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Anti-vector immune responses to an MVA vaccine

by

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A dissertation submitted in fulfilment of the requirements for the degree of MSc (Med)
in the Department of Clinical Laboratory Sciences, Division of Medical Virology,
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DECLARATION

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LIST OF ABBREVIATIONS

Ad26	Adenovirus type 26
Ad5	Adenovirus type 5
ADCC	Antibody dependent cell-mediated cytotoxicity
AIDS	Acquired immune deficiency syndrome
APC	Allophycocyanin
APC-Cy7	Allophycocyanin-Cy7
Ax700	Alexa Fluor 700
BCG	Bacille Calmette-Guérin
BFA	Brefeldin-A
CD	Cluster of differentiation
CEV	Cell-associated enveloped virus
CMV	Cytomegalovirus
CTL	Cytotoxic T lymphocyte
DMEM	Dulbecco's Modified Eagle Medium
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
EEV	Extracellular enveloped virus
ELISA	Enzyme-linked immunosorbent assay
ELISPOT	Enzyme-linked immunosorbent spot
Env	Envelope glycoprotein
FACS	Fluorescence-activated cell sorter
FBS	Foetal bovine serum
FITC	Fluorescein isothiocyanate
FSC	Forward scatter
g	gram
Gag	Group specific antigen
GFP/gfp	Green fluorescent protein
GM-CSF	Granulocyte macrophage - colony stimulating factor
gp	glycoprotein
GRTTN	Gag, RT, Tat, Nef
h	hour
HAART	Highly active antiretroviral treatment
HIV/HIV-1	Human immunodeficiency virus type 1
ICS	Intracellular cytokine staining

IEV	Intracellular enveloped virus
IFN-γ	Interferon- γ (gamma)
IL-2	Interleukin 2
IMV	Intracellular mature virion particle
LST	Leucosep separation tubes
mg	milligram
MHC	Major histocompatibility complex
min	minutes
ml	millilitre
mm	millimetre
MOI	Multiplicity of infection
MVA	Modified Vaccinia Ankara virus
Nef	Negative regulatory factor
NYVAC	Attenuated Vaccinia virus based on Copenhagen strain
pfu	plaque forming unit
PBMC	Peripheral blood mononuclear cells
PBS	Phosphate Buffered Saline
PE	Phycoerythrin
PE-Cy7	Phycoerythrin-Cy5.5
PERCP-Cy5.5	Peridininchlorophyll protein-Cy5.5
PHA	Phytohaemagglutinin
Pol	Polymerase protein
PRNT	Plaque reduction neutralisation test
PVA	Polyvinyl alcohol
PVP	Polyvinylpyrrolidone
Qdot	Quantum dot
r	recombinant
R1	RPMI 1640 medium (Gibco) containing 1% FBS (Delta Bioproducts) and 50mg/ml Penicillin-Streptomycin (Gibco)
R10	RPMI 1640 medium (Gibco) containing 10% FBS (Delta Bioproducts) and 50mg/ml Penicillin-Streptomycin (Gibco)
R20	RPMI 1640 medium (Gibco) containing 20% FBS (Delta Bioproducts) and 50mg/ml Penicillin-Streptomycin (Gibco)
Rev	Regulator of virion
RNA	Ribonucleic acid
RPMI	Roswell Park Memorial Institute cell culture medium

RT	Room temperature
SAAVI	South African AIDS Vaccine Initiative
SFU	Spot forming units
SHIV	Simian human immunodeficiency virus
SIV	Simian immunodeficiency virus
SSC	Side scatter
Tat	Transactivator of transcription
TB	Tuberculosis
T_{CM}	T-cell central memory
T_{EM}	T-cell effector memory
TNF-α	Tumor necrosis factor alpha
VIG	Reference vaccinia immune globulin
Vivid	Violet-fluorescent reactive dye
μg	microgram
μl	microlitre
%	percent
$^{\circ}$C	Degrees celsius
x g	times gravity (centrifugation speed)

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ABSTRACT

The need to improve our understanding of vector-specific immunity of HIV vaccine candidates was recently highlighted by the STEP trial. This study characterised the humoral and cellular immune responses to MVA from a candidate MVA-vectored HIV vaccine in non-human primates, and examined the effect of anti-vector immunity on the response to the HIV immunogens. The MVA construct, termed SAAVI-MVA-C, contains multiple genes of HIV-1 subtype C, and is currently in Phase I clinical trials. Eight Chacma baboons were vaccinated with a DNA-MVA regimen. Whilst strong and broad anti-HIV responses were generated, we investigated whether anti-vector responses may have dampened responses that would otherwise have been boosted to even greater levels after successive vaccinations.

Neutralising antibodies to MVA were detected using a MVA-GFP neutralisation assay in all animals, as well as binding antibody responses to vaccinia virus envelope proteins A33 and B5R, detected by ELISA. In addition, high magnitude IFN- γ ELISPOT responses to MVA were induced in all animals. These rose dramatically from a median of 497 SFU/10⁶ PBMC after the first MVA vaccination, to 4455 SFU/10⁶ PBMC after the third MVA, given over a year later. Responses to the HIV immunogens showed similar magnitudes and kinetics of boosting, reaching a median of 4459 SFU/10⁶ PBMC after the third MVA vaccination. Interestingly, MVA responses remained at similar levels 7 months later, whilst HIV responses waned. There was no relationship between MVA-specific responses and HIV-specific responses. Multiparameter flow cytometry was used to detect IFN- γ , TNF- α and IL-2 responses to MVA and HIV, and to characterise the differentiation phenotype of memory T-cells, using CD28 and CD95. MVA-specific CD8⁺ responses were predominately single function IFN- γ producing T-cells, while lower magnitude CD4⁺ T-cell responses largely produced TNF- α and IL-2. Greater polyfunctionality to the HIV immunogens was evident, which may have been the result of earlier recombinant DNA prime vaccinations. Phenotypically there were no differences in memory responses to HIV and MVA, with antigen-specific CD4⁺ T-cells being of the central memory phenotype, and CD8⁺ T-cells showing a more balanced central and effector memory profile.

Thus, high magnitude cellular responses were generated to the vector component of a candidate MVA-HIV vaccine. This anti-vector immunity, however, did not prevent the generation of potent, polyfunctional CD4⁺ and CD8⁺ responses to the HIV immunogens contained in the vector, or the boosting of these responses after successive vaccinations. These data support further development and testing of this candidate MVA vaccine.

CHAPTER 1

Literature Review

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Chapter 1

Literature Review

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1.1 Introduction

AIDS-related death rates are falling worldwide, however HIV remains a global pandemic that has claimed the lives of more than 1.3 million people in 2009 in Sub-Saharan Africa alone (UNAIDS 2010). World-wide, there are 32.8 million people living with HIV, and although antiretroviral therapy is becoming more widely available, 2.6 million people became newly infected with HIV in 2009, 69% of whom were in Sub-Saharan Africa (UNAIDS 2010). A prophylactic HIV vaccine remains the best long-term hope for preventing new infections.

This Chapter discusses challenges for developing an HIV vaccine and progress in preclinical and clinical trials of vaccine candidates, with a specific focus on recombinant MVA as a vaccine vector and the issue of anti-vector immunity and how this may impact vaccine antigen immunity.

1.2 HIV Vaccines

The development of an HIV-1 vaccine presents a huge challenge. Many highly successful vaccines, such as those against smallpox, polio and measles viruses, have been developed, leading to eradication of the pathogen or vast reductions in morbidity and mortality from these diseases. Many traditional vaccines have been developed using live attenuated forms of virus, inactivated (killed) virus or protein subunits (as reviewed in Girard, 2006). These approaches are not suitable for developing a vaccine against HIV. Both attenuated and inactivated virus have major safety concerns, in that attenuated HIV may mutate and regain its pathogenicity after inoculation (as reviewed in Letvin, 2006), and inactivated virus may still contain enough live virus to pose a risk (as reviewed in Burgers and Williamson, 2005). Subunit vaccines have been successful for other viruses such as Hepatitis B, and it was initially thought that this approach may also work for HIV. However, an HIV envelope subunit vaccine was shown not to be protective in two large Phase III efficacy trials in humans (Gilbert *et al.*, 2005; Flynn *et al.*, 2005).

The major challenges to developing an HIV vaccine include the high genetic variability of HIV-1, its ability to evade adaptive immune responses, establishment of viral reservoirs resulting in latency, the difficulty in generating broadly neutralising antibody responses, unknown correlates of protection, limitations of animal models and the difficulties in performing large clinical trials.

HIV-1 is genetically diverse

HIV-1 has an enormous genetic diversity, and consists of four groups (M, N, O and P; Plantier *et al.*, 2009; Vallari *et al.*, 2011), of which group M is the most prevalent worldwide and consists of nine subtypes/clades and multiple circulating recombinant forms (as reviewed in Korber *et al.*, 2009)). The genetic differences in HIV-1 subtypes result from errors in reverse transcription of single stranded RNA into proviral cellular DNA, recombination between two genomes, insertions and deletions, as well as gain and loss of glycosylation sites (as reviewed in Levy, 2009; Letvin, 2006; Korber *et al.*, 2009). In addition to the global variability, HIV can mutate through intersubtype recombination in individuals that are infected with more than one subtype (superinfection) leading to different recombinant HIV strains circulating in one person (as reviewed in Korber *et al.*, 2009; Girard, 2006). In terms of vaccine development, this means that any single candidate HIV vaccine will need to be able to induce immune responses to multiple subtypes or recombinants to be globally effective (as reviewed in Letvin 2006).

There are several ways in which vaccine designers are attempting to cater for the huge genetic diversity of the virus. One of the ways is by developing polyepitope vaccines, where a large number of epitopes based on those commonly recognised in diverse HLA backgrounds are expressed by the vaccine. Another method involves the use of central sequences, which include consensus, most recent ancestor or centre of tree, which are phylogenetically determined (Nickle *et al.*, 2003; Rolland *et al.*, 2007). More recent approaches involves the use of mosaic antigens (as reviewed in Korber *et al.*, 2009), where many variant forms of epitopes are included in a “mosaic” to encompass the most common variants of HIV epitopes. They have been shown to provide deeper depth and breadth of responses (Santra *et al.*, 2010), and include not only conserved regions, but conserved and immunologically vulnerable regions where immune escape is less likely, due to potential fitness costs (Dahirel *et al.*, 2011).

HIV evades the adaptive immune system

HIV is able to evade adaptive immune responses, termed viral ‘escape’. HIV has error prone reverse transcriptase which together with the high recombination rate results in viral variants that can resist efficient major histocompatibility complex (MHC) processing, presentation or recognition by T-cells (Moore *et al.*, 2002). In fact, these escape mutations are often in response to pressure generated by T-cell responses (Goulder *et al.*, 2004). The effects of escape mutations on HIV are varied. They can be beneficial to the virus, in that mutated virus is no longer recognised by T-cells, they can be deleterious with a fitness cost to the virus, or they can have no effect at all (Liu *et al.*, 2007). In rhesus macaques, early Tat-specific CD8 T-

cell responses were shown to correlate with early control of SIV (Friedrich *et al.*, 2004). These responses quickly selected escape mutations, then lessened or disappeared (Allen *et al.*, 2004). Immune escape by HIV also has implications for vaccination, and a recent study of participants from the STEP trial has shown that vaccine induced T-cell responses can drive HIV to escape, resulting in different viral variants in the vaccinated compared to unvaccinated individuals (Rolland *et al.*, 2010). Not only can escape variants evade recognition by T-cells, they can also evade neutralising antibody binding (Wei *et al.*, 2003).

HIV forms latent reservoirs

HIV-1 integrates as a latent proviral DNA into the genome of host cells and provides a persistent reservoir of the virus that does not undergo transcription and produces no proteins to be presented to immune system (as reviewed in Girard, 2006; Chun *et al.*, 1997). Latency occurs within days to weeks after infection and once established, cannot be eradicated (Finzi *et al.*, 1997). This means that an HIV vaccine would have to generate effective immune responses very quickly after infection, at relevant sites of exposure such as genital tissue, to prevent latent pool formation.

Neutralising antibodies to HIV

Neutralising antibodies produced by B-cells can potentially protect against infection by blocking viral entry into host cells (Gallarda *et al.*, 1992). In the first two to three weeks of HIV infection, binding antibodies against the outer coat protein, known as Env, are generated; however these are inefficient and fully neutralising antibodies are only generated much later (Gray *et al.*, 2009; Richman *et al.*, 2003). These early neutralising antibodies only target the infecting strain (as reviewed in Montefiori *et al.*, 2007) and it is likely that these autologous neutralising antibodies exert pressure on the envelope of HIV, resulting in escape viral variants that they are no longer recognised (Moore *et al.*, 2009). After several years of infection, broad antibodies capable of neutralising diverse viral strains develop in only 15 to 25% of infected individuals, and these appear too late to prevent or slow disease progression (Binley *et al.*, 2008; Euler *et al.*, 2010).

Broadly neutralising antibodies have been difficult to induce by vaccination, largely as a result of the high genetic variability of the Env capsid protein as well as shielding of antibody binding sites by complex glycan shields (Wyatt *et al.*, 1998). Until 2009, only a handful of broadly cross-neutralising antibodies had been isolated from HIV-infected individuals (as reviewed in Verkoczy *et al.*, 2011; Gray *et al.*, 2009). Recently, however, many more broadly cross-

neutralising antibodies have been identified, revealing a range of neutralisation abilities against viral isolates, such as HJ16 showing 40% (Corti *et al.*, 2010), PG9 and PG16 showing 70 to 80% (Walker *et al.*, 2009; Pejchal *et al.*, 2010) and VRC01, VRC02, VRC03 ranging from 57 to 90% (Wu *et al.*, 2010; Zhou *et al.*, 2010). In fact, VRC01 neutralises about 90% of almost 200 HIV-1 isolates from multiple clades (as reviewed in Poropatich and Sullivan, 2011). The discovery of these new broadly cross-neutralizing antibodies has led to structural analysis of their molecular features to determine why they are so effective at neutralisation (Pancera *et al.*, 2010). Understanding these broadly neutralising antibodies and viral binding sites may assist in the design of vaccine immunogens, which may involve the formation of stabilised Env trimers and building conserved neutralisation epitopes onto foreign proteins (as reviewed in Barouch, 2008).

Neutralising antibodies delivered passively can protect nonhuman primates from challenge intravenously or via the mucosa with SHIV (Mascola *et al.*, 1999; Shibata *et al.*, 1999; Baba *et al.*, 2000; Parren *et al.*, 2001). It has also been shown in humans that the passive transfer of broadly neutralising antibodies can delay viral loads returning to high levels after stopping anti-retroviral treatment in some individuals (Trkola *et al.*, 2005). Thus, preexisting antibodies generated by an HIV vaccine could neutralise and potentially protect against infection. Unfortunately, initial clinical efficacy trials developing an HIV vaccine directed against envelope protein (gp120), the AIDSVAX trials (discussed further in section 1.2.7) did not elicit neutralising antibodies and no protection was observed, and generating neutralising antibodies by vaccination remains elusive (Flynn *et al.*, 2005, Gilbert *et al.*, 2005; as reviewed in Korber, 2009).

There may be a role for non-neutralising antibodies in controlling HIV infection, such as those capable of directing antibody dependent cellular cytotoxicity (ADCC; as reviewed in Chung *et al.*, 2008). Cells of the innate immune system such as natural killer cells, neutrophils and macrophages can be triggered by ADCC to provide effector functions which may assist in viral control (Chung *et al.*, 2011). High levels of ADCC antibodies have been found in HIV-infected individuals and some studies have correlated them with slower progression of HIV infection (Nag *et al.*, 2004; Ahmad *et al.*, 2001). They have also been generated by vaccination in humans (Karnasuta *et al.*, 2005). Passive antibody studies in macaques have confirmed that ADCC can reduce viraemia and play a role in protection from disease progression (Hessell *et al.*, 2007). In addition, when induced by vaccination in macaques they have been associated with better viral control (Gómez-Román *et al.*, 2005). These are promising results, however it is evident that neutralising antibodies should still be the focus of research, as non-neutralising antibodies have recently shown to offer very limited or no protection (Burton *et al.*, 2011).

Design of an HIV-1 vaccine that induces broadly cross-neutralising antibodies or other antibody effector mechanisms may be critical for inducing sterilising immunity, although it has recently been shown that vaccine induced T-cell responses may also be able to provide exceptional control of HIV (Hansen *et al.*, 2011). An ideal vaccine will probably need to generate a combination of humoral and cellular responses.

Unknown correlates of protection

Perhaps the greatest challenge is that we do not know the correlates of protection against HIV infection. Until measurable correlates of protection are defined, there will be little to guide vaccine development in both animal or human studies, and the potential effectiveness of vaccine concepts and products have to be tested in large, lengthy clinical trials (as reviewed in Makedonas and Betts, 2011).

T cell responses have been observed to be associated with the decline in peak viraemia in early HIV and SIV infection (Koup *et al.*, 1994; Schmitz *et al.*, 1999) and particularly broad Gag-specific T cell responses but not Env-specific responses targeting multiple epitopes have been inversely associated with lower viral load and positively correlated with CD4+ counts in humans (Kiepiela *et al.*, 2007; Rolland *et al.*, 2008; Zuniga *et al.*, 2006; Geldmacher *et al.*, 2007). In vaccinated challenged macaques, high magnitude, broad Gag-specific CD8+ T-cell responses have been associated with reductions in peak and setpoint viral loads, decreased AIDS-related mortality and a reduction in immune escape variants (Liu *et al.*, 2009; Reece *et al.*, 2010).

In addition to producing broad, high magnitude Gag-specific responses, there are various functional attributes of a T-cell response that have been correlated with viral control. Polyfunctional CD8+ T-cells may be better at viral control. Betts *et al.* (2006) showed that individuals who control HIV infection have CD8+ T-cells that perform many functions simultaneously, including degranulation (CD107a mobilisation), cytokine (IFN- γ , TNF- α and IL-2) and chemokine production (MIP-1 β). The production of cytotoxic molecules such as perforin and granzyme-B are also important in viral control. Indeed rapid perforin upregulation was the earliest response detected in long-term non-progressors and elite controllers, and there was increased T-bet expression, which has been shown to correlate with levels of expression of cytolytic molecules, in elite controllers (Hersperger *et al.*, 2010; Hersperger *et al.*, 2011). In addition, the proliferative ability of HIV-specific CD4+ T-cells has also been correlated with viral control in long-term non-progressors, and proliferation of HIV-specific

CD8+ T cells has been inversely correlated with viral load in chronically infected, untreated individuals (Rosenberg *et al.*, 1997; Day *et al.*, 2007). Thus, HIV-specific T cell polyfunctionality and proliferative capacity may also be important in control of HIV replication.

The memory differentiation state of T-cell subsets may also be an important factor in viral control, not only because function and phenotype are linked, but different subsets have different longevity and homing capacities. The amount of central memory HIV-specific CD4+ T-cells was higher in elite controllers compared to viraemic individuals (Potter *et al.*, 2007; Ladell *et al.*, 2008). On the other hand, fast-acting effector memory T cells at the site of viral exposure may also be important for early viral control. Hansen *et al.* (2009) demonstrated that vaccination of macaques with a rhesus CMV vaccine expressing SIV Gag, Rev-Tat-Nef and Env protected 4 out of 12 from mucosal challenge with SIV, due to the presence of CD8+ T_{EM} cells at the mucosal site. These results were confirmed in a subsequent study of a larger group of animals and long-term protection of greater than 1 year was observed (Hansen *et al.*, 2011).

Thus, whilst important features of T cells have been identified as correlating with viral control, it is still not clear whether all or some of these features would be required for a successful HIV vaccine. A large effort is being undertaken to identify the correlates of protection in the RV144 trial, the only clinical trial of an HIV vaccine to date that has demonstrated efficacy in protection against HIV infection (Rerks-Ngarm *et al.*, 2009).

Relevance of animal models

Rhesus macaques are widely used as the animal model for HIV infection of humans. They are susceptible to infection with SIV, which shares many features of HIV, and infected animals progress to AIDS (albeit more rapidly), mirroring the disease course of HIV infection in humans (as reviewed in Koup *et al.* 2011; Mattapallil *et al.*, 2005). Thus, the non-human primate SIV challenge model has been widely used to study HIV vaccine concepts and vaccine candidates. Animal models can provide much useful information, however they do need to be interpreted with caution, as there are many differences between studies in the way challenges are performed. Challenge viruses may be administered at high doses via intravenous exposure (Haigwood *et al.*, 2009), which does not reflect the majority of human sexual exposures to HIV. It is likely that more meaningful interpretations can be made if these animals are exposed mucosally with repeated low-dose challenges (Keele *et al.*, 2009). In addition, the challenge virus used is often homogenous, using a virus containing sequences very similar to the vaccine sequence, which may be more easily controlled than a heterologous challenge, which is more relevant to natural human HIV infection (as reviewed in Wilson & Watkins, 2009). It is

more accurate to perform a heterologous challenge using a swarm virus such as SIVsmE660, with many sequence dissimilarities to vaccine inserts (Watkins *et al.*, 2008). Illustrating this, Wyand *et al.* (1999) showed that macaques immunised with live-attenuated SIV showed good control of viraemia after homologous SIVmac239 challenge, however when animals were challenged with heterologous SIVsmE660, there was reduced control of viraemia. Additionally, a recent candidate HIV vaccine showed disappointing results in the STEP trial in humans, even though it showed reduced viral loads in homogeneously challenged macaques (McElrath *et al.*, 2008; Buchbinder *et al.*, 2008; Shiver *et al.*, 2002). However, when vaccinated macaques were challenged with a more stringent heterologous virus, they showed no control of viral load, which is more in line with the results generated by the STEP trial (Casimiro *et al.*, 2005). Therefore, to provide meaningful information on whether a candidate vaccine should be advanced to human trials, monkey studies should be standardised as far as possible in terms of the SIV challenge virus, challenge route, challenge dose, species of monkey and endpoint measurements (Morgan *et al.*, 2008).

Human efficacy trials of candidate HIV vaccines

Testing candidate vaccines in humans remains the best way to evaluate protection against or control of HIV infection. Thus far, there have been four Phase IIb/III clinical trials that have tested the efficacy of candidate HIV vaccines that I will discuss in detail in this section.

The first two phase III clinical trials of an HIV vaccine were initiated in 1998 by VaxGen. The two vaccines consisted of a recombinant form of the glycoprotein-120 (gp120) portion of the HIV envelope administered with the adjuvant alum, in an attempt to generate neutralising antibodies. The first vaccine, AIDSVax B/B, consisting of two subtype B gp120s, was trialled in North America and the Netherlands in 5095 men who have sex with men and 308 heterosexual women (Gilbert *et al.*, 2005). The vaccine, which was given in seven immunisations over 30 months, elicited a gp-120-specific antibody response, but did not neutralise HIV-1 isolates from infected individuals, and showed no protection in an efficacy trial with 6.7% of the vaccinated individuals becoming infected versus 7.0% that received the placebo (Flynn *et al.*, 2005, Gilbert *et al.*, 2005). The second vaccine, AIDSVax E/B, consisting of subtype E (CRF_AE) and subtype B gp120s, was trialled in 2527 HIV-uninfected injection drug users in Thailand. Similarly, it showed no protection, with 8.4% of vaccine and 8.3% of placebo participants becoming infected with HIV during the trial (Pitisuttithum *et al.*, 2006). The failure of this trial signalled the move away from the 'antibody' vaccine concept, with the acknowledgement of the huge challenges in eliciting neutralising antibodies by vaccination, to a large amount of effort going into development of the 'T cell' vaccine concept.

The next large clinical trial, a proof of concept phase IIb trial known as the STEP trial, was initiated in late 2004 and focused on producing T-cell immune responses. The rationale for inducing T-cell responses by vaccination is discussed in section 1.2.1. The vaccine consisted of a replication-incompetent recombinant adenovirus serotype 5 (rAd5) vector expressing HIV-1 subtype B Gag, Pol and Nef. It showed good immunogenicity in non-human primate studies and vaccinated animals showed some viral control after challenge with SIVmac239 (Shiver *et al.*, 2002; Casimiro *et al.*, 2005; Wilson *et al.*, 2006). Immunogenicity trials in humans revealed that cellular immune responses were elicited in most individuals (Priddy *et al.*, 2008). The STEP trial set out to determine whether this vaccine given in a three dose regimen could protect from or control viraemia in a total of 3000 individuals from North America, the Caribbean, South America and Australia potentially exposed to subtype B HIV strains (McElrath *et al.*, 2008). A similar trial named 'Phambili', commenced in South Africa.

