producer cell. (C) Shows the budding in form of enveloped VLP. (D and E) Are electron micrographs of VLP budding from HighFive insect cells after infection with Gag-recombinant baculoviruses. CP: cytoplasma; EC: extra cellular; EM: electron micrograph. (Taken from Deml *et al*, 2005)



The process of $Pr55^{gag}$ VLP formation involves the expression of HIV-1 *gag* gene in appropriate eukaryotic cell, synthesis of $Pr55^{gag}$ polyprotein, which is then directed to the plasma membrane, where it acquires a lipid envelope from the plasma membrane of host cell and buds in form of VLP.

By use of recombinant technology, HIV VLP can be engineered to contain and display more than one HIV antigen, immunologically relevant or immunostimulatory foreign antigens or epitopes such as V-3 loop or CD4 binding region of HIV gp120, Nef-derived or Pol-derive epitope (Buonaguro *et al,* 2002; Griffiths *et al,* 1993; Luo *et al,* 1992; Wagner *et al,* 1994). Two types of particles can be formed when such polypeptides or molecules are introduced and displayed by the native HIV $Pr55^{gag}$ VLP. These are type 1 or type 2 particles (reviewed in Deml *et al,* 2005). Type 1 particles are formed when small epitopes are integrated or fused within the $Pr55^{gag}$ polypeptide as a single construct. In contrast, type 2 particles are formed when the foreign proteins are incorporated at the outer surface of VLP. This incorporation normally requires the co-infection using particle-forming $Pr55^{gag}$ polypeptide construct together with the one containing the foreign protein, resulting into co-expression. A number of different types of Env proteins have been successfully incorporated at the outer surface of the HIV-1 $Pr55^{gag}$ VLP (**Fig 1.7**). This includes: the unprocessed and processed forms of HIV Env precursor gp160 (Haffer *et al,* 1990; Rovinski *et al,* 1992; Vzorov *et al,* 1991); chimeric HIV gp120 protein, which has been linked to transmembrane domain of the Epstein-Barr virus (EBV) gp 220/350 (Deml *et al,* 1997a); the pseudorabies virus gD protein (Garnier *et al,* 1995); and the equine herpesvirus type-1 gp14 (Osterrieder *et al,* 1995) and influenza hemaglutinin antigen, HA (Guo *et al,* 2003).

1.5.4 Immune responses induced by HIV-1 $Pr55^{gag}$ VLP

A number of studies in rodents and non-human primates have shown that $Pr55^{gag}$ VLP elicits strong humoral and cellular responses as reviewed by Deml *et al* (2005) and Doan *et al* (2005). Examples of animal studies in which HIV-1-derived antigens and/or $Pr55^{gag}$ VLP were used to induce immune responses are outlined in **Table 1.5**.

Recent *in vitro* studies have shown that $Pr55^{gag}$ VLP can induce "danger signals" in dendritic cells resulting in activation of innate immune responses. It has been reported that yeast-derived HIV-1 $Pr55^{gag}$ VLP (Tsenetsugu-Yokota *et al,* 2003) and baculovirus-derived SHIV VLP (Zhang *et al,* 2004) were efficiently take up by DC, leading to maturation of DC and enhanced cytokine production. Also, VLP-treated APC were shown to re-stimulate antigen specific CD4+ and CD8+ T cells in whole blood of HIV-infected individuals resulting in production of intracellular cytokines (Sester *et al,* 2000; Heintel *et al,* 2002).

1.5.5 Features influencing efficient induction of immune responses

As already mentioned, $Pr55^{gag}$ VLP are able to stimulate potent innate and adaptive immune responses in the absence of adjuvants. The adaptive immune responses induced by VLP involve both Th-1 (cellular) and Th-2 (humoral) arms of immune system. It has been further shown that VLP-induced Th-1 responses include both CD4+ and CD8+ T-cell mediated responses, indicating that the antigens in VLP are introduced to both MHC class 1 and class 2 processing and presentation

pathways (Deml *et al*, 2005; Doan *et al*, 2005). Generally, exogenously administered antigens are displayed on MHC class 2 molecules to stimulate T-helper (CD4+) cells whereas endogenously synthesised antigens are degraded for loading on MHC class 1 molecules to stimulate CTL (CD8+) responses. Thus, the exogenously administered VLP must provide antigens that are presented to MHC class 2 molecules in the classical pathway as well as provide some antigens that are processed and cross-presented through the alternative antigen processing pathway for MHC class 1 presentation (Moron *et al*, 2002 & 2003). This process by which the exogenous VLP are taken up and processed by professional antigen presenting cells (APC) for dual presentation is not well understood. However, studies have shown dendritic cells to process exogenous antigens from different origins, including VLP for cross-presentation to MHC class 1 molecules (Reinmann & Schirmbeck, 1999; Heath & Carbone, 2001; Tsenetsugu-Yokota *et al*, 2003).

Fig. 1.7: Schematic representation of strategies that are used to produce type 1 VLP (by introducing small epitopes) and type 2 VLP (by incorporating the complete envelope proteins) into Gag particles. (A) The foreign epitopes such as the V3-loop (V3), the CD4 binding region (CD4BR) of HIV gp120 or a Nef-derived epitope are inserted into mutant Gag proteins to replace deleted sequences within p24(CA) or within the p6(LI) moiety within Gag. The integration sites of foreign epitopes are indicated as checkered rectangles. (B) Type 2 VLP can be generated by co-expressing the particle forming $Pr55^{gag}$ protein and Env proteins. Examples of types of Env proteins, which have been incorporated at the outer surface of the Gag VLP, are shown. PrC: protease cleavage site; EBV: Epstein-Barr virus; PRV: pseudorabies virus; EHV-1: equine herpesvirus type-1. (Taken from Deml *et al*, 2005)

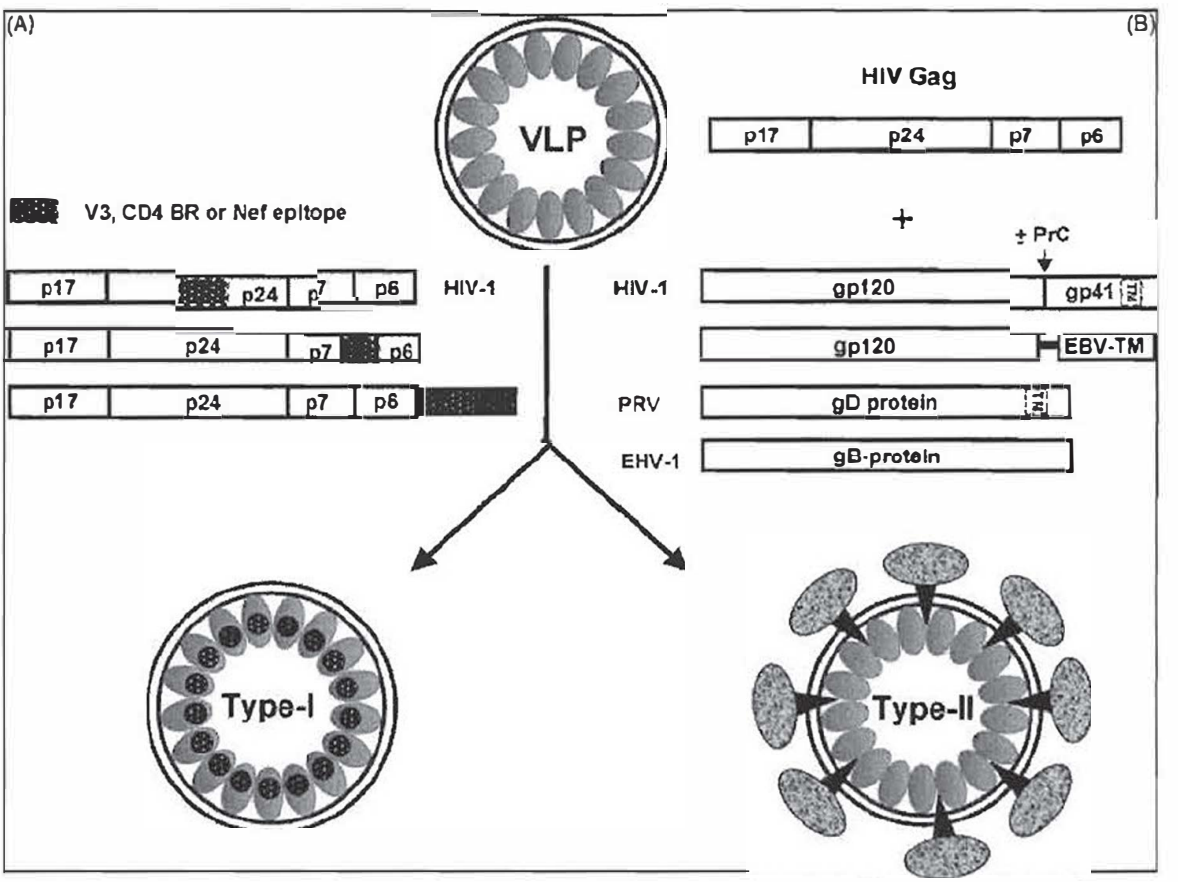


Table 1.5: Induction of humoral and cellular responses by HIV VLP immunogens in animal studies (Adapted and updated from Deml *et al*, 2005).

Immunogen	Animal model	Antibodies	Neutralising antibodies	CTL	Reference(s)
Naked HIV-1 Gag	Macaques	ND	ND	Strong	Paliard *et al*, 2000
HIV-2 Gag/V3, Gag/CD4BR, Gag/V3+CD4BR	Rabbits	Detectable	Weak	ND	Luo *et al*, 1992
HIV-1 Gag/V3	Mice, rats	Strong/Weak	Weak	Strong	Griffiths *et al*, 1993
HIV-1 Gag/V3	Mice	Strong/Weak	Weak	Strong	Wagner *et al*, 1996b
HIV-1 Gag/gp160	Rabbits	Strong	Medium	ND	Haffar *et al*, 1991
HIV-1 Gag/chimeric HIV-1 LAI/MN gp160	Mice	Strong	Medium	ND	Rovinski *et al*, 1992
HIV-1 Gag/gp120TM	Mice, rabbits	Strong	Medium	Medium (mice only)	Deml *et al*, 1997a & 1997b
HIV-1 Gag/gp160 + QS21	Macaques	Detectable	Weak	Weak, transient	Montefiori *et al*, 2001
HIV-1 (Gag-Pol-Nef)/gp 120TM	Mice	Strong	Weak	Strong	Buonaguro *et al*, 2002
SHIV Gag/HIV-1gp120, Gag/HIV-1 gp160	Macaques	Strong	ND	Weak, transient	Notka *et al*, 1999
HPV/SHIV Gag/HIV-1 tat, Gag/HIV-1 rev (SFV prime)	Macaques	Weak (DNA prime only)	ND	Weak (DNA prime only)	Dale *et al*, 2002
Chimeric SHIV Gag (with influenza HA protein)	Mice	Strong	Medium	Strong	Guo *et al*, 2003
SHIV Gag/HIV-189.6 Env + inactivated influenza virus	Mice	Strong	Medium	Medium	Kang *et al*, 2004
Naked HIV-1 Gag (DNA prime)	Mice	ND	ND	Strong	Jaffrey *et al*, 2004
Naked HIV-1 Gag (DNA + gp140 prime)	Baboons	Strong	Weak	(Strong CD4+ T)	Leung *et al*, 2004

VLP: virus-like particles; ND: not defined; HIV: human immunodeficiency virus; gp: glycoprotein; V3: third variable loop of HIV-1 gp120; TM: transmembrane domain; QS21: natural saponin adjuvant derived from the tree Quillaja saponaria Molina; Pol: polymerase; Nef: negative regulatory factor; SFV: Semliki-Forest viruses expressing proteins corresponding to VLP construct; DNA: DNA vaccine expressing proteins corresponding to VLP construct

A few pathways for the processing of exogenous proteins for MHC class 1 presentation have been proposed. One theory proposes that endocytosed exogenous proteins escape from the endosomes into the cytoplasm, followed by epitope loading on MHC class 1 molecules (Gromme & Neefjes, 2002; Zinkernagel *et al*, 2002; Melief *et al*, 2003). Other studies suggest a direct MHC class 1 loading of

epitopes derived from endocytosed polypeptides within the endosomal compartment (Harding & Song, 1994; Stober *et al*, 2002). In such a case, the association with lipid, the particulate nature, repetitive nature of antigen and the presence of fusogenic antigens, all of which are possessed by VLP, have been shown to enhance antigen uptake for MHC class 1 presentation (Schirmbeck *et al*, 1996; Jondal *et al*, 1996; Buseyne *et al*, 2001). Recent reports have shown an efficient uptake and MHC class 1 presentation of both yeast-derived (Tsenetsugu-Yokota *et al*, 2003; Zhang *et al*, 2004) and baculovirus-derived (Deml *et al*, 2005) $Pr55^{gag}$ VLP by human APC and the uptake was found to involve mannose recognition receptors (toll-like receptors) and membrane fusion respectively.

An efficient epitope presentation with MHC class 1 and 2 molecules by APC to the T cell generates an activation signal. However, the induction of an immune response requires a second signal, which is mediated by co-stimulatory molecules (e.g. B7-1 or CD80, and B7-2 or CD86, CD40) normally provided by the APC. These co-stimulatory molecules ligate with corresponding molecules on the T cells (e.g. CD28, CD49, CD40L) to generate a second activation signal. This can also be enhanced by pro-inflammatory cytokines like IL-12 and IL-18, which are normally provided by activated APC. Thus, the development of the second activation signal is initiated and enhanced by the innate arm of immune system via a variety of pathogen-associated molecular patterns (PAMP). A variety of microbial proteins and lipopolysaccharides, which are present in $Pr55^{gag}$ VLP and other microbes, make up the PAMP. To initiate the immune activation, PAMP are recognised by the pattern recognition receptors (such as toll-like receptors, TLR) that are found on the surface of macrophages, dendritic and epithelial cells. This recognition triggers a "danger signal" leading into maturation and activation of APC such as up-regulation of co-stimulatory molecules (Hartmann *et al*, 1999) and production of pro-inflammatory cytokines such as IL-12, IL-18 (Aderem & Ulevitch, 2000), thereby inducing the second activation signal.

In addition, VLP contain a varying composition of contaminating components derived from the expression system. These contaminants have been found to play a role in activation of innate immune system. Both yeast- and baculovirus- derived VLP preparations have been observed to stimulate human APC by upregulation of co-stimulatory molecules and maturation markers as well as induction of cytokines (Tsunetsugu-Yokota *et al*, 2003; Zhang *et al*, 2004; Deml *et al*, 2005). Moreover, yeast- and baculovirus- derived VLP have been found to contain substantial levels of yeast cell-derived membranes and baculovirus-derived lipids and proteins respectively. Indeed, baculovirus Env proteins, gp64, have been found incorporated at the outer surface of Gag VLP (Deml *et al*, 2005) and have been shown to play a major role in stimulatory activities of baculoviruses (Gronowski *et al*, 1999).

Generally, HIV $Pr55^{gag}$ VLP have been shown to be highly immunogenic class of sub-unit vaccines, with a potential of introducing antigens in the MHC class 1 and 2 processing and presentation pathway leading in CD4+ and CD8+ T-cell mediated immune responses. In addition, $Pr55^{gag}$ VLP have the potential to activate the innate immune response in addition to induction of potent cognitive responses. The importance of innate immune responses in protection against SIV/SHIV and HIV infections has been reported (Ahmad *et al*, 2005; Levy *et al*, 2003). These features make HIV-1 $Pr55^{gag}$ VLP very suited for development of HIV candidate HIV-1 vaccines.

1.6 RATIONALE AND SCOPE OF THE STUDIES

As part of an elaborate effort to develop affordable, effective and locally relevant HIV/AIDS vaccines for southern Africa, a number of vaccine approaches have been explored by our Vaccine Research Group based at the University of Cape Town. As numerous studies in animals have shown plasmid DNA, MVA, BCG and $Pr55^{gag}$ VLP to be safe and effective vectors in delivering vaccine immunogens to the immune system, these approaches were chosen. Previous studies in our Vaccine Research Group have demonstrated that a HIV-1 *gag* DNA (pTH-GagC) vaccine derived from a HIV-1 subtype C primary isolate (ZDU422) induced a potent, long-lived CTL response in BALB/c mice (van Harmelen *et al*, 2003). Subsequently, an rMVA (Burgers *et al*, unpublished) and a HIV-1 $Pr55^{gag}$ VLP (GagC VLP) vaccine (Jaffray *et al*, 2004), derived from the same HIV-1 isolate, were shown to efficiently boost immune responses that were induced by the pTH-GagC vaccine in the mouse model. The rMVA vaccine (SAAVI MVA-C) was multigenic, expressing a fusion gene comprising of modified portions of HIV-1 subtype C *gag*, *reverse transcriptase, tat* and *nef* and and *env* genes. The *gag* portion was derived from ZDU422 isolate. Similarly, two rBCG vaccines expressing HIV-1 subtype C *gag* gene (designated rBCG:RT106 and rBCG:RT108), also derived from ZDU422 isolate, demonstrated satisfactory immunogenicity potential in the mouse model (Thomas, 2005). These vaccines were selected for further evaluation in a non-human primate model. The objectives of the studies described here were therefore to (i) develop immunological assays for the baboon animal model and (ii) further assess the immunogenicity of these novel vaccines. A heterologous prime-boost immunisation protocol was chosen based on the reported successes of this regimen in enhancing vaccine-induced immune responses.

DNA vaccines are safe and very effective in inducing cellular immune responses, among other advantages. To assess the immunogenicity of pTH-GagC DNA-prime and GagC VLP-boost (Chapter 2), two experiments were conducted using the chacma baboon model. The first experiment was aimed at identifying immunologic reagents and subsequent development of immunological assays, namely the IFN-γ ELISPOT assay, intracellular IFN-γ staining assay and HIV-1 p24 antibody ELISA. The second experiment was intended to obtain further immunogenicity data by using a larger group size as well as using an immunisation regimen similar to those used in various clinical DNA vaccine trials,

whereby three DNA immunisations were given to prime the immune system followed by booster immunisation.

Recombinant BCG expressing HIV-1 or SIV antigens have been shown to have the capacity to induce long-lived antigen-specific cellular immune responses. Due to its low cost of production and safety profile among other advantages, rBCG could be developed to make safe, effective and affordable HIV-1 vaccines. Two rBCG HIV-1 vaccines, designated rBCG:RT106 and rBCG:RT108, were selected for further evaluation in the baboon model. Preliminary immunogenicity studies were done in the mouse model (Thomas, 2005) and further evaluation was recommended in the baboon model. However, there was paucity of published information on the immune responses of baboons to BCG, which could be used to guide the testing of rBCG in this model. Therefore, the main objective of the initial study (Chapter 3) was to investigate the components of immune response of chacma baboons in respect to BCG-specific cellular and humoral responses following vaccination with *Tokyo* and *Pasteur* strains of BCG. The *Tokyo* strain was chosen for this study as it is among the most widely used strain of BCG for vaccination against TB (WHO, 2001) and because the rBCG vaccines selected for further evaluation were constructed using this strain of BCG (Thomas, 2005). The *Pasteur* strain was chosen because it is a common laboratory strain of BCG.

To assess the immunogenicity of rBCG:RT106 and rBCG:RT108 vaccines in the baboon model, two experiments were conducted (Chapter 4). Based on the success of the pTH-GagC DNA and GagC VLP vaccines in the previous studies (Chapter 2), the prime-boost vaccination protocol was chosen, using either of the rBCG vaccines to prime and GagC VLP vaccine to boost. In the first experiment, two booster immunisations with GagC VLP were given 12 weeks apart. In the second experiment, animals were primed with rBCG:RT106 vaccine and boosted with a single inoculation of SAAVI MVA-C and single GagC VLP each, 12 weeks apart. Also, to investigate the effect of pre-existing anti-BCG immunity on the efficiency of rBCG vaccines, six of the baboons that had been inoculated with wild-type BCG in a previous experiment (see Chapter 3) were included in the second baboon experiment and used in rBCG prime-boost vaccination regimen.

Immunogenicity data obtained from these studies will be analysed and compared with other HIV-1 vaccine studies which have proceeded to clinical trials. These comparisons will assist to make decisions regarding the movement of the vaccines in the present study to phase 1 clinical trials.

CHAPTER TWO

IMMUNOGENICITY ASSESSMENT OF DNA AND VIRUS LIKE PARTICLE VACCINES

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2.1 INTRODUCTION

This chapter describes assessment of the immunogenicity of two candidate HIV-1 vaccines, a DNA vaccine and a $Pr55^{gag}$ virus-like particle (Gag VLP) vaccine in the chacma baboon. The vaccines, which were derived from HIV-1 Gag of a subtype C primary isolate (Williamson *et al*, 2003), were shown to be immunogenic in mice when given as a single DNA vaccine modality (van Harmelen *et al*, 2003) or as a DNA vaccine prime-Gag VLP boost modality (Jaffrey *et al*, 2004). In the present study, a DNA vaccine prime-boost vaccination modality was adopted. Also, this chapter will report the identification of reagents that were used to develop immunological methods for immunogenicity assessment in the studies described here and chapters 3 and 4.

2.1.1 DNA VACCINES

DNA vaccines encoding HIV or SIV/SHIV antigens have been studied extensively in vaccine research. As reviewed in section **1.3.7**, DNA vaccines have been shown to induce both humoral and cellular immune responses in animal models (Boyer *et al*, 1997; Haynes *et al*, 1994; Letvin *et al*, 1997; Lu *et al*, 1995; Yasutomi *et al*, 1996; Wang *et al*, 1993 & 1994) as well as in man (Boyer *et al*, 2000). However, DNA immunisation is seen to generate low and transient levels of immune responses in man and nonhuman primates (Boyer *et al*, 1999; Calarota *et al*, 1998, 1999 & 2001; MacGregor *et al*, 1998). Although these studies clearly support the safety of a HIV-1-DNA vaccine strategy, they also show that their potency in generating effective and protective immunity is not sufficient. Approaches of augmenting these responses induced by DNA vaccines include multiple DNA deliveries using biojectors (Raviprakash *et al*, 2003; Haynes *et al*, 1996), use of genetic adjuvants (Barouch & Letvin, 2000; Barouch *et al*, 2000; Seaman *et al*, 2004; Xin *et al*, 1999), inclusion of bacterial DNA sequences like unmethylated CpG motifs (Gursel *et al*, 2002; Hemmi *et al*, 2000 & 2003); and their use in heterologous prime-boost immunisation regimens (see review in section **1.3.11**). The DNA vaccine prime followed by a heterologous vaccine boost strategy has shown the greatest promise in animal vaccine studies.

In heterologous prime-boost vaccination protocols, the DNA is used as the priming vaccine while a protein or a live vector vaccine is used to boost the immune responses. Initial studies indicated that DNA prime and protein boost strategies, enhanced the antibody responses with little or no increase in CTL response upon protein boosting (Barnett *et al*, 1997; Letvin *et al*, 1997). Boosting the DNA immunisation with a recombinant viral vector boost resulted in excellent boosting of CTL response in many animal models (Hanke *et al*, 1998 & 1999; Allen *et al*, 2000; Robinson *et al*, 1999; Amara *et al*, 2001; Casimiro *et al*, 2003a, 2003b & 2005; Leung *et al*, 2004). In addition, DNA-prime viral-boost vaccination regimen has been shown to enhance the breadth of CD4+ T-cell immunity

in the murine animal model (Wu *et al*, 2005). The choice of $Pr55^{gag}$ virus-like particles for use in DNA vaccine prime-Gag VLP boost modalities is particularly attractive because of their demonstrated potential in inducing CD4+ and CD8+ T-cell mediated immune responses (Wagner *et al*, 1996b; Kang *et al*, 2003) as well as innate immune responses (Tsenetsugu-Yokota *et al*, 2003; Zhang *et al*, 2004).

2.1.2 VIRUS-LIKE PARTICLE VACCINES

Among the particulate sub-unit vaccines, virus-like particles (VLP) are excellent for use as sub-unit vaccines because VLP comprise of structural proteins of viruses, majority of which represent the immunologically relevant viral antigens. Because they lack the unwanted regulatory proteins and infectious genetic material, they are very safe. In addition, VLP possess numerous advantages over the conventional protein immunogens in terms of easy production and purification, potential of including chimeric antigens, excellent adjuvant properties and the ability of the immune system to respond well to particulate antigens that are the size of viruses (see review in section **1.5**).

As reviewed in section **1.5**, unprocessed HIV-1 $Pr55^{gag}$ polyprotein possesses an ability to self-assemble into non-infectious VLP (Deml *et al*, 1997b & 2004; Mergener *et al*, 1992; Sakuragi *et al*, 2002; Wagner *et al*, 1996b) and shown to elicits strong humoral and cellular responses in animals (Deml *et al*, 1997b; Wagner *et al*, 1996b) including non-human primates (Montefiori *et al*, 2001; Paliard *et al*, 2000; Wagner *et al*, 1996b). Also, studies with human subjects showed that VLP-treated APC were able to re-stimulate antigen specific CD4+ and CD8+ T cells in whole blood of HIV-infected individuals resulting in production of intracellular cytokines (Sester *et al*, 2000; Heintel *et al*, 2002). In addition to CD8+T and CD4+ T cells responses, recent *in vitro* studies have shown that $Pr55^{gag}$ VLP have a potential to induce "danger signals" in dendritic cells resulting in the activation of innate immune responses (Tsenetsugu-Yokota *et al*, 2003; Zhang *et al*, 2004). In view of the above, HIV-1 $Pr55^{gag}$ VLP are considered to be a novel class of sub-unit vaccines, which have the potential of stimulating both the adaptive and innate immune responses.

2.1.3 DEVELOPMENT OF IMMUNOLOGICAL ASSAYS

A key issue in evaluation of immunogenicity of HIV vaccines is the measurement of T-cell responses using reliable and reproducible assays. Currently, two cellular assays are widely available and frequently used for these measurements. These are (i) the enzyme-linked immunospot (ELISPOT) assay, which measures the release of cytokines from the cells (Mashishi & Gray, 2002; Mwau *et al*, 2002; Currier *et al*, 2002; Anthony & Lehmann, 2003) and (ii) intracellular cytokine staining (ICS) assay, which detects cytokines trapped artificially in the endoplasmic reticulum

(Suni *et al*, 1998; Hoffmeister *et al*, 2003) during specific-antigen stimulation. The most frequently measured cytokine is IFN-γ.

While measurement of IFN-γ alone provides the quantitative measure of a HIV-1 vaccine-induced cellular immune response, inclusion of additional functional cytokine assays such as IL-2 and TNF-α allows the assessment of qualitative features of the antigen-specific T cell response. Quantification of an array of cytokines using cytometric bead array (CBA) technique has recently become available using commercial kits (Becton Dikinson, 2003).

Measurement of neutralising antibodies is only relevant in vaccines that include HIV Env glycoprotein as an immunogen. Nonetheless, measurement of the binding antibodies to HIV Gag protein, for vaccines which include *gag* gene, may be important in monitoring the development of humoral responses in the immunised animals.

The use of chacma baboons (*Papio ursinus*) for immunogenicity studies has not been reported before. Therefore, this study started from scratch, initially, by identifying suitable immunological reagents and subsequently, by developing the relevant immunological assays for standard immunogenicity assessment of HIV-1 vaccines. Several reagents, including anti-rhesus monkey and anti-human monoclonal antibodies (mAB), were tested for cross-reactivity with baboon cells, cytokines and immunoglobulin G. Then, three immunological assays were established for the immunogenicity studies of HIV-1 vaccines in this baboon animal model. These assays are discussed in greater details in the Materials and Methods (this chapter and chapter 3) and Appendix sections. Also, additional data relating to the development of these methods are presented in Appendix section C. The following assays were established and optimised for use in vaccines studies that are presented in this thesis.

(i) IFN-γ ELISPOT assay to measure the magnitude of Gag peptide pool responses (Section **2.2.5.3**),

(ii) Pool-Matrix IFN-γ ELISPOT assay to measure the breadth of Gag-specific responses (Section **2.2.5.4**),

(iii) Intracellular IFN-γ staining and FACS analysis to determine the phenotype of Gag-specific T cell sub-populations (Section **2.2.5.5**),

(iv) Cytometric bead array (CBA) to quantifying the amounts of T-helper type 1 (IFN-γ, TNF-α and IL-2) and T-helper type 2 (IL-4, IL-5 and IL-6) cytokines (Section **2.2.5.6**), and

(v) HIV-1 p24 antibody ELISA to monitor the development of Gag-specific humoral response (Section **2.2.5.1**).

2.1.4 STUDY OBJECTIVES

Previous studies in our SAAVI-funded HIV vaccine development group have reported construction of a gag DNA vaccine expressing the *gag* gene from a South African HIV-1 subtype C primary isolate named ZDU422 (van Harmelen *et al*, 2003). Sequence analysis showed that this DNA vaccine (referred to as pTH-GagC) contained several CTL epitopes that are recognised by PBMC from HIV-1 subtype C-infected individuals. When tested in BALB/c mice, a single inoculation containing 100 μg of pTH-GagC per mouse induced potent, long-lived CTL response representing up to 76% specific lysis (effector to target ratio of 50:1) in ^{51}Chromium-release assay and up to 6.7% of antigen-specific IFN-γ producing CD8+ cells and this response was maintained for 15 weeks (van Harmelen *et al*, 2003). Subsequently, a $Pr55^{gag}$ VLP vaccine (referred to as GagC VLP), derived from the same HIV-1 isolate, was shown to efficiently boost the immune responses induced by this pTH-GagC DNA vaccine in the mouse model (Jaffray *et al*, 2004). A single inoculation of 100 μg of pTH-GagC per mouse and boosted with 2 ng of GagC VLP resulted in detection of 28.6% of antigen-specific CD8+/IFN-γ+ splenocytes after a pTH-GagC-prime followed by a GagC VLP inoculation compared with 9.4% of CD8+/IFN-γ+ splenocytes for mice inoculated with empty pTH-vector and GagC VLP. About 90% and 60% of specific lysis was demonstrated for the two groups respectively in ^{51}Chromium-release assay (Jaffray *et al*, 2004). In view of these data, the two vaccines were chosen for further pre-clinical evaluation. The aim of the present study was to extend this assessment of the pTH-GagC and GagC VLP vaccines in the chacma baboon using DNA-prime, Gag VLP-boost vaccination protocol.

To investigate this, two experiments were conducted. In the first experiment, 2 baboons were primed with six successive pTH-GagC injections while 2 others were mock primed with pTH empty DNA vector. All 4 baboons were subsequently boosted with baculovirus-produced GagC VLP vaccine. This experiment established and optimised immunological assays, namely the IFN-γ ELISPOT assay, intracellular IFN-γ staining assay and HIV-1 p24 antibody ELISA that could be used for this and future studies. A second experiment was then designed to investigate the immune response to a DNA vaccine regimen similar to that used in various clinical DNA vaccine trials. Three DNA immunisations were given as primary inoculations, 4 weeks apart, followed by two booster inoculations with GagC VLP.

Immunogenicity data from these experiments were then analysed and compared with other HIV-1 vaccine studies which have proceeded to clinical trials to assess the prospects of moving these vaccines to clinical evaluation.

2.2 MATERIALS AND METHODS

2.2.1 BABOONS

For the two experiments, 11 wild-caught chacma baboons of mixed sex and weighing between 6 and 13kg were used in these experiments. The baboons were housed at the South African Medical Research Council's animal facility at Delft in Cape Town, South Africa. Before the study, these baboons were shown to be healthy and serologically negative for SIV antibodies. Ketamine was used to sedate animals in all handlings. The experiments were reviewed and approved by the Animal Research Ethics Committee (Reference No. 00/03N) of the University of Cape Town.

2.2.2 VACCINE PREPARATIONS

The pTH-GagC DNA and GagC VLP vaccines were kindly provided by Drs Joanne van Harmelen and Ann Jaffray respectively (both of the SAAVI-funded UCT Vaccine Research Group) as frozen stocks. The DNA vaccine contained the HIV-1 subtype C *gag* gene from the South African HIV isolate ZDU422 (Williamson *et al*, 2003). The *gag* gene from the ZDU422 isolate was shown to have the closest amino acid similarity to both a derived South African subtype C consensus sequence (98.2%; Williamson *et al*, 2003) and the ancestral subtype C HIV-1. The cloning, transformation and production of the DNA vaccine has been described (van Harmelen *et al*; 2003). A DNA vaccine control comprising of the empty pTH DNA vaccine vector (pTH-vector) was prepared in a similar way as the corresponding pTH-GagC. Both DNA vaccine and DNA control were manufactured by Aldevron (GMP grade; Fargo, North Dakota, USA) for baboon inoculations.

The GagC VLP vaccine (experimental grade) was made using the HIV-1 subtype C *gag* gene from the ZDU422 isolate (Jaffray *et al*, 2004). Briefly, the HIV-1 *gag* gene was cloned into the multiple cloning site pFastBac1, transposed into competent *E. coli* DH10Bac cells and then into the baculovirus shuttle vector (bacmid). The GagC VLP were produced in *Spodoptera frugiperda* (Sf21) cells via recombinant baculovirus expressing the full-length myristylated $Pr55^{gag}$ precursor protein. GagC VLP were purified from culture fluid by centrifugation on sucrose gradient and then dialysed for 16h in PBS at 4 °C. The integrity of the GagC VLP were evaluated by Western blotting using anti-serum to HIV-1 p17 and also visualised by transmission electron microscopy. Finally, the GagC VLP were screened to ensure they were free of microbial contamination and the endotoxin level was <0.125 EU/mL. The inocula stocks were stored at -30 °C until use.

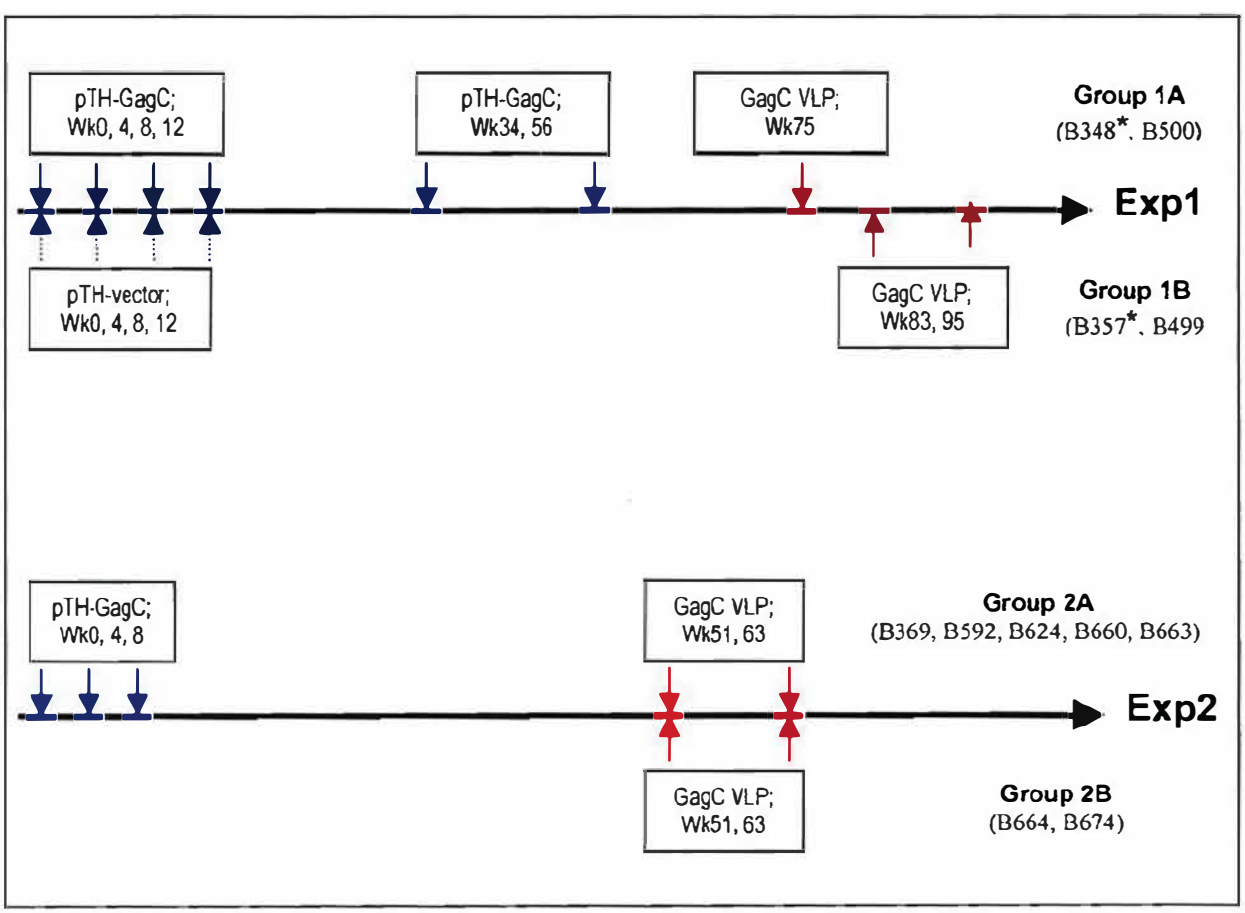
2.2.3 BABOON IMMUNISATION SCHEDULE

The immunisation schedule for the two experiments is shown in **Fig. 2.1.** Baboons 348 and 357 received an additional inoculation each with GagC VLP at week 164 (Baboon 348) or 127 (Baboon 357) post initial inoculation (not shown in **Fig. 2.1**). For the baboons that received pTH-GagC or

pTH-vector, 5mg DNA was given bilateraly in the quadriceps muscles by intramuscular injection using a hypodermic needle. GagC VLP vaccine was administered by intramuscular injection at a dosage rate of 24 μg (Exp1) and 29 μg (Exp2) per baboon.

Fig 2.1: Baboon immunisation schedule. Two separate experiments, Exp1 (n=4) and Exp2 (n=7) were undertaken. Each comprised of the experimental (Group A) and the control (Group B) groups. In Exp1, Group 1A and 1B (n=2, each group), the baboons were inoculated intramuscularly 4 times with 5mg of pTH-GagC DNA vaccine and pTH-vector DNA respectively, 4 weeks apart. Group 1A was given further inoculations with pTH-GagC at weeks 34 and 56 post-initial inoculation (p.i.). Both groups were subsequently inoculated intramuscularly with 24 μg GagC VLP at week 75 (1A) or 83 (1B) p.i. Group 1B received a second GagC VLP booster inoculation at week 95 at the same dosage rate. In Exp2, Group 2A (n=5) received 3 intramuscular inoculations with 5mg of pTH-GagC DNA vaccine 4 weeks apart. Group 2B (n=2) remained un-vaccinated. Both groups received two GagC VLP booster inoculations, 12 weeks apart at weeks 51 and 63. The GagC VLP vaccine was given intramuscularly at a dosage of 29μg per animal.

*: Baboons 348 and 357 were given an addition inoculation with GagC VLP (not shown on the diagram) at week 164 (B348) or 127 (B357) p.i.



2.2.4 BLOOD COLLECTION

Blood was collected at various time points before and after inoculation and processed to obtain serum and PBMC within 3 hours of collection as described in **Appendix B.1**. Serum aliquots were

stored frozen at –30 °C. The isolated PBMC were aliquoted for storage in liquid nitrogen as outlined in **Appendix B.2.** Cryo-preserved cells were used in the standard IFN-γ ELISPOT, ICS and CBA assays whereas freshly isolated cells were used in the Pool-Matrix IFN-γ ELISPOT assay.

2.2.5 LABORATORY ASSAYS

2.2.5.1 HIV-1 p24 ANTIBODY ELISA

An in-house ELISA method was developed to detect the presence of HIV-1 Gag-specific antibodies in the baboon sera. Maxisorp plates (Nalge-Nunc International, Denmark) were coated with 0.1 µg/well of HIV-1 recombinant p24 protein (Quality Biological Inc, MD, USA). Serum was diluted 1:40 and added in duplicate wells. Also, for selected key time-points, eleven 2-fold serial serum dilutions, starting at 1:40 or 1:60, were added in duplicate wells. A negative control serum, consisting of pre-immune baboon sera at 1:40 dilution, was included in each microtitre plate in quadruplicates. Serum immunoglobulin G was detected using rabbit anti-monkey IgG horseradish peroxidase (Sigma) diluted 1:2000 and developed with tetramethyl-benzidine (TMB, Kirkegaard & Perry Laboratories). The absorbance was measured at 450 nm and the results are given as mean optical density (OD) value at 1:40 serum dilution and as the endpoint antibody titre (for selected key time-points), which was defined as the reciprocal of the highest serum dilution that gave an absorbance that was equal or greater than the mean absorbance of the negative control serum plus 2 standard deviations. Negative control serum was prepared by pooling equal volumes of pre-immunisation sera from all the baboons in the experiment. A positive control serum was not available for this assay.

2.2.5.2 HIV WESTERN BLOT TEST

The presence of HIV-1 Gag antibodies in baboon sera was confirmed using the New LAV Blot 1™ kit (Bio-Rad Laboratories, CA, USA) following the manufacturer's instructions except that the anti-human IgG alkaline phosphatase conjugate solution (Reagent 5) was substituted with anti-monkey IgG conjugated to alkaline phosphatase (Sigma). Anti-monkey IgG alkaline phosphatase conjugated antibody was used at 1:500 dilution in Diluent buffer (Reagent 2 from the kit).

2.2.5.3 STANDARD IFN-γ ELISPOT ASSAY

HIV-1 Gag-specific T cells in baboon PBMC were quantified by an IFN-γ ELISPOT assay using a total of 66 synthetic peptides (donated by C Gray, National Institute for Communicable Diseases, Johannesburg, South Africa), which spanned the entire HIV-1 subtype C Gag protein sequence. The peptides (**Fig A.10**; Appendix D.2) were based on subtype C Gag consensus sequences, were 15-18 amino acids in length and overlapping by 10 amino acids.

Preparation of peptide pools:

The peptides were prepared (**Appendix A.3.7**) and arranged into 5 peptide pools (P1-P5), each pool containing 14 peptides as shown in **Table 2.1**. The peptide pools were constituted in PBS (Gibco) containing 10% DMSO (Sigma) and stored at –30 °C in aliquots of 80 μg/mL per peptide.

Antibodies:

Two sets of matched anti-IFN-γ monoclonal antibodies (set 1: anti-human IFN-γ clone 1-D1K and clone 7-B6-1; set 2: anti-rhesus monkey IFN-γ clone GZ-4 and clone 7-B6-1), were commercially obtained from Mabtech (Sweden) and tested for use in the IFN-γ ELISPOT assays. Both clones were tested by ELISA method for their ability to detect baboon IFN-γ produced in culture supernatants after 3 days of incubation of baboon PBMC with concanavalin A (Con A; Sigma). Based on the level of OD values in ELISA test (Appendix C.8, **Fig A.9**), clone 1-D1K was chosen for use in IFN-γ ELISPOT assays.

Table 2.1: Arrangement of HIV-1 subtype C Gag peptides in the peptide pools that were used for IFN-γ ELISPOT assays. The Gag peptides were 15-18 amino acids in length and overlapping by 10 amino acids. The peptide pools, which were constituted in PBS containing 10% DMSO, were further diluted 1:10 with R10 medium to yield 8 μg/mL per peptide containing 1% or 1.12% DMSO. These were added directly to the wells (along with medium containing PBMC) to a final concentration of 4 μg/mL per peptide and 0.5% or 0.56% DMSO (A final concentration of <1% DMSO has a negligible adverse effect in IFN-γ ELISPOT assay [Mashishi & Gray, 2002]). For the control wells in which R10 medium only (negative or background control) or PHA (positive control) was added, DMSO was included to give a final concentration of 0.5% in the wells.

Peptide Pool	Designated Gag peptides numbers	Total number of peptides in the pool	Gag domain represented (peptide number)
P1	1-14	14	p17
P2	15-28	14	p17 (15-18) p24 (19-28)
P3	29-42	14	p24
P4	43-56	14	p24 (43-48) p2p7p1p6 (49-56)
P5	57-66	10	p2p7p1p6

IFN-γ ELISPOT method:

The IFN-γ ELISPOT method was based on that described for human PBMC by Mashishi & Gray (2002). The ELISPOT plates (MultiScreen-IP, Millipore) were coated with 50μL/well of purified anti-human IFN-γ (5μg/mL in PBS; clone 1-D1K, Mabtech) then incubated at 4°C overnight. The wells were washed with PBS and the non-specific sites blocked by incubating the plates with R10 medium at room temperature for at least 2h. Shortly before the ELISPOT set-up, the peptide pools (see **Table 2.1**) were diluted 1:10 with R10 medium and added directly to the wells at a final concentration of 4 μg/mL along with $1x10^5$ or $2x10^5$ PBMC. If cryo-preserved PBMC were used,

the cells were revived as outlined in **Appendix B.3** and then rested overnight at 37°C, in 5%CO_2 incubator. They were then counted and washed once with warm R10 before being used in the ELISPOT assay. PBMC incubated with R10 medium only (background responses) and PHA at a final concentration of 4 μg/mL acted as negative and positive controls respectively. All reactions were done in triplicate. The plates were incubated at 37°C, in 5%CO_2 for 22-24h. After washing the wells with PBS-Tween (Sigma), the plates were incubated for 2h at 37°C with 50μL/well biotin-labelled anti-human IFN-γ (2μg/mL in PBS containing 10% FBS; clone 7-B6-1, Mabtech) followed by a further wash and 1h-incubation with 100μL/well of a 1:500 dilution of Streptavidin-HRP (BD-PharMingen) in PBS containing 10% FBS (Delta Bioproducts). Finally, the spots were developed by adding 100μL/well of horseradish peroxidase substrate (NovaRed™, Vector Laboratories) and incubating the plates in the dark at room temperature for 5-8 min. The colour reaction was stopped by washing the plates with tap water. An ImmunoSpot (Cellular Technology Ltd, Cleveland, Ohio) automated plate analyser together with ImmunoSpot Version 3.2 software was used to scan and count the spots in each well. The mean number of spots in triplicate wells were normalised to spots per 10^6 PBMC to give the IFN-γ spot-forming units (SFU) per 10^6 PBMC. The IFN-γ response to each peptide pool was determined by subtracting the background IFN-γ SFU/10^6 PBMC in the absence of peptides (background response) from the IFN-γ SFU/10^6 PBMC in the presence of peptides. Data was presented as the IFN-γ SFU/10^6 PBMC for each peptide pool. The cumulative IFN-γ response to Gag (sum of net IFN-γ responses for the 5 peptide pools) was calculated when necessary.

To determine if a response to Gag peptide pool was positive, a peptide pool cut-off value was established for each baboon experiment in the study (see **Appendix C.6**). The cut-off value was defined as the mean peptide pool response at pre-immunisation (after subtracting the background response in the absence of peptides) plus 4 standard deviations of the mean. A response to any peptide pool that was equal or greater than the peptide pool cut-off value was considered positive.

2.2.5.4 PEPTIDE MAPPING BY POOL-MATRIX IFN-γ ELISPOT ASSAY

This method was adapted from Anthony & Lehmann (2003) and Masemola *et al* (2004a). The assay utilised the same HIV-1 Gag peptides as a standard IFN-γ ELISPOT assay (section **2.2.5.4**) with the same arrangement of peptide pools as shown in **Table 2.1** and a second set of peptide pools containing 14 peptide pools (matrix pools, M1-M14) as shown in **Table 2.2**. Adjacent peptides in the first set of peptide pools (P1-P5) overlapped by 10 amino acids whereas the peptides in the second set (M1-M14) were non-overlapping. This arrangement of peptides (**Table 2.2**) in

which each peptide is contained in two separate pools is called 'Pool-Matrix' format and the assay referred to as Pool-Matrix IFN-γ ELISPOT assay. The two sets of pools were used together in IFN-γ ELISPOT assays, using freshly isolated PBMC. If only two peptide pools were reactive (a normal and a matrix pool), then only one peptide was targeted and it could be identified directly. In this case, no further testing was necessary. However, when there were responses to multiple peptide pools (both normal and matrix pools) were reactive, then more that one peptide was responsible for the observed responses in the pools and the identity of such peptides could only be predicted. Further testing of individual peptides in a subsequent (or confirmatory) IFN-γ ELISPOT assay using cryo-preserved PBMC was necessary to reveal the identity of the targeted peptides. The magnitude of response for the identified peptides in a confirmatory IFN-γ ELISPOT assay (using cryo-preserved PBMC) was calculated as the net IFN-γ SFU/10^6 PMBC above the background response (mean IFN-γ SFU/10^6 PBMC of wells without the Gag peptide). A positive response was defined as the IFN-γ SFU/10^6 PMBC that was equal or greater than the peptide pool cut-off value. Only data of positive responses are presented.

Table 2.2: Composition of Gag peptide pools and the arrangement of peptides in a Pool-Matrix format for use in peptide mapping by IFN-γ ELISPOT. The table shows the peptides that were used to reconstitute normal (rows, P1-P5) and matrix (columns, M1-M14) pools. Normal pools contained peptides overlapping by 10 amino acids whereas the matrix pools contained non-overlapping peptides. The pool-matrix format allowed each of the 66 peptides comprising the Gag protein to be included only once in the normal and the matrix pools, making it possible to predict a response to a peptide in any normal and matrix pool. Peptide responses were confirmed in subsequent IFN-γ ELISPOT assays using cryo-preserved PBMC.

	Gag peptides contained in the peptide pools													
	M1	**M2**	**M3**	**M4**	**M5**	**M6**	**M7**	**M8**	**M9**	**M10**	**M11**	**M12**	**M13**	**M14**
P1	1	2	3	4	5	6	7	8	9	10	11	12	13	14
P2	15	16	17	18	19	20	21	22	23	24	25	26	27	28
P3	29	30	31	32	33	34	35	36	37	38	39	40	41	42
P4	43	44	45	46	47	48	49	50	51	52	53	54	55	56
P5	57	58	59	60	61	62	63	64	65	66				

P, normal peptide pools (set 1; overlapping); M, matrix peptide pools (set 2; non-overlapping)

2.2.5.5 INTRACELLULAR IFN-γ STAINING (ICS) AND FACS ANALYSIS

The identification of cell surface markers is a key feature of ICS assay as it is crucial for identifying the cell phenotypes involved in an immune response. Several human- and monkey - derived monoclonal antibodies for detection of cell surface markers were tested for cross-reactivity with baboon PBMC.

It was not always possible to perform ICS using freshly isolated PBMC and the use of cryo-preserved PBMC was chosen. To investigate whether cryo-preservation of PBMC affected the expression of surface markers on the cells, CD3 and CD8 markers on fresh and revived cryo-

preserved PBMC were compared. Co-stimulatory antibodies, anti-CD28 and anti-CD49d, are often used in cytokine assays. Two anti-human CD28 and CD49d were commercially available from BD-Immunocytometry Systems (BD-IS) and BD-PharMingen (BD-Ph). These antibodies were also tested for cross-reactivity with baboon PBMC and the effect of cryo-preservation on their expression was investigated.

Anti-human CD69 monoclonal antibody is usually used to detect antigen-induced expression of CD69 marker on the surface of T cells in ICS assays. The utility of anti-human CD69 antibody was investigated with a view of incorporating it in baboon ICS assays.

For detection of IFN-γ in ICS assays, cryo-preserved PBMC were used after reviving and resting them overnight (as outlined in **Appendix B.3**). PBMC (2 x 10^6/mL) were incubated with either R10 medium only, R10 medium containing Gag peptides (4 μg/mL) or staphylococcal enterotoxin B (SEB; 1 μg/mL, Sigma) for 16h with addition of Brefeldin A (BFA; 10 μg/mL, Sigma) after 2h. Low endotoxin (<0.01 ng/μg) azide-free monoclonal antibodies to human CD28 (clone CD28.2, BD-PharMingen) and CD49d (clone 9F10, BD-PharMingen) were also added in the stimulation reactions at a final concentration of 1 μg/mL each. The cells were stained in a 2-step staining procedure with mouse anti-monkey CD3-FITC (Clone FN-18, BioSource), anti-human CD4-APC (clone SK3, BD), anti-human CD8-APC (Clone SK1, BD) or corresponding isotype controls (step 1) and stained again (step 2) with mouse anti-human IFN-γ-rPE (Clone 4S.B3, BD-PharMingen) or corresponding isotype control after the cells were permeabilized with FACS Permeabilizing Solution 2™ (BD). Cells were washed and re-suspended in fixing solution (CellFIX™, BD) then acquired using a BD FACSCalibur™ flow cytometer and analysed using CellQuest™ (BD) software. The gating strategy used for analysis is displayed in **Appendix B.5**, **Fig A.1**. Data is presented as the net percentage of CD4+ or CD3+CD8+ cells, which are IFN-γ positive after subtracting the background. The cut-off value was defined as twice the background response.

2.2.5.6 CYTOKINE ASSAY BY CYTOMETRIC BEAD ARRAY METHOD

Cytometric Bead Array (CBA) assay is a recently developed particle-based, flow cytometric multiplex sandwich immunoassay that is capable of simultaneously measuring levels of multiple molecules, including cytokines, in a single sample of biological fluid (Becton Dikinson, 2003). Multiple bead populations with distinct fluorescence intensities and coated with capture antibodies specific to the cytokines to be measured in the assay are used. These capture beads are mixed with the human-specific PE-conjugated detection antibodies, and incubated with recombinant standards or test samples to form sandwich complexes. These are the complexes that are analysed by flow

cytometry. By including serial dilution of standards of each cytokine to be measured, the CBA software is capable of generating a standard curve for each analyte to determine the concentration of each cytokine in the sample.

To further characterise the immune responses elicited by the vaccine in the immunised baboons, the concentration of cytokines released from PBMC during stimulation with Gag peptides was determined using the CBA technique. Baboons' PBMC (2-4 x 10^6/mL) were incubated for 16h (as described for ICS assay but in the absence of Brefeldin A; section **2.2.5.5**) with either culture medium only or medium containing individual Gag peptides (4 μg/mL). The Gag peptides that were used for PBMC stimulation in these assays were identified as being targeted by the baboon PBMC using the IFN-γ ELISPOT assay. The culture supernatants were harvested and stored frozen at -80 °C until the time of CBA assay. The BD™ CBA Non-Human Primate Th1/Th2 cytokine kit (BD, San Diego, CA) was used to quantitatively measure IFN-γ, TNF-α, IL-2, IL-4, IL-5 and IL-6 according to the manufacturer's instructions.

2.2.5.7 STATISTICAL ANALYIS

The t-test (Statistica®, StatSoft) was used for statistical analysis.

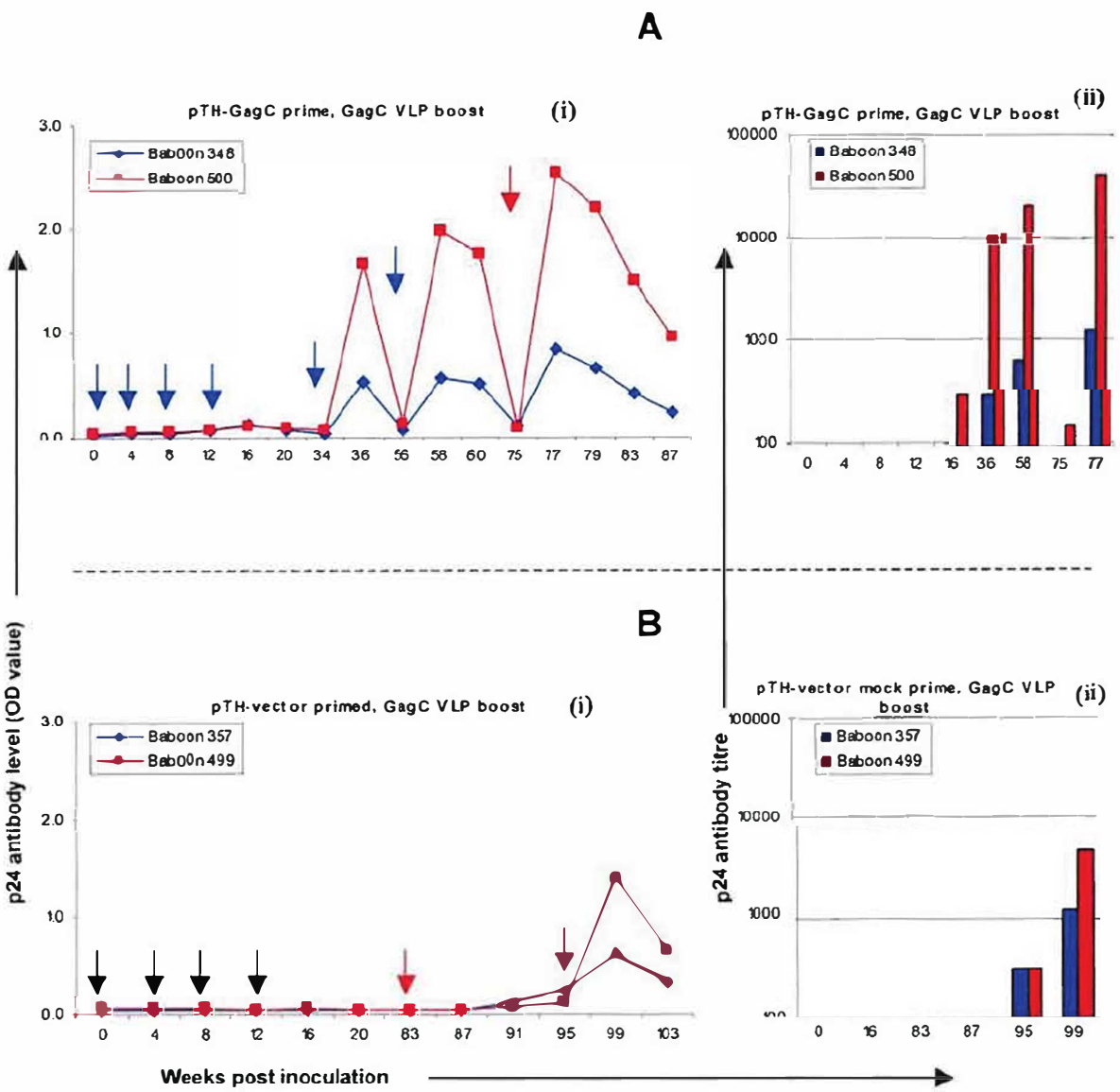
2.3 RESULTS

2.3.1 HUMORAL RESPONSES

2.3.1.1 HIV-1 p24 ANTIBODY ELISA

In Exp1, two baboons (Group 1A) were immunised six times with pTH-GagC and once with GagC VLP (**Fig 2.1**). Only weak humoral responses were induced after the first four successive immunisations with pTH-GagC and these responses were detected only after the 3rd or 4th pTH-GagC inoculation. Additional pTH-GagC inoculations at weeks 34 and 56 boosted these primary antibody responses substantially, to reach a peak antibody titre of 640 and 20480 for baboons 348 and 500 respectively (**Figs 2.2A(i)** and **2.2A(ii)**). The antibody levels dropped off quickly after each inoculation. Following a single GagC VLP inoculation, there was a dramatic increase of antibody levels with peak antibody titres at 1280 and 40960 for baboons 348 and 500 respectively. There was no humoral response that was observed after the first GagC VLP inoculation for the control baboons (Group 1B, baboons 357 and 499) which received pTH-vector mock-prime inoculation. For these baboons, an antibody response was only detected after a second GagC VLP inoculation. (**Figs 2.2B(i)** and **2.2B(ii)**).

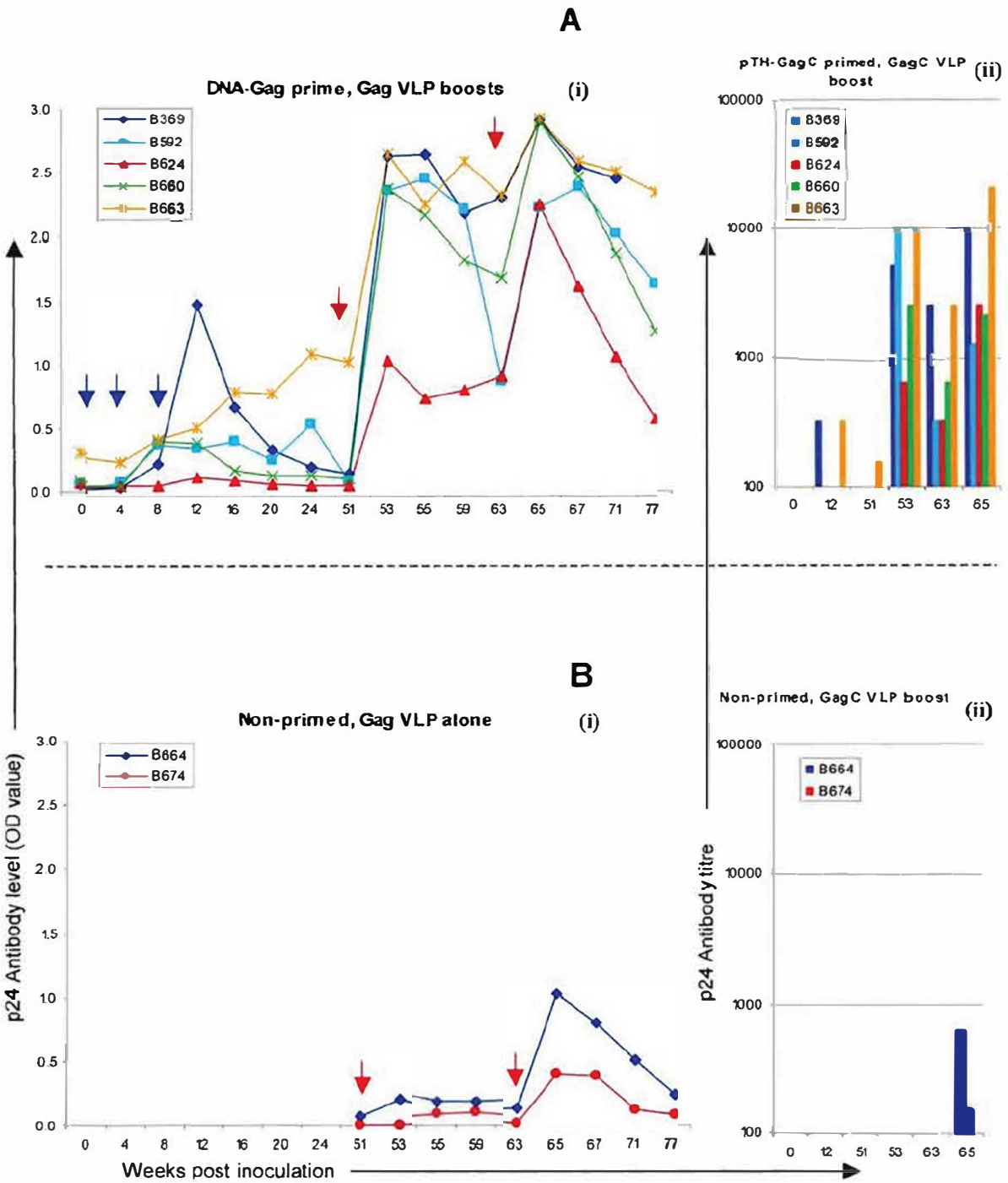
Fig 2.2: HIV-1 Gag-specific antibodies in serum of baboons in Exp1. Serum was obtained from baboons at various time points pre- (wk0) and post inoculation with pTH-GagC (**A**) or pTH-vector (**B**) and following booster immunisations with GagC VLP. The antibody levels and titres were determined by ELISA as previously described (Section **4.2.5.1**). Data points are (i) OD values (serum dilution 1:40) and (ii) antibody titres at various times post inoculation (only antibody titres that exceeded 100 are shown). Inoculation times are indicated with arrows as follows; pTH-GagC: ↓; pTH-vector: ↓; and GagC VLP: ↓.



In Exp2, five baboons (Group 2A) were immunised three times with pTH-GagC and twice with GagC VLP (Fig 2.1). Like for Exp1, only weak humoral responses were detected after three successive immunisations with pTH-GagC. However, these responses were more pronounced in two baboons (369 and 663) where they reached a peak titre of 320 (Figs 2.3A(i) and 2.3A(ii)). The antibody levels dropped off quickly after pTH-GagC immunisation except for baboon 663 where it was maintained. Following the first GagC VLP inoculation, dramatic increases in titres were observed in all the pTH-GagC-primed baboons (Group 2A) but not for the non-primed controls

(Group 2B), whose antibody levels increased only after the 2nd GagC VLP inoculation (Figs 2.3B(i) and 2.3B(ii)). For groups (2A and 2B), the antibody levels increased after the 2nd GagC VLP inoculation, peaking at titres between 1280 and 10240 for the pTH-GagC-primed baboons and 160 and 640 for the controls.

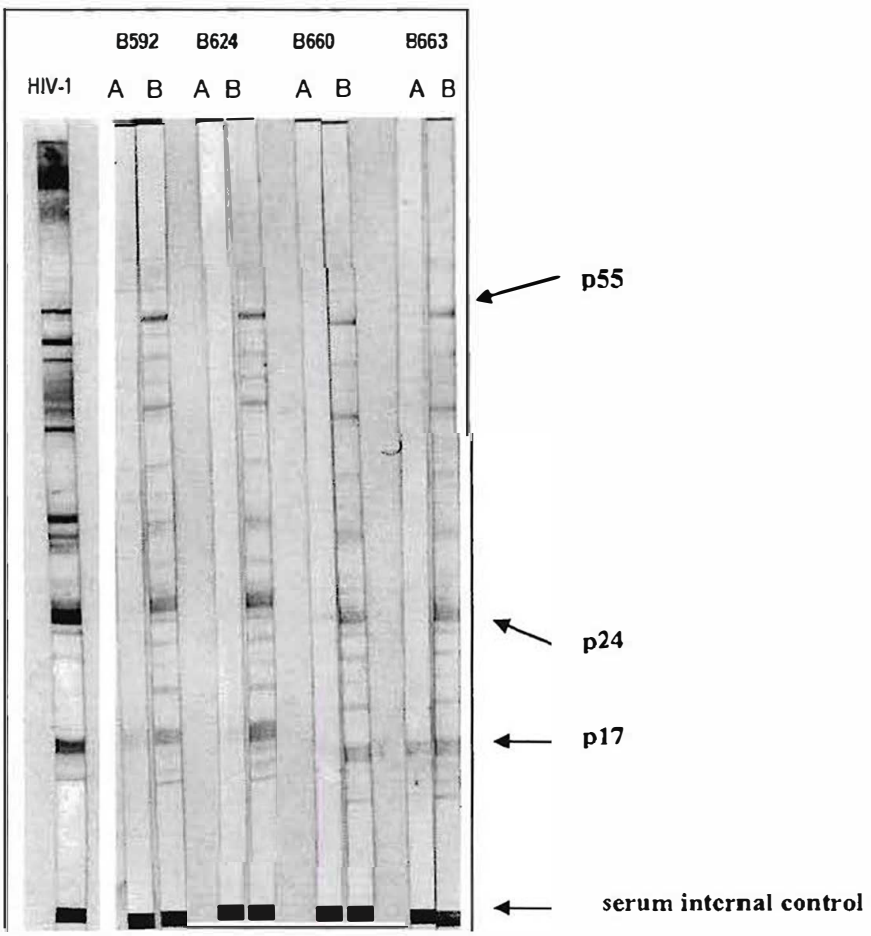
Fig 2.3: HIV-1 Gag-specific antibodies in serum of baboons in Exp2. Serum was obtained from baboons at various time points pre- (wk0) and post inoculation with pTH-GagC (**A**) or without (**B**) and following booster immunisations with GagC VLP. The antibody levels and titres were detennined by ELISA as previously described (Section **4.2.5.1**). Data points are (i) OD values (serum dilution 1:40) and (ii) antibody titres at various times post inoculation (only antibody titres that exceeded 100 are shown). Inoculation times are indicated with arrows as follows; pTH-GagC: ↓; and GagC VLP: ↓.



2.3.1.2 HIV-1 WESTERN BLOT

For a few selected serum samples (Exp2 only), HIV-1 specific antibodies were confirmed at peak antibody response (week 65) by western blot analysis using a commercially available HIV-1 kit (**Fig 2.4**). Western blot bands at positions of p17, p24 and p55 were detected on the strips that were incubated with post GagC VLP (lane B, **Fig 2.4**) but not with pre-inoculation (lane A, **Fig 2.4**) sera. The results provide evidence that the serum antibodies detected by ELISA method were indeed HIV-1 p24-specific antibodies and that antibodies to other regions of HIV-1 Gag were also present.

Fig 2.4: Western blot analysis. The strips in lane A and B were incubated with pre-inoculation (week 0 p.i) and post 2nd GagC VLP inoculation (week 65 p.i) respectively. The sera were used at 1:40 dilution.



2.3.2 CELLULAR RESPONSES

2.3.2.1 IFN-γ ELISPOT RESPONSES TO GAG PEPTIDE POOLS

Cellular immune responses were measured using an IFN-γ ELISPOT assay. The data from the 2 baboon experiments are given separately.

Experiment 1

In this experiment, 2 baboons (348 and 500; Group 1A) were inoculated six times at various intervals with pTH-GagC and boosted at week 75 with GagC VLP (see **Fig 2.1**). Two other baboons (357 and 499; Group 1B) served as controls and were inoculated four times with pTH-vector DNA and boosted twice with GagC VLP at weeks 83 and 95 (see **Fig 2.1**). The cellular immune response to Gag was measured in an IFN-γ ELISPOT assay (**Fig 2.5**). The threshold for a positive response to a Gag peptide pool for the assay after vaccination was determined using PBMC from these four baboons prior to immunisation in an IFN-γ ELISPOT assay using five Gag peptide pools. A cut-off value of 20 SFU/10^6 PBMC was determined (Appendix C.6, **Table A.10**). IFN-γ ELISPOT responses in PHA control wells were >500 SFU/10^6 PBMC (Appendix C.9, **Table A.11**), which were considered as positive.

For both baboons in Group 1A, no response to the 5 Gag peptide pools was detected after three primary inoculations with pTH-GagC. A weak Gag response (cumulative IFN-γ response of 99 SFU/10^6 PBMC) was detected for baboon 500 at four weeks after the 4th pTH-GagC inoculation. At two weeks after the 6th pTH-GagC inoculation, a cumulative IFN-γ response of 130 and 280 SFU/10^6 PBMC was detected for baboons 348 and 500 respectively. The Gag response for baboon 348 declined to 34 SFU/10^6 PBMC by week 75 but the response did not decline for baboon 500, which measured 312 SFU/10^6 PBMC at the same time point. Neither of the control baboons (Group 1B) developed an immune response to Gag in response to vaccination with pTH-vector (**Fig 2.5**).

A booster immunisation with GagC VLP given at week 75 to baboons in Group 1A (that had been primed with pTH-GagC) elicited a broad response to all 5 Gag peptide pools. For baboon 348 the peak response was reached by 2 weeks (Week 77) after the GagC VLP inoculation and the cumulative response to all 5 Gag peptide pools was 2254 SFU/10^6 PBMC. This was >17-fold increase over the peak response after pTH-GagC inoculation. The response to Gag for baboon 500 peaked at week 79 (4 weeks after the GagC VLP boost) reaching a cumulative response of 1262 SFU/10^6 PBMC, which was a 4-fold increase over peak response after pTH-GagC inoculation. By 20th week post GagC VLP inoculation, the cumulative Gag response in both baboons had fallen to 1095 and 520 SFU/10^6 PBMC for baboon 348 and 500 respectively (**Fig 2.5**).

For baboons in Group 1B (that had been mock-primed with pTH-vector), the GagC VLP inoculation was given at week 83. For baboon 357, the peak response to Gag was reached at week 87 (cumulative response of 854 SFU/10^6 PBMC) and was directed predominantly to Gag peptide pool 3. For baboon 499, only a weak response (cumulative response of 165 SFU/10^6 PBMC), which was directed to all peptide pools was detected at week 87. For both baboons, the cumulative

response to Gag did not decline and by week 95, when the 2nd GagC VLP inoculation was given, the cumulative response was 612 and 246 SFU/10^6 PBMC for baboons 357 and 499 respectively. After the 2nd GagC VLP booster inoculation, the cumulative response for both baboons increased only slightly, reaching a peak response of 943 and 327 SFU/10^6 PBMC at week 103 (**Fig 2.5**). No broadening of the response by the 2nd GagC VLP was achieved.

Additional GagC VLP booster inoculation was given to two baboons at week 127 (baboon 357) and 164 (baboon 348) post initial inoculation. Analysis of IFN-γ ELISPOT data showed that the cumulative IFN-γ response at the time of boosting was 62 SFU/10^6 PBMC (baboon 357) and 523 SFU/10^6 PBMC (baboon 348) (**Fig 2.5**). Three weeks after the third GagC VLP inoculation, the response for baboon 357 increased to cumulative IFN-γ response of 332 while that for baboon 348 decreased slightly to 460 SFU/10^6 PBMC. None of these responses reached the magnitude obtained at peak response after the first GagC VLP.