Robust T-cell responses were detected by IFN- γ ELISPOT in 77% of vaccinated individuals to two or three HIV proteins, with responses greater for Pol than Gag or Nef. Intracellular cytokine staining revealed similar results, with 73% of vaccinated individuals mounting CD8+ responses. Lower magnitude CD4+ T-cell responses were detected, in 41% of vaccinated individuals (Buchbinder *et al.*, 2008). Despite the high frequency of responders, 24 of 741 vaccinated individuals became infected with HIV, versus 21 of 762 individuals that received placebo. Thus, there was no protection against HIV, and viral load set points were not lowered in vaccinated individuals that became infected (McElrath *et al.*, 2008; Buchbinder *et al.*, 2008). Indeed, there was an increased risk of HIV infection in vaccinated individuals, and this trial and Phambili were halted. Failure of this vaccine led to some questioning the potential efficacy of T-cell generating vaccines, however it is important to note that vaccinees that became infected only recognised two to three epitopes, and that this response may not have been broad enough to recognise epitopes in the infecting virus (as reviewed in Wilson and Watkins, 2009; Fauci *et al.*, 2008). Also, the increased risk of HIV infection lasted for 18 months after immunisation, and occurred mainly in uncircumcised men with baseline Ad5 titres (Buchbinder *et al.*, 2008). The potential role of anti-vector immunity will be discussed in section 1.3.4. Recent results from the Phambili trial showed that the vaccination regimen had no protective effect, however there was no association between circumcision and Ad5 titres and lack of protection (Gray *et al.*, 2011).

The last efficacy trial was a Phase III trial, RV-144, initiated in Thailand in 2003, in 16 000 men and women at heterosexual risk of HIV infection (as reviewed in Vaccari *et al.*, 2010). The vaccine aimed to elicit both humoral and cellular immune responses to HIV by combining two

vaccines in a prime-boost strategy. There were four priming vaccinations of a canarypox-based- vector known as ALVAC-HIV (vCP1521), and two boosts with VaxGen AIDSVax B/E bivalent gp120 subunit vaccine (Rerks-Ngarm *et al.*, 2009). ALVAC-HIV expressed subtype B Gag and Protease, in addition to gp120 clade E, while the AIDSVax is the same gp120 envelope vaccine that failed in an earlier trial (as reviewed in Vaccari *et al.*, 2010; Gilbert *et al.*, 2005). RV144 was the first efficacy trial to show promising results, with 74 out of 8198 placebo recipients becoming infected with HIV compared to 51 out of 8197 vaccinated individuals, resulting in 31.2% vaccine efficacy ($p=0.04$; Rerks-Ngarm *et al.*, 2009). This protective effect was seen in the first year following start of vaccination, however the vaccinated individuals that acquired infection did not have improved viral control. The immunogenicity was measured by interferon- γ ELISPOT, intracellular cytokine staining, binding antibody to gp120 and lymphoproliferation (Rerks-Ngarm *et al.*, 2009). Gag-specific T-cell responses were detected in 7.6% and Env-specific responses in 11.1% of vaccinated individuals (Rerks-Ngarm *et al.*, 2009). Intracellular cytokine staining showed higher CD4+ Env responses in the vaccinated versus placebo group, at 34% (Rerks-Ngarm *et al.*, 2009). On the other hand only moderate neutralising activity detected against 3 of 5 tier 1 viruses, suggesting that antibody responses other than neutralisation may have contributed to protection (Gilbert *et al.*, 2010). These data provide the first evidence that a vaccine can prevent HIV infection. The reasons for protection provided by this vaccine are being studied to determine immunological correlates of protection.

The AIDSVax, STEP and RV-144 clinical trials show that testing a vaccine candidate in humans is the best way of determining vaccine efficacy. However, human clinical trials are complex, expensive and have traditionally followed a sequential approach consisting of phase I, phase IIa, phase IIb and phase III studies, which takes years to complete (as reviewed in Koup *et al.*, 2011). Vaccine development needs to be accelerated, possibly by performing more stringent macaque studies, focusing on early immune correlates of protection (Watkins *et al.*, 2008). Where possible, priority could be given only to vaccine candidates that show control of viraemia in heterologously challenged macaques, eliminating time spent on unlikely candidates (as reviewed in Barouch, 2008). Vaccine induced immune correlates of protection need to be determined rapidly so vaccine responses can be tested early in a trial, rather than waiting for post-trial analysis. This may require multiple efficacy trials of many different candidate vaccines in subjects who are at high risk of HIV infection, allowing more information to be gathered quickly and at less expense (Corey *et al.*, 2011). In addition, trials need to be faster, which may require new approaches, such as adaptive trial design, which is an iterative approach that allows changes in the study design in response to data being generated during the study, for example stopping the trial for lack of efficacy, or adding booster vaccinations if

efficacy reduces over time. This may allow cost effective evaluation of more approaches in a shorter amount of time.

In summary, an ideal HIV vaccine should be able to elicit both humoral (broadly neutralising or other types of antibodies), as well as broad, strong cellular (CD4+ and CD8+ T-cells), to provide long lasting protection.

1.3 T-cell vaccines

This literature review will now focus primarily on T-cell vaccines since this is the focus of my thesis.

1.3.1 What are the goals of a T-cell based HIV vaccine?

An ideal vaccine would elicit sterilising immunity through neutralising antibody production (Figure 1.1). However, as discussed in section 1.2.4, HIV-specific neutralising antibodies have not yet been produced by vaccination. It is thought that a T-cell vaccine could be partially effective, resulting in lower virus levels, less destruction of CD4+ T-cells in gut-associated lymphoid tissue during the acute phase, and a delay in disease progression (as reviewed in Barouch, 2008; Wilson and Watkins, 2009). This has the potential to reduce secondary transmission by herd effect (as reviewed in Girard, 2006; Hanke, 2007.) It would also be beneficial if a vaccine induced immune responses at the genital mucosa to block HIV transmission (as reviewed in Shacklett, 2008).

1.3.2 Evidence that a T-cell generating vaccine might control HIV

There is an association between the early appearance of HIV-specific CD8+ T-cells and the drop in peak viraemia to setpoint in acute infection both in SIV and HIV (Kuroda *et al.*, 1999; Koup *et al.*, 1994; as reviewed in Pantaleo *et al.*, 1996). Neutralising antibodies are only generated later in infection, and thus the cell-mediated rather than antibody responses are responsible for early control of viral replication (Koup *et al.*, 1994; Wei *et al.*, 2003; as reviewed in Letvin, 2006).

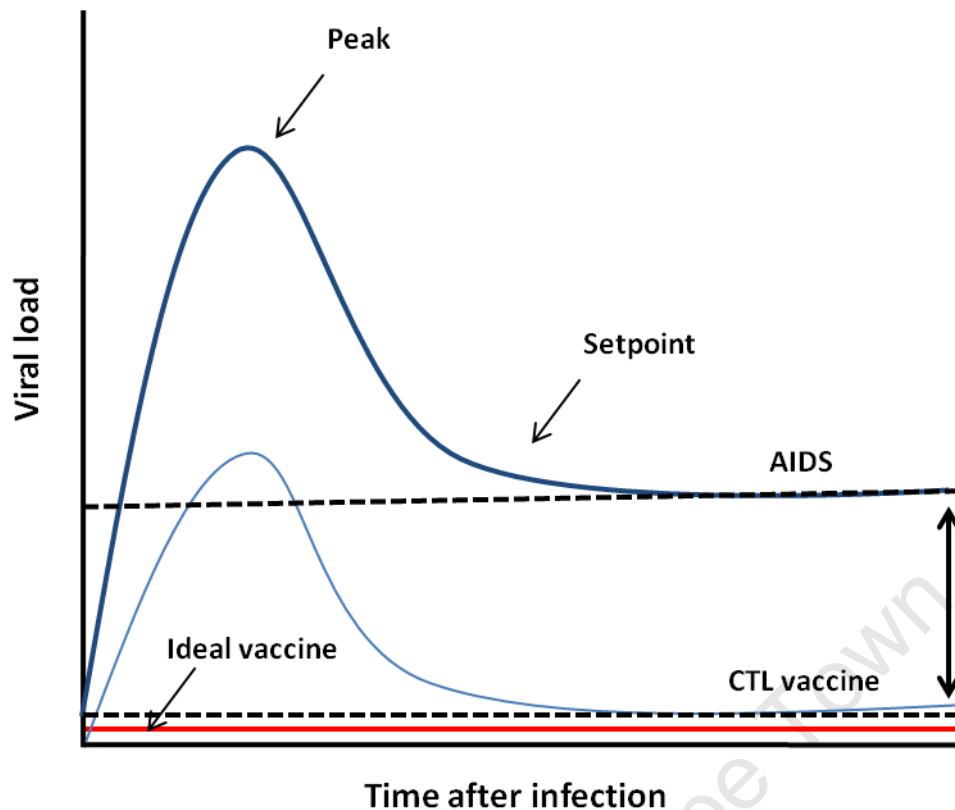


Figure 1.1 Goals of an HIV vaccine. After infection with HIV, replication as determined by viral load continues to a point, then drops to setpoint and is partially maintained (dark blue). An ideal vaccine (red) would completely protect against infection, while a vaccine that reduces peak viral replication during the acute phase and results in lower or undetectable setpoint viral load during chronic infection (light blue) may still be effective in preventing transmission and slowing or preventing disease progression (adapted from Wilson & Watkins, 2009 and Barouch, 2008).

Consistent with these studies, it has been shown that depletion of CD8⁺ T-cells by monoclonal antibodies led to loss of control of viraemia in macaques infected with SIV (Schmitz *et al.*, 1999; Okoye *et al.*, 2009). CD8⁺ T-cells are efficient killers that are capable of destroying HIV-infected cells through the Fas/Fas ligand-killing, or death receptor pathway or by the release of lytic granules (Poonia *et al.*, 2009; as reviewed in Suni *et al.*, 2005). Fas receptor, also known as CD95, is a protein of the TNF receptor family that induces apoptosis on binding Fas ligand. This binding sets a chain of events in motion by a system of proteases called caspases within the target cell itself and the cascade ends with the destruction of the target. In addition, CTLs have lytic granules containing perforin and granzymes, which induce apoptosis (as reviewed in Krensky and Clayberger, 2005). Besides these molecules, the lytic granules also consist of granulysin, which creates holes in the target cell membrane cells. During this process, the granule membrane fuses with the cell membrane and releases the killing molecules perforin and granzyme into target cells (as reviewed in Suni *et al.*, 2005).

Several non-human primate studies have shown that T-cell-based vaccines can provide immune control or HIV protection. In early HIV infection, decreased peak viraemia, and preserved CD4⁺ memory T-cells following high dose intravenous and mucosal challenge with pathogenic SIVmac239 have been described (Letvin *et al.*, 2006; Liu *et al.*, 2009; Mattapallil *et al.*, 2006; Horton *et al.*, 2002; Whitney *et al.*, 2009; Hansen *et al.*, 2009). Additionally, long-term control of infection has been shown in vaccinated macaques challenged multiple times intra-rectally or intra-vaginally with low dose virus, demonstrating that T-cell vaccination can elicit control even in chronic infection (Wilson *et al.*, 2006; Wilson *et al.*, 2009; Manrique *et al.*, 2011). In humans, the T-cell vaccine concept was challenged with the failure of the STEP trial, where no protection from infection or reduction of viral loads in infected individuals was seen (Buchbinder *et al.*, 2008; McElrath *et al.*, 2008). However, the modest protective effect seen in the RV-144 Thai trial has provided renewed encouragement that a successful vaccine is achievable (Rerks-Ngarm *et al.*, 2009). Although there was protection from infection in a proportion of individuals, there was no reduction in viral loads of those vaccinated individuals that did become infected. It also remains to be determined whether protection was a result of innate responses, T-cell responses or antibody-mediated (Rerks-Ngarm *et al.*, 2009).

A potential drawback of generating T-cell responses by vaccination is that SIV/HIV constantly mutates to escape epitopes recognised by CD8⁺ T cells in macaques and humans (Allen *et al.*, 2004; Brumme *et al.*, 2008; Goonetilleke *et al.*, 2009). In fact, suboptimal vaccine responses may in theory drive faster escape, which could reduce vaccine efficacy (as reviewed in Davenport *et al.*, 2008). Thus, the ultimate goal of an effective T cell vaccine may be to elicit immune responses that are early and target as many of the most conserved epitopes as possible, that may limit escape opportunities and inhibit viral replication, or result in a fitness cost to the virus, thereby reducing viral replication (as reviewed in Barouch, 2008; Reece *et al.*, 2010).

1.3.3 T-cell vaccine vectors

Current approaches for the development of HIV vaccines, and indeed several other major pathogens, use live viral vectors as “carriers” to deliver HIV antigens, in the hope of stimulating potent anti-HIV T cell immune responses which may protect against HIV infection or disease (as reviewed in Paris *et al.*, 2010). Vectors used most commonly include plasmid DNA and viruses, especially attenuated adenovirus serotypes and poxviruses (as reviewed in Pantaleo *et al.*, 2010). Viruses have the ability to infect cells where vaccine antigens are expressed and delivered into the MHC machinery, allowing the cells to effectively process and display these

antigens to the immune system and stimulate responses. Viral vectors are being tested as safe vaccines for malaria, tuberculosis, HIV, influenza, and various cancers (as reviewed in Brave *et al.*, 2006). These common and other novel vector systems will be discussed in the remainder of this review.

1.3.3.1 DNA

DNA-based vaccines allow direct gene transfer by 'naked' plasmid DNA, are not virus derived and do not rely on other pathogens to deliver antigens (as reviewed in Brave *et al.*, 2007). They are relatively cheap to produce, easy to work with and are very stable (as reviewed in Hanke, 2001). In addition, DNA vaccines allow the immune response to focus only on the immunogen, as no proteins other than the immunogen are present to be presented to the host cells. Thus, they are safe and have no risk of virulence (MacGregor *et al.*, 1998). Used alone, they generally elicit low magnitude responses of short duration (as reviewed in Lu *et al.*, 1998; MacGregor *et al.*, 1998; Graham *et al.*, 2006; Goepfert *et al.*, 2011). However, they have been shown to prime cellular responses to viral candidate vectors well, and are widely used in heterologous prime-boost vaccination regimens (as reviewed in Paris *et al.*, 2010; Harari *et al.*, 2008; Goepfert *et al.*, 2011). Improved delivery techniques such as electroporation are being explored to improve potency of immune responses to DNA vaccines (Otten *et al.*, 2004).

1.3.3.2 Adenovirus vectors

Adenoviruses of the genus Mastadenovirus are medium-sized (90–100 nm), nonenveloped icosahedral viruses composed of a nucleocapsid and a double-stranded linear DNA genome of 36–38 kb (as reviewed in Brave *et al.*, 2007). In humans, there are 56 accepted human adenovirus types (HAdV-1 to 56) in seven species (Human adenovirus A to G; Knipe and Howley, 2007). Adenovirus infections most commonly cause illness of the respiratory system. Symptoms of respiratory illness caused by adenovirus infection range from the common cold to pneumonia and bronchitis. Most humans have already been infected with adenoviruses, and thus wide-spread pre-existing immunity occurs that may influence vaccine vector responses (Mast *et al.*, 2010).

Adenovirus vectors, used alone or in combination with DNA prime vaccines, have been shown to be safe and generate strong T-cell responses (Shiver *et al.*, 2002; Priddy *et al.*, 2008). Studies in non-human primates have demonstrated induction of potent immune responses when using adenovirus type 5 (Ad5) vectors expressing clade B Gag, Pol and Nef, and reduction of SIV viral loads and slower disease progression (Casimiro *et al.*, 2005; Letvin *et al.*,

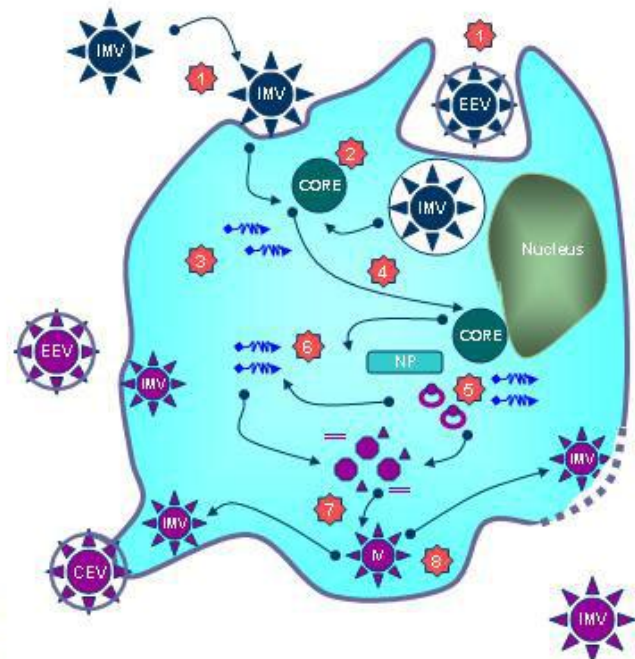
2006). In human trials an Ad5 vaccine candidate elicited good cellular immune responses and was safe, but failed to protect against HIV infection or to reduce viral loads in infected individuals in the STEP trial (Buchbinder *et al.*, 2008). Potential mechanisms of this will be discussed in section 1.3.5.2. There are also alternative Adenovirus vectors for which there is no significant pre-existing immunity in humans, such vector chimeras, other serotypes, and nonhuman Ads (as reviewed in Gamble and Matthews, 2010). One of these, Ad26, which is a serotype that does not infect humans, is currently in a Phase I clinical trial in a Ad26-Ad5 prime boost vaccination regimen (Baden *et al.*, 2009). In non-human primate studies, this vaccine regimen showed control of virus with a 2.4 log reduction in viral set-point and improved survival compared to unvaccinated controls, but preliminary results from a Phase I clinical trial indicate that it is not as immunogenic in humans (Liu *et al.*, 2009; Matthew Johnson, Keystone Symposia, Protection from HIV: Targeted Intervention Strategies (X8), Canada, 2011).

1.3.3.3 Poxvirus vectors

Poxviruses are the largest and most complex of all viruses. They are enveloped and contain complex DNA genomes of up to 360 kb that encode more than 200 genes (as reviewed in Brave *et al.*, 2007). They infect a wide range of hosts, and are divided into two subfamilies: Chordopoxviridae and Entomopoxviridae. All human poxviruses are in the Chordopoxviridae subfamily, and most of them belong to either the Orthopoxvirus (variola, vaccinia, cowpox) or the Parapoxvirus (Orf virus) genus (Knipe and Howley, 2007). Antigenically, poxviruses are very complex, inducing both specific and cross-reacting antibodies that have the ability to protect against related viruses, e.g. vaccinia virus is used to vaccinate against variola (smallpox).

Poxviruses enters cells mainly by cell fusion through an unknown receptor, and it is thought that three membrane proteins, A27L, D8L, and H3L, attach to host cell glycosaminoglycans (as reviewed in Sauter *et al.*, 2005). Poxviral replication results in the formation of virus containing early, intermediate and late genes transcribed by viral RNA polymerase and transcription factors (Figure 1.2). Virus replication occurs in the cytoplasm of infected cells and after late gene expression produces enveloped intracellular mature virion particles (IMV) that are transported to Golgi to be wrapped in additional membranes becoming intracellular enveloped virus (IEV). IEV is transported along microtubules to reach the cell periphery and fuse with plasma membrane to become cell-associated enveloped virus (CEV), that triggers actin tails on cell surfaces or is released as extracellular enveloped virus (EEV) (as reviewed in Smith *et al.*, 2002; Sauter *et al.*, 2005).

- 1 **Entry**
Intracellular mature virion (IMV) particles bind to unknown receptor(s) and fuse with the cell membrane. Extracellular enveloped virion (EEV) particles bind to unknown receptor(s) and are endocytosed into the cell.
- 2 **Initial Uncoating**
The viral core particle (CORE) containing the viral genome, the viral DNA-dependent RNA polymerase, and other enzymes is released into the cytoplasm.
- 3 **Early Transcription**
Early genes (including those coding for immunomodulatory proteins, enzymes, and replication and transcription factors) are transcribed and translated immediately upon core particle entry into the cytoplasm of the cell.
- 4 **Translocation**
The viral core particle translocates to the outside of the cell nucleus.
- 5 **Secondary Uncoating**
The viral nucleoprotein (NP) complex is released containing the viral genome. At this point the viral genome is replicated as a concatemer and transcription and translation of intermediate genes (mainly coding for transcription factors) occurs.
- 6 **Late Transcription**
The viral late genes (coding for structural proteins, enzymes and transcription factors) are transcribed and translated.
- 7 **Assembly**
Concatemeric intermediates are resolved into linear double stranded DNA and packaged with late viral proteins into immature virions (IV).
- 8 **Release**
IV's mature into IMV's via an undescribed mechanism which may include processing of the IV through the Golgi apparatus. The IMV's are transported to the periphery of the cell where they are released in one of three ways. IMV's released via cell lysis remain IMV's. Alternatively, IMV's can bud through to the cell surface, picking up a viral envelope from the cell plasma membrane. On the surface these cell-associated enveloped virions (CEV's) are pushed via an Actin tail into contact with a second cell. Lastly the IMV can bud through the plasma membrane picking up an envelope and becoming an EEV.



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Figure 1.2 Poxvirus replication cycle Replication of poxviruses occurs in the cytoplasm of the cell and contains steps shown graphically in the diagram (from Keckler *et al.*, 2005)

Poxviruses are ideal vectors for vaccine development for several reasons. Their large genome allows the insertion of multiple genes, their cytoplasm has all the tools required for replication without host cell integration, they are temperature stable, easily manipulated to generate recombinants and due to cessation of smallpox vaccination in the 1970s, there are a diminishing number of people with pre-existing vector immunity (as reviewed in Pantaleo *et al.*, 2010).

Fully replication competent vaccinia virus is not safe to use as a vaccine vector, however there are several other poxvirus vectors that have been used in vaccine development. These include the attenuated vaccinia virus strains, modified vaccinia Ankara (MVA) and NYVAC strain, and the attenuated canary poxvirus known as ALVAC (Belshe *et al.*, 1998). MVA will be discussed further in section 1.3.4, and ALVAC was used in the RV-144 trial (Rerks-Ngarm *et al.*, 2009) as discussed in section 1.2.7. NYVAC, based on the Copenhagen strain of vaccinia virus, was used in the EuroVacc 02 (EV02) phase I trial in combination with a DNA prime vaccine. It proved to be highly immunogenic with broad, strong, long-lasting polyfunctional T-

cell responses in 90% of vaccinees (Harari *et al.*, 2008). Polyfunctional vaccine-induced CD4+ T-cell responses were seen in 100% of responders and CD8+ T-cells in 50% of responders (Harari *et al.*, 2008). T-cell responses were largely targeted to Env (91 %) with Gag, Pol or Nef detected in 48 % of individuals.

Prime-boost vaccinations with DNA and NYVAC are safe, variably immunogenic and produce T-cell responses that are usually predominated by CD4+ T-cell responses directed towards Env (Harari *et al.*, 2008). This was also observed with MVA (discussed below), and appears to be a characteristic of poxviral vectors as a group (as reviewed in Paris *et al.*, 2010). Many factors relating to the immunogen such as dose, route, adjuvant and insert stability may influence the immunogenicity of the DNA/poxvirus regimen.

1.3.3.4 Other vectors

In light of the failure of the STEP trial, many vaccine vectors other than Ad5 are being studied. These include replication capable CMV, recombinant BCG and Yellow Fever Virus (Hansen *et al.*, 2009; Cayabyab *et al.*, 2009; Rosario *et al.*, 2010; Bonaldo *et al.*, 2010).

Picker and colleagues used a replicating simian cytomegalovirus (CMV) vector expressing SIV Gag, Rev, Tat, Nef and Env that controlled viraemia to undetectable levels in four of twelve repeatedly intrarectally challenged macaques (Hansen *et al.*, 2009). A follow-up study of 24 rhesus macaques receiving CMV vectors alone or CMV and an Ad5 boost showed early control of SIV in 13 animals and long term protection in 12 of these (Hansen *et al.*, 2011). Robust long-lasting polyfunctional cellular responses, predominantly by CD8+ effector memory T-cells, were elicited in all vaccinated animals, although no significant binding or neutralising antibodies were seen. Also encouraging was that recombinant CMV was given multiple times with no effect on immunogenicity (Hansen *et al.*, 2011). CMV causes persistent infection by remaining in host cells, and thus provides continuous antigen stimulation that maintains effector memory stimulation. It is thought that effector memory cells offer an advantage for protection, as they are readily available to kill infected cells at mucosal effector sites, the sites of viral entry. Central memory CD8+ T-cells, produced by current T-cell vaccine vectors such as MVA, although long lasting, would take longer to differentiate to effectors and be effective at viral entry sites. This suggests that a vaccine based on a persistent vector is important, or that repeated vaccinations of a waning vaccine will be necessary (as reviewed in Franchini, 2009; Hansen *et al.*, 2009; Hansen *et al.*, 2011). There is concern that it will not be safe to vaccinate CMV-negative, immunodeficient or pregnant individuals, however modifications to increase the

safety of CMV are currently being explored (Louis J. Picker, Keystone Symposia, Protection from HIV: Targeted Intervention Strategies (X8), Canada, 2011).