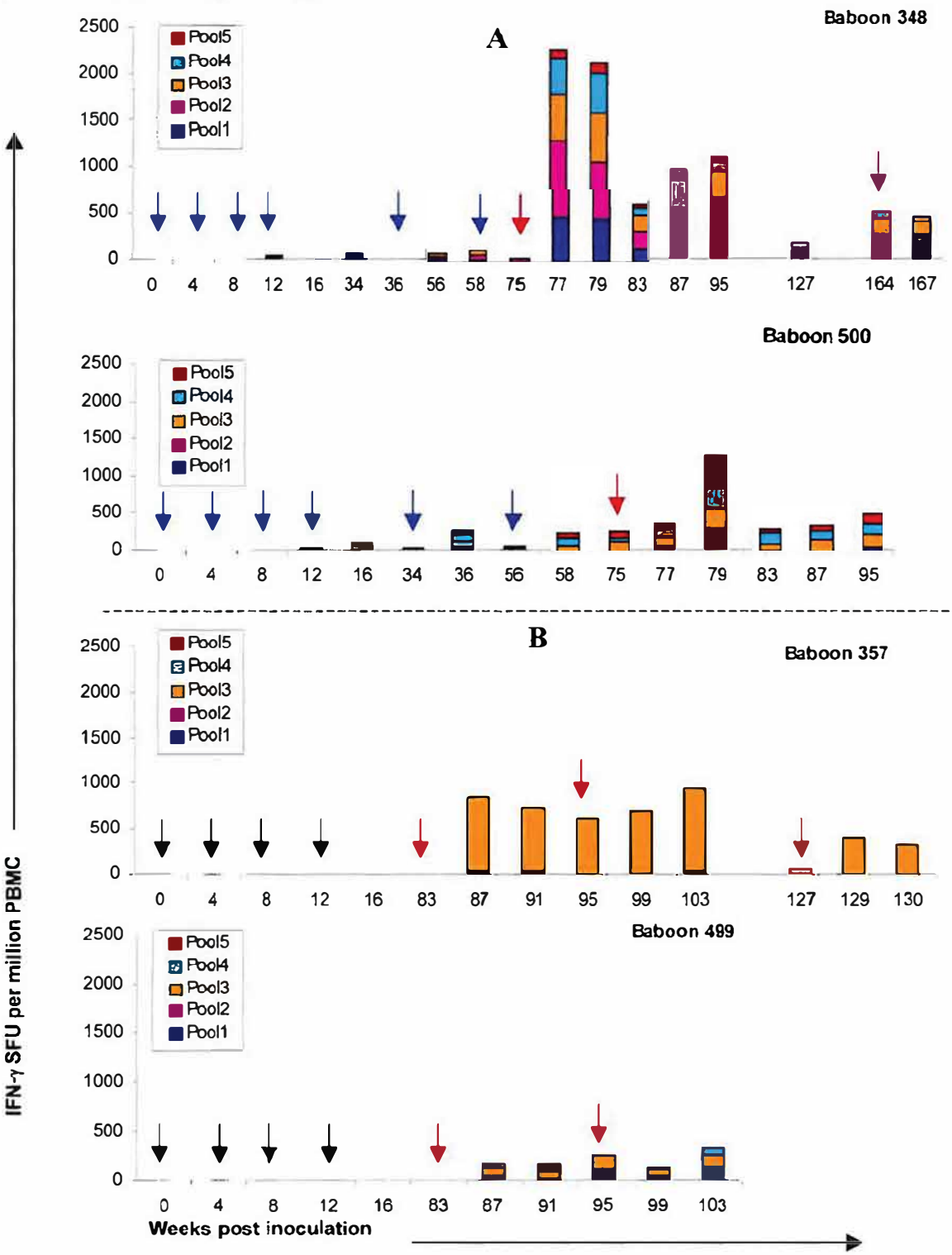
Experiment 2

For this experiment, five baboons (Group 2A) were inoculated 3 times, 4 weeks apart with pTH-GagC, and then boosted twice with GagC VLP vaccine. Two control baboons (Group 2B) were immunised with GagC VLP alone (see **Fig 2.1**). A cut-off value, for this experiment with 7 baboons was determined to be 45 SFU/10^6 PBMC (Appendix C.6, **Table A.10**). A response that was equal or greater than this value was considered positive. IFN-γ ELISPOT responses in PHA control wells were >200 SFU/10^6 PBMC (Appendix C.9, **Table A.11**), which were considered as positive.

For the baboons in Group 2A (pTH-GagC-primed; n=5), Gag-specific responses were detected in 3 of 5 baboons (baboons 592, 660 and 663) after three pTH-GagC inoculations (**Fig 2.6**). This response reached a peak at week 16 post initial inoculation (median: 393 SFU/10^6 PBMC range: 217-515 SFU/10^6 PBMC). Subsequently, this response decayed to pre-immunisation level by week 51 except for baboon 369 (92 SFU/10^6 PBMC). Two weeks following the 1st GagC VLP booster inoculation (at week 53), all the five baboons in the group responded to booster immunisation, giving a median cumulative Gag response of 967 SFU/10^6 PBMC (range: 775-3583 SFU/10^6 PBMC). PBMC obtained from baboons 624, 660 and 663 responded to all five Gag peptide pools while PBMC from baboons 369 and 592 recognised four peptide pools. The magnitude of cumulative Gag response reached the peak at 2 weeks after GagC VLP vaccination for the majority of the baboons. By 12th week post the 1st GagC VLP inoculation (week 63), the median cumulative Gag response had fallen to 455 SFU/10^6 PBMC (range: 222-616 SFU/10^6 PBMC).

Fig 2.5: IFN-γ ELISPOT responses (Exp1) to Gag peptide pools at various times pre- and post-inoculation with: A: pTH-GagC (weeks 0, 4, 8, 12, 34 & 56) and GagC VLP at weeks 75 (Baboons 348 and 500) & 164 (Baboon 348 only) or **B:** pTH-vector (weeks 0, 4, 8 & 12) and GagC VLP at weeks 83 & 95 (Baboons 357 and 499) & 127 (Baboon 357 only).

Five pools of Gag peptides (15-18 mers overlapping by 10 amino acids) spanning the entire Gag protein sequence were used in IFN-γ ELISPOT assays. The IFN-γ response to each Gag peptide pool was determined by subtracting the background (response to PBMC in the absence of peptides). Data shown for each baboon indicate the magnitudes of response to individual Gag peptide pools in which the value was equal or greater than the cut-off value of 20 IFN-γ SFU per 10^6 PBMC. The arrows indicate inoculation with pTH-GagC (↓), pTH-vector (↓) and GagC VLP (↓).



The control baboons 664 and 674 (Group 2B), which were inoculated with GagC VLP only, developed weak Gag-specific responses (median cumulative response of 440 SFU/10^6 PBMC; range: 415-465 SFU/10^6 PBMC) by two weeks post GagC VLP inoculation (**Fig 2.6**). This Gag response, which was more than 2 fold lower in magnitude than the one obtained for the pTH-GagC primed baboons, was directed mainly to 3 Gag peptide pools. By 12th week post the first GagC VLP inoculation (week 63), the Gag response had fallen to 45 SFU SFU/10^6 PBMC for one baboon (674) and nil for the other baboon (664).

Both Groups 2A (pTH-GagC prime) and 2B (control) were subsequently given a second GagC VLP inoculation at 12 weeks (week 63) after the first one. Two weeks after the 2nd GagC VLP inoculation (week 65), only a slight increase in cumulative response to Gag peptide pools was observed (**Fig 2.6**). At this time point, the median cumulative Gag response increased slightly from the previous level (12 weeks after the 1st GagC VLP inoculation) to 616 (range: 435-837) and 213 (range: 207-219) SFU/10^6 PBMC for the pTH-GagC-primed (Group 2A) and control (Group 2B) baboons respectively. Thereafter, the responses to Gag in both baboon groups declined and by week 71 when the study was terminated, the median cumulative response had fallen to 101 (range: 49-256) for 4 of 5 pTH-GagC-primed baboons and 58 SFU/10^6 PBMC for one control baboon. For one pTH-GagC-primed baboon (baboon 624) and one control (baboon 664), no response was detected at this time point.

2.3.2.2 PEPTIDE MAPPING BY POOL-MATRIX IFN-γ ELISPOT

It was of interest to identify the peptides in the five Gag peptide pools to which the PBMC were responding. An IFN-γ ELISPOT assay, using Gag peptides spanning the entire Gag protein sequences arranged in a 'pool-matrix' format (**Table 2.2**) was used for this. Peptides identified by this technique were then confirmed in a further IFN-γ ELISPOT assay using the individual Gag peptides. This confirmatory IFN-γ ELISPOT assay was only done at a single time point after the GagC VLP immunisation. The magnitude of the response to individual Gag peptides and the amino acid sequences in the Gag regions: p17, p24 and p2p7p1p6 are shown in **Tables 2.3A** (Exp1) and **2.3B** (Exp2). Several of the identified peptides are also peptides that are recognised by humans following infection with HIV-1 subtype C. A summary of the number of Gag peptides that were identified by IFN-γ ELISPOT assay as being targeted by PBMC from immunised baboons in both Exp1 and Exp2 is shown in **Table 2.4**.

As **Tables 2.3** and **2.4** show, PBMC obtained from the pTH-GagC-primed baboons in both Experiment 1 and 2 (Groups 1A and 2A) responded to an average of 8 peptides per baboon while PBMC from the mock-primed (Group 1B) or non-primed (Group 2B) controls responded to 2 or 3 peptides per baboon belonging to Gag peptide pool 3.

Fig 2.6: ELISPOT responses (Exp2) to Gag peptide pools at various times at pre- and -post inoculation with: A: pTH-GagC and GagC VLP (Baboons 369, 592, 624, 660 and 663) or **B:** GagC VLP alone (Baboons 664 and 674).

Five pools of Gag peptides (15-18 mers overlapping by 10 amino acids) spanning the entire Gag protein sequence were used in IFN-γ ELISPOT assays. The IFN-γ response to each Gag peptide pool was determined by subtracting the background (response to PBMC in the absence of peptides) from the Gag pool responses. Data shown for each baboon indicate the magnitudes of response to individual Gag peptide pools in which the value was equal or greater than the cut-off value of 45 IFN-γ SFU per 10^6 PBMC. The arrows indicate inoculation with pTH-GagC (black) and GagC VLP (red).

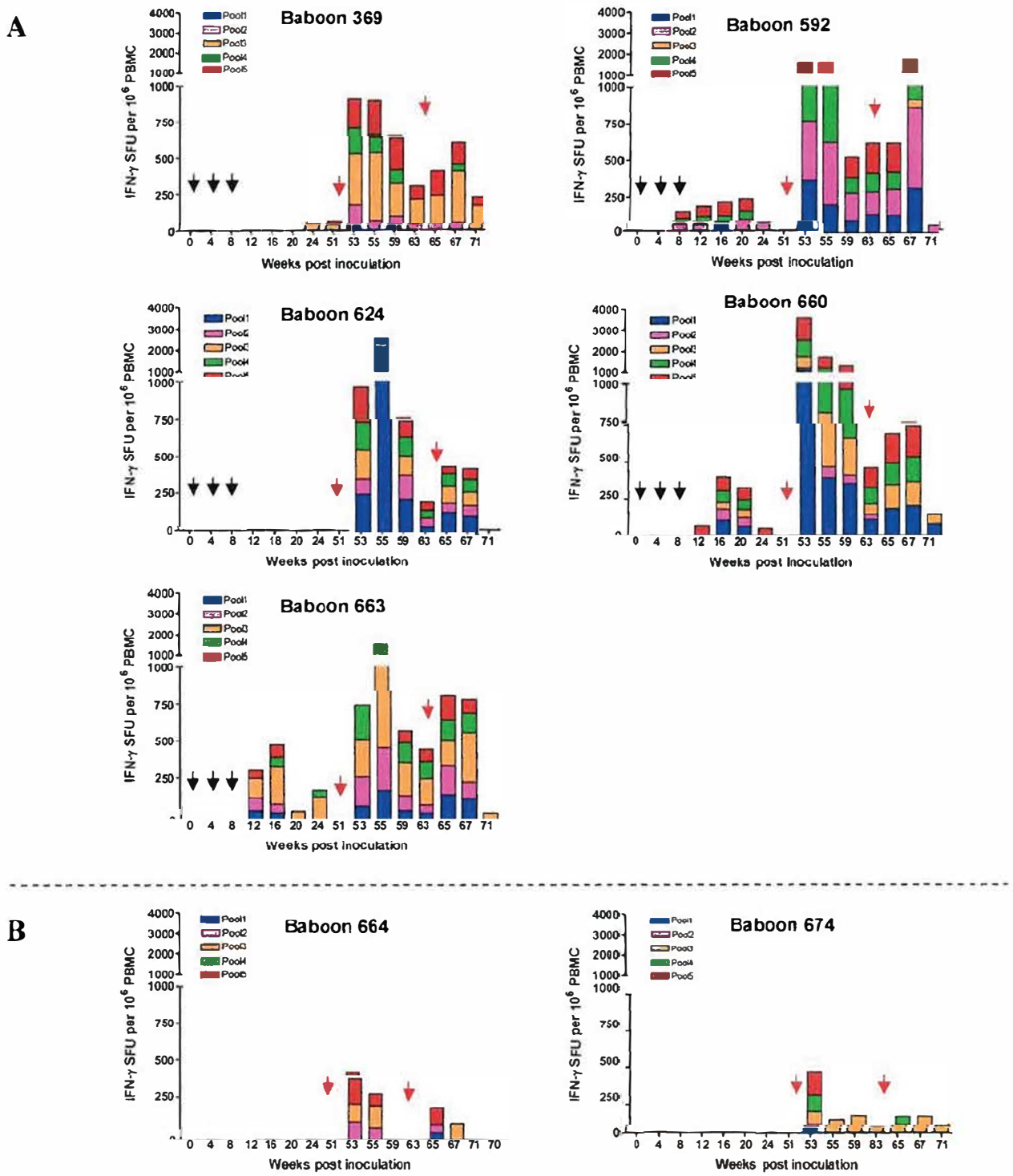


Table 2.3: The identity and amino acid sequences of Gag peptides that were identified by Pool-Matrix IFN-γ ELISPOT assay using PBMC obtained from immunised baboons in (A): Experiment 1 and (B): Experiment 2.

(A) Experiment 1: PBMC obtained at 4 weeks post the first GagC VLP inoculation were used in Pool-Matrix IFN-γ ELISPOT strategy to identify the peptides to which the PBMC were responding. The responses were confirmed by subsequent IFN-γ ELISPOT assay using cryo-preserved PBMC. The response is given as the net IFN-γ SFU/10^6 PBMC above the background response (PBMC in medium without peptides). A cut-off value of 20 IFN-γ SFU/10^6 PBMC was applied. The peptide sequences shown in **red** indicate the ones that have been reported as being targeted by PBMC from individuals with early HIV-1 subtype C infection (Masemola *et al*, 2004a and 204b). ↑: shows peptides that contain overlapping amino acid sequences with the adjacent peptide, indicating the possibility of sharing the same epitope.

Inoculation regimen	Baboon number	Gag peptides identified after GagC VLP inoculation				Response (IFN-γ SFU/10^6 PBMC)
		Gag region	Peptide pool	Peptide number	Amino acid sequence	
pTH-GagC/GagC VLP	**348**	p17	P1	6	**ASRELERFALNPGLL**	216
				9	EGCKQIMKQLQPALQTGT	66
				↑10	QLQPALQTGTEELRSLY	66
			P2	18	**GKVSQNYPIVQNLQGQMV**	358
		p24	P2	28	LKDTINEEAAEWDRLHPV	58
			P3	36	**PVGDIYWKRWIILGLNKIV**	266
				↑37	**WIILGLNKIVRMYSPVSI**	133
				40	**GPKEPFRDYVDRFFKTLR**	225
				↑41	**YVDRFFKTLRAEQATQDV**	83
			P4	46	TILRALGPGASLEEMMTA	58
				48	ACQGVGGPSHKARVLAEA	41
		p2p7p1p6	P4	55	HIARNCRAPRKKGCWK	83
	500	p17	P1	7	ERFALNPGLLETSEGCK	20
		p24	P2	23	**AFSPEVIPMFTALSEGA**	25
			P3	38	IVRMYSPVSILDIKQGPK	175
		p2p7p1p6	P4	51	QANSAIMMQRSNFKGPKR	84
			P5	63	TAPPAESFRFEETTPAPK	20
				↑64	RFEETTPAPLQEPKDREPL	30
pTH-vector/GagC VLP	**357**	p24	P3	40	**GPKEPFRDYVDRFFKTLR**	611
				↑41	**YVDRFFKTLRAEQATQDV**	624
	499	p24	P3	36	**PVGDIYWKRWIILGLNKIV**	94
				↑37	**WIILGLNKIVRMYSPVSI**	76

2.3.2.3 CYTOKINE ASSAY BY INTRACELLULAR IFN-γ STAINING (ICS)

The phenotype of the PBMC responding to Gag peptides was determined by flow cytometry after staining for CD3, CD4 and CD8 cell surface molecules and intracellular IFN-γ following stimulation of baboon PBMC in the presence of brefeldin A for 16h with HIV-1 Gag peptides. The individual Gag peptides to be used in these assays were identified by IFN-γ ELISPOT assay. With the exception of the anti-CD3 monoclonal antibody, which was derived from rhesus macaque all the other antibodies used in this assay were anti-human mAB and their cross-reactivity with baboon cells was confirmed prior to these experiments (see **Appendices C.1, C.2** and **C.4(i)**).

Cryo-preserved PBMC were used in ICS assays. Preliminary findings established that cryo-preservation of baboon PBMC did not significantly change the expression of CD3, CD8, CD28 and CD49d molecules on the cell surface (Appendix C.3, **Fig A.2**; Appendix C.4, **Fig A.4**).

(B) Experiment 2: PBMC obtained at 4 weeks post the first GagC VLP inoculation were used in Pool-Matrix IFN-γ ELISPOT strategy to identify the peptides to which the PBMC were responding. The responses were confirmed by subsequent IFN-γ ELISPOT assay using cryo-preserved PBMC. The response is given as the net IFN-γ SFU/10^6 PBMC above the background response (PBMC in medium without peptides). A cut-off value of 45 IFN-γ SFU/10^6 PBMC was applied. The peptide sequences shown in **red** indicate the ones that have been reported as being targeted by PBMC from individuals with early HIV-1 subtype C infection (Masemola *et al*, 2004a and 2004b). ↑: shows peptides that contain overlapping amino acid sequences with the adjacent peptide, indicating the possibility of sharing the same epitope.

Inoculation regimen	**Baboon number**	**Gag peptides identified after GagC VLP inoculation**				**Response (IFN-γ SFU/10^6 PBMC)**
		Gag region	Peptide pool	Peptide number	Amino acid sequence	
pTH-GagC/GagC VLP	**369**	p24	P3	40	**GPKEPFRDYVDRFFKTLR**	234
				↑41	**YVDRFFKTLRAEQATQDV**	350
	592	p17	P1	4	**GKKHYMLKHLVWASREL**	46
				6	**ASRELERFALNPGLL**	68
				↑7	ERFALNPGLLETSEGCK	113
				↑8	GLLETSEGCKQIMKQL	66
				10	QLQPALQTGTEELRSLY	65
		p24	P2	21	PRTLNAWVKVIEEKAF	386
				24	**PMFTALSEGATPQDLNTM**	238
			P4	46	TILRALGPGASLEEMMTA	55
		p2p7p1p6	P4	49	SHKARVLAEAMSQANSA	60
				51	QANSAIMMQRSNFKGPKR	63
			P5	62	FLQSRPEPTAPPAESFRF	103
				65	PKQEPKDREPLTSLK	63
	624	p17	P1	5	**KHLVWASRELERFAL**	68
		p24	P3	36	**PVGDIYWKRWIILGLNKIV**	238
			P4	46	TILRALGPGASLEEMMTA	63
		p2p7p1p6	P4	51	QANSAIMMQRSNFKGPKR	103
				54	CFNCGKEGHIARNCRAPR	63
			P5	65	PKQEPKDREPLTSLK	66
	660	p17	P1	6	**ASRELERFALNPGLL**	358
				9	EGCKQIMKQLQPALQTGT	303
				↑10	QLQPALQTGTEELRSLY	650
		p24	P3	34	STLQEQIAWMTSNPPVPV	211
				36	**PVGDIYWKRWIILGLNKIV**	451
				↑37	**WIILGLNKIVRMYSPVSI**	521
		p2p7p1p6	P4	51	QANSAIMMQRSNFKGPKR	275
			P5	63	TAPPAESFRFEETTPAPK	308
				↑64	RFEETTPAPLQEPKDREPL	396
	663	p17	P1	4	**GKKHYMLKHLVWASREL**	251
			P2	18	**GKVSQNYPIVQNLQGQMV**	247
		p24	P3	30	LHPVHAGPIAPGQMREPR	103
				32	MREPRGSDIAGTTSTL	100
				37	**WIILGLNKIVRMYSPVSI**	133
				39	SILDIKQGPKEPFRDYV	148
				↑40	**GPKEPFRDYVDRFFKTLR**	916
				↑41	**YVDRFFKTLRAEQATQDV**	1076
			P4	46	TILRALGPGASLEEMMTA	176
		p2p7p1p6	P4	51	QANSAIMMQRSNFKGPKR	173
				54	CFNCGKEGHIARNCRAPR	173
				↑55	HIARNCRAPRKKGCWK	201
			P5	58	QMKDCTERQANFLGKIW	100
GagC VLP alone	**664**	p24	P3	40	**GPKEPFRDYVDRFFKTLR**	229
				↑41	**YVDRFFKTLRAEQATQDV**	52
	674	p24	P3	36	**PVGDIYWKRWIILGLNKIV**	90
				40	**GPKEPFRDYVDRFFKTLR**	181
				↑41	**YVDRFFKTLRAEQATQDV**	253

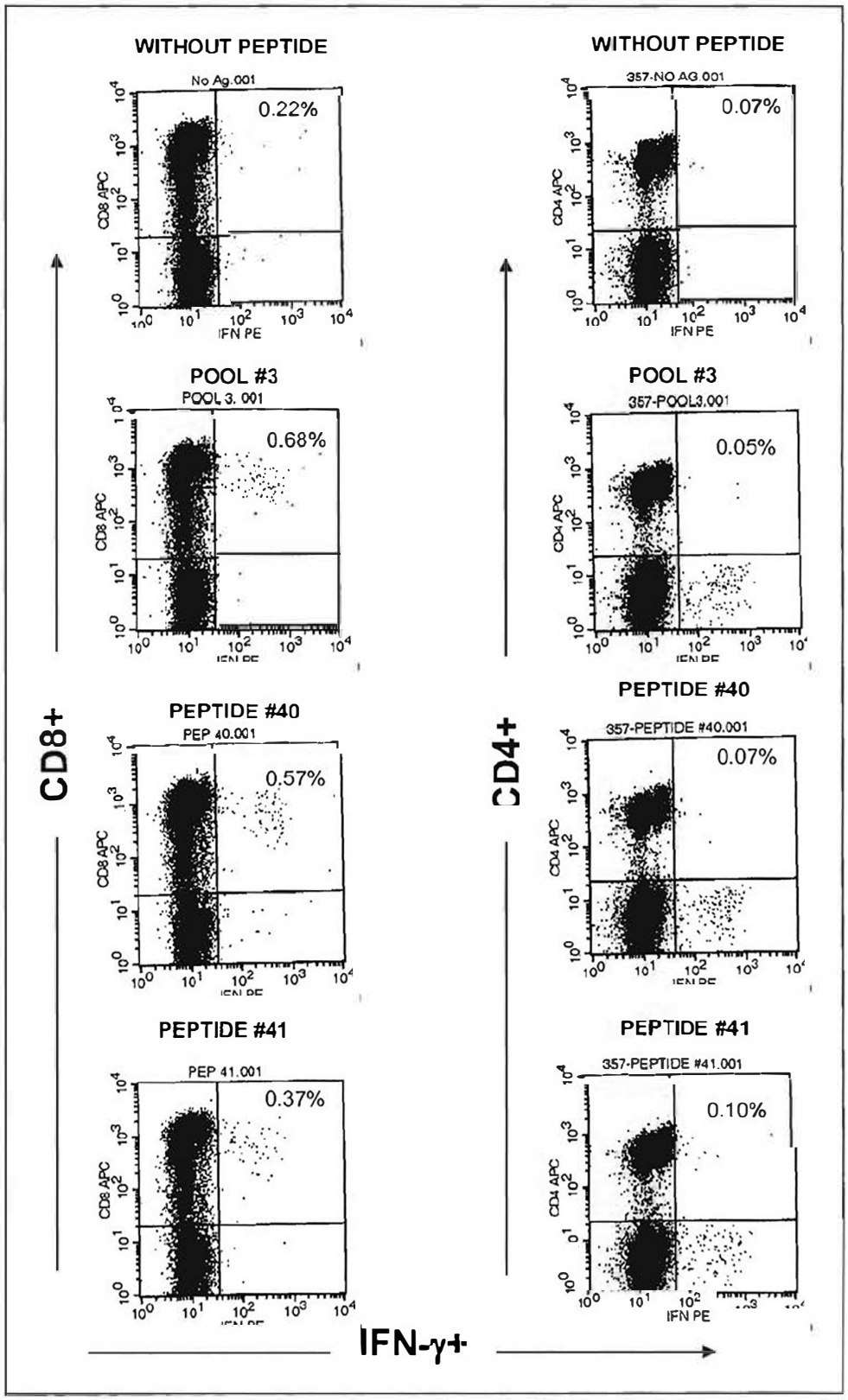
Table 2.4: Number of peptides identified by IFN-γ ELISPOT assay as being targeted by PBMC from immunised baboon (Exp1 and Exp2). PBMC obtained at 4 weeks post the 1st GagC VLP inoculation were used in IFN-γ ELISPOT assay to identify peptides to which PBMC were responding. The Gag peptides targeted by baboon PBMC (shown in **Fig 2.3**) have been categorised according to the position in the p17, p24 or p2p7p1p6 domains of HIV-1 Gag protein.

Experiment	Immunisation regimen	Baboon number	Number of targeted peptides in the Gag region			
			p17	p24	p2p7p1p6	**Total**
Exp1	pTH-GagC/GagC VLP	348	4	7	1	**12**
		500	1	2	3	**6**
	pTH-vector/GagC VLP	357	0	2	0	**2**
		499	0	2	0	**2**
Exp2	pTH-GagC/GagC VLP	369	0	2	0	**2**
		592	5	3	4	**12**
		624	1	2	3	**6**
		660	3	3	3	**9**
		663	2	7	4	**13**
	GagC VLP alone	664	0	2	0	**2**
		674	0	3	0	**3**

Co-stimulatory antibodies, anti-CD28 and anti-CD49d, are often used in cytokine assays due to their role in lowering the activation threshold for antigenic specific cells in a cellular immune response (Betts *et al*, 2004; Gauduin *et al*, 2004). Two anti-human CD28 and CD49d monoclonal antibodies were commercially available from BD-Immunocytometry Systems (BD-IS) and BD-PharMingen (BD-Ph). A preliminary study using baboon PBMC suggested that both monoclonal antibodies, in conjunction with SEB, were equally effective in inducing production of IFN-γ (Appendix C.4(iii), **Fig A.5**).

CD69 is an early activation marker that is expressed during *in vitro* stimulation (Testi *et al*, 1989). Thus, detection of CD69 marker on cytokine-producing cells in the ICS assay gives more assurance that the cells being defined as cytokine-positive have been activated by the antigen under investigation. The utility of anti-human CD69 antibody to detect cell activation was investigated for inclusion in the ICS assay. Preliminary results showed that the expression of the CD69 antigen by baboon PBMC upon stimulation with SEB reached the peak at 12 hours (Appendix C.5, **Fig A.6**). However, addition of BFA had a severe prohibitive effect on the expression of CD69 (Appendix C.5, **Fig A.7**) and therefore anti-CD69 mAB was not used in ICS assays. Permeabilising the cells prior to staining with anti-human CD69 mAB did not improve detection of CD69 antigen (data not shown).

Fig 2.7: Detection of IFN-γ in PBMC of baboon 357 by ICS assay and FAC analysis. Cro-preserved baboon PBMC from baboon 357 obtained at 4 weeks after the 2nd GagC VLP inoculation were incubated for 16h with R10 medium only (without peptide) or R10 containing Gag peptide pool 3, Gag peptide number 40 or number 41 (4 µg/mL) with addition of brefeldin A (10 µg/mL; Sigma) for the last 14h of incubation. The cells were stained with anti-monkey CD3-FITC (BioSource), anti-human IFN-γ-PE (BD) and anti-human CD4-APC or anti-human CD8-APC (BD) mAB. Data was acquired using FACSCalibur (BD) cytometer and analysed with CellQuest (BD) software. The figures given on the upper-right quadrants represent percentage of CD4+ or CD3+CD8+ IFN-γ-producing lymphocytes.



In the present study, CD3+CD8+ IFN-γ-producing lymphocytes were detected for PBMC obtained from one baboon (357; Exp1) at 4 weeks following the 2nd GagC VLP booster inoculation. For this baboon, the frequency of Gag-specific CD3+CD8+ lymphocytes producing IFN-γ ranged from 0.15% to 0.46% after 16h incubation with Gag peptide pool 3, Gag peptide number 40 or Gag peptide number 41 (**Fig 2.7**). The frequency of IFN-γ-producing CD4+ lymphocytes was almost negligible. For three other baboons (Exp2) where the ICS assay was performed, the frequency of IFN-γ-producing cells was negligible (data not shown).

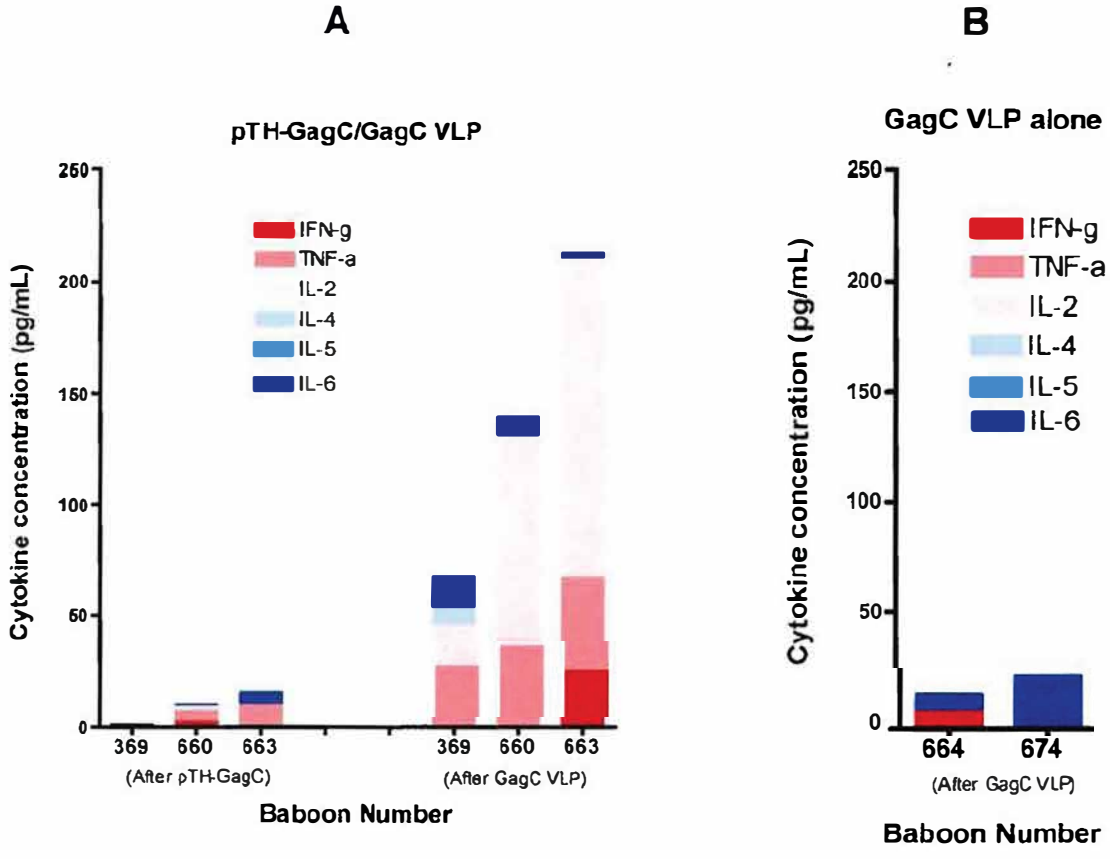
2.3.2.4 CYTOKINE DETERMINATION BY CBA ASSAY

The spectrum of cytokines produced by PBMC from selected baboons (Exp2 only) in response to Gag peptide stimulation was measured using a Non-human primate Th1/Th2 cytokine CBA kit (BD) that allows detection of IFN-γ, TNF-α, IL-2, IL-4, IL-5 and IL-6. The Gag peptides that were chosen for stimulation in the assays were identified by IFN-γ ELISPOT assay. A single peptide indicating the greatest magnitude of response in the IFN-γ ELISPOT assay was selected for each baboon PBMC stimulation.

For pTH-GagC-primed baboons (369, 660 and 663), the assay was done using PBMC obtained at week 12 (4 weeks after 3rd pTH-GagC DNA inoculation) and week 53 (2 weeks after 1st GagC VLP booster inoculation). At week 12, the total cytokines produced by PBMC were low (<25 pg/mL) for all the three baboons (**Fig 2.8**). However, for the PBMC obtained from these baboons at week 53 after the 1st GagC VLP inoculation, there was a marked increase in the total cytokines (up to 215 pg/mL for baboon 663) that were produced. This production was predominated by TNF-α and IL-2 cytokines for PBMC from baboons 369 and 660 while PBMC from baboon 663 produced predominantly IFN-γ, TNF-α and IL-2, with IL-2 going up to 143.3 pg/mL. Little or no production of IL-4, IL-5 and IL-6 was observed. Also, PBMC from baboons 369 and 660 did not produce any IFN-γ.

For the control baboons (664 and 674), the assay was done using PBMC obtained at week 65 (2 weeks post 2nd GagC VLP vaccination) only. For these baboons, only low levels of IFN-γ (baboon 664 only) and IL-6 were detected **Fig 2.8**).

Fig 2.8: Array of cytokines produced by baboon PBMC upon stimulation with specific Gag peptides and detection by CBA assay. Cryo-preserved baboon PBMC obtained: **(A)** at weeks 12 and 53 from baboons vaccinated with pTH-GagC and GagC VLP (Exp2), and **(B)** at week 65 from baboons vaccinated with GagC VLP alone (Exp2) were revived, rested and then incubated for 16h with R10 medium only or R10 medium containing Gag peptides (4 µg/mL), which were identified by the IFN-γ ELISPOT assay. Gag peptides that were used are Peptide 37 (baboon 660), Peptide 40 (baboons 369, 663 and 664) and Peptide 41 (baboon 674). The culture supernatants were collected and amounts of IFN-γ, TNF-α, IL-2, IL-4, IL-5 and IL-6 quantified simultaneously using a Non-human primate Th1/Th2 cytokine CBA kit (BD). Gag-specific cytokines were determined by subtracting the value measured for supernatants collected from cultures with R10 medium only from the corresponding cultures containing Gag peptides.



2.4 DISCUSSION

The present study presents immunological data from two experiments, which were conducted to test the immunogenicity of a HIV-1 Gag DNA and a $Pr55^{gag}$ VLP vaccine, given in a prime-boost vaccination modality to baboons. Both experiments utilised a DNA vaccine to prime the immune system and $Pr55^{gag}$ VLP vaccine to boost. However, the study objectives, group sizes and the number of vaccine inoculations for the two experiments differed. In the first experiment (Exp1) the principal objectives were to develop immunological assays for use in the chacma baboon HIV vaccine studies and to generate preliminary immunogenicity data. Therefore, a small group size comprising of 4 baboons was used. Two baboons received a total of six DNA vaccine inoculations followed by a single $Pr55^{gag}$ VLP vaccine whereas 2 control baboons received 4 inoculations with the DNA vector control

followed by two inoculations with $Pr55^{gag}$ VLP vaccine. After establishing the immunological assays, the second experiment (Exp2) was initiated with a group of 5 baboons using an immunisation regimen similar to that used in the majority of DNA vaccine clinical trials. Three DNA vaccine immunisations were given, 4 weeks apart followed by two successive inoculations with the $Pr55^{gag}$ VLP vaccine. Both groups were then immunised with two successive inoculations with the $Pr55^{gag}$ VLP vaccine. The control group, comprising of two baboons, were immunised with $Pr55^{gag}$ VLP vaccine only.

2.4.1 DEVELOPMENT OF METHODS

As the use of chacma baboons in HIV vaccine studies had not been reported, the present study started by developing immunological methods to be used for immunogenicity evaluation of HIV vaccines in this baboon model. Several anti-human monoclonal antibodies and a few anti-rhesus monkey monoclonals (see a list of reagents in Appendix A, **Table A.4**) were found to cross-react with baboon cells and cellular mediators such as IFN-γ and other cytokines and thus, they were used to develop various methods for use in these baboon vaccine studies. The cross-reactivity between anti-human monoclonal antibodies with baboon cells further illustrates the closeness in homology between the immune components of the baboon and humans, suggesting that data obtained from vaccine studies using the baboon model could be used to closely predict the outcome in clinical trials.

Cryo-preserved PBMC are often used in HIV vaccine studies because the use of freshly isolated PBMC is not readily feasible. The use of cryo-preserved baboon PBMC in the present study was chosen and it was therefore necessary to investigate the effect of cyro-preservation on the detection of important cell surface molecules on the baboon PBMC. The cryo-preservation of PBMC was shown not to alter the detection of CD3, CD8, CD28, CD49d and CD69 molecules on the surface of revived cryo-preserved PBMC, giving the confidence that the determination of cell phenotypic classification in ICS assays was not affected by cryo-preservation. The finding in the present study is consistent with the results of a recent study by Costantini *et al* (2003), which showed that cryo-preservation of human PBMC obtained from both healthy and HIV-infected donors did not induce significant changes in the proportions of CD4+ and CD8+ T cells, indicating that there was no selective death of specific T cells induced by the cryo-preservation and revival procedures. Other studies have investigated the impact of cryo-preservation of PBMC on immunologic assays, including production of IFN-γ in ICS and ELISPOT assays (Gauduin *et al*, 2004; Kreher *et al*, 2003; Kilani *et al*, 2005; Maecker *et al*, 2005) and found no significant difference in assay readouts between the fresh and cryo-preserved PBMC when peptide antigens were used. However, a 40% to 80% decrease in the frequency of antigen-specific CD4+ T cells, detected by ICS, was observed in cryo-preserved cells when using stimulation with viral proteins (Gauduin *et al*, 2004).

The use of co-stimulatory antibodies, anti-CD28 and anti-CD49d, in ICS assays has been shown to enhance the frequency of cytokine-producing cells (up to three-fold increases) following superantigen- or antigen-specific stimulation (Betts *et al*, 2004; Gauduin *et al*, 2004). The co-stimulatory effect by anti-CD28 and anti-CD49d antibodies is achieved via cross-linking of the corresponding CD28 and CD49d molecules on the T-cells, thereby providing a secondary activation signal. As cryo-preservation of baboon PBMC was shown not to affect the detection of CD28 and CD49d molecules by the anti-human CD28 and CD49d mAB, it gives confidence that the co-stimulatory potential of these human anti-CD28 and anti-CD49d antibodies is unaffected by the use of cryo-preserved cells in ICS assays.

CD69 molecule, which is not present on the surface of resting peripheral blood T cells, is an early activation molecule that is expressed on T cells during *in vitro* stimulation (Testi *et al*, 1989). Thus, the use of an anti-human CD69 monoclonal antibody in the ICS assay is beneficial as it gives more assurance that the cells being defined as cytokine-positive have been activated by the antigen under investigation. Also, the use of an anti-human CD69 antibody allows better clustering of cytokine-positive cells, making it easier to define a cytokine-positive population (Becton Dikinson, 2003). In the current study, the use of anti-human CD69 antibody was investigated with a view of including it in the ICS assays. However, its use was precluded by the finding that BFA, whose use is essential in ICS assays, prohibited the expression of CD69 antigen. It is possible that other factors could also inhibit CD69 expression.

2.4.2 IMMUNE RESPONSES INDUCED BY pTH-GagC AND GagC VLP VACCINES

The cellular immune responses to Gag were evaluated in the two baboon experiments and were found to be broad and strong in magnitude. In both experiments, the magnitudes of IFN-γ ELISPOT responses following three (Exp2) or six (Exp1) inoculations with pTH-GagC were low or absent. After a single GagC VLP booster immunisation, the cumulative IFN-γ response to the Gag peptide pools increased dramatically (7- to 9-fold) for the pTH-GagC-primed groups. In contrast, only a moderate cumulative IFN-γ response was observed for the mock-primed or non-primed controls. A second GagC VLP inoculation did not boost these responses in the majority of animals in both experiments, irrespective of whether a single or mixed vaccination regimen was given. Similarly, lack of boosting effect was not observed in two baboons that were inoculated further with GagC VLP at weeks 127 or 164 post initial inoculation. However, IFN-γ ELISPOT responses were still detectable in these two baboons 32 and 89 weeks post the last GagC VLP inoculation, demonstrating the longevity of these responses. Studies in HIV-1-infected humans and SIV- or SHIV- infected monkeys have shown that CTL activity correlate inversely with viral loads (Betts *et al*, 1999; Sindhu *et al*, 2003; Lubaki *et al*, 1999) and strong CTL activity could be associated with persons with stable non-progressing HIV-1

infection (Harrer *et al*, 1996; Edwards *et al*, 2002; Ogg *et al*, 1998). However, it not clear if the magnitude of a CTL response correlates with a delay in disease progression or non-progression status with some researchers reporting an inverse correlation with HIV-1 viraemia (Edwards *et al*, 2002; Ogg *et al*, 1998) while others found lack of such correlation (Addo *et al*, 2003; Betts *et al*, 2001). It is believed that a vaccine that induces a potent CTL response is beneficial in conferring protection (Robinson & Amara, 2005; Spearman, 2006).

The breadth of IFN-γ responses was investigated in the present study in both baboon experiments by mapping the Gag peptides to which the PBMC from the immunised baboons were responding. The specific CTL epitopes were not identified in this study. However, the Gag peptides which were identified by the peptide mapping technique that was employed in this study were assumed to contain the epitopes to which the responses were directed. These epitope-containing Gag peptides were then used to estimate the breadth of the IFN-γ responses. The data obtained clearly shows that the DNA-prime, Gag VLP boost modality was efficient at eliciting broad responses. Baboons that received a pTH-GagC DNA prime, followed by GagC VLP boost responded to an average of 9 Gag peptides compared to an average of 2 peptides for the groups receiving GagC VLP alone. A few of the targeted peptides had an overlap in amino acid sequence, indicating the possibility of a single epitope being the target in such adjacent peptides. This can be resolved only by complete epitope mapping using a technique that was beyond the scope of this study. Also, the peptides targeted by PBMC from the pTH-GagC primed animals were found in all three main Gag protein domains (i.e. p17, p24 and p2p7p1p6) while those targeted by PBMC from the control groups were restricted to a narrow portion of p24 (peptide pool 3 only).

An interesting outcome from this peptide mapping is the finding that several of these peptides targeted by PBMC of the GagC-immunised baboons have been identified as being targeted by PBMC from patients with early HIV-1 subtype C infection in southern Africa (Masemola *et al*, 2004a & 2004b). In their study, Masemola *et al* (2004a) found that when the overlapping amino acid consequences of the targeted peptides were co-joined, 10 epitopic regions across the Gag protein were identified. Of these, 5 epitopic regions were contained in the peptides to which the GagC-immunised baboons responded in the present study. A similar observation was made for the GagC-immunised baboons in a rBCG-prime, Gag VLP boost vaccination modality (Chapter 4). This finding provides evidence that ZDU422 isolate, the HIV-1 virus from which this vaccine was derived, is still relevant for designing vaccines against subtype C HIV-1 infections. Targeting of multiple CTL epitopes by PBMC from recipients of candidate HIV-1 vaccine may be considered an important outcome as it indicates that the vaccine-induced immunity is broad. Furthermore, the breadth of memory HIV-1 specific CTL cells has been found to correlate negatively with HIV-1 virus load and disease progression in a 5-year follow-up study of a cohort of chronically HIV-1-infected patients (Chouquet *et al*, 2002).

Virus-specific and vaccine-induced CD8+T cell responses have been shown to be crucial for the containment and suppression of viremia in HIV-1-infected humans and SIV/SHIV-infected monkeys (Amara *et al*, 2005a; Borrow *et al*, 1994; Jin *et al*, 1999; Kaul *et al*, 2000; Koup *et al*, 1994; Migueles *et al*, 2000; Rowland-Jones *et al*, 1995 & 1998; Schmitz *et al*, 1999 & 2005). In the present study, the phenotype of the vaccine-specific IFN-γ-producing cells was investigated by use of the ICS assay. A substantial proportion of the T-cell response (up to 0.46% CD3+CD8+ T cells) in the ICS assay was demonstrated in 1 of 11 GagC VLP-immunised baboons. For this baboon (357), the assay indicated that IFN-γ was produced almost solely by the CD8+ T cell population, and not by the CD4+ T cells. Attempts to characterise the phenotype of the IFN-γ-producing cells in three other baboons (in Exp2) materialised only negligible frequencies of IFN-γ-producing cells (data not shown) and similar characterisation was not possible for the majority of the other baboons because insufficient cells were available to perform the assay.

As stated above, only negligible frequencies of IFN-γ-producing cells (data not shown) could be detected in ICS assay for PBMC from three baboons in Exp2. The reason for these low frequencies in not clear but it was noted that the magnitude of the IFN-γ ELISPOT responses to individual Gag peptides, which were used for ICS assays was very low. Conversely, each of the two Gag peptides which were used for the ICS for baboon 357 (Exp1) gave an IFN-γ response of >600 SFU/10^6 PBMC out of a cumulative IFN-γ response to Gag of 821 SFU/10^6 PBMC when used individually for stimulation in the IFN-γ ELISPOT assay (which accounted for nearly 75% cumulative Gag response). The failure to demonstrate substantial proportions of IFN-γ-producing T cells for the other three baboon PBMC was probably caused by sub-optimal responses to the individual Gag peptides that were used in the ICS assay, resulting in too few IFN-γ-producing cells being detected. This may also explain the low levels of cytokines that were measured in their culture supernatants by CBA assays.

To further characterise the vaccine-induced immune response, the spectrum of cytokines produced by PBMC upon stimulation with specific Gag peptides was measured using a CBA assay. This assay was performed for PBMC samples from 3 of 5 pTH-GagC/GagC VLP prime-boost baboons in Exp2 at week 12 (post 3rd DNA prime) and week 53 (post VLP boost), and also PBMC from the non-primed controls at week 65 (post 2nd GagC VLP vaccination). The data obtained showed that low or no amounts of the 6 cytokines were produced in post DNA-prime, pre-GagC VLP PBMC samples (Wk12), where the total amounts of cytokines per baboon were <25 pg/mL. The levels of Th1 cytokines increased substantially, especially for IL-2 (up to 143 pg/mL for baboon 663), after GagC VLP booster inoculation for the pTH-GagC-primed baboons. For the non-primed controls, only modest levels (<25 pg/mL) of IFN-γ and IL-6 were detectable following GagC VLP immunisation.

Although the CBA data were of low magnitude, the results showed that Th1 cytokines (IFN-γ, IL-2 and TNF-α) were predominantly produced after the GagC VLP booster immunisation. A recent study by Mooij *et al* (2004) reported the important role of multiple cytokines in vaccine-induced immunity. Rhesus macaques that developed strong IFN-γ, IL-2 and IL-4 responses after immunisation with a vaccine containing Tat-Env-Gag combination were protected from disease progression after $SHV_{89.6P}$ challenge. Also, they observed that each individual antigen induced a different cytokine profile. Immunisation with Env or Tat induced mainly a Th2 type of response with marked IL-4 and IL-2 responses but low or absent IFN-γ whereas immunisation with Gag induced a Th1 type profile dominated with IFN-γ and IL-2 responses and lower IL-4 production. The cytokine data reported in the present study, in which the vaccine contained the Gag protein only is consistent with these findings.

In the study reported here, immunisation of baboons with pTH-GagC and GagC VLP in the two experiments resulted in development of similar patterns of humoral responses. Three inoculations with pTH-Gag C induced weak HIV-1 Gag-specific IgG binding antibodies. Further pTH-GagC inoculations (done in Exp1 only) were able to boost these primary humoral responses but these responses declined very quickly after vaccinations. Following immunisations with a single GagC VLP vaccine, high anti-Gag antibody titres developed for the baboons which received pTH-GagC-prime, GagC VLP-boost immunisation but not for those receiving a single vaccine modality. For these baboons (single vaccine modality), a second GagC VLP inoculation was required to induce a substantial Gag-specific antibody response. Neutralising antibodies were not assayed in these experiments since the vaccines used for immunisation did not contain any HIV envelope immunogen. While neutralising antibodies, given by passive immunisation, have been shown to be effective in providing protective immunity (Baba *et al*, 2000; Eda *et al*, 2006; Ferrantelli *et al*, 2004; Mascola *et al*, 2000; Nishimura *et al*, 2003; Parren *et al*, 2001; Veazey *et al*, 2003), only indirect evidence suggests that binding antibodies to Env proteins are helpful, probably by protecting uninfected bystander CD4+ T cells from gp120-mediated apoptosis (Amara *et al*, 2002). However, the value of binding antibodies to the Gag protein in protection against SIV/SHIV or HIV-1 protection has not been described. In the present study, the detection of HIV-1 Gag-specific antibodies served to monitor the development of a humoral immunity.

2.4.3 COMPARISON WITH OTHER VACCINE STUDIES IN NHP

Several studies using nonhuman primates in HIV vaccine research have reported induction of cellular responses in heterologous prime boost vaccine modalities (**Table 1.4**). The majority of these studies, using the macaque/SIV model, have shown the importance of DNA vaccine in heterologous prime-boost vaccination strategies (Allen *et al*, 2000; Amara *et al*, 2001, 2002 & 2005b; Barnett *et al*, 1997;

Boyer *et al*, 2005 & 2006; Casimiro *et al*, 2003a, 2003b & 2005; Coupar *et al*, 2006; Dale *et al*, 2004a & 2004b; Hanke *et al*, 1999; Kent *et al*, 2005; Letvin *et al*, 1997 & 2004; Mascola *et al*, 2005; Pal *et al*, 2006a; Puaux *et al*, 2005; Santra *et al*, 2004; Shiver *et al*, 2002; Someya *et al*, 2006; Stratov *et al*, 2005; Suh *et al*, 2006). So far, only two studies in the baboon model, which utilised heterologous prime-boost vaccination regimen, have been reported (Casimiro *et al*, 2003b; Leung *et al*, 2004).

In the first study, Casimiro *et al* (2003b) investigated the cellular responses of baboons that were primed with plasmid DNA expressing HIV-1 subtype B *gag* gene and boosted with replication-deficient adenovirus serotype 5 (Ad5) expressing a matched HIV-1 gene. Although they demonstrated a high level of response using a high dose of Ad5 alone, they showed that adjuvant-formulated DNA combined with an Ad5 booster resulted in the induction of T-cell immune response that was better than DNA or Ad5 vaccine alone. However, levels of Gag-specific T cells observed in the cohort that was primed with DNA-alone (without adjuvant) and boosted with Ad5 was comparable to the levels obtained in the Ad5 only control group. In this DNA-alone/Ad5 prime/boost group, three DNA vaccine prime inoculations induced low (<138 SFU/10^6 PBMC) level of Gag-specific response, which increased to 200-1500 SFU/10^6 PBMC after a single booster immunisation with 10^8 viral particles of Ad5 vaccine and 0.02-0.38% CD8+ T lymphocytes were shown to produce IFN-γ after Gag-specific stimulation.

In the present study, Exp2 is comparable to that by Casimiro *et al* (2003b) in terms of DNA vaccine prime regimen. In both studies, each baboon received three immunisations with 5mg of plasmid DNA by intramuscular route, four weeks apart. In the present study, the peak Gag-specific response ranged from 217-515 SFU/10^6 PBMC for the 3 of 5 baboons that responded after 3 DNA vaccine prime inoculations (Exp2). After a single $Pr55^{gag}$ VLP booster vaccination, all the five baboons responded and the peak response ranged from 775-3583 SFU/10^6 PBMC (median: 967 SFU/10^6 PBMC). Although, no substantial proportions of CD8+ IFN-γ-producing T lymphocytes could be detected in Exp2 of the present study, 0.15-0.46% CD3+CD8+ T lymphocytes were shown to produce IFN-γ after Gag-specific stimulation for one baboon in Exp1 after $Pr55^{gag}$ VLP booster vaccination. These comparisons demonstrate that the cellular immune responses that were generated in the present study were substantially stronger than those observed by Casimiro *et al* (2003b), strongly suggesting that our vaccines, pTH-GagC and GagC VLP, when used in prime-boost combination, are as good as, or probably better than, the DNA/Ad5 combination.

In the second study (Leung *et al*, 2004), groups of baboons were immunised with DNA vaccines expressing various subtype B HIV-1 Env and Gag proteins and boosted with matched oligomeric gp140 Env proteins or $Pr55^{gag}$ VLP. The baboon group that was primed with both Env- and Gag-expressing DNA plasmids and boosted with $Pr55^{gag}$ VLP developed only modest anti-gp120 antibody

and low lymphoproliferative responses following multiple DNA immunisations, which were enhanced several-fold after booster immunisation with the $Pr55^{gag}$ VLP vaccine. Although direct comparisons between this study and the present study are not possible because the vaccine formats and the immunogenicity assays are different, the two studies show that a $Pr55^{gag}$ VLP vaccine is highly immunogenic in the baboon model. Similar to the study by Leung *et al* (2004), the present study, showed that a $Pr55^{gag}$ VLP vaccine expressing subtype C HIV-1 *gag* gene, given as a booster vaccine in combination with a matched DNA vaccine, was able to induce a robust immune response.

As previously illustrated (**Table 1.3a**), the majority of HIV vaccine studies utilise the rhesus macaque model. It is therefore important to know if the data generated using the baboon model is comparable or not to that obtained using the rhesus macaque. This would help in making the decision whether or not to proceed to clinical trials basing on the immunogenicity data that is generated using the baboon model alone, as is the case with the present study. Two reports by the Casimiro *et al* (2003a and 2003b) in which rhesus macaques and baboons were immunised with the same vaccines in two separate experiments provide an excellent opportunity to address this issue. As summarised in **Table 2.5**, it can be observed that the immune responses generated in baboons are comparable to those generated in rhesus macaque. This strongly suggests that the baboon model is equally as good as the rhesus macaque model, if not better, for measuring the immunogenicity of HIV-1 vaccines, giving confidence in the data generated in the present study.

Table 2.5: Comparison of immunogenicity data from baboon and macaque HIV-1 vaccine studies.

Species	Vaccination regimen	Range of peak IFN-γ responses after DNA prime			Range of peak IFN-γ responses after boost	
		Adjuvant?	SFU/10^6 PBMC	%CD8+	SFU/10^6 PBMC	%CD8+
Rhesus[a]	5mg DNA at weeks 0, 4, 8, i.m; Ad5-gag, 10^7 at week 24, i.m	No (n=6)	111 ± 32	ND	~ 600[#]	ND
		Yes (n=6)	322 ± 46	0.06-0.25	1000-1500	0.36-1.32
Baboon[b]	5mg DNA at weeks 0, 4, 8, i.m; Ad5-gag, 10^8 at week 24, i.m	No (n=3)	<138	ND	200-1500	ND
		Yes (n=3)	293-705	ND	1800-2500	0.56[d]
Exp1[c] (n=2)	5mg DNA at weeks 0, 4, 8, 12, 34, 56, i.m; 24μg Gag VLP at week 75	No	130-280	ND	1262-2254	0.15-0.46[d]
Exp2[c] (n=5)	5mg DNA at weeks 0, 4, 8, i.m; 29μg Gag VLP at weeks 51, 63	No	217-515	ND	775-3583	ND

[#]: data available as an estimate; [a]: Casimiro *et al*, 2003a; [b]: Casimiro *et al*, 2003b; [c]: Present study; [d]: data for one animal; ND: not determined or data not available.

2.4.4 CONCLUSION

This study contributed immensely in identifying the immunological reagents to evaluate HIV vaccine immunogens in the chacma baboon, leading to development of appropriate immunological assays. In addition, the data obtained in this study strongly support the use of the chacma baboon model for testing the immunogenicity of HIV-1 vaccines.

The immunogenicity data that was generated in the present study demonstrated that a $Pr55^{gag}$ VLP derived from a subtype C HIV-1 is effective in boosting and broadening the immune response that is induced using a matched DNA vaccine. This response was shown to be of high magnitude and to be directed to Gag epitopes located in all the three regions of Gag protein. Importantly, several of these epitope-containing peptides are also targeted by the immune system of humans with early sub-type C HIV-1 infection, indicating the relevance of these vaccines. Also, the immune response in the vaccinated baboons was found to be mediated predominantly by CD8+ T lymphocytes and Th-1 bias was detected. Finally, these responses were comparable to other vaccine studies in NHP, especially the DNA/Ad5 vaccine study in baboons (Casimiro *et al*, 2003b).

Based on the immunogenicity data that was generated in the present study, it is not difficult to predict that pTH-GagC and GagC VLP vaccines could be as good as or probably better than other vaccines that are currently in clinical trials. There are two considerations that provide the reason to believe that these two vaccines, when used in DNA prime-VLP boost combination, have the potential to proceed to clinical trials. Firstly, the immunogenicity data in the present study appear to be as good as those generated by DNA-*gag*/rAd5-*gag* in the baboon model (Casimiro *et al*, 2003b). This DNA/Ad5 vaccine concept has demonstrated CTL responses that are capable of protecting macaques from pathogenic challenge with $SHIV_{89.6P}$ (Shiver *et al*, 2002) and a series of phase 1 clinical trials are already ongoing (Shiver & Emini, 2004; Spearman, 2006). The second consideration is that ZDU422 *gag* has been included in candidate HIV-1 vaccines which are currently in clinical trials, such as VEE replicon and adeno-associated virus vaccines (IAVI, 2005). The same *gag* gene is contained in multigene DNA (code-named SAAVI DNA-C) and rMVA (code-named SAAVI MVA-C) vaccines both of which are expected to enter phase 1 clinical trials in 2007 in South Africa and USA (A-L Williamson, personal communication).

The above considerations provide support for movement of both pTH-GagC and GagC VLP vaccine to clinical evaluation. Although this study promotes the evaluation of pTH-Gag in clinical trials, our group has already constructed a multigene DNA vaccine (SAAVI DNA-C), which has shown to be effective in the mouse (Burgers *et al*, 2006) and baboon (Burgers *et al*, manuscript in preparation; Williamson *et al*, 2005) models. Thus, it is realistic to move SAAVI DNA-C for clinical evaluation in favour of pTH-GagC. For the movement of GagC VLP to clinical evaluation, two options are feasible.

On one hand, the use of GagC VLP as a monovalent vaccine in combination with pTH-GagC as used in the present study or other vaccine formats may be considered. On the other hand, GagC VLP could be re-formatted to make a multivalent vaccine by incorporating other HIV-1 genes such as *env*, *tat*, *nef* and *pol* to match the immunogens in SAAVI DNA-C. Then the resultant chimeric $Pr55^{gag}$ VLP could be used as a boost for SAAVI DNA-C in a prime-boost protocol. This could have the advantage of maximising on intra- and cross-clade breadth of the vaccine-induced immunity. The feasibility of producing chimeric HIV-1 $Pr55^{gag}$ VLP has been demonstrated (Deml *et al*, 1997a; Griffiths *et al*, 1993; Luo *et al*, 1992; Rovinski *et al*, 1992; Vzorov *et al*, 1991; Wagner *et al*, 1994) but the cost of VLP vaccine production is likely to be an added consideration. However, the present study has shown that as little as 24 μg of GagC VLP was sufficient to induce a strong and long-lived immune response in baboons. The ability to boost a response with such a low dose of VLP could have a major impact on reducing the cost of HIV-1 $Pr55^{gag}$ VLP-based vaccine production.

CHAPTER THREE

ASSESSMENT OF IMMUNE RESPONSES FOLLOWING VACCINATION OF BABOONS WITH WILD-TYPE BACILLE CALMETTE GUERIN (BCG)

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3.1 INTRODUCTION

Mycobacterium bovis Bacille Calmette-Guerin (BCG) is used increasingly as an efficient live bacterial vector for delivery of vaccine immunogens, leading to development of several recombinant BCG (rBCG). This has given rise to the need for development of appropriate animal models in which to evaluate these candidate vaccines. The chacma baboon, an abundant baboon subspecies in southern Africa may be useful for testing the immunogenicity of such rBCG but this could be hindered by lack of knowledge of the immune response of this species to BCG. This chapter describes a study in which the components of the immune response of the chacma baboon to BCG were investigated. Both humoral and cellular BCG-specific immune responses were examined following vaccination of baboons with either *Tokyo* or *Pasteur* strains of BCG.

3.1.1 BCG VACCINE AND BABOON MODEL

As reviewed in section **1.4.2**, there are several advantages of using BCG as a live vector for vaccine delivery. These include (i) induction of a strong and long-lived immunity, (ii) strong adjuvant properties, (iii) a long safety profile and (iv) low cost of production. These features, among others, make BCG an attractive vaccine vector, particularly for HIV vaccines where the cost of production is a major consideration as majority of HIV-infected individuals are found in developing countries.

Chacma baboons are readily available in South Africa and could provide a cheap animal model for testing the immunogenicity of rBCG vaccines. The immune system of the baboon has close homology with the human immune system (reviewed in sections **1.2.2** and **1.4.6**) and thus can be a realistic animal model. Also, this baboon species has been reported to be infected with *M. tuberculosis* and *M. bovis* (Fourie & Odendall 1983; Keet *et al*, 2000; Thorel *et al*, 1998) but there are no published attempts to study immune responses to BCG and other mycobacterial infections.

3.1.2 STUDY OBJECTIVE

The objective of the present study was to document BCG-specific immune responses of chacma baboons inoculated with wild-type BCG in readiness to undertaking immunogenicity testing of rBCG vaccines expressing HIV-1 *gag* using the baboon as the animal model. The specific aim of this study therefore was to acquire knowledge of the immune responses of baboons to BCG. This study investigated the components of the immune response of chacma baboons in respect to BCG-specific cellular and humoral responses following vaccination with *Tokyo* and *Pasteur* strains of BCG. The *Tokyo* strain was chosen because the rBCG vaccines selected for further evaluation in a baboon animal model (Chapter 4) were constructed using this strain of BCG (Thomas, 2005) while the *Pasteur* strain was chosen as it is a common laboratory strain of BCG.

3.2 MATERIALS AND METHODS

3.2.1 BABOONS

Ten wild-caught young male or female chacma baboons (*Papio ursinus*) were used. The baboons were housed at the Medical Research Council's animal facility in Cape Town, South Africa for this study. These animals were free of parasites, negative for SIV antibodies and tuberculin skin test negative. Ketamine was used to sedate animals in all handlings. The experiments were reviewed and approved by the Animal Research Ethics Committee (Reference No. 00/03N) of the University of Cape Town.

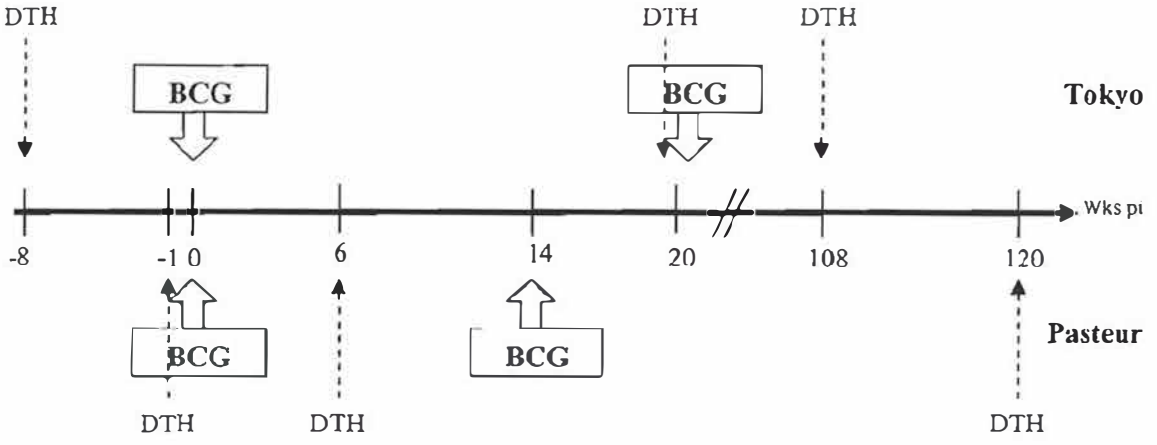
3.2.2 INOCULUM PREPARATION

BCG-*Tokyo* and BCG-*Pasteur* inocula were prepared from frozen stocks (gifts from Dr William Bourn and Prof Bernhard Ryffel of UCT's Vaccine Research Group and Division of Immunology respectively). A standard Sauton's medium was used to grow the bacteria for preparation of inocula stocks while Middlebrook 7H10 medium supplemented with oleic acid, albumen and dextrose complex (OADC) was used to grow the bacteria for viability counts. The inocula stocks were stored at -80 °C until use.

3.2.3 BABOON IMMUNISATIONS

Eight baboons were randomly selected for experimental inoculation with BCG- *Tokyo* (n=6) and *Pasteur* (n=2) as shown in **Fig 3.1**. Of the 6 baboons selected to receive BCG-*Tokyo*, baboon 388 and 428 were inoculated with 100μL saline containing 10^4 CFU (low dose), baboon 363 and 466 with 100μL saline containing 10^6 CFU (medium dose) and baboon 378 and 454 with 100μL saline containing 10^8 CFU (high dose). Each baboon was inoculated intradermally on a single site on the left chest after shaving the hairs. They were boosted at a uniform dosage of 10^6 CFU BCG-*Tokyo* per animal 20 weeks later. The BCG inocula were defrosted shortly before vaccination and the bacteria dispersed by passing the inocula through a 25G needle 10 times just prior to inoculation. The group selected to receive the BCG-*Pasteur*, comprising of 2 baboons (baboon 350 and 447), were each inoculated with 10^8 CFU BCG-*Pasteur* contained in 100 μL saline and boosted in the same manner 14 weeks later. All the groups were monitored for the development of local or systemic reactions by daily physical observations and regular complete physical examinations during blood collection.

Fig 3.1: Inoculation and DTH timelines. Baboons were inoculated with BCG-*Tokyo* or BCG-*Pasteur* at week 0 and boosted at week 14 (**Pasteur**) or 20 (**Tokyo**). DTH were performed at 1 (**Pasteur**) or 8 (**Tokyo**) weeks pre-immunisation and repeated twice at 6 and 120 (Pasteur) or 20 and 108 (Tokyo) weeks post initial inoculation by PPD skin test.



3.2.4 DELAYED TYPE HYPERSENSITIVITY TEST

At indicated time points (**Fig 3.1**), baboons were shaved on the chest and injected intradermally with 100 µL (1000 IU in saline) of bovine tuberculin Purified Protein Derivative (PPD, Institute of Animal Science and Health, Lelystad, The Netherlands) on the right chest. To control for non-specific indurations, a different site on the chest was injected with 100 µL of saline. At 48 hours, the diameter of the indurations was measured at the injection sites. No induration was observed on the saline injection site and thus any measurable induration on the PPD injection site was considered positive.

3.2.5 BLOOD COLLECTION

Blood was collected from each animal at the time of BCG inoculation and several times points post inoculation. From each baboon, 20mL of blood in heparin tubes and 3.5mL of blood in plain tubes were collected for PBMC isolation and serum separation respectively. The processing of blood was performed within 3h of blood collection as outlined in the **Appendix B.1**. Freshly isolated PBMC were used for all the assays except for intracellular cytokine assay, in which cryo-preserved cells were used.

3.2.6 LABORATORY ASSAYS

3.2.6.1 CELLULAR PROLIFERATION ASSAY

Freshly isolated PBMC were resuspended at $1x10^6$/mL in R10 (RPMI-1640 medium with Glutamax-1 [Gibco] containing 10% fetal bovine serum, [FBS, Delta Bioproducts] and 100

units/mL and 100 μg/mL Pencillin-G and Streptomycin respectively [Penicillin-Streptomycin, Gibco]). Triplicate reactions in 96-well round bottom wells (Nunc) were set up containing 100 μL PBMC ($1x10^5$) and 100 μL R10 with (test reactions) or without (background control reactions) $1x10^5$ live BCG or 1 μg PPD. Cultures were incubated at 37 °C in a humified atmosphere containing 5% CO_2 for 6 days. The cells were pulsed with 1 μCi of [methyl-^{3}H] thymidine (Amersham) per well for the last 18h of incubation and then harvested on fibre glass filters (Whatman International Ltd). Incorporation of the radio nucleotide into DNA of cells was measured by liquid scintillation counting. Proliferation was expressed as the proliferation index, which was calculated as the mean counts in triplicate test wells divided by the mean background counts in triplicate control wells.

3.2.6.2 PBMC STIMULATION AND IFN-γ ASSAY BY ELISA

Freshly isolated PBMC at were used for this assay. Triplicate reactions in 96-well round bottom wells (Nunc) were set up containing 100 μL PBMC ($1x10^5$) and 100 μL R10 with or without $1x10^5$ live BCG-*Tokyo*. Cultures were incubated at 37 °C in a humified atmosphere containing 5% CO_2 for 6 days. The culture supernatants were collected and the BCG-induced IFN-γ quantified using commercially available ELISA kits (Mabtech, Sweden) following the manufacturer's instructions. Recombinant human IFN-γ (BD-PharMingen) was used to generate a standard IFN-γ curve for interpolation of IFN-γ concentrations. The samples and standards were set up in triplicate and the results were reported as mean of the triplicate readings.

3.2.6.3 IFN-γ ELISPOT ASSAY

Freshly isolated PBMC obtained at week 136 post initial BCG immunisation were used. Triplicate reactions were set up with or without PPD (5 μg/well) and the IFN-γ ELISPOT assay was performed as previously described in section **2.2.5.3**. The average of triplicate counts was calculated and normalised to 10^6 PBMC to give IFN-γ SFU/10^6 PBMC. The results were reported as net IFN-γ SFU/10^6 PBMC after subtracting the background SFU/10^6 PBMC obtained in the absence of PPD stimulation. A response was considered positive if the net IFN-γ SFU/10^6 PBMC was equal or greater than the cut-off value, which was defined as the mean background response (in the absence of PPD) plus 3 standard deviations.

3.2.6.4 INTRACELLULAR IFN-γ STAINING ASSAY

Cryo-preserved PBMC obtained at week 136 post initial BCG inoculation were used. PBMC were retrieved from the liquid nitrogen freezer and revived as described in **Appendix B.3**. The cells were resuspended in R20 (RPMI medium containing 20% FBS) at 2-4x10^6 PBMC/mL and rested

overnight at 37 °C in a humified incubator containing 5% CO_2. After washing once with R10, the PBMC (at $1x10^6$/mL) were then incubated for 16h with either R10 medium only, bovine PPD (5 μg/mL) or staphylococcal enterotoxin B (1 μg/mL, SEB, Sigma). Brefeldin A (10 μg/mL; Sigma) was added after 2h. The staining of cells and FACS analysis were performed as previously described (section **2.2.5.5**). Data was presented as percentage of CD4+ or CD8+ lymphocytes, which are IFN-γ positive.

3.2.6.5 IMMUNO-PHENOTYPING OF PBMC AND DETECTION OF HLA-DR ACTIVATION MARKER

Freshly isolated PBMC were used for immuno-phenotyping or HLA-DR detection (week 20 only). PBMC were stained with the monoclonal antibodies: anti-monkey CD3-FITC (Clone FN-18, BioSource), anti-human CD4-RD1 (Coulter clone T4, Coulter), anti-human CD8-APC (Clone RPA-T8, BD-PharMingen), anti-human CD56-PE (Clone B159, BD-PharMingen), and anti-human CD20-RD1 (Coulter Clone B1, Coulter). For detection of the activation marker HLA-DR, freshly isolated PBMC and PMBC that had been cultured for 4 days with 1×10^6 cfu live BCG-*Tokyo*, were stained with mouse anti-monkey CD3-FITC (Clone FN-18, BioSource) and anti-human HLA-DR-Cy-Chrome (Clone G46-6, BD-PharMingen). Isotype matched control antibodies were also used. After staining, cells were fixed with 1% paraformaldehyde and data was acquired using FACSCalibur™ cytometer (BD). The lymphocyte gate was set by forward and side scatter and data was analyzed using CellQuest™ software (BD). HLA-DR positive cells were expressed as a percentage of the CD3+ cells.

3.2.6.6 ANTIBODY ASSAY BY ELISA

Levels of serum BCG-specific IgG at several time-points post inoculation were measured by ELISA using a lysate of BCG for the antibody capture. A lysate of BCG was prepared using a modification of the FastPrep (Q-BIOgene, Carlsbad, CA) method to extract BCG proteins and the protein concentration was quantified using the BIORAD protein kit (Biorad). Maxisorb microtitre plates (Nunc) were coated overnight at 4°C with the BCG protein extract (1 μg/mL) in carbonate-bicarbonate (Sigma) coating buffer (pH 9.6) then blocked with 2% nonfat milk powder in PBS for 3 h at 37°C. Serum samples were diluted 1:1600, loaded in duplicate wells and incubated overnight at 4°C. The detection of serum immunoglobulin G was done as previously described (section **2.2.5.1**). The mean of duplicate readings was calculated and results were represented as optical density (OD) units.

3.2.7 STATISTICAL ANALYSIS

Student's t-test and Wilcoxon matched pairs (Statistica™, StatSoft) were used for statistical analysis where appropriate.

3.3 RESULTS

3.3.1 LOCAL CUTANEOUS REACTIONS AT INJECTION SITE

The baboons were monitored for development of local or systemic reactions by daily physical observation during feeding times and regular complete physical examination during blood collection. All BCG-vaccinated baboons (except baboon 428, receiving the 10^4 CFU BCG-*Tokyo*) developed inflammation at the inoculation sites. The ulcerations developed within 2 weeks with doses of 10^6 and 10^8 CFU with the ulcerations occurring with a dose of 10^8 CFU being notably larger (10-20 mm) than that with a dose of 10^6 CFU. All ulcerations healed within 6 to 8 weeks. One baboon (378) developed lymphadenitis of one axillary lymph node at week 4 post inoculation. This resolved without medication. After a booster inoculation (given at weeks 14 [Pasteur group] and 20 [Tokyo group]), inflammation at the inoculation sites was notably less than that observed after the first inoculation. Ulcerations of 10 mm or lower occurred only in the baboons that received an initial dose of 10^8 CFU. These ulcerations resolved within 6 weeks after the booster injection. Except for these local tissue reactions at the injection sites, all the baboons maintained normal health parameters throughout the study period.

3.3.2 DELAYED TYPE HYPERSENSITIVITY POST VACCINATION

All the baboons in the study were PPD skin test negative pre-vaccination, indicating that they had no active mycobacterial infection or recent exposure to mycobacteria. Baboons inoculated with 10^4 CFU BCG-*Tokyo* did not respond to a skin test at 20 weeks post vaccination and one of them (baboon 428) was still non-responsive at 2 years, even though this baboon was given a booster injection after the skin test done at 20 weeks (**Table 3.2**). Doses of 10^6 and 10^8 CFU BCG-*Tokyo* induced a positive skin test at 20 weeks post the initial inoculation and a repeat skin test at 2 years after the start of the study was again positive. The baboons that received a dose of 10^8 CFU BCG-*Pasteur* were skin test positive at 6 weeks post the initial inoculation and gave a repeated positive skin test 120 weeks after the start of the study (**Table 3.2**).

Table 3.2: Delayed type hypersensitivity (DTH) reactions. Baboons were injected intradermally with PPD (1000 units in 100 µL saline) on the chest. The diameter (in millimitres) of the indurations at the PPD injection site was measured at 48h later.

Baboon #	Strain; dose (CFU)	Diameter (mm) of indurations at various time points post BCG inoculation				
		Pre-	6 weeks	20 weeks	108 weeks	120 weeks
388	Tokyo; 10^4	0	ND	0	16	ND
428	Tokyo; 10^4	0	ND	0	0	ND
363	Tokyo; 10^6	0	ND	11	5	ND
466	Tokyo; 10^6	0	ND	6	4	ND
378	Tokyo; 10^8	0	ND	6	5	ND
454	Tokyo; 10^8	0	ND	11	7	ND
350	Pasteur; 10^8	0	16	ND	ND	5
447	Pasteur; 10^8	0	15	ND	ND	4

ND: not determined; Pre-: pre-inoculation (1 and 8 weeks for *Pasteur* and *Tokyo* groups respectively)

3.3.3 PROLIFERATIVE LYMPOCYTE RESPONSES POST VACCINATION

Proliferative responses were measured at various times post vaccination to monitor development of lymphocyte recall responses to BCG or PPD. Prior to BCG inoculation, the proliferative index of PBMC (from all baboons in the study) to BCG was less than 5 (week 0, **Fig 3.2A** and **B**). After vaccination with either the BCG-*Tokyo* or BCG-*Pasteur*, 5 of 6 baboons inoculated with the BCG-*Tokyo* (**Fig 3.2B**) and 2 of 2 baboons inoculated with the BCG-*Pasteur* (**Fig 3.2A**) showed an overall significant ($p<0.05$) increase in proliferation index of PBMC to live BCG compared to the pre-inoculation level, irrespective of the BCG dose given at immunisation. In addition these PBMC were responsive to PPD (**Fig 3.2**). The proliferation index at various times post the initial inoculation for these baboons was significantly higher ($p<0.05$) than the pre-inoculation proliferation index but there was no significant difference in the indices between the groups. After the initial inoculation the proliferation index for all baboons reached a maximum at week 8 post inoculation then declined but this could be restored with a booster injection (**Fig 3.2A** and **B**). PBMC from all the baboons in the study had positive proliferative response to Concanavalin A (data not shown).

It was noted that PBMC from one baboon (baboon 454, 10^8 CFU BCG-*Tokyo*) had a high background proliferation up to 10,000 counts per minute (in the absence of antigen stimulation). The net proliferation above the background for this baboon and the other baboon (378), which received an equal BCG-*Tokyo* dose, was calculated instead of the proliferation index. This showed that the two baboons had similar magnitude of net proliferation to BCG and PPD (Appendix C.7, **Fig A.8**), indicating that PBMC from baboon 454 (with high background) also had specific proliferation to BCG and PPD.

The ability of PBMC from baboons post inoculation to proliferate to PPD was investigated. At 4 weeks post inoculation PBMC from only one baboon (baboon 378, inoculated with a dose of 10^8 CFU BCG-*Tokyo*) had a proliferation index to PPD significantly ($p<0.05$) above the pre-inoculation proliferation index (**Fig 3.2B**). After a booster injection at week 20, PBMC taken at week 26 from 3 of the 5 baboons that had been inoculated with BCG-*Tokyo*, proliferated to PPD with a proliferation index that was significantly higher ($p<0.05$) than the pre-inoculation proliferation index (**Fig 3.2B**). No proliferation could be detected at 4 weeks after inoculation with BCG-*Pasteur*. After a booster injection at week 14, PBMC taken at week 20 from both baboons inoculated with BCG-*Pasteur* had a proliferation index that was significantly above ($p<0.05$) the pre-inoculation proliferation index.

3.3.4 LYMPHOCYTE SUBSETS AND EXPRESSION OF HLA-DR ORTHOLOGUE

To monitor any changes in the distribution of lymphocyte sub-populations after BCG vaccination, fresh PBMC from vaccinated baboons were stained with anti-CD3, anti-CD4, anti-CD8, anti-CD20 and anti-CD56 monoclonal antibodies and analyzed the relative frequencies by flow cytometry. This revealed that vaccination with various doses of BCG Tokyo strain did not alter the distribution of lymphocyte subsets (Appendix C.2, **Table A.9**).

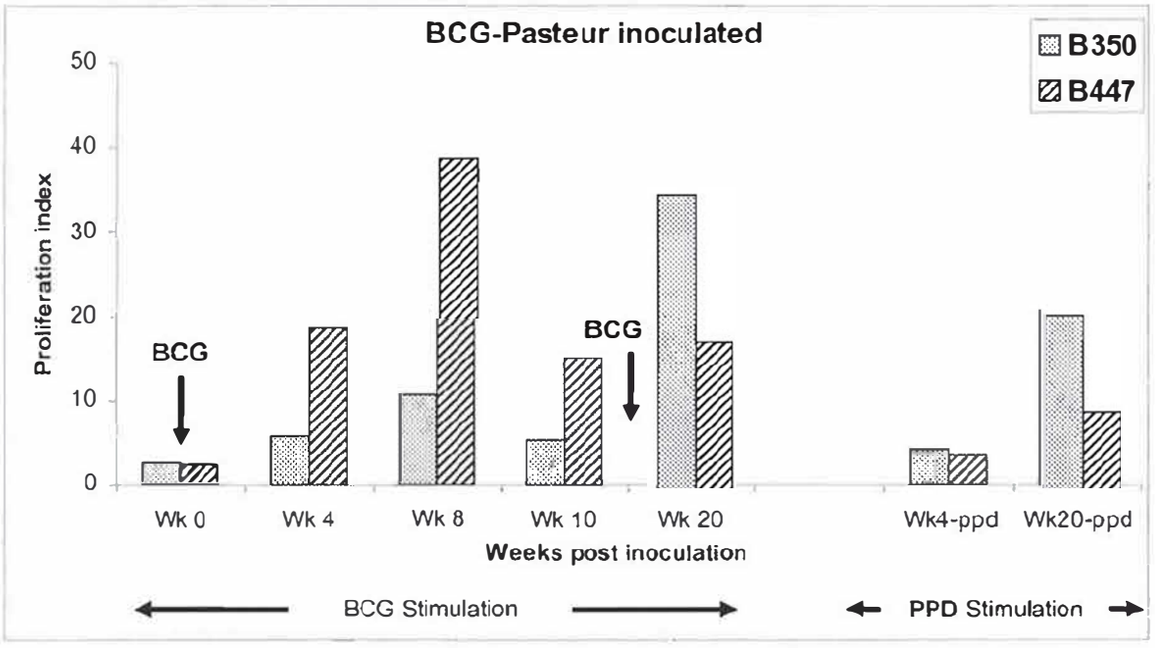
Activation marker, HLA-DR, is a class 2 major histocompatibility complex molecule, which is primarily expressed by antigen presenting cells (B cells, monocytes, macrophages) or by antigen-primed T cells following activation. At week 20 post BCG *Tokyo*–inoculation, the expression of HLA-DR orthologue (corresponding molecule in the baboon PBMC) by PBMC from immunised baboons was evaluated. Prior to *in vitro* stimulation with BCG, less than 5% of the CD3+ T cells expressed HLA-DR (**Fig 3.3**). Stimulation of PBMC from baboons post BCG inoculation with live BCG for 4 days resulted in a 2- to 8-fold increase in the percentage of CD3+ cells expressing the HLA-DR orthologue (**Fig 3.3**). The increase for baboons given 10^8 CFU BCG-*Tokyo* was significant ($p<0.05$). An increase of 1.5- to 2.5-fold in mean fluorescence intensity was also observed (data not shown).

Fig 3.2. Proliferation of baboon PBMC to live BCG and PPD at various times after intradermal inoculation of BCG.

A: **inoculation with 10^8 CFU BCG- *Pasteur*.**
B: **inoculation with 10^4 (i), 10^6 (ii), or 10^8 (iii) CFU BCG-*Tokyo*.**

The time of inoculation at week 0 and week 14 with BCG-*Pasteur* (A), and at week 0 and week 20 with the BCG-*Tokyo* (B) sub-strains is indicated by ↓. PBMC were isolated at these indicated times and incubated in test wells with live BCG (10^5 CFU) or PPD (5 μg/mL) or culture medium (background control reactions) for 6 days and proliferation measured as the incorporation of ^{3}H-thymidine. The proliferation index was calculated as the mean incorporation of ^{3}H-thymidine by PBMC in triplicate test wells in the presence of BCG or PPD divided by the mean background ^{3}H-thymidine incorporated by PBMC in triplicate control wells. The triplicates did not differ by more than 10% and incorporation of ^{3}H-thymidine by PBMC in the absence of BCG or PPD ranged from 400 to 9500 cpm. Proliferation was considered to be positive if the proliferation index was >5. The proliferation index in response to live BCG (A and B) at all times post the initial inoculation and the booster injection at week 20 was significantly greater ($p<0.05$) than the pre-inoculation proliferation index except for baboon 454. Proliferation to PPD was only significantly higher ($p<0.05$) at week 4 post inoculation for baboon 378 (B). By week 20 (A) and week 26 (B) post inoculation PBMC from all baboons except 428, 466 and 454 (B) proliferated significantly ($p<0.05$) to PPD.

A: inoculation with 10^8 CFU BCG- *Pasteur*.



B: inoculation with 10^4 (i), 10^6 (ii), or 10^8 (iii) CFU BCG-*Tokyo*.

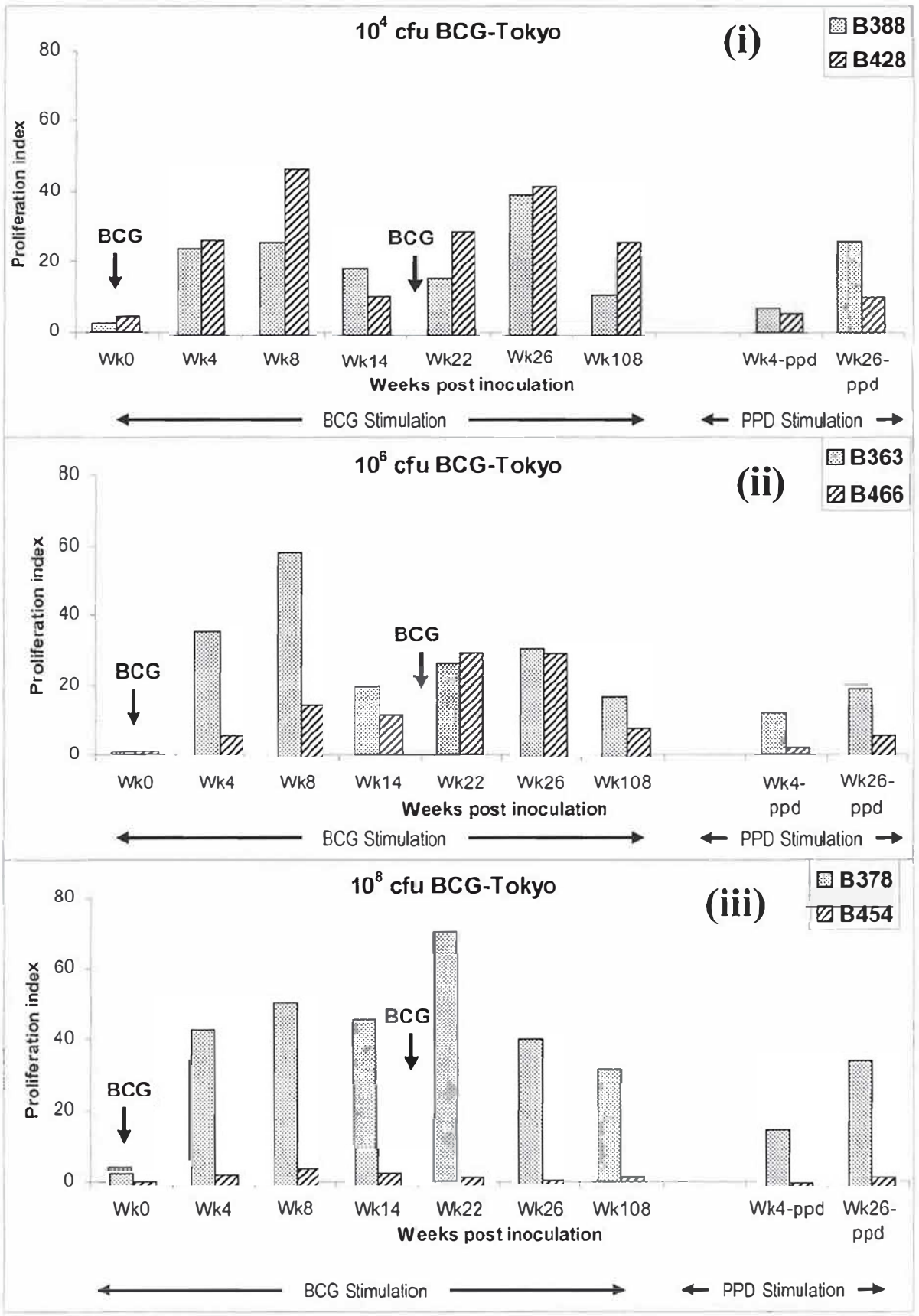
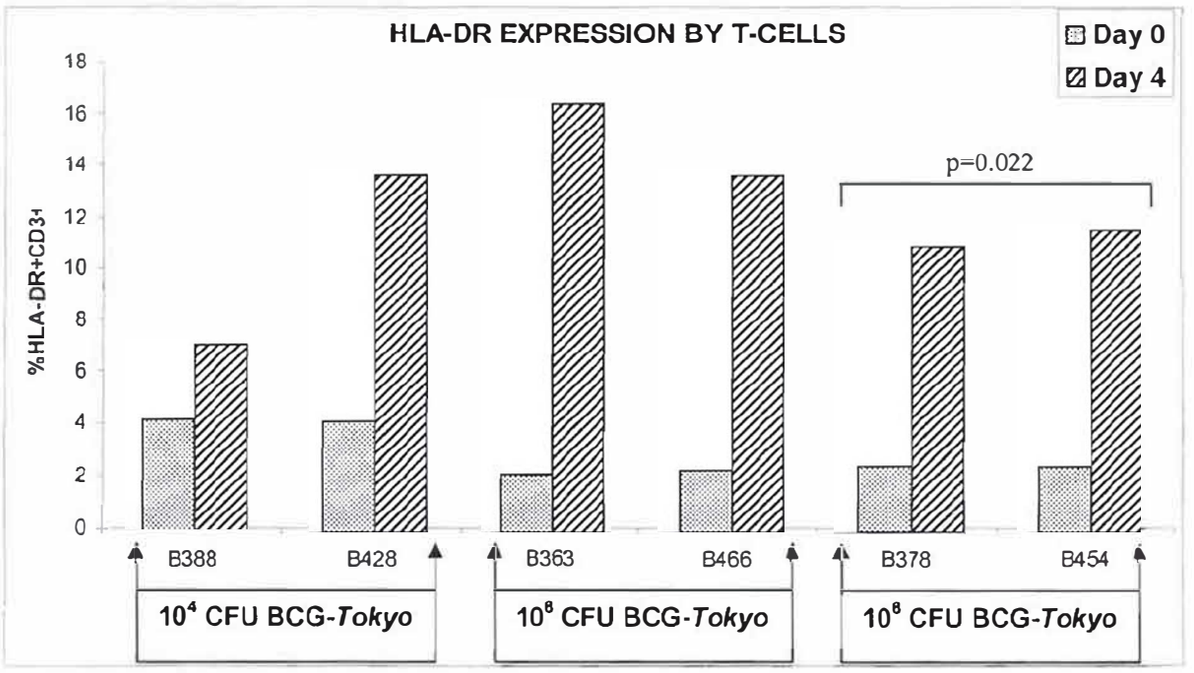


Fig 3.3: Up-regulation of a late activation marker orthologous to HLA-DR. Freshly isolated PBMC at 20 weeks post inoculation were stained prior to culture or after culture for 4 days (with 10^5 CFU live BCG) with anti-monkey CD3-FITC (BioSource) and anti-human HLA-DR-Cy-Chrome (BD-PharMingen), then analysed by flow cytometry. Data points are the percentage of CD3+ cells expressing HLA-DR orthologue. A p value is given where the difference in mean increase in the percentage of CD3+ cells expressing the HLA-DR orthologue between day 0 and day 4 cultures is significant.



3.3.5 IFN-γ PRODUCTION BY PBMC

Production of IFN-γ by PBMC during proliferation to live BCG was measured for baboons inoculated with BCG-*Tokyo*. As shown in **Fig 3.5**, BCG-specific IFN-γ production appeared to be dependent on the inoculation dose and time post inoculation. At week 8, the mean IFN-γ production for baboons vaccinated with BCG dose of 10^4, 10^6 and 10^8 CFU was 228 ±62, 550 ±70, and 1550 ±424 pg/mL. The overall mean level of IFN-γ production by PBMC at weeks 4, 6, 8, 10 and 12 for baboons inoculated with the different doses was significantly different ($p<0.01$; dose of 10^8 CFU > dose of 10^6 CFU > dose of 10^4 CFU). By week 12, production of IFN-γ by PBMC had declined for all baboons (**Fig 3.5**).

All baboons were boosted with a dose of 10^6 CFU at week 20 and the *in vitro* BCG-specific IFN-γ production by PBMC was measured at weeks 52 and 108 (**Fig 3.5**) i.e. 32 and 88 weeks after the boost. For those baboons that received an initial dose of 10^4 CFU, the mean IFN-γ production increased significantly ($p<0.05$) by 2.2-fold at week 52 and 6-fold at week 108 above that produced at week 8 after the initial inoculation. With a dose of 10^6 CFU the mean BCG-specific IFN-γ

production at weeks 52 and 108 after the boost was 1.2-fold and 1.5-fold above that produced at week 6 after the initial inoculation. No increase in mean IFN-γ production was measured when a booster dose of 10^6 CFU was given at week 20 to those baboons that received an initial dose of 10^8 CFU (**Fig 3.5**).

The frequency of PBMC producing IFN-γ was enumerated at week 136 post initial vaccination by use of an ELISPOT assay with PPD as a stimulant. The results are shown in **Table 3.3**. All the baboons displayed PPD-specific IFN-γ producing cells upon re-stimulation, but the frequency of cells producing IFN-γ varied and did not seem to be dependent on the dose of BCG used for initial vaccination (**Table 3.3**). An analysis of the subsets of cells producing IFN-γ was investigated for one baboon (baboon 388) utilising an intracellular IFN-γ staining technique using PBMC obtained at week 136 post vaccination. The data revealed that 3.19% of the CD4+ T lymphocytes expressed IFN-γ upon antigenic stimulation with PPD (**Fig 3.6**). No response was seen for CD8+ T lymphocytes.

Table 3.3: Frequency of PPD-specific T cells at week 136 post first BCG inoculation determined by an IFN-γ ELISPOT assay. The number of spots was analysed and counted automatically (CTL analyzer) and adjusted to SFU/10^6 PBMC. The results are expressed as mean of triplicate wells and given as net SFU/10^6 PBMC after subtracting the background (PBMC and medium only) SFU/10^6 PBMC. PBMC stimulated with PHA (4 μg/mL) were used as positive control (data not shown) and gave responses >1000 sfu/10^6. (Background stimulation: mean = 85 SFU/10^6 PBMC; standard deviation = 122; median = 40).

	Net IFN-γ spot forming units (SFU) per 10^6 PBMC					
Dose/baboon	Low Dose (10^4 CFU)		Medium Dose (10^6 CFU)		High Dose (10^8 CFU)	
Baboon number	B388	B428	B363	B466	B378	B454
PPD response	1310	367	230	154	276	2003

Fig 3.5: BCG-specific IFN-γ production by baboon PBMC at various time-points after intradermal inoculation with BCG (Tokyo strain) at week 0 and 20. PBMC isolated from baboons vaccinated with 10^4 (A), 10^6 (B), or 10^8 (C) CFU BCG-*Tokyo*, were stimulated *in vitro* for 6 days with R10 medium only or R10 medium containing 10^5 CFU live BCG-*Tokyo* per 10^5 PBMC in 200 µL/well (triplicate wells). Culture supernatants were harvested for IFN-γ determination by ELISA. Recombinant human IFN-γ (BD-PharMingen) was used to generate the standard curve for interpolation of IFN-γ concentrations. Results are the mean of triplicate values that did not differ by more than 10%. BCG-specific IFN-γ production was determined by subtracting background response (determinations for supernatants from wells with R10 medium and PBMC only). The time of BCG booster immunisations at week 20 post inoculation is indicated by↓. A p value is given where the difference in mean IFN-γ production between peak responses before and after boosting is significant.

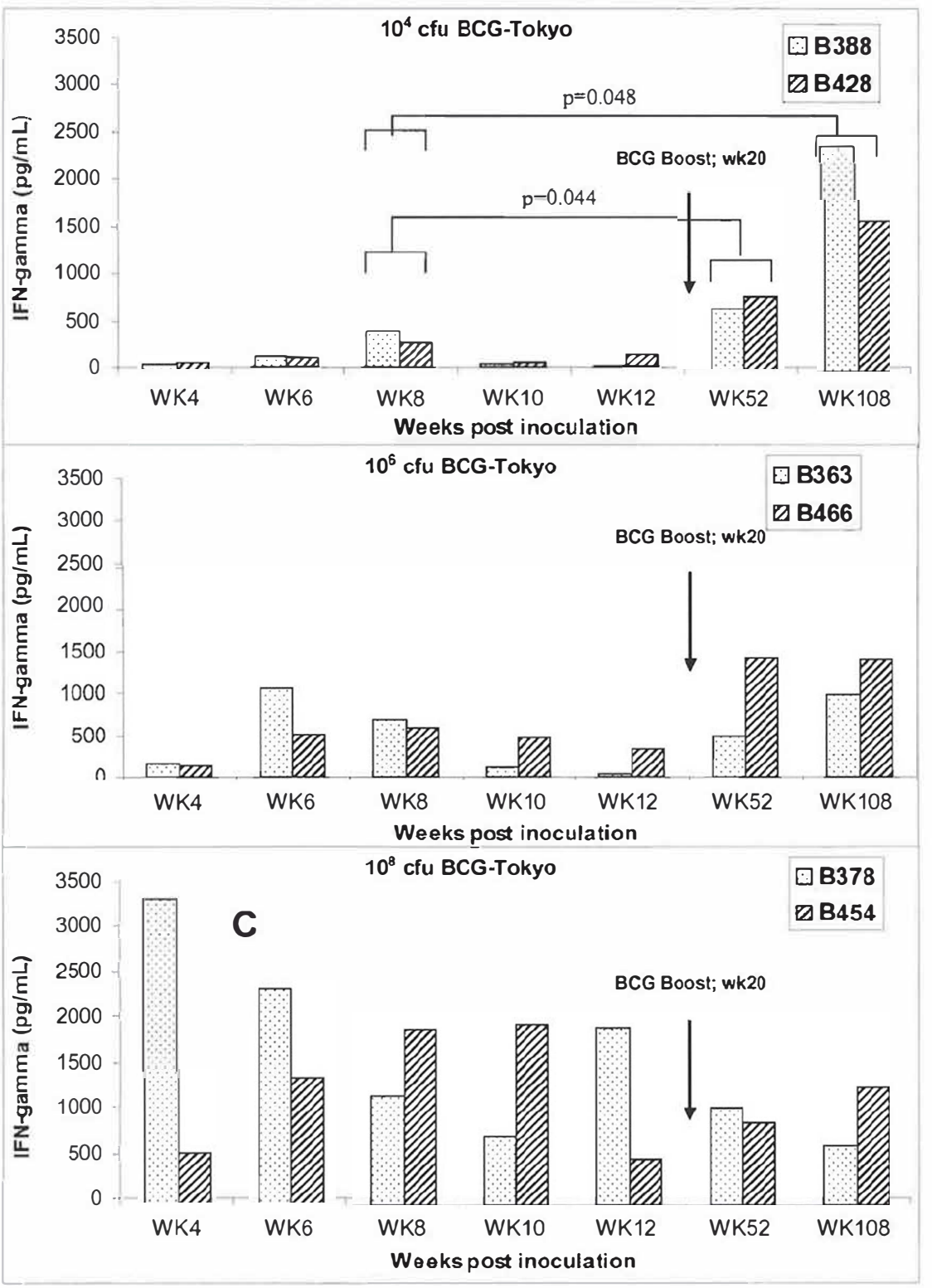
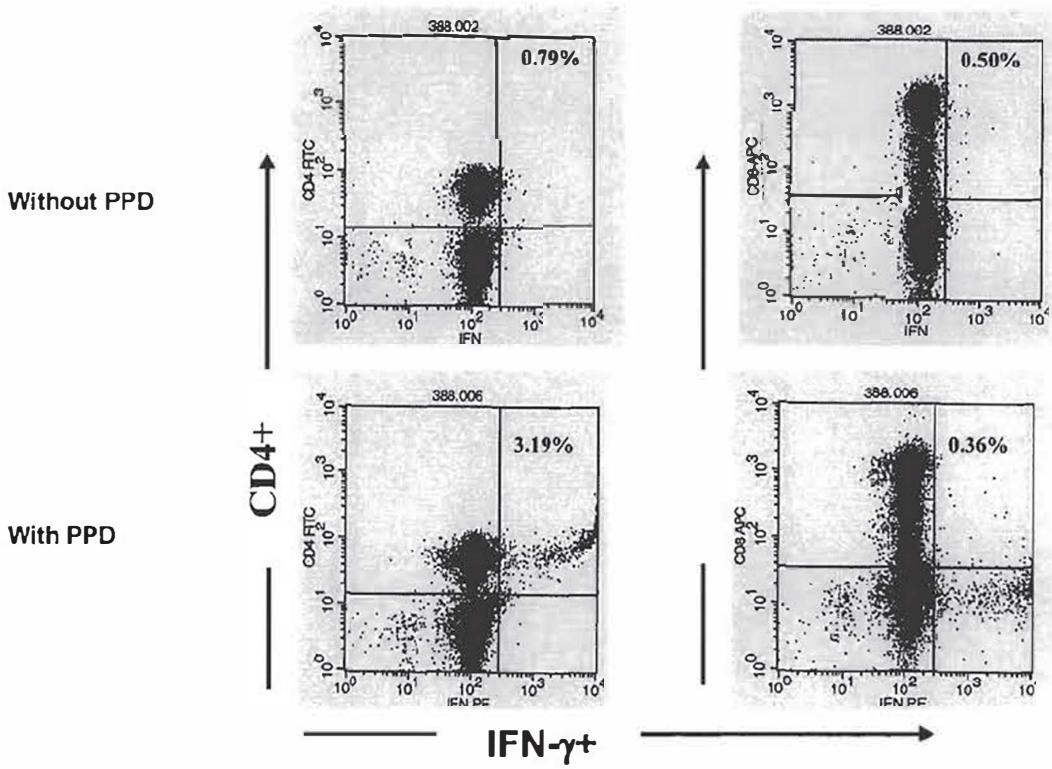


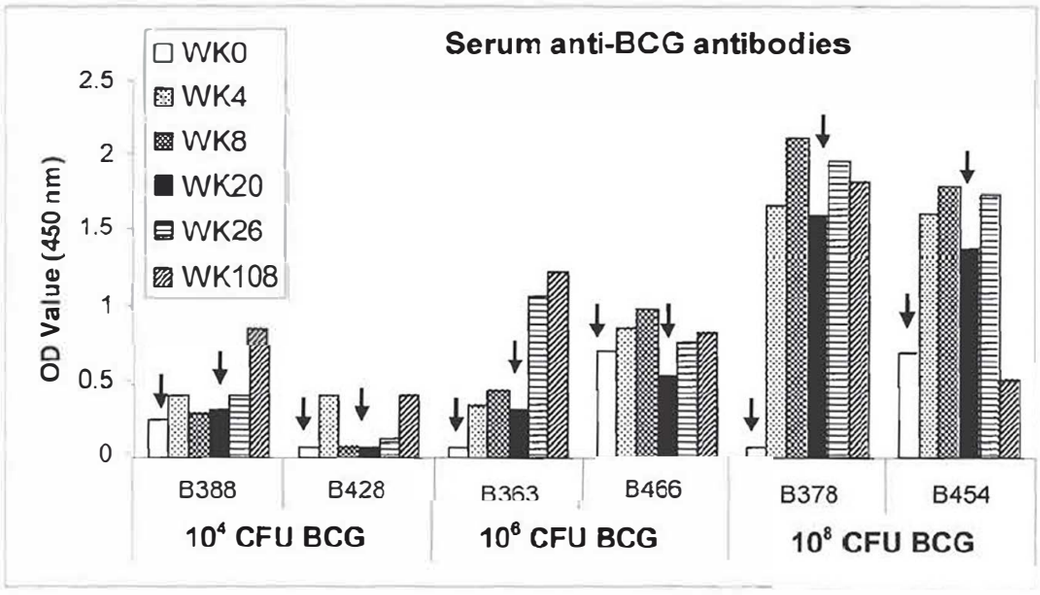
Figure 3.6: Intracellular IFN-γ staining assay. Cryo-preserved PBMC from baboon 388 at week 136 were incubated with either R10 medium only or R10 medium containing bovine PPD (5 μg/mL) for 16h, with addition of BFA after the first 2h. The cells were stained with anti-CD4-FITC (BD-PharMingen) and anti-CD8-APC (BD-PharMingen) human monoclonal antibodies. Cells were permeabilised then stained with human anti-IFN-γ-PE (BD-PharMingen) antibody. Cells were acquired using a BD FACSCalibur flow cytometer and analysis was done using CellQuest software. The figures in the upper right box are the percentage of either CD4+ or CD8+ cells producing IFN-γ.



3.3.6 ANTIBODIES TO BCG

The production of antibodies to BCG was investigated in the serum of baboons inoculated with various doses of BCG-*Tokyo*. The results are displayed in **Fig 3.7**. Three of the six baboons produced antibodies that were significantly higher than the pre-inoculation antibody level. These were baboon 363 (10^6 CFU dose), 378 and 454 (10^8 CFU dose for both). Two of these (baboon 363 and 378) maintained high antibody levels till week 108 post initial inoculation. Both of the baboons that were vaccinated with10^4 CFU BCG (baboon 388 and 428) and 1 of 2 (baboon 466) that received a dose of 10^6 CFU BCG did not develop significant antibody levels by week 20 post-inoculation or after a boost with a dose of 10^6 CFU BCG at week 20. Two baboons (466 and 454) were seen to have high pre-vaccination BCG antibodies relative to the others. However, one of them (baboon 454; receiving 10^8 CFU dose) showed an elevation of antibody level after vaccination with BCG. A booster inoculation at week 20 did not appear to increase the BCG antibody levels substantially for baboons that received a BCG dose of 10^8 CFU.

Figure 3.7: BCG-specific serum IgG antibodies at various time points post intradermal inoculation with various doses of BCG-*Tokyo* (10^4, 10^6, 10^8 CFU). Serum (diluted 1:1600) was incubated in duplicate wells on Maxisorb plates coated with BCG-*Tokyo* protein extract. Rabbit anti-monkey IgG conjugated to HRP in conjunction with tetramethyl-benzidine (TMB) substrate was used for detection of baboon BCG-specific antibodies. The OD values are the mean of duplicate values that did not differ by more than 10%. The time of BCG inoculation at weeks 0 and 20 is indicated by↓.



3.4 DISCUSSION

While the development of an efficacious HIV vaccine is an enormous challenge, the costs of the novel vaccine is an additional and concurrent challenge as the majority of the HIV-infected individuals reside in the developing world (UNAID, 2005). Recombinant BCG expressing immunogenic proteins to which immune responses develop in the host may offer a way to develop such an affordable vaccine. It has been shown that BCG transformed with viral, bacterial and parasitic genes have resulted in the development of cellular and/or humoral immune responses to the expressed gene in animal models (Matsuo *et al*,, 1990; Stover *et al*, 1991 & 1993; Aldovini & Young, 1991; Yasutomi *et al*, 1993; Connell *et al*, 1993; Abomoelak *et al*, 1999; Leung *et al*, 2000; Winter *et al*, 1991; Gheorghiu *et al*, 1994; Kameoka *et al*, 1994; Honda *et al*, 1995; Hiroi *et al*, 2001; Kawahara *et al*, 2002a & 2002b; Chujoh *et al*, 2002). The need to test vaccines and particularly vaccines against HIV infection has demanded the need for appropriate animal models. For such animal models to be rational, they should be readily available, inexpensive to maintain, afford vaccination regimens that are suitable for use in human, and give readouts that are useful in guiding clinical trials. It would be advantageous if they also have an immune system resembling that of man.

Extensive use has been made of the murine model for the evaluation of novel recombinant vaccines. While the regulatory authorities that approve vaccine trials in human may be willing to extrapolate immunogenicity and efficacy data from mice to humans, safety data on novel, live attenuated vaccines must be generated in nonhuman primates prior to approval. Thus, although the mouse model is important for screening candidate vaccines that show significant protective activity, nonhuman primates are essential for the final stages of preclinical evaluation. In addition, it may be necessary to conduct animal vaccines studies concurrently with the early stages of clinical trials for HIV candidate vaccines on "fast-track" development programme.

Chacma baboons are readily available in South Africa and could provide the model required for rBCG testing using vaccination regimens reflecting those to be used in human trials. This baboon species has been reported to be infected with *M. tuberculosis* and *M. bovis* (Fourie & Odendaal, 1983; Keet *et al*,, 2000; Thorel *et al*, 1998), but there are no published reports on immune responses to BCG and other mycobacterial infections in this species.

We show in this study that vaccination of Chacma baboons with various doses of either *Pasteur* or *Tokyo* BCG strain was well tolerated. The ulcerations that were induced at the inoculation site by these strains of BCG and the single case of lymphadenitis that was observed resolved without medication and within a few weeks. All the baboons remained healthy during the study period. This is not different from what has been reported in humans vaccinated intradermally with BCG (Ravn *et al*, 1997; Kemp *et al*, 1996; Hoft *et al*, 1999). In addition, no change in the proportion of circulating T cell subsets could be detected at any stage after the vaccination in these baboons.

Delayed-type hypersensitivity reaction is a cell-mediated response that is induced by several bacterial and viral antigens, including PPD. The reaction has been shown to be a CD4+ and CD8+ T cell-mediated memory effector mechanism (Black, 1999) in which the IFN-γ plays a major role (Seabrook *et al*, 2005). The DTH skin test is therefore an important *in vivo* test for measuring induction of cellular immune response to BCG vaccination. All the baboons in the study, except for one that received a dose of 10^4 CFU Tokyo strain, developed a positive PPD-induced DTH at some stage during the course of the study.

BCG inoculation induced a sustainable BCG-specific cellular immune response. Upregulation of the activation marker, the HLA-DR orthologue, on CD3+ cells and BCG-specific production of IFN-γ, which is an important cell-mediated response to intracellular pathogens, was observed. This T cell activation and IFN-γ production was accompanied by lymphocyte proliferation that was detectable beyond a year post the initial inoculation. Looking at the proliferative index as the

parameter for proliferation, an apparent lack of BCG-specific proliferation in one of the baboons (baboon 454) vaccinated with a high dose of BCG was observed. However, when the net proliferation above the background was considered, it was shown that this baboon indeed exhibited a BCG- and PPD-specific proliferation that was comparable to the proliferation obtained with the other baboon that received an equal dose of BCG. The reason for the high non-specific background proliferation exhibited by its PBMC in culture medium alone (in absence of BCG or PPD) was not known but could possibly be caused by prior sensitisation to an identified component in the culture medium especially fetal bovine serum.

All baboons inoculated with BCG produced BCG-specific IFN-γ response. The level and kinetics of BCG-specific IFN-γ production by PBMC after the initial BCG inoculation appeared to be dependent on the inoculating BCG dose. BCG-induced IFN-γ production took longer to develop and declined more quickly after a low dose than after a high dose. Boosting the baboons with BCG induced a strong BCG-specific IFN-γ production from PBMC from the majority of animals, which appeared to be independent of the initial inoculation dose. This response was long-lived and detectable more than a year post the boost. An IFN-γ ELISPOT assay detected BCG-specific IFN-γ producing cells beyond 2 years, further supporting the finding that long-lived BCG-specific memory was induced by the vaccination. Flow cytometric investigation of the subset producing this cytokine revealed the CD4+ T cell population to be predominantly responsible for producing the IFN-γ.

The findings reported in the present study are comparable with reported studies in macaques and humans. The vaccination of rhesus and cynomolgus macaques with BCG by parenteral routes (subcutaneous and intradermal) has demonstrated strong specific proliferative responses, PPD-induced IFN-γ cytokine production and cell-mediated cytotoxicity (Abolhassani *et al*, 2000). Similar findings and DTH responses have been reported in humans vaccinated intradermally with BCG (Kemp *et al*, 1996; Ravn *et al*, 1997; Hoft *et al*, 1998 & 1999). In addition, Kemp *et al*, (1996) showed that IFN-γ (a Th1 cytokine) and not IL-4 (a Th2 cytokine) was produced by cells from immunized subjects. Work by Ravn and colleagues (1997) also showed that BCG vaccination primed CD4+ cells with Th1-like cytokine profiles and cytotoxic effector functions. They showed production of IFN-γ but not IL-5 in volunteers vaccinated with BCG and identified the CD4 subset to be solely responsible for antigen-specific proliferation and IFN-γ production. Our data also show the CD4 subset to be the major producer of this cytokine and detection of a strong DTH response. However, other studies indicate that both CD4 and CD8 subsets produce IFN-γ in response to TB re-infection in the mouse model of tuberculosis (Serbina and Flynn 2001) and in humans suffering

from TB. Also, Lai and co-workers (2003) have reported production of PPD-induced IFN-γ by CD+ T cells from macaques vaccinated with BCG. In addition, the γδ T cells, a subset of T cells involved in presentation of non-protein ligands in mycobacterial infections (Carding & Egan, 2000; Poccia *et al*, 1999), has also been shown to proliferate during mycobacterial infections (Tsukaguchi *et al*, 1995; Hoft *et al*, 1998; Lai *et al*, 2003; Shen *et al*, 2002) and to produce IFN-γ upon restimulation with live *M. tuberculosis* and not with PPD (Tsukaguchi *et al*, 1995).

Early studies in mice vaccinated with BCG showed a shift towards protective cellular immune responses caused by low-dose immunisation (Bretscher *et al*, 1992; Bretscher, 1994), which suggested that small doses could induce the immune system into a Th1-like cell mediated mode, while higher doses would inhibit this response and shift it towards a Th2-like response. The Th1 helper T cells are associated with Th1 cytokines (IL-2, IFN-γ, TNF-α), which play a central role in protection against mycobacteria and other intracellular pathogens (Mosmann *et al*, 1986; Romagnani 1992; Flesch & Kaufmann 1987; Orme *et al*, 1993), while Th2 is associated with IL-4, IL-5 & IL-10, which up-regulate the less protective humoral responses (Surcell *et al*, 1994) and diminish Th1 responses (Schauf *et al*, 1993; Zhang *et al*, 1995). To determine the most appropriate BCG dose in the baboon model, vaccination with 3 varying doses (10^4, 10^6, and 10^8 CFU) of Tokyo BCG was investigated. Although no Th2 cytokine was measured in the present study, the data obtained in regard to IFN-γ production by PBMC from immunised baboons showed that vaccinating with high doses of BCG induces higher production of IFN-γ with peak production being reached earlier than vaccinating with low doses of BCG. PPD-induced DTH results also supported this view by demonstrating that bigger sizes of indurations were induced in animals vaccinated with higher doses than those given lower doses. Similarly, a study by Lowry *et al*, (1998) with human subjects immunized with 4 varying doses of BCG-*Tice* given by percutaneous method reported that lymphoproliferative responses were increased only in high-dose vaccinees and significant increases in IFN-γ expression were present only for persons given standard ($1.6x10^8$ CFU) or high ($3.2x10^8$ CFU) BCG and not very low ($1.6x10^5$ CFU) or low ($3.2x10^5$ CFU) doses. Also, vaccinees given standard- or high-dose BCG vaccination were significantly more likely to have a DTH response to PPD than those vaccinated with low or very low BCG dose. Like Lowry *et al* (1998), data from the present study do not support the view that a low dose BCG vaccination promotes a preferential Th1 type immune response. In contrast, both studies suggest that a high-dose BCG vaccination is necessary for the induction of a Th1 type immune response.

Development of a humoral immune response to BCG inoculation was not consistent for all baboons. Animals receiving the high BCG dose developed antibody responses while those receiving low dose did not. This finding is comparable to those of Lowry *et al*, (1998) whereby a

slight increase from baseline was detected in subjects given standard- or high-dose BCG but not those given a low- or very low-dose. The seeming existence of BCG antibodies in 1 of 2 baboons (baboon 466) prior to inoculation with 10^6 CFU may have influenced the development of antibodies post inoculation. However, a similar high pre-inoculation antibody level found in 1 of 2 baboons (baboon 454) receiving a dose of 10^6 CFU did not deter an increase in BCG-specific antibodies. Other investigators have described high pre-vaccination baseline sero-reactivity to BCG in olive baboons (Pehler *et al*, 2000) and human (Hoft *et al*, 1998; Andersen 2001). This has been attributed to prior immune sensitization by cross-reactive environmental mycobacteria. From this perspective, the finding of high pre-vaccination baseline sero-reactivity to BCG in some baboons suggests that these baboons may have been exposed to environmental mycobacteria in the wild, a situation that may resemble similar situations for the human population under field conditions. This mimicry further supports the view that a baboon model may give good predictive value for human trials with rBCG-based vaccines under field settings.

In conclusion, the results from this study indicate that chacma baboons develop a strong and sustained cell mediated immune response to both Tokyo and Pasteur BCG strains, which is characterised by strong DTH, BCG-specific proliferative and Th1 type cytokine responses. The data is comparable to those from studies with human and macaques vaccinated with BCG. These data support our view that the baboon may be a useful alternative animal model for testing rBCG vaccines intended for clinical use. This concept was investigated further by immunising baboons with rBCG vaccines expressing HIV-1 Gag derived from a subtype C virus. The results, which are described in the next chapter (Chapter 4), showed that the chacma baboon is a valuable and unique animal model for testing the immunogenicity of rBCG-based HIV candidate vaccines. This baboon sub-species is endemic in southern Africa and thus, its utility could translate into lower costs of conducting pre-clinical testing of HIV vaccines. Due to its large body size, it could provide the model required for rBCG testing using vaccination regimens reflecting those to be used in human trials.

CHAPTER FOUR

IMMUNOGENICITY STUDIES IN BABOONS FOLLOWING VACCINATION WITH RECOMBINANT BCG-HIV-1 *gag*, SAAVI MVA-C AND PR55^{gag} VLP VACCINES

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4.1 INTRODUCTION

In the previous chapter (Chapter 3), the induction of immune responses following the immunisation of chacma baboons with wild-type BCG (wt-BCG) was reported. The information gathered was very useful in initiating the immunogenicity studies of recombinant BCG (rBCG) vaccines, which are described in this chapter. In these immunogenicity studies, two experiments with baboons using rBCG expressing subtype C HIV-1 *gag* gene are described. A prime-boost vaccination strategy was adopted in which the rBCG vaccines were given as multiple priming inoculations, followed by booster vaccinations with either 2 successive inoculations of Gag VLP vaccine or single inoculation each of SAAVI MVA-C and Gag VLP. Six baboons from the study in Chapter 3, which were inoculated with wt-BCG, were included in one of these experiments to examine the effect of prior BCG sensitisation on the rBCG vaccine-induced responses.

4.1.1 RECOMBINANT BCG VACCINES

As reviewed in section **1.4**, a wide range of HIV-1 and SIV antigens have been successfully expressed in rBCG and reported to induce humoral and cellular responses to the heterologous viral antigens in mice and macaques (Aldovini & Young, 1991; Chujoh *et al*, 2002; Dellagostin *et al*, 1993; Fuerst *et al*, 1992; Gheorghiu *et al*, 1994; Hiroi *et al*, 2001; Honda *et al*, 1995; Kameoka *et al*, 1994; Kawahara *et al*, 2002a & 2002b; Lagaranderie *et al*, 1998; Leung *et al*, 2000; Matsuo *et al*, 1990; Mederle *et al*, 2003; Someya *et al*, 2005; Winter *et al*, 1991). Majority of these rBCG HIV-1 vaccines used viral antigens derived from subtype B and, in one case, a subtype E antigen (Chujoh *et al*, 2002). The present study extends this vaccine development concept to include a Gag immunogen derived from a HIV-1 subtype C, an HIV-1 clade that is responsible for over 50% of global HIV-1 infections (McCutchan, 2006).

Pre-existing immunity to BCG resulting from global TB vaccination programmes is considered a key concern over the success of future rBCG-based vaccines. Reports from BCG studies in animal models suggest that such a pre-existing immunity has a potential of lowering the efficiency of rBCG vaccines (Palmer & Long, 1966; Brandt *et al*, 2002; Buddle *et al*, 2002) while other studies seem to contradict this (Gheorghiu *et al*, 1994; Kameoka *et al*, 1994). The present study sought to clarify this ambiguity.

4.1.2 PRIME-BOOST VACCINATION STRATEGY

Given that prime-boost vaccination strategies have shown great success in enhancing the immune responses (see review in section **1.3.11**), it is in order to incorporate rBCG vaccines into such strategies. A few studies using wt-BCG as a prime to rMVA expressing antigen 85A in TB vaccine research have reported similar successes in the mouse (Goonetillleke *et al*, 2003) and guinea pig

(Williams *et al*, 2005) animal models and even in humans who were BCG-primed or who had a naturally acquired antimycobacterial immunity (McShane *et al*, 2004). Using rBCG expressing SIV Gag to prime the immune system and a replication-deficient vaccinia virus expressing SIV Gag, Ami *et al* (2005) have recently demonstrated induction of high levels of IFN-γ spot forming cells and effective immunity against mucosal challenge with pathogenic SHIV in cynomolgus monkeys that had been primed with rBCG and boosted with recombinant vaccinia virus but not in animals receiving opposite combination or single-modality vaccines.

HIV $Pr55^{gag}$ VLP have been shown to be highly immunogenic class of sub-unit vaccines (see review in section **1.5**). In the present study, a HIV-1 subtype C $Pr55^{gag}$ VLP (GagC VLP) was used to boost baboons that had been primed with a matched rBCG vaccine. GagC VLP was chosen as a boost vaccine due to our previous success in using this vaccine to boost and broaden the immune response of baboons that were primed with a DNA (pTH-GagC) vaccine (see Chapter 2).

Triple vector vaccine strategy, which involves utilising a combination of three different chimeric vectors that share specific vaccine immunogen(s), has been used in HIV vaccine studies (Heeney *et al*, 2000; Koopman *et al,* 2004; Michelini *et al*, 2004; Negri *et al*, 2004). Triple vector vaccine strategy is envisaged to overcome the common problem posed by induction of anti-vector immune responses to the vaccine vector itself, which may preclude sufficient priming or optimal boosting upon repeated immunisation with the same vector. In the present study, a triple vector vaccine strategy, comprising of rBCG-SAAVI MVA-C-GagC VLP vaccination modality was used in 1 of 2 experiments in this study. A boost with SAAVI MVA-C was chosen due to its excellent performance in boosting responses induced by a DNA vaccine using chacma baboons (Williamson *et al*, 2005; Burgers *et al*, unpublished results).

4.1.3 STUDY OBJECTIVES

As part of an elaborate programme in our research group to develop affordable, effective and locally relevant HIV-1 vaccines, a number of rBCG vaccines that express Gag from a South African HIV-1 subtype C isolate (ZDU422; Williamson *et al*, 2003) were developed using the BCG *Tokyo* vaccine strain (Thomas, 2005). Based on immunogenicity in mice, two of them, designated rBCG:RT106 and rBCG:RT108, were selected for evaluation in the baboon model. The *in vitro* stability of these rBCG:Gag vaccines (rBCG:RT106 and rBCG:RT108) and expression of HIV-1 p24 in culture was also investigated and shown that rBCG:RT106, although relatively less stable had higher expression of HIV-1 p24 antigen than rBCG:RT108.

Data from the study described in Chapter 2 showed that GagC VLP vaccine derived from the same HIV-1 subtype C isolate (ZDU422) efficiently boosted and broadened the immune responses primed with a DNA HIV-1 Gag (pTH-GagC) vaccine in a prime-boost vaccination modality. Accordingly, the same GagC VLP vaccine was chosen to boost the immune response to a primary inoculation with rBCG:Gag vaccines for the 1st baboon experiment.

In our research group, another study investigating the immune response in a DNA vaccine-prime and SAAVI MVA-C boost immunisation strategy using mice (Makhubela *et al*, 2005) and baboons (Williamson *et al*, 2005) showed that SAAVI MVA-C was very efficient in boosting the immune responses induced by DNA vaccine. This multigene SAAVI MVA-C vaccine, expressing the *gag* gene as well as 4 other genes (*RT, tat, nef* and *env*) derived from HIV-1 subtype C was incorporated in a triple vector vaccination protocol involving rBCG-SAAVI MVA-C-GagC VLP regimen for the 2nd baboon experiment. A single inoculation of SAAVI MVA-C was followed with a GagC VLP booster immunisation 12 weeks later.

As mentioned previously (section **1.4.4**), it is not clear if pre-existing immunity to BCG has a potential adverse effect on the ability of a rBCG vaccine to induce vaccine-specific immune responses. This is an important issue as the use of BCG vaccination to protect against TB is widespread in the developing world where a BCG-based HIV-1 vaccine would be most relevant. To address the role of pre-existing immunity to wild-type BCG in the induction of Gag-specific immune responses by rBCG:RT106 vaccine, six of the baboons that had been inoculated with wild-type BCG in a previous experiment (see Chapter Three) were included in the second baboon experiment and used in rBCG-SAAVI MVA-C-GagC VLP vaccination regimen.

4.2 MATERIALS AND METHODS

4.2.1 VACCINE PREPARATION

The following vaccine preparations were provided as frozen stocks (-80 °C) by the following people in our UCT Vaccine Research group;

(i) wild-type BCG, *Tokyo* substrain (wt-BCG) by Robin Thomas, grown from the human vaccine strain, Tokyo-172 (Thomas, 2005).

(ii) two rBCG:Gag vaccine constructs designated rBCG:RT106 and rBCG:RT108 by Robin Thomas. These were made by introducing *E. coli/Mycobacterium* shuttle vectors into BCG (Tokyo-172 vaccine strain) as episomal vectors. Both shuttle vectors expressed Gag of ZDU422 HIV-1 subtype C isolate using identical localisation signal sequence (19kDa lipoprotein) but different promoters. rBCG:RT106 used *mtrA* promoter while rBCG:RT108 used *katG* promoter. The two vaccine constructs differed in stability and antigen expression

with rBCG:RT106 being relatively less stable with higher expression of Gag and rBCG:RT108 being more stable but with a lower Gag expression (Thomas, 2005).

(iii) rBCG designated rBCG:CB119-L1e by James Maclean. This construct was made in an identical way as rBCG:RT106 except that it expressed the L1e gene derived from HPV type 16 (Maclean, 2005) and served as a rBCG control.

(iv) rMVA, a multigene vaccine code-named SAAVI MVA-C, supplied by Wendy Burgers. The vaccine construct expressed the *gag*, RT, *tat* and *nef* as a polyprotein and a truncated *env* (gp150) derived from HIV-1 subtype C (Williamson *et al*, 2005; Burgers *et al*, unpublished).

(v) $Pr55^{gag}$ virus-like particles (GagC VLP) by Ann Jaffray (Jaffray *et al*, 2004) and used as in Chapter 2.

The rBCG:CB119-L1e, rBCG:RT106, rBCG:RT108 and wt-BCG were cultured in 100mL of Sauton's medium containing tyloxapol detergent (0.25% w/v) as previously described (Maclean, 2005; Thomas, 2005). The inocula were provided in 200µL aliquots containing $1x10^9$ CFU/mL of bacteria. The vaccine stocks were stored at -80°C. After thawing and prior to inoculation, the inocula were passed through a syringe needle (Gauge 25) 5-10 times to disperse the clumps.

The GagC VLP were prepared as previously described (Section **2.2.2**). The endotoxin level was below 0.125 EU/mL.

The SAAVI MVA-C vaccine was manufactured by Therion Biologics Corporation (Cambridge, USA) using *env* gene and 'Grttn' polygene (supplied by W Burgers and C Williamson respectively) from HIV-1 subtype C. Grttn is a fusion gene comprising of modified portions of *gag*, *reverse transcriptase* (RT), *tat* and *nef*. The Gag component was derived from the South Africa isolate, ZDU422 (Williamson *et al*, 2003). The *env* and Grttn were inserted at 49/50 and deletion III sites respectively in the virus genome and their expressions regulated by vaccinia virus I3 and 40K promoters respectively. The inocula stocks were stored in 250 µL aliquots (at $7.6x10^{10}$ PFU/mL) at -80°C. At the time of inoculation, the vaccine stock was diluted with 1mM Tris (pH 9.0) to $5x10^8$/mL.

4.2.2 BABOONS

Twenty baboons that were housed in single cages at Medical Research Council's animal facility at Delft in Cape Town, South Africa were used for this study. Before the study, all animals were examined clinically, tuberculin skin-tested and screened serologically for SIV antibodies. The

experiments were reviewed and approved by the Animal Research Ethics Committee of University of Cape Town (Reference No. 00/03N; 03/021).

4.2.3 BABOON IMMUNISATIONS

Two separate experiments were conducted. The baboons were immunised as indicated below and monitored for development of local reactions at injection sites during feeding and bleeding times.

4.2.3.1 Experiment 1: rBCG prime, GagC VLP boost

In this experiment (Exp1), all 6 baboons used in the study were given rBCG as an intradermal inoculation at a dose of $1x10^8$ CFU and GagC VLP at a dose of 10.8 μg protein per animal (**Fig 4.1**). Group 1C served as the control.

4.2.3.2 Experiment 2: rBCG prime, rMVA and $Pr55^{gag}$ VLP boosts

In this experiment (Exp2), 6 of 12 baboons (group **2A** [baboons 363, 378 & 428] and group **2B**; [611, 632 & 661]) were primed twice with rBCG:RT106, 12 weeks apart, with an intradermal inoculation of $1x10^8$ CFU per animal. The control groups **2AC** (baboons 350, 447 & 466) and **2BC** (baboons 427, 449 & 672) were mock-primed twice with wild-type BCG, 12 weeks apart with an intradermal inoculation of $1x10^8$ CFU per animal. Six of the baboons in the study, Group **2A** (baboons 363, 378 & 428) and Group **2AC** (baboons 350, 447 & 466) had been experimentally exposured to wild-type BCG by two intradermal inoculations 3 years prior to the present study (see Chapter 3). All 12 baboons were boosted with SAAVI MVA-C by intramuscular injection of 10^9 PFU per animal and with GagC VLP at a dose of 29 μg protein per animal. The grouping and immunisation schedule is depicted in **Fig 4.1.**

4.2.4 BLOOD COLLECTION

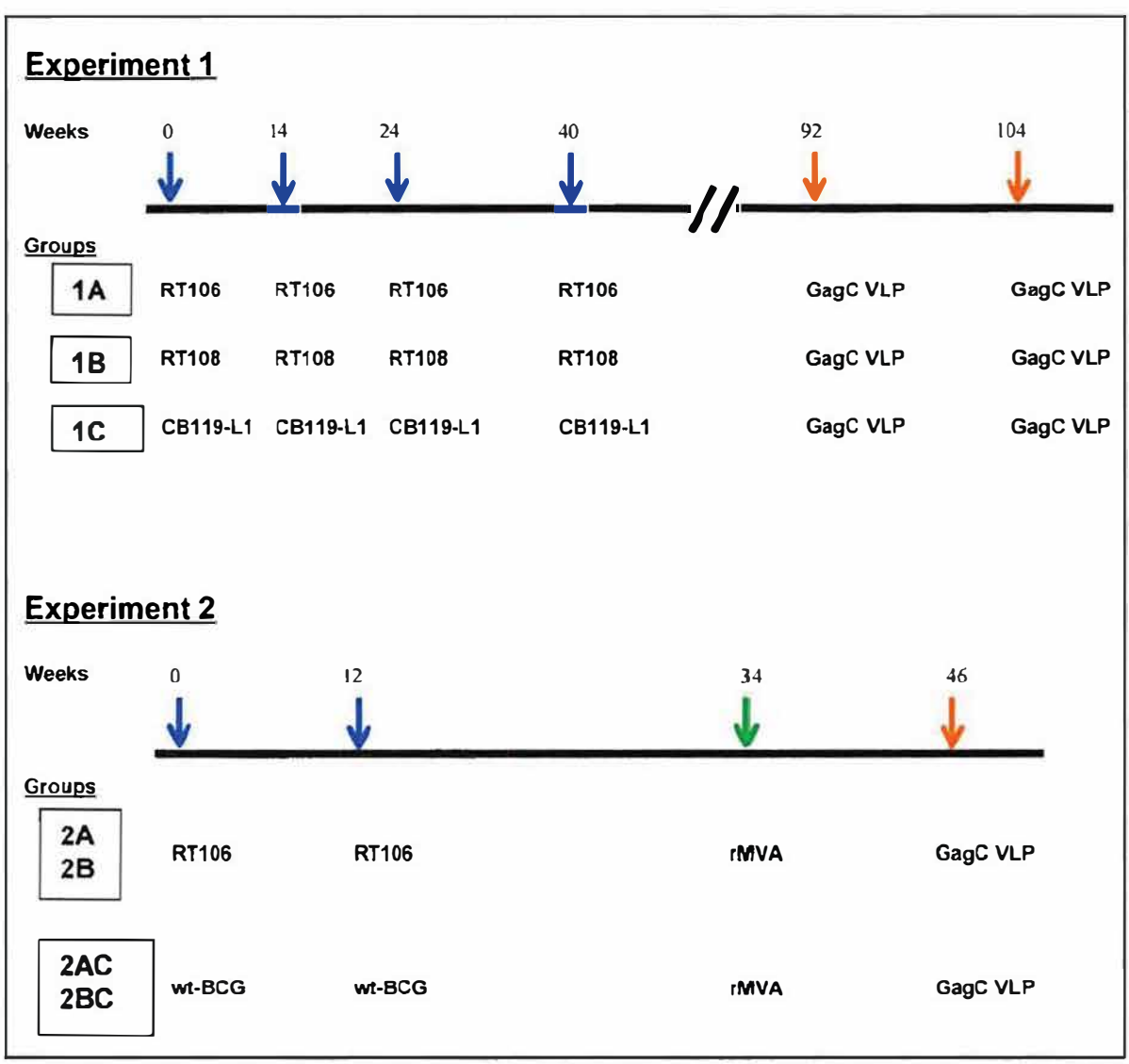
Blood samples for serum and PBMC isolation were obtained at pre-inoculation and various time points post inoculation. Ketamine were used to sedate animals for all procedures involving animal handlings. Blood was processed within 3 hours of collection as described in **Appendix B.1**. Serum aliquots were stored frozen at –30 °C. The isolated PBMC were prepared into aliquots for storage in liquid nitrogen as described in **Appendix B.2**. Aliquots of freshly isolated PBMC were used in Pool-Matrix IFN-γ ELISPOT assays in some selected time points.

Fig 4.1: Baboon immunisation time line. Baboons were divivded into groups and inoculated as indicated below.

Experiment 1: Groups **1A** (baboons 542 and 545) and **1B** (baboons 538 and 539) were primed with intradermal injections of rBCG:RT106 and rBCG:RT108 respectively at weeks 0, 14, 24 and 40 as indicated (↓). The control group (**1C;** baboons 546 and 548) was mock-primed with rBCG:CB119-L1e, which expressed HPV-16 L1 protein, at the same time points. All the baboons were subsequently boosted intramuscularly with 2 inoculations of GagC VLP at weeks 92 and 104 as indicated (↓). A third GagC VLP immunisation (not shown in the diagram) was given to baboons 539, 542, 545 and 548 at week 141 (baboons 542 and 548) or 154 (baboons 539 and 545). All the groups did not have prior experimental exposure to wt-BCG.

Experiment 2: Groups **2A** (baboons 363, 378 and 428) and **2B** (baboons 611, 632 and 661) were primed with rBCG:RT106 at weeks 0 and 12 as indicated (↓). Two control groups (**2AC**; baboons 350, 427 and 447) and (**2BC**; baboons 449, 466 and 672) were mock-primed similarly with wild type BCG. All the baboons were then inoculated intramuscularly with SAAVI MVA-C at week 34 (↓) and GagC VLP (↓) at week 46. The SAAVI MVA-C expressed the *gag*, RT, *tat*, *nef* and *env* genes of HIV-1 subtype C. Groups **2A** and **2AC** had had prior experimental exposure to wt-BCG via two intradermal inoculations three years before (see Chapter 3). Groups **2B** and **2BC** did not have any experimental exposure to wt-BCG.

The dosages per animal were as follow: wt-BCG and rBCG at 10^8 CFU; SAAVI MVA-C at 10^9 PFU; GagC VLP at either 10.8 µg (Groups **1A**, **1B** and 1C) or 29 µg (Groups **2A**, **2B**, **2AC** and **2BC**).



4.2.5 LABORATORY ANALYSIS

4.2.5.1 HIV-1 p24 ANTIBODY ELISA

Serum antibodies to HIV-1 p24 in the sera of immunised baboons were measured as previously described (section **2.2.5.1**). The results are given as optical density (OD) value at 1:40 serum dilution and as the endpoint antibody titre (selected time points).

4.2.5.2 HIV WESTERN BLOT TEST

The presence of Gag antibodies in baboon sera was confirmed using the New LAV Blot I™ kit (Bio-Rad Laboratories, CA, USA) following the manufacturer's instructions with modifications (section **2.2.5.2**).

4.2.5.3 IFN-γ ELISPOT ASSAY

Gag-specific T cells in cryopreserved baboon PBMC were quantified by an IFN-γ ELISPOT assay using pools of 15-18 mer Gag peptides overlapping by 10 amino acids spanning the entire Gag subtype C protein sequence as previously described (section **2.2.5.3**). In addition, the number of PPD-specific cells was determined, using PPD (Institute for Animal Science and Health, The Netherlands; final concentration of 5 μg/mL) as a stimulant. All reactions were set in triplicate. Data was presented as net IFN-γ spot-forming units (SFU) per 10^6 PBMC. As previously described (section **2.2.5.3**), a cut-off value was established for each of these two studies.

4.2.5.4 PEPTIDE MAPPING BY POOL-MATRIX IFN-γ ELISPOT ASSAY

To identify the peptides to which the IFN-γ responses were directed, the Pool-Matrix method was employed as previously described (section **2.2.5.4**).

4.2.5.5 INTRACELLULAR IFN-γ STAINING ASSAY AND FACS ANALYSIS

The proportion of the IFN-γ-producing PBMC and their phenotypic classification were determined using the ICS method as previously described (section **2.2.5.5**). Data is presented as percentage of CD4+ or CD3+CD8+ lymphocytes, which are IFN-γ positive.

4.2.5.6 CYTOMETRIC BEAD ARRAY (CBA) ASSAY

To measure Gag peptide-specific cytokines produced in the culture supernatant of PBMC from immunised baboons, the antigenic stimulations with specific Gag peptides were set up as previously described (section **2.2.5.6**). The CBA (Non-human primate Th1/Th2 kit, BD, San Diego, CA) assay was used to quantify the amount of IFN-γ, TNF-α, IL-2, IL-4, IL-5 and IL-6, according to the manufacturer's instructions.

4.2.6 STATISTICAL ANALYSIS

The t-test (Statistica®, StatSoft) was used for statistical analysis.

4.3 RESULTS

4.3.1 LOCAL CUTANEOUS REACTIONS AT INJECTION SITES

The baboons were monitored for development of local cutaneous reactions by physical observation during feeding and bleeding times. All baboons developed ulcerations (5-20mm) within 2 weeks of inoculation with either wt-BCG or rBCG. These ulcerations healed within 8 weeks post inoculation without medication. There was no apparent difference between the size of ulcerations induced by wt-BCG, rBCG:RT106, rBCG:RT108 and rBCG:CB119-L1e. Pre-immunisation with wt-BCG (Groups 2A and 2AC) did not appear to influence these cutaneous reactions. Repeated inoculations with these BCG inocula induced similar ulcerations with similar healing times. Inoculation with SAAVI MVA-C and GagC VLP did not induce any observable reaction at the injection site. Apart from these local tissue reactions at injection sites, all the baboons maintained good health throughout the study period.

4.3.2 HUMORAL RESPONSES TO HIV-1 GAG

The levels of IgG antibodies to HIV-1 p24 protein in the sera of baboons at various time points pre- and post- immunisation are shown in **Figs 4.2** (Exp1) and **4.3** (Exp2).

In Exp1, only a weak Gag-specific antibody response (from a titre of 80 to 160) could be detected for one baboon (545; Group 1A) after inoculation with rBCG:RT106. This weak response was observed in a single time point (Wk92) just prior to GagC VLP booster inoculation (**Fig 4.2**). However, the first GagC VLP booster inoculation at week 92 (exactly 1 yr after the last rBCG prime inoculation) induced a low Gag antibody titre in the serum of baboons primed with rBCG:RT106 or rBCG:RT108 and not in the serum of mock-primed (with rBCG:CB119-L1e) baboons. A 2nd GagC VLP booster inoculation induced dramatic increases in antibody titres in both rBCG:RT106- and rBCG:RT108-primed baboons and only moderate increases in the rBCG:CB119-L1e-primed controls. For all 3 groups, the peak titres were reached by 4 weeks after the 2nd GagC VLP inoculation after which these titres started to decline (**Fig 4.2**). Using a commercial HIV-1 western blot assay, antibodies to p55 and p24 were strongly detected at peak response (Week 108) thereby confirming the antibodies detected by ELISA method as Gag-specific (**Fig 4.4**). However, antibodies to p17 were absent or weakly detected in the western blot assay.

In Exp2, HIV-1 p24 antibodies were undetectable after rBCG:RT106 or wt-BCG primary inoculations for all the baboons (**Figs 4.3A,** and **4.3B**). After a booster inoculation with SAAVI

MVA-C, which was given at week 34 (22 weeks after the last primary inoculation), the p24 antibodies remained undetectable and antibody titres unchanged in all the groups. A modest rise in both antibody level and titre were detected in a few baboons after the GagC VLP booster immunisation, which was given at week 46 (12 weeks after SAAVI MVA-C inoculation).

Fig 4.2: HIV-1 p24 IgG antibody titres (Exp1). Serum was obtained from baboons (groups 1A, 1B and 1C) at pre- (Wk0) and various post-immunisation times. The antibody titres were determined by ELISA as described (section **4.2.5.1**) in serial dilutions of serum starting at 1:40. Data points are antibody titres that exceeded 100.

Pre-: pre-immunisation (Wk0); 2nd BCG: 6 weeks post 2nd rBCG immunisation; 3rd BCG: 4 weeks post 3rd rBCG immunisation; 4th BCG: 4 weeks post 4th rBCG immunisation; Pre-VLP: 0 weeks pre-GagC VLP immunisation; 1st VLP: 4 weeks post 1st GagC VLP immunisation; Pre-2nd VLP: 0 weeks pre-2nd GagC VLP immunisation; 2nd VLP-a: 4 weeks post 2nd GagC VLP immunisation; 2nd VLP-b: 8 weeks post 2nd GagC VLP immunisation.