Mycobacterium bovis BCG (rBCG) has long been used to vaccinate against tuberculosis, with over 3 billion doses given since the 1920s, and therefore has a proven record of safety and persistence (as reviewed in Joseph *et al.*, 2004). Thus, it is being considered as an HIV vaccine vector. Recent non-human primate studies have shown that vaccinated animals developed only modest SIV-specific cellular responses, however responses were boosted with a range of other vectors, such as Ad5, MVA and yellow fever virus (Bonaldo *et al.*, 2010). These boosted responses were durable, of high magnitude responses and higher than those generated by the other vectors alone (Cayabyab *et al.*, 2009; Rosario *et al.*, 2010; Bonaldo *et al.*, 2010). This suggests that rBCG may be a good prime candidate.

Several other viral vectors are in development as HIV vaccine candidates, such as live recombinant vesicular stomatitis virus (VSV) and Semliki Forest virus (SFV), and these have shown protection in mucosally challenged and vaccinated macaques (Schell *et al.*, 2011).

1.3.4 MVA

1.3.4.1 What is MVA?

Modified Vaccinia Ankara (MVA) is a highly attenuated strain of vaccinia virus that was developed towards the end of the campaign for the eradication of smallpox (McCurdy *et al.*, 2004; as reviewed in Sutter and Staib, 2003). MVA has lost about 10% of the vaccinia genome by hundreds of passages of vaccinia virus in chicken cells. It has lost the ability to replicate efficiently in cells, rendering it safe for use in humans (as reviewed in Pantaleo *et al.*, 2010). Like all the poxviruses, it has a large genome which allows easy gene insertion making ideal to act as a vaccine vector (Blanchard *et al.*, 1998; as reviewed in Sutter and Staib, 2003).

1.3.4.2 Why is MVA a desirable vector?

In the 1970s MVA demonstrated its safety and efficacy in over 120 000 people vaccinated against smallpox (as reviewed in Paris *et al.*, 2010). It is even safe in immunocompromised persons receiving HAART (Dorrell *et al.*, 2007). A variety of antigens from human pathogens have been expressed in MVA, and potent immune responses have been generated (Smith *et*

et al., 2005; Hanke *et al.*, 2007; Sandstrom *et al.*, 2008; Goepfert *et al.*, 2011). A number of promising experimental MVA vaccines expressing HIV, TB, malaria and tumour antigens are currently being evaluated in clinical trials in human volunteers (Hanke *et al.*, 2007; Scriba *et al.*, 2010).

Vaccination against HIV using MVA has shown moderate success in non-human primate models. Researchers have described lower and/or delayed acute viraemia and better survival times after challenge with SIV in vaccinated macaques compared to unvaccinated controls (Im *et al.*, 2006; Horton *et al.*, 2002). Indeed, mucosal vaccination via the nasal route with DNA and MVA-based vaccines elicited stronger immune responses and longer survival in macaques (Ourmanov *et al.*, 2009; Manrique *et al.*, 2011).

The most advanced HIV vaccine candidate regimen based on MVA that has been tested in humans is a DNA prime-MVA boost HIV vaccine candidate, tested in safety trials and in larger phase II studies by Oxford University and the International AIDS Vaccine Initiative (IAVI; as reviewed in Paris *et al.*, 2010). The HIVA immunogen used in the plasmid DNA and recombinant MVA vaccines consisted of consensus clade A Gag p24p17 sequences and various CD8+ T-cell epitopes. This and subsequent studies proved that the vaccine was safe, but did not induce antibody responses and only moderate cellular responses (Cebere *et al.*, 2006; Hanke *et al.*, 2007; Guimaraes-Walker *et al.*, 2008). Interestingly, all responses were dominated by CD4+ T-cells, with few individuals having CD8+ T-cell responses (Hanke *et al.*, 2007; Goonetilleke *et al.*, 2006). In addition, responses to the DNA and MVA combination were of higher magnitude and breadth than those generated by the rMVA alone (Goonetilleke *et al.*, 2006). This vaccine candidate is currently undergoing testing in larger clinical trials (IAVI Report 2010).

Promising results have been reported by another Phase I study of a multigene, multiclade DNA-MVA prime boost vaccine candidate (Sandstrom *et al.*, 2008). A DNA and MVA vaccination regimen was given in combination with recombinant granulocyte macrophage colony-stimulating factor (GM-CSF). Encouragingly, this vaccination regimen was safe and highly immunogenic with responses detected in the majority of individuals in both the CD4+ and CD8+ T-cell compartments (Sandstrom *et al.*, 2008; as reviewed in Paris *et al.*, 2010). Humoral responses were however poor. Another candidate DNA-MVA vaccine regimen having undergone a Phase I trial produced CD4+ and CD8+ cellular responses in addition to antibody responses induced to trimeric Env that was included in the vaccine construct (Goepfert *et al.*, 2011).

These Phase 1 trials have proven that MVA is safe, immunogenic and that priming with DNA generates higher T-cell responses in higher dose MVA regimens (Goonetilleke *et al.*, 2006; Sandstrom *et al.*, 2008; Goepfert *et al.*, 2011). It is also evident that T-cell responses are often dominated by CD4+ T-cells, although CD8+ T-cells responses have also been generated, and that the magnitude of responses are variable, according to the vaccine inserts. The HIVA immunogen expressing strings of CD8 epitopes elicited much lower responses than those that were observed when expressing whole proteins (Hanke *et al.*, 2007; Sandstrom *et al.*, 2008; Goepfert *et al.*, 2011). In addition to T-cell responses, antibody responses can be generated by MVA vectors with envelope inserts, although it appears that higher antibody responses are seen in individuals having fewer or no DNA primes (Goepfert *et al.*, 2011). The importance of these antibody responses have been shown in macaque studies, where high avidity anti-Env binding antibody responses have been associated with control of peak viraemia following high-dose intrarectal challenges (Zhao *et al.*, 2009). Thus, DNA priming induces better cellular immunity but lower humoral immunity.

1.3.4.3 MVA-specific immune responses

MVA is being considered as an alternative to Dryvax, a live vaccinia virus, as a smallpox vaccine in the era of bioterrorism. MVA cannot replicate in most human cells and has fewer complications in high risk individuals such as the immunocompromised, very young or elderly individuals (Meseda *et al.*, 2005). MVA readily produces binding and neutralising antibodies in individuals vaccinated with varying doses and routes comparable to those produced by Dryvax (Meseda *et al.*, 2005; Wilck *et al.*, 2010). Strong, predominantly CD8+ T-cell responses were also generated to MVA (Frey *et al.*, 2002; Ennis *et al.*, 2002). These responses are long-lived and detectable in nonhuman primates as long as 3 years after vaccination (Nigam *et al.*, 2007). Goepfert *et al.* (2011) recently showed that MVA-specific CD4+ and CD8+ T-cells of high magnitude were generated in humans.

1.3.5 Anti-vector immunity

1.3.5.1 What is anti-vector immunity?

Anti-vector immunity refers to immune responses directed at the vector itself. These anti-vector immune responses can potentially dampen the immune response to the vaccine antigens by rapid clearance of the vaccine vector, and possibly shorter duration of antigen presentation to T cells as a result of cleared infection (as reviewed in Duerr *et al.*, 2006). It is important to make the following distinction between 'anti-vector' immunity. Anti-vector

immunity may refer to pre-existing immunity to the vector, for example, with adenovirus, it is common that a person may have been naturally exposed to adenovirus previously. Pre-existing immunity to Adenovirus type 5 has been well described and may have been one of the contributing factors leading to the recent failure of the Merck Adenovirus HIV vaccine (McCoy *et al.*, 2007; Sekaly *et al.*, 2008). With poxviruses such as MVA, the presence of pre-existing immunity to vaccinia virus in the adult population is the result of smallpox vaccination programs. It is hoped that MVA will be resistant to this kind of anti-vector immunity, as routine vaccinia immunisation was stopped in the early 1970s, and the majority of the HIV at-risk population will have no pre-existing immunity.

A few studies have looked at the effect of pre-existing vaccinia immunity on the generation of responses to vaccinia virus and MVA by vaccination. Humoral responses and cellular responses following vaccination with vaccinia Lister strain were not affected by pre-existing vaccinia immunity, with comparable levels of both achieved in vaccinia naïve and immune individuals (Kim *et al.*, 2005; Kim *et al.*, 2006). Similarly, MVA used as a vaccine showed no significant difference in the vaccinia-specific CD4+ and CD8+ T-cell responses elicited in pre-immunised compared to vaccinia-naïve individuals (Parrino *et al.*, 2007). In two non-human primate studies of vaccinia-naïve animals, MVA cellular responses were generated by one vaccination, but were not significantly higher after the second MVA or subsequent vaccinations (Earl *et al.*, 2004; Grandpre *et al.*, 2009). Dose and timing of immunisations very likely affects the responses. Humoral responses generated to MVA appear to be affected by the dose, with higher doses generating higher binding and neutralising antibody levels, while cellular responses do not appear as dose dependent (Wilck *et al.*, 2010; Keefer *et al.*, 2011). In other studies, however, it has been shown that MVA-specific cellular responses are boosted by subsequent vaccinations and are long-lasting (Smith *et al.*, 2005; Nigam *et al.*, 2007; Precopio *et al.*, 2007). MVA produces predominantly CD8+ T-cell responses and only low frequency and magnitude CD4+ T-cell responses (Parrino *et al.*, 2007; Kim *et al.*, 2006; Smith *et al.*, 2005; Nigam *et al.*, 2007; Earl *et al.*, 2004). MVA also generates binding and neutralising antibody responses by two or more vaccinations that are equivalent to those generated by Dryvax (Earl *et al.*, 2004; Nigam *et al.*, 2007; Wilck *et al.*, 2010). Interestingly, it has been suggested that using MVA as an HIV vaccine vector may have a dual role in protection against HIV and smallpox (Nigam *et al.*, 2007).

A DNA-MVA prime boost vaccination regimen found that HIV-specific responses were elicited after the MVA vaccination was given in the majority of individuals (Sandstrom *et al.*, 2008). However, individuals with pre-existing vaccinia-virus immunity had the same frequency of responses, but they were of a moderately lower magnitude than in those individuals who were

vaccinia-naïve (Sandstrom *et al.*, 2008; Gudmundsdotter *et al.*, 2009). They speculate that this was caused not only by pre-existing antibodies in these individuals, but they were also older and that age had a moderate negative effect on HIV responses. In a study by Howles *et al.* (2010) this was not seen, and there was no difference in responses to HIV inserts between those individuals that did or did not have pre-existing vaccinia immunity, however they used a much lower dose of rMVA (5×10^7). A non-human primate study, where some animals received Dryvax 17 months prior to receiving a DNA-MVA vaccination regimen containing SIV inserts, showed that vaccinia-naïve animals developed SIV responses after the first MVA vaccination that were boosted to higher levels after the second MVA vaccination. However, vaccinia-immune animals developed over twofold lower responses after the first MVA, responses were not boosted by the second MVA, and were twelvefold lower than in naïve animals (Kannanganat *et al.*, 2010). However, even though the magnitude of the SIV-specific responses was affected by pre-existing vaccinia immunity, upon challenge these animals did not show poorer control of virus than the vaccinia-naïve animals.

Anti-vector immunity also refers to immunity generated by the vaccination regimen itself. Individuals may have never been exposed to the vector before, so the first vaccination may generate immune responses to the vaccine antigens presented, as well as the vector itself, and upon subsequent or boost vaccinations with the same vector, vector-specific immune responses generated after the first immunisation may dampen the effect of the second or third immunisations to the inserts. It has been suggested that poxviral immune responses, even in the absence of prior smallpox vaccination, are always immunodominant over vector inserts (Smith *et al.*, 2005). Even if this is the case, it is interesting to study what happens to the insert specific immune responses when MVA is given more than once in individuals with no pre-existing vaccinia immunity before vaccination. It has indeed been observed that vector-specific responses can negatively impact upon the immunogenicity of later vaccinations if they are given repeatedly (Sharpe *et al.*, 2001). In other non-human primate studies, SIV-specific CD4+ and CD8+ responses were generated to a DNA-MVA vaccine vaccination regimen, with CD4+ T-cell elicited at higher magnitude. Cellular responses peaked after the first MVA vaccination, and were not boosted to higher levels by the second MVA vaccinations (Horton *et al.*, 2002; Lai *et al.*, 2011). Notably though in the study by Lai *et al.* (2011), even though SIV-specific cellular responses were not boosted by a second MVA vaccination, 71% of these animals were protected from 12 rectal challenges with SIVsmE660. This protective effect was shown to correlate with the avidity of the anti-Env antibody responses. Lack of boosting of insert specific responses to subsequent MVA vaccines has also been described in human studies, where the magnitude of HIV-specific responses reached their peak after the first MVA vaccination and did not increase after the second MVA (Goepfert *et al.*, 2011; Guimaraes-

Walker *et al.*, 2008). Possibly combining the DNA-MVA vaccination regimen with a boost of a different viral vector could result in increased insert specific responses.

1.3.5.2 Ad5 and STEP

Adenovirus serotype 5, Ad5, is endemic around the world, with 30 – 70 % of people in the United States, and as many as 90 % of individuals in sub-Saharan Africa, having neutralising antibodies due to natural exposure (as reviewed in Sauter *et al.*, 2005). Anti-vector neutralising antibodies limit repeat administration of Ad5 vaccines, as they bind the virus and prevent the infection of host cells and thus delivery of vaccine antigens (Chirmule *et al.*, 1999).

The STEP trial (discussed in section 1.2.7) showed that a homologous Ad5 vaccine expressing HIV-1 Gag, Pol and Nef genes elicited frequent INF- γ responses with a limited breadth of response, but failed to protect against HIV (McElrath *et al.*, 2008; Buchbinder *et al.*, 2008). Indeed, vaccinated individuals with pre-existing Ad5-specific neutralising antibodies (titres > 18) showed a 2.3-fold higher incidence of HIV-1 acquisition (Buchbinder *et al.*, 2008; as reviewed in Corey *et al.*, 2009). It was also shown that uncircumcised men, mainly those that were Ad5 seropositive, had almost a four-fold higher incidence of HIV infection (as reviewed in Corey *et al.*, 2009). There was no increase in HIV acquisition seen in vaccinated Ad5 seronegative individuals. It is thought that the lack of efficacy may be due to the immune response generated by the vaccine being too narrow, where on average only one epitope in each of the Gag, Pol and Nef genes in the vaccine were produced in most vaccinated individuals (as reviewed in Corey *et al.*, 2009). This may be because continued boosting with the same vector (homologous boosting) can enhance immunodominance of the T-cell response (Smith *et al.*, 2005).

In addition to determining lack of protective effect of the vaccine, investigations are on-going to determine the cause of increased incidence of HIV acquisition among vaccinated individuals. The effect of circumcision and other epidemiologic confounders make it difficult to determine the cause, however a number of mechanisms have been suggested (as reviewed in Corey *et al.*, 2009). Notably this effect was not seen in the Phambili trial in South Africa with the same vaccine (Gray *et al.*, 2011). First, it is thought that vaccination in individuals with previous immunity to Ad5 have activation and expansion of their Ad5-specific CD4+ T-cells resulting in an increase in target cells for HIV to infect (Sekaly *et al.*, 2008). Activation was speculated to be mediated by the formation of Ad5 immune complexes (Perreau *et al.*, 2008). Hutnick *et al.* (2009) studied Ad5-specific CD4+ T-cell responses and Ad5 neutralising antibody titres in blood. However, their results do not support this theory, as they found no significant difference

in the magnitude, phenotype, function, proliferative capacity or circulating numbers of Ad5-specific CD4⁺ T-cells between Ad5 seropositive and seronegative individuals. Ad5 responses may, however, need to be assessed in mucosal tissues also. Finally, pre-existing Ad5 immunity may have other effects, such as production of enhancing antibodies that facilitate HIV infection (as reviewed in Corey *et al.*, 2009). There are likely other causes that need to be identified, and it is probably a result of a combination of many factors.

1.3.5.3 Attempts to reduce anti-vector immunity

Heterologous prime-boost strategies involve sequential immunisation with a common antigen incorporated into different vectors, such as a DNA prime vaccine and viral vector boost vaccine. This regimen has been shown to result in larger numbers of antigen-specific CD8 T cells than achieved by a single vaccine administration or homologous boosting by focusing the boost response on the vaccine antigens expressed in the vectors and preventing anti-vector immunity arising as a result of repeated administration of the same viral vector (Goonetilleke *et al.*, 2006; Sandstrom *et al.*, 2008; Goepfert *et al.*, 2011; Harro *et al.*, 2009; Gudmundsdotter *et al.*, 2009). DNA priming has been shown to overcome pre-existing vaccinia immunity in macaques vaccinated with an MVA-Ebola Glycoprotein vaccination (Yang *et al.*, 2003).

In addition to heterologous prime-boosts researchers are attempting to reduce pre-existing immunity by using different Adenoviral serotypes (section 1.3.3.2), developing alternative vaccine vectors (section 1.3.3.4) or modifying existing vaccine vectors (Naito *et al.*, 2007; Roberts *et al.*, 2006). Roberts *et al.* (2006) engineered Ad5 to remove neutralising epitopes on the viral surface. They replaced these epitopes with corresponding regions from Ad48, which is uncommon in humans. The vaccine showed good immunogenicity in nonhuman primates, even in the presence of pre-existing Ad5-specific immunity (Roberts *et al.*, 2006). Modification of the capsid structure of recombinant MVA or deletion of antigenically complex genes has shown that MVA can also be engineered to be resistant to pre-existing immunity (Naito *et al.*, 2007; Garber *et al.*, 2009).

1.4 Aims and Objectives

The HIV vaccine group at UCT has developed a candidate HIV vaccine, consisting of MVA expressing several genes from HIV-1 subtype C. This vaccine is a component of an immunisation regimen that is currently in Phase I clinical trials, and has been given as multiple vaccinations to boost a recombinant DNA vaccine expressing the same HIV genes. HIV-specific immune responses generated by the vaccines have been evaluated in preclinical trials in non-human primates. These studies reveal that the candidate vaccines generate potent HIV-specific immune responses that peak one week after MVA immunisation (Burgers *et al.*, 2009). An interesting observation from this study was that MVA vaccinations given in close succession do not appear to boost responses to HIV antigens further, although these responses are boosted when MVA is given one year after the previous MVA vaccination. It is possible that anti-MVA immunity, i.e. immunity to the vector itself, limits the magnitude of the HIV response or the ability to boost responses after successive immunisations.

1.4.1 Aim

The aim of this study was to determine whether anti-vector immunity to MVA inhibits the ability to boost immune responses to the HIV antigens expressed by the vector in successive immunisations, and investigate the nature of MVA-specific immunity.

1.4.2 Hypothesis

Immune responses to the MVA vector inhibit immunity to the HIV antigens it expresses.

1.4.3 Specific Objectives

1. To optimise methods for detecting immune responses generated against the MVA vector.
2. To investigate humoral responses to MVA, including binding and neutralising antibodies.
3. To investigate cellular responses to MVA, including their magnitude, functional nature and memory phenotypes.
4. To determine the effect of MVA responses on HIV-specific responses from the same vector.

CHAPTER 2

Materials and Methods

University of Cape Town

Chapter 2

Materials and Methods

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2.1 Introduction

There were two aims to this study. The first was to measure humoral responses to MVA in preclinical immunogenicity studies in non-human primates, including both binding and neutralising antibody responses, and the second was to determine the magnitude, phenotype and function of T-cell responses to MVA in these animals.

2.2 Study design, samples and antigens

2.2.1 Animals

Nine wild-caught Chacma baboons (*Papio ursinus*) were used in a previous study describing the immunogenicity of candidate HIV vaccines (Burgers *et al.*, 2009). Archived cryopreserved PBMC and serum samples from eight of these animals from multiple time points were used for this study. One animal (B630) was excluded from the study because of insufficient sample availability. Animals were housed in the South African Medical Research Council (MRC) Primate Facility in Cape Town. This study also included limited analyses (only determination of neutralising antibody titres) on 22 macaques from a similar study using the same vaccine candidates. The macaques were housed at the same facility. All studies were approved by the Animal Ethics Committee of the Faculty of Health Sciences, University of Cape Town.

Chacma baboons are not widely used as the animal model of choice in vaccine research. However, at the time these experiments were performed, they were the only non-human primates available in Cape Town to perform preclinical immunogenicity studies for candidate HIV vaccines, prior to the subsequent establishment of a breeding colony for rhesus macaques. There are numerous advantages to this model, such as the high homology between human and baboon immune system components (Damian *et al.*, 1971), lack of sensitivity to infection with Herpes B virus (Kennedy *et al.*, 1997) and thus a lower safety risk for handlers, and their larger size which allows greater blood draw volumes for use in immunological testing. They are also outbred, which ensures a diverse genetic background between animals, similar to the situation in humans tested with vaccines (Murthy *et al.*, 2006). They have also been used in various safety and immunogenicity studies of HIV vaccine candidates, and it has been shown that they can present HIV peptides containing epitopes recognised in HIV-infected humans (Burgers *et al.*, 2009). Immune responses have been generated in baboons in response to vaccination with a DNA and MVA expressing HIV proteins (Burgers *et al.*, 2009) and to DNA and HIV Gag virus-like particles (VLP) (Chege *et*

al., 2008), as well as a rBCG prime and Gag VLP vaccine (Chege *et al.*, 2009). All these results suggest that the Chacma baboon may be used as a non-human primate model for immunogenicity studies.

2.2.2 Vaccines and immunisations

Two vaccine constructs were given in a prime-boost regimen. They consisted of DNA (termed SAAVI DNA-C) and recombinant MVA (SAAVI MVA-C), as depicted in Figure 2.1. SAAVI DNA-C contained an equal mixture of two plasmids, pThr.grttnC and pThr.gp150CT, and expressed five human codon-optimised HIV-1 subtype C genes, namely Gag, RT, Tat, Nef (GRTTN) and a C-terminal truncated Envelope gp150CT under the early promoter enhancer element from human cytomegalovirus. All genes were derived from subtype C strains Du151 and Du422, and modified to be non-functional to increase safety and immunogenicity (Burgers *et al.*, 2006). SAAVI MVA-C consisted of a live recombinant MVA that expressed the same five codon-optimised, non-functional HIV-1 subtype C genes (GRTTN and truncated Env gp150CT). The MVA was obtained by Therion Biologics (Cambridge, MA, USA) from Dr. Anton Meyer, University of Munich, Germany. It was three times purified on primary chicken embryo dermal (CED) cells, amplified and used to generate a master virus stock, TBC-MVA MVS lot 1. This was used to generate the recombinant MVA. Construction of the recombinant MVA has been described (Burgers *et al.*, 2008). Briefly, Env was expressed by the weaker vaccinia virus promoter I3, to increase stability of the virus, and GRTTN under the control of the vaccinia early-late-40K promoter. For the 22 vaccinated macaques, eight were primed with SAAVI DNA-C2, which differed from DNA-C in the regulatory elements, in that it contained a T-cell leukaemia virus type 1 regulatory (HTLV1-R) element in addition to the CMV promoter (Barouch *et al.*, 2005). This was shown to enhance the immunogenicity of HIV DNA vaccines in mice and nonhuman primates (Barouch *et al.*, 2005). Since there were no differences in HIV-specific responses between the macaques that received DNA-C or DNA-C2, these were grouped together as 'DNA-MVA' animals for the purposes of this thesis.

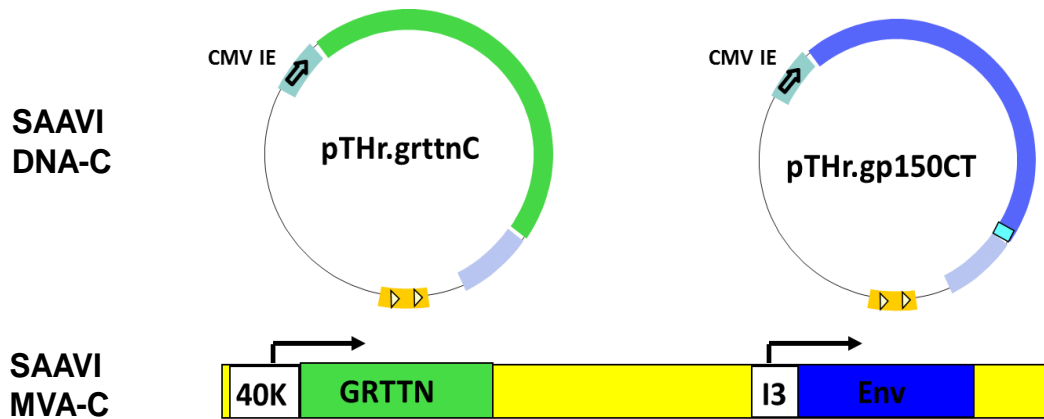


Figure 2.1 SAAVI DNA-C and SAAVI MVA-C vaccine constructs. The constructs contain genes encoding HIV-1 subtype C Gag, RT, Tat, Nef and truncated Env. SAAVI DNA-C encodes the genes under control of the CMV intermediate-early promoter, whereas the SAAVI DNA-C2 construct given to macaques (not depicted) contained the HTLV1-R enhancer. SAAVI MVA-C expressed the same genes under control of the I3 and 40K promoters in a single recombinant virus.

Five of the eight baboons (B515, B534, B531, B549 and B575) received three SAAVI DNA-C priming vaccinations of 4 mg one month apart, delivered intramuscularly. The remaining three animals (B533, B623 and B629) received empty DNA with no insert according to the same regimen. All eight animals received SAAVI MVA-C (10^9 pfu) 33 weeks later (week 41), and two months later at week 49. One year after the second MVA boost, at week 105, four animals (B515, B549, B575 and B623) received a third MVA-C boost (Figure 2.2 A). The immunisation regimen for the macaques was slightly different, as it was based on the proposed schedule for Phase I clinical trials (Figure 2.2 B). Eight macaques (P18, P23, P24, P27, P37, P41, P45 and P67) received three SAAVI DNA-C priming vaccinations of 4 mg one month apart, delivered intramuscularly. A further eight macaques (P3, P28, P30, P31, P32, P34, P36 and P38) received three SAAVI DNA-C2 primes. The remaining six animals (P1, P2, P5, P25, P36A and P66) received no DNA vaccination. All 22 macaques received SAAVI MVA-C (10^9 pfu) eight weeks later (week 16), and again four weeks later at week 20.

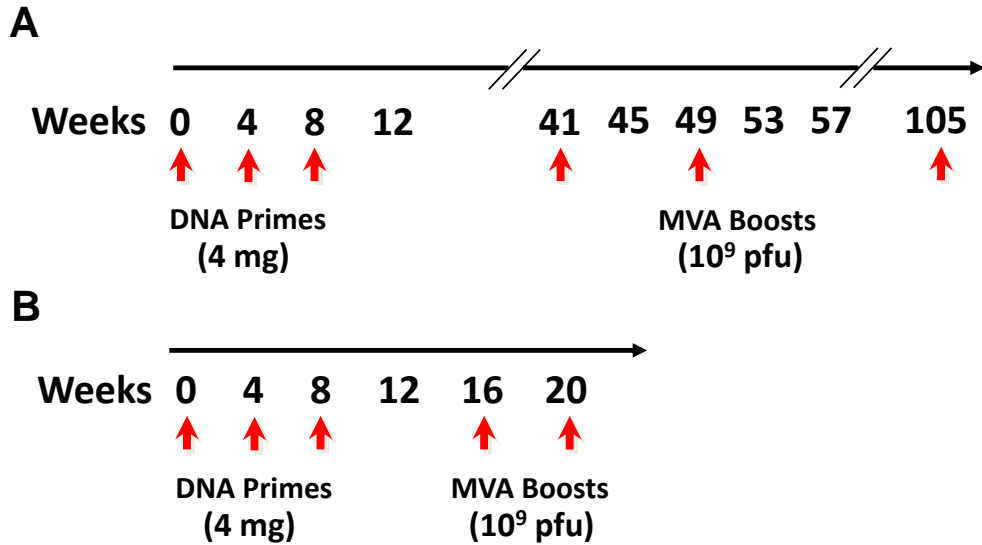


Figure 2.2 Immunisation schedules. (A) Five baboons received three SAAVI DNA-C (4 mg) priming vaccinations and three received DNA with no vector. All baboons received SAAVI MVA-C (10^9 pfu) at week 41 and 49. At week 105, four baboons received a third MVA-C boost. (B) Sixteen macaques received three SAAVI DNA-C (4mg) or SAAVI DNA-C2 (4mg) priming vaccinations and six received DNA with no vector. All macaques received SAAVI MVA-C at week 16 and 20.

2.2.3 Sample collection

Blood samples were collected from the animals at various time points pre- and post-immunisation. This included 20 – 60 ml whole blood, not exceeding 6.6 ml/kg body mass per animal per occasion, collected by venepuncture of the femoral vein into Heparin vacutainer tubes (BD) and 5 – 10 ml into serum separator tubes (SST; BD). Heparin blood samples were used for isolation of peripheral blood mononuclear cells (PBMC). The SST samples were used to prepare sera aliquots that were stored at -20°C for ELISA to be performed at a later date.

2.2.4 Antigens

A range of antigens were used in the immunological assays (Table 2.1). Purified vaccinia virus membrane proteins A27, A33, B5R and L1R were obtained from Dr Gary H. Cohen and Dr Roselyn J. Eisenberg, School of Dental Medicine, University of Pennsylvania, for use in ELISA. These vaccinia proteins were produced using a baculovirus expression system and have been used previously for detection of MVA antibodies (Aldaz-Carrol *et al.*, 2005; Cosma *et al.*, 2007; Earl *et al.*, 2007; Parrino *et al.*, 2007). A27 and L1R are intracellular mature virion (IMV) membrane proteins involved with viral replication and cell attachment leading to viral spread

among hosts, while A33 and B5R are membrane proteins involved in the formation of extracellular enveloped virus (EEV) which is involved in cell to cell viral spread (Smith *et al.*, 2002).

For MVA-*gfp* (*green fluorescent protein*) neutralisation assays, recombinant MVA-*gfp* was obtained from Michael Cottingham, University of Oxford. MVA used in T-cell assays (ELISPOT and intracellular cytokine staining) was obtained from Bernard Moss, National Institutes of Health. MVA and MVA-*gfp* were grown on the chorioallantoic membranes (CAMs) of 9-12 day old fertilised hens' eggs (modification of method of Westwood *et al.*, 1957) and the titre determined in baby hamster kidney (BHK-21) cells. A rabbit anti-vaccinia antibody (Biogenesis Ltd) and swine anti-rabbit HRP (Dako) were used for immunostaining to determine the viral titres expressed as plaque forming units per ml (pfu/ml). Niki Douglass and Anke Binder, University of Cape Town, grew up the virus and performed the titrations, respectively. The titre of MVA-*gfp* was 2×10^8 pfu/ml and the titre of MVA was 8.5×10^8 pfu/ml. Aliquots of each were stored at -70°C . MVA-*gfp* and MVA were used at a multiplicity of infection (MOI) that was optimised as described in Chapter 3. MOI is the ratio of virus to the ratio of target cells (Knipe and Howley, 2007).

Synthetically synthesized HIV-1 subtype C peptides overlapping by 10-11 amino acids each were used to simulate HIV-specific responses for detection by intracellular cytokine staining. The peptides were synthesized using 9-Fluorenylmethoxy carbonyl chemistry and standard based solid phase techniques (Masemola *et al.*, 2004). They varied in length between 15 and 18 amino acids, overlapping by 10-11 amino acids to ensure that when combined into pools, the whole gene is expressed and no epitopes are lost. Gag consisting of 121 15-18 mer peptides from Du422, was obtained from the NIH Research and Reference Reagent Repository, USA. Nef consisting of 50 15-18 mer peptides from Du151 was kindly supplied by Prof. Clive Gray of the National Institute of Communicable Diseases, South Africa. The Du HIV strains were used because they are close to a South African consensus sequence (Williamson *et al.*, 2003). They were all received in lyophilised form and resuspended in dimethyl sulfoxide (DMSO; Sigma-Aldrich) at a concentration of 10 mg/ml. One aliquot of each peptide was further diluted in RPMI (Invitrogen) to a concentration of 40 $\mu\text{g/ml}$, and stored at -70°C . HIV peptides were combined into a Gag and a Nef pool, and used at a final concentration of 2 $\mu\text{g/ml}$.

TABLE 2.1 Antigens used in immunological assays

Reagent	Description	Working concentration	Manufacturer
A27	IMV (Intracellular mature virion) purified vaccinia membrane protein	1 mg/ml	University of Pennsylvania
A33	EEV (Extracellular enveloped virus) purified vaccinia membrane protein	1 mg/ml	University of Pennsylvania
B5R	EEV purified vaccinia membrane protein	1 mg/ml	University of Pennsylvania
L1R	IMV purified vaccinia membrane protein	2 mg/ml	University of Pennsylvania
MVA-gfp	MVA expressing green fluorescent protein (GFP) reporter gene	MOI optimised (Chapter 3)	In-house
MVA	Wild type virus grown up in chick embryos	MOI optimised (Chapter 3)	In-house
HIV Gag superpool	A pool of 121 15-18 mer Gag peptides overlapping by 10 amino acids: HIV-1 subtype C Du422	2 µg/ml	Natural and Medical Sciences Institute, University of Tübingen, Tübingen, Germany
HIV Nef superpool	A pool of 50 15-18 mer Gag peptides overlapping by 10 amino acids: HIV-1 subtype C Du151	2 µg/ml	Natural and Medical Sciences Institute, University of Tübingen, Tübingen, Germany

2.3 Sample processing

2.3.1 PBMC Isolation

Cell based immunology assays require the isolation of peripheral blood mononuclear cells (PBMC) from freshly anticoagulated whole blood. The method involves layering of whole venous blood onto Ficoll (Ficoll-Hypaque; Sigma-Aldrich), a polysucrose and sodium

diatrizoate solution with a density of 1.077 g/ml. Upon centrifugation, this solution aggregates the erythrocytes and granulocytes, causing them to sediment. Mononuclear cells, consisting mainly of lymphocytes, do not aggregate and remain at the plasma-Ficoll interface. Contamination by erythrocytes is minimal, and most platelets are removed during subsequent washing steps (Detrick *et al.*, 2006).

Heparinised whole blood (20-60 ml) was collected from each animal by venepuncture and density gradient centrifugation performed within 5 h. All processing was performed at room temperature. Briefly, 15 ml Ficoll was added to 50 ml Leucosep tubes (Greiner Bio-one) and centrifuged at 1000 x g for 1 min to force the Ficoll below the separation filter. Blood (not exceeding 30 ml) was poured into the Leucosep tubes on top of the separation disk and centrifuged for 15 min at 1000 x g to separate the erythrocytes, mononuclear cells and plasma (Figure 2.3). Cells from the interface were removed carefully being sure not to remove too much supernatant containing plasma proteins and platelets, or too much of the Ficoll containing residual granulocytes. The cells were washed twice in wash buffer, consisting of PBS containing 1% FBS, resuspended in a fixed volume of wash buffer and counted (section 2.4.2) to determine cell number and viability. They were then cryopreserved (section 2.4.3) and stored in liquid nitrogen.

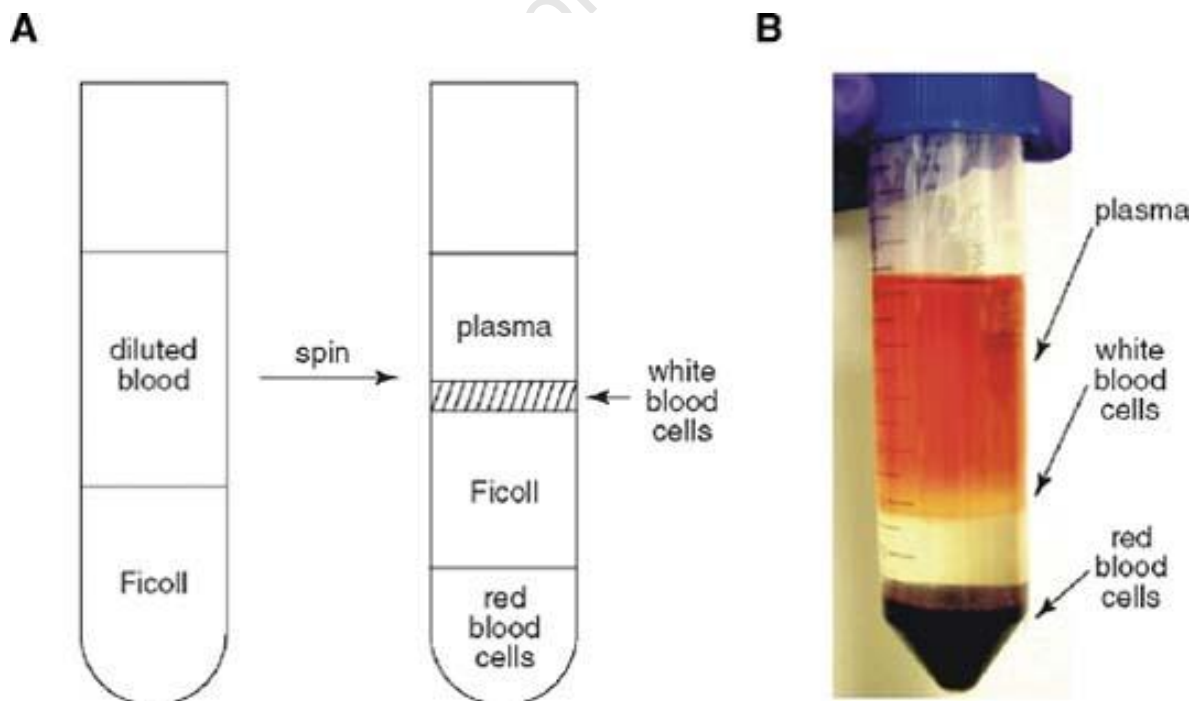


Figure 2.3 Density gradient centrifugation for PBMC isolation using the Leucosep method. (A) After centrifugation, separation of blood components into plasma, mononuclear cells and erythrocytes occurs. (B) An actual separation of whole blood (taken from Lan *et al.*, 2007).

2.3.2 Cell Counting

Two cell counting techniques were performed. Manual counting of freshly isolated PBMC was performed by trypan blue (Sigma-Aldrich) staining to determine the optimal concentration for long term storage of cells. Trypan blue is used in dye exclusion procedures for viable cell counts. Non-viable cells take up the trypan blue because of damage to the cell membrane, and stain dark blue, while viable cells remain refractile. An automated cell counter (Guava; Millipore) later became available, and automated counting of thawed PBMC was subsequently performed. The ViaCount reagent (Millipore) contains two fluorescent DNA-binding dyes that differentially stain viable and non-viable cells based on their permeability to the dyes.

Manual cell counting

Counting was performed using a haemocytometer, as shown in Figure 2.4. The count was performed by loading a 1:1 dilution of cell suspension in 0.4% trypan blue into the well of an Improved Neubauer counting chamber (Hausser Scientific). The numbers of viable nucleated cells and nonviable cells that appeared in the top-left square (A) and bottom-right square (B) of the grid were counted using a light microscope.

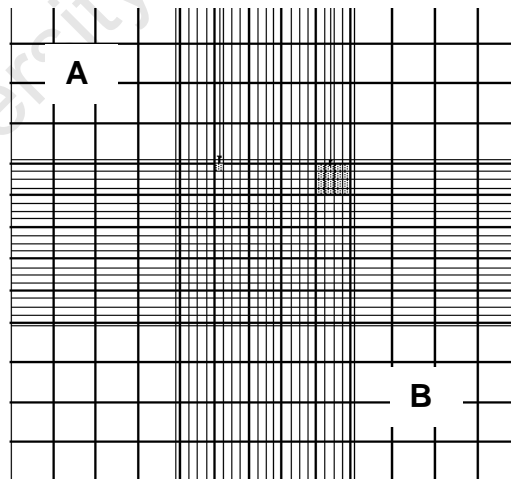


Figure 2.4 Improved Neubauer counting chamber. The grid is divided into 9 large squares, each 1mm x 1mm, by triple lines. There are four large outer squares that consist of 16 smaller squares. Viable and non-viable cells in outer squares A and B were counted (image adapted from Experimental Biosciences handout, Rice University, USA).

The Neubauer chamber is designed so that the total number of cells in one set of 16 corner squares is equivalent to the number of cells per μl (1000 cells per ml). Thus, to obtain the concentration of viable cells, the count of viable cells from the two sets of 16 corner squares (A and B in Figure 2.3) was added, divided by two to average them and then multiplied by two to adjust for the 1:2 dilution in trypan blue. Viability is the percentage of viable cells to total cells counted.

Automated cell counting

Automated cell counting was performed using the Guava ViaCount assay and Guava Personal Cell Analysis system (PCA; Millipore) that contains a green laser. The ViaCount assay is performed using the ViaCount application that is part of the CytoSoft software, version 2.1.4. A 1:20 dilution of cells in Guava ViaCount reagent was incubated in 2 ml microtubes (QSP) for 8 min at room temperature in the dark. All stained nucleated cells were counted and FSC properties used to separate cell debris from actual cells and provide absolute cell counts and viability. Once these counts were obtained, the appropriate cell concentration adjustments required for the assay were made.

2.3.3 Cryopreservation and thawing of PBMC

After PBMC were isolated and counted, they were ready for cryo-preservation and storage in liquid nitrogen for long periods of time before use. This process involves freezing cells in a media containing DMSO, which is cryoprotectant agent that protects the cells by eliminating formation of ice crystals that can damage cell membranes and result in cell death (Weinberg *et al.*, 2000). In addition, the temperature of the samples is gradually lowered in cryo freezing containers (Mr Frosty; Nalgene) that contain isopropanol (Sigma-Aldrich) which controls the rate of freezing to $-1^{\circ}\text{C}/\text{min}$ and a -70°C freezer. This protects protect the samples from release of energy in the form of heat when it changes in state from liquid to crystalline form.

All cryo-preservation work was carried out on crushed ice to limit the damage to the cells by the toxicity of DMSO. After cell counting, the PBMC were centrifuged at $320 \times g$ for 10 min. They were then resuspended carefully drop-wise in fresh ice-cold freezing to a final concentration of $10\text{-}15 \times 10^6$ cells/ml. Aliquots of one ml were pipetted into pre-cooled labelled cryovials (Greiner Bio-one) and transferred into cooled Mr Frosty containers. The Mr Frosty container was kept at -70°C overnight, and the cells transferred within 24 h to liquid nitrogen for long term storage.

PBMC were carefully and quickly thawed to protect them against damage by osmotic swelling upon rehydration, as well as the toxicity of DMSO. PBMC vials were removed from liquid nitrogen and kept on dry ice until ready to be thawed. They were placed in a waterbath at 37°C until the cell suspension was almost completely melted or a small bit of ice remained. Cells were added to a 50 ml centrifuge tube (Sterilin) and 10 ml of pre-warmed R1 (Table 2.2) was added drop-wise while swirling the cells. Cells were washed by topping up to 25 ml with R1 and centrifuging at 230 x g for 10 min to remove the DMSO. The supernatant was decanted and cell pellet resuspended in 500 µl of 0.02 mg/ml DNase (Roche) for 2 min. DNase prevents clumping of the cells by digesting DNA and hydrolysing the phosphodiester linkages resulting in a mixture of oligo- and mononucleotides. Cells were washed as before then resuspended in R20 at a concentration of between 2 and 4 x 10⁶ cells/ml without exceeding 5 ml in a tube and left overnight at 37°C with 5% CO₂. The following morning cells were resuspended cells gently and the cell count and viability determined. Cells were washed in 25 ml R10 then adjusted to 2 x 10⁶ cells/ml for use in the IFN-γ ELISPOT assay.

2.4 Detection of antibody responses to MVA

2.4.1 Binding Antibody responses by Indirect ELISA

Enzyme-Linked ImmunoSorbent Assay (ELISA) can detect the presence of antibody or antigen in a sample (Hornbeck *et al.*, 2001). In indirect ELISAs, an antigen of known concentration is adsorbed onto wells of a plastic plate. After washing to remove excess antigen, the test item, such as serum containing an unknown amount of antibody, is incubated with antigen. If present, antibody binds to adsorbed antigen and this is detected by means of a secondary antibody conjugated to an enzyme specific for a particular substrate. This then results in a colour change that is used to quantify the relative amount of specific antibodies present.

MaxiSorp flat bottom plates (96-well; Nalgene) were coated overnight at 4°C with purified vaccinia virus proteins in carbonate buffer (pH 9.6; Sigma-Aldrich) at 1 mg/ml (A27, A33 and B5R) or 2 mg/ml (L1R). Plates were washed six times with sterile phosphate-buffered saline (PBS, pH 7.4; Sigma-Aldrich) using an AutoStrip Elix50 Plate Washer (BioTek), then incubated at room temperature for 2 h with 0.5% polyvinyl alcohol (PVA; Sigma-Aldrich) to remove any unbound antigen (Studentsov *et al.*, 2002). Frozen sera samples were thawed and diluted 1:40 in 0.5% PVA solution. Plates were washed a further six times and 100µl diluted serum

added to the wells in duplicate. After incubation at 37°C in 5% CO₂ for 2 h, plates were washed as before, followed by incubation with a 1:6000 dilution of anti-monkey IgG-peroxidase conjugate (Sigma-Aldrich) in 0.8% polyvinylpyrrolidone (PVP; Sigma-Aldrich) at 37°C for 1 h. After washing to remove unbound conjugate 100 µl TMB (tetramethylbenzidine) peroxidase substrate (KPL) was added per well for 10 min at room temperature in the dark. The reaction was stopped by adding 50 µl 2N sulphuric acid (Sigma-Aldrich) to each well. Optical densities were determined within 30 min at a wavelength of 450 nm by using a VersaMax microplate reader (Molecular Devices) and SOFTmax PRO software (Version 4.3.1).

Negative and positive controls were included on each plate. There were three negative controls, namely PVA only wells, wells that received no secondary antibody and wells that received no substrate. These negative controls were included to ensure that there was no background interference by the sample diluent, secondary antibody or substrate. Negative controls receiving no secondary antibody and no substrate were not to exceed the optical density of the PVA negative control. The positive control consisted of reference vaccinia immune globulin (VIG; kindly provided by Dr Christine Anderson, Food and Drug Administration, USA). All controls and samples were set up in duplicate and the optical density of the negative PVA control subtracted from each sample. Sera were initially screened for reactivity at 1:40, and positive samples subsequently titred using two-fold dilutions starting at 1:40. A positive response was taken as a response greater than twice the response for the pre-immune sera of the same animal run concurrently. Results are expressed as end-point titres, which is the reciprocal of the highest dilution whose optical density value was greater than twofold over the pre-immunisation sera at the lowest dilution.

2.4.2 Neutralising antibody responses by MVA-*gfp* neutralisation assay

Virus neutralisation assays measure a reduction in viral infectivity as a result of neutralising antibodies. Traditionally this was measured by means of plaque reduction neutralisation tests (PRNT), which showed fewer foci of infection in mammalian cell monolayers in the presence of neutralising activity. Disadvantages of PRNTs are that they are time-consuming, labour-intensive and subject to visual user interpretation that can be a source of error (Earl *et al.*, 2003). Newer assays have been developed that use MVA expressing green fluorescent protein reporter gene (MVA-*gfp*), and MVA-*gfp* infected cells are quantified by flow cytometry (Earl *et al.*, 2003; Cosma *et al.*, 2004). This assay can be performed more rapidly than traditional PRNTs, and flow cytometric analysis excludes some of the subjectivity and user error. This assay yields results equivalent to that of PRNT with the advantage that it can be

performed more rapidly than traditional PRNT, and flow cytometric analysis excludes some of the subjectivity and user error. Decreased fluorescence relative to the maximum percentage of GFP expressing cells indicates that virus has been neutralised by some degree by the sera being tested (Earl *et al.*, 2003; Cosma *et al.* 2004). The protocol described below is based closely on that described in Earl *et al.*, 2003, although they used vaccinia virus and not MVA. HeLa cells were chosen as the cell line to use since they are easy to culture and MVA undergoes a block in assembly that does not allow it to undergo replication or grow permissively in these cells (Sancho *et al.*, 2002). Thus, there is no secondary infection of cells with new MVA virus particles, and the MOI can be controlled.

All tissue culture reagents used are summarised in Table 2.2. HeLa cells were maintained in culture using D10 in 200 ml tissue culture flasks (Corning). Cell cultures were split every two to three days when they reached confluency to maintain healthy, viable cell cultures at a density of between 2 - 5 x 10⁵ cells/ml.

TABLE 2.2 Commonly used tissue culture reagents

Reagent	Description	Manufacturer
RPMI-1640	Cell culture growth media containing Glutamax-I and 25 mM HEPES	Invitrogen
DMEM	Dulbeccos modified Eagle's medium, containing amino acids and vitamins for cell culture	Invitrogen
PBS	Phosphate buffered saline pH 7.2 without CaCl ₂ or MgCl ₂	Invitrogen
FBS	Heat Inactivated foetal bovine serum	Delta bioproducts
Penicillin	Antibiotic mix containing 5000 units/ml Penicillin and 5000	Invitrogen
Streptomycin	units/ml Streptomycin	
Fungin	Soluble form of Pimaricin, an anti-fungal agent	Invitrogen
Trypsin	A serine protease that hydrolyses proteins and is used for dissociation of tissues and cells	Sigma-Aldrich
R1	1% FBS in RPMI + 50 mg/ml Penicillin Streptomycin	In-house
R10	10% FBS in RPMI + 50 mg/ml Penicillin Streptomycin	In-house
R20	20% FBS in RPMI + 50 mg/ml Penicillin Streptomycin	In-house
D10	10% FBS in DMEM + 50 mg/ml Penicillin Streptomycin + 0.8 mg/ml Fungin	
Freezing solution	10% DMSO + 90% FBS	In-house

Once HeLa cells reached at least 80% confluence, they were treated with 5 ml of 2.5 mg/ml trypsin for 2 min at 37°C to dislodge them from the culture flask. Cells were then well resuspended and viewed under a light microscope to confirm that detachment had taken place

and that they were in suspension. The trypsin was deactivated by adding 2 ml FBS. Cells were counted manually as described in section 2.4.2 below and adjusted to a count of 5×10^6 cells/ml in D10. Frozen serum samples were thawed, heat inactivated for 30 min at 56°C, and serially diluted (starting at 1:40) in D10. A pre-determined dilution of VIG was prepared as a positive control (see Chapter 3). Each dilution was performed in duplicate. MVA-*gfp*, at an optimised MOI (see Chapter 3), was added to 50 µl of each sera dilution or control in 96-well round bottom tissue culture plates (Nalgene). The plate was incubated for 2 h at 37°C. During this incubation step, the plate was gently agitated by hand every 15-20 min to allow maximum contact between any neutralising antibodies present in the sera and the viral particles. After this period, 50 µl of the HeLa cell suspension was added to the wells and the plate incubated for 2 h at 37°C to allow cell infection to take place. Next, the plate was washed twice with 100 µl per well of D10 by centrifuging for 10 min at 320 x g and excess supernatant removed carefully with a pipette. The plates were then incubated at 37°C for a pre-determined length of time (see Chapter 3). Plates were washed with 100 µl per well PBS by centrifuging for 3 min at 1000 x g. Cells were removed by treatment with trypsin as before, with 50 µl per well and 10 µl FBS to halt the reaction. Cells were then fixed with 100 µl CellFIX (BD) and transferred to FACS tubes for flow cytometric acquisition within 24 h.