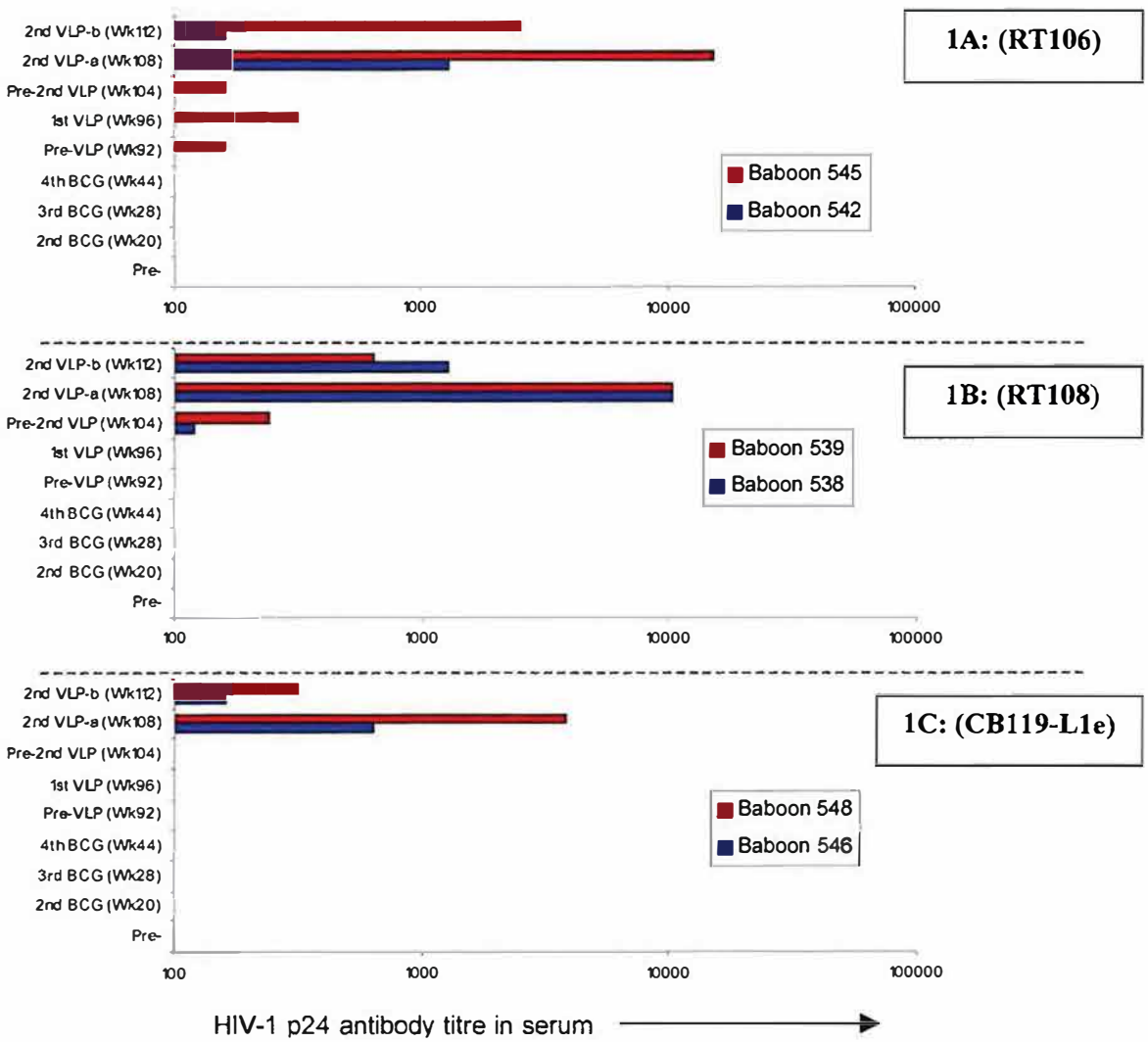


Fig 4.3A: HIV-1 p24 IgG antibody levels (Exp2). Serum from baboons which had prior experimental BCG sensitisation was obtained at various time points pre- (wk0) and post inoculation (wk12) with rBCG:RT106 (**2A**) or wt-BCG (**2AC**) and following booster immunisations with SAAVI MVA-C (wk34) and GagC VLP (wk46). The antibody levels and titres were determined by ELISA method as previously described (Section **4.2.5.1**). Data points are OD values (serum dilution 1:40) and antibody titres at various times post inoculation. Inoculation times are indicated with arrows as follows; rBCG:RT106: ↓; wt-BCG: ↓; SAAVI MVA-C: ↓; GagC VLP: ↓.

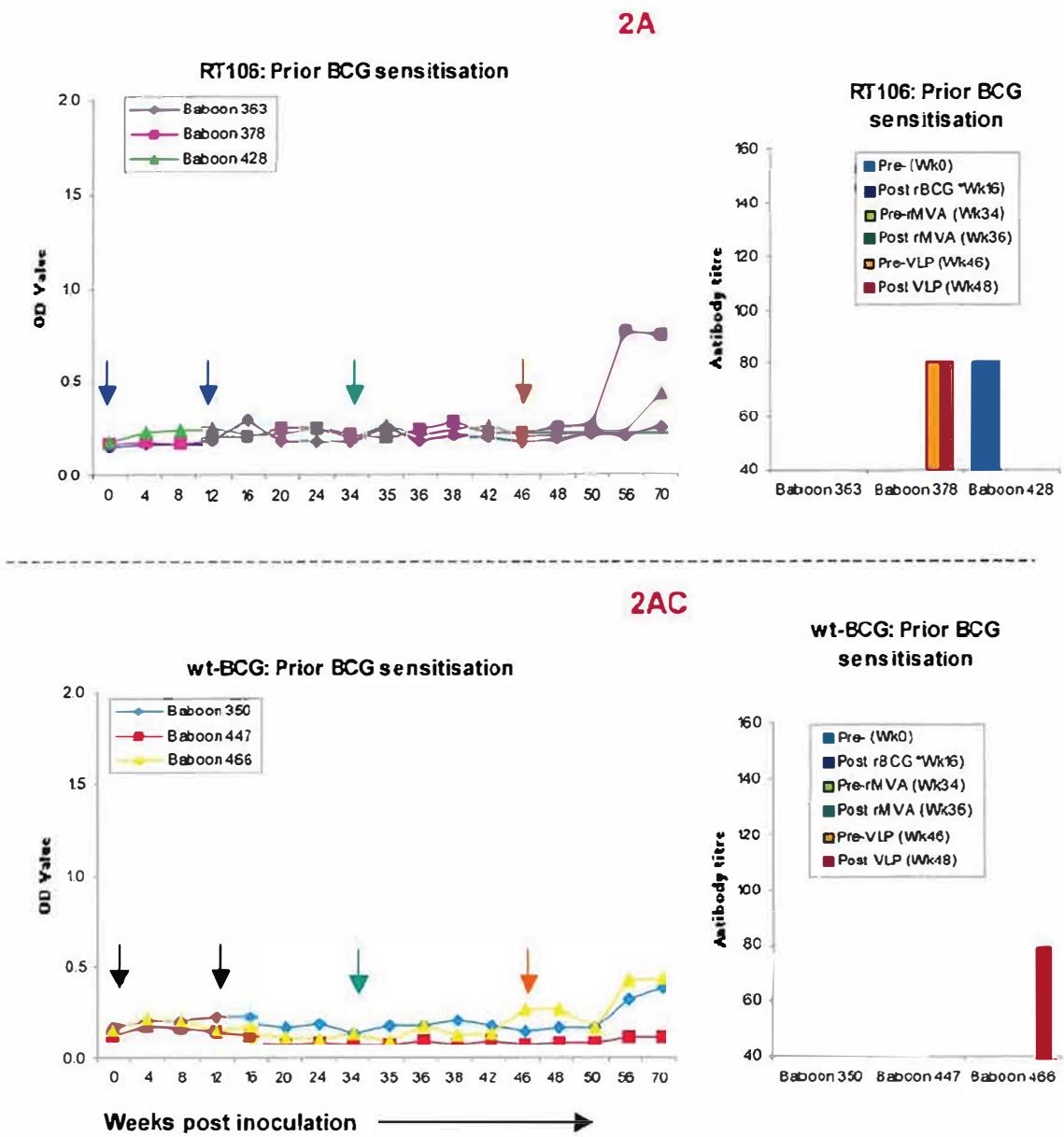


Fig 4.3B: HIV-1 p24 IgG antibody levels (Exp2). Serum from baboons which had no prior BCG sensitisation was obtained at various time points pre- (wk0) and post inoculation (wk12) with rBCG:RT106 (**2B**) or wt-BCG (**2BC**) and following booster immunisations with SAAVI MVA-C (wk34) and GagC VLP (wk46). The antibody levels and titres were determined by ELISA method as previously described (Section **4.2.5.1**). Data points are OD values (serum dilution 1:40) and antibody titres at various times post inoculation. Inoculation times are indicated with arrows as follows; rBCG:RT106: ↓; wt-BCG: ↓; SAAVI MVA-C: ↓; GagC VLP: ↓.

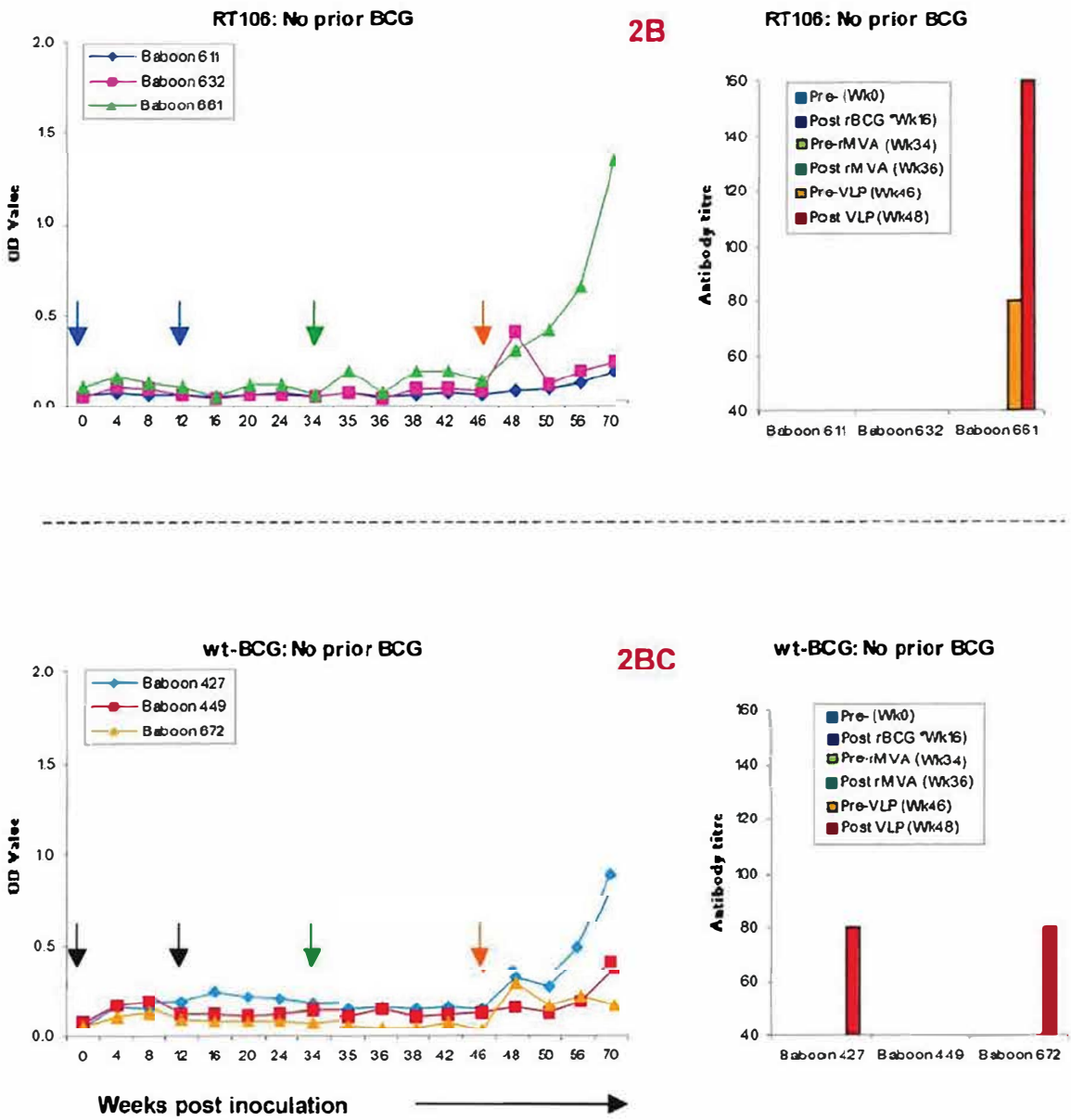
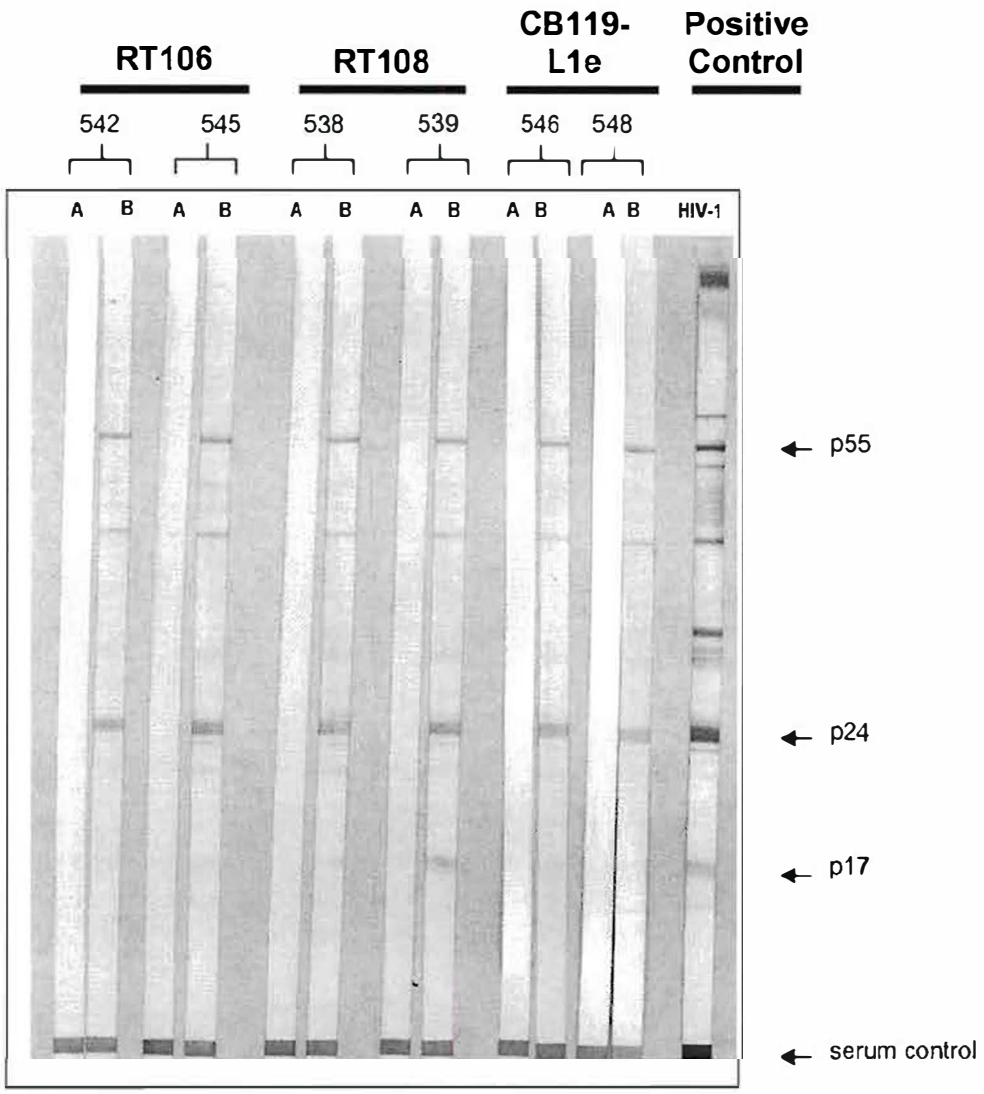


Figure 4.4: Western blot analysis (Exp1). Baboon sera taken at pre-inoculation (Lane A) and 4 weeks post the 2nd GagC VLP inoculation (Lane B) from baboons primed with rBCG:RT106 or rBCG:RT108, or mock-primed with rBCG:CB119-L1e were used at a 1:40 dilution. A positive control (HIV-1) comprised of a human serum positive for anti-HIV-1 antibodies (provided in the kit) used at a dilution of 1:100. The positions of the Gag proteins and the internal serum control for the strips are indicated by the arrows (←). (RT106: rBCG:RT106; RT108: rBCG:RT108; CB119-L1e: rBCG:CB119-L1e)



4.3.3 CELLULAR RESPONSES

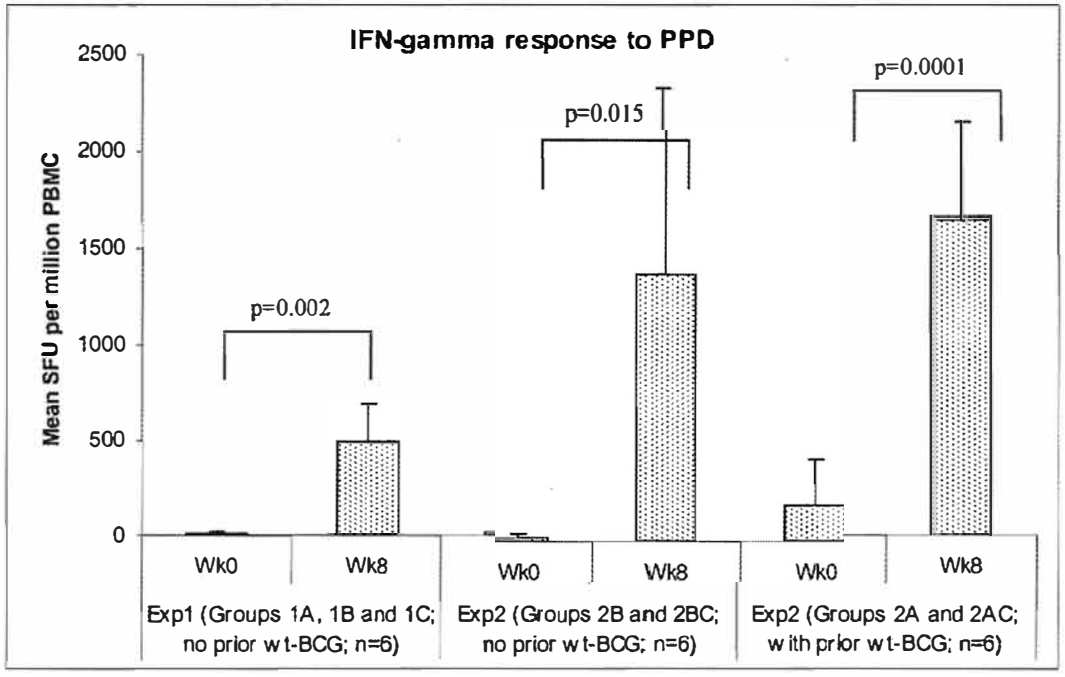
4.3.3.1 IFN-γ ELISPOT RESPONSE

IFN-γ response to PPD

PPD-specific responses were quantified by an IFN-γ ELISPOT assay at two time points, pre-immunisation (Wk0) and at 8 weeks post inoculation for all the baboons in both experiments (**Fig 4.5**). At week 0 (pre-immunisation), the mean IFN-γ response to PPD was 5, (median: 0; maximum range: 27), 16 (median: 7; range: 3-63) and 191 (median: 89; range: 23-668) SFU/10^6 PBMC for the baboons in Exp1 (Groups 1A, 1B and 1C; n=6) and Exp2 (Groups 2B and 2BC; n=6) which had no prior

exposure to wt-BCG and those with prior wt-BCG exposure in Exp2 (Groups 2A and 2AC; n=6) respectively. At 8 weeks post the 1st rBCG or wt-BCG inoculation, the mean IFN-γ response to PPD rose significantly ($p<0.05$) to 517 (median: 535; range: 206-750; Groups 1A, 1B and 1C), 1390 (median: 1485; range: 165-2457; Groups 2B and 2BC) and 1668 (median: 1447; range: 1247-2483; Groups 2A and 2AC) SFU/10^6 PBMC.

Fig 4.5: IFN-γ response to PPD. Baboons that had prior (Exp2; Groups 2A and 2AC) or no prior (Expl, all Groups, and Exp2; Groups 2B and 2BC) experimental exposure to wt-BCG were inoculated with rBCG:RT106, rBCG:RT108, rBCG:CB119-L1e or wt-BCG before boosting with SAAVI MVA-C or/and GagC VLP as indicated in **Fig 4.1**. Experimental exposure to wt-BCG was done 3 years before the start of the present study by two intradermal inoculations (see Chapter 3). In the present study, cryo-preserved PBMC obtained from baboons at pre- (Wk0) and post- inoculation (Wk8) were used in a standard IFN-γ ELISPOT assay using bovine PPD at a final concentration of 5 μg/mL. Data points are mean IFN-γ responses to PPD and error bars represent the standard deviation of mean response in each group. A p value is given to indicate that the difference between week 0 and week 8 post inoculation mean IFN-γ response is significant. (Exp: Experiment; Wk: week)



IFN-γ response to Gag

HIV-1 Gag-specific responses were quantified by an IFN-γ ELISPOT assay at pre-immunisation and several different time points post inoculation. The results are shown in **Figs 4.6** (Exp1) and **4.7** (Exp2). The responses were recorded as the net IFN-γ SFU per million PBMC across all the 5 Gag peptide pools. The two experiments are discussed separately below.

Experiment 1: IFN-γ response to Gag

In the first baboon experiment (Exp1), the two baboons in each group were primed with rBCG:RT106, rBCG:RT108 or mock-primed with rBCG:CB119-L1e vaccines respectively by 4 successive intradermal inoculations at weeks 0, 14, 24 and 40 post inoculation (**Fig 4.1**). A cut-off value for a

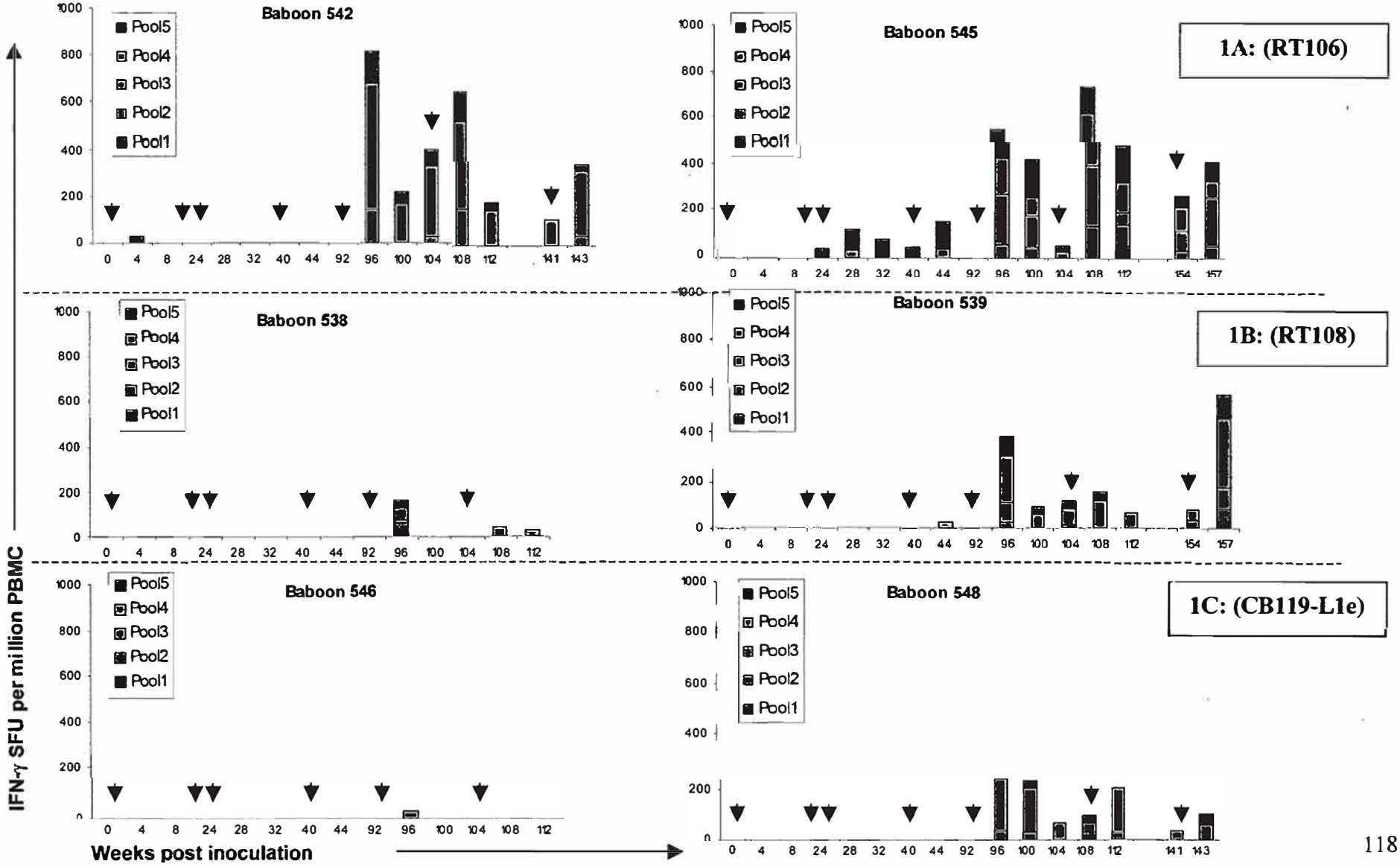
positive response to each peptide pool in the IFN-γ ELISPOT assay was 24 IFN-γ SFU/10^6 PBMC (established as previously described in **Appendix C.6**). A consistent but weak Gag-specific cellular immune response was elicited in only one baboon (545) that had been primed with rBCG:RT106 vaccine (**Fig** 4.6). For this baboon, the cumulative IFN-γ response rose to a peak of 157 SFU/10^6 PBMC at Wk 44, four weeks post the 4th rBCG:RT106 inoculation. This response was directed to Gag peptide pools number 4 and 5. The response declined to pre-immunisation level by week 92. Two other baboons (542 and 539), had weak response to Gag peptide pool number 4 only at weeks 4 and 44 respectively. None of the other baboons in this study developed Gag-specific response (**Fig 4.6**).

A GagC VLP booster inoculation was given to all baboons at week 92 (1yr after the last primary inoculation). By Wk 96, all the baboons had developed a response to Gag (**Fig 4.6**). The median cumulative IFN-γ response was 677 (range: 542-812) and 274 (range: 156-391) SFU/10^6 PBMC for rBCG:RT106- and rBCG:RT108-primed baboons respectively at this time point and the difference in magnitude of response between the two groups was not significant (p=0.15). This response was directed to 3 or 4 Gag peptide pools. For the mock-primed baboons, the median cumulative Gag response at week 96 was a maximum of 240 SFU/10^6 PBMC (range: 30-240) and predominantly directed to Gag peptide 3. For all the baboons, the response declined quickly following the peak response at week 96 and by week 104 (when the 2nd GagC VLP inoculation was given), the cumulative IFN-γ response had waned considerably and undetectable for some baboons (**Fig 4.6**).

A second GagC VLP booster immunisation was given to all the baboons at week 104. At 4 weeks (Wk108) after the 2nd Gag VLP booster, the median cumulative IFN-γ responses increased substantially to 686 (range: 642-730) for rBCG:RT106-primed baboons and slightly to 98 (range: 40-155) SFU/10^6 PBMC for the rBCG:RT108-primed baboons. For the baboons that had received a CB119-L1 primary inoculation, 1 of 2 baboons (baboon 546) did not respond to 2nd GagC inoculation and the peak response in the sole responder (baboon 548) delayed to Wk112.

A third GagC VLP booster inoculation was given to 4 of 6 baboons at week 141 (baboons 542 and 548) and 154 (baboons 539 and 545) post initial inoculation. Analysis of IFN-γ ELISPOT data showed that the median cumulative IFN-γ response at the time of boosting was 73 SFU/10^6 PBMC (range: 35-110) for baboons 542 and 548 and 174 SFU/10^6 PBMC (range: 76-271) for baboons 539 and 545 (**Fig 4.6**). Following the third GagC VLP inoculation, these responses increased to median cumulative IFN-γ response of 222 (range: 102-341) and 488 (range: 411-564) SFU/10^6 PBMC for the two groups respectively. These data represents a boosting effect for baboon 539 (increase from 76 to 564 SFU/10^6 PBMC) and slight to moderate increases in cumulative IFN-γ for the other three baboons.

Fig 4.6: Experiment 1: IFN-γ response to Gag. Cryo-preserved PBMC obtained from baboons at pre- (Wk0) and post- inoculation with rBCG:RT106 (**1A**), rBCG:RT108 (**1B**) or rBCG:CB119-L1e (**1C**) and GagC VLP were used in a standard IFN-γ ELISPOT assay. Five Gag peptide pools (Pool 1-5) were used for antigenic stimulation for 22-24h at a final concentration of 4 μg/mL as previously described (section **2.2.5.3**). Data points are the magnitudes of IFN-γ responses to individual Gag peptide pools in which the value was equal or greater than the cut-off value of 24 IFN-γ SFU/10^6 PBMC. The arrows indicate inoculation times with rBCG:RT106 or rBCG:RT108 (↓), rBCG:CB119:L1e (↓) and GagC VLP (↓).



Experiment 2: IFN-γ response to Gag

Although there was no significant difference (p=0.15; probably the group size was too small to have good statistical power) between the mean cumulative IFN-γ response to Gag in rBCG:RT106- and rBCG:RT108-primed baboons in Exp1 at peak response (at week 96 following GagC VLP immunisation), the overall IFN-γ ELISPOT data strongly suggested that rBCG:RT106 vaccine induced better IFN-γ responses to Gag than the rBCG:RT108 vaccine (**Fig 4.6**). Moreover, rBCG:RT106 alone, before immunisation with the GagC VLP vaccine, induced consistent IFN-γ responses following the second prime immunisation in 1 of 2 baboons. Therefore, the rBCG:RT106 vaccine was chosen as the primary vaccine to prime the immune response in the second baboon experiment (Exp2). Six baboons (groups **2A** and **2B**) received 2 successive primary inoculations with rBCG:RT106 while 6 control baboons (groups **2AC** and **2BC**) were given wt-BCG in the same way. Three animals from each group had had 2 wt-BCG inoculations (groups **2A** and **2AC**) 3 years before the start of the current experiment (see **Table 4.1**). Subsequently, all baboons were boosted with SAAVI MVA-C (Wk34) and GagC VLP (Wk46). A cut-off value for mean IFN-γ response to Gag peptide pools at pre-immunisation was 32 SFU/10^6 PBMC.

Following immunisation with rBCG:RT106 or wt-BCG at weeks 0 and 12, no Gag-specific response could be detected for any of the 12 baboons (**Figs 4.7A** and **4.7B**). One week (Wk35) following SAAVI MVA-C booster immunisation, 4 of 6 rBCG:RT106-primed baboons (combined groups **2A** and **2B**) responded modestly to Gag (range of cumulative IFN-γ response: 33-223 IFN-γ SFU/10^6 PBMC). This response declined by week 36 with only 2 of 6 rBCG:RT106-primed baboons showing a response (range: 92-238 IFN-γ SFU/10^6 PBMC). For the wt-BCG-primed baboons (combined groups **2AC** and **2BC**), 3 of 6 baboons initially responded to SAAVI MVA-C inoculation at week 35 (range of cumulative IFN-γ response: 72-555 SFU/10^6 PBMC) and all 6 of 6 baboons showed varying magnitudes of response to Gag by week 36 (ranging from 44-1420 IFN-γ SFU/10^6 PBMC). The peak responses to Gag were reached between 1 and 2 weeks following SAAVI MVA-C inoculation. At this time points, the difference in the mean cumulative IFN-γ response between the rBCG:RT106-primed and wt-BCG mock-primed groups was not significant (p=0.36). The response to Gag was undetectable for 8 of 12 baboons by week 46. All the baboons showed a response to Gag following GagC VLP booster immunisation at week 46. At week 48 (2 weeks after GagC VLP inoculation), the median cumulative IFN-γ response was 441 (range: 92-1880) and 588 (range: 86-3852) SFU/10^6 PBMC for rBCG:RT106- and wt-BCG- primed baboons respectively and the difference between the two groups was not significant (=0.90). The peak responses were reached between 2 and 4 weeks after GagC VLP inoculation in majority of baboons. By week 70 when the study was terminated, the response to Gag, although it had declined, was still detectable in 6 of 12 baboons in the study.

Fig 4.7A: Experiment 2: IFN-γ response to Gag. Baboons that had prior experimental exposure to wt-BCG were inoculated twice (at weeks 0 and 12) with either rBCG:RT106 **(2A)** or wt-BCG **(2AC)** and boosted with SAAVI MVA-C (Week 34) and GagC VLP (Week 46) as indicated by arrows. Experimental exposure to wt-BCG was done 3 years before the start of the current study by intradermal inoculation (see Chapter 3). Cryo-preserved PBMC obtained from baboons at pre- (Wk0) and post- inoculations were used in a standard IFN-γ ELISPOT assay as described (section **2.2.5.3**). Data points are the magnitudes of IFN-γ responses to individual Gag peptide pools in which the value was equal or greater than the cut-off value of 32 IFN-γ SFU/10^6 PBMC.

(RT106: rBCG:RT106; rMVA: SAAVI MVA-C)

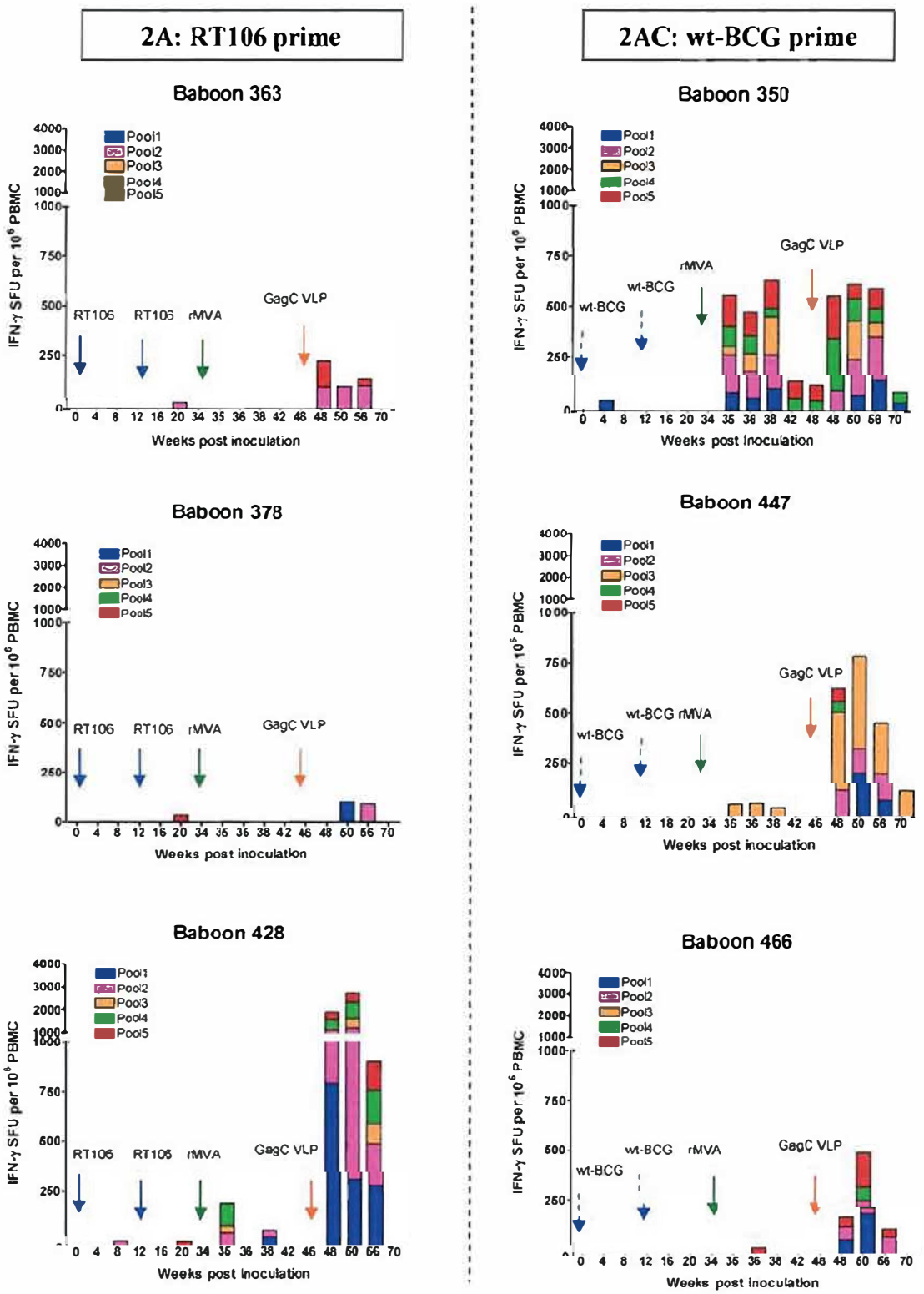
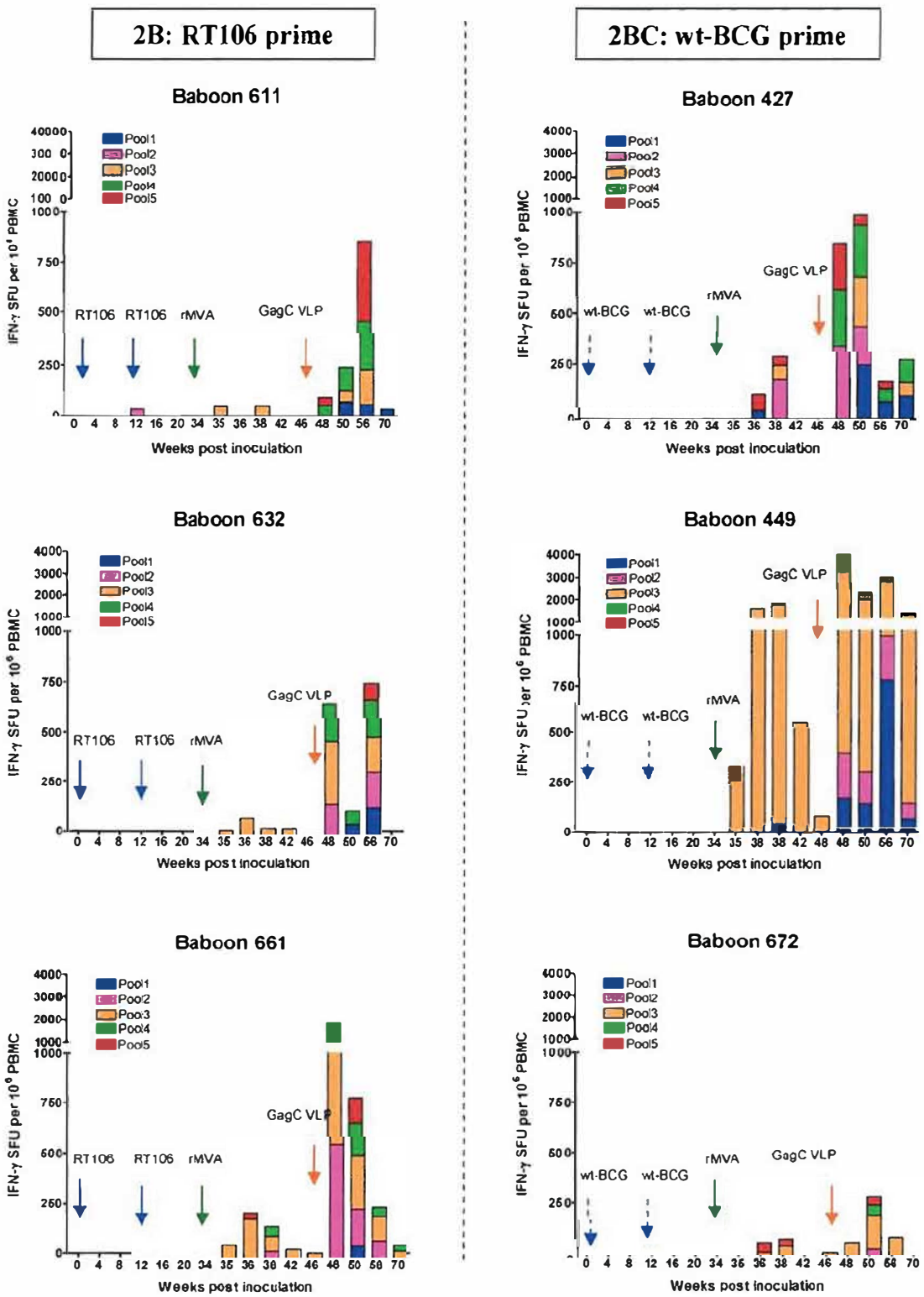


Fig 4.7B: Experiment 2: IFN-γ response to Gag. Baboons that had not been experimentally exposed to wt-BCG were inoculated twice (at weeks 0 and 12) with either rBCG:RT106 (**2B**) or wt-BCG (**2BC**) and boosted with SAAVI MVA-C (Week 34) and GagC VLP (Week 46) as indicated by arrows. Cryo-preserved PBMC obtained from baboons at pre- (Wk0) and post- inoculations were used in a standard IFN-γ ELISPOT assay as described (section **2.2.5.3**). Data points are the magnitudes of IFN-γ responses to individual Gag peptide pools in which the value was equal or greater than the cut-off value of 32 IFN-γ SFU/10^6 PBMC.

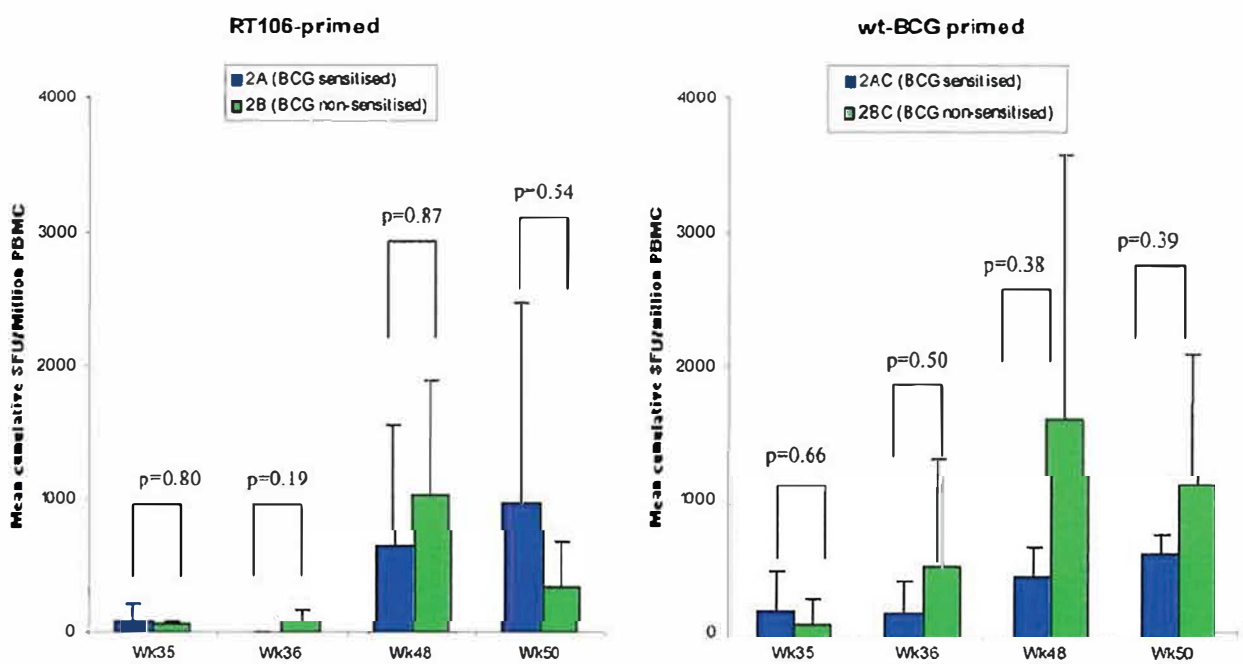
(RT106: rBCG:RT106; rMVA: SAAVI MVA-C)



Comparison of IFN-γ responses to Gag: BCG sensitised versus non-sensitised

Following a boost with SAAVI MVA-C and GagC VLP vaccines, comparisons of mean cumulative IFN-γ responses to Gag were made between the baboon groups that had prior experimental exposure to BCG and those that did not. Since the peak responses to Gag were found to occur in the majority of baboons at 1-2 weeks and 2-4 weeks following SAAVI MVA-C and GagC VLP inoculations respectively, IFN-γ ELISPOT data obtained at weeks 35, 36, 48 and 50 were chosen for this comparison. The following groups were compared: group **2A** versus **2B** (both primed with rBCG:RT106) and group **2AC** versus **2BC** (both mock-primed with wt-BCG). Groups 2A and 2AC were experimentally exposed to BCG prior to start of the experiment whereas groups **2B** and **2BC** were not exposed (see **Table 4.1**). The mean cumulative IFN-γ responses for each group at the chosen time points are shown in **Fig 4.8**. As shown, the difference in mean cumulative IFN-γ responses between the BCG-exposed (**2A** & **2AC**) and the non-exposed (**2B** & **2BC**) baboons were insignificant ($p>0.05$) at all the 4 time points following SAAVI MVA-C and GagC VLP inoculations.

Fig 4.8: Comparison of cumulative IFN-γ responses between BCG sensitised and non-sensitised groups. The differences in mean cumulative IFN-γ responses to Gag at the indicated time points for groups **2A** versus **2B** and **2AC** versus **2BC** were analysed by t-test. The p values for the t test are shown.



4.3.3.2 PEPTIDE MAPPING BY POOL-MATRIX IFN-γ ELISPOT ASSAY

Further investigations were carried out to identify the Gag peptides to which the IFN-γ responses (observed in Section **4.3.2.1**) were directed. This was done for baboons in Exp1. After the 1st GagC VLP immunisation the Pool-Matrix IFN-γ ELISPOT assay was employed as previously described. The

identity of peptides to which the PBMC were responding, including the magnitude of response are shown in **Table 4.1**.

Table 4.1: Identity and amino acid sequences of Gag peptides that were recognised by baboon PBMC (Exp1) in an IFN-γ ELISPOT assay. Freshly isolated PBMC obtained at Week 96 (4 weeks after the 1st GagC VLP inoculation) were used in a Pool-Matrix IFN-γ ELISPOT strategy to identify the peptides to which baboon PBMC were responding. The responses were confirmed in a subsequent IFN-γ ELISPOT assay using cryo-preserved PBMC. The data is give as the net IFN-γ SFU/10^6 after subtracting the background response (in the absence of peptide) and a cut-off value of 24 IFN-γ SFU/10^6 PBMC was used. The peptide sequences shown in red indicate the ones that have been reported as being targeted by PBMC from individuals with early HIV-1 subtype C infection (Masemola *et al*, 2004a and 2004b).

Inoculation regimen	Baboon number	Gag peptides identified after GagC VLP inoculation				Response (IFN-γ SFU/106 PBMC)
		Gag region	Peptide pool	Peptide number	Amino acid sequence	
RT106/GagC VLP	542	p24	P3	30	LHPVHAGPIAPGQMREPR	245
		p2p7p1p6	P4	53	KGPKRIVKCFNCGKEGHI	90
				↑54	CFNCGKEGHIARNCRAPR	613
				↑55	HIARNCRAPRKKGCWK	255
				↑56	RAPRKKGCWKCGKEGHQM	250
			P5	↑57	WKCGKEGHQMKDCTERQA	185
				↑58	QMKDCTERQANFLGKIW	110
				66	KDREPLTSLKSLFGSDPLSQ	260
	545	p24	P2	23	AFSPEVIPMFTALSEGA	25
			P3	36	PVGDIYWKRWIILGLNKIV	70
				↑37	WIILGLNKIVRMYSPVSI	55
				↑38	IVRMYSPVSILDIKQGPK	105
				40	GPKEPFRDYVDRFFKTLR	70
				↑41	YVDRFFKTLRAEQATQDV	43
		p2p7p1p6	P4	54	CFNCGKEGHIARNCRAPR	160
			P5	66	KDREPLTSLKSLFGSDPLSQ	150
RT108/GagC VLP	538	p24	P3	38	IVRMYSPVSILDIKQGPK	42
	539	p24	P3	36	PVGDIYWKRWIILGLNKIV	27
		p2p7p1p6	P4	54	CFNCGKEGHIARNCRAPR	342
				56	RAPRKKGCWKCGKEGHQM	27
			P5	↑57	WKCGKEGHQMKDCTERQA	110
				66	KDREPLTSLKSLFGSDPLS	155
CB119-L1e/GagC VLP	546*	NT	NT	NT	NT	NT
	548	p24	P3	40	GPKEPFRDYVDRFFKTLR	222
				↑41	YVDRFFKTLRAEQATQDV	287

*: Gag-specific response for baboon 546 was too small in magnitude to allow detection of individual Gag peptide; NT: not tested: RT106: rBCG:RT106 vaccine; RT108: rBCG:RT108 vaccine; CB119-L1e: rBCG:CB119-L1e vaccine (control); P: Gag peptide pool; p: protein; ↑: shows peptides that contain amino acid sequences that overlap with an adjacent one, indicating the possibility of sharing the same epitope.

For Exp2, due to shortage of cells, only a few of the responses to individual Gag peptides predicted by the Pool-Matrix IFN-γ ELISPOT assay were confirmed in further IFN-γ ELISPOT assay. The Gag peptides that were predicted by the Pool-Matrix IFN-γ ELISPOT assay and those confirmed in a further IFN-γ ELISPOT assay are shown in **Table 4.2**.

Table 4.2: Gag peptides predicted in a Pool-Matrix IFN-γ ELISPOT assay as being recognised by PBMC from immunised baboons in Exp2. Fresh PBMC obtained at week 53 (2 weeks after the 1st GagC VLP inoculation) were used in Pool-Matrix IFN-γ ELISPOT assay. A response was considered positive if the net IFN-γ SFU/10^6 PBMC, after subtracting the background response (in the absence of peptides) was equal or greater than the cut-off value of 32 SFU/10^6 PBMC.

Inoculation regimen	Baboon group	Baboon number	Peptide pools recognised by PBMC	Predicted Gag peptides	Confirmed Gag peptides
RT106/ SAAVI MVA-C / GagC VLP	2B	632	P3,M8,M9,M12	36,37,40	Peptide 36
		661	P2,P3,P4,M9,M10,M13	23,24,27,37,38,41,40, 51,52,55	Peptide 38
wt-BCG/ SAAVI MVA-C / GagC VLP	2BC	449	P3,P4, M12, M13	40, 41, 54, 55	Peptide 40
		672	P3, M7, M8, M12, M13	35, 36, 40, 41, 42	Peptide 41

P: normal peptide pool; M: matrix peptide pool.

4.3.3.3 PHENOTYPES OF CELLS RESPONDING TO GAG PEPTIDES

Experiment 1

Characterisation of the phenotype of the cells responding to individual Gag peptides after GagC VLP booster immunisation was done using anti-CD3-FITC, anti-CD8-APC and intracellular IFN-γ staining followed by flow cytometry. The proportion of CD3+CD8+ cells producing IFN-γ ranged from 0.13 to 1.12% (**Table 4.3**). Only minimal proportions of CD3+CD8- IFN-γ-producing cells (representing CD4+ IFN-γ-producing cells) were observed, indicating that the majority of the IFN-γ-producing T lymphocytes in response to stimulation with the peptides being used in ICS assay belonged to the CD8+ phenotype.

Experiment 2

For this experiment, characterisation of the phenotype of the cells responding to individual Gag peptides after GagC VLP immunisation was done using anti-CD3-FITC, anti-CD4-APC, anti-CD8-APC and intracellular IFN-γ staining followed by flow cytometry. Due to a shortage of cells, not all the individual Gag peptides identified in the Pool-Matrix IFN-γ ELISPOT assay were confirmed by a further ELISPOT assay. Only the Gag peptides that were confirmed were used in an intracellular cytokine assay to identify the phenotype of responding lymphocytes. The proportion of IFN-γ-producing lymphocytes were very low except in baboon 449 (**Table 4.4**). For this baboon (449), following SAAVI MVA-C and GagC VLP immunisations, 0.35% and 2.32% of CD3+CD8+ cells respectively produced IFN-γ in response to stimulation with Gag peptide number 40 (**Fig 4.9**). The CD4+ lymphocytes showed no IFN-γ response.

Table 4.3: Phenotype of baboon PBMC responding to individual Gag peptides (Exp1). Cryo-preserved baboon PBMC obtained at week 96 were incubated for 16h with selected Gag peptides in an ICS assay. The cells were labelled with anti-CD3-FITC (BioSource), anti-CD8-APC (BD) and anti- IFN-γ-PE (BD), then subjected to flow cytometry to detect CD3+CD8+ cells producing IFN-γ. IFN-γ responses by ELISPOT assay and ICS assays are shown in the table.

Primary inoculation	Baboon number	Identified peptide	Peptide pool of identified peptide	IFN-γ response		
				ELISPOT[a] *(SFU/10⁶ PBMC)*	*ICS assay*[a] *%CD3+CD8+ producing IFN-γ*	*ICS assay*[a] *%CD3+CD8- producing IFN-γ*
RT106	542	54	P4	613	0.41	0.07
	545	54	P4	160	0.13	0.00
		66	P5	150	0.36	0.00
RT108	538	38	P3	42	NT	NT
	539	54	P4	342	1.12	0.32
		66	P5	155	0.61	0.19
CB119-L1e	546	NT	NT	NT	NT	NT
	548	40	P3	222	0.40	0.00
		41	P3	287	0.47	0.00

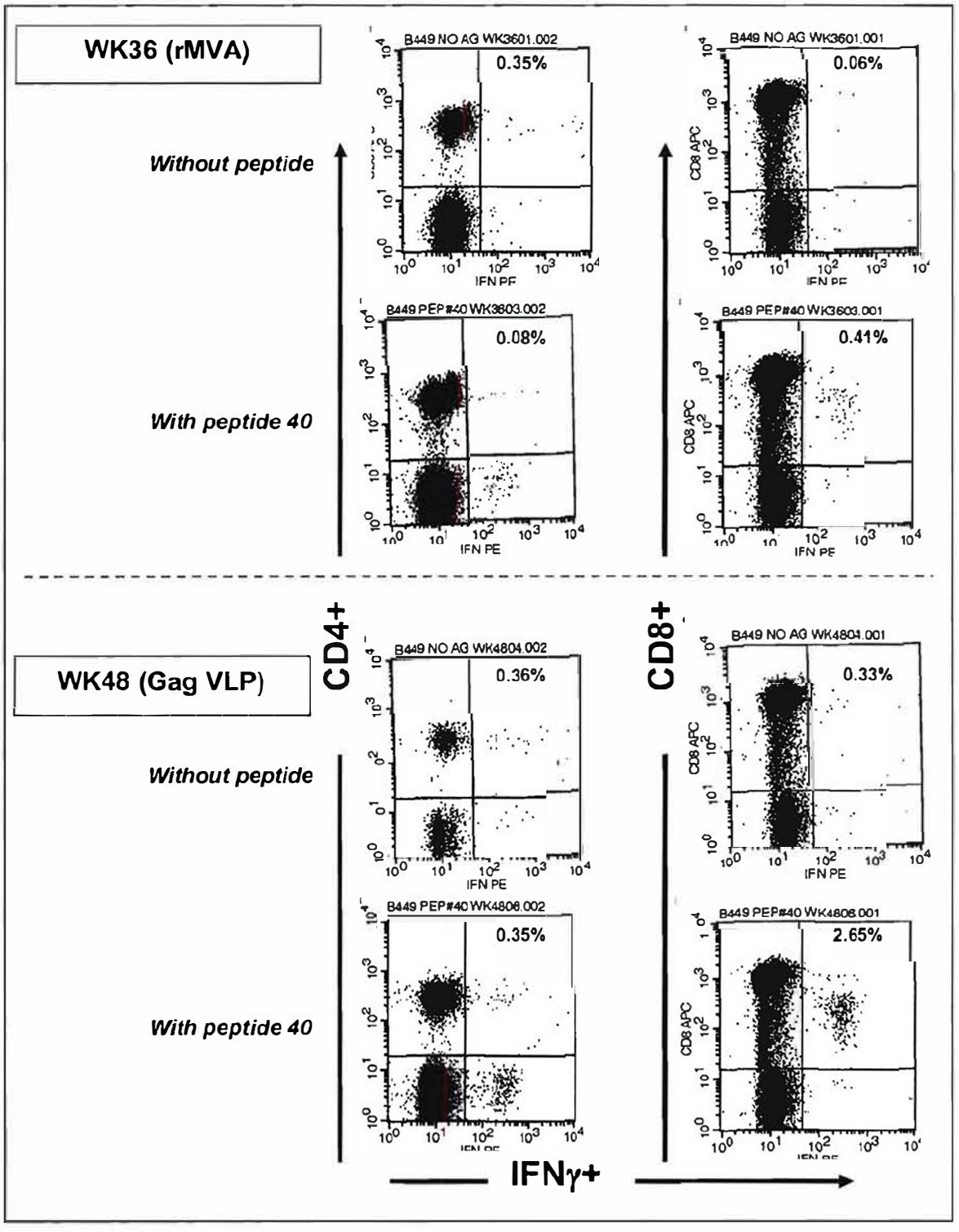
[a]: Net IFN-γ response after subtracting background response; NT: not tested

Table 4.4: Phenotype of baboon PBMC responding to individual Gag peptides (Exp2). Cryo-preserved baboon PBMC obtained at weeks 36 and 48 (2 weeks post SAAVI MVA-C and GagC VLP inoculations respectively) were incubated for 16h with selected Gag peptides in an ICS assay. The cells were labelled with anti-CD3-FITC (BioSource), anti-CD4-APC (BD), anti-CD8-APC (BD) and anti- IFN-γ-PE (BD), then subjected to flow cytometry to detect CD3+CD8+ cells producing IFN-γ. IFN-γ responses by ELISPOT assay and ICS assays are shown in the table.

Primary inoculation	Baboon number	Time point	Identified peptide	IFN-γ response		
				ELISPOT[a] *(SFU/10⁶ PBMC)*	*ICS assay*[a] *%CD4+ producing IFN-γ*	*ICS assay*[a] *%CD3+CD8+ producing IFN-γ*
RT106	632	Wk36	36	73	0.00	0.00
		Wk48	36	117	0.12	0.00
	661	Wk36	38	103	0.06	0.00
		Wk48	38	117	0.10	0.00
wt-BCG	449[b]	Wk36	40	1393	0.00	0.35
		Wk48	40	3047	0.00	2.32
	672	Wk36	41	72	0.11	0.04
		Wk48	41	83	0.03	0.09

[a]: Net IFN-γ response after subtracting background response; [b]: more details on ICS data for baboon 449 shown in **Fig 4.9**; Wk36: 2 weeks post SAAVI MVA-C inoculation; Wk48: 2 weeks post GagC VLP inoculation.

Fig 4.9: Intracellular IFN-γ staining and FACS analysis (Baboon 449-Exp2). Baboon 449, which had no prior BCG exposure, was inoculated with wt-BCG at weeks 0 and 12 and then boosted with SAAVI MVA-C and GagC VLP vaccines at weeks 34 and 48 respectively. Cryo-preserved PBMC obtained at 2 weeks post- SAAVI MVA-C (Wk36) or GagC VLP (Wk48) inoculation were incubated with or without Gag peptide number 40 (identified to be strongly responsive in an IFN-γ ELISPOT assay – see **Table 4.4**) for 16h with addition of Brefeldin A for the last 14h. The cells were stained as previously described and data acquired and analysed by FACSCalibur cytometer and CellQuest software (BD) respectively. The data is presented dot-plot graphs. The figures in the upper right box of each graph give the percentage of CD4+ or CD3+CD8+ IFN-γ-producing cells (also see **Table 4.8**).



4.3.3.4 CYTOKINE ASSAY BY CBA

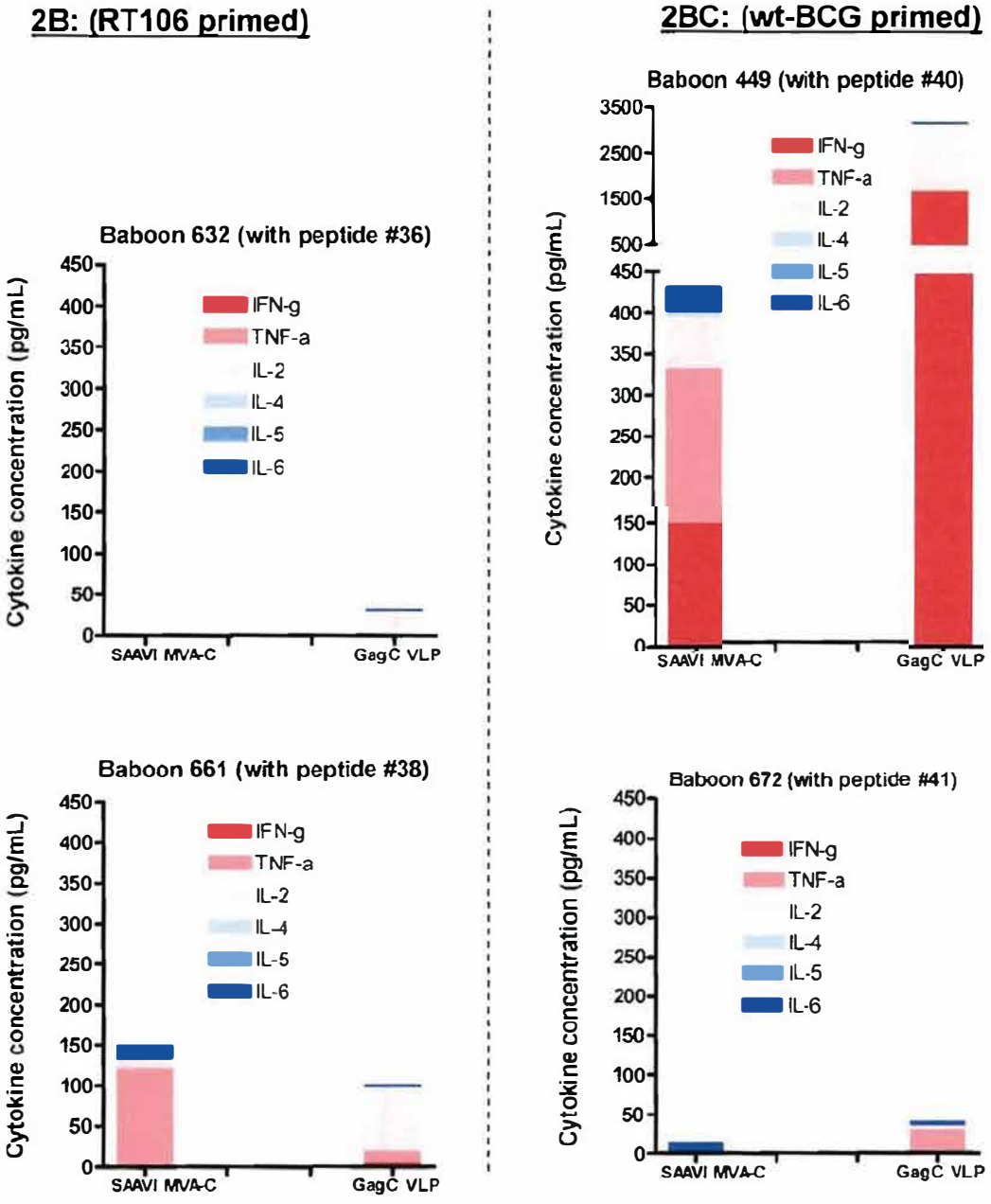
The spectrum of cytokines (IFN-γ, TNF-α, IL-2, IL-4, IL-5 and IL-6) released into the extracellular medium by PBMC during stimulation with specific Gag peptides was evaluated using the CBA assay kit (Nonhuman primate Th1/Th2™, BD). Individual Gag peptides identified using Pool-Matrix IFN-γ ELISPOT assays were used to stimulate PBMC in 16-h incubations. The results for PBMC from four baboons, 632 and 661 (group 2B- rBCG:RT106 primary inoculation) and 449 and 672 (group 2BC- wt-BCG primary inoculation) that were tested are shown in **Fig 4.10**.

For baboon 449, low amounts of IFN-γ and IL-2 (151 and 61 pg/mL respectively) were produced by PBMC obtained at week 36 (2 weeks following SAAVI MVA-C immunisation) prior to GagC VLP. A substantial amount of TNF-α (183 pg/mL) was also produced after SAAVI MVA-C inoculation but not after GagC VLP immunisation. A high level of IFN-γ (1703 pg/mL) was measured for PBMC obtained at week 48 (2 weeks following immunisation with GagC VLP). At the same time point, a high level of IL-2 (1453 pg/mL) was also measured for this baboon (449). Less than 30 pg/mL of IL-4, IL-5 or IL-6 were produced in both time points. For the PBMC from the other 3 baboons (baboons 632, 661 & 672), only modest amounts of TNF-α (32 & 123 pg/mL for baboons 672 and 661 respectively) and IL-2 (31 & 77 pg/mL for baboons 632 and 661 respectively) were produced following SAAVI MVA-C or GagC VLP immunisations. Almost negligible amounts (<16 pg/mL) of IL-4, IL-5 or IL-6 were produced by PBMC from these baboons. In general, the CBA data showed that only Th1 cytokines (IFN-γ, TNF-α and IL-2) and little or negligible amounts of Th2 cytokines (IL-4, IL-5 and IL-6) were produced by baboon PBMC upon Gag peptide stimulation following immunisation with SAAVI MVA-C and GagC VLP vaccines.

4.4 DISCUSSION

This study investigated the potential use of rBCG HIV-1 vaccines, expressing subtype C HIV-1 Gag in a prime-boost vaccination strategy with $Pr55^{gag}$ VLP and rMVA expressing matched HIV-1 Gag protein. The rBCG vaccines, rBCG:RT106 and rBCG:RT108, were made in an identical manner except that two different promoters were used to drive the expression of HIV-1 Gag in the two shuttle vectors. rBCG:RT106 used the *mtrA* promoter while rBCG:RT108 used the *katG* promoter. However, both vectors were episomal and they used an identical localisation signal sequence, 19kDa lipoprotein from *M. tuberculosis* (Thomas, 2005). The $Pr55^{gag}$ VLP vaccine (GagC VLP) was produced via the baculovirus expression system using the standard procedures (Jaffray *et al*, 2004). The rMVA vaccine (SAAVI MVA-C) was manufactured by Therion Biologics (USA) for pre-clinical evaluation using a Good Manufacturing Practice (GMP) protocol. SAAVI MVA-C is a multigene expressing *gag* (identical to the gene in the rBCG and $Pr55^{gag}$ VLP vaccines), *pol* (RT), *tat*, *nef* and *env* of HIV-1 subtype C.

Fig 4.10: Array of cytokines produced by baboon PBMC upon stimulation with specific Gag peptides and detection by CBA assay (Exp2). Cryo-preserved PBMC obtained from rBCG:RT106 primed (baboons 632 and 661; group 2B) or wt-BCG mock primed (baboons 449 and 672; group 2BC) baboons at 2 weeks post- SAAVI MVA-C (Wk36) or GagC VLP (Wk48) inoculation were incubated with individual Gag peptides (identified using the Pool-Matrix IFN-γ ELISPOT assay) as indicated. The supernatants were harvested after a16h-incubation and the amounts of IFN-γ, TNF-α, IL-2, IL-4, IL-5 and IL-6 quantified simultaneously using a commercially available CBA kit (Nonhuman primate Th1/Th2, BD).



4.4.1 REACTOGENICITY AFTER VACCINATION

Two baboon experiments were conducted in which the experimental groups were vaccinated intradermally with either rBCG:RT106 or rBCG:RT108 as the primary rBCG vaccines. The control groups were vaccinated with either rBCG expressing HPV-16 L1 capsid protein (Exp1) or wt-BCG (Exp2). Both experimental and control groups were then boosted with either two successive

inoculations of HIV-1 GagC VLP (Exp1) or with a single inoculation each of SAAVI MVA-C and Gag VLP (Exp2) vaccines. These vaccinations were well tolerated and no systemic reactions were noted in any baboon. Both wt-BCG and various rBCG vaccines induced similar local tissue reactions, which were characterised by development of ulcerations at the injection sites. These ulcerations resolved without need for medication. Local cutaneous reactions and ulcerations, which are usually well tolerated, are also known to develop at inoculation sites in humans vaccinated intradermally with BCG (Ravn *et al*, 1997; Kemp *et al*, 1996; Hoft *et al*, 1999).

4.4.2 IMMUNE RESPONSES INDUCED BY rBCG, SAAVI MVA-C and GagC VLP VACCINES

4.4.2.1 HUMORAL RESPONSE TO GAG

Gag-specific humoral responses in both Exp1 and Exp2 were largely undetectable after immunisation with either rBCG:RT106 or rBCG:RT108 vaccines. However, after the 1st Gag VLP immunisation, anti-HIV-1 p24 antibodies were consistently detected in Exp1 for the baboons receiving prime-boost (mixed modality) immunisation. In contrast, the Gag humoral responses for the mock-primed controls (single modality; rBCG:CB119-L1e-inoculated) were detectable only after the 2nd GagC VLP immunisation. This finding suggests that both rBCG:RT106 and rBCG:RT108 were able to prime the immunised animals for GagC VLP immunisation although the responses were undetectable by ELISA method before the GagC VLP inoculation. In Exp2, the humoral response was not detected until after the GagC VLP immunisation and even then, the response was weak and detectable in only a third of the immunised baboons.

Antibodies to p55 and p24 were readily detected by western blot assay but antibodies to p17 were either absent or weakly detected. This is in contrast to the finding in the pTH-GagC/GagC VLP study whereby all the three proteins were strongly detected. This finding suggests that p17 was not readily expressed by rBCG:RT106 and rBCG:RT108.

4.4.2.2 IFN- ELISPOT RESPONSE TO PPD

In both experiments, it was shown by IFN-γ ELISPOT assay that vaccination with either rBCG vaccines or wt-BCG induced strong PPD responses by the 8th week after the initial inoculation. Prior sensitisation to wt-BCG (Exp2) did not inhibit the induction of these responses and baboons with prior BCG sensitisation were observed to develop similar magnitudes of IFN-γ responses to PPD as the non-sensitised baboons. In addition, these responses to PPD were long-lived and detectable more than 2 years after initial inoculation (data not shown). These results served to show that the intradermal inoculation route was successful in delivering the rBCG vaccines to the immune system.

4.4.2.3 IFN-γ ELISPOT RESPONSE:- Exp1

Development of cellular immune responses was observed in both Exp1 and Exp2 but detected at varying degrees. In Exp1, the rBCG:RT106, but not rBCG:RT108, induced very weak but consistently detectable Gag-specific IFN-γ ELISPOT responses in 1 of 2 immunised baboons. However, strong responses were elicited by a single GagC VLP immunisation after 1 year and they were directed broadly to peptides in 3 or 4 peptide pools. A second GagC VLP inoculation did not appear to improve the primary cellular immune response. The identification of peptides in the major peptide pools that were being recognised by baboon cells revealed that 3 of 4 rBCG:Gag primed baboons responded to a wider range of Gag peptides (5-8 peptides contained in 2-4 different peptide pools) than the mock-primed controls where the response was restricted to 2 peptides in one peptide pool for the one baboon (548) that developed a sufficient cellular immune response to Gag to permit peptide mapping. The ELISPOT data provide evidence that the rBCG:Gag vaccine elicits a strong Gag-specific immune response that is broadly directed when used with GagC VLP in a prime-boost vaccination modality. In addition, the data show that the immune memory induced by the rBCG:Gag prime vaccination as well as the GagC VLP inoculation were long-lived as it must have been maintained for a year between the last rBCG:Gag prime (Wk40) and the 1st GagC VLP boost (Wk92) and also for 37-50 weeks after the 2nd GagC VLP inoculation.

4.4.2.4 IFN-γ ELISPOT RESPONSE:- Exp2 (Triple vector vaccination)

In Exp2, a triple vector vaccine strategy comprising of rBCG, SAAVI MVA-C and GagC VLP was tested as well as the possible role of pre-existing anti-BCGr immunity. In this experiment, baboons with or without prior experimental exposure to wt-BCG were inoculated twice with either rBCG:Gag (rBCG:RT106) or wt-BCG (control), followed by sequential immunisation with SAAVI MVA-C and GagC VLP. In the experimental group, the rBCG:RT106 vaccine, which appeared to be more immunogenic in Exp1, was given twice 12 weeks apart. No cellular response to Gag was detectable with this inoculation regimen. Even after the SAAVI MVA-C booster immunisation, the cellular response could be detected in only half of the immunised animals. In these baboons where a cellular response was detected, the response was weak except for one baboon (449) where the magnitude was quite high (>1400 SFU/10^6). A consistent positive cellular immune response was obtained for all the immunised baboons only after GagC VLP immunisation. Surprisingly, there was no statistical difference in the mean cumulative IFN-γ response to Gag between the rBCG:RT106-primed and the controls (wt-BCG-primed) after SAAVI MVA-C or GagC VLP immunisation, suggesting that rBCG:RT106 was not effective in priming the immune system.

A triple vector vaccine regimen is expected to result in generation of potent immune responses as it overcomes the potential adverse effect of anti-vector immunity which may be elicited by repeated immunisation with the same vector (Heeney *et al*, 2000). Contrary to this expectation, the triple vector

vaccine regimen (Exp2) in the present study failed to demonstrate any superiority over the double vector prime-boost immunisation protocol (Exp1) in terms of the magnitude of cellular or humoral responses. The reasons of these are not clear but the difference in the number of primary immunisations with rBCG vaccines may provide a possible explanation. The baboons in Exp2 (triple vector) received two immunisations with rBCG:RT106 whereas those in Exp 1 (double vector) were immunised four times. The rBCG:RT106 that was used in both experiments was from the same inoculum stock. Therefore, the difference in the magnitude of IFN-γ ELISPOT response to Gag between the two experiments could possibly be a result of the difference in the number of inoculations of rBCG:RT106 that were used to prime the immune system, suggesting that more than 2 immunisations with rBCG:RT106 were required to confer effective priming of the immune system. This is supported by data from Exp1 which showed that a consistent response to Gag in the sole responder to rBCG:RT106 immunisation was achieved after the 3rd rBCG:RT106 inoculation (Wk24). Moreover, the lack of statistical difference in the mean cumulative IFN-γ response to Gag between the rBCG:RT106-primed and the wt-BCG mock-primed baboons after SAAVI MVA-C or GagC VLP immunisation seems to agree with the explanation that two primary inoculations with rBCG:RT106 is not sufficient to prime the baboon immune system. The implication of this is that further efforts to optimise the expression of vaccine immunogens in rBCG in order to achieve sufficient priming effects with fewer immunisations are justified. Moreover, a single priming immunisation with a BCG-based vaccine would be preferred to multiple immunisations in a clinical setting as this would improve the vaccination compliance among the vaccinees. Besides, BCG vaccination causes substantial local cutaneous ulcerations on injection sites and thus more than one injection with rBCG vaccine might be unacceptable.

An interesting outcome in Exp2 is the observation that baboons in the control group which received wt-BCG, followed by SAAVI MVA-C and finally by GagC VLP developed substantial immune responses. In this baboon cohort, SAAVI MVA-C alone induced a mean cumulative IFN-γ ELISPOT response of 370 ± 538 (range: 44-1420) SFU/10^6 PBMC, which increased to 1025 ± 1413 (range: 86-3852) SFU/10^6 PBMC. This implies that SAAVI MVA-C, a poxvirus vectored vaccine, acted as an effective primary vaccine for the GagC VLP in a prime-boost modality, supporting the value of GagC VLP in boosting immune responses induced by a poxvirus-vectored vaccine. This has the same vaccine concept as ALVAC-HIV and AIDSVAX B/E, the two candidate HIV-1 vaccines currently in phase 3 clinical trials (Pitisuttithum, 2005; Trinvuthipong, 2004), except that the vaccines in the latter case are aimed at inducing neutralising antibodies. This supports the use of GagC VLP in clinical evaluation. Moreover, it is feasible to produce chimeric HIV-1 $Pr55^{gag}$ VLP that include other antigens such as Nef, Pol, V3 loop or CD4 binding region of gp120 (Griffiths *et al*, 1993; Luo *et al*, 1992; Wagner *et al*, 1994) or even the full-length gp160 and gp120 (Deml *et al*, 1997a; Rovinski *et al*, 1992; Vzorov *et al*, 1991) to match the antigens in SAAVI MVA-C, making the results obtained in the

present study both exciting and relevant for development of a multigene VLP-based vaccine as a boost in a prime-boost vaccination regimen with SAAVI MVA-C. In this regard, further development of subtype C HIV-1 $Pr55^{gag}$ VLP may be considered.

4.4.2.5 IFN-γ ELISPOT RESPONSE:- PRIOR BCG SENSITISATION

The question of whether pre-existing immunity to BCG could limit the induction of immune responses by rBCG vaccines has often been asked. To address this, six of the baboons that had been inoculated with wild-type BCG in a previous study (see Chapter 3) were included in the second baboon experiment and used in either rBCG:RT106 prime, SAAVI MVA-C/Gag VLP boost or wt-BCG mock-prime, SAAVI MVA-C/Gag VLP vaccination regimens. Immunisation with rBCG:RT106 did not induce any measurable immune response in the baboons, thus comparison of IFN-γ ELISPOT responses was possible only after boosting with SAAVI MVA-C and GagC VLP vaccines. The mean cumulative IFN-γ responses between the BCG-exposed and the non-exposed baboons did not demonstrate any statistical significant differences in both rBCG:RT106-primed and the wt-BCG mock-primed control groups, suggesting that pre-existing immunity to BCG does not significantly affect the induction of vaccine-specific immune responses by rBCG vaccines. However, due to the failure of the rBCG:RT106 to induce any measurable immune response in any of the baboons before immunisation with SAAVI MVA-C, these results are not conclusive. Further investigation to confirm this finding is desirable. A few animal studies by other researchers have suggested that pre-existing immunity to BCG could adversely affect the efficiency of rBCG vaccines (Palmer & Long, 1966; Brandt *et al*, 2002; Buddle *et al*, 2002) while others have demonstrated enhanced immunologic effects resulting from prior BCG sensitisation (Gheorghiu *et al*, 1994; Kameoka *et al*, 1994).

4.4.2.6 PEPTIDE MAPPING:- RELEVANCE OF THE GAG VACCINES

PBMC from subtype C HIV-1-infected people in southern Africa recognise several epitopes in the conserved regions of Gag protein (Masemola *et al*, 2004a & 2004b). In the present HIV-1 vaccine study, it is shown that several regions of Gag that are targeted by PBMC from humans with early HIV-1 subtype C infection were also targeted by PBMC from baboons immunised with rBCG HIV-1 and $Pr55^{gag}$ VLP vaccines. For the 5 of 6 baboons (Exp1) in which the Gag responses were mapped, 3 of these identified epitope-containing peptide stretches are located in p24 and one in p2p7p1p6 Gag regions. These are: ^{163}AFSPEVIPMFTALSEGA^{179} (Peptide #23; p24; 1 of 5 baboons), ^{257}PVGDIYWKRWIILGLNKIVRMYSPVSI^{282} (Peptides #36 & #37; p24; 2 of 5 baboons), ^{288}GPKEPFRDYVDRFFKTLRAEQATQDV^{313} (Peptides #40 & #41; p24; 2 of 5 baboons) and ^{384}KGPKRIVKCFNCGKEGHI^{401} (Peptide #53; p2p7p1p6; 1 of 5 baboons). The amino acid positions are based on Gag from HIV-1 HXBc2 isolate.

It is noteworthy that all the 5 Gag VLP-vaccinated baboons targeted at least one epitope-containing peptide in p24. However, it was observed that none of the immunised baboons targeted any peptides in the p17 region. This contrasts with data from a previous DNA-prime, Gag VLP-boost vaccine study (described in Chapter 2) and what has been reported in humans infected with subtype C HIV-1 (Masemola *et al*, 2004a) where the greatest cumulative magnitude in Gag-specific IFN-γ ELISPOT response was observed to be directed to epitope-containing peptides in the order p24>p17>p2p7p1p6. This finding suggests that rBCG:RT106 and rBCG:RT108 are not expressing sufficient levels of p17, a view that is reinforced by the observation that antibodies to p17 were largely undetectable in western blot assay. Further investigation aimed at improving the expression of p17 is thus needed.

Another noteworthy finding is that the greatest cumulative magnitude of IFN-γ ELISPOT response for the 3 of 4 baboons (Exp1) that were primed with either rBCG:RT106 or rBCG:RT108 was directed to peptides in p2p7p1p6 region. It is not clear whether this is a reflection of better expression of p2p7p1p6 protein by rBCG and if this is characteristic of cellular response has any beneficial effects. This is a question that may be worth investigating in the future.

In agreement with the findings in Chapter 2, the data from the present study show that ZDU422 isolate from which these vaccines were derived is relevant as a vaccine strain for subtype C HIV-1 infections. More importantly, the data strengthen the view that the baboon is a valuable and unique model for pre-clinical testing of HIV vaccines.

4.4.2.7 PHENOTYPE OF GAG-SPECIFIC CELLS AND CYTOKINE ASSAYS

It has been shown that CD8+ T lymphocytes play a central role in the control of viremia in HIV-1-infected patients and SIV or SHIV-infected monkeys (Amara *et al*, 2005a; Borrow *et al*, 1994; Jin *et al*, 1999; Kaul *et al*, 2000; Koup *et al*, 1994; Migueles *et al*, 2000; Rowland-Jones *et al*, 1995 & 1998; Schmitz *et al*, 1999 & 2005). It is therefore desirable for a HIV vaccine to elicit strong CD8+ T cell response. The phenotype of the cells responding to individual Gag peptides at peak responses following GagC VLP immunisation was investigated in this study by flow cytometry techniques. The Gag peptides used in ICS assays were identified using the Pool-Matrix IFN-γ ELISPOT technique. Although the majority of the baboons that were investigated did not demonstrate strong IFN-γ responses to individual Gag peptides, this study showed that these responses were contributed largely by the CD8+ lymphocytes.

IFN-γ is a crucial cytokine that is associated with CD4+ and CD8+ T cell-mediated immune responses which are key in suppression of virus replication in HIV-1, SIV and SHIV infections (Boaz *et al*, 2002 & 2003; Migueles *et al*, 2000; Rosenberg *et al*, 1997). As such, it has been used widely to assess the

immunogenicity of HIV candidate vaccines both in pre-clinical and human trials. However, more data has emerged recently to show that other cytokines play an important role in synergy with IFN-γ in the control of viral replication in lentiviral infections (Mooij *et al*, 2004; Sadagopal *et al*, 2005; Harari *et al*, 2004; Iyasere *et al*, 2003; Pantaleo & Koup, 2004; Robinson & Amara, 2005). Cell populations that are dual IFN-γ and IL-2 secreting cells have been shown to confer CD4-independent proliferation of HIV-1 specific CD8 T cells (Zimmerli *et al*, 2005) and viral replication control in non-progressive HIV-1 (Emu *et al*, 2005; Harari *et al*, 2004) and $SHIV_{89.6P}$ infection (Amara *et al*, 2005a; Mooij *et al*, 2004). It has become necessary therefore to measure the induction of other cytokines produced by immune cells in vaccinated individuals or animals for further evaluation of candidate vaccines. In this study, a CBA technique was employed to quantify 6 cytokines, 3 of which were Th-1-like (IFN-γ, TNF-α, IL-2) cytokines. Data from 4 baboon PBMC samples were analysed (Exp2) and showed production of IFN-γ, TNF-α and IL-2 and very little or negligible amounts of IL-4, IL-5 and IL-6 (Th-2 cytokines) following SAAVI MVA-C and GagC VLP vaccination. For one baboon (449), as much as 1703 and 1453 pg/mL of IFN-γ and IL-2 respectively were produced during a 16-h incubation of Gag peptide number 40 (p24 region) with PBMC obtained at 2 weeks post immunisation with GagC VLP. This data point to the multi-functionality of the lymphocytes produced in the immunised baboons. Further investigation is desirable to identify the frequency of cells producing TNF-α and IL-2 and possibly enumerate multi-functional cells, especially IFN-γ and IL-2 dual producers.

4.4.3 COMPARISON OF IMMUNE RESPONSES WITH OTHER NHP STUDIES

Humoral and cellular immune responses to various HIV antigens expressed in rBCG have previously been shown (Aldovini & Young, 1991; Chujoh *et al*, 2002; Fuerst *et al*, 1992; Kameoka *et al*, 1994; Kawahara *et al*, 2002a; Someya *et al*, 2005; Winter *et al*, 1995). Only a few of these studies utilised the macaque model (Kawahara *et al*, 2002a; Someya *et al*, 2005) and so far no study has reported the use of the baboon model. Moreover, both of these macaque studies used rBCG expressing HIV-1 Env V3 protein, not Gag. This makes it difficult to compare the data generated in the present study with other rBCG studies in NHP. However, a recent study by Ami *et al* (2005), using rBCG expressing SIV Gag to prime and a replication-deficient vaccinia virus expressing SIV Gag to boost, may be used for comparison purposes. In this study, Ami and colleagues (2005) demonstrated induction of high levels of IFN-γ spot forming cells and effective immunity against mucosal challenge with pathogenic SHIV in rBCG-primed cynomolgus monkeys but not in animals receiving opposite combination or single-modality vaccines. The present study advances the prospects of using rBCG HIV-1 and VLP vaccines in a prime-boost vaccination modality in clinical trials. A comparison of the magnitude of ELISPOT data that were generated in the present study and that of Ami *et al* (2005) is shown in **Table 4.5**.

Table 4.5: Comparison between the IFN-γ ELISPOT data generated in cynomolgus macaques (Ami *et al*, 2005) and baboons (present study). Cynomolgus macaques were immunised with rBCG (rBCG-SIVgag) or recombinant replication-deficient vaccinia virus (rIDs-SIVgag) expressing SIV gag or corresponding control constructs in combinations as indicated in the table. rBCG-SIVgag or corresponding control (rBCG-pSO246) was inoculated intradermally at wk0 if given as a prime or at wk54 if given as a boost at a dosage of 10mg per macaque. rIDs-SIVgag or corresponding control (rIDs-LacZ) was given intravenously at a dosage of 10^6 PFU per macaque at weeks 0 and 8 if given as a prime or weeks 47 and 54 if given as a boost. Baboons were immunised as previously shown (Fig. 4.1).

Experiment	Group	Vaccination regimen	Peak IFN-γ ELISPOT response (SFU/10^6 PBMC)	
			after primary inoculations	after heterologous boost
Ami *et al* (2005)	rBCG/rIDs (n=3)	**Prime-boost:** 1x rBCG-SIVgag (i.d); 2x rIDs-SIVgag (i.v)	~ 200*	1020 ± 360#
	rIDs/rBCG (n=3)	**Prime-boost:** 2x rIDs-SIVgag (i.v); 1x rBCG-SIVgag (i.d)	~ 500*	380 ± 35#
	rBCG (n=2)	**Single:** 1x rBCG-SIVgag (i.d); 2x mock rIDs (i.v)	~ 200*	NA
	rIDs (n=2)	**Single modality:** 2x rIDs-SIVgag (i.v); 1x mock rBCG (i.d)	~ 500*	NA
Exp1	RT106/VLP (n=2)	**Prime-boost:** 4x rBCG-HIVgag (i.d); 2x HIVgag VLP (i.m)	157 (1 of 2 baboons)	679 ± 163 (582-812)
	RT108/VLP (n=2	**Prime-boost:** 4x rBCG-HIVgag (i.d); 2x HIVgag VLP (i.m)	No response	274 ± 166 (156-391)
	VLP (n=2)	**Single modality:** 4x mock rBCG (i.d); 2x HIVgag VLP (i.m)	135 ± 148 (30-240)	NA
Exp2	RT106/MVA/VLP (n=6)	**Prime-boost (triple):** 2x rBCG-HIVgag (i.d); 1x SAAVI MVA-C (i.m); 1x HIVgag VLP (i.m)	No response	96 ± 87 (33-223) [post-MVA] 933 ± 857 (92-1880) [post-VLP]
	wt-BCG/MVA/VLP (n=6)	**Prime-boost (double$^{\Psi}$):** 2x mock wt-BCG (i.d); 1x SAAVI MVA-C (i.m); 1x HIVgag VLP (i.m)	370 ± 538 (44-1420) [post-MVA]	1025 ± 1413 (86-3852) [post-VLP]

*: estimated values from graphs; #: range not available; $^{\Psi}$: double modality in respect to Gag; NA: not applicable; i.d: intradermally; i.m: intramuscularly; i.v: intravenously

As illustrated (**Table 4.5**), the magnitudes of IFN-γ responses after booster immunisation in both the macaque (Ami *et al*, 2005) and baboon (present study) studies are comparable. A striking similarity in both studies is the ability of the recombinant poxviral vectors (replication-deficient vaccinia and

MVA) alone to elicit an immune response that is substantially stronger than the corresponding rBCG. In the same way, the $Pr55^{gag}$ VLP alone was able to elicit strong immune response (Exp1, present study). However, there are two notable differences. Firstly, the efficacy of rBCG:RT106 and rBCG:RT108 (present study) in priming the immune system is substantially lower than the rBCG-SIV*gag* vaccine that was used in the macaque study, suggesting a lower level of HIV-1 Gag protein expression in rBCG:RT106 and rBCG:RT108. This finding recommends more work to be directed at optimising the BCG shuttle vectors in order to improve the expression level of heterologous proteins while maintaining good stability of the recombinants. Such efforts could lead to development of rBCG vaccines with the capacity to prime effective immune responses with a single inoculation and this could greatly benefit the progress of the current rBCG HIV-1 subtype C vaccines. Currently, no rBCG-based HIV-1 vaccine is in the pipeline for clinical trials, indicating that although this vaccine strategy holds great promise, there are serious challenges in this field. A number of these challenges have been identified (Dennehy & Williamson, 2005) and are currently receiving intense investigations.

The second notable difference in these comparisons (**Table 4.5**) is that rIDs-SIVgag/rBCG-SIVgag prime-boost regimen in the macaque study did not lead to boosting of the immune responses whereas the SAAVI MVA-C/GagC VLP prime-boost regimen (Exp2, present study) resulted in substantial boosting of the immune responses. This finding underscores the value $Pr55^{gag}$ VLP as a booster vaccine and the potential of poxviral vectored vaccine/VLP prime-boost regimen.

Animal studies utilising a triple vector vaccination regimen have been reported (Heeney *et al*, 2000; Koopman *et al*, 2004; Michelini *et al*, 2004; Negri *et al*, 2004). In one of these studies, Negri *et al* (2004) investigated the immunogenicity and efficacy of a polyvalent triple vector based vaccine comprising of plasmid DNA, recombinant Semliki Forest virus (rSFV) and rMVA using cynomolgus monkeys. Both DNA and recombinant viral vectors expressed SIV Gag, Pol, Tat, Rev, Env and Nef proteins. Animals were primed with DNA, boosted twice with rSFV and finally with rMVA. Although vaccinated monkeys developed SIV-specific T helper proliferative and IFN-γ ELISPOT responses after the last boost with rMVA, the beneficial effect of triple vector vaccination, compared to homologous prime boost (Negri *et al*, 2001), became evident only after pathogenic SIV_{mac251} challenge. IFN-γ ELISPOT responses were generally of low magnitude before the challenge, ranging from 222-605 SFU/10^6 PBMC (directed against the whole inactivated virus) and <110 SFU/10^6 PBMC Gag- or Nef-specific at two weeks after the last vaccination. Surprisingly, virus replication was rapidly brought under control in 3 of 4 vaccinated macaques, becoming undetectable by week 4 and remained negative for over 35 weeks. In the present study, triple vector vaccination regimen generated Gag-specific IFN-γ ELISPOT responses in the range of 92-1880 (mean: 933 ± 857) SFU/10^6 PBMC, which are substantially higher than those generated in the study by Negri *et al* (2004). These data suggest that although triple vector vaccine regimen (present study) appeared to be less robust than the double

vector vaccine regimen, may confer important vaccine-induced protective benefits and hence further exploration should be continued in the future.

4.4.4 CONCLUSION

Work presented here is the first to report the use of baboon for evaluation of rBCG-based candidate HIV-1 vaccines. The present study demonstrates that the chacma baboon (*Papio ursinus*), which is endemic in southern Africa, is a suitable animal model for immunogenicity testing of candidate HIV vaccines. Taken together, these data demonstrate that the chacma baboon could be used as an alternative animal model for evaluation of HIV vaccines and especially BCG-based vaccines. The use of chacma baboons could benefit pre-clinical HIV vaccine studies in southern Africa, where this baboon species can be acquired cheaply from the wild. This would exclude the need to set up extensive baboon breeding programs to supply the necessary research animals, further lowering the cost of pre-clinical studies in Africa.

The present study reports induction of immune responses by rBCG:*gag* vaccines and a multigene rMVA vaccine given as primary vaccines and the boosting potential of these responses by $Pr55^{gag}$ VLP vaccine. The data from this study demonstrated that rBCG:RT106, rBCG:RT108 and SAAVI MVA-C have the capacity to prime the immune system for GagC VLP protein boost. Broad, strong and long-lived cellular responses were generated after the $Pr55^{gag}$ VLP boost. These cellular responses were found to be mediated chiefly by the CD8+ T lymphocytes with IFN-γ, IL-2 and TNF-α being the main cytokines that were involved. Also, several of the epitope-containing peptides were identified and that they were identical to some of those recognised by PBCM from HIV-1 subtype C-infected individual, indicating the relevance of the vaccine strain. The magnitude of the cellular responses was found to compare well with a limited number of published results, indicating the vaccine concept in the present study is promising. A triple vector vaccine regimen yielded lower magnitude of responses, not unlike other reported studies, but showed great promise. At the same time, the potential of SAAVI MVA-C, a poxvirus vectored vaccine as a primary vaccine in prime-boost regimen with GagC VLP was observed. Like the studies described in Chapter two, this data further justifies the movement of GagC VLP to clinical phase of evaluation. However, the need to improve the expression of the heterologous antigens in rBCG vaccine constructs was suggested by the data in the present study as a way of improving the immunogenicity of these vaccines.

Finally, this study showed that as little as 10.8 μg of GagC VLP, given a booster immunisation, was efficient in eliciting strong and long-lived cellular immune responses in the baboons. The finding that such a low dose was effective indicates that this could impact on reducing the cost of $Pr55^{gag}$ VLP-based HIV-1 vaccine.

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5.1 INTRODUCTION

This project was part of elaborate efforts by the SAAVI-funded HIV vaccine development group at UCT to develop safe, effective and affordable HIV-1 vaccines, which are relevant to southern Africa where C subtype causes over 90% of HIV-1 infections. This group is mandated to conduct research, which is geared towards discovery and pre-clinical development of HIV-1 subtype C vaccines. These activities, which started in earnest in the year 2000, are multi-pronged using multiple vaccine strategies that include DNA, bacterial and viral vectors and sub-unit vaccines. Towards this end, various first generation HIV gag vaccines that have been developed and tested in the mouse model include pTH-GagC (van Harmelen *et al*, 2003), GagC VLP (Jaffrey *et al*, 2004) and rBCG:GagC (Thomas, 2005).

The development of a non-human primate model started in 2000 and the chacma baboon was chosen based on its availability as it is an endemic baboon species in South Africa. In addition, a primate facility, which was being used to house chacma baboons for surgical experiments, was available and staffed with personnel experienced in handling baboons. Moreover, the prospect of acquiring Indian macaques, a monkey model that is well characterised for HIV research, was hindered by high costs and a reported severe shortage (Cohen, 2000).

The work presented in this thesis was the first to utilise the chacma baboon model to evaluate the immunogenicity of HIV vaccines. Thus, due to lack of published information regarding this baboon species, the immunisation schedules used in the series of experiments that are

described here varied widely as the results and information obtained in the early phase of these studies was often used to guide the next phase. Another factor that affected the immunisation schedules was the technical obstacles that hindered the timely production of GagC VLP vaccine for boosting the baboons. Despite these early difficulties, care was taken to harmonise the animal and laboratory protocols. The use of cryo-preserved PBMC for most of the laboratory assays helped in minimising inter-assay variations as it was possible to batch together samples obtained from various time points.

5.2 SUMMARY OF KEY FINDINGS

5.2.1 DEVELOPMENT OF METHODS

At the initial stages of these studies, various immunological reagents were identified and tested for cross-reactivity *in vitro* with baboon PBMC, cytokines or immunoglobulin G. Reagents with cross-reactivity were found, and then used to develop immunological methods for these studies. The following immunological assays were developed; (i) IFN-γ ELISPOT, (ii) intracellular IFN-γ staining (iii) lymphocyte proliferation assays using radio-active thymidine for detection and (iv) HIV-1 p24 ELISA. Investigation of expression of various cell surface markers showed that cryo-preservation of PBMC did not alter the expression of these markers. However, the expression of CD69 by PBMC upon specific antigen stimulation was adversely affected by addition of brefeldin A, an essential protein translocation inhibitor that is used in ICS assays. This precluded the utility of anti-CD69 monoclonal antibody in ICS assay.

The immunological methods that were developed in the present study were used to evaluate the immunogenicity of the various HIV-1 immunogens that are described in this thesis. In addition, these methods were successfully adapted to assess the immunogenicity of multigene DNA and rMVA HIV-1 subtype C-derived candidate vaccines (Burgers *et al*, unpublished; Williamson *et al*, 2005) which are scheduled to enter into Phase 1 clinical trials in the USA and South Africa in 2007 (A-L Williamson, personal communication).

5.2.2 INOCULATION WITH DNA AND PR55^{gag} VLP VACCINES

Two experiments, DNA Exp1 and DNA Exp2, were conducted using these pTH-GagC DNA and GagC VLP vaccines in prime-boost immunisation protocol. Baboons immunised with both vaccines developed stronger and broader immune responses compared to those receiving the single vaccine modality. The pTH-GagC vaccination was found to induce weak responses, which were boosted several folds by a single GagC VLP vaccination. This was comparable to other HIV-1 vaccine studies in non-human primates. Mapping of IFN-γ responses showed

that peptides that were recognised by baboon PBMC were located in all the three main regions of Gag protein i.e. p17, p24 and p2p7p1p6 domains in the order p24>p2p7p1p6>p17. Several of these peptides that were targeted by baboon PBMC have been reported in the literature as being recognised by PBMC from patients with early HIV-1 subtype C infection in southern Africa. The ICS assay performed with PBMC from one of the baboons indicated that these IFN-γ responses were mediated by CD8+ T lymphocytes. Analysis of the spectrum of Gag-specific cytokines that were produced by PBMC showed that the Th1 type (IL-2, IFN-γ and TNFα) were the predominant cytokines that were released by PBMC. A second GagC VLP inoculation was given to two baboons in DNA Exp1 (mock-primed with pTH-vector) and all baboons in DNA Exp2, twelve weeks after the first GagC VLP immunisation. This immunisation did not boost the IFN-γ ELISOPOT responses that were induced by the first GagC VLP vaccination. A further GagC VLP inoculation given to two baboons at 34 or 89 weeks after the second GagC VLP confirmed the inability of GagC VLP to boost the response. But IFN-γ responses were detectable for both baboons before and after the third GagC VLP inoculation, indicating that these responses that were induced by the second GagC VLP inoculation were long-lived.

5.2.3 INOCULATION WITH rBCG, rMVA AND PR55^{gag} VLP VACCINES

Evaluation of these vaccines was preceded by an investigation of baboon responses to wild-type BCG, which was aimed at providing some base-line information. Both BCG-*Tokyo* and BCG-*Pasteur* were shown to confer long-lived BCG-specific cell-mediated responses, which were characterised by strong DTH, proliferative and IFN-γ responses whose magnitude appeared to be BCG dose dependent with baboons receiving a high dose showing increased magnitudes. These IFN-γ responses were shown to be mediated by CD4+ lymphocytes. Humoral response was detected consistently only in baboons receiving a high dose of BCG (10^6 and 10^8 CFU).

The first vaccine evaluation comprised of rBCG:GagC vaccines (either rBCG:RT106 or rBCG:RT108) and GagC VLP, which were given in a rBCG-prime and GagC VLP boost regimen. Four inoculations of rBCG:GagC over a period of 40 weeks elicited only a weak Gag cellular response that was detected consistently in only 1 of 4 immunised baboons. After the first GagC VLP inoculation with a dose of 10.8 μg, which was given 52 weeks (1 year) after the last rBCG vaccine, strong and broad IFN-γ responses to Gag developed in 3 of 4 baboons. The 4th baboon developed a weak and narrow response. Mapping of these responses revealed that the majority of peptides which were recognised by the baboon PBMC belonged mainly to p24 and p2p7p1p6 regions of Gag protein but not the p17 region. One of 2 control

baboons that were mock-primed with rBCG:CB119-L1 targeted only two peptides in the p24 region after the GagC VLP boost. It was not possible to map the response in the other control baboon because it developed only a weak transient IFN-γ response. Phenotypic determination of the cells producing IFN-γ following the GagC VLP boost indicated that the responses were mediated principally by CD8+ T lymphocytes. Also, GagC VLP induced a weak Gag-specific antibody response in the serum of baboons, which received the mixed vaccine modality and this response was further boosted by the 2nd GagC VLP vaccination. Control baboons that received a mock-prime inoculation with rBCG:CB119-L1 developed an antibody response only after the 2nd GagC VLP.

A third GagC VLP inoculation was given to four baboons, 37 or 50 weeks after the 2nd GagC VLP. Two of these baboons had been primed with rBCG:RT106, one with rBCG:RT108 and one was mock-primed with rBCG:CB119-L1 . Prior to this boost all four baboons still had a substantial detectable response to Gag in the IFN-γ ELISPOT assay, illustrating the longevity of the response induced by the original inoculation regimen.

This study was then followed by a triple vector vaccine regimen that maybe used in human clinical trials. Six baboons were inoculated twice with rBCG:RT106, boosted with a single inoculation of SAAVI MVA-C followed finally with a single inoculation of GagC VLP. The control group (six baboons; double vector vaccine regimen) received wt-BCG/SAAVI MVA-C/GagC VLP. Inoculation with rBCG:RT106 did not generate a detectable IFN-γ ELISPOT response in any of the 6 immunised baboons. After the SAAVI MVA-C vaccination, weak Gag IFN-γ responses were detectable in some baboons while a strong IFN-γ response was seen in only 1 of 12 immunised baboons. IFN-γ ELISPOT responses were consistently detected in all baboons after the GagC VLP inoculation. No significant difference in mean cumulative IFN-γ ELISPOT response between the baboons receiving a triple vector vaccine regimen and those receiving a double vector vaccine regimen was found at peak response after SAAVI MVA-C or GagC VLP boost. Although complete mapping of IFN-γ responses was not achieved for baboons in this study, Gag-specific stimulation of baboon PBMC indicated that the IFN-γ response was mediated by a CD8+ T cell population. The spectrum of cytokines produced upon stimulation of PBMC from 4 of 12 baboons with specific Gag peptides showed that Th1 type cytokines (IFN-γ, IL-2 and TNFα) were the predominant cytokines produced by these cells.

In this study the potential adverse effect of pre-existing anti-BCG immunity on the vaccine-induced immune responses was investigated. Six baboons in this study had been immunised

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with wt-BCG during a previous study investigating the response of baboons to wtBCG (Chapter 3). By comparing the mean cumulative IFN-γ responses at four time points post SAAVI MVA-C and GagC VLP inoculation, it was shown that there was no significant difference between baboons with prior BCG sensitisation and those without. However, the failure of rBCG:RT106 immunisation to induce detectable immune response in these baboons prevented deduction of a firm conclusion. Conversely, PPD-specific IFN-γ responses were detected for the baboons with prior BCG sensitisation at the time of vaccination with rBCG and these responses did not hinder further development of PPD-specific IFN-γ responses following inoculation with rBCG:RT106 or wt-BCG. Further investigation using a rBCG exhibiting an improved immunogenicity is desirable.

Of note, in all these experiments involving inoculation with wt-BCG or rBCG, the baboons developed ulcerations on the inoculation sites but these reactions were well tolerated and healed within 6 weeks without medication. This is comparable to what is observed in humans after receiving intradermal BCG vaccination.

5.3 FINAL CONCLUSIONS

The studies reported here were successful in identifying the appropriate immunological reagents that allowed the development of immunological assays for use in HIV vaccine research using chacma baboons. An IFN-γ ELISPOT assay was developed to detect cell mediated immune responses and suitable antibodies were identified that could be used in ELISA assays for detection of humoral immune responses. A commercially available non-human primate CBA kit was able to successfully detect cytokines released from in vitro stimulated PBMC. It would be desirable for future studies to develop other assays that allow detection of IFN-γ/IL-2 dual producing cells. Recent studies have shown that IFN-γ/IL-2 dual producing cells are associated with effective control of HIV replication in HIV-1 infected individuals (Emu *et al*, 2005; Zimmerli, *et al*, 2005) and provide better protection of vaccinated macaques upon pathogenic virus challenge (Mooij *et al*, 2004; Sadagopal *et al*, 2005). Development of an ELISPOT assay for other cytokines such as IL-4, IL-5 and IL-6 as well as chemokines such as RANTES, MIP-1α and MIP-1β would allow detection of cells producing Th2 type cytokines and soluble factors associated with innate immunity. Moreover, current HIV vaccine studies appear to emphasise the evaluation of the quality, rather than quantity, of vaccine-induced memory cells. Such type of evaluations requires the measurements of phenotypes associated with effector functions (e.g. CD45RA, CD27, CCR7) as well as proliferation potential using flow cyotometric methods (e.g. via the use of carboxyl fluorescein diacetate succinimidyl ester [CFSE]). Several of these cell features can be

measured simultaneously using polychromatic flow cytometry. Development of methods towards this direction could benefit immensely the use of the chacma baboon model for future immunogenicity studies.

With the exception of SAAVI MVA-C, the vaccines tested in these studies were first generation vaccines, hence comprising of one gene (HIV-1 *gag*). Gag is a good choice for a single gene as HIV infected humans develop strong CTL response to Gag. The DNA prime/$Pr55^{gag}$ VLP boost vaccination protocol offered the best immune responses in terms of magnitude and breadth of IFN-γ ELISPOT responses. Similarly, $Pr55^{gag}$ VLP was able to boost responses that were induced by primary vaccination with rBCG:*gag*, multigene rMVA and rBCG:*gag*/multigene rMVA prime-boost combination. The magnitudue of IFN-γ ELISPOT responses that were induced following boost immunisation with GagC VLP in the present studies were as good as those elicited by other candidate vaccines which have already proceeded to clinical evaluation. Data from the present study therefore supports GagC VLP for consideration for phase 1 clinical trials in combination with other vaccine formats especially DNA vaccines. The fact that GagC VLP was able to boost rBCG:GagC-induced responses which were below detection level prior to booster immunisation, lends further support for the movement of GagC VLP to clinical evaluation.

The present study showed that as little as 10.8 μg of GagC VLP was able to efficiently boost the immune responses that were induced by the priming vaccine in these baboon studies. Considering that the body mass of an adult baboon is almost half of the weight of an adult human being, a similar GagC VLP dose could be effective in clinical trials, indicating that this could have a major impact in reducing the cost per dose. This prospect further justifies the need to advance GagC VLP to clinical evaluation.

It is feasible to develop chimeric $Pr55^{gag}$ VLP by incorporating other relevant structural and non-structural HIV-1 genes, including *env* gene. This could lead to induction of immune responses with increased potency and breadth. Our vaccine group has already developed a multigene DNA (Burgers *et al*, 2006) and rMVA (Burgers *et al*, unpublished) vaccines by incorporating *env*, *RT*, *tat* and *nef* genes of HIV-1 subtype C, in addition to HIV-1 *gag*. Thus, development of chimeric $Pr55^{gag}$ VLP to match these genes could make a SAAVI MVA-C/$Pr55^{gag}$ VLP prime-boost combination possible for consideration for phase 1 clinical trials.

Data from the present studies suggest that although rBCG:RT106 and rBCG:RT108 were able to prime the immune response for a heterologous boost, these rBCG vaccines on their own are

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not sufficiently immunogenic to be considered for further evaluation in clinical trials. Further improvement of the expression level of HIV genes in these rBCG vaccines could benefit this HIV vaccine strategy since higher vaccine expression could lead to improved immunogenicity. This in turn could lead to use of lower rBCG doses and less number of inoculations. Moreover, a vaccination regimen comprising of more than one immunisation with rBCG vaccine is likely to be unacceptable in clinical settings, as BCG may cause substantial local ulceration on injection sites. However, in the present studies this did not pose a problem that required medication.

Although a triple vector vaccine regimen did not show any advantage over a double vector vaccine protocol in terms of the magnitude of responses, it is a strategy worth further investigation. Furthermore, immunisation of macaques with a triple vector-based vaccine has been shown to confer a rapid control of viral replication after pathogenic SIV challenge (Negri *et al*, 2004). It would be envisaged that a successful HIV-1 triple vector vaccination regimen that includes rBCG as a prime vaccine would confer special benefits to childhood immunisation. BCG, the tuberculosis vaccine is one of the childhood vaccines, which has been shown to be safe and unaffected by maternal antibody. An rBCG HIV-1 vaccine would confer the double benefit by affording protection against TB and post natal HIV infections. Later in life, two booster immunisations, comprising different vectors, could then be given during the adolescent age to provide protection against sexual HIV transmission.

In conclusion, the present studies succeeded in developing a cheap and readily available baboon model for testing the immunogenicity of HIV-1 vaccines. The immunogenicity data generated using this model showed that GagC VLP, a $Pr55^{gag}$ VLP vaccine derived from subtype C HIV-1, should be considered for clinical evaluation to be used as a boost in prime-boost immunisation protocols with a matched DNA vaccine. In addition, the potential use of GagC VLP as a booster vaccine in prime-boost combination with either rBCG:RT106, rBCG:RT108, SAAVI MVA-C or both rBCG:RT106 and SAAVI MVA-C (triple vector) was demonstrated, further supporting the movement of GagC VLP to clinical evaluation. Data from the present study further showed that the rBCG vaccines, rBCG:RT106 and rBCG:RT108, were not sufficiently immunogenic on their own to warrant consideration for clinical evaluation at this stage but results from the present study strongly suggest the potential use of rBCG with enhanced expression of the recombinant protein to prime the immune system needs to be further explored.

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5.2.1 DEVELOPMENT OF METHODS

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Evaluation of these vaccines was preceded by an investigation of baboon responses to wild-type BCG, which was aimed at providing some base-line information. Both BCG-*Tokyo* and BCG-*Pasteur* were shown to confer long-lived BCG-specific cell-mediated responses, which were characterised by strong DTH, proliferative and IFN-γ responses whose magnitude appeared to be BCG dose dependent with baboons receiving a high dose showing increased magnitudes. These IFN-γ responses were shown to be mediated by CD4+ lymphocytes. Humoral response was detected consistently only in baboons receiving a high dose of BCG (10^6 and 10^8 CFU).

The first vaccine evaluation comprised of rBCG:GagC vaccines (either rBCG:RT106 or rBCG:RT108) and GagC VLP, which were given in a rBCG-prime and GagC VLP boost regimen. Four inoculations of rBCG:GagC over a period of 40 weeks elicited only a weak Gag cellular response that was detected consistently in only 1 of 4 immunised baboons. After the first GagC VLP inoculation with a dose of 10.8 μg, which was given 52 weeks (1 year) after the last rBCG vaccine, strong and broad IFN-γ responses to Gag developed in 3 of 4 baboons. The 4th baboon developed a weak and narrow response. Mapping of these responses revealed that the majority of peptides which were recognised by the baboon PBMC belonged mainly to p24 and p2p7p1p6 regions of Gag protein but not the p17 region. One of 2 control

baboons that were mock-primed with rBCG:CB119-L1 targeted only two peptides in the p24 region after the GagC VLP boost. It was not possible to map the response in the other control baboon because it developed only a weak transient IFN-γ response. Phenotypic determination of the cells producing IFN-γ following the GagC VLP boost indicated that the responses were mediated principally by CD8+ T lymphocytes. Also, GagC VLP induced a weak Gag-specific antibody response in the serum of baboons, which received the mixed vaccine modality and this response was further boosted by the 2nd GagC VLP vaccination. Control baboons that received a mock-prime inoculation with rBCG:CB119-L1 developed an antibody response only after the 2nd GagC VLP.

A third GagC VLP inoculation was given to four baboons, 37 or 50 weeks after the 2nd GagC VLP. Two of these baboons had been primed with rBCG:RT106, one with rBCG:RT108 and one was mock-primed with rBCG:CB119-L1 . Prior to this boost all four baboons still had a substantial detectable response to Gag in the IFN-γ ELISPOT assay, illustrating the longevity of the response induced by the original inoculation regimen.

This study was then followed by a triple vector vaccine regimen that maybe used in human clinical trials. Six baboons were inoculated twice with rBCG:RT106, boosted with a single inoculation of SAAVI MVA-C followed finally with a single inoculation of GagC VLP. The control group (six baboons; double vector vaccine regimen) received wt-BCG/SAAVI MVA-C/GagC VLP. Inoculation with rBCG:RT106 did not generate a detectable IFN-γ ELISPOT response in any of the 6 immunised baboons. After the SAAVI MVA-C vaccination, weak Gag IFN-γ responses were detectable in some baboons while a strong IFN-γ response was seen in only 1 of 12 immunised baboons. IFN-γ ELISPOT responses were consistently detected in all baboons after the GagC VLP inoculation. No significant difference in mean cumulative IFN-γ ELISPOT response between the baboons receiving a triple vector vaccine regimen and those receiving a double vector vaccine regimen was found at peak response after SAAVI MVA-C or GagC VLP boost. Although complete mapping of IFN-γ responses was not achieved for baboons in this study, Gag-specific stimulation of baboon PBMC indicated that the IFN-γ response was mediated by a CD8+ T cell population. The spectrum of cytokines produced upon stimulation of PBMC from 4 of 12 baboons with specific Gag peptides showed that Th1 type cytokines (IFN-γ, IL-2 and TNFα) were the predominant cytokines produced by these cells.

In this study the potential adverse effect of pre-existing anti-BCG immunity on the vaccine-induced immune responses was investigated. Six baboons in this study had been immunised

with wt-BCG during a previous study investigating the response of baboons to wtBCG (Chapter 3). By comparing the mean cumulative IFN-γ responses at four time points post SAAVI MVA-C and GagC VLP inoculation, it was shown that there was no significant difference between baboons with prior BCG sensitisation and those without. However, the failure of rBCG:RT106 immunisation to induce detectable immune response in these baboons prevented deduction of a firm conclusion. Conversely, PPD-specific IFN-γ responses were detected for the baboons with prior BCG sensitisation at the time of vaccination with rBCG and these responses did not hinder further development of PPD-specific IFN-γ responses following inoculation with rBCG:RT106 or wt-BCG. Further investigation using a rBCG exhibiting an improved immunogenicity is desirable.

Of note, in all these experiments involving inoculation with wt-BCG or rBCG, the baboons developed ulcerations on the inoculation sites but these reactions were well tolerated and healed within 6 weeks without medication. This is comparable to what is observed in humans after receiving intradermal BCG vaccination.

5.3 FINAL CONCLUSIONS

The studies reported here were successful in identifying the appropriate immunological reagents that allowed the development of immunological assays for use in HIV vaccine research using chacma baboons. An IFN-γ ELISPOT assay was developed to detect cell mediated immune responses and suitable antibodies were identified that could be used in ELISA assays for detection of humoral immune responses. A commercially available non-human primate CBA kit was able to successfully detect cytokines released from in vitro stimulated PBMC. It would be desirable for future studies to develop other assays that allow detection of IFN-γ/IL-2 dual producing cells. Recent studies have shown that IFN-γ/IL-2 dual producing cells are associated with effective control of HIV replication in HIV-1 infected individuals (Emu *et al*, 2005; Zimmerli, *et al*, 2005) and provide better protection of vaccinated macaques upon pathogenic virus challenge (Mooij *et al*, 2004; Sadagopal *et al*, 2005). Development of an ELISPOT assay for other cytokines such as IL-4, IL-5 and IL-6 as well as chemokines such as RANTES, MIP-1α and MIP-1β would allow detection of cells producing Th2 type cytokines and soluble factors associated with innate immunity. Moreover, current HIV vaccine studies appear to emphasise the evaluation of the quality, rather than quantity, of vaccine-induced memory cells. Such type of evaluations requires the measurements of phenotypes associated with effector functions (e.g. CD45RA, CD27, CCR7) as well as proliferation potential using flow cyotometric methods (e.g. via the use of carboxyl fluorescein diacetate succinimidyl ester [CFSE]). Several of these cell features can be

measured simultaneously using polychromatic flow cytometry. Development of methods towards this direction could benefit immensely the use of the chacma baboon model for future immunogenicity studies.

With the exception of SAAVI MVA-C, the vaccines tested in these studies were first generation vaccines, hence comprising of one gene (HIV-1 *gag*). Gag is a good choice for a single gene as HIV infected humans develop strong CTL response to Gag. The DNA prime/$Pr55^{gag}$ VLP boost vaccination protocol offered the best immune responses in terms of magnitude and breadth of IFN-γ ELISPOT responses. Similarly, $Pr55^{gag}$ VLP was able to boost responses that were induced by primary vaccination with rBCG:*gag*, multigene rMVA and rBCG:*gag*/multigene rMVA prime-boost combination. The magnitudue of IFN-γ ELISPOT responses that were induced following boost immunisation with GagC VLP in the present studies were as good as those elicited by other candidate vaccines which have already proceeded to clinical evaluation. Data from the present study therefore supports GagC VLP for consideration for phase 1 clinical trials in combination with other vaccine formats especially DNA vaccines. The fact that GagC VLP was able to boost rBCG:GagC-induced responses which were below detection level prior to booster immunisation, lends further support for the movement of GagC VLP to clinical evaluation.

The present study showed that as little as 10.8 μg of GagC VLP was able to efficiently boost the immune responses that were induced by the priming vaccine in these baboon studies. Considering that the body mass of an adult baboon is almost half of the weight of an adult human being, a similar GagC VLP dose could be effective in clinical trials, indicating that this could have a major impact in reducing the cost per dose. This prospect further justifies the need to advance GagC VLP to clinical evaluation.

It is feasible to develop chimeric $Pr55^{gag}$ VLP by incorporating other relevant structural and non-structural HIV-1 genes, including *env* gene. This could lead to induction of immune responses with increased potency and breadth. Our vaccine group has already developed a multigene DNA (Burgers *et al*, 2006) and rMVA (Burgers *et al*, unpublished) vaccines by incorporating *env*, *RT*, *tat* and *nef* genes of HIV-1 subtype C, in addition to HIV-1 *gag*. Thus, development of chimeric $Pr55^{gag}$ VLP to match these genes could make a SAAVI MVA-C/$Pr55^{gag}$ VLP prime-boost combination possible for consideration for phase 1 clinical trials.

Data from the present studies suggest that although rBCG:RT106 and rBCG:RT108 were able to prime the immune response for a heterologous boost, these rBCG vaccines on their own are

not sufficiently immunogenic to be considered for further evaluation in clinical trials. Further improvement of the expression level of HIV genes in these rBCG vaccines could benefit this HIV vaccine strategy since higher vaccine expression could lead to improved immunogenicity. This in turn could lead to use of lower rBCG doses and less number of inoculations. Moreover, a vaccination regimen comprising of more than one immunisation with rBCG vaccine is likely to be unacceptable in clinical settings, as BCG may cause substantial local ulceration on injection sites. However, in the present studies this did not pose a problem that required medication.

Although a triple vector vaccine regimen did not show any advantage over a double vector vaccine protocol in terms of the magnitude of responses, it is a strategy worth further investigation. Furthermore, immunisation of macaques with a triple vector-based vaccine has been shown to confer a rapid control of viral replication after pathogenic SIV challenge (Negri *et al*, 2004). It would be envisaged that a successful HIV-1 triple vector vaccination regimen that includes rBCG as a prime vaccine would confer special benefits to childhood immunisation. BCG, the tuberculosis vaccine is one of the childhood vaccines, which has been shown to be safe and unaffected by maternal antibody. An rBCG HIV-1 vaccine would confer the double benefit by affording protection against TB and post natal HIV infections. Later in life, two booster immunisations, comprising different vectors, could then be given during the adolescent age to provide protection against sexual HIV transmission.

In conclusion, the present studies succeeded in developing a cheap and readily available baboon model for testing the immunogenicity of HIV-1 vaccines. The immunogenicity data generated using this model showed that GagC VLP, a $Pr55^{gag}$ VLP vaccine derived from subtype C HIV-1, should be considered for clinical evaluation to be used as a boost in prime-boost immunisation protocols with a matched DNA vaccine. In addition, the potential use of GagC VLP as a booster vaccine in prime-boost combination with either rBCG:RT106, rBCG:RT108, SAAVI MVA-C or both rBCG:RT106 and SAAVI MVA-C (triple vector) was demonstrated, further supporting the movement of GagC VLP to clinical evaluation. Data from the present study further showed that the rBCG vaccines, rBCG:RT106 and rBCG:RT108, were not sufficiently immunogenic on their own to warrant consideration for clinical evaluation at this stage but results from the present study strongly suggest the potential use of rBCG with enhanced expression of the recombinant protein to prime the immune system needs to be further explored.

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APPENDIX A: BUFFERS AND REAGENTS

APPENDIX A1: ELISA REAGENTS

A.1.1 ELISA Coating solution, pH9.6: Carbonate bicarbonate capsules [Sigma; Cat: C-3041] were dissolved in de-ionised water according to manufacturer's instructions.

A.1.2 ELISA Coating antigen: Recombinant HIV-1_{NY5} p24 protein [produced in baculovirus; Quality Biologicals; Product Code: 303649] was stored in -80 °C in original concentrations. At the time of using, the protein was diluted with coating solution to 1 μg/mL. 100 μL/well (0.1 μg) was used to coat the ELISA plates (Maxisorb immunoplates [Nunc, Denmark]).

A.1.3 ELISA Wash solution, pH7.4: One PBS-Tween 20 powder sachet [Sigma; Cat: Cat: P3563] was dissolved in 1L de-ionised water according to manufacturer's instructions.

A.1.4 ELISA Diluent solution: PBS Solution, pH7.4 containing 1% non-fat skim milk (w/v). To make PBS solution, 1 PBS powder sachet [Sigma; Cat: P-3813] was dissolved in 1L de-ionised water as per manufacturer's instructions.

A.1.5 ELISA Blocking buffer: PBS Solution, pH 7.4 containing 2% non-fat skim milk (w/v).

A.1.6 ELISA Conjugate solution: Dilution solution containing Rabbit anti-monkey IgG HRP polyclonal antibody [Sigma; Cat:] in 1:2000 dilution.

A.1.7 ELISA Substrate solution: Prepared from TMB Microwell Peroxidase Substrate kit [KPL; Product Number: 50-76-00] according to manufacturer's instructions.

A.1.8 ELISA Stop solution: 4N sulphuric acid.

APPENDIX A.2: TISSUE CULTURE MEDIA

A.2.1 **R1 medium:** RPMI-1640 medium with Glutamax-1 [Gibco; Ref: 72400-021] supplemented with Penicillin-G [100 units/mL], Streptomycin [100 µg/mL] and 1% FBS [Delta Bioproducts; Cat: 14-501AI]. FBS was heat-inactivated at 56 °C for 30 min before being used.

A.2.2 **R10 medium:** RPMI-1640 medium with Glutamax-1 supplemented with 10% FBS, Penicillin-G [100 units/mL] and Streptomycin [100 µg/mL]. FBS was heat-inactivated at 56 °C for 30 min before being used.

A.2.2 **R20 medium:** RPMI-1640 medium with Glutamax-1 supplemented with 20% FBS, Penicillin-G [100 units/mL] and Streptomycin [100 µg/mL]. FBS was heat-inactivated at 56 °C for 30 min before being used.

A.2.3 **DNAse medium:** R1 medium containing DNAse-1 [Roche; Cat: 1284932] at 0.02mg/mL.

APPENDIX A.3: IFN-γ ELISPOT REAGENTS

A.3.1 **ELISPOT Plate Coating solution:** PBS solution, pH7.2 [Gibco; Ref: 14190-094].

A.3.2 **Capture antibody:** Purified anti-human IFN-γ [clone 1-D1K, Mabtech; HRP kit Cat: 3420-1H] diluted with capture antibody coating solution to 5 µg/mL. 50 µL/well was used.

A.3.3 **Detection (secondary) antibody:** Biotinylated anti-IFN-γ [clone 7-B6-1, Mabtech; HRP kit Cat: 3420-1H] diluted with diluent solution to 2 µg/mL. 50 µL/well was used.

A.3.4 **Streptavidin conjugate solution:** Streptavidin-HRP [BD-PharMingen; Cat: 554066] diluted 1:500 with diluent solution. 100 µL/well was used.

A.3.5 **Diluent solution:** PBS solution, pH7.2 containing 10% FBS.

A.3.6 **Substrate solution:** This was prepared from the NovaRed substrate kit [Vector; Cat: SK-4800] according to manufacturer's instructions. It was prepared just prior to use and kept protected from light.

A.3.7 Normal Gag peptide pools: These were made from 66 synthetic HIV-1 subtype C Gag peptides (kind donation of Dr C Gray, National Institute for Communicable Diseases [NICD], Johannesburg), which encompassed the entire Gag protein. Each peptide was supplied in lyophilised form at 1 mg. The peptides contained 15-18 amino acids with adjacent peptides overlapping by 10 amino acids. Each peptide was dissolved by adding 100 µL DMSO (tissue culture grade) to give 10mg/mL stocks. The peptides were pooled into 5 normal peptide pools as shown in **Table A.1** and diluted further with PBS to a final concentration of 80 µg/mL. Aliquots of 100 µL were kept in -80 °C until use. Shortly before use in IFN-γ ELISPOT assays, the peptide pool stocks were diluted 1:10 with R10 medium.

Table A.1: Normal peptide pools

Pool name	Peptide number	Total peptides (pool)	Volumes (µL) making the peptide pool stocks			Final concentration (in assay[f])	
			Total peptides	DMSO added	PBS added	µg/mL/peptide	%DMSO
P1	1-14	14	140	0	1110	4	0.56
P2	15-28	14	140	0	1110	4	0.56
P3	29-42	14	140	0	1110	4	0.56
P4	43-56	14	140	0	1110	4	0.56
P5	57-66	10	100	25	1125	4	0.50

f: final concentrations in the well (assay) after diluting peptide pool stocks 1:10 with R10 medium and a further dilution of 1:2 with the PBMC suspension, which is added to the ELISPOT plate wells.

A.3.8 Matrix peptide pools: These were made in the same manner as the normal peptide pools using the 10mg/mL peptide stocks but the number and arrangement of peptides in the matrix pools were different. Fourteen peptide pools were made from the 66 individual peptides comprising the Gag protein. Each pool contained 5 (or 4 in a few pools) individual and not overlapping peptides. The peptides were pooled as shown in **Table A.2** and diluted further with PBS to a final concentration of 80 µg/mL. Aliquots of 100 µL were kept in -80 °C. Shortly before use in Pool-Matrix IFN-γ ELISPOT assays, the pools were diluted 1:10 with R10 medium.

A.3.9 Individual Gag peptides: These were made from the 10mg/mL stocks by diluting them further to 0.4 mg/mL (for ICS) or 80 µg/mL (for IFN-γ ELISPOT assays) with PBS. Aliquots were kept in -80 °C until use. Shortly before use in ICS or IFN-γ ELISPOT assays, the peptides were diluted appropriately with R10 medium to yield a final concentration of 4 µg/mL in the stimulation reactions.

Table A.2: Matrix peptide pools.

Matrix pool[χ]	Peptide numbers contained in the matrix peptide pool					Total peptides in the pool
M1	1	15	29	43	57	5
M2	2	16	30	44	58	5
M3	3	17	31	45	59	5
M4	4	18	32	46	60	5
M5	5	19	33	47	61	5
M6	6	20	34	48	62	5
M7	7	21	35	49	63	5
M8	8	22	36	50	64	5
M9	9	23	37	51	65	5
M10	10	24	38	52	66	5
M11	11	25	39	52		4
M12	12	26	40	54		4
M13	13	27	41	55		4
M14	14	28	42	56		4
Normal pools [χ]	P1	P2	P3	P4	P5	

χ: Columns represent peptides in the normal peptide pool while rows represent peptides in the matrix peptide pools. Thus, each peptide is present only twice, in one normal and one matrix peptide pool. This arrangement is referred to as 'Pool-Matrix' format.

A.3.10 Positive control antigen: PHA-P [Sigma; Cat: L9132] stock solutions at 1mg/mL and stored at -30 °C in aliquots. A working solution was made by adding DMSO the stock solution and diluting with R10 medium to 8 µg/mL PHA and 1% DMSO to yield final concentrations of 4 µg/mL PHA and 0.5% DMSO in the stimulation reactions.

A.3.11 Negative 'No Antigen' control: R10 medium containing 1% DMSO, yielding a final concentration of 0.5% DMSO in the assay.

A.3.12 PPD antigen: Bovine PPD [Institute for Animal Science and Health, The Netherlands] at 1mg/mL stocks were stored at +4 °C until use. A working solution was made by adding DMSO and R10 medium to 10 µg/mL PPD and 1% DMSO. A final concentration of 5 µg/mL PPD and 0.5% DMSO was used in the stimulation reactions.

APPENDIX A.4: FACS REAGENTS

A.4.1 FACS Wash Solution: PBS, pH7.4, containing 1% FBS and 0.01% sodium azide.

A.4.2 FACS Lysing Solution: FACS Lysing Buffer [BD; Cat: 349202] diluted 1:10 with de-ionised water as per manufacturer's instructions.

A.4.3 Permeabilising Solution: FACS Permeabilizing Solution 2 [BD; Cat: 340973] diluted 1:10 with de-ionised water as per manufacturer's instructions.

A.4.4 Fixing Solution: CellFix [BD; Cat: 340181] diluted 1:10 with de-ionised water as per manufacturer's instructions.

A.4.5 Brefeldin A Solution: Brefeldin A (BFA; 5mg powder, Cat: B7651, Sigma) was reconstituted in 500 µL DMSO to make 10 mg/mL stock solution. Aliquots of 10 µL were kept frozen at -20 °C until use. 1 µL of stock solution was added to 1 mL PBMC suspension in ICS assay to give a final concentration of 10 µg/mL brefeldin A and 0.1% DMSO (or dilute BFA stock 1:10 with R10 and dispense 5 µL to 0.5 mL PBMC suspension).

A.4.6 EDTA Solution (20mM): 3.7g EDTA ($EDTA.2H_2O$; FW = 372.24) was dissolved in 50mL PBS, and adjusted to pH 9. Subsequently, a further dilution of 1:10 in PBS was done (to give 20mM EDTA) and pH adjusted to 7.0. After filter sterilising the solution, aliquots were made and stored at +4 °C. 50 µL per 1 mL cell suspension was used.

A.4.7 Positive Control: Staphylococcal Enterotoxin B (SEB, 1mg; Sigma Cat S4881) was reconstituted in 2 mL sterile PBS to give 0.5 mg/mL. A further dilution of 1:10 was done using PBS (50 µg/mL). Aliquots were stored in +4°C. 20 µL per 1 mL PBMC suspension was used for stimulation (final concentration of 1 µg/mL SEB and 0.1% DMSO).

A.4.8 Monoclonal antibodies: A panel of monoclonal antibodies that were used for staining the cells are shown in **Table A.3**

Table A.3: A panel of monoclonal antibodies that was used in ICS assay.

Name	Clone	Label	Source	Use
Anti-human CD28	CD28.2	Purified	BD-PharMingen	Co-stimulation
Anti-human CD49d	9F10	Purified	BD-PharMingen	Co-stimulation
Anti-monkey CD3	FN18	FITC	BioSource	CD3 surface marker
Mouse IgG1	MOPC21/321	FITC	BioSource	Isotypic control
Anti-human CD4	SK3	APC	BD-IS	CD4 surface marker
Anti-human CD8	SK1	APC	BD-IS	CD8 surface marker
Mouse IgG1	X40	APC	BD-IS	Isotypic control
Anti-human IFN-γ	4S.B3	PE	BD-PharMingen	IFN-γ intracellular marker
Mouse IgG1	MOPC-21	PE	BD-PharMingen	Isotypic control

APC: Allophycocyanin; FITC: Fluorescein isothiocyanate; PE: Phycoerythrin; BD: Becton Dickinson; IS: Immunocytometry Systems.

Table A.4: Details of antibodies used in immunological assays utilising baboon samples during the course of the studies described in this thesis.

Assay Category	Antibody name and description	Clone	Isotype and clone	Manufacturer	Use
FACS	Anti-monkey CD3; FITC labelled	FN18	IgG1; MOPC-21/321	BioSource	Detection of CD3 molecule (T cell)
	Anti-human CD3; PE labelled	SP34	IgG3; ???	BD-Ph	Detection of CD3 molecule (T cell)
	Anti-human CD4; PerCP labelled	L200	IgG1; MOPC-21	BD-Ph	Detection of CD4 molecule (T-helper)
	Anti-human CD4; APC labelled	SK3	IgG1; X40	BD-IS	Detection of CD4 molecule (T-helper)
	Anti-human CD4; RD1 (PE) labelled	SFC112T4D11	IgG1; 2T8-2F5	Coulter	Detection of CD4 molecule (T-helper)
	Anti-human CD8; APC labelled	RPA-T8	IgG1; MOPC-21	BD-Ph	Detection of CD8 molecule (CTL)
	Anti-human CD8; APC labelled	SK1	IgG1; X40	BD-IS	Detection of CD8 molecule (CTL)
	Anti-human CD16; FITC labelled	3G8	IgG1; MOPC-31C	BD-Ph	Detection of CD16 molecule (NK cell)
	Anti-human CD20; FITC labelled	H299	IgG2a; 7T4-1F5	Coulter	Detection of CD20 molecule (B cell)
	Anti-human CD28; purified	CD28.2	NA	BD-Ph	Co-stimulation
	Anti-human CD28; purified	L293	NA	BD-IS	Co-stimulation
	Anti-human CD49d; purified	9F10	NA	BD-Ph	Co-stimulation
	Anti-human CD49d; purified	L25	NA	BD-IS	Co-stimulation
	Anti-human CD56; PE labelled	B159	IgG1; MOPC-21	BD-Ph	Detection of CD56 molecule (NK cell)
	Anti-human CD69; FITC labelled	FN50	IgG1; MOPC-21	BD-IS	Detection early activation marker, CD69
	Anti-human HLA-DR; cy-chrome	G46-6	IgG2a; G155-178	BD-Ph	Detection late activation marker, HLA-DR
	Anti-human IFN-γ; PE labelled	4S.B3	IgG1; MOPC-21	BD-Ph	Detection of intracellular IFN-γ
ELISPOT	Anti-human IFN-γ; purified	1-D1K	NA	Mabtech	Capture antibody for IFN-γ
	Anti-monkey IFN-γ; purified	GZ-4	NA	Mabtech	Capture antibody for IFN-γ
	Anti-human IFN-γ; biotinylated	7-B6-1	NA	Mabtech	Detection of captured IFN-γ
ELISA	Anti-monkey IgG; HRP conjugated	Not defined	NA	Sigma	Detection of IgG antibodies in serum
	Anti-monkey IgG; AP conjugated	Not defined	NA	Sigma	Detection of IgG antibodies in serum
	Anti-human IFN-γ; purified	1-D1K	NA	Mabtech	Capture antibody for IFN-γ
	Anti-monkey IFN-γ; purified	GZ-4	NA	Mabtech	Capture antibody for IFN-γ
	Anti-human IFN-γ; biotinylated	7-B6-1	NA	Mabtech	Detection of captured IFN-γ
Western blot	Anti-monkey IgG; AP conjugated	Not defined	NA	Sigma	Detection of IgG antibodies in serum
CBA	Non-human primate cytokine kit containing anti-human IFN-γ, TNF-α, IL-2, IL-4, IL-5 & IL-6; PE labelled	Not defined	NA	BD	Detection of multiple cytokines secreted in the extra-cellular medium

AP: alkaline phosphatise; APC: allophycocyanin; BD-IS: Becton Dikinson - Immunocytometric Systems; BD-Ph: Becton Dikinson – PharMingen; CBA: cytometric bead array; CTL: cytotoxic T lymphocytes; FACS: fluorochrome activated cell sorting; FITC: fluorescein isothiocyanate; HRP: horseradish peroxidase; NA: not applicable; NK: natural killer cell; PE: phycoerythrin; PerCP: peridinin chlorophyll.

APPENDIX B: GENERAL TECHNIQUES

APPENDIX B.1: BLOOD PROCESSING AND PBMC ISOLATION

PBMC isolation by gradient centrifugation

Blood was collected in 10 mL BD Vacutainer™ tubes containing lithium heparin (170 I.U. per tube). Leucosep tubes (LST) were prepared by adding 15 mL ficoll (which is allowed to stabilise to room temperature) to corresponding number of tubes and centrifuged at 1000xg (2300 rpm Heraeus 1.0R centrifuge) for 1 min to make the ficoll pass through the membrane. Then, 20 mL (contents of 2 tubes) was poured on directly onto the LST tubes after gently inverting the blood tubes in an arc several times. The LST tubes containing the blood were spanned in the centrifuge for 15 min at 1000xg. Centrifugation resulted in the blood tube contents dividing into four distinct layers:

(i) Packed red blood cells (RBC), granulocytes and "debris" at the bottom

(ii) Ficoll layer with disc separating RBC from rest of Ficoll

(iii) PBMC band, and

(iv) Plasma

The plasma was removed (and discarded), being careful not to disturb the PBMC layer beneath the plasma, and transferred carefully into a 50 mL polypropylene centrifuge tube. Wash solution (1%FBS in PBS) was added to PBMC, followed by centrifugation for 10 minutes at 250xg. The supernatant was decanted carefully without disturbing the cell pellet. The cell pellet was re-suspended in 10 mL wash solution using disposable pipettes after which more wash solution was added to 25 mL. After further centrifugation as above, the pellet was re-suspended in 10 mL wash solution and an aliquot of 50µL taken into a labelled eppendorf tube for cell counting using the Coulter MD18™ cell counter. After final centrifugation, the PBMC were finally re-suspended either at 10 million cells per mL using R10 for use in assays or prepared for cryo-preservation as described in **Appendix B.2**.

Separation of serum

Blood was collected in 3.5 mL SST II BD Vacutainer™ tubes. Blood was left to clot in the tubes for at least 2 hours at room temperature. The tubes were span at 1000xg for 10 min and serum aspirated into cryovials. Serum stocks and aliquots of 15 µL were stored at -30 °C for use in serology.

APPENDIX B.2: PBMC STORAGE

Cryo-preservation of PBMC for long-term storage

The principle was to lower the temperature of the samples gradually to -80 °C before transferring them to liquid nitrogen freezer for long-term storage. The gradual and controlled rate of cooling may be provided by Mr. Frosty™ (Nalgene) when placed in -80 °C freezers. DMSO helps to maintain the integrity of the cell membranes, by eliminating ice formation (which would otherwise break up the cell membranes).

Directions for using Mr Frosty™

This instrument provides a slow rate of cooling at approximately -1°C/min when used correctly.

(i) It should be stored at room temperature when not in use.

(ii) Isopropanol should be replaced after every 5th use.

(iii) To change isopropanol, the high-density polyethylene vial holder and the foam insert should be removed from the polycarbonate unit. Then, 250 mL of 100% isopropyl alcohol should be added to the 250-mL fill line and the foam inserted and vial holder replaced carefully.

(iv) Mr. Frosty should be stored at 4°C before use by placing in ice (or at 4 °C) for at least 30 min before use.

Cell freezing solution

Cell freezing solution was made just before use by mixing 3 parts of FBS with 2 parts DMSO (to give 40% DMSO) in a 15 or 50-mL screw cap centrifuge tube (using **Table A.5** as a guide). The freezing solution was allowed to cool on ice for at least 10 minutes before use (as DMSO is toxic to cells and the toxicity increases at higher temperatures.

Table A.5: Freezing Solution - Ratio of FBS to DMSO

Final volume (mL)	Max Number of cryovials	FBS (mL)	DMSO (mL)
2.5	10	1.5	1
5	20	3	2
10	40	6	4
15	60	9	6
20	80	12	8

Preparation of PBMC for cryo-preservation.

After the last washing, PBMC were re-suspended in ice-cold FBS, in a 50mL screw cap centrifuge tube. The amount of FBS to be added should give a final concentration of 10-20x10^6 cells per mL (after addition of freezing solution) so that a mono-dispersion of cells is attained (Table 2 below used as a guide). The cells were re-suspended using a pipette. Then, an appropriate volume of ice-cold freezing solution wash added to the cell suspension in the ratio of 3 parts PBMC suspension to 1 part freezing solution (as shown in **Table A.6** below).

Aliquots of 1 mL suspension were placed into pre-prepared cryovials, which were then placed into the pre-cooled Mr. Frosty. Mr. Frosty was place on ice for not longer than 10 min before transferring to -80°C for a maximum of 24 hours.

Table A.6: Details of cell suspension in FBS, freezing solution, and cryovials, based on final number of PBMC to be frozen.

Cell count (10^6 PBMC)	FBS to resuspend PBMC (µL)	Freezing Solution added (µL)	Final Vol (mL)	Volume per Cryovial (µL)	Number of Cryovials
<11	300	100	0.4	400	1
11-20	750	250	1	1000	1
21-40	1500	500	2	1000	2
31-60	2250	750	3	1000	3
41-80	3000	1000	4	1000	4
51-100	3750	1250	5	1000	5
61-120	4550	1500	6	1000	6
71-140	5250	1750	7	1000	7
81-160	6000	2000	8	1000	8
91-180	6750	2250	9	1000	9
101-200	7500	2500	10	1000	10

Transfer of frozen cells to liquid nitrogen (on the following day)

The cryovials containing the frozen cells were transferred from the Mr Frosty into pre-cooled boxes and left for a further 1h at -80 °C. The boxes were then carried on dry ice and transferred to liquid nitrogen freezers. The storage details were recorded in a logbook.

APPENDIX B.3: REVIVAL OF CRYO-PRESERVED PBMC

This is a STERILE procedure and sterility techniques were applied. PBMC samples in cryovials were transferred from the liquid nitrogen tank onto dry ice in a cold box and moved to the laboratory. The tubes were placed in the water bath at 37°C to thaw the cells. Care was taken to avoid shaking the vials as micro-crystals of ice may damage the cell membranes resulting into loss of viability. Working in the biosafety cabinet, cells were transferred to a 50 mL tube and 10 mL of R1 at 37°C added drop-wise while swirling the tube. Then more R1

was added to 25 mL and centrifuged at 1100 rpm (250xg) for 10 minutes. The supernatants were discarded and the cell pellet re-suspend with 5 mL of R1/DNAse medium (0.002% of DNAse). The cells were washed again with 25 mL of R1 and centrifuged for 10 minutes at 1100 rpm once again. After discarding the supernatant, the cells were re-suspended in 5mL of R20 medium and an aliquot for cell count was removed. Trypan blue solution (Sigma) and a hemocytometer were used for cell counting. Samples with cell viability of less than 66% were discarded. Using R20 medium, cell concentration was adjusted to 2-4x10^6 cells/mL, without exceeding a final volume of 5 mL per tube. The cells left to rest overnight in CO_2 incubator (set at 37°C, 5% CO_2).

On the the following day, the cells were re-counted to determine the cell viability. Samples with viability of is less than 66% were discarded. The cells were then washed with warm R10 medium as before and re-suspended at 4x10^6 cells/mL using R10 medium. The cells were then used in ELISPOT, ICS or CBA assays.

APPENDIX B.4: IFN-γ ELISPOT ASSAY

Day 1: Coating plates and reviving PBMC

Coating plates

This is a STERILE procedure and sterile techniques were used. Working in the biosafety cabinet, the human IFN-γ capture mAb (1-D1K, Mabtech) was diluted in sterile PBS to 5μg/mL (for 1 plate, 6mL was needed i.e. 30 μL antibody + 5970 μL PBS). The antibody solution was poured into sterile 90mm petri dish. Still working in the biosafety cabinet, the wells of 96-well Multi-Screen ELISPOT plates (Millipore; Cat: MAIP NOB50) were coated with capture antibody by dispensing 50μL/well of the antibody solution and gently tapping to ensure that the entire membrane surface was completely covered with coating antibody. The plates were then sealed with the sterile microplate sealer (applied over the wells before replacing the plate cover) **or** parafilm tape (applied around on the outside after replacing plate cover) to prevent evaporation of antibody solution from the wells during the incubation period. Plates were incubated overnight at +4°C.

Reviving cryo-preserved PBMC

The cells to be analysed were revived as described in **Appendix B.3**. The cells were left overnight in CO_2 incubator (set at 37°C, 5% CO_2).

Day 2: ELISPOT Plate preparation and plating cells

Plate set-up

An appropriate template was drawn detailing the plate layout. The ELISPOT plates were removed from the fridge and labelled appropriately (e.g. name or initials of operator, date & plate number). The plates were washed 3 times to remove excess coating antibody by adding 200µL/well of sterile PBS from a sterile 90mm petri dish. A multi-channel pipette and sterile pipette tips were used to dispense. The PBS was flicked out from the plates into a plastic discard dish that was placed inside the biosafety hood. To block non-specific sites on the plate, 100µL R10 medium per well was added and plates incubated at room temperature for at least 2h.

Preparation of PBMC

During the blocking period, the revived cells (in the previous day) were re-counted and the cell viability calculated. Samples whose viability was less than 66% were discarded. The cells were washed with R10 medium as before and re-suspend at $4x10^6$ cells/mL using R10 medium. 50µL (ie $2x10^5$ cells) was added per well. (If the recovered cells were too few, the cells were re-suspend in $3x10^6$cells/mL or $2x10^6$cells/mL (ie $1.5x10^5$ or $1x10^5$ per well respectively).

Set-up of stimulation reactions

When ready to plate the PBMC, the blocking solution (R10 medium) was discarded from the ELISPOT plate by flicking out the medium without draining out the wells completely. Using a clean pipette tip for each reagent, 50µL of '**No Antigen**' (background control) and 50µL of Gag peptide or peptide pools were added to triplicate wells (using the template as a giude) followed by 50µL of the PBMC suspension. PHA (and PPD) was added last to avoid splashing or carry-over into the test wells. The plates were then incubated at 37° C with 5% CO_2 for 22-24 h.