Negative and positive control wells were included in each assay. The negative control consisted of uninfected cells where no MVA-*gfp* was added to ensure that there was no fluorescence generated. There were two positive controls, namely an infection control where wells received no VIG or serum, and a neutralisation control, where wells received VIG. All controls were performed in duplicate.

Samples were acquired on a FACSCalibur flow cytometer (BD) using CellQuest Pro software (Version 5.2). The percentage GFP expression was determined by gating cells on forward scatter (FSC) and side scatter (SSC), and GFP expression analysed in the FL-1 channel. A minimum of 100 000 total events were captured per sample. Results are expressed as neutralising end-point titres which is the reciprocal for the highest dilution of sera that can inhibit 50% or more GFP expression.

The following protocol parameters were optimised; (1) the optimal time period to infect HeLa cells with MVA-*gfp*; (2) the optimal MOI of MVA-*gfp* for infection; and (3) the optimal concentration of VIG to use as a positive neutralisation control. Results are described in Chapter 3.

2.5 Detection of Cellular T cell responses to MVA

2.5.1 Interferon- γ ELISPOT Assay

The IFN- γ Enzyme-linked immunosorbent spot (ELISPOT) is an ELISA-based technique that was developed for the detection of antibody-secreting B cells (Czerkinsky *et al.*, 1983). It is widely used for studying immune responses, particularly in vaccine trials, and is robust, easy to perform and quality control. It can determine both the type of response (what cytokine) and the magnitude of the response (number of responding cells). Each spot that develops represents a single reactive cell, thus providing information at a single-cell level (Masemola *et al.*, 2004; Streeck *et al.*, 2009). Antibodies commonly used for detecting IFN- γ in human samples were previously shown to cross-react and detect IFN- γ from baboons (Chege *et al.*, 2005).

Ninety-six well polyvinylidene difluoride plates (Millipore) were coated overnight at 4°C with 50 μ l per well of 5 μ g/ml anti-IFN- γ monoclonal capture antibody (clone 1-D1K; Mabtech). The following morning, the plates were washed three times with 200 μ l sterile PBS and then incubated at room temperature for 2 h with 100 μ l/well of R10 to remove any unbound capture antibody. Cryopreserved PBMC were thawed and adjusted to a concentration of 2×10^6 cells/ml. They were infected with MVA for a pre-determined length of time, and added to the wells at a final concentration of 100 000 cells per well, in triplicate. MVA MOI and infection times were optimised and are described in Chapter 3. After incubation at 37°C in 5% CO₂ for 20-24 h, plates were washed six times with 0.05 % PBS-Tween20 (Sigma-Aldrich, St. Louis, MO, USA), followed by incubation with a biotinylated anti-IFN- γ monoclonal antibody (2 μ g/ml; clone 7-B6-1; Mabtech) at 37°C for 2 h. After washing, 2 μ g/ml of Streptavidin-horseradish peroxidase (BD) was added to the wells and incubated for 1 h at room temperature in the dark. Nova Red substrate (Vector Laboratories) was prepared by adding three drops of reagent 1, two drops of reagent 2 and two drops of hydrogen peroxide in 15 ml sterile distilled water. After mixing well, 100 μ l was added to each well and left at room temperature in the dark until spots became visible and before background began to darken (approximately 6 min).

Duplicate wells containing uninfected PBMC for each sample were used as negative controls, as well as duplicate wells containing PBMC treated with 8 μ g/ml phytohaemagglutinin (PHA, Sigma-Aldrich) as a positive control. PHA is a lectin found in plants that induces mitosis in

cells and stimulates cytokine production and hence spot formation in an ELISPOT assay. The number of spots per well were counted using with an Immunospot (Cellular Technology) automated plate counter. The plates are first scanned using Image Acquisition software 3B and the spots are then counted using Immunospot version 3.1. Results are reported as net spot forming units per million PBMC (SFU/10⁶ PBMC), where uninfected PBMC wells were subtracted from stimulated wells. In addition, a response was only considered to be positive when values exceeded a cut-off of three times greater than the background, and greater than or equal to 60 net SFU/10⁶ PBMC.

2.5.2 Intracellular cytokine staining (ICS) and flow cytometry

Flow Cytometry measures the size, granularity and fluorescence intensity of a stream of single cells as they pass through a beam of laser light (Detrick *et al.*, 2006). Cells are labelled with antibody-conjugated fluorochromes and aspirated into the sample port of a flow cytometer. As they pass through the laser in single file in an isotonic fluid, light is scattered and fluorochromes excited. These fluorescent chemicals emit light of a different wavelength to the laser light source. The analyser has a number of detectors that measure FSC and SSC. The FSC detector is in line with the light beam and the SSC detector is perpendicular to it. FSC gives an indication of cell volume, whilst SSC reveals the inner complexity of the cell, such as granules in the cytoplasm. A FACSCalibur flow cytometer (BD) has two lasers and four detectors and was used for neutralisation assays described above, whereas the LSR II (BD) used in ICS has three lasers and 14 detectors, allowing more complex staining panels (Figure 2.5). The analysers both have optical coupling systems that convert the detected light signals into electronic (FACSCalibur) or digital (LSR II) signals by means of the PMT (photomultiplier tube).

Antigen stimulation

Cytokines may be produced in response to antigenic stimulation and can be measured by ICS and flow cytometry (Chattopadhyay *et al.*, 2008). In addition to the antigen stimulation, other molecules are necessary to induce cytokine production. This includes the co-stimulatory molecules anti-CD28 and anti-CD49d, which work together with presentation of the antigen to provide a signal to the T-cells via the T-cell receptor (TCR). This triggers the induction of transcription factors and subsequent cytokine production by the activated T-cell (Waldrop *et al.*, 1997). Brefeldin-A (BFA), an antibiotic produced by the fungus *Penicillium brefeldianum*, causes the cytokines produced to be accumulated inside the cell. It does this by inhibiting

golgi transport of newly synthesised proteins out of the cell (Klaussner *et al.*, 1992) which allows their production to be detected by ICS.

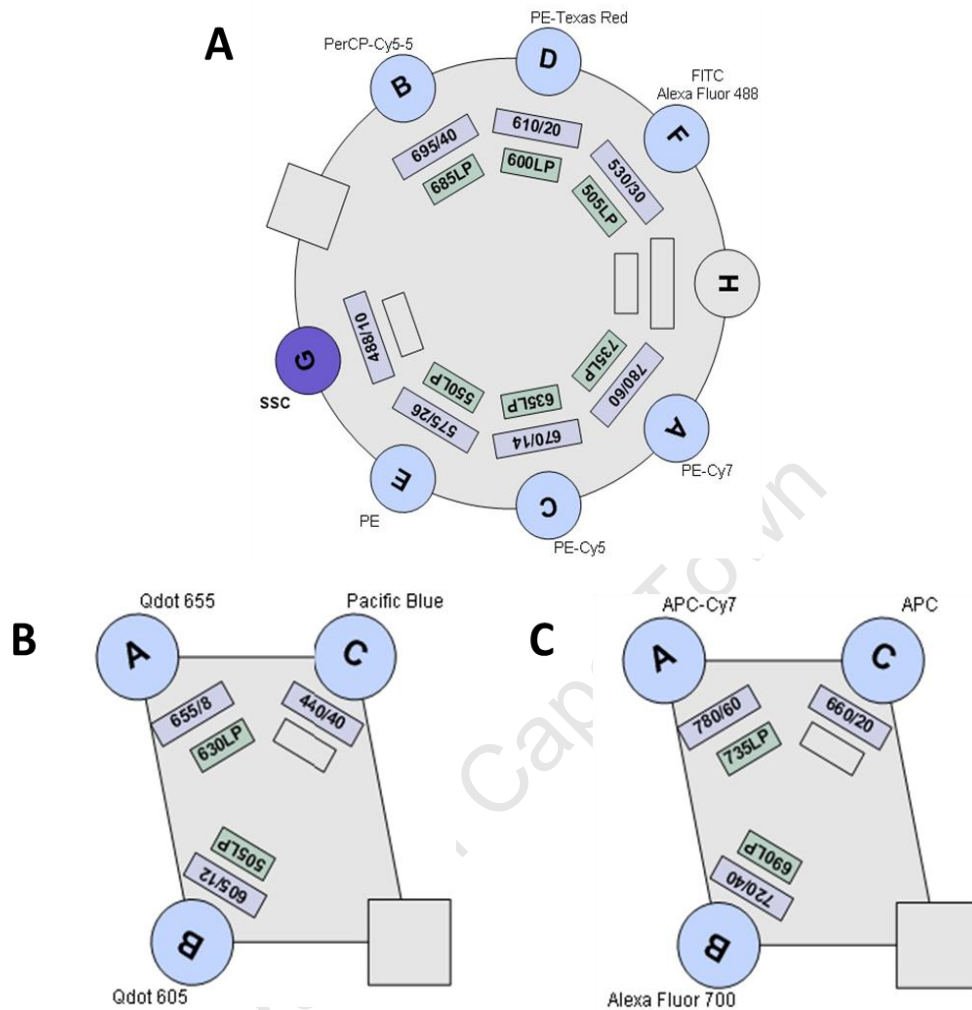


Figure 2.5 Configuration of the detectors of the LSRII used in these studies. (A) Detectors for the blue laser (octagon), (B) detectors for the violet laser (trigon) and (C) detectors for the red laser (trigon).

Cryopreserved PBMC were thawed and rested overnight to reduce non-specific background responses (Horton *et al.*, 2007), and adjusted to a concentration of 1×10^6 cells/ml. Some of each cell suspension was removed and left unstimulated to act as a negative control, while the remainder was stimulated with MVA or with HIV peptide mixes (described in section 2.2.3). All reagents used are described in Table 2.3. Peptide stimulations were performed in v-bottom tissue culture plates (Nalgene, Rochester, NY, USA) by resuspending cell pellets in $100 \mu\text{l}$ a stimulation mix consisting of R10, $0.02 \mu\text{g/ml}$ DNase, $1 \mu\text{g/ml}$ of both anti-CD49d and anti-CD28, $0.5 \mu\text{g/ml}$ BFA and $2 \mu\text{g/ml}$ Gag or Nef HIV peptides. Gag or Nef peptides were used based on the immunodominant peptide pool response as measured by IFN- γ ELISPOT and reported previously (Burgers *et al.*, 2009). Cells were incubated at 37°C in $5\% \text{CO}_2$ for 16-18 h

before washing and staining. MVA stimulations were performed in 15 ml tubes by incubating 1×10^6 cells/ml with MVA at 37°C. The cells were washed in R10 to remove excess virus, transferred to v-bottom 96-well plates, and incubated for a pre-determined period (Chapter 3), after which 1 µg/ml BFA was added. The plate was incubated a further 16 h before staining.

TABLE 2.3 Common reagents used in stimulation and staining

Reagent	Composition	Usage	Supplier
anti-CD28	Co-stimulatory molecule that promotes T-cell receptor (TCR) signalling	1 µg/ml	BD
anti-CD49d	Co-stimulatory molecule that promotes TCR signalling	1 µg/ml	BD
DNase I	Enzyme that prevents cells from clumping	0.002%	Roche
Brefeldin-A (BFA)	Inhibits Golgi transport of newly synthesised proteins which allows newly produced cytokines to accumulate in the cell	0.5 µg/ml	Sigma-Aldrich
FACS wash buffer	1% FBS, 0.001% sodium azide (Sigma-Aldrich). Sodium azide prevents bacterial growth	All washes and antibody master mixes, excluding washes after permeabilisation	Prepared in lab
Cytofix/Cytoperm (1x)	Formaldehyde and saponin. Saponin is a detergent that prevents reversal of permeabilisation	Permeabilisation of cells prior to intracellular cytokine staining step	BD
Perm Wash (1x)	FBS, sodium azide and saponin	Washes after permeabilisation	BD
CellFIX (1x)	1 % w/v formaldehyde and sodium azide	Final re-suspension. Immobilises bound antibodies on stained cells and fixes the cells	BD

Antibody staining

The phenotype and functional nature of T cell responses to MVA and HIV stimulations were determined by staining with the antibody panels depicted in Table 2.4. The staining panels were optimised in our laboratory by Agano Kiravu and Rubina Bunjun. The memory

phenotype panel included three cytokines (IFN- γ , IL-2 and TNF- α) on one channel, PE, since for the purpose of phenotyping it was only necessary to detect antigen-specific cells, and not each individual cytokine, which was determined by the functional panel performed in parallel. It was also important to include a viability stain since this study used cryopreserved PBMC samples, which often contain a percentage of dead or dying cells due to the cryopreservation and thawing processes. A violet viability stain ('Vivid') was used, and it enters dead or dying cells through damaged cell membranes, reacts with amine groups in the cytoplasm and enhances fluorescence, whereas it cannot enter viable cells. Dead cells that can non-specifically bind antibodies and increase background responses were thus excluded (Horton *et al.*, 2007; Perfetto *et al.*, 2006). All the antibodies used have been tested and published to be cross-reactive with baboons (Casimiro *et al.*, 2003; Locher *et al.*, 2003; Chege *et al.*, 2008; Burgers *et al.*, 2009). The procedure involves first staining for antibodies expressed on the surface of the cell, followed by permeabilisation of cells before staining for intracellular markers, such as cytokines. The permeabilisation reagent contains saponin which creates holes in the cells and allows antibodies to enter. Washes were performed with a buffer also containing saponin, which prevented the reversal of permeabilisation. Before acquisition on the flow cytometer, cells were fixed to preserve the staining.

TABLE 2.4 Phenotype and functional panels used in the study

Laser	Bandpass filters	Longpass filters	Fluorochrome detected	Memory Panel	Functional Panel
Blue 488nm			SSC		
Blue 488nm	780/60	735LP	CY7PE		TNF- α
Blue 488nm	695/40	685LP	PerCPCy5.5	CD4	CD8
Blue 488nm	575/26	550LP	PE	TNF- α , IFN- γ , IL-2	
Blue 488nm	530/30	505LP	FITC	CD28	CD3
Violet 407nm	655/8	630LP	Qdot-655		CD4
Violet 407nm	440/40		Pacific Blue	Vivid	Vivid
Violet 407nm	605/12	505LP	Qdot-605	CD8	
Red 633nm	780/60	735LP	CY7APC	CD3	
Red 633nm	720/40	690LP	Alexa700		IFN- γ
Red 633nm	660/020		APC	CD95	IL-2

Throughout the staining procedure, wash steps were performed in plates by centrifuging at 1000 x g for 3 min at 4°C, while incubation steps were carried out for 20 min at room temperature in the dark. The details of the reagents and antibodies used are listed in Tables 2.3 and 2.5. Cells were washed twice with PBS, then resuspended and incubated in 50 µl PBS containing a pre-titrated volume of Vivid. Samples were then washed twice, with 150 µl FACS wash buffer for the first wash and 200 µl FACS wash buffer for the second wash. Cell pellets were incubated in 50 µl of a surface staining master mix, consisting of CD4-PerCP-Cy5.5, CD8-QDot-605, CD95-APC, CD28-FITC and FACS wash buffer for the phenotype panel, or CD4-QDot-655, CD8-PerCP-Cy5.5 and FACS wash buffer for the functional panel. The cell pellets were washed twice and incubated with 100 µl Cytotfix/Cytoperm. Two washes using Perm/Wash buffer were performed before incubating the cell pellets in 50 µl of an intracellular cytokine staining master mix, consisting of CD3-APC-Cy7, IFN-γ-PE, TNF-α-PE, IL-2-PE and FACS wash buffer for the phenotype panel or CD3-FITC, IFN-γ-Ax700, TNF-α-PE-Cy7 and IL-2-APC for the functional panel. After two washes with Perm/Wash buffer they were resuspended in 150 µl 1X CellFIX and transferred to FACS tubes for acquisition within 24 h on an LSRII flow cytometer using FACSDiva software version 6.0 (BD). Between 300 000 and 1 000 000 events were collected per sample.

TABLE 2.5 Antibodies used in the study

Antibody	Fluorochrome	Clone	Volume (µl)*	Supplier[#]
CD4	Peridininchlorophyll Cy5.5 (PerCP-Cy5.5)	L200	0.5	BD
CD8	Quantum Dot nanocrystal 605 (QDot-605)	(7Pt-3F9)	0.25	NIH NHPRR
CD95	Allophycocyanin (APC)	DX2	1	BD
CD28	Fluorescein isothiocyanate (FITC)	CD28.2	5	BD
CD3	Allophycocyanin Cy7 (APC-Cy7)	SP34-2	5	BD
IFN-γ	Phycoerythrin (PE)	4S.B3	5	BD
TNF-α	Phycoerythrin (PE)	MAb11	5	BD
IL-2	Phycoerythrin (PE)	MQ1-17H12	2.5	BD
CD4	Quantum Dot nanocrystal 655 (QDot-655)	(19Thy5D7)	0.5	NIH NHPRR
CD8	Peridininchlorophyll Cy5.5 (PerCP-Cy5.5)	SK-1	0.25	BD
CD3	Fluorescein isothiocyanate (FITC)	HIT3a	5	BD
IFN-γ	Alexa Fluor 700 (Ax-700)	B27	1	BD
TNF-α	Phycoerythrin Cy7 (PE-Cy7)	MAb11	5	BD
IL-2	Allophycocyanin (APC)	MQ1-17H12	1	BD

* Titrated volume used per reaction [#] NIH NHPRR – National Institutes of Health, Nonhuman Primate Reagent Resource

Gating strategy and data analysis

The gating strategy was as follows; singlets, lymphocytes, live CD3+, followed by CD4+ and CD8+. For determining the memory phenotype of antigen-specific cells, CD4+ and CD8+ cells were then gated to determine the total amount of cytokine produced (IL-2 or TNF- α or IFN- γ) and the expression of CD28 and CD95 within these cytokine-producing cells was determined. To differentiate memory subsets, CD28+CD95+ cells represented central memory cells and CD28-CD95+ represented effector memory cells (Sun *et al.*, 2005). Naïve cells (CD28+CD95-) were excluded. For functional characterisation, CD4+ and CD8+ were gated to determine IL-2, TNF- α and IFN- γ produced, and boolean combinations thereof. All results are reported as net responses once the background value (unstimulated tube) was subtracted.

Compensation

Spectral overlap, where fluorescence from a single fluorochrome may be detected by more than one detector, can occur (Roederer *et al.*, 2004). To overcome this, compensation was performed. This involved staining a single tube of anti-mouse or anti-rat Igk CompBeads (BD), depending on the antibody origin, per fluorochrome used in the panels. Polymer amine beads (Bangs Laboratories, Fishers, IN, USA) were used for compensation of Vivid, and a tube of negative CompBeads (BD) was included. Briefly, 100 μ l of Perm Wash (BD) was added to CompBeads (BD), with one tube per fluorochrome. Corresponding volumes of antibodies as used in the staining panels were added to the beads, incubated at room temperature for 15 min, washed and acquired.

Each compensation tube was acquired on the LSRII at the same voltages and settings as test samples, making sure that they were as bright as or brighter than the same fluorochrome on the cells being studied. During analysis in FlowJo (Tree Star), colour compensation for each panel was performed automatically by subtraction of fluorescence spill-over using the compensation wizard. This resulted in a compensation matrix that was applied to all samples so that spectral overlap between the fluorochromes was minimised.

2.6 Statistical and data analyses

All statistical analyses were performed using GraphPad Prism version 5.0. Median values, interquartile ranges and ranges are presented. For non-parametric data (such as differences between groups), non-paired medians were compared using the nonparametric Mann-Whitney test, while paired data was compared using the Wilcoxon Ranks test. Correlations were

determined using the Spearman rank correlation test. P-values of less than 0.05 were considered significant.

Analysis of flow cytometry data was performed using FlowJo version 8.8.1 (Tree Star), GraphPad Prism version 5 (GraphPad Software), Pestle version 1.6.2 and Spice version 5.1 (provided by M. Roederer and J. Nozzi, NIAID, NIH; Roederer *et al.*, 2011).

University of Cape Town

CHAPTER 3

**Optimisation of techniques to determine
humoral and cellular responses**

University of Cape Town

Chapter 3

Optimising the detection of MVA-specific immunity

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3.1 Introduction

Several of the methods described in Chapter 2 required optimisation, in order to ensure accurate results with study samples, lab environment and MVA constructs used in this study.

3.2 Optimisation of MVA-*gfp* neutralisation assay

Optimisation had to be carried out to optimise three steps of the assay, described in Chapter 2, section 2.2.2. First, it was necessary to determine the optimal time period to infect HeLa cells with MVA-*gfp* to detect efficient expression of GFP by flow cytometry. Second, it was important to determine the optimal MOI of MVA-*gfp* for infection, and finally, the concentration of Vaccinia Immune Globulin (VIG), used as a positive neutralisation control, needed to be determined.

3.2.1 Time-course to determine the optimal infection time for MVA-*gfp*

A time course experiment was performed using three different MOI of MVA-*gfp* to determine the length of culture time that was required for detection of MVA infection of HeLa cells, as measured by expression of GFP by flow cytometry. This infection time refers to the length of the culture period of HeLa cells with an appropriate MOI of MVA-*gfp* after the initial 2 h infection period, following the washing step to remove excess free virus particles, and prior to harvesting the cells for flow cytometric detection of GFP expression. Infection times of 1 h, 2 h, 3 h, 4 h, 5 h and 17 h were tested at MOI of 5, 0.1 and 0.05. Figure 3.1A shows the gating strategy used to determine the proportion of cells infected by MVA and expressing GFP. HeLa cells were first gated on side scatter and forward scatter, and then the amount of GFP expression was detected in FL-1, the channel where GFP fluoresces. There was an increase in GFP expression over time, from barely detectable background expression after 1 h of culture, to 47.4% expression after 17 h. Figure 3.1B shows the amount of GFP expression at three different MOI for culture periods from 1 h to 17 h. After a 1 h culture for all MOI, no viral infection was detectable, as was evident from the lack of GFP expression. At an MOI of 5, expression of GFP was detected as early as 2 h (17.8%), and increased slightly every hour, until it reached 58.6% at 5 h. Although GFP expression levels from the lower MOI of 0.1 and 0.05 followed the same upward trend as the higher MOI, GFP expression levels at 5 h were markedly lower at these lower virus concentrations. The greatest increase was seen after 17 h of culture, where GFP expression reached 60.2% for an MOI of 0.1 and 47.4% for 0.05. Since

stocks of MVA-*gfp* were limited and a 17 h culture time post infection yielded sufficient detectable GFP expression from a lower MOI, and this was also a practical time period from a logistical stand point, this incubation period was chosen to perform the assay.