Day 3: Detection and membrane development

ELISPOT Wash Solution (PBS solution containing 0.05% tween-20) and ELISPOT Diluent Solution (PBS solution containing 10% FBS) were prepared before hand. The plates were washed 6 times with Wash Solution using an automatic plate washer. The plates were tapped firmly on absorbent paper to remove excess wash fluid and 50 µL/well of anti-IFN-γ biotinylated antibody (clone 7-B6-1, Mabtech; diluted to 2µg/mL in Diluent Solution) was added. The plates were incubated at 37° C for 2 h after which they were washed again as

before. Streptavidin-HRP was diluted to 1:500 in Diluent Solution and then added (100 µL/well) to the plates. The plates were incubated at room temperature for 1 h followed by a wash as before., Freshly prepared NovaRed substrate was added at a rate of 100µL/well and plates left to stand for 5-8 min (or until spots appear clearly) at room temperature in the dark. The reactions were stopped by emptying the wells and rinsing with cold tap water. The plastic bottoms covering the plates were removed and the plates left to air-dry by placing them up side down in the dark overnight.

Day 4: Spots analysis

The plates were scanned automatically using the CTL Spot Analyser (Cellular Technology Ltd, CTL) and the spots were analysed using the CTL Immunospot software version 3.2. Average spots per well for each antigen was calculated and normalised to spots per million PBMC input (or spot-forming units, SFU/10^6 PBMC). The background stimulation was removed from 'Test' stimulations by subtracting the '**No antigen**' read-out from each test reaction.

APPENDIX B.5: ICS ASSAY

Day 1: Reviving of cryo-preserved PBMC

The cells to be analysed were retrieved from the liquid nitrogen tank and revived as described in **Appendix B.3**.

Day 2: Preparation of PBMC and stimulation set-up

The revived cells were counted and the cell viability calculated. Samples whose viability was less than 66% were discarded The cells were washed with R10 medium as before and re-suspend at 2-4x10^6 cells/mL using R10 medium. (If the recovered cells were too few, the cells were re-suspended 1x10^6cells/mL). 0.5 mL per tube of cell suspension was dispensed into 5-mL poly propylene tubes, which were marked as follows; No antigen, SEB, Test 1, Test 2 etc depending on the number of test stimuli to be used in the stimulation reactions. The following reagents were added to the tubes as shown in **Table A.7**. The tubes were vortexed briefly on low setting and incubated for 2 h in 37 °C, CO_2 incubator. The BFA stock (at 10mg/mL) was diluted 1:10 with R10 medium and 5 µL per tube dispensed (to a final concentration of 10 µg/mL BFA). After brief mixing by vortexing, the tubes were incubated overnight for a further 14 h.

Table A.7: Reagents used in setting up ICS stimulation reactions.

Reagent	No Antigen tube	Test tube	SEB tube
PBMC suspension, 2-4x10^6/mL (µL)	450	450	450
Anti-CD28/49d mixture, 1mg/mL each (µL)	2	2	2
10% DMSO in PBS (µL)	2	0	2
Gag peptide, 0.4mg/mL (µL)	0	5	0
SEB, 50 µg/mL (µL)	0	0	10
R10 medium (µL)	46	43	36
Total volume (µL)	**500**	**500**	**500**

Day 3: Cell staining and data acquisition

The reactions were stopped by adding 50 µL EDTA (20 mM) to each tube followed by mixing and incubation at RT for 15 minutes. After vigorous vortexing, 1.5 mL FACS wash solution was added and the contents of each tube split into 4 equal volumes and transferred to 4 new 5-mL polystyrene FACS tubes. , Two tubes of cells were for staining for CD4 and CD8 and the other two tubes for isotype control antibody staining. The volume was made up to 2 mL with FACS wash solution and the cells were pelleted by centrifugation for 5 minutes at 500xg (1600 rpm). The supernatant was decanted and cells re-suspended in the residual volume by vortexing. A 2-step staining procedure with the monoclonal antibodies (see **Table A.3**) was performed. In the first step, the monoclonal antibodies for surface markers (CD3, CD4 & CD8) and their isotope controls were added (10 µL each per tube) to appropriate tubes, mixed then incubated on wet ice in the dark for 30 min. The cells were washed as before and the pellets re-suspended in 1 mL FACS lysing solution. After 10 min incubation at RT, the cells were washed again and re-suspended in 0.5 mL Permebealising solution. Cells were incubated again at RT for 10 min and washed again as before. The cell pellets were re-suspended in the residual volume and 10 µL of anti-human IFN-γ and isotope control antibodies were added to appropriate tubes. The tubes were incubated on wet ice for 30 min in the dark then washed as before and the pellets re-suspended in 0.5 mL Fixing solution.

Data acquisition

BD FACSCalibur™ (BD) flow cytometer was used for FACS data acquisition. Before the data acquisition, the photomultiplier tube (PMT) voltages, fluorescence, compensation and instrument sensitivity were set using BD CaliBrite beads™ (BD). Further instrument set-up was performed manually to tune the PMT voltages and compensation percentages using baboon PBMC stained with single fluorochromes i.e. CD3-FITC, CD8-APC and IFN-γ PE. Once the appropriate instrument settings were established, an instrument settings file was saved and used for FACS data acquisition for these studies.

Data acquisition was performed by recalling the instrument settings file. An acquisition template was created by using forward scatter (FSC) versus side scatter (SSC) and drawing a region 1 (R1) around the lymphocyte population on the dot plot graph. A histogram graph for each of CD3-FITC, CD4- or CD8-APC and IFN-γ-PE. For each sample, at least 100,000 events in R1 were collected.

Data analysis

Using BD CellQuest™ (BD) software, analysis templates were created for CD4 and CD8 (see **Fig A.1**). The CD8 template was created as follows:

(i) A FSC versus SSC dot plot with no gate was created and a region, R1, drawn around the lymphocyte population.

(ii) A CD3-FITC versus CD8-APC dot plot was created gating on Gate 1, G1 (where G1=R1) and a region, R2, was drawn to encompass all CD3 positive cells. A quadrant statistics box was also created for this dot plot graph.

(iii) A CD3-FITC histogram and histogram statistics box gating on G1=R1 were created.

(iv) A CD8-APC histogram and histogram statistics box gating on G1=R1 were created.

(v) A IFN-γ-PE histogram and histogram statistics box were created gating on G1=R1.

(vi) A logical gate named G3 (where G3=R1 and R2) was created. Gating on G3, a CD8-APC versus IFN-PE dot plot was created. A quadrant statistics box was created for this dot plot. The upper right (UR) quadrant gives the statistics for CD4 (or 8) and IFN- double positive events.

A CD4 template was created in a similar way except for creating a CD3-FITC versus CD4-APC and logical gate G3 since the anti-monkey CD3 antibody was not used for staining these cells. Thus, a CD4 template only included the following:

(i) A FSC versus SSC dot plot with no gate and with a region, R1, drawn around the lymphocytes.

(ii) A CD4-APC histogram and histogram statistics box gated on G1=R1.

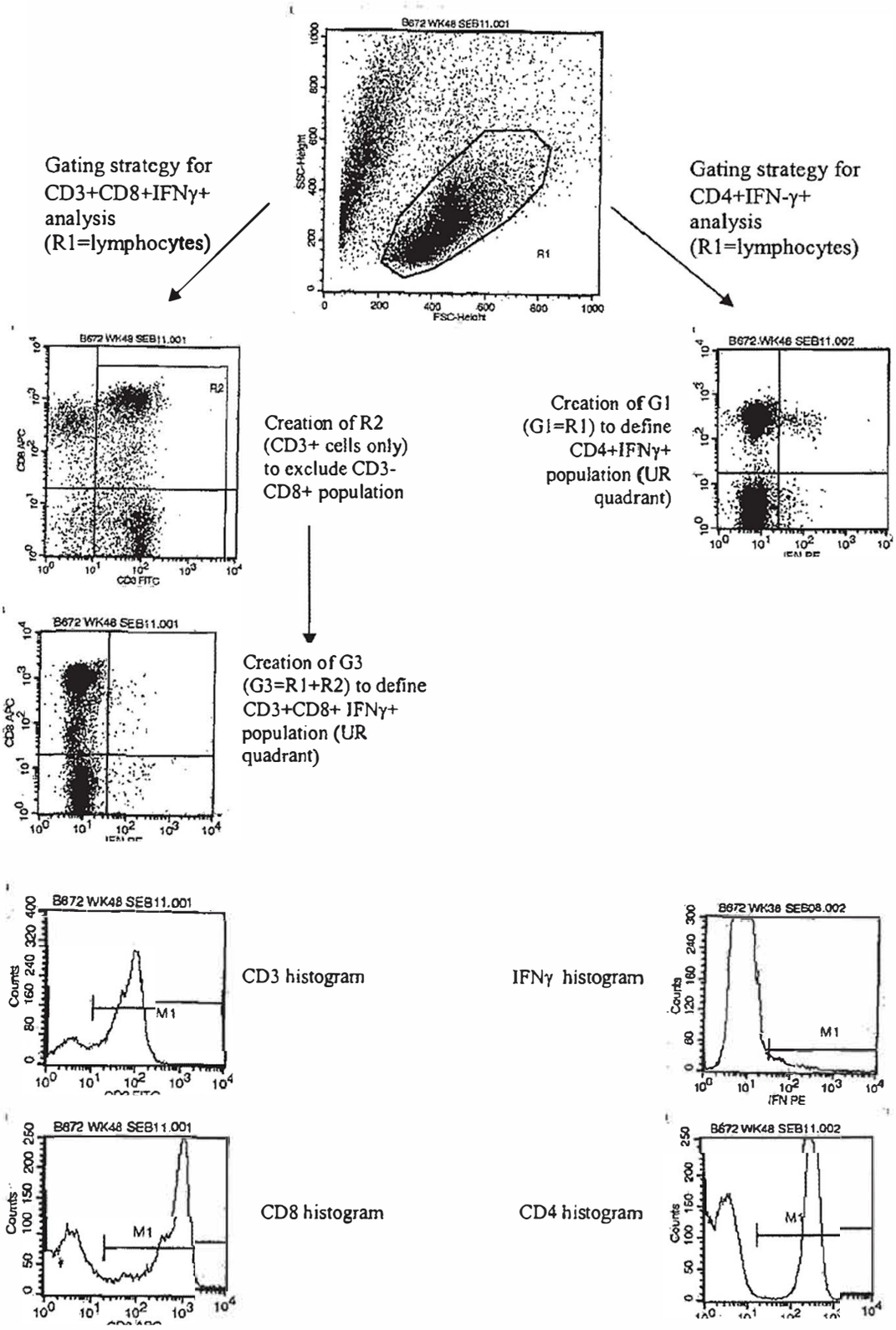
(ii) IFN-γ-PE histogram and histogram statistics box gated on G1=R1.

(iii) A CD4-APC versus IFN-PE dot plot gated on G1=R1. A quadrant statistics box was created for this dot plot. The upper right (UR) quadrant gives the statistics for CD4 and IFN-γ double positive events.

The positive population in the SEB control sample rather than the negative or isotype control sample was used to define a response region around the CD4 (or CD3/CD8) and IFN-γ double positive events on the CD4 or CD8 -APC versus IFN-γ-PE dot plot.

The percentage of IFN-γ producing CD4+ or CD8+ cells was calculated by dividing the events in the UR (double positive for IFN-γ and CD4 or CD3/CD8) by the sum of events in UR and LR (lower right; CD4+ or CD3+/CD8+ which was negative for IFN-γ). The specific IFN-γ response was obtained by subtracting the percentage positive events in the unstimulated (No antigen) sample from the percentage positive events in the activated sample

Fig A.1: Gating strategy for data analysis. A forward versus side scatter dot plot separated the cells into a distinct lymphocyte population (R1). Creating a CD4 versus IFNγ dot plot and gating it on G1 (=R1) defined a CD4+IFN+ cell population on the upper right (UR) quadrant. For CD8 analysis, a CD3 versus CD8 dot plot was created and a region R2 drawn on CD3 positive cells to exclude CD8+ cells which were not T lymphocytes (which are largely NK cells). Creating a CD8 versus IFNγ dot plot which was gated on G3 (=R1 + R2) defined a CD3+CD8+IFN+ cell population on the UR quadrant.



APPENDIX C: SUPPLEMENTARY DATA

APPENDIX C.1: SURFACE MARKERS ON NAÏVE BABOON PBMC

Table A.8: Freshly isolated PBMC from naïve baboons were stained with anti-monkey CD3-FITC (BioSource), anti-human CD4-PerCP and anti-human CD8-APC (BD) and fixed with CellFix (BD). Data was acquired using FACSCalibur (BD) cytometer and analysed with CellQuest (BD) software. Data is presented as percentage of positive cells relative to lymphocyte gate.

Baboon Number	Sex	Weight (kg)	Surface markers (% lymphocyte gate)				
			CD3+	CD3+CD4+	CD3+CD8+	CD3-CD8+	CD8+
491	F	19.0	85.7	29.9	56.3	4.6	61.0
493	F	16.4	68.7	24.1	62.1	6.5	68.7
495	M	23.4	69.3	34.2	34.2	20.5	54.7
498	M	24.0	72.9	26.9	46.5	16.8	63.3
507	F	19.1	72.9	18.7	54.3	21.7	76.0
508	F	16.3	78.6	42.7	33.4	4.6	38.1
514	F	13.5	88.5	46.3	39.8	2.6	42.5
515	M	11.6	77.1	40.8	31.8	4.7	36.7
519	F	9.0	80.8	38.0	37.2	5.9	43.1
526	F	21.3	82.2	44.9	33.5	7.2	40.8
528	F	20.0	76.3	40.1	37.6	3.3	40.9
531	F	18.7	81.9	40.7	40.9	2.9	43.9
533	M	11.6	77.6	35.6	39.6	12.7	52.4
534	F	18.8	81.1	40.9	40.8	9.5	50.3
541	F	19.0	89.0	36.0	53.9	4.4	58.3
543	M	19.5	89.9	30.7	61.2	4.8	66.0
549	F	14.1	84.2	36.2	49.8	7.3	57.1
575	M	14.5	81.7	30.1	48.4	11.7	60.2
592	M	6.2	70.4	30.7	36.9	6.1	43.0
611	M	9.0	79.8	35.2	43.7	7.6	51.4
623	F	8.3	76.9	41.8	36.0	7.6	43.7
624	F	6.7	58.3	31.5	25.6	13.7	39.4
629	M	9.1	67.4	28.4	38.3	12.4	50.9
630	M	10.4	58.7	20.2	34.4	12.5	47.1
632	M	4.0	65.1	30.8	32.3	8.2	40.4
659	F	4.0	73.0	38.4	30.1	3.0	33.2
660	F	5.4	63.5	20.4	40.9	6.8	47.8
661	F	4.1	64.2	27.8	34.1	3.4	47.6
662	M	5.2	59.8	20.9	39.2	24.1	63.4
663	F	6.4	74.0	30.9	39.0	9.0	48.0
664	F	4.4	63.3	25.6	33.1	11.3	44.5
672	M	5.7	64.4	31.7	32.9	22.4	55.3
673	F	5.0	79.8	40.3	41.5	9.3	50.8
Mean		**12.2**	**74.4**	**33.1**	**40.6**	**9.4**	**50.3**
Standard deviation		6.5	9.0	7.5	9.0	6.0	10.2
Median		**11.6**	**76.3**	**31.7**	**39.0**	**7.6**	**48.0**
Minimum		4.0	58.3	18.7	25.6	2.6	33.2
Maximum		24.0	89.9	46.3	62.1	24.1	68.7

APPENDIX C.2: SURFACE MARKERS ON BCG-IMMUNISED BABOON PBMC

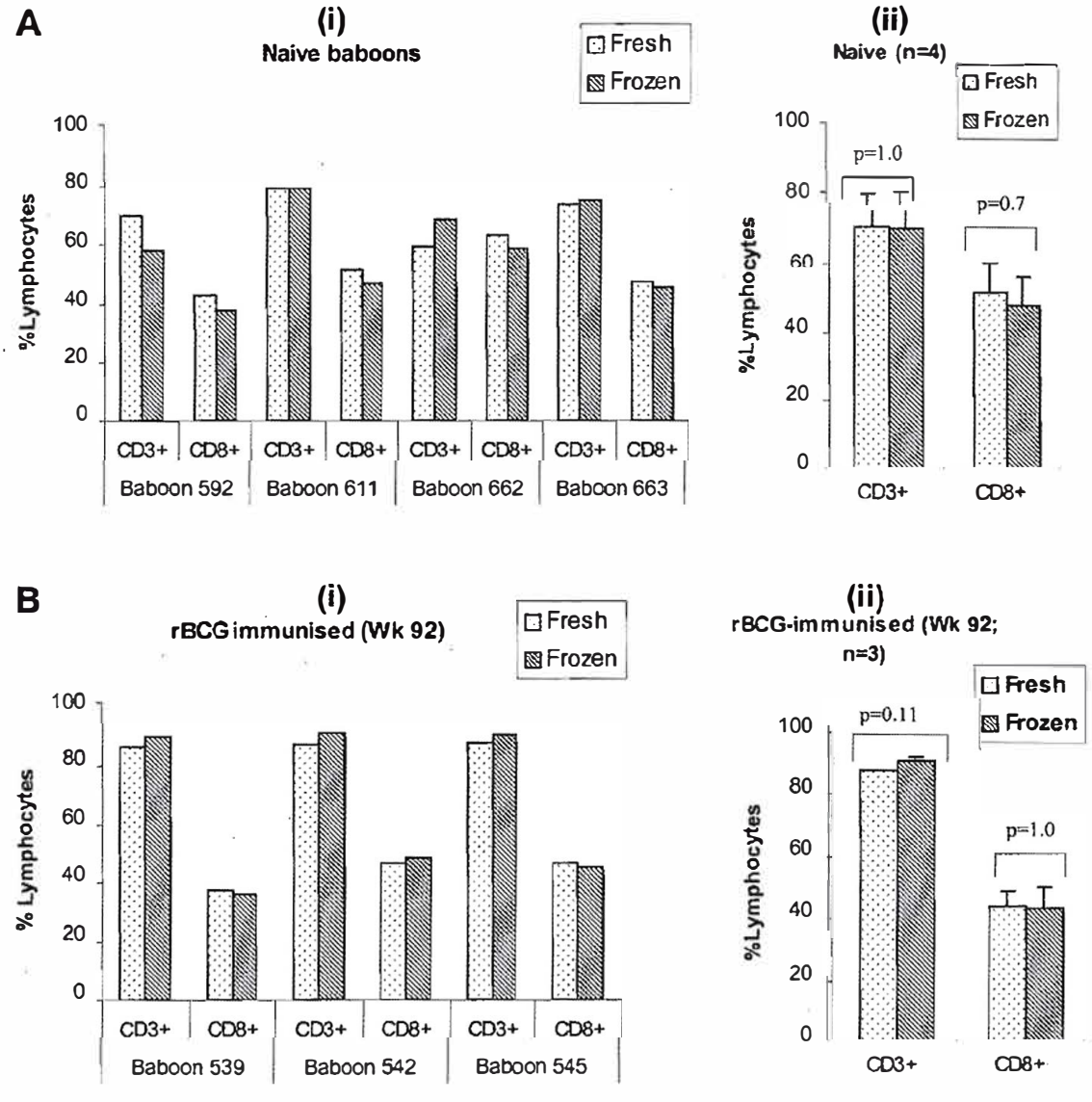
Table A.9: PBMC were obtained from baboons prior (Wk0) and post inoculation with wild-type BCG or saline and stained freshly with anti-monkey CD3-FITC (BioSource), anti-human CD4-RD1 (Coulter) and anti-human CD8-APC or with anti-human CD20-FITC (Coulter) and anti-human CD56-PE (BD-Ph) in a 2-test staining procedure. The cells were fixed with FACS lysing solution (BD). Data was acquired using FACSCalibur (BD) flow cytometer and analysed with CellQuest (BD) software. Data is presented as percentage of positive cells relative to lymphocyte gate.

BCG strain (and dose)	Baboon number	Surface marker	Wk0	Wk4	Wk10	Wk 20	Mean	Stdev
	350	CD3+	51.7	80.7	85.5	86.3	***76.0***	***16.4***
Pasteur		CD4+	20.4	26.2	32.5	27.9	***26.7***	***5.0***
		CD8+	NT	NT	NT	60.1	***NA***	***NA***
		CD20+	5.8	6.2	2.6	4.5	***4.8***	***1.6***
(10^6 CFU)		CD56+	2.0	2.7	2.2	2.8	***2.4***	***0.4***
	447	CD3+	82.4	78.0	83.0	84.1	***81.9***	***2.7***
		CD4+	44.1	40.0	41.2	42.0	***41.8***	***1.7***
		CD8+	NT	NT	NT	46.6	***NA***	***NA***
		CD20+	5.6	10.4	5.4	5.7	***6.8***	***2.4***
		CD56+	2.8	4.0	2.4	2.9	***3.0***	***0.7***
	378	CD3+	77.4	90.1	85.1	83.7	***84.1***	***5.2***
Tokyo		CD4+	39.8	52.6	49.0	45.6	***46.7***	***5.4***
		CD8+	NT	NT	30.7	34.1	***32.4***	***2.4***
		CD20+	4.3	1.4	3.0	5.3	***3.5***	***1.7***
(10^8 CFU)		CD56+	2.1	1.1	3.1	2.2	***2.1***	***0.8***
	454	CD3+	77.0	78.0	82.4	76.2	***78.4***	***2.8***
		CD4+	47.6	54.2	50.2	47.5	***49.9***	***3.2***
		CD8+	NT	NT	28.2	26.8	***27.5***	***1.1***
		CD20+	6.4	5.0	6.0	6.6	***6.0***	***0.7***
		CD56+	4.2	4.2	6.2	2.9	***4.4***	***1.4***
	363	CD3+	75.3	79.9	77.0	66.9	***74.8***	***5.6***
Tokyo		CD4+	42.8	51.3	47.7	37.4	***44.8***	***6.0***
		CD8+	NT	NT	28.2	28.9	***28.6***	***0.5***
		CD20+	5.0	3.6	4.4	10.5	***5.9***	***3.1***
(10^6 CFU)		CD56+	2.9	1.4	5.4	3.0	***3.2***	***1.6***
	466	CD3+	83.0	83.2	78.8	79.3	***81.1***	***2.4***
		CD4+	45.5	44.4	45.5	40.9	***44.1***	***2.2***
		CD8+	NT	NT	34.1	37.8	***36.0***	***2.6***
		CD20+	9.5	3.7	5.2	7.8	***6.6***	***2.6***
		CD56+	4.9	2.4	3.3	2.6	***3.3***	***1.1***
	388	CD3+	87.2	86.4	77.5	76.9	***82.0***	***5.5***
Tokyo		CD4+	45.6	48.2	35.6	38.1	***41.9***	***6.0***
		CD8+	NT	NT	42.7	39.9	***41.3***	***2.0***
		CD20+	4.7	2.0	4.7	5.5	***4.2***	***1.5***
(10^4 CFU)		CD56+	4.4	2.6	3.5	3.0	***3.4***	***0.8***
	428	CD3+	84.9	81.9	79.9	73.7	***80.1***	***4.8***
		CD4+	50.4	45.2	44.7	38.6	***44.7***	***4.8***
		CD8+	NT	NT	30.1	32.0	***31.0***	***1.3***
		CD20+	3.7	2.3	5.6	7.7	***4.8***	***2.3***
		CD56+	2.2	1.2	2.9	2.6	***2.2***	***0.7***
	427	CD3+	88.0	89.3	86.7	81.8	***86.5***	***3.3***
Saline		CD4+	55.6	56.4	50.3	49.3	***52.9***	***3.6***
		CD8+	NT	NT	31.4	29.9	***30.7***	***1.0***
		CD20+	3.4	2.5	5.5	6.5	***4.4***	***1.9***
		CD56+	2.6	1.0	2.9	3.0	***2.4***	***0.9***
	449	CD3+	75.7	78.9	75.2	74.5	***76.1***	***2.0***
		CD4+	46.4	43.8	41.3	41.7	***43.3***	***2.3***
		CD8+	NT	NT	31.3	30.4	***30.8***	***0.6***
		CD20+	8.8	4.3	5.5	6.0	***6.1***	***1.9***
		CD56+	5.3	2.2	5.0	3.4	***4.0***	***1.5***

APPENDIX C.3: SURFACE MARKERS: FRESH VERSUS FROZEN PBMC

The cell surface markers are important in identification of cell phenotypes involved in an immune response. This characterisation is a key feature of ICS assay. However, it was not always possible to perform ICS using freshly isolated PBMC, and often, cryo-preserved PBMC were used. Thus, it was important to establish if cryo-preservation of PBMC affected the expression or detection of these markers in any significant way.

Fig A.2: PBMC were obtained from 3 naïve (**A**) and 4 rBCG-immunised (**B**) baboons and divided into 2 aliquots per sample. Cells in one sample were stained freshly with anti-monkey CD3-FITC (BioSource) and anti-human CD8-APC in a single staining step and analysed by FACS. The other aliquots were stored in liquid nitrogen as described before (Appendix B.2). After four weeks, the cells were retrieved, as described in Appendix B.3. The cells were stained and analysed by FACS as described for fresh cells. Data points are percentages of positive cells in the lymphocyte gate for individual baboons (**i**) and mean values per group with error bars indicating the standard deviations of means (**ii**). P values were >0.05, indicating that there was no significant difference in expression/detection of CD3 and CD8 surface markers between fresh and cryo-preserved PBMC.



APPENDIX C.4: CD28 AND CD49d CO-STIMULATORY MOLECULES

Co-stimulatory antibodies, anti-CD28 and anti-CD49d, are often used in ICS assays due to their role in lowering the activation threshold for antigenic specific cells in a cellular immune response (Betts *et al*, 2004; Gauduin *et al*, 2004). This co-stimulatory effect is achieved by anti-CD28 and –CD49d via cross-linking of the corresponding CD28 and CD49d molecules on the T-cells, thereby providing a secondary activation signal. Two anti-human CD28 and CD49d were commercially available from BD-Immunocytometry Systems (BD-IS) and BD-PharMingen (BD-Ph).Three issues were investigated, namely

(i) if these human antibodies recognised baboon PBMC (**Fig A.3**)

(ii) if cryo-preservation of PBMC significantly changed the proportion of cells expressing these molecules (**Fig A.4**) and,

(iii) if antibodies from the two sources (BD-IS & BD-Ph) had similar effects in co-stimulating baboon PBMC (**Fig A.5**).

APPENDIX C.5: CD69 ACTIVATION MARKER

CD69 molecule, which is not present on the surface of resting peripheral blood T cells, is an early activation molecule that is expressed on T cells during *in vitro* stimulation (Testi *et al*, 1989). Thus, the use of an anti-human CD69 monoclonal antibody in the ICS assay is beneficial as it gives more assurance that the cells being defined as cytokine-positive have been activated by the antigen under investigation. Also, the use of an anti-human CD69 antibody allows better clustering of cytokine-positive cells, making it easier to define a cytokine-positive population (BD Application Handbook, 2003). The utility of anti-human CD69 antibody was investigated with a view of including it in the ICS assays. Firstly, the kinetics of CD69 antigen expression by baboon PBMC was examined and whether it could be detected using the anti-human CD69 monoclonal antibody clone FN50 (BD-IS; **Fig A.6**). Secondly, the prohibitive effect of BFA on expression of CD69 (previously observed; **Fig A.6**) and production of IFN-γ was further investigated by adding BFA at various times during a 16-h incubation of baboon PBMC with SEB (**Fig A.7**). These data showed that the anti-human CD69 antibody could be used to detect CD69 molecules on the baboon PBMC and that the expression of CD69 was acutely inhibited by brefeldin A.

Fig A.3: PBMC were obtained from baboon 536 and a human donor and incubated on ice for 30 min with or without pure anti-human CD28 (clone L293, BD-IS) or CD49d (clone L25, BD-IS). After 2 washes with FACS wash solution, the cells were incubated again on ice for 30 min with goat anti-mouse IgG-PE (DAKO, diluted 1:30 in PBS). The cells were fixed with FACS lysing solution (BD), washed twice as before and then re-suspended in FACS wash solution. Data was acquired using FACSCalibur (BD) cytometer and analysed with CellQuest (BD) software. The results are presented as histograms. The figures represent percentage of positive cells within the lymphocyte gate.

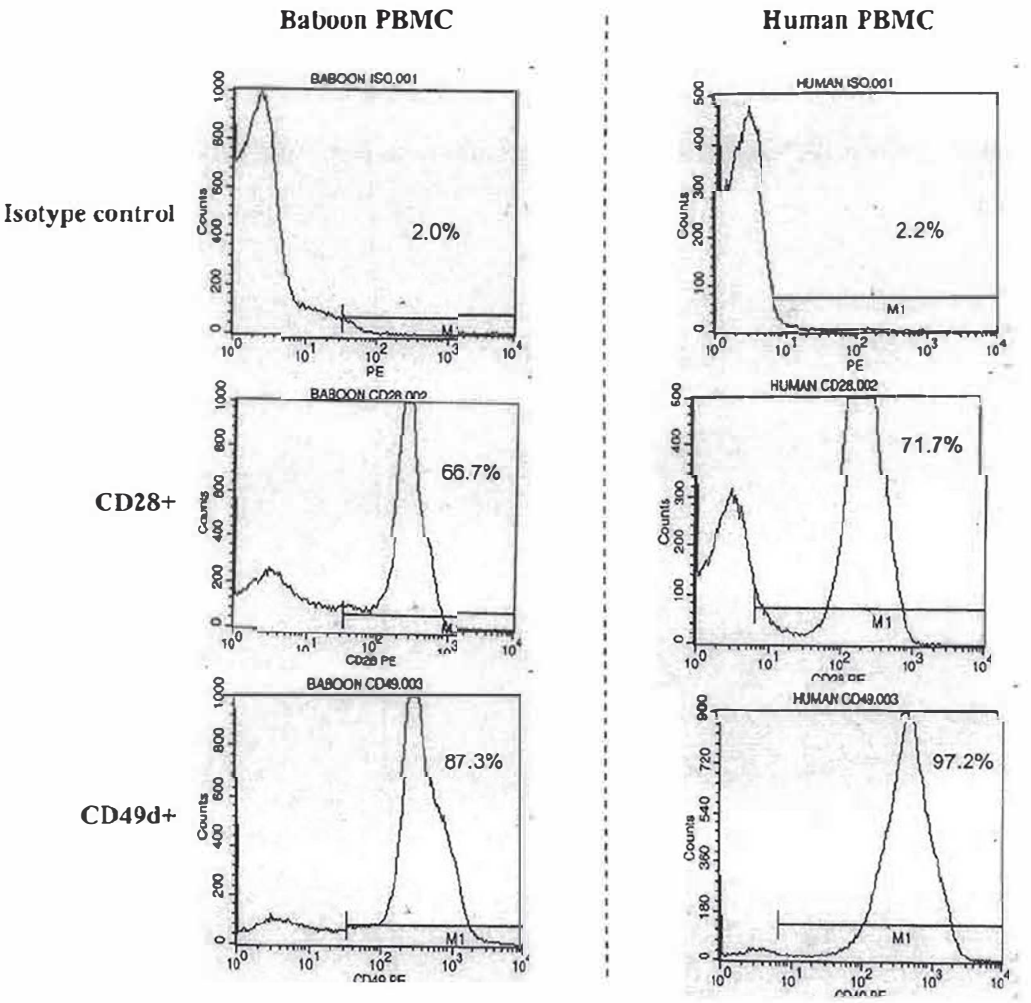


Fig A.4: PBMC were obtained from 3 baboons, which had been inoculated four times with RT106 (baboons 542 and 545) or RT108 (baboon 539) and once with GagC VLP vaccine. Aliquots of freshly isolated PBMC and aliquots of PBMC that had been frozen (Appendix B.2) for 4 weeks then revived (Appendix B.3) were stained with pure anti-human CD28 (clone 28.2, BD-Ph) or CD49d (clone 9F10, BD-Ph) mAB and incubated on ice for 30 min. After 2 washes with FACS wash solution, the cells were incubated again on ice for 30 min with goat anti-mouse IgG-PE (DAKO, diluted 1:30 in PBS). The cells were fixed with FACS lysing solution (BD), washed twice as before and then re-suspended in FACS wash solution. Data was acquired using a FACSCalibur (BD) flow cytometer and analysed with CellQuest (BD) software. Data points are percentages of positive cells in the lymphocyte gate for individual baboons (i) and mean values per group with error bars indicating the standard deviations of means (ii). P values are greater than 0.05, indicating that there was no significant difference in expression/detection of CD28 and CD49d surface molecules between fresh and cryo-preserved PBMC.

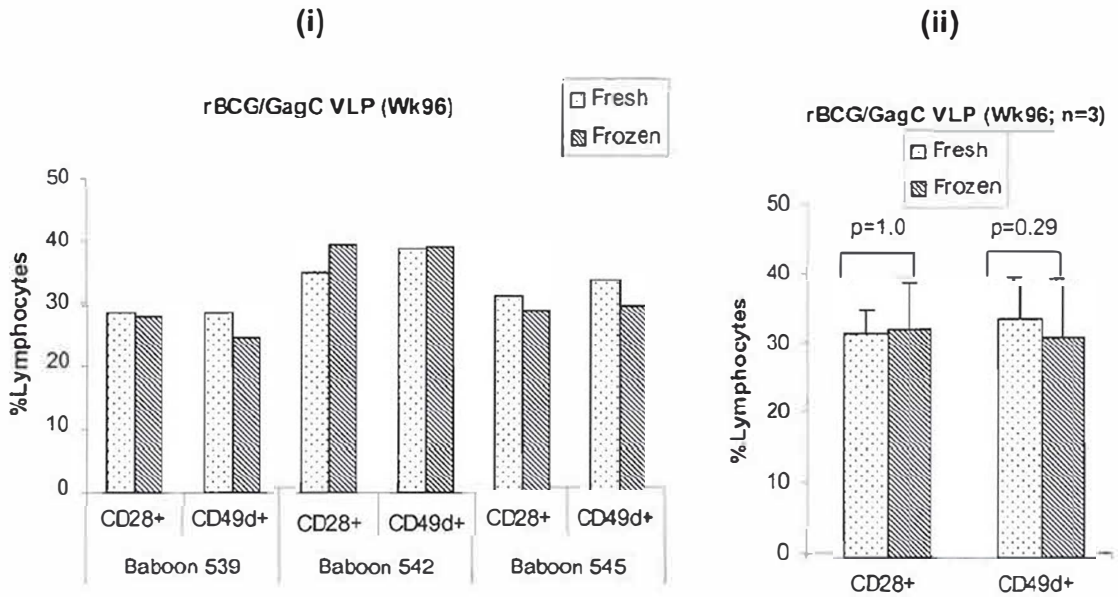


Fig A.5: PBMC were obtained from baboon 536 and incubated for 16 h with or without SEB, with or without addition of anti-human CD28 and CD49d monoclonal antibodies from either BD-IS or BD-Ph. Brefeldin A (Sigma, 10 µg/mL) was added in the last 14 h of incubation. Cells were stained for CD3, CD8 and IFN-γ in a 2-step staining protocol as described (Appendix B.5). Data was acquired using a FACSCalibur (BD) flow cytometer and analysed with CellQuest (BD) software. The data points are the percentage of CD3+ or CD8+ T cells that are positive for IFN-γ. These data indicate that the enhancement of the frequency of cytokine producing cells by the two mAB is comparable.

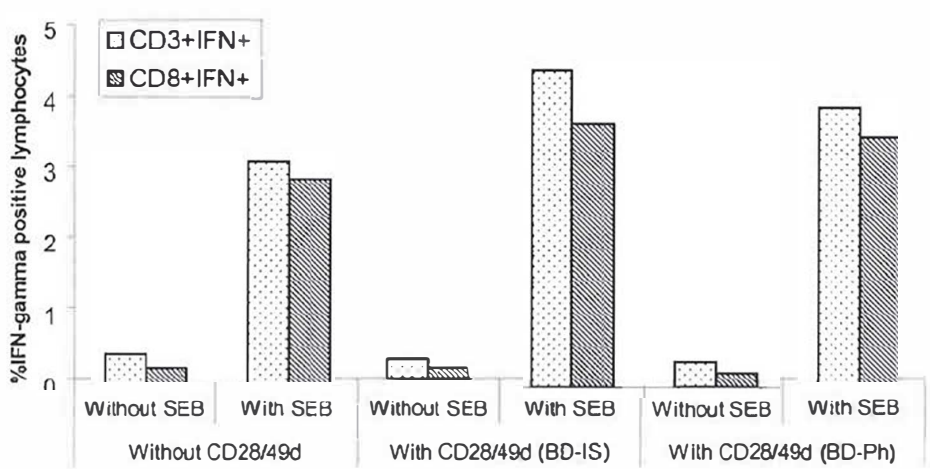


Fig A.6: PBMC were obtained from baboon 537 and incubated with or without SEB for various times or phorbol myristate acetate/calcium ionophore (25 ng/mL; PMA/I; Sigma) for 4 h. In one stimulation reaction, brefeldin A (Sigma) was added at the beginning of the incubation. Cells were washed once and stained with anti-human CD69 FITC-labelled antibody (BD-IS). Data was acquired using FACSCalibur (BD) flow cytometer and analysed with CellQuest software. Data points are percentage of lymphocytes expressing CD69 molecule at various times of incubation. These data show that the expression of CD69 molecule is dependent on the length of incubation, reaching the peak between 12 and 20h. Also, the data shows that addition of BFA at the beginning of incubation time completely inhibited the expression of CD69 molecule. Permeabilising the cells prior to staining with anti-human CD69 produced the same results (data not shown).

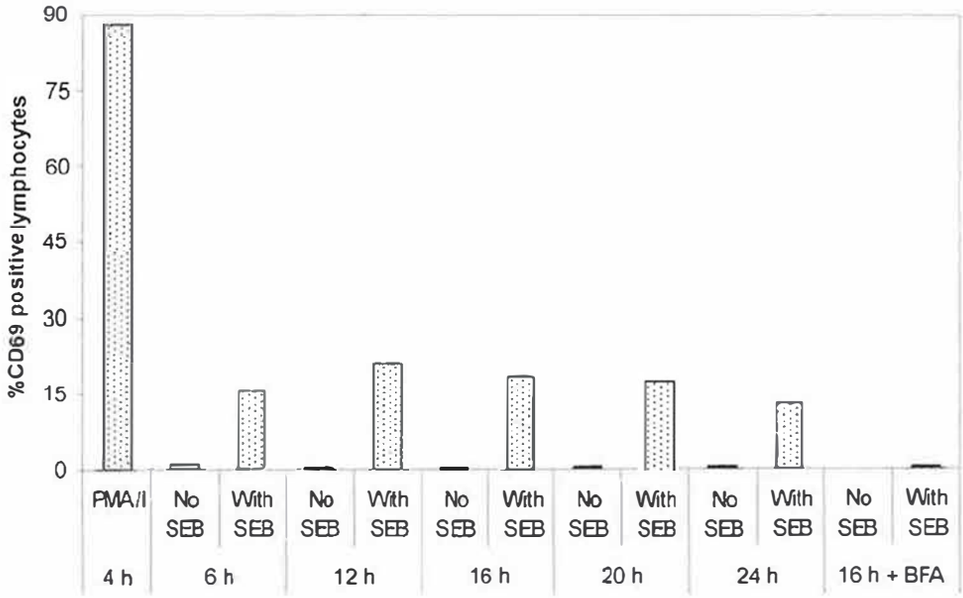
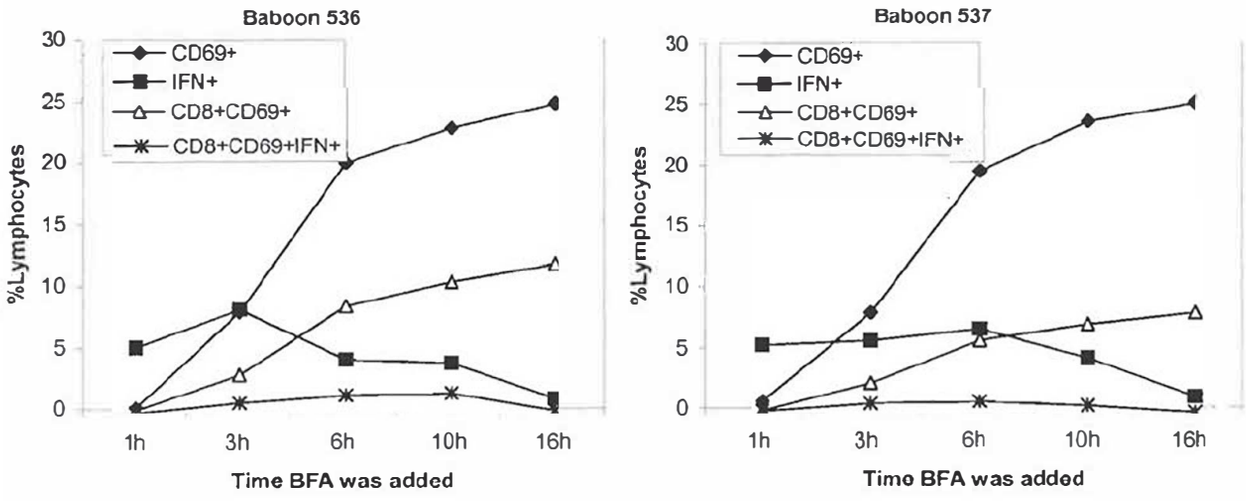


Fig A.7: PBMC were obtained from baboons 536 and 537 and incubated for 16h with SEB and anti-human CD28 and CD49d (BD-IS). Brefeldin A (BFA; Sigma) was added at 1h, 3h, 6h, 10h or 16h during the course of the incubation. Cells were stained with anti-human CD8-APC (clone SK1; BD-IS) CD69-FITC (clone FN50; BD-IS) and IFN-γ-PE (clone 4S.B3; BD-Ph) in a 2-step staining protocol (Appendix B.5). Data was acquired using a FACSCalibur (BD) flow cytometer and analysed with CellQuest software. Data points are percentages of cells in the lymphocyte gate that were expressing CD69, IFN-γ, CD8/CD69 or CD8/CD69/ IFN-γ at various incubation times with BFA.



APPENDIX C.6: IFN-γ ELISPOT ASSAY: CUT-OFF VALUES

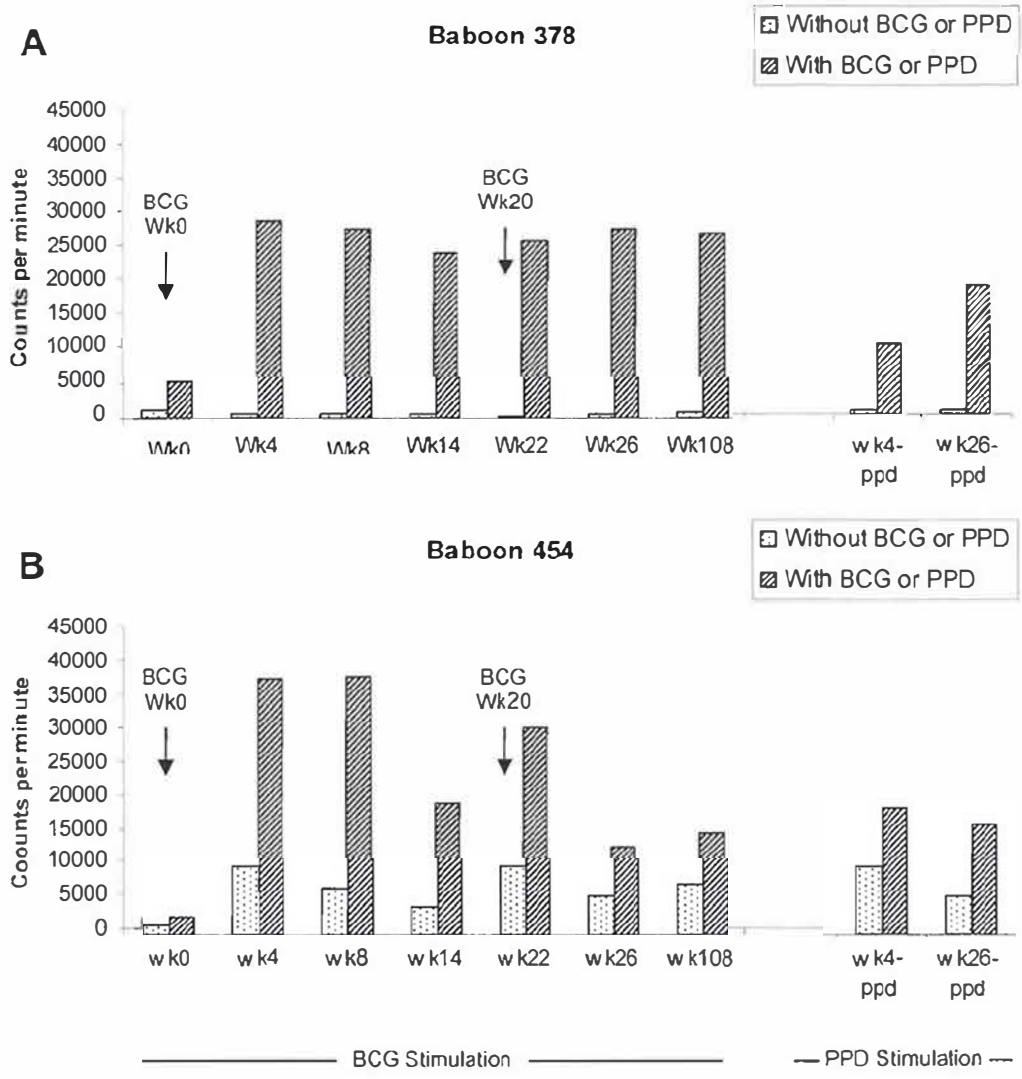
Table A.10: The response of the baboons in each study to the five Gag peptide pools in the IFN-γ ELISPOT assay prior to immunisation was used to determine the cut-off value for a positive response after the immunisation. The mean IFN-γ response (after subtracting the background IFN-γ response in the absence of peptides) obtained in response to the five Gag peptide pools was calculated in each study pre-immunisation. The cut-off value was set at a value equal to this mean IFN-γ response plus four standard deviations of the mean. A response to peptide pools after immunisation was considered positive if it was equal or greater than this cut-off value. Any peptide pool IFN-γ response that was less than the cut-off value was assigned an IFN-γ ELISPOT value of zero.

Name of Experiment	Baboon number	Net IFN-γ ELISPOT response (SFU/10^6 PBMC)							Cut-off value (SFU/10^6 PBMC)
		P1	P2	P3	P4	P5	Mean	4 Stdev	
DNA-VLP Exp1	348	14	4	0	4	10	3.85	16.22	**20**
	357	0	0	0	0	0			
	499	5	5	8	0	0			
	500	5	0	8	7	5			
DNA-VLP Exp2	369	0	0	3	6	6	6.23	38.42	**45**
	592	0	0	0	0	0			
	624	0	0	0	0	2			
	660	0	9	2	0	12			
	663	5	22	9	14	25			
	664	21	36	1	6	31			
	674	5	0	0	3	0			
BCG-VLP Exp1	538	0	0	0	0	0	3.17	20.61	**24**
	539	0	0	0	0	0			
	542	0	0	0	2	0			
	545	20	5	15	5	5			
	546	3	0	8	0	0			
	548	0	13	6	0	8			
BCG-VLP Exp2	350	7	27	10	17	14	3.57	28.11*	**32**
	363	0	0	7	7	24			
	378	3	3	3	7	3			
	427	0	0	0	0	0			
	428	0	13	0	6	3			
	447	0	1	3	5	5			
	449	0	0	0	0	0			
	466	7	0	0	2	0			
	611	3	8	5	3	3			
	632	0	0	0	0	0			
	661	0	1	0	0	1			
	672	0	2	2	7	2			

*: value represents 5 standard deviations (this was increased due to the high pre-immunisation background displayed by baboon 350 to make it at least 2x greater that the mean background response of 15 SFU/10^6 PBMC); P: peptide pool; Stdev: standard deviation; DNA: pTH-GagC vaccine; VLP: GagC VLP vaccine; Exp: experiment; BCG: RT106 or RT108 vaccines; SFU: spot-forming units; PBMC: peripheral blood mononuclear cells.

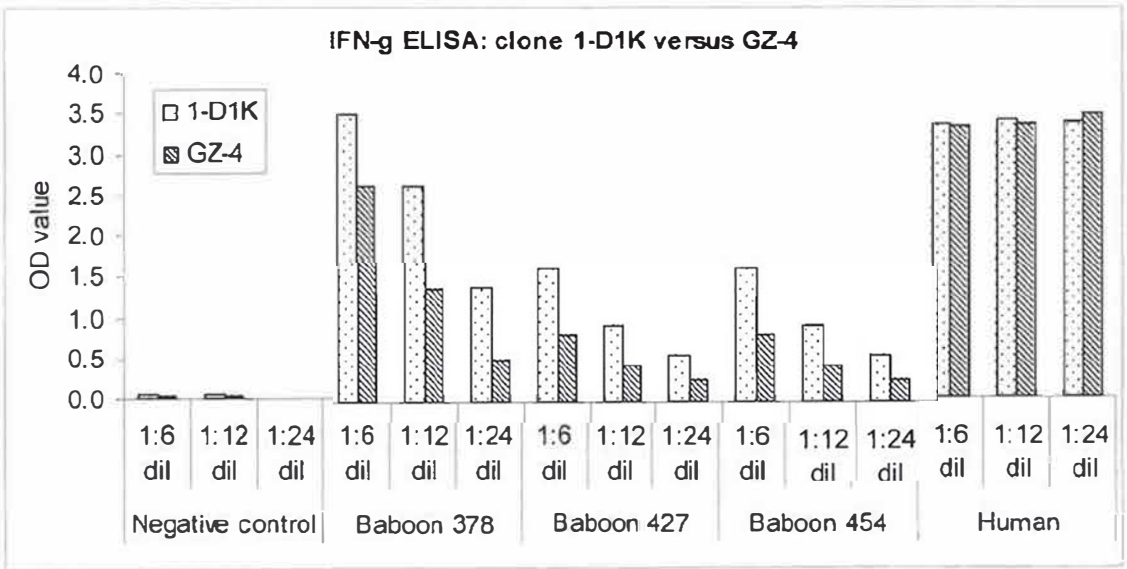
APPENDIX C.7: CELLULAR PROLIFERATION

Fig A.8: Comparison of actual (in the presence of live BCG or PPD) and background (without BCG or PPD) proliferation for baboon 378 (A) and 454 (B) pre- and post inoculation with 10^8 CFU BCG-*Tokyo* strain. Freshly isolated PBMC were stimulated *in vitro* for 6 days with or without 10^6 CFU/mL live BCG -*Tokyo* strain. At two time-points (Wk4-ppd* and Wk26-ppd*), a parallel proliferation assay was done using bovine PPD (5 μg/mL) as the stimulant. The data points are given as actual counts per minute with and without BCG or PPD.



APPENDIX C.8: IFN-γ ELISA: CLONE 1-D1K VERSUS CLONE GZ-4

Fig A.9: PBMC from 3 baboons (numbers 378, 427, 454) and a human donor were cultured for 3 days in R10 medium containing 5 µg/mL concanavalin A (Con A; Sigma). The culture supernatant was harvested, diluted with R10 medium to 1:6; 1:12 and 1:24 and then used in an IFN-γ ELISA assay (as described in section **3.2.6.2**) using either anti-human IFN-γ (clone 1-D1K; Mabtech) or anti-rhesus monkey IFN-γ (clone GZ-4; Mabtech) monoclonal antibodies for coating the ELISA plates. R10 medium was use as negative control for culture supernatants. Data points are given as the absorbance (OD) at 405 nm wavelength. Higher OD values were obtained with clone 1-D1K for baboon samples, indicating that this clone had better cross-reactivity with baboon IFN-γ than clone GZ-4. (dil: dilution).



APPENDIX C.9: IFN-γ ELISPOT ASSAY: RESPONSES TO PHA

Table A.11: Cryo-preserved PBMC obtained from baboons at various time points pre (0) and post-inoculation with pTH-GagC and GagC VLP (Groups 1A and 2A), pTH-vector and GagC VLP (Group 1B) or GagC VLP alone (Group 2B) were used in a standard IFN-γ ELISPOT assay using PHA (Sigma) at a final concentration of 4 μg/mL. The mean IFN-γ response (in SFU/10^6 PBMC) of triplicate wells is given in the table.

Group 1A

Baboon ID	Weeks post initial inoculation														
	0	4	8	10	16	34	36	56	58	75	77	79	83	87	95
348	2330	2603	1997	2667	2283	3640	2457	2143	908	900	1607	1282	1730	1417	1793
500	2035	1797	2143	2103	2777	3250	3303	1830	2285	1712	1818	728	2270	1338	1842

Group 1B

Baboon ID	Weeks post initial inoculation											
	0	4	56	58	75	79	83	87	91	95	99	103
357	ND	ND	1440	1210	1413	797	1502	1468	1345	1793	2025	1663
499	ND	ND	2143	1145	722	570	1203	1055	1130	1433	1339	1517

Group 2A

Baboon ID	Weeks post initial inoculation														
	0	4	8	12	16	34	36	51	53	55	59	63	65	67	71
389	830	1792	1655	260	353	585	1778	1760	1752	1835	2217	1875	2133	2058	1208
592	1875	1913	1755	1507	1958	1913	1715	1653	2015	1882	1633	1675	1527	1973	778
624	1815	2188	1833	1180	2048	2112	1978	1470	1743	1995	2127	3343	3260	2253	438
660	1043	1397	453	970	1698	1740	1027	957	1653	1572	1617	997	1272	1675	723
663	1802	1782	1387	370	1940	820	1870	247	1223	1673	1730	2870	1633	1898	240

Group 2B

Baboon ID	Weeks post initial inoculation														
	0	4	8	12	16	34	36	51	53	55	59	63	65	67	71
664	ND	ND	ND	ND	ND	ND	ND	1167	1218	1203	1140	1305	1092	945	872
674	ND	ND	ND	ND	ND	ND	ND	737	1133	1190	1160	1180	1203	1547	782

APPENDIX D: HIV-1 SUBTYPE C GAG PEPTIDES AMINO ACID SEQUENCES

APPENDIX D.1: AMINO ACID CODE

Table A.11: Amino acids and their 3-letter and single-letter codes.

Amino acid	3-letter code	1-letter code	Amino acid	3-letter code	1-letter code
Alanine	Ala	A	Leucine	Leu	L
Arginine	Arg	R	Lysine	Lys	K
Aspargine	Asn	N	Methionine	Met	M
Aspartic acid	Asp	D	Phenylalanine	Phe	F
Cysteine	Cys	C	Proline	Pro	P
Glutamine	Gln	Q	Serine	Ser	S
Glutamic cid	Glu	E	Threonine	Thr	T
Clycine	Gly	G	Tryptophan	Trp	W
Histidine	His	H	Tyrosine	Tyr	Y
Isoleucine	Ile	I	Valine	Val	V

APPENDIX D.2: GAG PEPTIDES: AMINO ACID SEQUENCES

Fig A.10: Amino acid sequences of HIV-1 subtype C Gag peptides number 1-66. The amino acids of adjacent peptides overlap (shaded sequences) by 10 amino acids (except where indicated). The peptides were a kind donation by Dr Clive Gray of National Institute for Communicable Diseases (NICD), Johannesburg, South Africa.

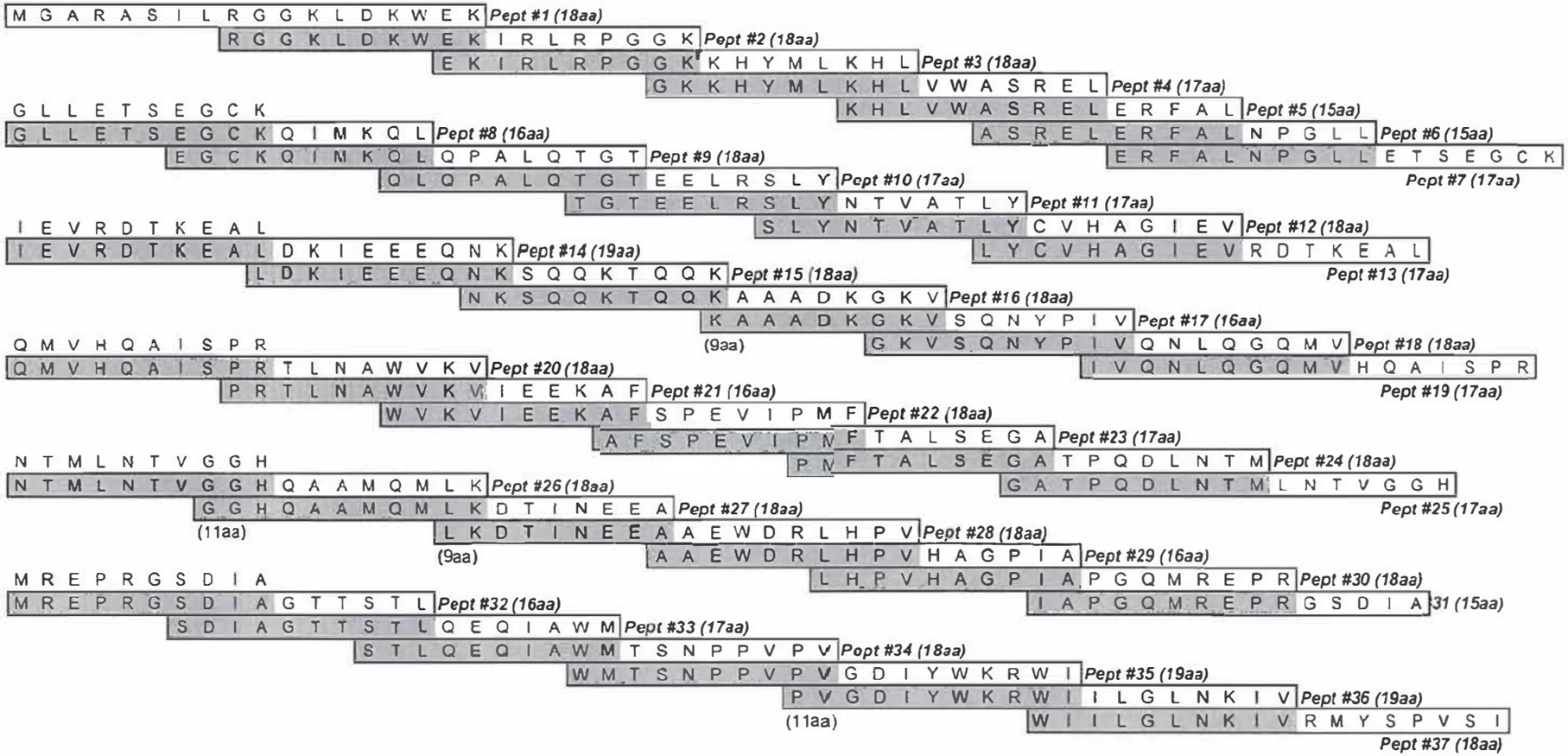
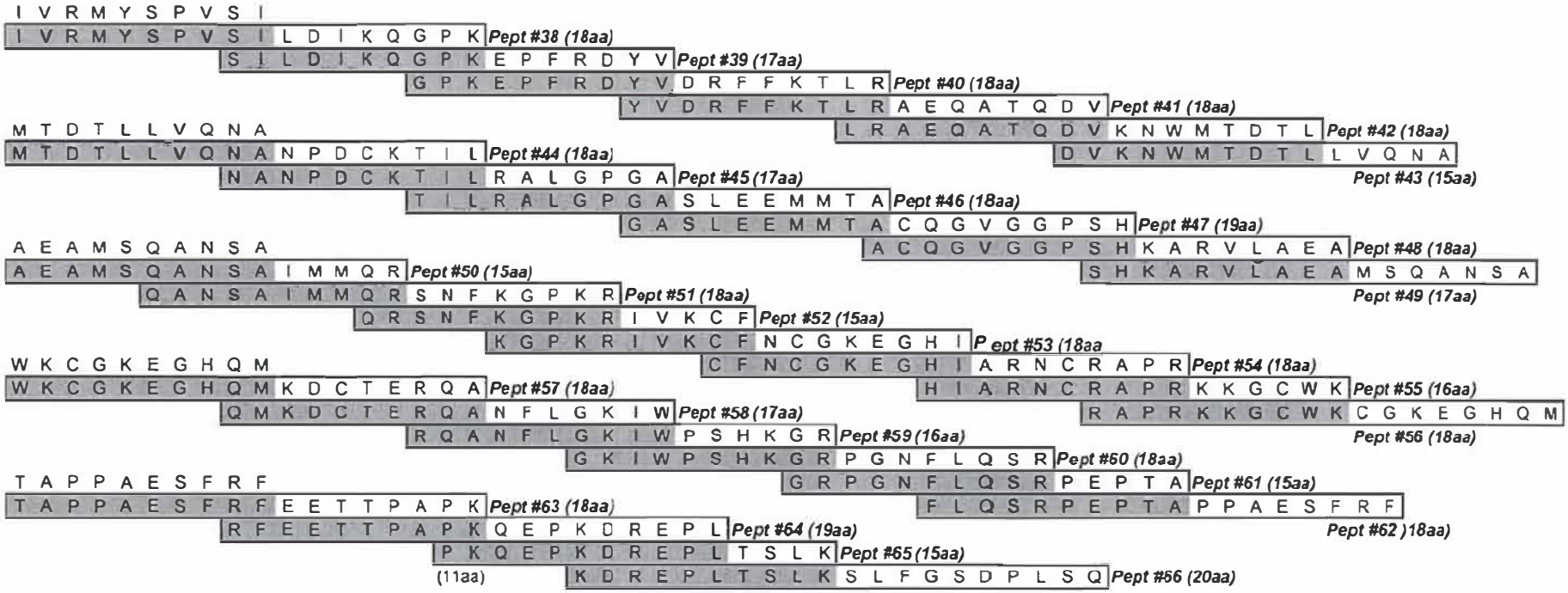


Fig A.10 Continued.....



References

Abaitua F, Rodriguez JR, Garzon A, Rodriguez D, and Esteban M (2006). Improving recombinant MVA immune responses: potentiation of the immune responses to HIV-1 with MVA and DNA vectors expressing Env and the cytokines IL-12 and IFN-gamma. *Virus Research* **116**, 11-20.

Abolhassani M, Lagranderie M, Chavarot P, Balazuc AM, and Marchal G (2000). *Mycobacterium bovis* BCG induces similar immune responses and protection by rectal and parental immunization routes. *Infection and Immunity* **68**, 5657-5662.

Abomoelak B, Huygen K, Kremer L, Turneer M, and Locht C (1999). Humoral and cellular immune responses in mice immunized with recombinant *Mycobacterium bovis* Bacillus Calmette-Guerin producing a pertussis toxin-tetanus toxin hybrid protein. *Infection and Immunity* **67**, 5100-5105.

Adams EJ and Parham P (2001). Species-specific evolution of MHC class I genes in the higher primates. *Immunological Reviews* **183**, 41-64.

Addo MM, Yu XG, Rathod A, Cohen D, Eldridge RL, Strick D, Johnston MN, Corcoran C, Wurcel AG, Fitzpatrick CA, Feeney ME, Rodriguez WR, Basgoz N, Draenert R, Stone DR, Brander C, Goulder PJR, Rosenberg ES, Altfeld M, and Walker BD (2003). Comprehensive epitope analysis of human immunodeficiency viurs type 1 (HIV-1)-specific T-cell responses directed against the entire expressed HIV-1 genome demonstrate broadly directed responses but no correlation to viral load. *Journal of Virology* **77**, 2081-2092.

Aderem A and Ulevitch RJ (2000). Toll-like receptors in the induction of the innate immune response. *Nature* **406**, 782-787.

Agy MB, Frumkin LR, Corey L, Coombs RW, Wolinsky SM, Koehler J, Morton WR, and Katze MG (1992). Infection of Macaca nemestrina by human immunodeficiency virus type-1. *Science* **257**, 103-106.

Agy MB, Schmidt A, Florey MJ, Kennedy BJ, Schaefer G, Katze MG, Corey L, Morton WR, and Bosch ML (1997). Serial in vivo passage of HIV-1 infection in Macaca nemestrina. *Virology* **238**, 336-343.

Ahmad S, Lohman B, Marthas M, Giavedoni L, el-Amad Z, Haigwood NL, Scandella CJ, Gardner MB, Luciw PA, and Yilma T (1994). Reduced virus load in rhesus macaques immunized with recombinant gp160 and challenged with simian immunodeficiency virus. *AIDS Research and Human Retroviruses.* **10**, 195-204.

Ahmed R, Biberfeld G, and Thorstensson R (2005). Innate immunity in experimental SIV infection and vaccination. *Molecular Immunology* **42**, 251-258.

Al-Zarouni M and Dale JW (2002). Expression of foreign genes in *Mycobacterium bovis* BCG strains using different promoters reveals instability of the *hsp60* promoter for expression of foreign genes in *Mycobacterium bovis* BCG strains. *Tuberculosis* **82**, 283-291.

Aldovin A and Young RA (1991). Humural and cell-mediated immune responses to live recombinant BCG-HIV vaccines. *Nature* **351**, 479-482.

Alexander L, Illyinskii PO, Lang SM, Means RE, Lifson J, Mansfield K, and Desrosiers RC (2003). Determinants of increased replicative capacity of serially passaged simian immunodeficiency virus with nef deleted in rhesus monkeys. *Journal of Virology* **77**, 6823-6835.

Allan JS, Ray P, Broussard S, Whitehead E, Hubbard G, Butler T, Brasky K, Luciw P, Cheng-Meyer C, and Levy JA (1995). Infection of baboons with simian/human immunodeficiency viruses. *Journal of Acquired Immune Deficiency Syndromes and Human Retrovirology* **15**, 429-441.

Allen TM, Vogel TU, Fuller DH, Mothe BR, Steffen S, Boyson JE, Shipley T, Fuller J, Hanke T, Sette A, Altman JD, Moss B, McMichael AJ, and Watkins DI (2000). Induction of AIDS virus-specific CTL activity in fresh, unstimulated peripheral blood lymphocytes from rhesus macaques vaccinated with a DNA prime/modified vaccinia virus Ankara boost regimen. *Journal of Immunology* **164**, 4968-4978.

Almond N, Kent K, Cranage M, Rud E, Clarke B, and Stott EJ (1995). Protection by attenuated simian immunodeficiency virus in macaques against challenge with virus-infected cells. *Lancet* **345**, 1342-1344.

Amara R R, Villinger F, Altman JD, Lydy SL, O'Neil SP, Staphrans SI, Montefiori DC, Xu Y, Herndon JG, Wyatt LS, Candido MA, Kozyr NL, Earl PL, Smith JM, Ma HL, Grimm BD, Hulsey ML, Miller J, McClure HM, McNicholl JM, Moss B, and Robinson HL (2001). Control of a mucosal challenge and prevention of AIDS by a multiprotein DNA/MVA vaccine. *Science* **292**, 69-74.

Amara R R, Ibegbu C, Villinger F, Montefiori DC, Sharma S, Nigam P, Xu Y, McClure HM, and Robinson HL (2005a). Studies using a viral challenge and CD8 T cell depletions on the roles of cellular and humoral immunity in the control of an SHIV-89.6P challenge in DNA/MVA-vaccinated macaques. *Virology* **343**, 246-255.

Amara R R, Patel K, Niedziela G, Nigam P, Sharma S, Staprans SI, Montefiori DC, Chenareddi L, Herndon JG, Robinson HL, McClure HM, and Novembre FJ (2005b). A combination DNA and attenuated simian immunodeficiency virus vaccine strategy provides enhanced protection from simian/human immunodeficiency virus-induced disease. *Journal of Virology* **79**, 15356-15367.

Amara RR, Smith JM, Staprans SL, Montefiori DC, Villinger F, Altman JD, O'Neil SP, Kozyr NL, Xu L, Wyatt LS, Earl PL, Herndon JG, McNicholl JM, McClure HM, Moss B, and Robinson HL (2002). Critical role for Env as well as Gag-Pol in control of a simian-human immunodeficiency virus 89.6P challenge by a DNA prime/recombinant modified vaccinia virus Ankara vaccine. *Journal of Virology* **76**, 6138-6146.

Ami Y, Izumi Y, Matsuo T, Someya K, Kanekiyo M, Horibata S, Yoshino N, Sakai K, Shinohara K, Matsumoto S, Yamada T, Yamazaki S, Yamamoto N, and Honda M (2005). Priming-boosting vaccination with recombinant Mycobacterium bovis bacillus Calmette-Guerin and a nonreplicating vaccinia virus recombinant leads to long-lasting and effective immunity. *Journal of Virology* **79**, 12871-12879.

Andersen P (2001). TB Vaccines:progress and problems. *Trends in Immunology* **22**, 160-168.

Anderson R and Hanson M (2005). Potential public health impact of imperfect HIV type 1 vaccines. *Journal of Infectious Diseases* **191 (Suppl 1)**, S85-S96.

Andersson S, Makitalo B, Thorstensson R, Franchini G, Tartaglia J, Limbach K, Paoletti E, Putkonen P, and Biberfeld G (1996). Immunogenicity and protective efficacy of a human immunodeficiency virus type 2 recombinant canarypox (ALVAC) vaccine candidate in cynomolgus monkeys. *Journal of Infectious Diseases* **174**, 977-985.

Anthony DD and Lehmann PV (2003). T-cell epitope mapping using the ELISPOT approach. *Methods* **29**, 260-269.

Apetrei C, Gormus B, Pandrea I, Metzger M, ten Haaft P, Martin LN, Bohm R, Alvarez X, Koopman G, Murphey-Corb M, Veazey RS, Lackner AA, Baskin G, Heeney J, and Marx PA (2004). Direct inoculation of simian immunodeficiency virus from sooty mangabeys in black mangabeys (Lophocebus aterrimus): first evidence of AIDS in a heterologous African species and different pathologic outcomes of experimental infection. *Journal of Virology* **78**, 11506-11518.

Arthur LO, Bess JW Jr, Waters DJ, Pyle SW, Kelliher JC, Nara PL, Krohn K, Robey WG, Langlois AJ, Gallo RC, and Fischinger PJ (1989). Challenge of chimpanzees (Pan troglodytes) immunized with human immunodeficiency virus envelope glycoprotein gp120. *Journal of Virology* **63**, 5046-5053.

Arthur LO, Bess JW Jr, Urban RG, Strominger JL, Morton WR, Mann DL, Henderson LE, and Benveniste RE (1995). Macaques immunized with HLA-DR are protected from challenge with simian immunodeficiency virus. *Journal of Virology* **69**, 3117-3124.

Baba TW, Liska V, Hofmann-Lehmann R, Vlasak J, Xu W, Ayehunie S, Cavacini LA, Posner MR, Katinger H, Stiegler G, Bemacky BJ, Rizvi TA, Schmidt R, Hill LR, Keeling ME, Lu Y, Wright JE, Chuo TC, and Ruprecht RM (2000). Human neutralizing monoclonal antibodies of the IgG1 subtype protect against mucosal simian-human immunodeficiency virus infection. *Nature Medicine* **6**, 200-206.

Barnett SW, Murthy KK, Herndier BG, and Levy JA (1994). An AIDS-like condition induced in baboons by HIV-2. *Science* **266**, 642-646.

Barnett SW, Rajasekar S, Legg H, Doe B, Fuller DH, Haynes JR, Walker CM, and Steimer KS (1997). Vaccination with HIV-1 gp120 DNA induces immune responses that are boosted by a recombinant gp120 protein subunit. *Vaccine* **15**, 869-873.

Barnett SW, Lu S, Srivastava I, Cherpelis S, Gettie A, Blanchard J, Wang S, Mboudjeka I, Leung L, Lian Y, Fong A, Buckner C, Ly A, Hilt S, Ulmer J, Wild CT, Mascola JR, and Stamatatos L (2001). The ability of an oligomeric human immunodeficiency virus type 1 (HIV-1) envelope antigen to elicit neutralizing antibodies against primary HIV-1 isolates is improved following partial deletion of the second hypervariable region. *Journal of Virology* **75**, 5526-5540.

Barouch DH and Letvin NL (2000). DNA vaccination for HIV-1 and SIV. *Intervirology* **43**, 282-287.

Barouch DH, Santra S, Schimtz JE, Kuroda MJ, Fu TM, Wagner W, Bliska M, Craiu A, Zheng XX, Krivulka GR, Beaudry K, Lifton MA, Nickerson EC, Trigona WL, Punt K, Freed DC, Guan L, Dubey S, Casimiro D, Simon A, Davies ME, Chastain M, Strom TB, Gelman RS, Montefiori DC, Lewis MG, Emini EA, Shiver JW, and Letvin NL (2000). Control of viremia and prevention of clinical AIDS in rhesus monkeys by cytokine-augmented DNA vaccination. *Science* **290**, 486-492.