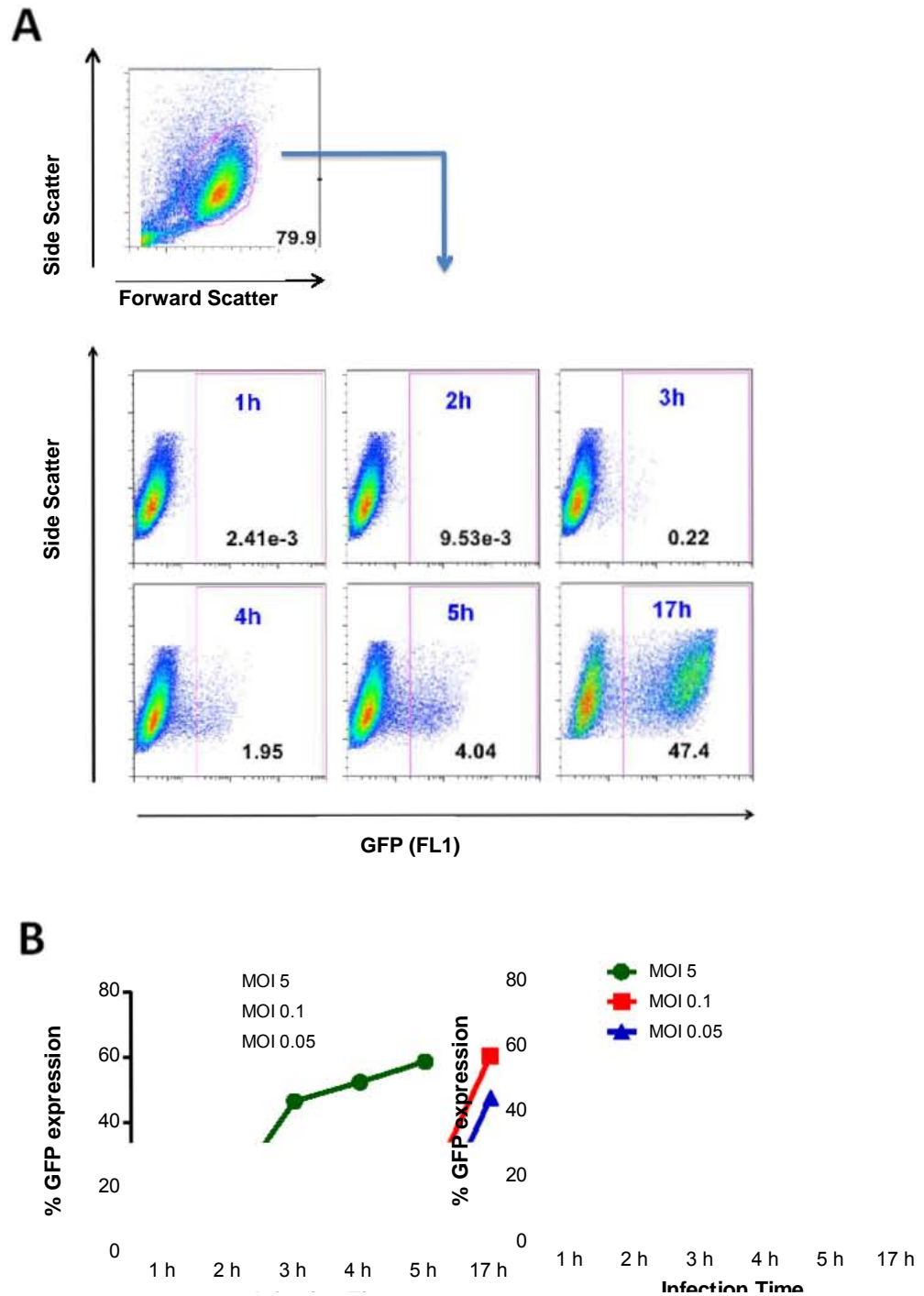


Figure 3.1 Optimal incubation period for MVA-*gfp* expression in HeLa cells. (A) Flow plot showing HeLa cell infection with MVA-*gfp* and time-course at an MOI of 0.05. **(B)** GFP expression at an MOI of 5 (green), 0.1 (red) and 0.05 (blue) over time.

3.2.2 Determining the optimal MOI for MVA-gfp

Once the optimal incubation time post infection had been established, the next step was to determine the optimal MOI. Eight MOI ranging from 0.005 to 0.5 were prepared by serial dilution to yield measurable GFP expression by flow cytometry. An uninfected control was also included. Figure 3.2A shows representative flow cytometry plots of GFP-positive cells at the different MOI, with the same data represented graphically in Figure 3.2B. This experiment was performed in duplicate, and results were highly reproducible at each MOI tested. In the uninfected control, no background expression of GFP was evident. GFP expression was initially low at an MOI of 0.005 (3.53%), and increased with each increase in MOI. At an MOI of 0.5, GFP expression reached 82.8%. GFP expression of between 20% and 30% was considered ideal because it was readily detectable, but not too high that neutralisation of infection was hindered. Thus, an MOI of between 0.025 (15.1% GFP expression) and 0.05 (29% GFP expression) was optimal, and an MOI of 0.04 was chosen for further experiments.

3.2.3 Determining the optimal amount of VIG to use as a positive assay control

To maintain good quality control of assays performed, it was necessary to introduce a positive neutralisation control for MVA. Reference Vaccinia Immune Globulin (VIG) was obtained from Dr Christine Anderson (FDA, USA), and was titrated in order to establish the optimal concentration to be used as a positive control. A series of dilutions of VIG ranging from 1:5 to 1:320 were performed. Each dilution was incubated with MVA-gfp at an MOI of 0.04 for 2 h at 37°C, agitating gently every 15 min, prior to adding the HeLa cells at 0.5×10^6 per well and incubating overnight. Neutralisation (%) was calculated as the ratio of GFP expression of each VIG dilution to the GFP expression of the control where no VIG was added. The results are shown in Figure 3.3. GFP expression increased at higher VIG dilutions, as neutralisation of MVA decreased. This relationship was reciprocal, as the more dilute the VIG the less capable it was of neutralising MVA infection, and therefore GFP expression. A VIG dilution of 1:20 (represented by the dashed line in Figure 3.3) was considered optimal for use as a positive control as it neutralised almost close to 100% of infection, yielding GFP expression of 1 - 3%. This dilution was included in every assay performed. In summary, the MVA-gfp neutralisation assay was carried out as illustrated by Figure 3.4, including a 17 h incubation time post the initial 2 h infection, at an MOI of 0.04, using a VIG dilution of 1:20 as a positive control. This optimised technique was then used to analyse 8 baboons and 22 macaques at various time-points to determine the magnitude and kinetics of neutralising antibody responses to MVA responses over time.

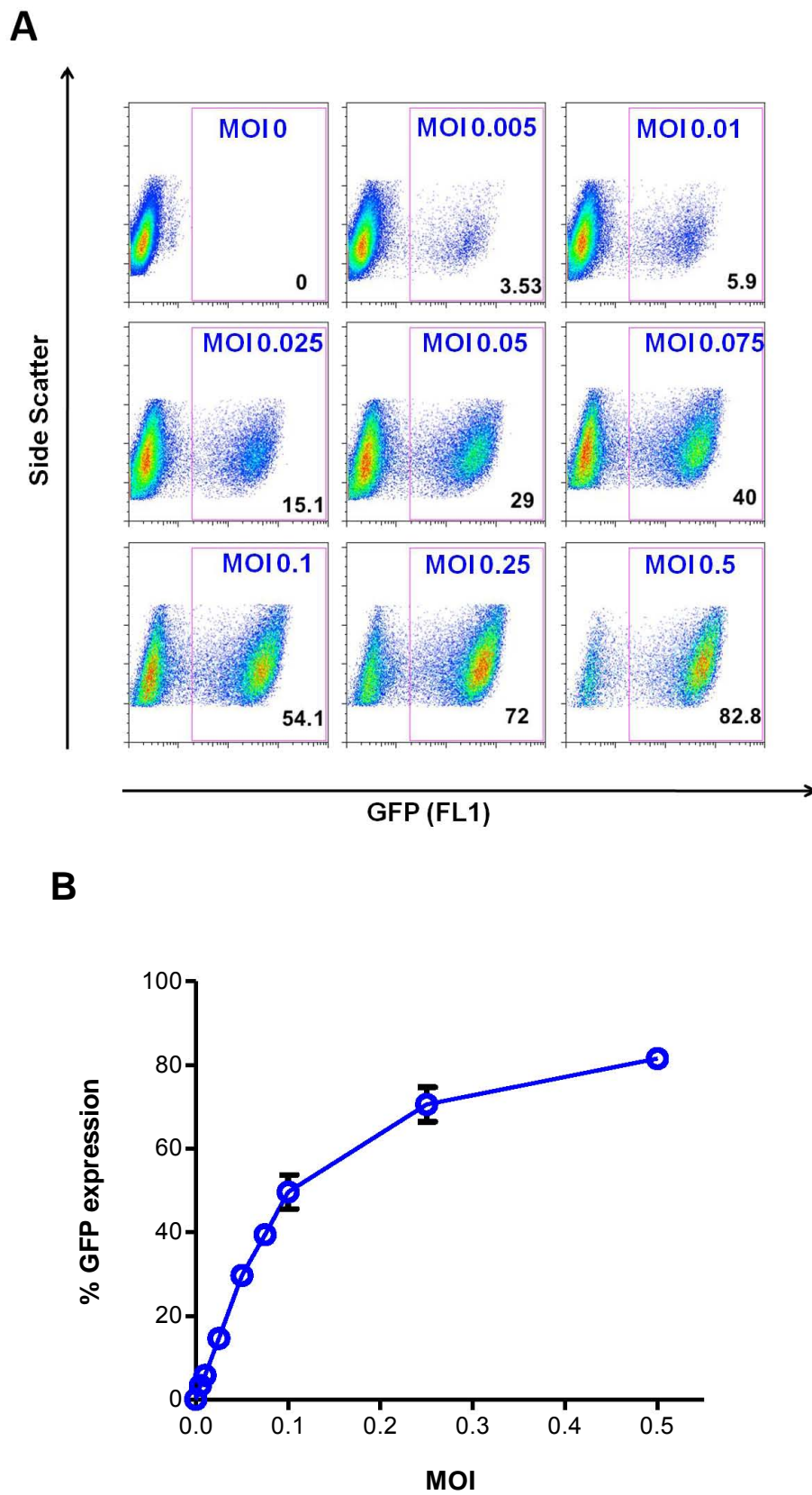


Figure 3.2 Titration of MVA-*gfp* to determine optimal MOI for MVA-*gfp* expression in HeLa cells (A) Representative flow plots for MOI ranging from 0 to 0.5. (B) GFP expression (%) at different MOI. Values are an average of two assays, and error bars are shown.

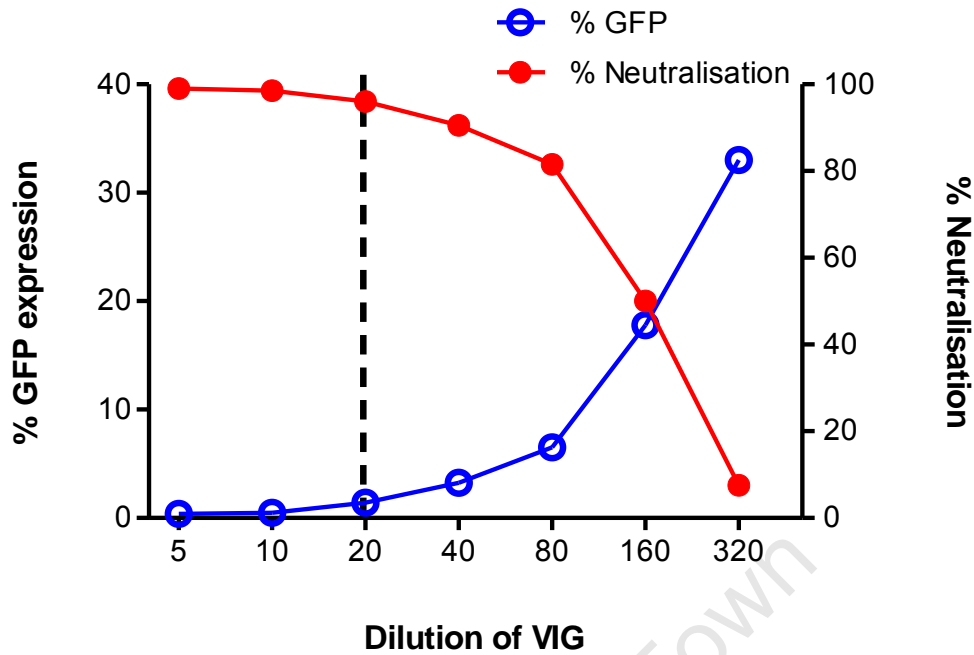


Figure 3.3 Titration of VIG to demonstrate neutralisation activity. The percentage of GFP expression (*blue*) vs. the percentage of MVA neutralisation (*red*) plotted against serial dilutions of VIG.

3.3 Optimisation of IFN- γ ELISPOT assay for detection of cellular responses to MVA

The basic IFN- γ ELISPOT technique performed was based on that described by Masemola *et al.* (2004). This assay was designed to analyse cellular responses to HIV. However, for this study, it was adapted and optimised to detect responses to whole MVA wild type viral particles.

In previous studies using MVA as a stimulus in the ELISPOT assay, three major differences in techniques used to infect cells with MVA were noted. Firstly, the technique for infecting the cells with MVA was carried out either by mixing virus and cells directly in the wells of an ELISPOT plate (Smith *et al.*, 2005), or by mixing the cells and virus in tubes, washing off the excess virus, and then adding the infected cells to the wells of an ELISPOT plate for incubation (Speller and Warren, 2002; Kim *et al.*, 2007). Secondly, the MOI of MVA ranged from 0.1 (Hammarlund *et al.*, 2003) to 10 (Speller and Warren, 2002). Finally, the length of time that the cells were infected with MVA prior to incubation in the ELISPOT plate ranged from 1 h (Speller and Warren, 2002) to overnight (Cosma *et al.*, 2007, Earl *et al.*, 2007). Therefore, optimisation was required to establish which infection technique, which MOI and which infection time would work optimally in our hands.

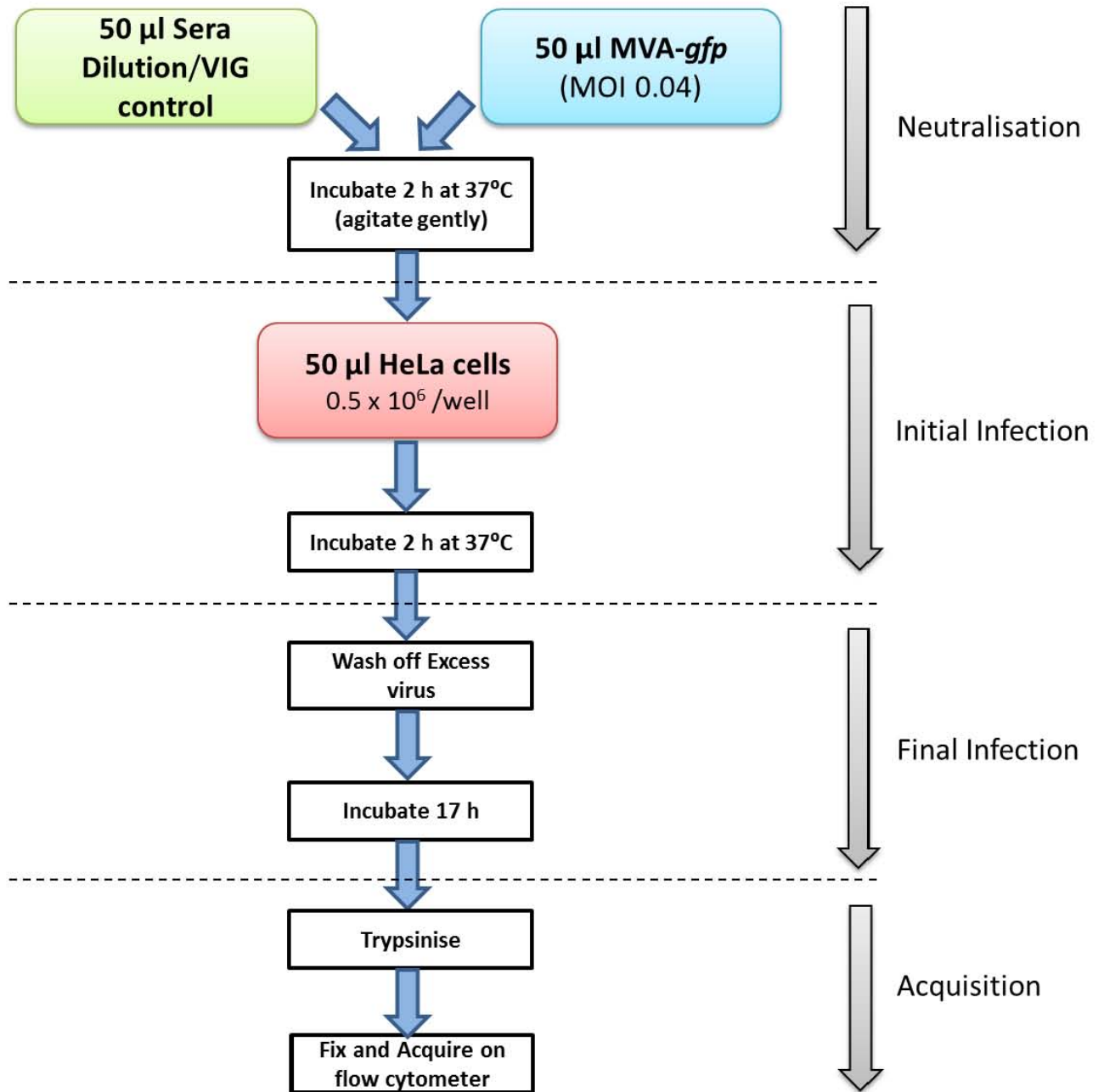


Figure 3.4 Schematic of MVA-gfp neutralisation assay technique. Coloured blocks represent serum (green), MVA-gfp (blue) and cells (red), while processing steps are shown in black.

3.3.1 Determination of optimal infection technique

The first investigation performed was to determine whether cells could be infected directly with MVA in the well of ELISPOT plate, or whether it was necessary to pre-infect cells with MVA in tubes, wash off excess virus, and then transfer the infected cells into the wells of an ELISPOT plate for incubation and subsequent detection of responses. Three animals (B623, B629 and B549), each at a different time-point following MVA vaccination, were used to investigate the infection technique. The B623 sample was from a time point one week following the first MVA vaccination, B629 one week following the second MVA vaccination, and B549 one week following the third MVA vaccination. By assaying different time-points, the chances of detecting a range of MVA-specific responses were maximised. Figure 3.5A shows duplicate wells of an IFN- γ ELISPOT assay performed to compare direct infection to pre-infection, at an MOI of 0.1 in the same animal. Responses to MVA were detected in all three animals. A sample from a time-point prior to MVA vaccination was also included in the assay, and no spots were present (data not shown). Figure 3.5B shows that although responses to MVA differed considerably between animals, most likely due to different time-points studied, it was clear that the pre-infection technique was consistently superior to direct infection in all three animals, with a 36% to 86% (median 58%) increase in net SFU/10⁶ PBMC. Therefore, T-cell responses were readily detectable to MVA, and the pre-infection technique was considered optimal.

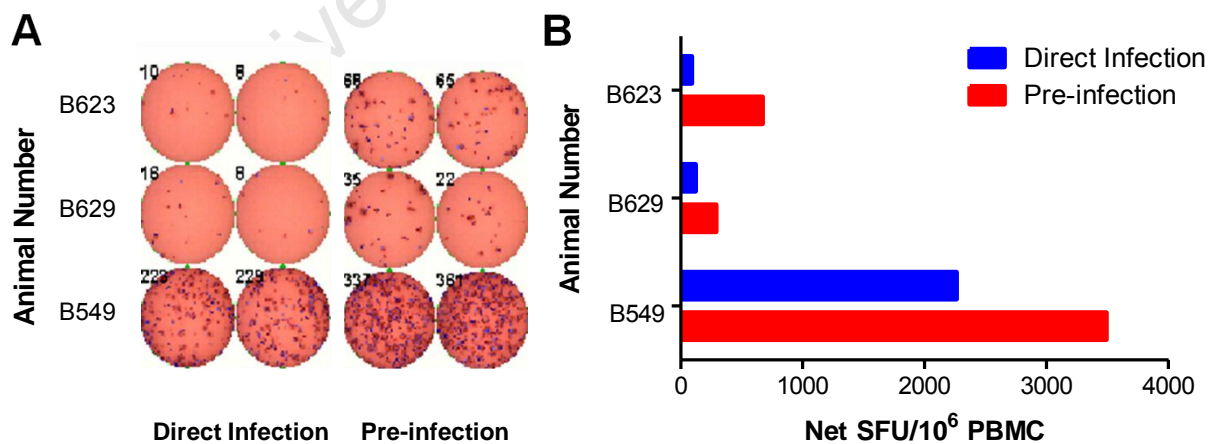


Figure 3.5 Determination of optimal infection technique. (A) IFN- γ ELISPOT assay for comparison of direct infection (*left panel*) and pre-infection (*right panel*). (B) Net SFU/10⁶ PBMCs for direct infection (*blue*) and pre-infection (*red*).

3.3.2 Determination of optimal MOI

The same three animals were used to determine the optimal MOI to pre-infect the cells. Figure 3.6A shows IFN- γ ELISPOT assays performed in duplicate at MOI of 10, 5, 1 and 0.1 in the same animal. Responses to MVA were detected in all three animals at all MOI used, although they were very low in B629 at an MOI of 10. Samples from a time-point prior to MVA vaccination were also assayed in each animal and no spots were detected (data not shown). Figure 3.6B shows that although responses to MVA differed considerably between animals and MOI, it was clear that an MOI of 0.1 resulted in greater responses in two of the three animals, with a two- to threefold increase in detectable responses between an MOI of 1 and 0.1. The third animal, B549, showed similar results at MOI of 1 and 0.1 with net SFU/10⁶ PBMCs of 3575 at an MOI of 1, compared to 3585 at an MOI of 0.1, but this is most likely due to the limit of detection being reached at greater spot numbers. Thus, interestingly, lower MOI were better at detecting responses to MVA than higher MOI. This may be due to higher MOI causing an increase in the number of dead and apoptotic cells (higher cytopathic effect) or impairment of antigen presentation capacity (Norder *et al.*, 2010). Therefore, T-cell responses were readily detectable to MVA and an MOI of 0.1 was selected for subsequent assays.

3.3.3 Determination of optimal length of infection time

Pre-infection with an MOI of 0.1 was selected for the assay however the optimal infection time required to measure MVA responses needed to be determined. Two of the animals used previously (B623 and B629, both at one week after the second MVA vaccination) were initially tested over an infection time period of 30 min, 90 min, 3 h, 6 h, 12 h and 16 h. As shown in Figure 3.7A, B623 and B629 both revealed no advantage in infection times of longer than 6 h, with 12 h and 16 h infection times resulting in a two- to threefold reduction in response in B623 and no difference in B629. Thus, it was determined that there was no benefit in infection for 12 h and 16 h, and a further four animals were tested at various time-points after the second and third MVA vaccinations for an infection period of 90 min, 3 h and 6 h only. An infection time of 3 h showed the highest response for five of six animals tested. There was a trend towards a 3 h incubation period resulting in the greatest responses (Figure 3.7B). Thus, a 3 h infection period was chosen as the optimal infection time.

In summary, the IFN- γ ELISPOT assay to detect MVA-specific T cell responses was carried out as described in 2.4.5, using the pre-infection technique at an MOI of 0.1 for 3 h, before transferring the cells to an ELISPOT plate for incubation and subsequent spot detection.

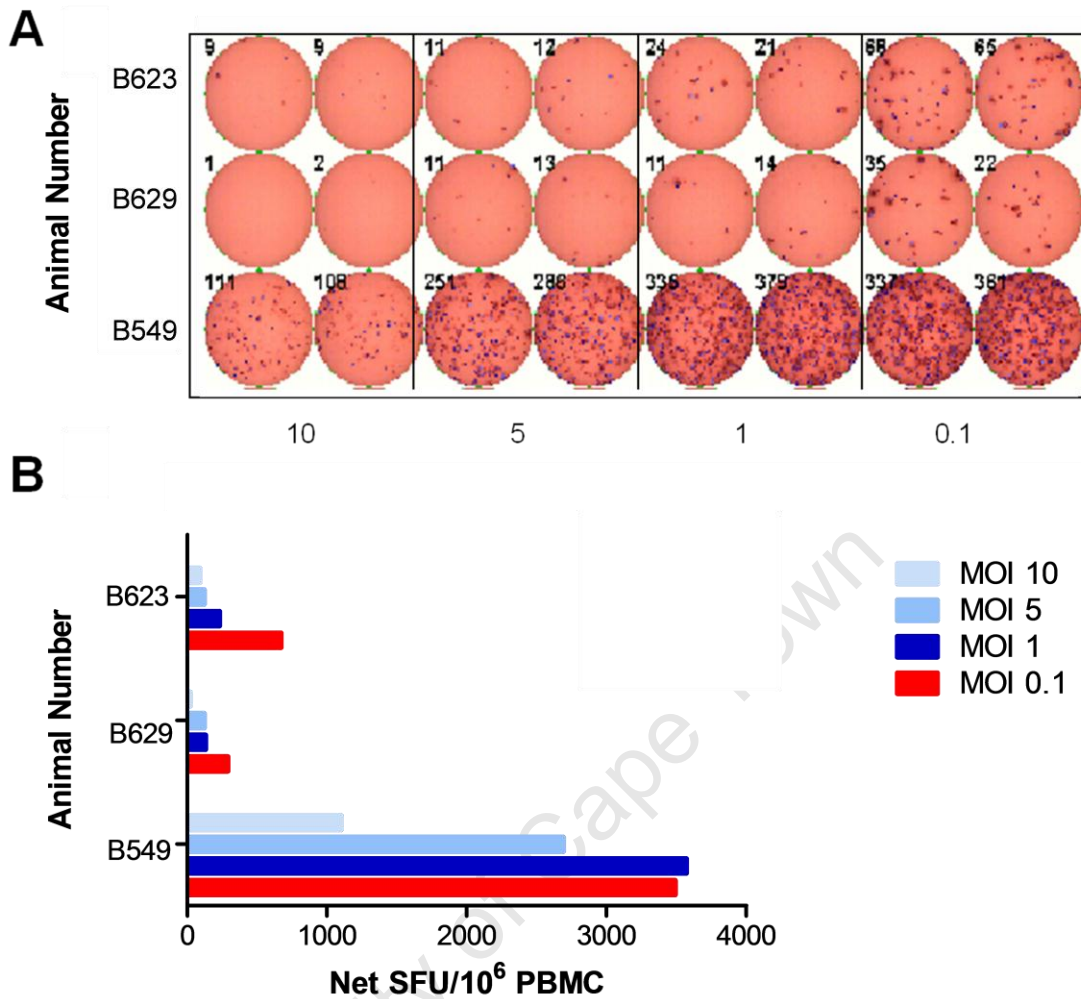


Figure 3.6 Determination of optimal MOI to infect cells with MVA to detect responses in the ELISPOT assay. (A) IFN- γ ELISPOT assay for comparison of direct infection at four different MOI, of 10, 5, 1 and 0.1. (B) Net SFU/ 10^6 PBMCs for direct infection at MOI of 0.1 (red), 1 (dark blue), 5 (medium blue) and 10 (light blue).

3.4 Optimisation of stimulation of T-cells with MVA in the ICS assay

In order to determine the length of time that led to the optimal expression of T-cell cytokines after the addition of BFA, an experiment was performed in one animal (B549) at one week after the second MVA vaccination, using three different MOI at three different infection times. MVA infections were performed by mixing cells with MVA at an MOI of 0.1, 1.0 and 2.0, and incubating for 3 h at 37°C in 5% CO₂. Cells were centrifuged and washed in R10 to remove any excess virus, transferred to wells of a 96-well tissue culture plate, and then harvested immediately or incubated for a further 3 h or 5h (total time of 3 h, 6 h or 8 h), after which 1 μ g/ml BFA was added. The plate was incubated a further 16 h before antibody staining with

CD3-FITC and IFN- γ -PE, to determine cytokine production by T-cell lymphocytes. Figure 3.8A shows the gating strategy used to determine the levels of IFN- γ produced by CD3+ lymphocytes in response to MVA stimulation. The gating strategy was as follows: lymphocytes identified by SSC against FSC, CD3+ lymphocytes and finally IFN- γ + cytokine production. A 3 h incubation before the addition of BFA induced an IFN- γ response of 0.1%, while a total of 6 h or 8 h incubation induced a twelve-fold higher net IFN- γ response, of 1.2% (Figure 3.8B). There was little difference in the level of IFN- γ detected between 6 h and 8 h incubation before the addition of BFA, yielding 1.2% vs. 1.3% IFN- γ expression. There was no benefit in longer incubation periods, as 16 h incubation showed a marked reduction in IFN- γ production (0.25%).

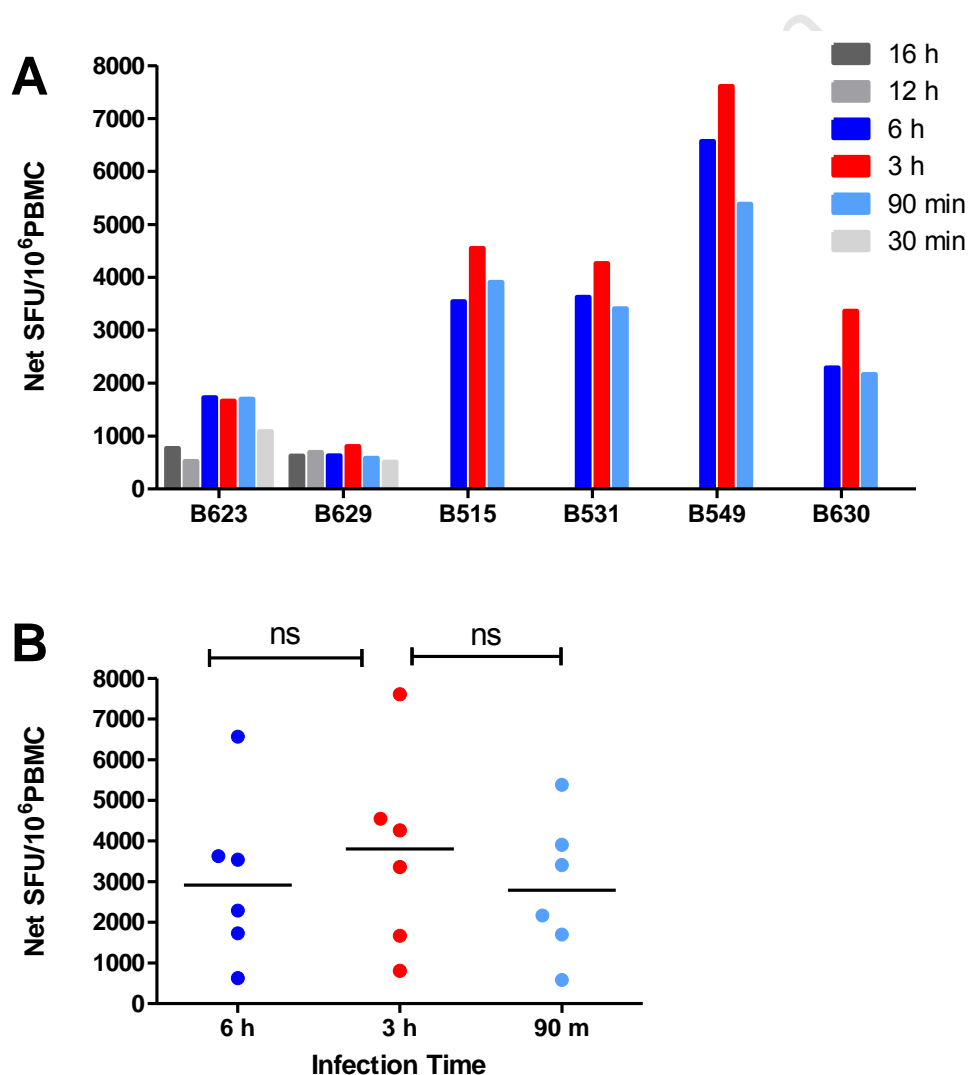


Figure 3.7 Determination of optimal infection time. (A) Net SFU/10⁶ PBMCs for different infection times in six animals. (B) Infection times of 3 h versus 6 h, and 3 h versus 90 min, with medians and statistical significance determined by the Mann-Whitney test.

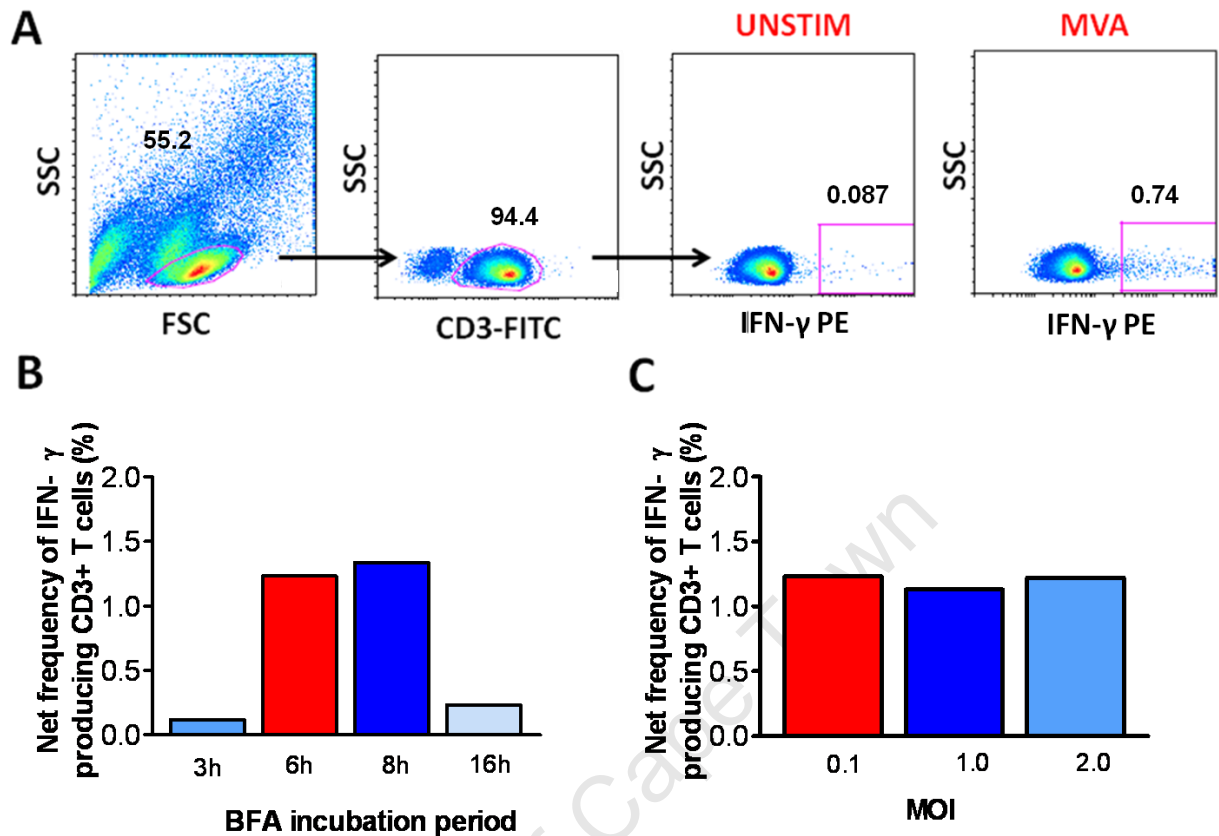


Figure 3.8 Optimisation of ICS assay for detecting MVA responses. (A) Representative flow plots of IFN- γ expression from MVA-stimulated or unstimulated T cells. (B) Net IFN- γ responses by ICS for different incubation periods with BFA, after infection with MVA at an MOI of 0.1. (C) Net IFN- γ responses by ICS after infection with three MOI after 6 h of BFA incubation.

According to Speller and Warren (2002), exposure to vaccinia virus requires a chain of intracellular steps to facilitate antigenic presentation prior to the addition of a golgi transport inhibitor such as BFA. Their study revealed that infection of between 5 h and 7 h was optimal to detect cytokines, whereas 3 h was insufficient and 16 h was too long, as cytokine had likely already been secreted. My results support this, and 6 h incubation at an MOI of 0.1 was considered optimal and used in all further experiments.

These methods were applied to measure neutralising antibody and T-cell responses to MVA generated by vaccination in non-human primates that had been vaccinated with candidate HIV vaccines, as described in the next chapter.

CHAPTER 4

Investigation of humoral and T-cell responses to MVA and their comparison to HIV-specific responses

University of Cape Town

Chapter 4

Results

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4.1 Introduction

The aim of this study was to investigate immune responses to the MVA vector of an HIV candidate vaccine, SAAVI MVA-C, expressing five genes from HIV-1 subtype C, namely Gag, RT, Tat, Nef and Env. The need to improve our understanding of vector-specific immunity of HIV vaccine candidates was highlighted recently by the STEP trial, where pre-existing Ad5 immunity was implicated in increased HIV-1 acquisition after vaccination (Buchbinder *et al.* 2008). Cellular and humoral responses to MVA were investigated in non-human primates vaccinated at multiple time-points pre- and post-MVA vaccination, and the effect of the vector-specific immunity on immune responses to the HIV immunogens was determined. Binding antibody responses to vaccinia virus capsid proteins A27, A33, B5R and L1R were assessed by ELISA, and MVA-specific neutralising antibody responses were quantified using a MVA-*gfp* neutralisation assay. T cell responses to MVA were assessed using an IFN- γ ELISPOT assay, and further characterised by intracellular cytokine staining and flow cytometry, to measure a range of cytokine responses and memory phenotypes of vector-specific T cells. Data on HIV-specific responses shown in Section 4.4.1 - 4.4.4 were provided by Dr Wendy Burgers and have been published previously (Burgers *et al.*, 2009).

4.2 Humoral Responses to MVA

Binding antibody responses to vaccinia virus membrane proteins A27, A33, B5R and L1R were assessed in eight baboons by ELISA. In addition, neutralising antibody responses were analysed in the same eight baboons and in 22 macaques using an MVA-*gfp* flow cytometry-based neutralisation assay.

4.2.1 MVA elicits low titre binding antibody responses

Sera from eight baboons were tested for the presence of antibodies to vaccinia virus capsid proteins A27, A33, B5R and L1R at multiple time-points pre- and post-MVA vaccination. No antibody responses were detected to A27 and L1R proteins. End-point antibody titres were determined for sera positive for A33 and B5R proteins.

No responses to vaccinia virus capsid proteins were detectable prior to MVA vaccination. Antibodies were first detectable in a proportion of animals 8 weeks after the first MVA vaccination. One week later, after the second MVA vaccination, binding antibody responses to A33 were detectable in all eight animals and to B5R in six of the eight animals tested. Antibody

responses to A33 reached the highest titres after the second SAAVI MVA-C vaccination, ranging from 160 to 5120 (median 480; Figure 4.1A). These responses subsequently waned but by 12 weeks after the second MVA-C vaccination (week 61) were still detectable at low levels in all but one animal (B575). Two of the four animals that received a third MVA vaccination (B515 and B549) still had low, but detectable levels of antibodies on the day of the third MVA vaccination (week 104), which was 10 months after the second MVA. All animals showed boosting of their responses 1 week after the third MVA vaccination, to levels very similar, but not higher than those after the second MVA, with a median titre of 320. Antibody responses to B5R showed similar kinetics as the A33 responses, but were of lower magnitude, at a median of 160, 1 week after the second MVA vaccination (Figure 4.1B). End-point titres to B5R in a single animal (B623) were boosted to a higher level after the third MVA-C vaccination. A33 titres were significantly higher than B5R responses 8 weeks after the first MVA vaccination, and 1 week after the second MVA vaccination ($p=0.012$ and 0.043 , respectively; Figure 4.1C), and although still a trend towards higher A33 responses 1 week after the third MVA vaccinations, this was not significant. There were no differences in anti-MVA antibody responses in those animals that had received priming immunisations with DNA prior to MVA vaccinations (data not shown).

4.2.2 Neutralising antibody responses to MVA

Having established that binding antibodies to MVA were elicited by vaccination, neutralising antibody responses to MVA were assessed in the same eight baboons, in order to determine whether levels of these antibodies had any impact on the ability of the MVA and HIV-specific cellular responses to be boosted. Twenty-two macaques that were undergoing pre-clinical testing of the same vaccines in a similar vaccination regimen were included in this analysis. Based on available sample numbers and due to limited serum availability, one baboon (B623) was selected to perform neutralisation assays at multiple time-points to ascertain which time-point would be best suited to detect neutralising antibody responses to MVA. This assay was also performed on one macaque sample to confirm the assay sensitivity and the peak time-point, as this may have differed in the two species.

No neutralising antibodies were detected prior to the first MVA vaccination at 41 weeks, or indeed prior to the second MVA vaccination at 49 weeks, in animal B623 (Figure 4.2A). One week after the second MVA vaccination was given, neutralising antibodies were detected at a titre of 320. This response subsequently waned, and by 12 weeks after the second MVA (week 61) there was an eight-fold lower neutralising titre.

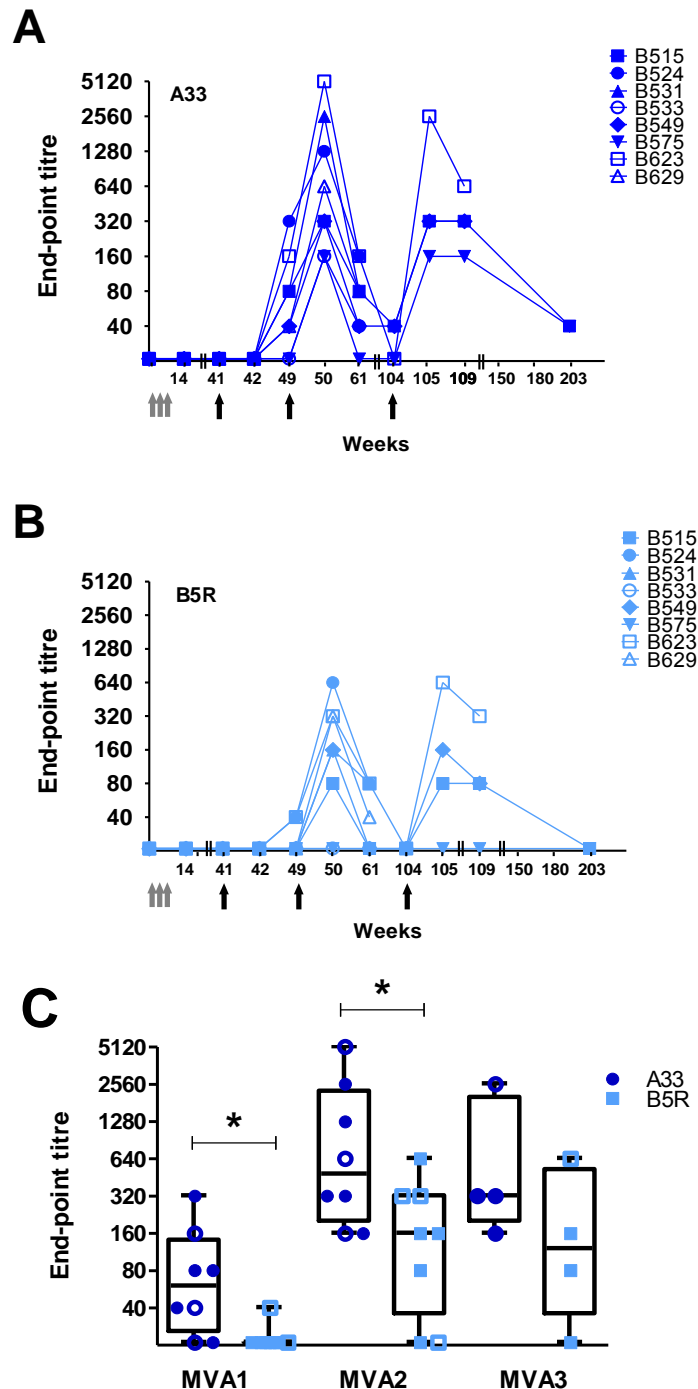


Figure 4.1 Binding antibody responses to MVA. Eight baboons were immunised with SAAVI MVA-C. Five of the animals had previously received three vaccinations with DNA-C (B515, B524, B531, B549, B575). ELISA responses are expressed as end-point titres. The DNA-C prime vaccinations are represented by grey arrows and the MVA-C vaccinations by black arrows. Four animals (B515, B549, B575, and B623) received a third MVA-C vaccination. **(A)** Kinetics of responses to vaccinia virus envelope protein A33. **(B)** Kinetics of responses to vaccinia virus envelope protein B5R. **(C)** Magnitude of peak binding antibody responses to vaccinia virus envelope proteins A33 and B5R 8 weeks after one, and 1 week after two or three MVA-C vaccinations. Filled symbols represent DNA-MVA vaccinated animals and open symbols indicate animals that received MVA only. Box and whisker plots show medians and ranges. Significant differences as determined by a Mann Whitney test are shown by an asterisk. * indicates $p < 0.05$.

On the day of the third MVA vaccination at week 105, no neutralising antibodies were detected, however the response rapidly reappeared to a titre greater than that induced by the second MVA vaccination, of 960. The single macaque sample tested longitudinally (P3) exhibited an identical pattern, peaking at 1 week after the second MVA vaccination (no third MVA vaccination was given in these animals; data not shown). Thus, sera from all eight baboons and 22 macaques were tested at one time-point pre-immunisation, on the day of the second MVA vaccination, and 1 week after the second MVA. No baboons had any detectable neutralising antibodies prior to the second MVA vaccination, whereas all animals subsequently developed neutralising antibodies 1 week later, with end-point titres ranging from 40 to 640, with a median of 80 (Figure 4.2B). Macaque neutralisation titres ranged from 40 to 640, with a median of 320. Neutralising antibody responses in the 22 macaques tested were significantly higher than the levels found in the baboons ($p=0.04$; Figure 4.2C). There were no differences in neutralising antibody responses in those animals that had received priming immunisations with DNA prior to MVA vaccinations (data not shown). The relationship between B5R and A33 antibody responses and neutralising titres were examined in baboons, to investigate whether responses to these capsid proteins were related to MVA neutralising activity. There was no relationship between B5R responses and neutralising antibodies at matching time-points ($p=0.46$, $r=0.29$; Figure 4.3A). There was, however, a trend towards a positive correlation between A33 antibodies and neutralisation ($p=0.096$, $r=0.65$; Figure 4.3B), indicating that some of the neutralising activity may be mediated by antibodies specific for A33.

4.3 Cellular responses to MVA

Cellular responses to MVA were measured by the IFN- γ ELISPOT assay in eight vaccinated baboons at multiple time-points pre- and post-MVA vaccination, using cryopreserved PBMC samples. The group included five animals that received three SAAVI DNA-C prime vaccinations and two (B524 and B531) or three (B515, B549 and B575) SAAVI MVA-C boost vaccinations, and three animals that received two (B533 and B623) or three (B629) SAAVI MVA-C vaccinations only. In four animals, cellular responses were further characterised at three time-points by ICS and multiparameter flow cytometry to investigate a broader range of cytokine production and memory phenotypes of vector-specific responses.

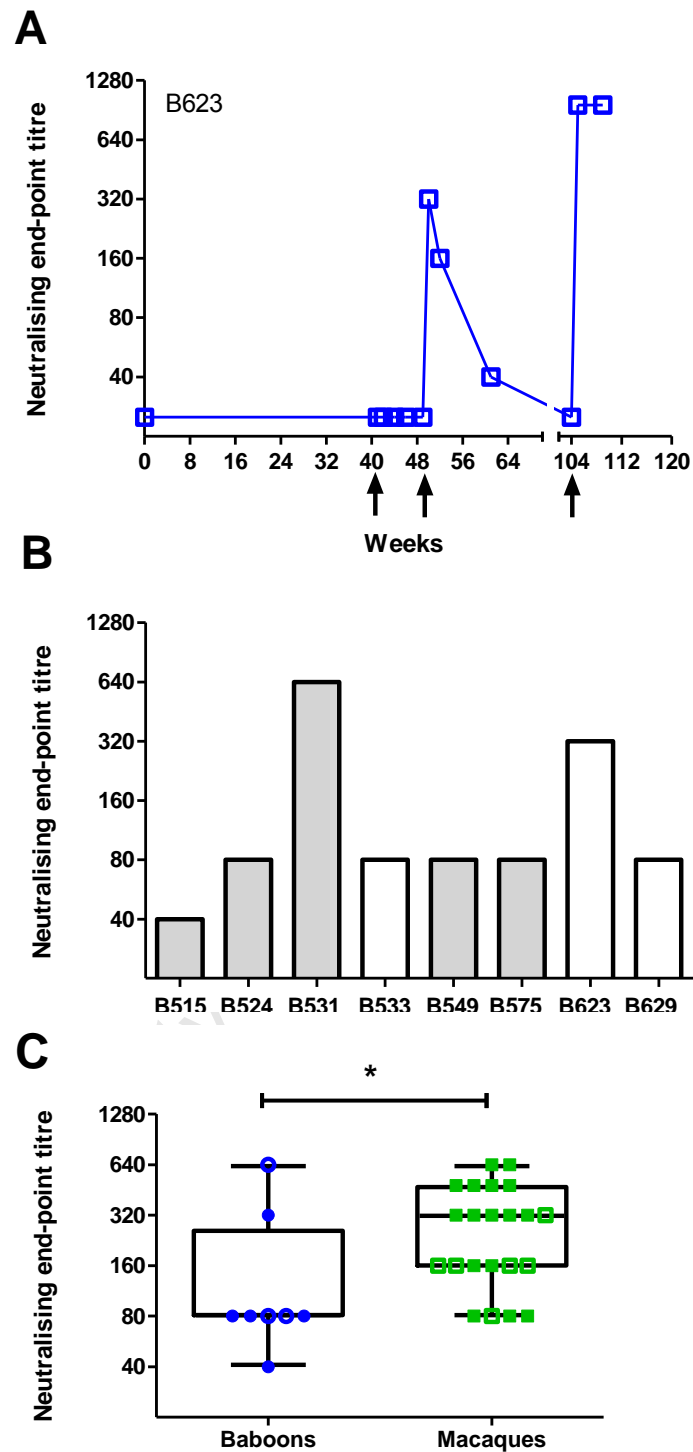


Figure 4.2 Neutralising antibody responses to MVA. Eight baboons and 22 macaques were immunised with SAAVI MVA-C. Five baboons and 16 macaques had previously received three vaccinations with DNA-C and are represented by closed bars and symbols, while animals that received only MVA-C are represented by open bars and symbols. Neutralising antibody responses are expressed as IC₅₀ end-point titres. The MVA-C vaccinations are represented by black arrows. **(A)** Kinetics of neutralising antibody responses in B623. **(B)** Magnitude of neutralising antibody responses to MVA in baboons 1 week after the second MVA vaccination. **(C)** Magnitudes of neutralising antibody responses after the second MVA vaccination in baboons compared to macaques, showing medians and ranges. Differences were tested for significance by the Mann-Whitney test. * indicates $p < 0.05$.