Barouch DH, Pau MG, Custers JH, Koudstaal W, Kostense S, Havenga MJ, Truitt DM, Sumida SM, Kishko MG, Arthur JC, Korioth-Schmitz B, Newberg MH, Gorgone DA, Lifton MA, Panicali DL, Nabel GJ, Letvin NL, and Goudsmit J (2004). Immunogenicity of recombinant adenovirus serotype 35 vaccine in the presence of pre-existing anti-Ad5 immunity. *Journal of Immunology* **172**, 6290-6297.

Barratt-Boyes SM, Soloff AC, Garcia-Moll M, Nwanegbo E, Liu X, Rajakumar PA, Brown KN, Robbins PD, Murphey-Corb M, Day RD, and Gambotto A (2006). Broad cellular immunity with robust memory responses to simian immunodeficiency virus following serial vaccination with adenovirus 5- and 35-based vectors. *Journal of General Virology* **87**, 139-149.

Bean AGD, Roach DR, Briscoe H, France MP, Korner H, Segwick JD, and Britton WJ (1999). Structural deficiencies in granuloma formation in TNF gene-targeted mice underlie the heightened susceptability to aerosol *Mycobacterium tuberculosis* infection, which is not compensated for by lyphotoxin. *Journal of Immunology* **162**, 3504-3511.

Becton Dikinson (2003). BD Cytometric bead array (CBA) multiplex assays. *In: Techniques for Immune Function Analysis: Application Handbook 1st Edition* p35-p48.

Beddows S, Schulke N, Kirschner M, Barnes K, Franti M, Michael E, Ketas T, Sanders RW, Maddon PJ, Olson WC, and Moore JP (2005). Evaluating the immunogenicity of a disulfide-stabilised, cleaved, trimeric form of the envelope glycoprotein complex of human immunodeficiency virus type 1. *Journal of Virology* **79**, 8812-8827.

Behar SM, Dascher CC, Grusby MJ, Wang CR, and Brenner MB (1999). Susceptible of mice dificient in CD1D or TAP1 to infection with Mycobacterium tuberculosis. *Journal of Experimental Medicine* **189**, 1973-1980.

Benson J, Chougnet C, Robert-Guroff M, Montefiori D, Markham P, Shearer MH, Gallo RC, Cranage M, Paoletti E, Limbach K, Venzon D, Tartaglia J, and Franchini G (1998). Recombinant vaccine-induced protection against the highly pathogenic simian immunodeficiency virus SIV(mac251): dependence on route of challenge exposure. *Journal of Virology* **72**, 4170-4182.

Berglund P, Quesada-Rolander M, Putkonen P, Biberfeld G, Thorstensson R, and Liljestrom P (1997). Outcome of immunization of cynomolgus monkeys with recombinant Semliki Forest virus encoding human immunodeficiency virus type 1 envelope protein and challenge with a high dose of SHIV-4 virus. *AIDS Research and Human Retroviruses.* **13**, 1487-1495.

Berman PW, Gregory TJ, Riddle L, Nakamura GR, Champe MA, Porter JP, Wurm FM, Hershberg RD, Cobb EK, and Eichberg JW (1990). Protection of chimpanzees from infection by HIV-1 after vaccination with recombinant glycoprotein gp120 but not gp160. *Nature* **345**, 622-625.

Bertley FM, Kozlowski PA, Wang SW, Chappelle J, Patel J, Sonuyi O, Mazzara G, Montefiori D, Carville A, Mansfield KG, and Aldovini A (2004). Control of simian/human immunodeficiency virus viremia and disease progression after IL-2-augmented DNA-modified vaccinia virus Ankara nasal vaccination in nonhuman primates. *Journal of Immunology* **172**, 3745-3757.

Betts MR, Krowka JF, Kepler TB, Davidian M, Christopherson C, Kwok S, Louie L, Eron J, Sheppard H, and Frelinger JA (1999). Human immunodeficiency virus type 1-specific cytotoxic T lymphocyte activity is inversely correlated with HIV type 1 viral load in HIV type 1-infected long-term survivors. *AIDS Research and Human Retroviruses.* **15**, 1219-1228.

Betts MR, Ambrozak DR, Douek DC, Bonhoeffer S, Brenchley JM, Casazza JP, Koup RA, and Picker LJ (2001). Analysis of total human immunodeficiency virus (HIV)-specific CD4+ and CD8+ T-cell responses: relationship to viral load in untreated HIV infection. *Journal of Virology* **75**, 11983-11991.

Betts MR, Price DA, Brenchley JM, Lore K, Guenaga FJ, Smed-Sorensen A, Ambrozak DR, Migueles SA, Connors M, Roederer M, Douek DC, and Koup RA (2004). The functional profile of primary human antiviral CD8+ T cell effector activity is dictated by cognate peptide concentration. *Journal of Immunology* **172**, 6407-6417.

Bibollet-Ruche F, Galat-Luong A, Cuny G, Sarni-Manchado P, Galat G, Durand JP, Pourrut X, and Veas F (1996). Simian immunodeficiency virus infection in a patas monkey (Erythrocebus patas): evidence for cross-species transmission from African green monkeys (Cercopithecus aethiops sabaeus) in the wild. *Journal of General Virology* **77**, 773-781.

Bibollet-Ruche F, Bailes E, Gao F, Pourrut X, Barlow KL, Clewley JP, Mwenda JM, Langat DK, Chege GK, McClure HM, Mpoudi-Ngole E, Delaporte E, Peeters M, Shaw GM, Sharp PM, and Hahn BH (2004). New simian immunodeficiency virus infecting De Brazza's monkeys (Cercopithecus neglectus): evidence for a cercopithecus monkey virus clade. *Journal of Virology* **78**, 7748-7762.

Binley JM, Sanders RW, Clas B, Schuelke N, Master A, Guo Y, Kajumo F, Anselma DJ, Maddon PJ, Olson WC, and Moore JP (2000). A recombinant human immunodeficiency virus type 1 envelope glycoprotein complex stabilized by an intermolecular disulfide bond between the gp120 and gp41 subunits is an antigenic mimic of the trimeric virion-associated structure. *Journal of Virology* **74**, 627-643.

Black, C. A. (1999). Delayed type hypersensitivity: current theories with an historic perspective. *Dermatology Online Journal* **5**, 7.

Blackbourn DJ, Locher CP, Ramachandran B, Barnett SW, Murthy KK, Carey KD, Brasky KM, and Levy JA (1997). CD8+ cells from HIV-2-infected baboons control HIV replication. *AIDS* **11**, 737-746.

Boaz MJ, Waters A, Murad S, Eastebrook PJ, and Vyakarnam A (2002). Presence of HIV-1 Gag specific IFN-gamma+IL-2+ and CD28+IL-2+ CD4 T cell responses is associated with nonprogression in HIV-1 infection. *Journal of Immunology* **169**, 6376-6385.

Boaz MJ, Waters A, Murad S, Eastebrook PJ, D'Sousa E, van Wheeley C, and Vyakarnam A (2003). CD4 responses to conserved HIV-1 T helper epitopes show both negative and positive associations with virus load in chronically infected subjects. *Clinical Experimental Immunology* **134**, 454-463.

Bogers WM, Dubbes R, ten Haaft P, Niphuis H, Cheng-Mayer C, Stahl-Hennig C, Hunsmann G, Kuwata T, Hayami M, Ranjbar S, Almond N, Stott J, Rosenwirth B, and Heeney J (1997). Comparison of in vitro and in vivo infectivity of different clade B HIV-1 envelope chimeric simian/human immunodeficiency viruses in Macaca mulatta. *Virology* **236**, 110-117.

Bontrop RE, Otting N, Slierendregt BL, and Lanchburg JS. (1995). Evolution of major histocompatibility complex polymorphisms and T-cell receptor diversity in primates. *Immunological Reviews* **143**, 33-62.

Bontrop RE, Otting N, de Groot NG, and Doxiadis GG (1999). Major histocompatibility complex class II polymorphism in primates. *Immunological Reviews* **167**, 339-350.

Bontrop RE and Watkins DI (2005). MHC polymorphism: AIDS susceptibility in non-human primates. *Trends in Immunology* **26**, 227-233.

Bonyhadi ML and Kaneshima H (1997). The SCID-hu mouse: an in vivo model for HIV-1 infection in humans. *Molecular Medicine Today* **3**, 246-253.

Borrow P, Lewicki H, Hahn BH, Shaw GM, and Oldstone MB (1994). Virus-specific CD8+ cytotoxic T-lymphocyte activity associated with control of viremia in primary human immunodeficiency virus type 1 infection. *Journal of Virology* **68**, 6110.

Bosch ML, Schmidt A, Agy MB, Kimball LE, and Morton WR (1997). Infection of Macaca nemestrina neonates with HIV-1 via different routes of inoculation. *AIDS* **11**, 1555-1563.

Boyer JD, Ugen KE, Wang B, Agadjanyan M, Gilbert L, Bagarazzi ML, Chattergoon M, Frost P, Javadian A, Williams WV, Rafaeli Y, Ciccarelli RB, McCallus D, Coney L, and Weiner DB (1997). Protection of chimpanzes from high dose heterologous HIV-1 challenge by DNA vaccination. *Nature Medicine* **3**, 526-532.

Boyer JD, Chattergoon MA, Ugen KE, Shah A, Bennett M, Cohen A, Nyland S, Lacy KE, Bagarazzi ML, Higgins TJ, Baine Y, Ciccarelli RB, Ginsberg RS, MacGregor RR, and Weiner DB (1999). Enhacement of cellular immune response in HIV-1 seropositive individuals: a DNA-based trial. *Clinical Immunology* **90**, 100-107.

Boyer JD, Cohen AD, Vogt S, Schumann K, Nath B, Ahn L, Lacy K, Bagarazzi ML, Higgins TJ, Baine Y, Ciccarelli RB, Ginsberg RS, MacGregor RR, and Weiner DB (2000). Vaccination of seronegative volunteers with a human immunodeficiency virus type 1 env/rev DNA vaccine induces antigen-specific proliferation and lymphocyte production of beta-chemokines. *Journal of Infectious Diseases* **181**, 476-483.

Boyer JD, Robinson TM, Maciag PC, Peng X, Johnson RS, Pavlakis G, Lewis MG, Shen A, Siliciano R, Brown CR, Weiner DB, and Paterson Y (2005). DNA prime Listeria boost induces a cellular immune response to SIV antigens in the rhesus macaque model that is capable of limited suppression of SIV239 viral replication. *Virology* **333**, 88-101.

Boyer JD, Maciag PC, Parkinson R, Wu L, Lewis MG, Weiner DB, and Paterson Y (2006). Rhesus macaques with high levels of vaccine induced IFN-gamma producing cells better control viral set-point following challenge with SIVmac239. *Vaccine* **24**, 4498-4502.

Boyson JE, Shufflebotham C, Cadavid LF, Urvater JA, Knapp LA, Hughes AL, and Watkins DI (1996). The MHC class I genes of the rhesus monkey. Different evolutionary histories of MHC class I and II genes in primates. *Journal of Immunology* **156**, 4656-4665.

Brandt L, Feino CJ, Weinreich OA, Chilima B, Hirsch P, Appelberg R, and Andersen P (2002). Failure of the mycobacterium bovis BCG vaccine: some species of environmental mycobacteria block multiplication of BCG and induction of protective immunity to tuberculosis. *Infection and Immunity* **70**, 672-678.

Breitburd F, Kimbauer R, Hubbert NL, Nonnenmacher B, Trin Dinh Desmarquet C, Orth G, Schiller JT, and Lowy DR (1995). Immunization with virus-like particles from rabbit cottontail rabbit papillomavirus (CRPV) can protect against experimental CRPV infection. *Journal of Virology* **69**, 3959-3963.

Bretscher PA, Wei G, Menon JN, and Bielefeldt-Ohmann H (1992). Establishment of stable, cell-mediated immunity that makes "susceptible" mice resistant to Leishmania major. *Science* **257**, 539-542.

Bretscher PA (1994). Prospects for low dose BCG vaccination against tuberculosis. *Immunobiology* **191**, 548-554.

Buddle BM, Wards BJ, Aldwell FE, Collins DM, and De Lisle GW (2002). Influence of sensitisation to environmental mycobacteria on subsequent vaccination against bovine tuberculosis. *Vaccine* **20**, 1126-1133.

Buonaguro L, Buonaguro FM, Tornesello ML, Mantas D, Beth-Giraldo E, Wagner R, Michelson S, Prevost MC, Wolf H, and Giraldo G (2001). High efficient production of Pr55(gag) virus-like particles expressing multiple HIV-1 epitopes, including a gp120 protein derived from an Ugandan HIV-1 isolate of subtype. *Antiviral Research* **49**, 35-47.

Buonaguro L, Racioppi L, Tornesello ML, Arra C, Visciano ML, Biryahwaho B, Sempala SD, Giraldo G, and Buonaguro FW (2002). Induction of neutralizing antibodies and cytotoxic T lymphocytes in Balb/c mice immunized with virus-like particles presenting a gp120 molecule from a HIV-1 isolate of clade A. *Antiviral Research* **54**, 189-201.

Burgers WA, van Harmelen JH, Shephard E, Adams C, Mgwebi T, Bourn W, Hanke T, Williamson A-L, and Williamson C (2006). Design and preclinical evaluation of a multigene human immunodeficiency virus type 1 subtype C DNA vaccine for clinical trial. *Journal of General Virology* **87**, 399-410.

Burton DR (1997). A vaccine for HIV type 1: The antibody perspective. *Proceedings of the National Academy of Sciences of the USA* **94**, 10018-10023.

Buseyne F, Le Gall S, Boccaccio C, Abastado JP, Lifson JD, Arthur LO, Riviere Y, Heard JM, and Schwartz O (2001). MHC-1-restricted presentation of HIV-1 virion antigens without viral replication. *Nature Medicine* **7**, 344-349.

Calarota S, Bratt G, Nordlund S, Hinkula J, Leandersson AC, Sandstrom E, and Wahren B (1998). Cellular cytotoxic response induced by DNA vaccination in HIV-1-infected patients. *Lancet* **351**, 1320-1325.

Calarota SA, Leandersson AC, Bratt G, Hinkula J, Klinman D M, Weinhold KJ, Sandstrom E, and Wahren B (1999). Immune responses in asymptomatic HIV-1-infected patients after HIV-DNA immunization followed by highly active antiretroviral treatment. *Journal of Immunology* **163**, 2330-2338.

Calarota SA, Kjerrström A, Islam KB, and Wahren B (2001). Gene combination raises broad human immunodeficiency virus-specific cytotoxicity. *Human Gene Therapy* **12**, 1637.

Carding SR and Egan PJ. (2000). The importance of gamma delta T cells in the resolution of pathogen-induced inflammatory immune response. *Immunological Reviews* **173**, 98-108.

Carrington M and Bontrop RE (2002). Effects of MHC class I on HIV/SIV disease in primates. *AIDS* **16 (Suppl 4)**, S105-S114.

Casimiro DR, Chen L, Fu TM, Evans RK, Caulfied MJ, Davies ME, Tang A, Chen M, Huang L, Harris V, Freed DC, Wilson KA, Dubey S, Zhu DM, Nawrocki D, Mach H, Troutman R, Isopi L, Williams D, Hurni W, Xu Z, Smith JG, Wang S, Liu X, Guan L, Long R, Trigona W, Heidecker GJ, Perry HC, Persaud N, Toner TJ, Su Q, Liang X, Youil R, Chastain M, Bett AJ, Volkin DB, Emini EA, and Shiver JW (2003a). Comparative immunogenicity in rhesus monkeys of DNA plasmid, recombinant vaccinia virus, and replication-defective adenovirus vectors expressing a human immunodeficiency virus type 1 *gag* gene. *Journal of Virology* **77**, 6305-6313.

Casimiro DR, Tang A, Chen TM, Fu RK, Evans ME, Davies DC, Freed W, Hurni JM, Aste-Amezaga, Guan L, Long R, Huang L, Harris V, Nawrocki DK, Mach H, Troutman RD, Isopi LA, Murthy KK, Rice K, Wilson KA, Volkin DB, Emini EA, and Shiver JW (2003b). Vaccine-induced immunity in baboons by using DNA and replication-incompetent adenovirus type 5 vectors expressing a human immunodeficiency virus type 1 *gag* gene. *Journal of Virology* **77**, 7663-7668.

Casimiro DR, Bett AJ, Fu TM, Davis ME, Tang A, Wilson KA, Chen M, Long R, McKelvery T, Chastain M, Gurunathan S, Tartaglia J, Emini EA, and Shiver J (2004). Heterologous human immunodeficiency virus type 1 priming-boosting immunization strategies involving replication-defective adenovirus and poxvirus vaccine vectors. *Journal of Virology* **78**, 11434-11438.

Casimiro DR, Wang F, Schleif WA, Liang X, Zhang ZQ, Tobery TW, Davies ME, McDermott AB, O'Connor DH, Fridman A, Bagchi A, Tussey LG, Bett AJ, Finnefrock AC, Fu TM, Tang A, Wilson KA, Chen M, Perry HC, Heidecker GJ, Freed DC, Carella A, Punt KS, Sykes KJ, Huang L, Ausensi VI, Bachinsky M, Sadasivan-Nair U, Walkins DI, Emini EA, and Shiver JW (2005). Attenuation of simian immunodeficiency virus SIVmac239 infection by prophylactic immunisation with DNA and recombinant adenovirus vaccine vectors expressing Gag. *Journal of Virology* **79**, 15547-15555.

Castro BA, Nepomuceno M, Lerche NW, Eichberg JW, and Levy JA (1991). Persistent infection of baboons and rhesus monkeys with different strains of HIV-2. *Journal of Virology* **184**, 219-226.

Cawthon Lang KA (2006). Primate Factsheets: Olive baboon (Papio anubis) Taxonomy, Morphology, & Ecology. *http://pin.primate.wisc.edu/factsheets/entry/olive_baboon* [accessed: 5 May 2006].

CDC (Centers for Disease Control) (2006). Vaccine development. *http://www.cdc.gov/hiv/vaccine/vudev.htm* [accessed: 19 March 2006].

Center RJ, Lebowitz J, Leapman RD, and Moss B (2004). Promoting trimerization of soluble human immunodeficiency virus type 1 (HIV-1) Env through the use of HIV-1/simian immunodeficiency virus chimeras. *Journal of Virology* **78**, 2265-2276.

Chakrabarti BK, Kong WP, Wu BY, Yang ZY, Friborg J, Ling X, King SR, Montefiori DC, and Nabel GJ (2002). Modifications of the human immunodeficiency virus envelope glycoprotein enhance immunogenicity for genetic immunization. *Journal of Virology* **76**, 5357-5368.

Chakrabarti L, Guyader M, Alizon M, Desrosiers RC, Tiollais P, and Sonigo P (1987). Sequence of simian immunodeficiency virus from macaque and its relationship to other human and simian retroviruses. *Nature* **328**, 543-547.

Chakrabarti LA, Metzner KJ, Ivanovic T, Cheng H, Louis-Virelizier J, Connor RI, and Cheng-Mayer C (2003). A truncated form of Nef selected during pathogenic reversion of simian immunodeficiency virus SIVmac239Deltanef increases viral replication. *Journal of Virology* **77**, 1245-1256.

Chan J, Xing Y, Magliozzo R, and Bloom BR (1992). Killing of virulent *Mycobacterium tuberculosis* by reactive nitrogen intermediates produced by activation of murine macrophages. *Journal of Experimental Medicine* **175**, 1111-1122.

Chen X, Rock MT, Hammonds J, Tartaglia J, Shintani A, Currier J, Slike B, Crowe JR Jr, Marovich M, and Spearman P (2005). Pseudovirion particle production by live poxvirus human immunodeficiency virus vaccine vector enhances humoral and cellular immune responses. *Journal of Virology* **79**, 5537-5547.

Chen Z, Zhou P, Ho DD, Landau NR, and Marx PA (1997). Genetically divergent strains of simian immunodeficiency virus use CCR5 as a co-receptor for entry. *Journal of Virology* **71**, 2705-2714.

Chen Z, Huang Y, Zhao X, Skulsky E, Lin D, Ip J, Gettie A, and Ho DD (2000). Enhanced infectivity of an R5-tropic simian/human immunodeficiency virus carrying human immunodeficiency virus type 1 subtype C envelope after serial passages in pig-tailed macaques (Macaca nemestrina). *Journal of Virology* **74**, 6501-6510.

Cheng-Mayer C, Shioda T, and Levy JA (1991). Host range, replicative, and cytopathic properties of human immunodeficiency virus type 1 are determined by very few amino acid changes in tat and gp120. *Journal of Virology* **65**, 6931-6941.

Cherpelis S, Shrivastava I, Gettie A, Jin X, Ho DD, Barnett SW, and Stamatatos L (2001). DNA vaccination with the human immunodeficiency virus type 1 SF1DeltaV2 envelope elicits immune responses that offer partial protection of CD8(+) T-cell-depleted rhesus macaques. *Journal of Virology* **75**, 1547-1550.

Cho MW, Kim YB, Lee MK, Gupta KC, Ross W, Plishka R, Buckler-White A, Igarashi T, Theodore T, Byrum R, Kemp C, Montefiori DC, and Martin MA (2001). Polyvalent envelope glycoprotein vaccine elicits a broader neutralizing antibody response but is unable to provide sterilizing protection against heterologous Simian/human immunodeficiency virus infection in pigtailed macaques. *Journal of Virology* **75**, 2224-2234.

Chouquet C, Autran B, Gomard E, Bouley J-M, Calvez V, Katlama C, Costagliola D, Riviere Y, and IMMUNOCO Study Group (2002). Correlation between breadth of memory HIV-specific cytotoxic T cells, viral load and disease progression in HIV infection. *AIDS* **16**, 2399-2407.

Chujoh Y, Matsuo K, Yoshizaki H, Nakasatomi T, Someya K, Okamoto Y, Naganawa S, Haga S, Yoshikura H, Yamazaki S, and Honda M (2001). Cross-clade neutralizing antibody production against human immunodeficiency virus type 1 clade E and B strains by recombinant *Mycobacterium bovis* BCG-based candidate vaccine. *Vaccine* **20**, 797-804.

Clerici M, Levin JM, Kessler HA, Harris A, Berzofsky JA, Landay AL, and Shearer GM (1994). HIV-specific T-helper activity in seronegative health care workers exposed to contaminated blood. *JAMA* **271**, 42-46.

Cohen J (2000). AIDS research: vaccine studies stymied by shortage of animals. *Science* **287**, 959-960.

Cohen J (2003). Public health: AIDS vaccine trial produces disappointment and confusion. *Science* **299**, 1290-1291.

Comstock GW, Edwards LB, and Nabangxang H (1971). Tuberculin sensitivity eight to fifteen years after BCG vaccination. *American Review of Respiratory Diseases* **103**, 572-575.

Connel ND, Medina-Acosta E, McMaster WR, Bloom BR, and Russell DG. (1993). Effective immunization against cutaneous leishmaniasis with recombinant bacille Calmette-Guerin expressing the *Leishmania* surface proteinase gp63. *Proceedings of the National Academy of Sciences of the USA* **90**, 11473-11477.

Connor RI, Montefiori DC, Binley JM, Moore JP, Bonhoeffer S, Gettie A, Fenamore EA, Sheridan KE, Ho DD, Dailey PJ, and Marx PA (1998). Temporal analyses of virus replication, immune responses, and efficacy in rhesus macaques immunized with a live, attenuated simian immunodeficiency virus vaccine. *Journal of Virology* **72**, 7501-7509.

Constant P, Davodeau F, Peyrat MA, Poquet Y, Puzo G, Bonneville M, and Fournie JJ. (1994). Stimulation of human gamma delta T cells by nonpeptidic mycobacterial ligands. *Science* **264**, 267-270.

Cooper AM, Dalton DK, Stewart TA, Griffen JP, Russel DG, and Orme IM (1993). Disseminated tuberculosis in IFN-γ gene-disrupted mice. *Journal of Experimental Medicine* **178**, 2243-2248.

Cooper AM, Magram J, Ferrante J, and Orme IM (1997). Interleukin 12 (IL-12) is crucial to the development of protective immunity in mice intravenously infected with *Mycobacterium tuberculosis*. *Journal of Experimental Medicine* **186**, 39-45.

Costantini A, Mancini S, Giuliodoro S, Butini L, Regnery CM, Silvestri G, and Montroni M (2003). Effects of cryopreservation on lymphocyte immunophenotype and function. *Journal of Immunological Methods* **278**, 145-155.

Coupar BE, Purcell DF, Thomson SA, Ramshaw IA, Kent SJ, and Boyle DB (2006). Fowlpox virus vaccines for HIV and SHIV clinical and pre-clinical trials. *Vaccine* **24**, 1378-1388.

Cranage MP, Whatmore AM, Sharpe SA, Cook N, Polyanskaya N, Leech S, Smith JD, Rud EW, Dennis MJ, and Hall GA (1997). Macaques infected with live attenuated SIVmac are protected against superinfection via the rectal mucosa. *Virology* **229**, 143-154.

Crotty S, Miller CJ, Lohman BL, Neagu MR, Compton L, Lu D, Lu FX, Pritts L, Lifson JD, and Andino R (2001). Protection against simian immunodeficiency virus vaginal challenge by using Sabin poliovirus vectors. *Journal of Virology* **75**, 7435-7452.

Crotty S and Andino R (2004). Poliovirus vaccine strains as mucosal vaccine vectors and their potential use to develop an AIDS vaccine. *Advanced Drug Delivery Review* **56**, 835-852.

Currier JR, Kuta EG, Turk E, Earhart LB, Loomis-Price L, Janetzki S, Ferrari G, Birx DL, and Cox JH (2002). A panel of MHC class 1 restricted viral peptides for use as a quality control for vaccine trial ELISPOT assays. *Journal of Immunological Methods* **260**, 157-172.

Dale CJ, Liu XS, De Rose R, Purcell DF, Anderson J, Xu Y, Leggatt GR, Frazer IH, and Kent SJ (2002). Chimeric human papilloma virus-simian/human immunodeficiency virus virus-like-particle vaccines: immunogenicity and protective efficacy in macaques. *Virology* **301**, 176-187.

Dale CJ, De Rose R, Stratov I, Chea S, Montefiori DC, Thomson S, Ramshaw IA, Coupar BE, Boyle DB, Law M, and Kent SJ (2004a). Efficacy of DNA and fowlpox virus priming/boosting vaccines for simian/human immunodeficiency virus. *Journal of Virology* **78**, 13819-13828.

Dale CJ, De Rose R, Wilson KM, Croom HA, Thomson S, Coupar BE, Ramsay A, Purcell DF, French R, Law M, Emery S, Cooper DA, Ramshaw IA, Boyle DB, Kent SJ, and Australian Thai HIV Vaccine Consortium (2004b). Evaluation in macaques of HIV-1 DNA vaccines containing primate CpG motifs and fowlpoxvirus vaccines co-expressing IFNgamma or IL-12. *Vaccine* **23**, 188-197.

Damian RT, Greene ND, and Katler SS (1971). IgG subclasses of baboon (*Papio cynocephalus*). *Journal of Immunology* **106**, 246-257.

Daniel MD, Letvin NL, King NW, Kannagi M, Sehgal PK, Hunt RD, Kanki PJ, Essex M, and Desrosiers RC (1985). Isolation of T-cell tropic HTLV-III-like retrovirus from macaques. *Science* **228**, 1201-1204.

Daniel MD, Kirchhoff F, Czajak SC, Sehgal PK, and Desrosiers RC (1992). Protective effects of a live attenuated SIV vaccine with a deletion in the nef gene. *Science* **258**, 1938-1941.

Daniel MD, Mazzara GP, Simon MA, Sehgal PK, Kodama T, Panicali DL, and Desrosiers RC (1994). High-titre immune responses elicited by recombinant vaccinia virus priming and particle boosting are ineffective in preventing virulent SIV infection. *AIDS Research and Human Retroviruses.* **10**, 839-851.

Davenport MP, Ribeiro RM, Chao DL, and Perelson AS (2004). Predicting the impact of a nonsterilizing vaccine against human immunodeficiency virus. *Journal of Virology* **78**, 11340-11351.

Davids V, Hanekom WA, Mansoor N, Gamieldien H, Gelderbloem SJ, Hawkridge A, Hussey GD, Hughes EJ, Soler J, Murray RA, Ress SR, and Kaplan G (2006). The effect of bacille Calmette-Guerin vaccine strain and route of administration on induced immune responses in vaccinated infants. *Journal of Infectious Diseases* **193**, 531-536.

Davis HL (2000). CpG motifs for optimization of DNA vaccines. *Developments in Biologicals* **104**, 165-169.

Davis NL, Caley IJ, Brown KW, Betts MR, Irlbeck DM, McGrath KM, Connell MJ, Montefiori DC, Frelinger JA, Swanstrom R, Johnson PR, and Johnson RE (2000). Vaccination of macaques against pathogenic simian immunodeficiency virus with Venezuelan equine encephalitis virus replicon particles. *Journal of Virology* **74**, 371-378.

de Groot NG, Otting N, Arguello R, Watkins DI, Doxiadis GG, Madrigal JA, and Bontrop RE (2000). Major histocompatibility complex class I diversity in a West African chimpanzee population: implications for HIV research. *Immunogenetics* **51**, 398-409.

De Rose R, Chea S, Dale CJ, Reece J, Fernandez CS, Wilson KM, Thomson S, Ramshaw IA, Coupar BE, Boyle DB, Sullivan MT, and Kent SJ (2005). Subtype AE HIV-1 DNA and recombinant Fowlpoxvirus vaccines encoding five shared HIV-1 genes: safety and T cell immunogenicity in macaques. *Vaccine* **23**, 1949-1956.

Delchambre M, Gheysen D, Thines D, Thiriart D, Jacobs E, Verdin E, Horth M, Burny A, and Bex F (1989). The GAG precursor of simian immunodeficiency virus assembles into virus-like particles. *The European Molecular Biology Organization Journal* **8**, 2653-2660.

Dellagostin OA, Wall S, Norman E, O'Shaughnessy T, Dale JW, and Mcfadden JJ (1993). Construction and use of integrative vectors to express foreign genes in mycobacteria. *Molecular Microbiology* **10**, 983-993.

Deml L, Kratochwill G, Osterrieder N, Knuchel R, Wolf H, and Wagner R (1997a). Increased incorporation of chimeric human immunodeficiency virus type 1 gp120 proteins into Pr55gag virus-like particles by an Epstein-Barr virus gp220/350-derived transmembrane domain. *Virology* **235**, 10-25.

Deml L, Schirmbeck R, Reimann J, Wolf H, and Wagner R (1997b). Recombinant human immunodeficiency Pr55gag virus-like particles presenting chimeric envelope glycoproteins induced cytotoxic T-cells and neutralizing antibodies. *Virology* **235**, 26-39.

Deml L, Schirmbeck R, Reimann J, Wolf H, and Wagner R (1999). Purification and characterization of hepatitis B virus surface antigen particles produced in Drosophila Schneider-2 cells. *Journal of Virological Methods* **79**, 205-217.

Deml L, Wild J, and Wagner R (2004). Virus-like particles: a novel tool for the induction and monitoring of both T-helper and cytotoxic T-lymphocyte activity. *Methods in Molecular Medicine* **94**, 133-157.

Deml L, Speth C, Dierich MP, Wolf H, and Wagner H (2005). Recombinant HIV-1 Pr55gag virus-like particles: potent stimulators of innate and acquired immune responses. *Molecular Immunology* **42**, 259-277.

Dennehy M and Williamson A-L (2005). Factors influencing the immune response to foreign antigen expressed in recombinant BCG vaccines. *Vaccine* **23**, 1209-1224.

Desrosiers RC, Wyand MS, Kodama T, Ringler DJ, Arthur LO, Sehgal PK, Letvin NL, King NW, and Daniel MD (1989). Vaccine protection against simian immunodeficiency virus infection. *Proceedings of the National Academy of Sciences of the USA* **86**, 6353-6357.

Disotell TR, Honeycutt RL, and Ruvolo M (1992). Mitochondrial DNA phylogeny of the Old-World monkey tribe Papionini. *Molecular Biology and Revolution* **9**, 1-13.

Disotell TR (1994). Generic level relationships of the Papionini (Cercopithecoidea). *American Journal of Physical Anthropology* **94**, 47-57.

Doan LX, Li M, Chen C, and Yao Q (2005). Virus-like particles as HIV-1 vaccines. *Reviews of Medical Virology* **15**, 75-88.

Doxiadis GG, Otting N, de Groot NG, and Bontrop RE (2001). Differential evolutionary MHC-class II strategies in humans and rhesus macaques:relevance for biomedical studies. *Immunological Reviews* **183**, 76-85.

Earl PL, Sugiura W, Montefiori DC, Broder CC, Lee SA, Wild C, and Moss B (2001). Immunogenicity and protective efficacy of oligomeric human immunodeficiency virus type 1 gp140. *Journal of Virology* **75**, 645-653.

Eda Y, Murakami T, Ami Y, Nakasone T, Takizawa M, Someya K, Kaizu M, Izumi Y, Yoshino N, Matsushita S, Higuchi H, Matsui H, Shinohara K, Takeuchi H, Koyanagi Y, Yamamoto N, and Honda M (2006). Anti-V3 humanized antibody KD-247 effectively suppresses ex vivo generation of human immunodeficiency virus type 1 and affords sterile protection of monkeys against a heterologous simian/human immunodeficiency virus infection. *Journal of Virology* **80**, 5563-5570.

Edwards BH, Bansal A, Sabbaj S, Bakari J, Mulligan MJ, and Goepfert PA (2002). Magnitude of functional CD8+ T-cell responses to the Gag protein of human immunodeficiency virus type 1 correlates inversely with viral load in plasma. *Journal of Virology* **76**, 2298-2305.

Egan MA, Chong SY, Rose NF, Megati S, Lopez KJ, Schadeck EB, Johnson JE, Massod A, Piacente P, Druihet RE, Barras PW, Hasselschwert DL, Reilly P, Mishkin EM, Montefiori DC, Lewis MG, Clarke DK, Hendry RM, Marx PA, Eldridge JH, Udem SA, Israel ZR, and Rose JK (2004). Immunogenicity of attenuated vesicular stomatitis virus vectors expressing HIV type 1 Env and SIV Gag peptides: comparison of intranasal and intramuscular vaccination routes. *AIDS Research and Human Retroviruses.* **20**, 989-1004.

Egan MA, Chong SY, Megati S, Montefiori DC, Rose NF, Boyer JD, Sidhu MK, Quiroz J, Rosati M, Schadeck EB, Pavlakis GN, Weiner DB, Rose JK, Israel ZR, Udem SA, and Eldridge JH (2005). Priming with plasmid DNAs expressing interleukin-12 and simian immunodeficiency virus gag enhances the immunogenicity and efficiency of an experimental AIDS vaccine based on recombinant vesicular stomatitis virus. *AIDS Research and Human Retroviruses.* **21**, 629-643.

Emini EA, Schleif WA, Nunberg JH, Conley AJ, Eda Y, Tokiyoshi S, Putney SD, Matsushita S, Cobb KE, and Jett CM (1992). Prevention of HIV-1 infection in chimpanzees by gp120 V3 domain-specific monoclonal antibody. *Nature* **355**, 728-730.

Emory University (2006). Primate taxonomy. *http://www.emory.edu/LIVING_LINKS/Taxonomy.html* [accessed: 4 May 2006].

Emu B, Sinclair E, Favre D, Moretto WJ, Hsue P, Hoh R, Martin JN, Nixon DF, McCune JM, and Deeks SG (2005). Phenotypic, functional and kinetic parameters associated with apparent T-cell control of human immunodeficiency virus replication in individuals with and without antiretroviral treatment. *Journal of Virology* **79**, 14169-14178.

Esparza J and Bhamarapravati N (2000). Accelerating the development and future availability of HIV-1 vaccines: why, when, where, and how? *Lancet* **355**, 2061-2066.

Estcourt MJ, McMichael AJ, and Hanke T (2004). DNA vaccines against human immunodeficiency virus type 1. *Immunological Reviews* **199**, 144-155.

Evans DT, Chen LM, Gillis J, Lin KC, Harty B, Mazzara GP, Donis RO, Mansfield KG, Lifson JD, Desrosiers RC, Galan JE, and Johnson RP (2003). Mucosal priming of simian immunodeficiency virus-specific cytotoxic T-lymphocyte responses in rhesus macaques by the Salmonella type III secretion antigen delivery system. *Journal of Virology* **77**, 2400-2409.

Evans DT, Bricker JE, Sanford HB, Lang S, Carville A, Richardson BA, Piatak M Jr, Lifson JD, Mansfield KG, and Desrosiers RC (2005). Immunization of macaques with single-cycle simian immunodeficiency virus (SIV) stimulates diverse virus-specific immune responses and reduces viral loads after challenge with SIVmac239. *Journal of Virology* **79**, 7707-7720.

Fang ZY, Kuli-Zadel I, and Spearman P (1999). Efficient human immunodeficiency virus (HIV)-1 Gag-Env pseudovirion formation elicited from mammalian cells by a canarypox HIV vaccine candidate. *Journal of Infectious Diseases* **180**, 1122-1132.

Feng CG and Britton WJ (2000). CD4+ and CD8+ T cells mediate adoptive immunity to aerosol infection of *Mycobacterium bovis* bacillus Calmette-Guerin. *Journal of Infectious Diseases* **181**, 1846-1849.

Ferrantelli F, Rasmussen RA, Buckley KA, Li PL, Wang T, Montefiori DC, Katinger H, Stiegler G, Anderson DC, McClure HM, and Ruprecht RM (2004). Complete protection of neonatal rhesus macaques against oral exposure to pathogenic simian-human immunodeficiency virus by human anti-HIV monoclonal antibodies. *Journal of Infectious Diseases* **189**, 2167-2173.

Flesch I and Kaufmann SH (1987). Mycobacterial growth inhibition by interferon-gamma-activated bone marrow macrophages and differential susceptability among strains of Mycobacterium tuberculosis. *Journal of Immunology* **138**, 4408-4413.

Flesch I and Kaufmann SH (1990). Activation of tuberculostatic macrophage function by gamma interferon, interlukin-4, and tumour necrosis factor. *Infection and Immunity* **558**, 2675-2677.

Flynn JL, Goldstein MM, Triebold KJ, Koller B, and Bloom BR (1992). Major histocompatibility complex class 1-restricted T cells are required for resistance to *Mycobacterium tuberculosis* infection. *Proceedings of the National Academy of Sciences of the USA* **89**, 12013-12017.

Flynn JL, Chan J, Triebold KJ, Stewart TA, and Bloom BR (1993). An essential role for interferon-γ in resistance to Mycobacterium tuberculosis infection. *Journal of Experimental Medicine* **178**, 2249-2254.

Flynn JL, Goldstein MM, Triebold KJ, Sypek J, Wolf S, and Bloom BR (1995a). IL-12 increases resistance of BALB/c mice to *Mycobacterium tuberculosis* infection. *Journal of Immunology* **155**, 2515-2525.

Flynn JL, Goldstein MM, Chan J, Triebold KJ, Pfeffer L, Lowenstein CJ, Schreiber R, Mak TW, and Bloom BR (1995b). Tumour necrosis factor-α is required in the protective immune response against *M. tuberculosis* in mice. *Immunity* **2**, 561-572.

Flynn NM, Forthal DN, Harro CD, Judson FN, Mayer KH, Para MF, and The rgp120 HIV Vaccine Study Group (2005). Placebo-controlled phase 3 trial of a recombinant glycoprotein 120 vaccine to prevent HIV-1 infection. *Journal of Infectious Diseases* **191**, 654-665.

Fourie PB and Odendaal MW (1983). Mycobacterium tuberculosis in a closed colony of baboons (*Papio ursinus*). *Laboratory Animal* **17**, 125-128.

Franchini G, Robert-Guroff M, Tartaglia J, Aggarwal A, Abimiku A, Benson J, Markham P, Limbach K, Hurteau G, Fullen J, Aldrich K, Miller N, Sadoff J, Paoletti E, and Gallo RC (1995). Highly attenuated HIV type 2 recombinant poxviruses but not HIV-2 recombinant Salmonella vaccines induce long-lasting protection in rhesus macaques. *AIDS Research and Human Retroviruses.* **11**, 909-920.

Franchini G, Gurunathan S, Baglyos L, Plotkin S, and Tartaglia J (2004). Poxvirus-based vaccine candidates for HIV: two decades of experience with special emphasis on canarypox vectors. *Expert Review of Vaccines* **3(suppl 1)**, S75-S88.

Freed EO (1998). HIV-1 gag proteins: diverse functions in the virus life cycle. *Virology* **251**, 1-15.

Frumkin LR, Agy MB, Coombs RW, Panther L, Morton WR, Koehler J, Florey MJ, Dragavon J, Schmidt A, Katze MG, and Corey L (1993). Acute infection of *macaca nemestrina* by human immunodeficiency virus type 1. *Virology* **195**, 422-431.

Fuerst TR, De la Cruz VF, Bansal GP, and Stover CK (1992). Development and analysis of recombinant BCG vector systems. *AIDS Research and Human Retroviruses.* **8**, 1451-1455.

Fukasawa M, Miura T, Hasegawa A, Morikawa S, Tsujimoto H, Miki K, Kitamura T, and Hayami M (1988). Sequence of simian immunodeficiency virus from African green monkey, a new member of the HIV/SIV group. *Nature* **333**, 457-461.

Fultz PN (1992). Immunization and challenge of chimpanzees with HIV-1. *AIDS Research and Human Retroviruses.* **8**, 1517-1519.

Fultz PN (1993). Nonhuman primate models for AIDS. *Clinical Infectious Diseases* **17 (Suppl 1)**, S230-S235.

Fultz PN, Stallworth J, Porter D, Novak M, Anderson MJ, and Morrow CD (2003). Immunogenicity in pig-tailed macaques of poliovirus replicons expressing HIV-1 and SIV antigens and protection against $SHIV_{89.6P}$ disease. *Virology* **315**, 425-437.

Gao F, Bailes E, Robertson DL, Chen Y, Rodenburg CM, Michael SF, Cummins LB, Arthur LO, Peeters M, Shaw GM, Sharp PM, and Hahn BH (1999). Origin of HIV-1 in the chimpanzee Pan troglodytes troglodytes. *Nature* **397**, 436-441.

Garcia I, Miyaza Y, Marchal G, Lesslauer W, and Vassalli P (1997). High sensitivity of transgenic mice expressing soluble TNFR1 fusion protein to mycobacterial infections: synergistic action of TNF and IFN-gamma in the differentiation of protective granulomas. *European Journal of Immunology* **27**, 3182-3190.

Gardner MB. (2003). Simian AIDS: an historical perspective. *Journal of Medical Primatology* **32**, 180-186.

Garnier L, Ravallec M, Blanchard P, Bossy JP, Devauchelle G, Jestin A, and Cerutti M (1995). Incorporation of pseudorabies virus gD into human immunodeficiency virus type 1 Gag particles produced in baculovirus-infected cells. *Journal of Virology* **69**, 4060-4068.

Gauduin MC, Glickman RL, Means R, and Johnson RP (1998). Inhibition of simian immunodeficiency virus (SIV) replication by CD8(+) T lymphocytes from macaques immunized with live attenuated SIV. *Journal of Virology* **72**, 6315-6324.

Gauduin MC, Kaur A, Ahmad S, Yilma T, Lifson JD, and Johnson RP (2004). Optimization of intracellular cytokine staining for the quantitation of antigen-specific CD4+ T cell responses in rhesus macaques. *Journal of Immunological Methods* **288**, 61-79.

Geluk A, Elferink DG, Slierendregt BC, van Meijgaarden KE, de Vries RR, Pottenhoff TH, and Bontrop RE. (1993). Evolutionary conservation of major histocompatibility complex-DR/peptide/T cell interactins in primates. *Journal of Experimental Medicine* **177**, 979-987.

Genain CP and Hauser SL (2001). Experimental allergic encephalomyelitis in the New World monkey Callithrix jacchus. *Immunological Reviews* **183**, 159-172.

Gheorghiu M, Lagranderie MR, Gicquel BM, and Leclerc CD (1994). Mycobacterium bovis BCG priming induces a strong potentiation of the antibody response induced by recombinant BCG expressing a foreign antigen. *Infection and Immunity* **62(10)**, 4287-4295.

Gherardi MM, Najera JL, Perez-Jimenez E, Guerra S, Garcia-Sastre A, and Esteban M (2003). Prime-boost immunization schedules based on influenza virus and vaccinia virus vectors potentiate cellular immune responses against human immunodeficiency virus Env protein systemically and in the genitorectal draining lymph nodes. *Journal of Virology* **77**, 7048-7057.

Gheysen D, Jacobs E, de Foresta F, Thiriart C, Francotte M, Thines D, and De Wilde M (1989). Assembly and release of HIV-1 precursor Pr55gag virus-like particles from recombinant baculovirus-infected insect cells. *Cell* **59**, 103-112.

Giavedoni L, Ahmad S, Jones L, and Yilma T (1997). Expression of gamma interferon by simian immunodeficiency virus increases attenuation and reduces postchallenge virus load in vaccinated rhesus macaques. *Journal of Virology* **71**, 866-872.

Gibbs JS, Regier DA, and Desrosiers RC (1994). Construction and in vitro properties of SIVmac mutants with deletions in "nonessential" genes. *AIDS Research and Human Retroviruses.* **10**, 607-616.

Girard M, Meignier B, Barre-Sinoussi F, Kieny MP, Matthews T, Muchmore E, Nara PL, Wei Q, Rimsky L, Weinhold K, and Fultz PN (1995). Vaccine-induced protection of chimpanzees against infection by a heterologous human immunodeficiency virus type 1. *Journal of Virology* **69**, 6239-6248.

Girard M, van der Ryst E, Barre-Sinoussi F, Nara P, Tartaglia J, Paoletti E, Blondeau C, Jennings M, Verrier F, Meignier B, and Fultz PN (1997). Challenge of chimpanzees immunized with a recombinant canarypox-HIV-1 virus. *Virology* **232**, 98-104.

Giri M, Ugen KE, and Weiner DB (2004). DNA vaccines against human immunodeficiency virus type 1 in the past decade. *Clinical Microbiology Reviews* **17**, 370-389.

Gluck R and Metcalfe IC (2002). New technology platforms in the development of vaccines for the future. *Vaccine* **20**, 10-16.

Goldstein S, Elkins WR, London WT, Hahn A, Goeken R, Martin JE, and Hirsch VM (1994). Immunization with whole inactivated vaccine protects from infection by SIV grown in human but not macaque cells. *Journal of Medical Primatology* **23**, 75-82.

Gonzalo RM, Rodriguez D, Garcia-Sastre A, Rodriguez JR, Palese P, and Esteban M (1999). Enhanced CD8+ T cell response to HIV-1 env by combined immunization with influenza and vaccinia virus recombinants. *Vaccine* **17**, 887-892.

Goonetilleke NP, McShane H, Hannan CM, Anderson RJ, Brooks RH, and Hill AV (2003). Enhanced immunogenicity and protective efficiency against *Mycobacterium tuberculosis* of Bacille Calmette-Guerin vaccine using mucosal administration and boosting with a recombinant modified vaccinia virus Ankara. *Journal of Immunology* **171**, 1602-1609.

Gorelick RJ, Benveniste RE, Lifson JD, Yovandich JL, Morton WR, Kuller L, Flynn BM, Fisher BA, Rossio JL, Piatak M Jr, Bess JW Jr, Henderson LE, and Arthur LO (2000). Protection of Macaca nemestrina from disease following pathogenic simian immunodeficiency virus (SIV) challenge: utilization of SIV nucleocapsid mutant DNA vaccines with and without an SIV protein boost. *Journal of Virology* **74**, 11935-11949.

Griffiths JC, Berrie EL, Holdworth LN, Moore JP, Harris SJ, Senior JM, Kingsman SM, Kingsman AJ, and Adams SE (1991). Induction of high-titre neutralizing antibodies, using hybrid human immunodeficiency virus V3-Ty viruslike particles in a clinically relevant adjuvant. *Journal of Virology* **65**, 450-456.

Griffiths JC, Harris SJ, Layton GT, Berrie EL, French TJ, Burns NR, Adams SE, and Kingsman AJ (1993). Hybrid human immunodeficiency virus Gag particles are an antigen carrier system: induction of cytotoxic T-cell and humoral responses by a Gag:V3 fusion. *Journal of Virology* **67**, 3191-3198.

Groenink M, Andeweg AC, Fouchier RA, Broersen S, van der Jagt RC, Schuitemaker H, de Goede RE, Bosch ML, Huisman HG, and Tersmette M (1992). Phenotype-associated env gene variation among eight related human immunodeficiency virus type 1 clones: evidence for in vivo recombination and determinants of cytotropism outside the V3 domain. *Journal of Virology* **66**, 6175-6180.

Gromme M and Neefjes J (2002). Antigen degradation or presentation by MHC class 1 molecules via classical and non-classical pathways. *Molecular Immunology* **39**, 181-202.

Gronowski AM, Hilbert DM, Sheehan KC, Garotta G, and Schreiber RD (1999). Baculovirus stimulates antiviral effects in mammalian cells. *Journal of Virology* **73**, 9944-9951.

Guan Y, Whitney JB, Liang C, and Wainberg MA (2001). Novel, live attenuated simian immunodeficiency virus constructs containing major deletions in leader RNA sequences. *Journal of Virology* **75**, 2776-2785.

Gundlach BR, Linhart H, Dittmer U, Sopper S, Reiprich S, Fuchs D, Fleckenstein B, Hunsmann G, Stahl-Hennig C, and Uberla K (1997). Construction, replication and immunogenic properties of a simian immunodeficiency virus expressing interleukin 2. *Journal of Virology* **71**, 2225-2232.

Guo L, Lu X, Kang SM, Chen C, Compans RW, and Yao Q (2003). Enhancement of mucosal immune responses by chimeric influenza HA/SHIV virus-like particles. *Virology* **313**, 502-513.

Gursel M, Verthelyi D, Gursel I, Ishii K J, and Klinman D M (2002). Differential and competitive activation of human immune cells by distinct classes of CpG oligodeoxynucleotide. *Journal of Leukocyte Biology* **71**, 813-820.

Haffar ●, Garrigues J, Travis B, Moran P, Zarling J, and Hu SL (1990). Human Immunodefiency virus-like, nonreplicating, gag-env particles assemble in a recombinant vaccinia virus expression system. *Journal of Virology* **64**, 2653-2659.

Hainsey BM, Hubbard B, Leland MM, and Brasky KM. (1993). Clinical parameters of the normal baboons (Papio species) and chimpanzes (*Pan troglyodytes*). *Laboratory Animal Science* **43**, 236-243.

Hammonds J, Chen X, Ding L, Fouts T, De Vico A, zur Megede J, Barnett S, and Spearman P (2003). Gp120 stability on HIV-1 virions and Gag-Env pseudovirions is enhanced by an uncleaved Gag core. *Virology* **314**, 636-649.

Hammonds J, Chen X, Fouts T, De Vico A, Montefiori D, and Spearman P (2005). Induction of neutralizing antibodies against human immunodeficiency virus type 1 primary isolates by Gag-Env pseudovirion immunization. *Journal of Virology* **79**, 14804-14814.

Hanke T, Blanchard TJ, Schneider J, Hannan CM, Becker M, Gilbert SC, Hill AV, Smith GL, and McMichael A (1998). Enhancement of MHC class 1-restricted peptide-specific T cell induction by a DNA prime/MVA boost vaccination regime. *Vaccine* **16**, 439-445.

Hanke T, Samuel RV, Blanchard TJ, Neumann VC, Allen TM, Boyson JE, Sharpe SA, Cook N, Smith GL, Watkins DI, Cranage MP, and McMichael A J (1999). Effective induction of simian immunodeficiency virus-specific cytotoxic T lymphocytes in macaques by using a multiepitope gene and DNA prime-modified vaccinia Ankara boost vaccination regimen. *Journal of Virology* **73**, 7524-7532.

Hanke T, Barnfield C, Wee EG, Agren L, Samuel RV, Larke N, and Liljestrom P (2003). Construction and immunogenicity in a prime-boost regimen of a Semliki Forest virus-vectored experimental HIV clade A vaccine. *Journal of General Virology* **84**, 361-368.

Harari A, Petitpierre S, Vallelian F, and Pantaleo G (2004). Skewed representation of functionally distinct populations of virus-specific CD4 T cells in HIV-1-infected subjects with progressive disease: changes after antiretroviral therapy. *Blood* **103**, 966-972.

Harding CV and Song R (1994). Phagocytic processing of exogenous particulate antigens by macrophages for presentation by class 1 MHC molecules. *Journal of Immunology* **153**, 4925-4933.

Harouse JM, Gettie A, Tan RCH, Blanchard J, and Cheng-Mayer C (1999). Distinct pathogenic sequela in rhesus macaques infected with CCR5 or CXCR4 utilizing SHIVs. *Science* **284**, 816-819.

Harrer T, Harrer E, Kalams SA, Elbeik T, Staprans SI, Feinberg MB, Cao Y, Ho DD, Yilma T, Caliendo AM, Johnson RP, Buchbinder SP, and Walker BD (1996). Strong cytotoxic T cell and weak neutralizing antibody responses in a subset of persons with stable nonprogressing HIV type 1 infection. *AIDS Research and Human Retroviruses.* **12**, 585-592.

Harris SJ, Gearing AJ, Layton GT, Adams SE, and Kingsman AJ (1992). Enhanced poliferative cellular responses to HIV-1 V3 peptide and gp120 following immunization with V3:Ty virus-like particles. *Immunology* **77**, 315-321.

Hartmann G, Weiner GJ, and Krieg AM (1999). CpG DNA: a potent signal for growth, activation,and maturation of human dendritic cells. *Proceedings of the National Academy of Sciences of the USA* **96**, 9305-9310.

Hawgood BJ (1999). Doctor Albert Calmette 1863-1933: founder of antivenomous serotherapy and antituberculous BCG vaccination. *Toxicon* **37**, 1241-1258.

Haynes BF (1996). HIV vaccines: where we are and where we are going. *Lancet* **348**, 933-937.

Haynes JR, Fuller DH, Eisenbraun MD, Ford MJ, and Pertmer TM (1994). Accell® particle-mediated DNA immunization elicits humoral,cytotoxic and protective responses. *AIDS Research and Human Retroviruses.* **10**, S43-S45.

Haynes JR, Fuller DH, McCabe D, Swain WF, and Widera G (1996). Induction and characterization of humoral and cellular immune responses elicited via gene gun-mediated nucleic acid immunization. *Advanced Drug Delivery Review* **21**, 3-18.

Heath WR and Carbone FR (2001). Cross-presentation, dendritic cells, tolerance and immunity. *Annual Review of Immunology* **19**, 47-64.

Heeney J, Koopman G, Rosenwirth B, Bogers W, van Dijk J, Nieuwenhuis I, Niphuis H, ten Haaft P, Hanke T, Rhodes G, Berglund P, Burny A, Bex F, Sutter G, and Liljestrom P (2000). A vaccine strategy utilising a combination of three different chimeric vectors which share specific vaccine antigens. *Journal of Medical Primatology* **29**, 268-273.

Heeney JL (1996). Primate models for AIDS vaccine development. *AIDS* **10 (Suppl A)**, S115-S122.

Heintel T, Sester M, Rodriguez MM, Krieg C, Sester U, Wagner R, Pees HW, Gartner B, Maier R, and Meyerhans A (2002). The fraction of perforin-expressing HIV-specific CD8 T cells is a marker for disease progression in HIV infection. *AIDS* **16**, 1497-1501.

Hel Z, Tsai WP, Thornton A, Nacsa J, Giuliani L, Tryniszewska E, Poudyal M, Venzon D, Wang X, Altman J, Watkins DI, Lu W, von Gegerfelt A, Felber BK, Tartaglia J, Pavlakis GN, and Franchini G (2001). Potentiation of simian immunodeficiency virus (SIV)-specific CD4+ and CD8+ T cell responses by a DNA-SIV and NYVAC-SIV prime/boost regimen. *Journal of Immunology* **167**, 7180-7191.

Hemmi H, Takeuchi O, Kawai T, Kaisho T, Sato S, Sanjo H, Matsumoto M, Hoshino K, Wagner H, Takeda K, and Akira S (2000). A Toll-like receptor recognizes bacteria DNA. *Nature* **408**, 740-745.

Hemmi H, Kaisho T, and Akira S (2003). The roles fo Toll-like receptor 9, MyD88, and DNA-dependent protein kinase catalytic subunit in the effects of two distinct CpG DNAs on dendritic cell subsets. *Journal of Immunology* **170**, 3059-3064.

Henderson RA, Watkins SC, and Flynn JL (1997). Activation of human dendritic cells following infection with *Mycobacterium tuberculosis*. *Journal of Immunology* **159**, 635-643.

Hill CM, Deng H, Unutmaz D, KewalRamani VN, Bastiani L, Gorny MK, Zolla-Pazner S, and Littman DR (1997). Envelope glycoproteins from human immunodeficiency virus types 1 and 2 and simian immunodeficiency virus can use human CCR5 as a coreceptor for viral entry and make direct CD4-dependent interactions with this chemokine receptor. *Journal of Virology* **71**, 6304.

Hill CP, Worthylake D, Bancroft DP, Christensen AM, and Sundquist WI (1996). Crystal structures of the trimetric human immunodeficiency virus type 1 matrix protein: implications for membrane association and assembly. *Proceedings of the National Academy of Sciences of the USA* **93**, 3099-3104.

Hiroi T, Goto H, Someya K, Yanagita M, Honda M, Yamanaka N, and Kiyono H (2001). HIV mucosal vaccine: nasal immunization with rBCG-V3J1 induces a long term V3J1 peptide-specific neutralizing immunity in Th1- and Th2-deficient conditions. *Journal of Immunology* **167**, 5862-5867.

Hirsch VM, Fuerst TR, Sutter G, Carroll MW, Yang LC, Goldstein S, Piatak M Jr, Elkins WR, Alvord WG, Montefiori DC, Moss B, and Lifson JD (1996). Patterns of viral replication correlate with outcome in simian immunodeficiency virus (SIV)-infected macaques: effect of prior immunization with a trivalent SIV vaccine in modified vaccinia virus Ankara. *Journal of Virology* **70**, 3741-3752.

Hoffmeister B, Kiecker F, Tesfa L, Volk H-D, Picker LJ, and Kern F (2003). Mapping T cell epitopes by flow cytometry. *Methods* **29**, 270-281.

Hoft DF and Gheorghiu M. (1996). Mucosal immunity induced by oral administration of Bacille Calmette-Guerin. *In:'Mucosal Vaccines', Kiyono H, Ogra PL, McGhee JR (eds) Academic Press, San Diego CA* p269-279.

Hoft DF, Brown RM, and Roodman ST (1998). Bacille Calmette-Guerin vaccination enhances human γδT cell responsiveness to Mycobacteria suggestive of a memory-like phenotype. *Journal of Immunology* **161**, 1045-1054.

Hoft DF and Tennant JM (1999). Persistent and boosting of bacille Calmette-Guerin-induced delayed-type hypersensitivity. *Annals of Internal Medicine* **131**, 32-36.

Hoft DF, Kemp EB, Marinaro M, Cruz I, Kiyono H, McGhee JR, Belisle JT, Milligan TW, Miller JP, and Belshe RB (1999). A double-blind, placebo-controlled study of *Mycobacterium*-specific human immune responses induced by intradermal bacille Calmette-Guerin vaccination. *Journal of Laboratory Clinical Medicine* **134**, 244-252.

Hoft DF, Kemp EB, Marinaro M, Cruz O, Kiyono H, McGhee JR, Belisle JT, Milligan TW, Miller JP, and Belshe RB (1999). A double-blind, placebo-controlled study of Mycobacterium-specific human immune responses induced by intradermal bacille Calmette-Guerin vaccination. *Journal of Laboratory and Clinical Medicine* **134**, 244-252.

Honda M, Matsuo K, Nakasone T, Okamoto Y, Yoshizaki H, Kitamura K, Sugiura W, Watanabe K, Fukushima Y, Haga S, Katsura Y, Tasaka H, Komuro K, Yamada T, Asano T, Yamazaki A, and Yamazaki S (1995). Protective immune responses induced by secretion of a chimeric soluble protein from a recombinant Mycobacterium bovis bacillus Calmette-Guerin vector candidate vaccine for human immunodeficiency virus type 1 in small animals. *Proceedings of the National Academy of Sciences of the USA* **92**, 10693-10697.

Horiuchi R, Akahata W, Kuwata T, Enose Y, Ido E, Suzuki H, Miyake A, Saito N, Ibuki K, Goto T, Miura T, and Hayami M (2006). DNA vaccination of macaques by a full-genome SHIV plasmid that has an IL-2 gene and produces non-infectious virus particles. *Vaccine* **24**, 3677-3685.

Horwitz MA., Harth G, Dillon BJ, and Maslesa-Galic S (2000). Recombinant bacillus Calmette-Guerin (BCG) vaccines expressing the *Mycobacterium tuberculosis* 30-kDa major secretory protein induces greater protective immunity against tuberculosis than conventional BCG vaccines in a highly susceptible animal model. *Proceedings of the National Academy of Sciences of the USA* **97**, 13853-13858.

Hu SL, Abrams K, Barber GN, Moran P, Zarling JM, Langlois AJ, Kuller L, Morton WR, and Benveniste RE (1992). Protection of macaques against SIV infection by subunit vaccines of SIV envelope glycoprotein gp160. *Science* **255**, 456-459.

Huh JW, Hong KW, Yi JM, Kim TH, Takenala O, Lee WH, and Kim HS (2003). Molecular phylogeny and evolution of the human endogenous retrovirus HERV-W LTR family in hominoid primates. *Molecules and Cells* **15**, 122-126.

HVTN (HIV Vaccine Trials Network) (2006). Phases of clinical trials. *http://www.hvtn.org/science/phases.html* [accessed: 19 March 2006].

IAVI (International AIDS Vaccine Initiative) (2006). AIDS Vaccine Candidate Trials 2005-Early 2006: A Review. *http://www.iavi.org/* [accessed: 11 May 2006].

Igarashi T, Endo Y, Englund G, Sadjadpour R, Matano T, Buckler C, Buckler-White A, Plishka R, Theodore T, Shibata R, and Martin M (1999). Emergence of a highly pathogenic simian/human immunodeficiency virus in a rhesus macaque treated with anti-CD8 mAb during a primary infection with a nonpathogenic virus. *Proceedings of the National Academy of Sciences of the USA* **96**, 14049-14054.

Inaba K, Inaba M, Naiton M, and Steinman RM (1993). Dendritic cell progenitors phagocytose particulates, including bacillus Calmette-Guerin organisms, and sensitize mice to mycobacterial antigens in vivo. *Journal of Experimental Medicine* **178**, 479-488.

Iyasere C, Tilton JC, Johnson AJ, Younes S, Yassine-Diab B, Sekaly RP, Kwok WW, Migueles SA, Laborico AC, Shupert WL, Hallahan CW, Davey RT, Dybul M, Vogel S, Metchalf J, and Connors M (2003). Diminished proliferation of human immunodeficiency virus-specific CD4+ T cells is associated with diminished interleukin-2 (IL-2) production and is recovered by exogenous IL-2. *Journal of Virology* **77**, 10900-10909.

Jaffray A, Shephard E, van Harmelen J, Williamson C, Williamson A-L, and Rybicki EP (2004). Human immunodeficiency virus type 1 subtype C Gag virus-like particle boost substantially improves the immune response to a subtype C *gag* DNA vaccine in mice. *Journal of General Virology* **85**, 409-413.

Janicki BW, Good RC, Minden P, Affronti LF, and Hymes WF (1973). Immune responses in rhesus monkeys after bacillus Calmette-Guerin vaccination and aerosol challenge with *Mycobacterium tuberculosis*. *American Review of Respiratory Diseases* **107**, 359-366.

Jin MJ, Rogers J, Phillips-Conroy JE, Allan JS, Desrosiers RC, Shaw GM, Sharp PM, and Hahn BH (1994). Infection of a yellow baboon with simian immunodeficiency virus from African green monkeys: evidence for cross-species transmission in the wild. *Journal of Virology* **68**, 8454-8460.

Jin X, Bauer DE, Tuttleton SE, Lewin S, Gettie A, Blanchard J, Irwin CE, Safrit JT, Mittler J, Weinberger L, Kostrikis LG, Zhang L, Perelson AS, and Ho DD (1999). Dramatic rise in plasma viremia after CD8+ T cell depletion in simian immunodeficiecy virus-infected macaques. *Journal of Experimental Medicine* **189**, 991-998.

Joag SV, Li Z, Foresman L, Stephens EB, Zhao LJ, Adany I, Pinson DM, McClure HM, and Narayan O (1996). Chimeric simian/human immunodeficiency virus that causes progressive loss of CD4+ T cells and AIDS in pig-tailed macaques. *Journal of Virology* **70**, 3189-3197.

Joag SV, Li Z, Wang C, Jia F, Foresman L, Adany I, Pinson DM, Stephens EB, and Narayan O (1998). Chimeric SHIV that causes CD4+ T cell loss and AIDS in rhesus macaques. *Journal of Medical Primatology* **27**, 59-64.

Joag SV (2000). Primate models of AIDS. *Microbes and Infection* **2**, 223-229.

Johnson RP, Lifson JD, Czajak SC, Cole KS, Manson KH, Glickman R, Yang J, Montefiori DC, Montelaro R, Wyand MS, and Desrosiers RC (1999). Highly attenuated vaccine strains of simian immunodeficiency virus protect against vaginal challenge: inverse relationship of degree of protection with level of attenuation. *Journal of Virology* **73**, 4952-4961.

Johnston RE, Johnson PR, Connell MJ, Montefiori DC, West A, Collier ML, Cecil C, Swanstrom R, Frelinger JA, and Davis NL (2005). Vaccination of macaques with SIV immunogens delivered by Venezuelan equine encephalitis virus replicon particle vectors followed by a mucosal challenge with SIVsmE660. *Vaccine* **23**, 4969-4979.

Jondal M, Schirmbeck R, and Reimann J (1996). MHC class 1-restricted CTL responses to exogenous antigens. *Immunity* **5**, 295-302.

Kameoka M, Nishino Y, Matsuo K, Ohara N, Kimura T, Yamazaki A, Yamada T, and Ikuta K (1994). Cytotoxic T lymphocyte response in mice induced by a recombinant BCG vaccination which produces an extracellular α antigen that fused with the human immunodeficiency virus type 1 envelope immunodominant domain in the V3 loop. *Vaccine* **12**, 153-158.

Kang SM, Yao Q, Guo L, and Compans RW (2003). Mucosal immunization with virus-like particles of simian immunodeficiency virus conjugated with cholera toxin subunit B. *Journal of Virology* **77**, 9823-9830.

Kang SM, Guo L, Yao Q, Skountzou I, and Compans RW (2004). Intranasal immunization with inactivated influenza virus enhances immune responses to coadministered simian-human immunodeficiency virus-like particle antigens. *Journal of Virology* **78**, 9624-9632.

Kano M, Matano T, Kato A, Nakamura H, Takeda A, Suzuki Y, Ami Y, Terao K, and Nagai Y (2000a). Primary replication of a recombinant Sendai virus vector in macaques. *Journal of General Virology* **83**, 1377-1386.

Kano M, Matano T, Nakamura H, Takeda A, Kato A, Ariyoshi K, Mori K, Sata T, and Nagai Y (2000b). Elicitation of protective immunity against simian immunodeficiency virus infection by a recombinant Sendai virus expressing the Gag protein. *AIDS* **14**, 1281-1282.

Karacostas V, Nagashima K, Gonda MA, and Moss B (1989). Human immunodeficiency virus-like particles produced by a vaccinia virus expression vector. *Proceedings of the National Academy of Sciences of the USA* **86**, 8964-8967.

Kaul R, Plummer FA, Kimani J, Dong T, Kiama P, Rostron T, Njagi E, MacDonald KS, Bwayo JJ, McMichael AJ, and Rowland-Jones SL. (2000). HIV-1-specific mucosal CD8+ lymphocyte responses in the cervix of HIV-1 resistant prostitutes in Nairobi. *Journal of Immunology* **164**, 1602-1611.

Kawada M, Igarashi H, Takeda A, Tsukamoto T, Yamamoto H, Dohki S, Takiguchi M, and Matano T (2006). Involvement of multiple epitope-specific cytotoxic T-lymphocyte responses in vaccine-based control of simian immunodeficiency virus replication in rhesus macaques. *Journal of Virology* **80**, 1949-1958.

Kawahara M, Hashimoto A, Toida I, and Honda M (2002a). Oral recombinant *mycobacterium bovis* bacillus Calmette-Guerin expressing HIV-1 antigens as a freeze-dried vaccine induces long-term, HIV-specific mucosal and systemic immunity. *Clinical Immunology* **105**, 326-331.

Kawahara M, Matsuo K, Nakasone T, Hiroi T, Kiyono H, Yamada T, Yamamoto N, and Honda M (2002b). Combined intrarectal/intradermal innoculation of recombinant *Mycobacterium bovis* bacillus Calmette-Guerin (BCG) induces enhanced immnue responses against the inserted HIV-1 V3 antigen. *Vaccine* **21**, 158-166.

Keet DF, Kriek NP, Bengis RG, Grobler DG, and Michel A. (2000). The rise and fall of tuberculosis in a free-ranging chacma baboon troop in the Kruger National Park. *Onderstepoort Journal of Veterinary Research* **67**, 115-122.

Kemp EB, Belshe RB, and Hoft DF (1996). Immune responses stimulated by percutaneous and intradermal bacille Calmette-Guerin. *Journal of Infectious Diseases* **174**, 113-119.

Kenedy RC, Shearer MH, and Hildebrand W. (1997). Nonhuman primate models to evaluate vaccine safety and immunogenicity. *Vaccine* **15**, 903-908.

Kent S J, Zhao A, Best S J, Chandler J D, Boyle D B, and Ramshaw I A (1998). Enhanced T-cell immunogenicity and protective efficacy of a human immunodeficiency virus type vaccine regimen consisting of consecutive priming with DNA and boosting with recombinant fowlpox virus. *Journal of Virology* **72**, 10180-10188.

Kent SJ, Dale CJ, Ranasinghe C, Stratov I, De Rose R, Chea S, Montefiori DC, Thomson S, Ramshaw IA, Coupar BE, Boyle DB, Law M, Wilson KM, and Ramsay AJ (2005). Mucosally-administered human-simian immunodeficiency virus DNA and fowlpoxvirus-based recombinant vacines reduce acute phase viral replication in macaques following vaginal challenge with CCR5-tropic $SHIV_{SF162P3}$. *Vaccine* **23**, 5009-5021.

University of Cape Town

Kestler H, Kodama T, Ringler D, Marthas M, Pedersen N, Lackner A, Regier D, Sehgal P, Daniel M, and King N (1990). Induction of AIDS in rhesus monkeys by molecularly cloned simian immunodeficiency virus. *Science* **248**, 1109-1112.

Kestler HW 3rd, Li Y, Naidu YM, Butler CV, Ochs MF, Jaenel G, King NW, Daniel MD, and Desrosiers RC (1988). Comparison of simian immunodeficiency virus isolates. *Nature* **331**, 619-622.

Kestler HW 3rd, Ringler DJ, Mori K, Panicali DL, Sehgal PK, Daniel MD, and Desrosiers RC (1991). Importance of the nef gene for maintenance of high virus loads and for development of AIDS. *Cell* **65**, 651-662.

Kilani RT, Delehanty M, Shankowsky HA, Ghahary A, Scott P, and Tredget EE (2005). Fluorescent-activated cell-sorting analysis of intracellular interferon-γ and interleukin-4 in fresh and frozen human peripheral blood T-helper cells. *Wound Repair and Regeneration* **13**, 441-449.

Kindler V, Sappino A-P, Grau GE, Piquet P-F, and Vassalli P (1989). The inducing role of tumour necrosis factor in the development of bactericidal granulomas during BCG infection. *Cell* **56**, 731-740.

Kirnbauer R (1996). Papillomavirus-like particles for serology and vaccine development. *Intervirology* **39**, 54-61.

Kitchen SG and Zack JA (1998). HIV type 1 infection in lymphoid tissue: natural history and model systems. *AIDS Research and Human Retroviruses.* **14 Suppl 3**, S235-S239.

Klinger JM, Himathongkham S, Legg H, Luciw PA, and Barnett SW (1998). Infection of baboons with a simian immunodeficiency virus/HIV-1 chimeric virus constructed with an HIV-1 Thai subtype E envelope. *AIDS* **12**, 849-857.

Kong WP, Huang Y, Yang ZY, Chakrabarti BK, Moodie Z, and Nabel GJ (2003). Immunogenicity of multiple gene and clade human immunodeficiency virus type 1 DNA vaccines. *Journal of Virology* **77**, 12764-12772.

Koopman G, Mortier D, Hofman S, Niphuis H, Fagrouch Z, Norley S, Sutter G, Liljestrom P, and Heeney JL (2004). Vaccine protection from CD4+ T-cell loss caused by simian immunodeficiency virus (SIV) mac251 is afforded by sequential immunization with three unrelated vaccine vectors encoding multiple SIV antigens. *Journal of General Virology* **85**, 2915-2924.

Kostense S, Koudstaal W, Sprangers M, Weverling GJ, Penders G, Helmus N, Vogels R, Bakker M, Berkhout B, Havenga M, and Goudsmit J (2004). Adenovirus types 5 and 35 seroprevalence in AIDS risk groups supports type 35 as a vaccine vector. *AIDS* **18**, 1213-1216.

Koup RA, Safrit JT, Cao Y, Andrews CA, McLeod G, Borkowsky W, Farthing C, and Ho DD (1994). Temporal association of cellular immune responses with the initial control of viremia in primary human immunodeficiency virus type 1 syndrome. *Journal of Virology* **68**, 4650-4655.

Kreher CR, Dittrich MT, Guerkov R, Boehm BO, and Tary-Lehmann M (2003). CD4+ and CD8+ cells in cryopreserved human PBMC maintain full functionality in cytokine ELISPOT assays. *Journal of Immunological Methods* **278**, 79-93.

Kuate S, Stahl-Hennig C, ten Haaft P, Heeney J, and Uberla K (2003). Single-cycle immunodeficiency viruses provide strategies for uncoupling in vivo expression levels from viral replicative capacity and for mimicking live-attenuated SIV vaccines. *Virology* **313**, 653-662.

Kumar A, Lifson JD, Silverstein PS, Jia F, Sheffer D, Li Z, and Narayan O (2000). Evaluation of immune responses induced by HIV-1 gp120 in rhesus macaques: effect of vaccination on challenge with pathogenic strains of homologous and heterologous simian human immunodeficiency viruses. *Virology* **274**, 149-164.

Kumar D, Srivastava BS, and Srivastava R (1998). Genetic rearrangements leading to disruption of heterologous gene expression in mycobacteria: an observation with Escherichia coli-β-galactosidase in *Mycobacterium smegmatis* and its implication in vaccine development. *Vaccine* **16**, 1212-1215.

Labidi AH, Estes RC, David HL, and Bollon AP. (2001). Mycobacterium recombinant vaccines. *La Tunisie Medicale* **79**, 65-81.

Lackner AA, Rodriguez MH, Bush CE, Munn RJ, Kwang HS, Moore PF, Osborn KG, Marx PA, Gardner MB, and Lowenstine LJ (1988). Distribution of a macaque immunosuppressive type D retrovirus in neural, lymphoid and salivary tissues. *Journal of Virology* **62**, 2134-2142.

Ladel CH, Szalay G, Reidel D, and Kaufmann SH (1997). Interlukin-12 secretion by *Mycobacterium tuberculosis*. *Infection and Immunity* **65**, 1936-1938.

Lagaranderie M, Winter N, Balazuc A, Gicquel B, and Gheorghiu M (1998). A cocktail of *mycobacterium bovis* BCG recombinants expressing the SIV Nef, Env, and Gag antigens induces antibody and cytotoxic responses in mice vaccinated by different mucosal routes. *AIDS Research and Human Retroviruses.* **14**, 1625-1633.

Lai X, Shen Y, Zhou D, Sehgal P, Shen L, Simon M, Qiu L, Letvin NL, and Chen ZW (2003). Immune biology of macaque lymphocyte population during mycobacterial infection. *Clinical Experimental Immunology* **133**, 182-192.

Lalvani A, Brookes R, Wilkinson RJ, Malin AS, Pathan AA, Andersen P, Dockrell H, Pasvol G, and Hill AV (1998). Human cytolytic and interferon gamma-secreting CD8+ T lymphocytes specific for *Mycobacterium tuberculosis*. *Proceedings of the National Academy of Sciences of the USA* **95**, 270-275.

Langermann S (1996). Recombinant BCG as vector for mucosal immunity. *In:'Mucosal Vaccines', Kiyono H, Ogra PL, McGhee JR (eds) Academic Press, San Diego CA* p129-136.

Langermans JA, Andersen P, van Soolingen D, Vervenne RA, Frost PA, van der Laan T, van Pinxteren LA, van den Hombergh J, Kroon S, Peekel I, Florquin S, and Thomas AW. (2001). Divergent effect of bacillus Calmette-Guerin (BCG) vaccination of *Mycobacterium tuberculosis* infection in highly related macaque: Implications for primate models in tuberculosis vaccine research. *Proceedings of the National Academy of Sciences of the USA* **98**, 11497-11502.

Langlois AJ, Desrosiers RC, Lewis MG, KewalRamani VN, Littman DR, Zhou P, Manson K, Wyand MS, Bolognesi DP, and Montefiori DC (1998). Neutralizing antibodies in sera from macaques immunized with attenuated simian immunodeficiency virus. *Journal of Virology* **72**, 6950-6955.

le Grand R, Vogt G, Vaslin B, Roques P, Theodoro F, Aubertin AM, and Dormont D (1992). Specific and non-specific immunity and protection of macaques against SIV infection. *Vaccine* **10**, 873-879.

Letvin NL, Montefiori DC, Yasutomi Y, Perry HC, Davies ME, Lekutis C, Alroy M, Freed DC, Lord CI, Handt LK, Liu MA, and Shiver JW (1997). Potent, protective anti-HIV immune responses generated by bimodal HIV envelope DNA plus protein vaccination. *Proceedings of the National Academy of Sciences of the USA* **94**, 9378-9383.

Letvin NL, Robinson S, Rohne D, Axthelm MK, Fanton JW, Bilska M, Palker TJ, Liao HX, Haynes BF, and Montefiori DC (2001). Vaccine-elicited V3 loop-specific antibodies in rhesus monkeys and control of a simian-human immunodeficiency virus expressing a primary patient human immunodeficiency virus type 1 isolate envelope. *Journal of Virology* **75(9):4165-75**, 4165-4175.

Letvin NL, Huang Y, Chakrabarti BK, Xu L, Seaman MS, Beaudry K, Korioth-Schmitz B, Yu F, Rohne D, Martin KL, Miura A, Kong WP, Yang ZY, Gelman RS, Golubeva OG, Montefiori DC, Mascola JR, and Nabiel GJ (2004). Heterologous envelope immunogens contribute to AIDS vaccine protection in rhesus monkeys. *Journal of Virology* **78**, 7490-7497.

Leung L, Srivastava IK, Kan E, Legg H, Sun Y, Greer C, Montefiori DC, zur Megede J, and Barnett SW (2004). Immunogenicity of HIV-1 Env and Gag in baboons using a DNA prime/protein boost regimen. *AIDS* **18**, 991-1001.

Leung NJ, Aldovini A, Young R, Jarvis MA, Smith JM, Meyer D, Anderson DE, Carlos MP, Gardner MB, and Torres JV (2000). The kinetics of specific immune responses in rhesus monkeys inoculated with live recombinant BCG expressing SIV gag, Pol, Env and Nef proteins. *Virology* **268**, 94-103.

Levy JA, Scott I, and Mackewicz C (2003). Protection from HIV/AIDS: the importance of innate immunity. *Clinical Immunology* **108**, 167-174.

Lewis MG, Bellah S, McKinnon K, Yalley-Ogunro J, Zack PM, Elkins WR, Desrosiers RC, and Eddy GA (1994). Titration and characterization of two rhesus-derived SIVmac challenge stocks. *AIDS Research and Human Retroviruses.* **10**, 213-220.

Lewis MG, Yalley-Ogunro J, Greenhouse JJ, Brennan TP, Jiang JB, VanCott TC, Lu Y, Eddy GA, and Birx DL (1999). Limited protection from a pathogenic chimeric simian-human immunodeficiency virus challenge following immunization with attenuated simian immunodeficiency virus. *Journal of Virology* **73**, 1262-1270.

Li J, Lord CI, Haseltine W, Letvin NL, and Sodroski J (1992). Infection of cynomolgus monkeys with a chimeric HIV-1/SIVmac virus that expresses the HIV-1 envelope glycoproteins. *Journal of Acquired Immune Deficiency Syndromes* **5**, 639-646.

Lifson JD, Rossio JL, Piatak M Jr, Bess J Jr, Chertova E, Schneider DK, Coalter VJ, Poore B, Kiser RF, Imming RJ, Scarzello AJ, Henderson LE, Alvord WG, Hirsch VM, Benveniste RE, and Arthur LO (2004). Evaluation of the safety, immunogenicity, and protective efficacy of whole inactivated simian immunodeficiency virus (SIV) vaccines with conformationally and functionally intact envelope glycoproteins. *AIDS Research and Human Retroviruses.* **20**, 772-787.

Lin YL, Borenstein LA, Ahmed R, and Wettstein FO (1993). Cottontail rabbit papillomavirus L1 protein-based vaccines:protection is achieved only with a full-length, nondenatured product. *Journal of Virology* **67**, 4154-4162.

Ling B, Apetrei C, Pandrea I, Veazey RS, Lackner AA, Gormus B, and Marx PA (2004). Classic AIDS in a sooty mangabey after an 18-year natural infection. *Journal of Virology* **78**, 8902-8908.

Liu Z, Singh DK, Sheffer D, Smith MS, Dhillon S, Chebloune Y, Hegde R, Buch S, and Narayan O (2006). Immunoprophylaxis against AIDS in macaques with a lentiviral DNA vaccine. *Virology* **IN PRESS**.

Locher CP, Blackbourn DJ, Barnett SW, Murthy KK, Cobb EK, Rouse S, Greco G, Reyes-Teran G, Brasky KM, Carey KD, and Levy JA (1997). Superinfection with human immunodeficiency virus type 2 can reactivate virus production in baboons but is contained by a CD8 T cell antiviral response. *Journal of Infectious Diseases* **176**, 948-959.

Locher CP, Blackbourn DJ, Herndier BG, Reyes-Teran G, Barnett SW, Murthy KK, and Levy JA (1998a). Transient virus infection and pathogenesis of a new HIV type 2 isolate, UC12, in baboons. *AIDS Research and Human Retroviruses*. **14**, 79-82.

Locher CP, Barnett SW, Herndier BG, Blackbourn DJ, Reyes-Teran G, Murthy KK, Brasky KM, Hubbard GB, Reinhart TA, Haase AT, and Levy JA (1998b). Human immunodeficiency virus-2 infection in baboons is an animal model for human immunodeficiency virus pathogenesis in humans. *Archives of Pathology and Laboratory Medicine* **122**, 523-533.

Locher CP, Witt SA, Herndier BG, Tenner-Racz K, Racz P, and Levy JA (2001). Baboons as an animal model for human immunodeficiency virus pathogenesis and vaccine development. *Immunological Reviews* **183**, 127-140.

Locher CP, Witt SA, Hemdier BG, Abbey NW, Tenner-Racz K, Racz P, Kiviat NB, Murthy KK, Brasky K, Leland M, and Levy JA. (2003). Increased virus replication and virulence after serial passage of human immunodeficiency virus type 2 in baboons. *Journal of Virology* **77**, 77-83.

Locher CP, Witt SA, Ashlock BM, Polacino P, Hu SL, Shiboski S, Agy MB, Anderson DM, Staphrans SI, zur Megede J, and Levy JA (2004). Human immunodeficiency virus type 2 DNA vaccine provides partial protection from acute baboon infection. *Vaccine* **22**, 2261-2272.

Lorin C, Mollet L, Delebecque F, and et al (2004). A single injection of recombinant measles virus vaccines expressing human immunodeficieny (HIV) type 1 clade B envelope glycoproteins induces neutralizing antibodies and cellular immune responses to HIV. *Journal of Virology* **78**, 146-157.

Lotte A, Wasz-Hockert O, Poisson N, Dumistrescu N, Verron M, and Couvet E (1984). BCG complications: Estimates of the risks among vaccinated subjects and statistical analysis of their main characteristics. *Advanced Tuberculosis Research* **21**, 107-193.

Lowry PW, Ludwing TS, Adams JA, Fitzpatrick ML, Grant SM, Andrle GA, Offerdahl MR, Cho SN, and Jacobs DR Jr (1998). Cellular immune responses to four doses of percutaneous bacille Calmette-Guerin in healthy adults. *Journal of Infectious Diseases* **178**, 138-146.

Lu S, Santoro JC, Fuller DH, Haynes JR, and Robinson HL (1995). Use of DNAs expressing HIV-1 env and non-infectious HIV-1 particles to raise antibody responses in mice. *Virology* **209**, 147-154.

Lu Y, Pauza CD, Montefiori DC, and Miller CJ (1998). Rhesus macaques that become systemically infected with pathogenic SHIV 89.6-PD after intravenous, rectal, or vaginal inoculation and fail to make an antiviral antibody response rapidly develop AIDS. *Journal of Acquired Immune Deficiency Syndromes and Human Retrovirology* **19**, 6-18.

University of Cape Town

Lubaki NM, Shepherd ME, Brookmeyer RS, Hon H, Quinn TC, Kashamuka M, Johnson M, Gottle R, Devers J, Lederman HM, and Bollinger RC (1999). HIV-1-specific cytolytic T-lymphocyte activity correlates with lower viral load, higher CD4 count, and CD8+CD38-DR-phenotype: comparison of statistical methods for measurement. *Journal of Acquired Immune Deficiency Syndromes* **22**, 19-30.

Lubeck MD, Natuk R, Myagkikh M, Kalyan N, Aldrich K, Sinangil F, Alipanah S, Murthy SC, Chanda PK, Nigida SM Jr, Markham PD, Zolla-Pazner S, Steimer K, Wade M, Reitz MS Jr, Arthur LO, Mizutani S, Davis A, Hung PP, Gallo RC, Eichberg J, and Robert-Guroff M (1997). Long-term protection of chimpanzees against high-dose HIV-1 challenge induced by immunization. *Nature Medicine* **3**, 651-658.

Luciw PA, Pratt-Lowe E, Shaw KES, Levy JA, and Cheng-Mayer C (1995). Persistent infection of rhesus macaques with T-cell-line-tropic and macrophage-tropic clones of simian/human immunodeficiency viruses (SHIV). *Proceedings of the National Academy of Sciences of the USA* **92**, 7490-7494.

Luciw PA, Shaw KE, Shacklett BL, and Marthas ML (1998). Importance of the intracytoplasmic domain of the simian immunodeficiency virus (SIV) envelope glycoprotein for pathogenesis. *Virology* **252**, 9-16.

Luciw PA, Mandell CP, Himathongkham S, Li J, Low TA, Schmidt KA, Shaw KES, and Cheng-Mayer C (1999). Fatal immunopathogenesis by SIV/HIV-1 (SHIV) containing a variant form of the HIV-1_{SF33} *env* gene in juvenile and newborn rhesus macaques. *Virology* **263**, 112-127.

Luo L, Li Y, Cannon PM, Kim S, and Kang CY (1992). Chimeric gag-V3 virus-like particles of human immunodeficiency virus induce virus-neutralizing antibodies. *Proceedings of the National Academy of Sciences of the USA* **89**, 10527-10531.

Luo L, Li Y, and Yong Kang C (2003). Budding and secretion of HIV Gag-Env virus-like particles from recombinant human adenovirus infected cells. *Virus Research* **92**, 75-82.

MacGregor RR, Boyer JD, Ugen KE, Lacy KE, Gluckman SJ, Bagarazzi ML, Chattergoon MA, Baine Y, Higgins TJ, Ciccarelli RB, Coney LR, Ginsberg RS, and Weiner DB (1998). First human trial of a DNA-based vaccine for treatment of human immunodeficiency virus type 1 infection: safety and host response. *Journal of Infectious Diseases* **178**, 92-100.

Maclean JM (2005). Investigation of the use of recombinant BCG, expressing the major protein (L1) of human papillomavirus type16, as a candidate vaccine for cervical cancer. *PhD Thesis, University of Cape Town.*

Maecker HT, Moon J, Bhatia S, Ghanekar SA, Maino VC, Payne JK, Kuus-Reichel K, Chang JC, Summers A, Clay TM, Morse MA, Lyerly HK, DeLaRosa C, Ankerst DP, and Disis ML (2005). Impact of cryopreservation on tetramer, cytokine flow cytometry, and ELISPOT. *BioMed Central Immunology* **6**, available from: http://www.biomedcentral.com/1471-2172/6/17-[accessed: 4 May 2006].

Makhubela S, Binder A, Shephard E, Burgers W, van Harmelen J, Williamson C, Monroe J, Greenhalgh T, and Williamson A-L (2005). A rMVA expressing five HIV-1 subtype C genes is immunogenic in mice and boosts the response to a matched DNA vaccine. *AIDS VACCINES 2005 CONFERENCE* **Abstract 173PA**, 6-9 September 2005, Montreal, Quebec, Canada.

Malkevitch N, Rohne D, Pinczewski J, Aldrich K, Kalyanaraman VS, Letvin NL, and Robert-Guroff M (2004). Evaluation of combination DNA/replication-competent Ad-SIV recombinant immunization regimens in rhesus macaques. *AIDS Research and Human Retroviruses* **20**, 235-244.

Mandell CP, Reyes RA, Cho K, Sawai ET, Fang AL, Schmidt KA, and Luciw PA (1999). SIV/HIV Nef recombinant virus (SHIVnef) produces simian AIDS in rhesus macaques. *Virology* **265**, 235-251.

Mansfield KG, Lerche NW, Gardner MB, and Lackner AA (1995). Origins of simian immunodeficiency virus infection in macaques at the New England Regional Primate Research Center. *Journal of Medical Primatology* **24**, 116-122.

Marthas ML, Sutjipto S, Higgins J, Lohman B, Torten J, Luciw PA, Marx PA, and Pedersen NC (1990). Immunization with a live, attenuated simian immunodeficiency virus (SIV) prevents early disease but not infection in rhesus macaques challenged with pathogenic SIV. *Journal of Virology* **64**, 3694-3700.

Martin LN (1982). Chromatographic fractionation of rhesus monkey (*Macaca mulatta*) IgG subclasses using DEAE cellulose and protein A-sepharose. *Journal of Immunological Methods* **50**, 319-329.

Mascola JR, Stiegler G, VanCott TC, Katinger H, Carpenter CB, Hanson CE, Beary H, Hayes D, Frankel SS, Birx DL, and Lewis MG (2000). Protection of macaques against vaginal transmission of a pathogenic HIV-1/SIV chimeric virus by passive infusion of neutralizing antibodies. *Nature Medicine* **6**, 207-210.

Mascola JR (2003). Defining the protective antibody response for HIV-1. *Current Molecular Medicine* **3**, 209-216.

Mascola JR, Sambor A, Beaudry K, Santra S, Welcher B, Louder MK, VanCott TC, Huang Y, Chakrabarti BK, Kong WP, Yang ZY, Xu L, Montefiori DC, Nabel GJ, and Letvin NL (2005). Neutralizing antibodies elicited by immunization of monkeys with DNA plasmids and recombinant adenoviral vectors expressing human immunodeficiency virus type 1 proteins. *Journal of Virology* **79**, 771-779.

Masemola A, Mashishi T, Khoury G, Bredell H, Paximadis M, Mathebula T, Barkhan D, Puren A, Vardas E, Colvin M, Zijenah L, Katzenstein D, Musonda R, Allen S, Kumwenda N, Taha T, Gray G, McIntyre J, Karim SA, Sheppard HW, Gray CM, and HIVNET 028 Study Team (2004a). Novel and promiscuous CTL epitopes in conserved regions of GAg by individuals with early subtype C HIV type 1 infection from Southern Africa. *Journal of Immunology* **173**, 4607-4617.

Masemola A, Mashishi T, Khoury G, Mohube P, Mokgotho P, Vardas E, Colvin M, Zijenah L, Katzenstein D, Musonda R, Allen S, Kumwenda N, Taha T, Gray G, McIntyre J, Karim SA, Sheppard HW, Gray CM, and HIVNET 028 Study Team. (2004b). Hierarchial targeting of subtype C human immunodeficiency virus type 1 proteins by CD8+ T cells: correlation with viral load. *Journal of Virology* **78**, 3233-3243.

Mashishi T and Gray CM (2002). The ELISPOT assay: an easily transferable method for measuring cellular responses and identifying T cell epitopes. *Clinical and Chemical Laboratory Medicine* **40**, 903-910.

Matano T, Kano M, Nakamura H, Takeda A, and Nagai Y (2001). Rapid appearance of secondary immune responses and protection from acute CD4 depletion after a highly pathogenic immunodeficiency virus challenge in macaques vaccinated with a DNA prime/Sendai virus vector boost regimen. *Journal of Virology* **75**, 11891-11896.

Matsuo K, Yamaguchi R, Yamazaki A, Tasaka H, Terasaka K, Totsuka M, Kobayashi K, Yukitake H, and Yamada T (1990). Establishment of a foreign antigen secretion system in Mycobacteria. *Infection and Immunity* **58**, 4049-4054.

McCutchan FE (2000). Understanding the genetic diversity of HIV-1. *AIDS* **14 (Suppl 3)**, S31-S44.

McCutchan FE (2006). Global epidemiology of HIV. *Journal of Medical Virology* **78**, S7-S12.

McGettigan JP, Koser ML, McKenna PM, Smith ME, Marvin JM, Eisenlohr LC, Dietzschold B, and Schnell MJ (2006). Enhanced humoral HIV-1-specific immune responses generated from recombinant rhabdoviral-based vaccine vectors co-expressing HIV-1 proteins and IL-2. *Virology* **344**, 363-377.

McKenna PM, Aye PP, Dietzchold B, Montefiori DC, Martin LN, Marx PA, Pomerantz RJ, Lackner A, and Schnell MJ (2004). Immunogenicity study of glycoprotein-deficient rabies virus expressing simian/human immunodeficiency virus $SHIV_{89.6P}$ envelope in a rhesus macaque. *Journal of Virology* **78**, 13455-13459.

McMichael A J and Hanke T (2003). HIV vaccines 1983-2003. *Nature Medicine* **9**, 874-880.

McMurray DN (2000). A nonhuman primate model for preclinical testing of new tuberculosis vaccines. *Clinical Infectious Diseases* **30(Suppl 3)**, S210-S212.

McNeil JG, Johnston MI, Birx DL, and Tramont EC (2004). Policy rebuttal. HIV vaccine trial justified. *Science* **303**, 961.

McShane, H., Pathan, A. A., Sander, C. R., Keating, S. M., Gilbert, S. C., Huygen, K., Fletcher, H. A., and Hill, A. V. (2004). Recombinant modified vaccinia virus Ankara expressing antigen 85A boosts BCG-primed and naturally acquired antimycobacterial immunity in humans. *Nature Medicine* **10**, 1240-1244.

Melief CJ (2003). Mini-review:regulation of cytotoxic T lymphocyte responses by dendritic cells: peaceful coexistence of cross-priming and direct priming? *European Journal of Immunology* **33**, 2645-2654.

Menzies R and Vissandjee B (1992). Effect of bacille Calmette-Guerin vaccination on tuberculin reactivity. *American Review of Respiratory Diseases* **145**, 621-625.

Mergener K, Facke M, Welker R, Brinkmann, V, Gelderblom HR, and Krausslich HG (1992). Analysis of HIV particle formation using transient expression of subviral constructs in mammalian cells. *Virology* **186**, 25-39.

Méderlé I, Bourguin I, Ensergueix D, Badell E, Moniz-Peireira J, Gicquel B, and Winter N (2002). Plasmidic versus insertional cloning of heterologous genes in *Mycobacterium bovis* BCG: impact on in vivo antigen persistence and immune responses. *Infection and Immunity* **70**, 314.

Méderlé I, le Grand R, Vaslin B, Badell E, Vingert B, Dormont D, Gicquel B, and Winter N (2003). Mucosal adiministration of three recombinant *Mycobacterium bovis* BCG-SIVmac251 strains to cynomolgus macaques induces rectal IgAs and boosts systemic cellular immune responses that are primed by intradermal vaccination. *Vaccine* **21**, 4153-4166.

Michelini Z, Negri DRM, Baroncelli S, Catone S, Comini A, Maggiorella MT, Sernicola L, Crostarosa F, Belli R, Mancini MG, Farcomeni S, Fagrouch Z, Ciccozzi M, Rovetto C, Liljestrom P, Norley S, Heeney J, and Titti F (2004). T-cell-mediated protective efficacy of a systemic vaccine approach in cynomolgus monkeys after SIV mucosal challenge. *Journal of Medical Primatology* **33**, 251-261.

Migueles SA, Sabbaghian MS, Shupert WL, Bettinotti MP, Marincola FM, Martino L, Hallahan CW, Selig SM, Schwartz D, Sullivan J, and Connors M (2000). HLA B*5701 is highly associated with restriction of virus replication in a subgroup of HIV-infected long-term non-progressors. *Proceedings of the National Academy of Sciences of the USA* **97**, 2709-2714.

Mohan VP, Scanga CA, Yu K, Scott HM, Tanaka KE, Tsang E, Flynn JL, and Chan J (2001). Effects of tumour necrosis factor alpha on host immune response in chronic persistent tuberculosis: possible role of limiting pathology. *Infection and Immunity* **69**, 1847-1855.

Montefiori DC, Safrit JT, Lydy SL, Barry AP, Bilska M, Vo HT, Klein M, Tartaglia J, Robinson, HL, and Rovinski B (2001). Induction of neutralizing antibodies and gag-specific cellular immune responses to an R5 primary isolate of human immunodeficiency virus type 1 in rhesus macaques. *Journal of Virology* **75**, 5879-5890.

Mooij P, Nieuwenhuis IG, Knoop CJ, Doms RW, Bogers WMJM, ten Haaft PJF, Niphuis H, Koornstra W, Bieler K, Kostler J, Morein B, Cafaro A, Ensoli B, Wagner R, and Heeney JL (2004). Qualitative T-Helper responses to multiple viral antigens correlate with vaccine-induced immunity to simian/human immunodeficiency virus infection. *Journal of Virology* **78**, 3333-3342.

Mori K, Yasutomi Y, Ohgimoto S, Nakasone T, Takamura S, Shioda T, and Nagai Y (2001). Quintuple deglycosylation mutant of simian immunodeficiency virus SIVmac239 in rhesus macaques: robust primary replication, tightly contained chronic infection, and elicitation of potent immunity against the parental wild-type strain. *Journal of Virology* **75**, 4023-4028.

Moron VG, Rueda P, Casal I, and Leclerc C (2002). CD8-alpha-CD11b+ dendritic cells present exogenous virus-like particles to CD8+ T cells and subsequently express CD8-alpha and CD205 molecules. *Journal of Experimental Medicine* **195**, 1233-1245.

Moron VG, Rueda P, Sedlik C, and Leclerc C (2003). *In vivo,* dendritic cells can cross-present virus-like particles using an endosome-to-cytosol pathway. *Journal of Immunology* **171**, 2242-2250.

Mosmann TR, Cherwinski H, Bond MW, Giedlin MA, and Coffman RL (1986). Two types of murine helper T cell clone 1. Definition according to profiles of lymphokine activities and secreted proteins. *Journal of Immunology* **136**, 2348-2357.

Mossman SP, Pierce CC, Robertson MN, Watson AJ, Montefiori DC, Rabin M, Kuller L, Thompson J, Lynch JB, Morton WR, Benveniste RE, Munn R, Hu SL, Greenberg P, and Haigwood NL (1999). Immunisation against SIVmne in macaques using multigenic DNA vaccines. *Journal of Medical Primatology* **28**, 206-213.

Mossman SP, Pierce CC, Robertson MN, Montefiori DC, Kuller L, Richardson BA, Bradshaw JD, Munn RJ, Hu SL, Greenberg PD, Benveniste RE, and Haigwood NL (2004). Protective immunity to SIV challenge elicited by vaccination of macaques with multigenic DNA vaccines producing virus-like particles. *AIDS Research and Human Retroviruses.* **20**, 425-434.

Murphey-Corb M, Martin JN, Davidson-Fairburn B, Montroni M, Miller M, West M, Ohkawa S, Baskin GB, Zhang JY, Putney SD, and et al (1989). A formalin-inactivated whole SIV vaccine confers protection in macaques. *Science* **246**, 1293-1297.

Murphy CG, Lucas WT, Means RE, Czajak S, Hale CL, Lifson JD, Kaur A, Johnson RP, Knipe DM, and Desrosiers RC (2000). Vaccine protection against simian immunodeficiency virus by recombinant strains of herpes simplex virus. *Journal of Virology* **74**, 7745-7754.

Murthy KK, Salas MT, Carey KD, and Patterson JL (2006). Baboon as a nonhuman primate model for vaccine studies. *Vaccine* **24**, 4622-4624.

Muthumani K, Bagarazzi M, Conway D, Hwang DS, Manson K, Ciccarelli R, Israel Z, Montefiori DC, Ugen K, Miller N, Kim J, Boyer J, and Weiner DB (2003). A Gag-Pol/Env-Rev SIV239 DNA vaccine improves CD4 counts, and reduce viral loads after pathogenic intrarectal SIV(mac)251 challenge in rhesus Macaques. *Vaccine* **21**, 629-637.

Mwau M, McMichael A J, and Hanke T (2002). Design and validation of an enzyme-linked immunospot assay for use in clinical trials of candidate HIV vaccines. *AIDS Research and Human Retroviruses.* **18**, 611-618.

Myagkikh M, Alipanah S, Markham PD, Tartaglia J, Paoletti E, Gallo RC, Franchini G, and Robert-Guroff M (1996). Multiple immunizations with attenuated poxvirus HIV type 2 recombinants and subunit boosts required for protection of rhesus macaques. *AIDS Research and Human Retroviruses.* **12**, 985-992.

Myers P, Espinosa R, Parr CS, Jones T, Hammond GS, and Dewey TA (2006). Order Primates. *The Animal Diversity Web at http://animaldiversity.org* [cited by: http://primates.com/primate/index.html; accessed: May 04, 2006].

Nabel GJ (2002). HIV vaccine strategies. *Vaccine* **20**, 1945-1947.

Nath BM, Schumann KE, and Boyer JD (2000). The chimpanzee and other non-human-primate models in HIV-1 vaccine research. *Trends in Microbiology* **8**, 426-431.

Ndung'u T, Lu Y, Renjifo B, Touzjian N, Kushner N, Pena-Cruz V, Novitsky VA, Lee T-H, and Essex M (2001). Infectious simian/human immunodeficiency virus with human immunodeficiency virus type 1 subtype C from an African isolate: rhesus macaque model. *Journal of Virology* **75**, 11417-11425.

Negri DR, Baroncelli S, Michelini Z, Macchia I, Belli R, Catone S, Incitti F, ten Haaft P, Corrias F, Cranage MP, Polyanskaya N, Norley S, Heeney J, Verani P, and Titti F (2001). Effect of vaccination with recombinant modified vaccinia virus Ankara expressing structural and regulatory genes of SIV(macJ5) on the kinetics of SIV replication in cynomolgus monkeys. *Journal of Medical Primatology* **30**, 197-206.

Negri DRM, Baroncelli S, Catone S, Comini A, Michelini Z, Maggiorella MT, Sernicola L, Crostarosa F, Belli R, Mancini MG, Farcomeni S, Fagrouch Z, Ciccozzi M, Boros S, Liljestrom P, Norley S, Heeney J, and Titti F (2004). Protective efficacy of a multicomponent vector vaccine in cynomolgus monkeys after intrarectal simian immunodeficiency virus challenge. *Journal of General Virology* **85**, 1191-1201.

Newman TK, Jolly CJ, and Rogers J (2004). Mitochondrial phylogeny and systematics of baboons (Papio). *American Journal of Physical Anthropology* **124**, 17-27.

NIAID (National Institute of Allergy and Infectious Diseases) (2005). Clinical research on HIV vaccines. *http://www.niaid.nih.gov/factsheets/clinrsch.htm* [accessed: 19 March 2006].

Nilsson C, Makitalo B, Berglund P, Bex F, Liljestrom P, Sutter G, Erfle V, ten Haaft P, Heeney J, Biberfeld G, and Thorstensson R (2001). Enhanced simian immunodeficiency virus-specific immune responses in macaques induced by priming with recombinant Semliki Forest virus and boosting with modified vaccinia virus Ankara. *Vaccine* **19**, 3526-3536.

Nilsson C, Sutter G, Walther-Jallow L, ten Haaft P, Akerblom L, Heeney J, Erfle V, Bottiger P, Biberfeld G, and Thorstensson R (2002). Immunization with recombinant modified vaccinia virus Ankara can modify mucosal simian immunodeficiency virus infection and delay disease progression in macaques. *Journal of General Virology* **83**, 807-818.

Nishimura Y, Igarashi T, Haigwood NL, Sadjadpour R, Donau OK, Buckler C, Plishka RJ, Buckler-White A, and Martin MA (2003). Transfer of neutralizing IgG to macaques 6 h but not 24 h after SHIV infection confers sterilizing protection: implications for HIV-1 vaccine development. *Proceedings of the National Academy of Sciences of the USA* **100**, 15131-15136.

Nishimura Y, Igarashi T, Donau OK, Buckler-White A, Buckler C, Lafont BA, Goeken RM, Goldstein S, Hirsch VM, and Martin MA (2004). Highly pathogenic SHIVs and SIVs target different CD4+ T cell subsets in rhesus monkeys, explaining their divergent clinical courses. *Proceedings of the National Academy of Sciences of the USA* **101**, 12324-12329.

Nishimura Y, Brown CR, Mattapallil JJ, Igarashi T, Buckler-White A, Lafont BA, Hirsch VM, Roederer M, and Martin MA (2005). Resting naive CD4+ T cells are massively infected and eliminated by X4-tropic simian-human immunodeficiency viruses in macaques. *Proceedings of the National Academy of Sciences of the USA* **102**, 8000-8005.

Nixon DF, Donahoe SM, Kakimoto WM, Samuel RV, Metzner KJ, Gettie A, Marx PA, and Connor RI (2000). Simian immunodeficiency virus-specific cytotoxic T lymphocytes and protection against challenge in rhesus macaques immunized with a live attenuated simian immunodeficiency virus vaccine. *Virology* **266**, 203-210.

Notka F, Stahl-Hennig C, Dittmer U, Wolf H, and Wagner R (1999). Accelerated clearance of SHIV in rhesus monkeys by virus-like particle vaccines is dependent on induction of neutralizing antibodies. *Vaccine* **18**, 291-301.

Novembre FJ, Saucier M, Anderson DC, Klumpp SA, O'Neil SP, Brown CR 2nd, Hart CE, Guenthner PC, Swenson RB, and McClure HM (1997). Development of AIDS in a chimpanzee infected with human immunodeficiency virus type 1. *Journal of Virology* **71**, 4086-4091.

Novembre FJ, de Rosayro J, Nidtha S, O'Neil SP, Gibson TR, Evans-Strickfaden T, Hart CE, and McClure HM (2001). Rapid CD4(+) T-cell loss induced by human immunodeficiency virus type 1(NC) in uninfected and previously infected chimpanzees. *Journal of Virology* **75**, 1533-1539.

Nwanegbo E, Vardas E, Gao W, Whittle H, Sun H, Rowe D, Robbins PD, and Gambotto A (2004). Prevalence of neutralizing antibodies to adenoviral serotypes 5 and 35 in the adult populations of The Gambia, South Africa, and the United States. *Clinical and Diagnostic Laboratory Immunology* **11**, 351-357.

O'Neill E, Martinez I, Villinger F, Rivera M, Gascot S, Colon C, Arana T, Sidhu M, Stout R, Montefiori DC, Martinez M, Ansari AA, Israel ZR, and Kaiselburd E (2002). Protection by SIV VLP DNA prime/protein boost following mucosal SIV challenge is markedly enhanced by IL-12/GM-CSF co-administration. *Journal of Medical Primatology* **31**, 217-227.

Oettinger T, Jorgensen M, Ladefoged A, Haslov K, and Andersen P. (1999). Development of the Mycobacterium bovis BCG vaccine: review of the historical and biochemical evidence for a genealogical tree. *Tubercle and Lung Disease.* **79(4)**, 243-250.

Ogg GS, Jin X, Bonhoeffer S, Dunbar PR, Nowak MA, Monard S, Segal JP, Cao Y, Rowland-Jones SL, Cerundolo V, Hurley A, Markowitz M, Ho DD, Nixon DF, and McMichael A J (1998). Quantitation of HIV-1-specific cytotoxic T lymphocytes and plasma load of viral RNA. *Science* **279**, 2103-2106.

Ono A, Waheed AA, Joshi A, and Freed EO (2005). Association of human immunodeficiency virus type 1 gag with membrane does not require highly basic sequences in the nucleocapsid: use of a novel Gag multimerization assay. *Journal of Virology* **79**, 14131-14140.

Orme IM, Roberts AD, Griffin JP, and Abrams JS (1993). Cytokine secretion by CD4 T lymphocytes acquired in response to mycobacterium tuberculosis infection. *Journal of Immunology* **151**, 518-525.

Osterrieder N, Wagner R, Brandmuller C, Schmidt P, Wolf H, and Kaaden OR (1995). Protection against EHV-1 challenge infection in the murine model after vaccination with various formulations of recombinant glycoprotein gp14(gB). *Virology* **208**, 500-510.

Ottenhof TH, Kumararantne D, and Casanova JL (1998). Novel human immunodeficiencies reveal the essential role of type-1 cytokines in immunity to intracellular bacteria. *Immunology Today* **19**, 491-494.

Ourmanov I, Brown CR, Moss B, Carroll M, Wyatt L, Pletneva L, Goldstein S, Venzon D, and Hirsch VM (2000). Comparative efficacy of recombinant modified vaccinia virus Ankara expressing simian immunodeficiency virus (SIV) Gag-Pol and/or Env in macaques challenged with pathogenic SIV. *Journal of Virology* **74**, 2740-2751.

Pal R, Reitz Jr MS, Tschachler E, Gallo RC, Sarngadharan MG, and Veronese FD (1990). Myristoylation of gag proteins of HIV-1 plays an important role in virus assembly. *AIDS Research and Human Retroviruses.* **16**, 273-282.

Pal R, Venzon D, Letvin NL, Santra S, Montefiori DC, Miller NR, Tryniszewska E, Lewis MG, VanCott TC, Hirsch V, Woodward R, Gibson A, Grace M, Dobratz E, Markham PD, Hel Z, Nacsa J, Klein M, Tartaglia J, and Franchini G (2002). ALVAC-SIV-gag-pol-env-based vaccination and macaque major histocompatibility complex class I (A*01) delay simian immunodeficiency virus SIVmac-induced immunodeficiency. *Journal of Virology* **76**, 292-302.

Pal R, Wang S, Kalyanaraman VS, Nair BC, Whitney S, Keen T, Hocker L, Hudacik L, Rose N, Mboudjeka I, Shen S, Wu-Chou TH, Montefiori D, Mascola J, Markham P, and Lu S (2006a). Immunisation of rhesus macaques with a polyvalent DNA prime/protein boost human immunodeficiency virus type 1 vaccine elicits protective antibody response against simian human immunodeficiency virus of R5 phenotype. *Virology* **IN PRESS**.

Pal R, Venzon D, Santra S, Kalyanaraman VS, Montefiori DC, Hocker L, Hudacik L, Rose N, Nacsa J, Edghill-Smith Y, Moniuszko M, Hel Z, Belyakov IM, Berzofsky JA, Parks RW, Markham PD, Letvin NL, Tartaglia J, and Franchini G (2006b). Systemic immunization with an ALVAC-HIV-1/protein boost vaccine strategy protects rhesus macaques from CD4+ T-cell loss and reduces both systemic and mucosal simian-human immunodeficiency virus SHIVKU2 RNA levels. *Journal of Virology* **80**, 3732-3742.

Paliard X, Liu Y, Wagner R, Wolf H, Baenziger J, and Walker CM (2000). Priming of strong, broad, and long-lived HIV type 1 p55gag-specific CD8+ cytotoxic T cells after administration of a virus-like particle vaccine in rhesus macaques. *AIDS Research and Human Retroviruses.* **16**, 273-82.

Palmer CE and Long MW (1966). Effects of infection with atypical mycobacteria on BCG vaccination and tuberculosis. *American Review of Respiratory Diseases* **94**, 553-568.

Pantaleo G and Koup RA (2004). Correlates of immune protection in HIV-1 infection: what we know, what we don't know, what we should know. *Nature Medicine* **10**, 806-810.

Parren PW, Marx PA, Hessell AJ, Luckay A, Harouse J, Cheng-Mayer C, Moore JP, and Burton DR (2001). Antibody protects macaques against vaginal challenge with a pathogenic R5 simian/human immunodeficiency virus at serum levels giving complete neutralization *in vitro*. *Journal of Virology* **75(17):8340-7**, 8340-8347.

Pehler K, Brasky KM, Butler TM, and Attanasio R. (2000). *Mycobacterium tuberculosis*-secreted protein antigens: immunogenicity in baboons. *Journal of Clinical Immunology* **20**, 306-316.

Pinto LA, Sullivan J, Berzofsky JA, Clerici M, Kessler HA, Landay AL, and Shearer GM (1995). ENV-specific cytotoxic T lymphocyte responses in HIV seronegative health care workers occupationally exposed to HIV-contaminated body fluids. *Journal of Clinical Investigation* **96**, 867-876.

Pitisuttithum P. (2005). HIV-1 prophylactic vaccine trials in Thailand. *Current HIV Research* **3**, 17-30.

Poccia F, Malkovsky M, Pollak A, Colizzi V, Sireci G, Salerno A, and Dieli F. (1999). In vivo gamadelta T cell priming to mycobacterial antigens by primary Mycobacterium tuberculosis infection and exposure to nonpeptiditic ligands. *Molecular Medicine* **5**, 471-476.

Poon B, Hsu JF, Gudeman V, Chen IS, and Grovit-Ferbas K (2005a). Formaldehyde-treated, heat-inactivated virions with increased human immunodeficiency virus type 1 env can be used to induce high-titer neutralizing antibody responses. *Journal of Virology* **79**, 10210-10217.

Poon B, Safrit JT, McClure H, Kitchen C, Hsu JF, Gudeman V, Petropoulos C, Wrin T, Chen IS, and Grovit-Ferbas K (2005b). Induction of humoral immune responses following vaccination with envelope-containing, formaldehyde-treated, thermally inactivated human immunodeficiency virus type 1. *Journal of Virology* **79**, 4927-4935.

Porcelli S, Morita CT, and Brenner MB. (1992). CD1b restricts the response of human CD4-8-T lymphocytes to a microbial antigen. *Nature* **360**, 593-597.

Puaux AL, Delache B, Marconi S, Huerre M, le Grand R, Riviere Y, and Michel ML (2005). Loss of reactivity of vaccine-induced CD4 T cells in immunised monkeys after SIV/HIV challenge. *AIDS* **19**, 757-765.

Putkonen P, Bottiger B, Warstedt K, Thorstensson R, Albert J, and Biberfeld G (1989). Experimental infection of cynomolgus monkeys (Macaca fascicularis) with HIV-2. *Journal of Acquired Immune Deficiency Syndromes* **2**, 366-373.

Putkonen P, Thorstensson R, Ghavamzadeh L, Albert J, Hild K, Biberfeld G, and Norrby E (1991). Prevention of HIV-2 and SIVsm infection by passive immunization in cynomolgus monkeys. *Nature* **352**, 436-438.

Radaelli A, Zanotto C, Perletti G, Elli V, Vicenzi E, Poli G, and de Giuli C (2003). Comparative analysis of immune responses and cytokine profiles elicited in rabbits by the combined use of recombinant fowlpox viruses, plasmids and virus-like particles in prime-boost vaccination protocols against SHIV. *Vaccine* **21**, 2052-2064.

Ramsburg E, Rose NF, Marx PA, Mefford M, Nixon DF, Moretto WJ, Montefiori D, Earl P, Moss B, and Rose JK (2004). Highly effective control of an AIDS virus challenge in macaques by using vesicular stomatitis virus and modified vaccinia virus Ankara vaccine vectors in a single-boost protocol. *Journal of Virology* **78**, 3930-3940.

Ranjbar S, Jones S, Stott EJ, and Almond N (1997). The construction and evaluation of SIV/HIV chimeras that express the envelope of European HIV type 1 isolates. *AIDS Research and Human Retroviruses.* **13**, 797-800.

Rasmussen RA, Hofmann-Lehmann R, Montefiori DC, Li PL, Liska V, Vlasak J, Baba TW, Schmitz JE, Kuroda MJ, Robinson HL, McClure HM, Lu S, Hu SL, Rizvi TA, and Ruprecht RM (2002). DNA prime/boost vaccine strategy in neonatal macaques against simian human immunodeficiency virus. *Journal of Medical Primatology* **31**, 40-60.

Raviprakash K, Ewing D, Simmons M, Porter KR, Jones TR, Hayes CG, Stout R, and Murphy GS (2003). Needle-free Biojector injection of a dengue virus type 1 DNA vaccine with human immunostimulatory sequences and the GM-CSF gene increases immunogenicity and protection from virus challenge in Aotus monkeys. *Virology* **315**, 345-352.

Ravn P, Boesen H, Pedersen BK, and Andersen P. (1997). Human T cell responses induced by vaccination with *Mycobacterium bovis* bacille Calmette-Guerin. *Journal of Immunology* **158**, 1949-1955.

Reimann J and Schirmbeck R (1999). Alternative pathways for processing exogenous and endogenous antigens that can generate peptides for MHC class I-restricted presentation. *Immunological Reviews* **172**, 131-152.

Reimann KA, Li TJ, Veazey R, Halloran M, Park IW, Karlsson GB, Sodroski J, and Letvin NL (1996a). A chimeric simian/human immunodeficiency virus expressing a primary patient human immunodeficiency virus type 1 isolate env causes an AIDS-like disease after in vivo passage in rhesus monkeys. *Journal of Virology* **70**, 6922-6928.

Reimann KA, Li JT, Voss G, Lekutis C, Tenner-Racz K, Racz P, Lin W, Montefiori DC, Lee-Parritz DE, Lu Y, Collman RG, Sodroski J, and Letvin NL (1996b). An env gene derived from a primary human immunodeficiency virus type 1 isolate confers high in vivo replicative capacity to a chimeric simian/human immunodeficiency virus in rhesus monkeys. *Journal of Virology* **70**, 3198-3206.

Ribi E, Anacker RL, Barclay WR, Brehmer W, Harris SC, Leif WR, and Simmons J (1971). Efficacy of mycobacterial cell walls as a vaccine against airborne tuberculosis in the Rheusus monkey. *Journal of Infectious Diseases* **123**, 527-538.

Robertson DL, Anderson JP, Bradac JA, Carr JK, Foley B, Funkhouser RK, Gao F, Hahn BH, Kalish ML, Kuiken C, Learn GH, Leitner T, McCutchan F, Osmanov S, Peeters M, Pieniazek D, Salminen M, Sharp PM, Wolinsky S, and Korber B (2000). HIV-1 nomenclature proposal. *Science* **288**, 55-57.

Robinson HL, Montefiori DC, Johnson RP, Manson KH, Kalish ML, Lifson JD, Rizvi TA, Lu SL, Mazzara GP, Panicali DL, Herndon JC, Glickman R, Candido MA, Lydy SL, Wyand MS, and McClure HM (1999). Neutralizing antibody-independent containment of immunodeficiency virus challenges by DNA priming and recombinant pox virus booster immunizations. *Nature Medicine* **5**, 526-534.

Robinson HL and Amara R R (2005). T cell vaccines for microbial infections. *Nature Medicine* **11**, S25-S32.

Rogers J and Hixson JE. (1997). Baboons as an animal model for genetic studies of common human diseases. *American Journal of Human Genetics* **61**, 489-493.

Rolph MS, Raupach B, Kobernick HH, Collins HL, Perarnau B, Lemonnier FA, and Kaufmann SH (2001). MHC class 1a-restricted T cells partially account for beta2-microglobulin-dependent resistance to *Mycobacterium tuberculosis*. *European Journal of Immunology* **31**, 1944-1949.

Romagnani S (1992). Induction of TH1 and TH2 responses: a key role for the 'natural' immune response? *Immunology Today* **13**, 379-381.

Rose NF, Marx PA, Luckay A, Nixon DF, Morreto WJ, Donahoe SM, Montefiori D, Roberts A, Buonocore L, and Rose KL (2001). An effective AIDS vaccine based on live attenuated vesicular stomatitis virus recombinants. *Cell* **106**, 539-549.

Rosenberg ES, Billingsley JM, Caliendo AM, Boswell SL, Sax PE, Kalams SA, and Walker BD (1997). Vigorous HIV-1-specific CD4+ T cell responses associated with control of viremia. *Science* **278**, 1447-1450.

Rossio JL, Esser MT, Suryanarayana K, Schneider DK, Bess JW Jr, Vasquez GM, Wiltrout TA, Chertova E, Grimes MK, Sattentau Q, Arthur LO, Henderson LE, and Lifson JD (1998). Inactivation of human immunodeficiency virus type 1 infectivity with preservation of conformational and functional integrity of virion surface proteins. *Journal of Virology* **72**, 7992-8001.

Rovinski B, Haynes JR, Cao SX, James O Sia C, Zolla Pazner S, Matthews TJ, and Klein MH (1992). Expression and characterization of genetically engineered human immunodeficiency virus-like particles containing modified envelope glycoproteins: implications for development of a cross-protective AIDS vaccine. *Journal of Virology* **66**, 4003-4012.

Rowland-Jones S, Sutton J, Ariyoshi K, Dong T, Gotch F, McAdam S, Whitby D, Sabally S, Gallimore A, Corrah T, Takiguchi M, Schultz T, McMichael A, and Whittle H (1995). HIV-specific cytotoxic T cells in HIV-exposed but unifected Gambian women. *Nature Medicine* **1**, 59-64.

Rowland-Jones SL, Dong T, Fowke KR, Kimani J, Krausa P, Newell H, Blanchard T, Ariyoshi K, Oyugi J, Ngugi E, Bwayo J, MacDonald KS, McMichael A J, and Plummer FA (1998). Cytoxic T cell responses to multiple conserved HIV epitopes in HIV-resistant prostitutes in Nairobi. *Journal of Clinical Investigation* **164**, 1602-1611.

Rueda P, Martinez-Torrecuadrada JL, Sarraseca J, Sedlik C, del Barriro M, Hurtado A, Leclerc C, and Casal JI (1999). Engineering parvovirus-like particles for the induction of B-cell, CD4(+) and CTL responses. *Vaccine* **18**, 325-332.

Sadagopal S, Amara R R, Montefiori DC, Wyatt LS, Staprans SI, Kozyr NL, McClure HM, Moss B, and Robinson HL (2005). Signature for long-term vaccine-mediated control of a simian and human immunodeficiency virus 89.6P challenge: stable low-breadth and low-frequency T-cell response capable of coproducing gamma interferon and interleukin-2. *Journal of Virology* **79**, 3243-3253.

Sakate R, Osada N, Hida M, Sugano S, Hayasaka I, Shimohira N, Yanagi S, Suto Y, Hashimoto K, and Hirai M (2003). Analysis of 5'-end sequences of chimpanzee cDNAs. *Genome Research* **13**, 1022-1026.

Sakuragi S, Shibata R, Mukai R, Komatsu T, Fukasawa M, Sakai H, Sakuragi J, Kawamura M, Ibuki K, and Hayami M (1992). Infection of macaque monkeys with a chimeric human and simian immunodeficiency virus. *Journal of General Virology* **73**, 2983-2987.

Sakuragi S, Goto T, Sano K, and Morikawa Y (2002). HIV type 1 Gag virus-like particle budding from spheroplasts of Saccharomyces cerevisiae. *Proceedings of the National Academy of Sciences of the USA* **99**, 7956-7961.

Sanders RW, Vesanen M, Schuelke N, Master A, Schiffner L, Kalyanaraman R, Paluch M, Berkhout B, Maddon PJ, Olson WC, Lu M, and Moore JP (2002). Stabilization of the soluble, cleaved, trimeric form of the envelope glycoprotein complex of human immunodeficiency virus type 1. *Journal of Virology* **76**, 8875-8889.

Sano K, Handea K, Tamura G, and Shirato K (1999). Ovalbumin (OVA) and *Mycobacterium tuberculosis* bacilli cooperatively polarized anti-OVA T-helper cells towards a Th1-diminant phenotype and ameriolate murine tracheal eosinophilia. *American Journal of Respiratory Cell and Molecular Biology* **20**, 1260-1267.

Santiago ML, Rodenburg CM, Kamenya S, Bibollet-Ruche F, Gao F, Bailes E, Meleth S, Soong SJ, Kilby JM, Moldoveanu Z, Fahey B, Muller MN, Ayouba A, Nerrienet E, McClure HM, Heeney JL, Pusey AE, Collins DA, Boesch C, Wrangham RW, Goodall J, Sharp PM, Shaw GM, and Hahn BH (2002). SIVcpz in wild chimpanzees. *Science* **295**, 465.

Santra S, Barouch DH, Korioth-Schmitz B, Lord CI, Krivulka GR, Yu F, Beddall MH, Gorgone DA, Lifton MA, Miura A, Philippon V, Manson K, Markham PD, Parrish J, Kuroda MJ, Schmitz JE, Gelman RS, Shiver JW, Montefiori DC, Panicali D, and Letvin NL (2004). Recombinant poxvirus boosting of DNA-primed rhesus monkeys augments peak but not memory T lymphocyte responses. *Proceedings of the National Academy of Sciences of the USA* **101**, 11088-11093.

Santra S, Seaman MS, Xu L, Barouch DH, Lord CI, Lifton MA, Gorgone DA, Beaudry KR, Svehla K, Welcher B, Chakrabarti BK, Huang Y, Yang ZY, Mascola JR, Nabel GJ, and Letvin NL (2005). Replication-defective adenovirus serotype 5 vectors elicit durable cellular and humoral immune responses in nonhuman primates. *Journal of Virology* **79**, 6516-6522.

Sapolsky RM and Else JC (1987). Bovine tuberculosis in a wild baboon population: epidemiological aspects. *Journal of Medical Primatology* **16**, 229-235.

Schauf V, Rom WN, Smith KA, Sampaio EP, Meyn PA, Tramontana JM, Cohn ZA, and Kaplan G (1993). Cytokine gene activation and modified responsiveness to interleukin-2 in the blood of tuberculosis patients. *Journal of Infectious Diseases* **168**, 1056-1059.

Schirmbeck R, Melber K, Mertens T, and Reimann J (1994). Selective stimulation of murine cytotoxic T cell and antibody responses by particulate or monomeric hepatitis B virus surface (S) antigen. *European Journal of Immunology* **24**, 1088-1096.

Schirmbeck R, Bohm W, and Reimann J (1996). Virus-like particles induce MHC class I-restricted T-cell responses. Lessons learned from the hepatitis B small surface antigen. *Intervirology* **39**, 111-119.

Schmitz JE, Kuroda MJ, Santra S, Sasseville VG, Simon MA, Lifton MA, Racz P, Tenner-Racz K, Dalesandro M, Scallon BJ, Ghrayeb J, Forman MA, Montefiori DC, Rieber EP, Letvin NL, and Reimann KA (1999). Control of viremia in simian immunodeficiency virus infection by CD8+ lymphocytes. *Science* **283**, 857-860.

Schmitz JE, Kuroda MJ, Santra S, Simon MA, Lifton MA, Lin W, Khunkhun R, Piatak M, Lifson JD, Grosschupff G, Gelman RS, Racz P, Tenner-Racz K, Mansfield KA, Letvin NL, Montefiori DC, and Reimann KA (2003). Effect of humoral immune responses on controlling vireamia during primary infection of rhesus monkeys with simian immunodeficiency virus. *Journal of Virology* **77**, 2165-2173.

Schmitz JE, Johnson RP, McClure HM, Manson KH, Wyand MS, Kuroda MJ, Lifton MA, Khunkhun RS, McEvers KJ, Gillis J, Piatak M, Lifson JD, Grosschupff G, Racz P, Tenner-Racz K, Rieber EP, Kuus-Reichel K, Gelman RS, Letvin NL, Montefiori DC, Ruprecht RM, Desrosiers RC, and Reimann KA (2005). Effect of CD8+ lymphocyte depletion on virus containment after simian immunodeficiency virus SIVmac251 challenge of live attenuated SIVmac239delta3-vaccinated rhesus macaques. *Journal of Virology* **79**, 8131-8141.

Schwimmer B (1996). Primate taxonomy. *http://www.umanitoba.ca/anthropology/courses/121/primatology/taxonomy.html* [accessed: 4 May 2006].

Seabrook TJ, Borron PJ, Dudler L, Hay JB, and Young AJ (2005). A novel mechanism of immune regulation: interferon-g regulates retention of CD4+ T cells during delayed type hypersensitivity. *Immunology* **116**, 184-192.

Seaman MS, Peyerl FW, Jackson SS, Lifton MA, Gorgone DA, Schimtz JE, and Letvin NL (2004). Subsets of memory cytotoxic T lymphocytes elicited by vaccination influence the efficiency of secondary expansion in vivo. *Journal of Virology* **78**, 206-215.

Sedlik C, Saron M, Sarraseca J, Casal JI, and Leclerc C (1997). Recombinant parvovirus-like particles as an antigen to elicit protective antiviral cytotoxic T cells. *Proceedings of the National Academy of Sciences of the USA* **94**, 7503-7508.

Serbina NV and Flynn JL (1999). Early emergence of CD8+ T cells primed for production of type 1 cytokine in the lungs of *Mycobacterium tuberculosis*-infected mice. *Infection and Immunity* **67**, 3980-3988.

Serbina NV and Flynn JL (2001). CD8(+) T cells participate in the memory immune response to Mycobacterium tuberculosis. *Infection and Immunity* **69**, 4320-4328.

Sester M, Sester U, Kohler H, Schneider T, Deml L, Wagner R, Mueller-Lantzsch N, Pees HW, and Meyerhans A (2000). Rapid whole blood analysis of virus-specific CD4 and CD8 T cell responses in persistent HIV infections. *AIDS* **14**, 2653-2660.

Simon F, Mauclere P, Roques P, Loussert-Ajaka I, Muller-Trutwin MC, Saragosti S, Georges-Courbot MC, Barre-Sinoussi F, and Brun-Vezinet F (1998). Identification of a new human immunodeficiency virus type 1 distinct from group M and group O. *Nature Medicine* **4**, 1032-1037.

Sindhu ST, Ahmad R, Blagdon M, Ahmad A, Toma A, Morisset R, and Menezes J (2003). Virus load correlates inversely with the expression of cytotoxic T lymphocyte activation markers in HIV-1-infected/AIDS patients showing MHC-unrestricted CTL-mediated lysis. *Clinical Experimental Immunology* **132**, 120-127.

Singh DK, Liu Z, Sheffer D, Mackay GA, Smith M, Dhillon S, Hegde R, Jia F, Adany I, and Narayan O (2005). A noninfectious simian/human immunodeficiency virus DNA vaccine that protects macaques against AIDS. *Journal of Virology* **79**, 3419-3428.

Smith RJ and Blower SM (2004). Could disease-modifying HIV vaccines cause population-level perversity? *Lancet Infectious Diseases* **4**, 636-639.

Smith SM, Khoroshev M, Marx PA, Orenstein J, and Jeang KT (2001). Constitutively dead, conditionally live HIV-1 genomes. Ex vivo implications for a live virus vaccine. *Journal of Biological Chemistry* **276**, 32184-32190.

Smith SM (2002). HIV vaccines development in the nonhuman primate models of AIDS. *Journal of Biomedical Science* **9**, 100-111.

Someya K, Dayaraj C, Ami Y, Nakasone T, Matsuo K, Burda S, Yamamoto H, Yoshino N, Kaizu M, Ando S, Okuda K, Zolla-Pazner S, Yamazaki S, Yamamoto N, and Honda M (2005). Vaccination of rhesus macaques with recombinant *mycobacterium bovis* bacillus Calmette-Guérin Env V3 elicits neutralizing antibody-mediated protection against simian-human immunodeficiency virus with a homologous but not as hererologous V3 motif. *Journal of Virology* **79**, 1452-1462.

Someya K, Ami Y, Nakasone T, Izumi Y, Matsuo K, Horibata S, Xin KQ, Yamamoto H, Okuda K, Yamamoto N, and Honda M (2006). Induction of positive cellular and humoral immune responses by a prime-boost vaccine encoded with simian immunodeficiency virus gag/pol. *Journal of Immunology* **176**, 1784-1795.

Sousa AO, Mazzaccaro RJ, Russell RG, Lee FK, Turner OC, Hong S, van Kaer L, and Bloom BR (2000). Relative contributions of distinct MHC class 1-dependent cell populations in protection to tuberculosis infection in mice. *Proceedings of the National Academy of Sciences of the USA* **97**, 4204-4208.

Spearman P (2006). Current progress in the development of HIV vaccines. *Current Pharmaceutical Design* **12**, 1147-1167.

Srivastava IK, VanDorsten K, Vojtech L, Barnett SW, and Stamatatos L (2003). Changes in the immunogenic properties of soluble gp140 human immunodeficiency virus envelope constructs upon partial deletion of the second hypervariable region. *Journal of Virology* **77**, 2310-2320.

Stahl-Hennig C, Dittmer U, Nisslein T, Petry H, Jurkiewicz E, Fuchs D, Wachter H, Matz-Rensing K, Kuhn EM, Kaup FJ, Rud EW, and Hunsmann G (1996). Rapid development of vaccine protection in macaques by live-attenuated simian immunodeficiency virus. *Journal of General Virology* **77**, 2969-2981.

Stahl-Hennig C, Gundlach BR, Dittmer U, ten Haaft P, Heeney J, Zou W, Emilie D, Sopper S, and Uberla K (2003). Replication, immunogenicity, and protective properties of live-attenuated simian immunodeficiency viruses expressing interleukin-4 or interferon-gamma. *Virology* **305**, 473-485.

Tan GS, McKenna PM, Koser ML, McLinden R, Kim JH, McGettigan JP, and Schnell MJ (2005). Strong cellular and humoral anti-HIV Env immune responses induced by a heterologous rhabdoviral prime-boost approach. *Virology* **331**, 82-93.

Tarara R, Suleman MA, Sapolsky R, Wabomba MJ, and Else JG. (1985). Tuberculosis in wild olive baboons, *Papio cynocephalus anubis* (Lesson), in Kenya. *Journal of wildlife Diseases* **21**, 137-140.

ten Haaft P, Murthy K, Salas M, McClure HM, Dubbes R, Koornstra W, Niphuis H, Davis D, van der Groen G, and Heeney J (2001). Differences in early virus loads with different phenotypic variants of HIV-1 and SIV(cpz) in chimpanzees. *AIDS* **15**, 2085-2092.

Testi R, Phillips JH, and Lanier LL (1989). T cell activation via Leu-23 (CD69). *Journal of Immunology* **143**, 1123-1128.

Theon CO, Beluhan FZ, Himes EM, Capek V, and Bernett T. (1977). Mycobacterium bovis infection in baboons (*Papio Papio*). *Archives of Pathology and Laboratory Medicine* **101**, 291-293.

Thomas R (2005). Recombinant BCG expressing HIV-1 C Gag: selection of the vaccine gene and construction and evaluation as a vaccine candidate. *PhD Thesis, University of Cape Town.*

Thomson MM, Perez-Alvarez I, and Najera R (2002). Molecular epidemiology of HIV-1 genetic forms and its significance for vaccine development and therapy. *Lancet Infectious Diseases* **2**, 461-471.

Thorel MF, Karoui C, Varnerot A, Fleury C, and Vincent V. (1998). Isolation of Mycobacterium bovis from baboons, leopards, and a sea-lion. *Veterinary Research* **29**, 207-212.

Trinvuthipong C (2004). Letters. Thailand's prime-boost HIV Vaccine phase III. *Science* **303**, 954-955.

Tsukaguchi K, Balaji KN, and Boom WH (1995). CD4+ alpha beta T cell and gamma delta R cell responses to *Mycobacterium tuberculosis*. Similarities and differences in Ag recognition, cytotoxic effector function, and cytokine production. *Journal of Immunology* **154**, 1786-1796.

Tsunetsugu-Yokota, Morikawa Y, Kawana-Tachikawa A, Odawara T, Nakamura T, Grassi F, Autran B, and Iwamoto A (2003). Yeast-derived human immunodeficiency virus type 1 p55(gag) virus-like particles activate dendritic cells (DCs) and induce perforin expression in Gag-specific CD8(+) T cells by cross-presentation of DCs. *Journal of Virology* 77, 10250-10259.

Uberla K (2005). Efficacy of AIDS vaccine strategies in nonhuman primates. *Medical Microbiology and Immunology* **194**, 201-206.

Ulmer JB, Donnelly JJ, Parker SE, Rhodes GH, Felgner PL, Dwarki VJ, Gromkowski SH, Deck RR, DeWitt CM, and Friedman A (1993). Heterologous protection against influenza by injection of DNA encoding a viral protein. *Science* **259**, 1745-1749.

UNAIDS (2004). 2004 report on the global AIDS epidemic. *http://www.unaids.org/bangkok2004/report.html* [accessed: 7 May 2006].

UNAIDS (2005). AIDS epidemic update: December 2005. *http://www.unaids.org/epi/2005/doc/report_pdf.asp* [accessed: 7 May 2006].

von Gegerfelt AS, Liska V, Li PL, McClure HM, Horie K, Nappi F, Montefiori DC, Pavlakis GN, Marthas ML, Ruprecht RM, and Felber BK (2002). Rev-independent simian immunodeficiency virus strains are nonpathogenic in neonatal macaques. *Journal of Virology* **76**, 96-104.

Vzorov AN, Bukrinsky MI, Grigoriev VB, Tentsov YY, and Bukrinskaya AG (1991). Highly immunogenic human immunodeficiency virus-like particles are produced by recombinant vaccinia virus-infected cells. *AIDS Research and Human Retroviruses.* **7**, 29-36.

Wada N, Ohara N, Kameoka M, Nishino Y, Matsumoto S, Nishiyama T, Naito M, Yukitake H, Okada Y, Ikuta K, and Yamada T (1996). Long-lasting immune response induced by recombinant bacillus Calmette-Guérin (BCG) secretion system. *Scandinavian Journal of Immunology* **43**, 202-209.

Wagner R, Fliessbach H, Wanner G, Motz M, Niedrig M, Deby G, Von Brunn A, and Wolf H (1992). Studies on processing, particle formation, and immunogenicity of the HIV-gag gene product: a possible component of a HIV vaccine. *Archives of Virology* **127**, 117-137.

Wagner R, Deml L, Fliessbach H, Wanner G, and Wolf H (1994). Assembly and extracellular release of chimeric HIV-1 Pr55gag retrovirus-like particles. *Virology* **200**, 162-175.

Wagner R, Deml L, Schirmbeck R, Niedrig M, Reimann J, and Wolf H (1996a). Construction, expression and immunogenicity of chimeric HIV-1 virus-like particles. *Virology* **220**, 128-140.

Wagner R, Deml L, Notka F, Wolf H, Schirmbeck R, Reimann J, Teeuwsen V, and Heeney J (1996b). Safety and immunogenicity of recombinant human immunodeficiency virus-like particles in rodents and rhesus macaques. *Intervirology* **39**, 93-103.

Walsh GP, Tan EV, dela Cruz EC, Abalos RM, Villahermosa LG, Young LJ, Cellona RV, Nazareno JB, and Horwitz MA. (1996). The Philippine Cynomolgus Monkey (*Macaca fasicularis*) provides a new nonhuman primate model of tuberculosis that resembles human disease. *Nature Medicine* **2**, 430-436.

Wang B, Ugen KE, Srikantan V, Agadjanyan MG, Dang K, Refaeli Y, Sato AI, Boyer J, Williams WV, and Weiner DB (1993). Gene inoculation generates immune responses against human immunodeficiency virus type 1. *Proceedings of the National Academy of Sciences of the USA* **90**, 4156-4160.

Wang B, Merva M, Dang K, Ugen KE, Boyer J, Williams WV, and Weiner DB (1994). DNA innoculation induces protective in vivo immune responses against cellular challenge with HIV-1 antigen-expressing cells. *AIDS Research and Human Retroviruses.* **10 (suppl 2)**, S35-S41.

Wang SW, Kozlowski PA, Schmelz G, Manson K, Wyand MS, Glickman R, Montefiori DC, Lifson JD, Johnson RP, Neutra MR, and Aldovini A (2000). Effective induction of simian immunodeficiency virus-specific systemic and mucosal immune responses in primates by vaccination with proviral DNA producing intact but noninfectious virions. *Journal of Virology* **74**, 10514-10522.

Warren J (2002). Preclinical AIDS vaccine research: survey of SIV, SHIV and HIIV challenge studies in vaccinated nonhuman primates. *Journal of Medical Primatology* **31**, 237-256.

Wee EG, Patel S, McMichael AJ, and Hanke T (2002). A DNA/MVA-based candidate human immunodeficiency virus vaccine for Kenya induces multi-specific T cell responses in rhesus macaques. *Journal of General Virology* **83**, -75.

Whetter LE, Ojukwu IC, Novembre FJ, and Dewhurst S (1999). Pathogenesis of simian immunodeficiency virus infection. *Journal of General Virology* **80**, 1557-1568.

Xu R, Srivastava IK, Kuller L, Zarkikh I, Kraft Z, Fagrouch Z, Letvin NL, Heeney JL, Barnett SW, and Stamatatos L (2006). Immunization with HIV-1 SF162-derived Envelope gp140 proteins does not protect macaques from heterologous simian-human immunodeficiency virus SHIV89.6P infection. *Virology* **349**, 276-289.

Yamshchinok GV, Ritter GD, Vey M, and Compans RW (1995). Assembly of SIV virus-like particles containing envelope proteins using a baculovirus expression system. *Virology* **214**, 50-58.

Yang X, Lee J, Mahony EM, Kwong PD, Wyatt R, and Sodroski J (2002). Highly stable trimers formed by human immunodeficiency virus type 1 envelope glycoproteins fused with the trimeric motif of T4 bacteriophage fibritin. *Journal of Virology* **76**, 4634-4642.

Yang ZY, Wyatt LS, Kong WP, Moodie Z, Moss B, and Nabel GJ (2003). Overcoming immunity to a viral vaccine by DNA priming before vector boosting. *Journal of Virology* **77**, 799-803.

Yao Q, Zhang R, Guo L, Li M, and Chen C (2004). Th cell-independent immune responses to chimeric hemagglutinin/simian human immunodeficiency virus-like particles vaccine. *Journal of Immunology* **173**, 1951-1958.

Yasutomi Y, Koeing S, Haun SS, Stover CK, Jackson RK, Conard P, Conley AJ, Emini EA, Fuerst TR, and Letvin NL (1993). Immunization with recombinant BCG-SIV elicits SIV-specific cytotoxic Tlymphocytes in rhesus monkey. *Journal of Immunology* **150**, 3101-3107.

Yasutomi Y, Robinson HL, Lu S, Mustafa F, Lekutis C, Arthos J, Mullins JI, Voss G, Manson K, Wyand M, and Letvin NL (1996). Simian immunodeficiency virus-specific cytotoxic T-lymphocyte induction through DNA vaccination of rhesus monkeys. *Journal of Virology* **70**, 678-681.

Zhang M, Lin Y, Iyer DV, Gong J, Abrams JS, and Barnes PF (1995). T-cell cytokine responses in human infection with Mycobacterium tuberculosis. *Infection and Immunity* **63**, 3231-3234.

Zhang R, Li M, Chen C, and Yao Q (2004). SHIV virus-like particles bind and activate human dendritic cells. *Vaccine* **23**, 139-147.

Zhang Y, Lou B, Lal RB, Gettie A, Marx PA, and Moore JP (2000). Use of inhibitors to evaluate coreceptor usage by simian and simian/human immunodeficiency viruses and human immunodeficiency virus type 2 in primary cells. *Journal of Virology* **74**, 6893-6910.

Zhou X, Vink M, Klaver B, Verhoef K, Marzio G, Das AT, and Berkhout B (2006). The genetic stability of a conditional-live HIV-1 variant can be improved by mutations in the Tet-On regulatory system that restrain evolution. *Journal of Biological Chemistry* **In Press**.

Zimmerli SC, Harari A, Cellerai C, Vallelian F, and Bart P-A (2005). HIV-1-specific IFN-γ/IL-2-secreting CD8 cells support CD4-independent proliferation of HIV-1-specific CD8 T cells. *Proceedings of the National Academy of Sciences of the USA* **102**, 7239-7244.

Zinkernagel RM (2002). On cross-priming of MHC class I-specific CTL: rule or exception? *European Journal of Immunology* **32**, 2385-2392.

Zurbriggen R (2003). Immunostimulating reconstituted influenza virosomes. *Vaccine* **21**, 921-924.