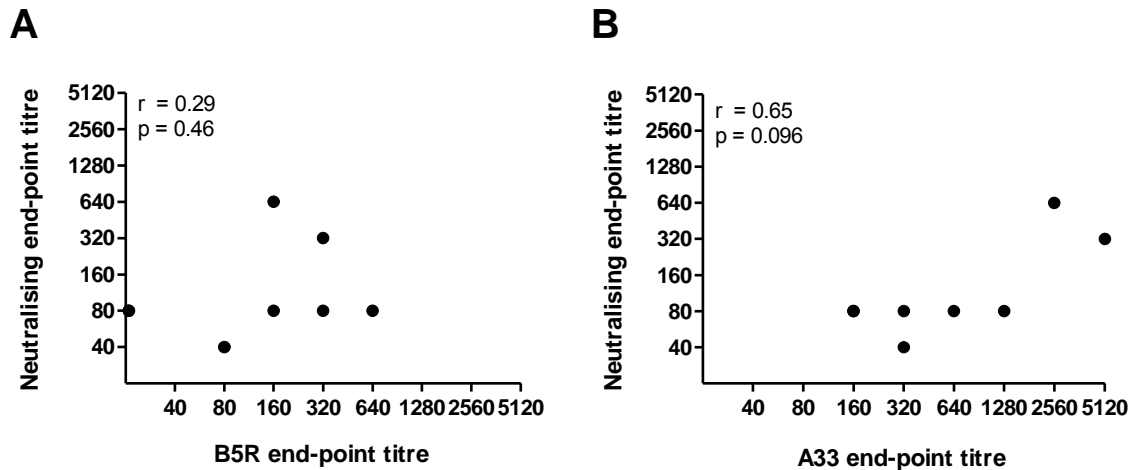


Figure 4.3 Relationship between MVA neutralising antibody responses and binding antibody responses to the vector. Eight baboons were immunised with SAAVI MVA-C. Five of the animals had previously received three vaccinations with DNA-C (B515, B524, B531, B549 and B575). Correlations between the neutralising end-point titre 1 week after the second MVA-C vaccination and matching end-point binding antibody titres to vaccinia capsid proteins B5R (**A**) and A33 (**B**) were examined. Relationships were determined by Spearman's rank correlation.

4.3.1 MVA elicits high magnitude IFN- γ ELISPOT responses

The IFN- γ ELISPOT assay was used to determine whether T-cell responses were generated to the MVA vector. Indeed, strong T-cell responses to MVA were elicited in all eight animals, and first detected by 1 week after the first MVA-C vaccination (Figure 4.4A). Responses ranged from 318 to 838 net SFU/10⁶ PBMC, with a median of 497 net SFU/10⁶ PBMC. These responses continued to rise 7 weeks later, and were boosted approximately three-fold after the second MVA vaccination in all animals, to a median of 1518 net SFU/10⁶ PBMC (range, 728 to 2762 net SFU/10⁶ PBMC). Responses were long-lasting, with all animals showing similar or higher T-cell responses 12 weeks after the second MVA vaccination (week 61), with a median of 1658 net SFU/10⁶ PBMC. Four animals (B515, B549, B575 and B623) received a third MVA vaccination. Surprisingly, responses were boosted even further, reaching magnitudes of between 2772 (B515) and 5245 net SFU/10⁶ PBMC (B549), with a median of 4455 net SFU/10⁶ PBMC, 1 week after the third MVA vaccination. These responses persisted to similar or higher frequencies (median 4679 SFU/10⁶ PBMC) at the final time-point assessed, of 8 months after the third MVA vaccination (week 137). Thus, T cell responses as measured by IFN- γ release, increased significantly in magnitude with each successive MVA vaccination in all eight animals vaccinated (Figure 4.4B). There were no significant differences in magnitudes of MVA-specific responses between those animals that had been primed with DNA compared to those that received MVA only.

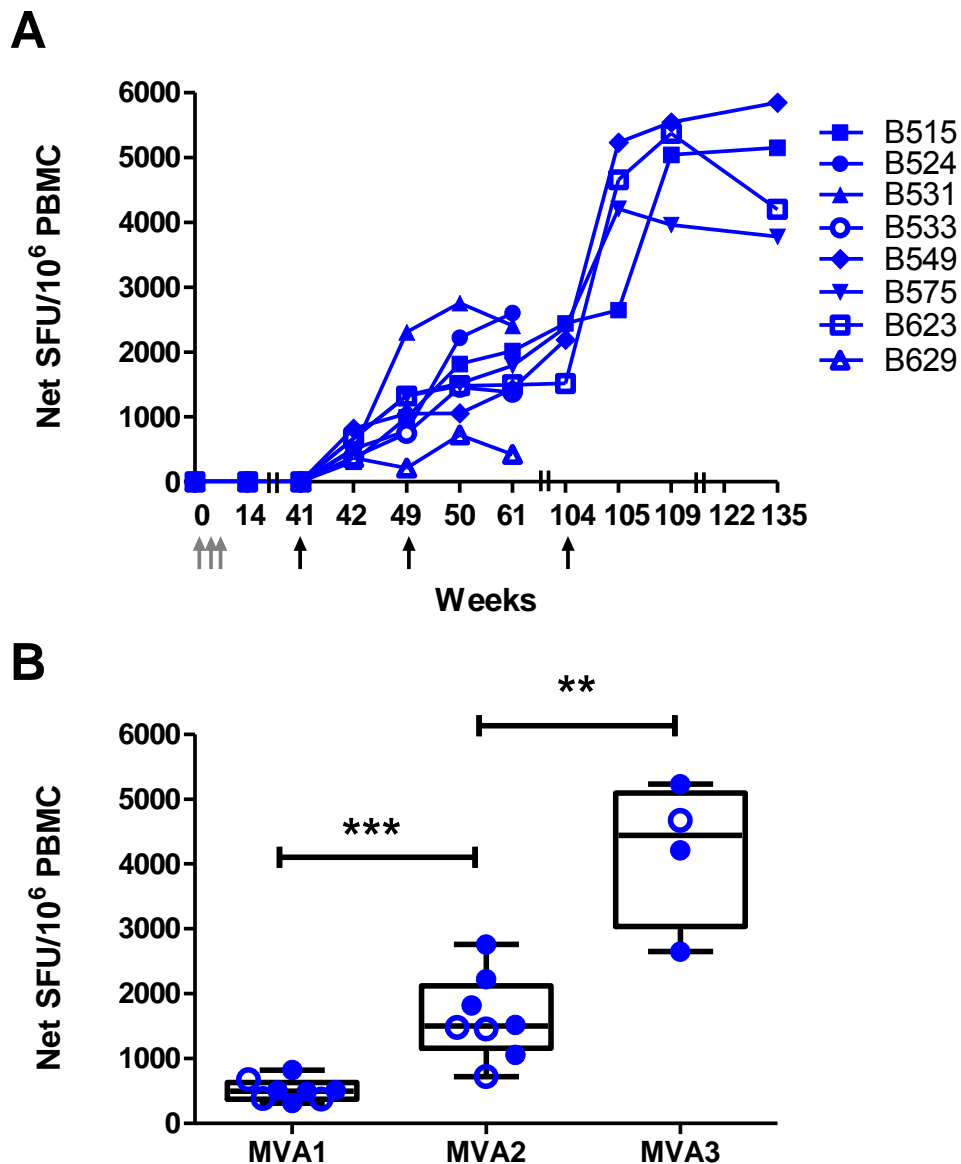


Figure 4.4 IFN- γ ELISPOT responses to MVA. Eight baboons were immunised with SAAVI MVA-C. Five of the animals had previously received three vaccinations with DNA-C (B515, B524, B531, B549 and B575). ELISPOT responses are expressed as net spot forming units (SFU)/ 10^6 PBMC. The DNA-C prime vaccinations are represented by grey arrows and the MVA-C vaccinations by black arrows. Four animals (B515, B549, B575 and B623) received a third MVA vaccination. **(A)** Kinetics of MVA responses over the course of the study. **(B)** Magnitude of MVA responses 1 week after one, two, or three MVA vaccinations. Filled symbols represent DNA-MVA vaccinated animals and open symbols indicate animals that received MVA only. Box and whisker plots show medians and ranges. Significant differences detected by the Mann Whitney non-parametric test are shown. ** indicates $p < 0.01$, *** indicates $p < 0.001$.

4.3.2 The function and phenotype of MVA-specific T cell responses

Cellular responses detected in the ELISPOT assay were further characterised at three time-points by ICS and multiparameter flow cytometry to determine further functional characteristics, phenotypes and memory differentiation status. Responses were investigated in the four animals that received a third MVA vaccination (B515, B549, B575 and B623), one of which received no DNA prime (B623). Time-points were selected based on sample availability, namely 1 week after the second MVA vaccination (week 50), 12 weeks after the second MVA vaccination (week 61) and 7 months after the third MVA vaccination (week 135).

MVA-specific responses are predominantly mediated by CD8+ T cells

The magnitudes of the total MVA-specific cytokine responses were determined (including all cells secreting one or any combination of IFN- γ , IL-2 and TNF- α). A representative example is shown in Figure 4.5A, indicating the gating strategy used to quantify production of IFN- γ , IL-2 and TNF- α cytokines by CD4+ and CD8+ T cells in response to MVA. Cells were gated on singlets, lymphocytes, live CD3+, CD4+ and CD8+. Cytokine responses were elicited by CD8+ T-cells in response to MVA, and detected 1 week after the second MVA-C vaccination, at a median response of 0.465% (range, 0.059 to 0.95%; Figure 4.5B). These responses increased two-fold in frequency by week 12 following the second MVA vaccination (median 0.865%, range 0.334 to 1.84%), and a further three-fold (median 2.55%, range 1.831 to 7.43%) by 7 months after the third MVA vaccination (week 137). There was a significant increase in total cytokine produced by CD8+ T-cells between 1 week after the second MVA and 7 months after the third MVA ($p=0.029$). CD4+ T-cell responses were detectable but at a much lower frequency, except for B575, that had magnitudes ranging from 2.22 to 2.6% over the time-points measured. Figure 4.5C shows that the magnitude of IFN- γ , IL-2 and TNF- α produced by CD8+ T-cells increased at each time-point, while cytokine frequencies by CD4+ T-cells remained relatively unchanged over the same time period. Thus, multiple cytokine responses are readily elicited in response to MVA, are predominantly mediated by CD8+ T cells, and increase in magnitude over successive immunisations.

MVA-specific responses are predominantly CD8-mediated and single cytokine-producing

The CD4+ and CD8+ T-cell responses to MVA were further analysed to determine the magnitude and proportion of cells producing combinations of IFN- γ , TNF- α or IL-2. CD8+ T-cell responses were present in all four animals, reaching high magnitudes (Figure 4.6A; top panel), as observed previously by the IFN- γ ELISPOT assay.

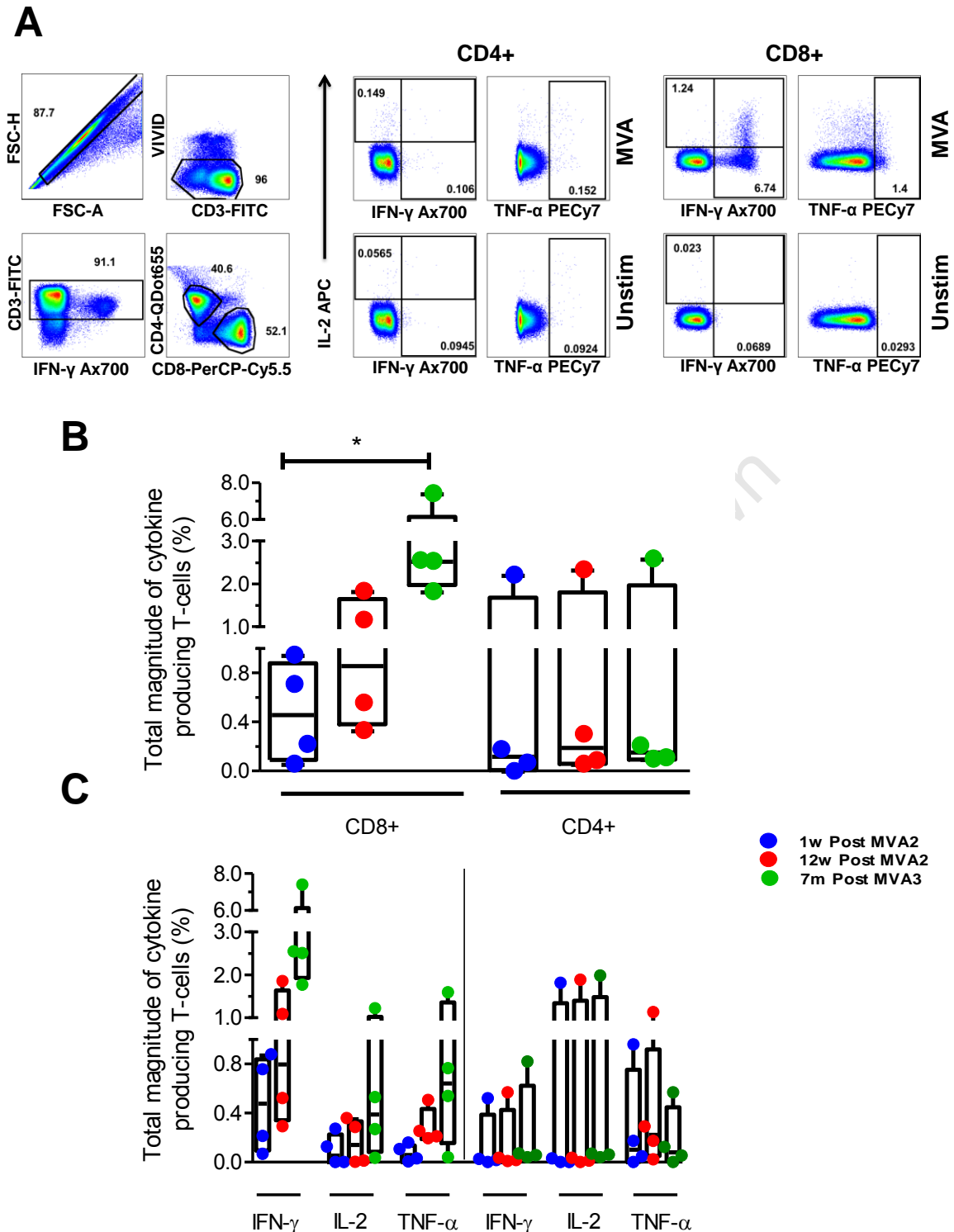


Figure 4.5 MVA-specific responses are skewed towards CD8⁺ T-cells. Cytokine responses were characterised in four baboons that received three MVA-C vaccinations, and IFN- γ , IL-2 and TNF- α responses 1 and 12 weeks after the second MVA-C vaccination, and 7 months after the third MVA vaccination, were measured. (A) Representative flow plots showing gating strategy and CD4⁺ and CD8⁺ responses, in response to MVA and an unstimulated control. (B) Total cytokine responses at each time-point by CD8⁺ and CD4⁺ T-cells. (C) Total IFN- γ , IL-2 and TNF- α cytokine responses at each time-point by CD8⁺ and CD4⁺ T-cells. Box and whisker plots show medians and ranges.

CD8⁺ T-cell responses increased over time, and by 7 months following the third MVA vaccination, the cells were producing combinations of three cytokines (IFN- γ , IL-2 and TNF- α), two cytokines (IFN- γ and TNF- α or IL-2) or single cytokine (IFN- γ). The CD8⁺ T-cell compartment was dominated by single cytokine-producing cells, with IFN- γ being the predominant cytokine produced (Figure 4.6A; *bottom panel*). CD8⁺ T-cells remained predominantly single cytokine-producing at all time-points tested, at a mean of 63% of the response. One of the four animals (B575) had high magnitude CD4⁺ T-cell responses at all time-points tested, with the other three animals having detectable but low magnitude responses (Figure 4.6B; *top panel*). Apart from the high CD4⁺ responder, and unlike the CD8⁺ T-cell responses, the CD4⁺ T-cell responses in the remaining animals were relatively constant over time. The CD4⁺ T-cell response was also dominated by single cytokine producing cells, predominantly IL-2 or TNF- α (Figure 4.6B; *bottom panel*).

Memory phenotypes of MVA-specific T-cells

MVA-specific responses were further analysed to investigate the memory differentiation phenotype of these cells. CD28 and CD95 were used to differentiate central memory (T_{CM}; CD28⁺CD95⁺ cells) and effector memory (T_{EM}; CD28⁻CD95⁺ cells) from naïve T cells (CD28⁺CD95⁻ cells; Sun *et al.*, 2005). The memory differentiation status of total cytokine producing cells (IFN- γ , IL-2 and TNF- α) after MVA stimulation was assessed. All three cytokines were placed on one fluorochrome in the staining panel, as the individual cytokine contributions had already been determined using a functional panel, and due to limitations of the three-laser LSRII flow cytometer that were used. Total cytokine responses measured by the two panels correlated closely ($p=0.0013$, $r=0.83$ and $p=0.0016$, $r=0.82$, for the CD4⁺ and CD8⁺ responses, respectively; data not shown).

Cells were gated on singlets, lymphocytes, live CD3⁺, CD4⁺ and CD8⁺, and cytokine-producing cells were then overlaid onto the memory populations to determine their distribution within the different memory subsets (Figure 4.7A). As reported above, CD8⁺ T-cells increased in magnitude at each time-point (Figure 4.7B). For example, B515 showed low levels MVA-specific CD8⁺ T-cells 1 week after the first MVA vaccination (0.2%). This increased by 12 weeks after the second MVA vaccination (0.5%), and increased even further by 7 months following the third MVA vaccination, to 2.6%. The other three animals followed a similar pattern. CD4⁺ T-cell responses were generally of lower magnitude than CD8⁺ responses, with the exception of B575, as reported above (Figure 4.7B). In B549, MVA-specific CD4⁺ responses were not detected until 7 months after the third MVA. CD8⁺ T-cell responses to MVA were relatively evenly distributed between T_{CM} and T_{EM} subsets (Figure 4.7C, *top panel*),

and these proportions changed little over time as the response increased, with a mean of 50.5% of CD8+ cells being T_{CM}. MVA-specific CD4+ T-cells were almost exclusively T_{CM} in animals with detectable cytokine (Figure 4.7C, *bottom panel*), with a mean of 91% T_{CM} over the three time-points. In fact, in all three animals with detectable CD4+ responses at the earlier time-points, the proportion of MVA-specific T_{EM} made up less than 5% of the CD4+ response, and this increased to 22% in these animals by 7 months after the third MVA vaccination.

These data reveal that cellular responses to MVA were predominantly CD8+ IFN- γ + cells, distributed evenly between T_{CM} and T_{EM} subsets, with lower magnitude single cytokine CD4+ T_{CM} responses.

4.4 The influence of MVA-specific cellular and humoral immunity on cellular responses to HIV antigens

The humoral and cellular responses to MVA described above were compared to the HIV-specific responses that were generated in the same animals, reported previously (Burgers *et al.*, 2009), in order to determine whether vector-specific responses limited response to the HIV immunogens that the vector expressed, in the context of successive vaccinations. These comparisons focused on vaccinated baboons, but since neutralising antibody responses were also measured in vaccinated macaques, these data are also presented. Unpublished HIV-specific responses in macaques were provided by Dr Wendy Burgers.

4.4.1 Cellular responses to HIV in response to DNA-MVA vaccination

HIV-specific responses measured by IFN- γ ELISPOT in baboons to the vaccination regimen are shown in Figure 4.8. HIV-specific responses to DNA priming were undetectable. However, at week 42, one week after the first MVA boost, four out of five animals that had been primed with DNA responded vigorously (B515, B524, B549 and B575). These responses ranged from 2357 to 5970 net SFU/10⁶ PBMC per animal (median 2575 SFU/10⁶ PBMC). Responses waned, but were then boosted by one week after the second immunisation, ranging from 320 to 4452 net SFU/10⁶ PBMC per animal (median 2352 SFU/10⁶ PBMC). In three of these four animals (B524, B549, B575), the peak response after the second MVA vaccination was lower than that after the first MVA vaccination, with a median of 1399 SFU/10⁶ PBMC, compared to a median of 2357 SFU/10⁶ PBMC.

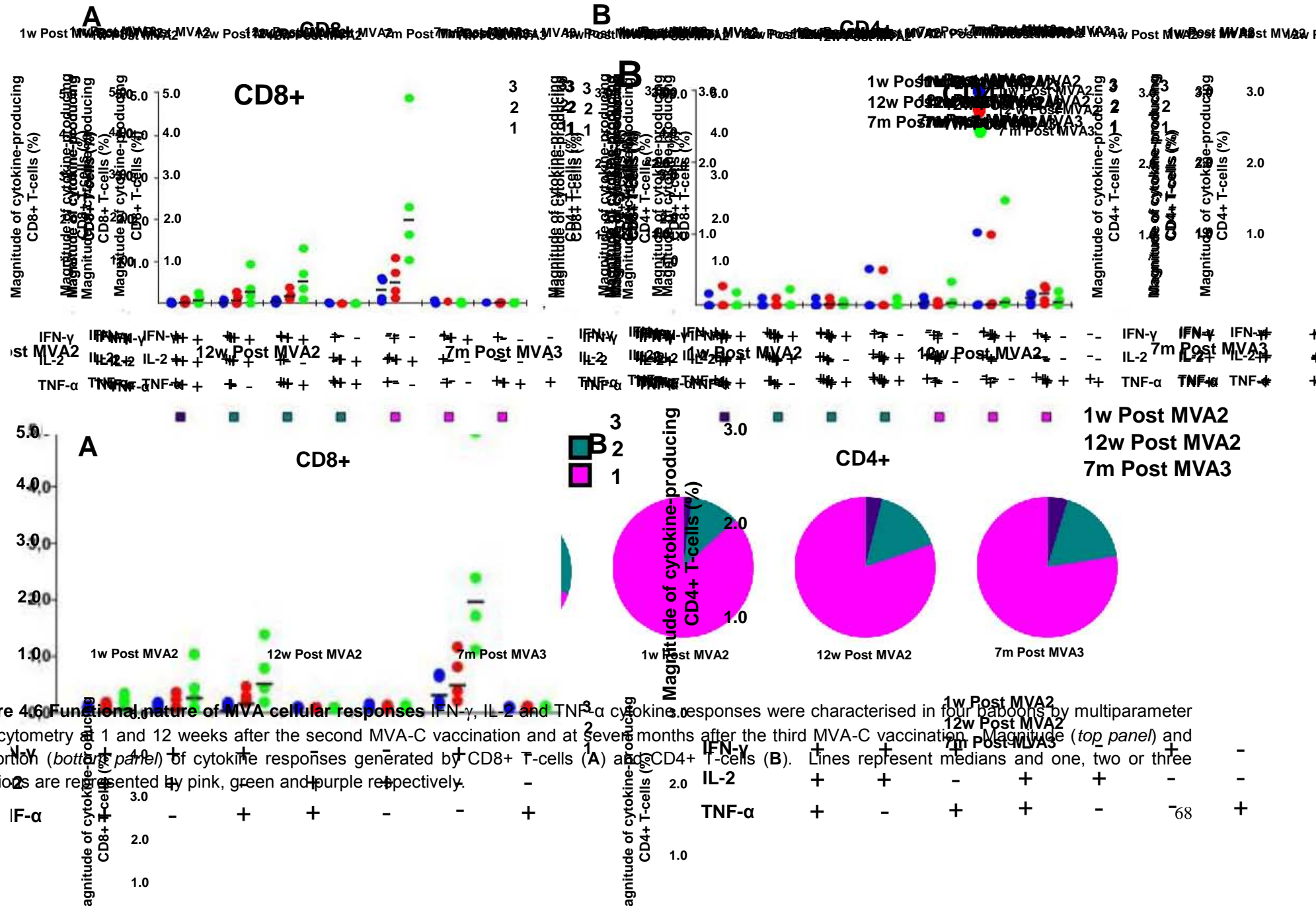


Figure 4.6 Functional nature of MVA cellular responses IFN- γ , IL-2 and TNF- α cytokine responses were characterised in four baboons by multiparameter flow cytometry at 1 and 12 weeks after the second MVA-C vaccination and at seven months after the third MVA-C vaccination. Magnitude (top panel) and proportion (bottom panel) of cytokine responses generated by CD8+ T-cells (A) and CD4+ T-cells (B). Lines represent medians and one, two or three functions are represented by pink, green and purple respectively.

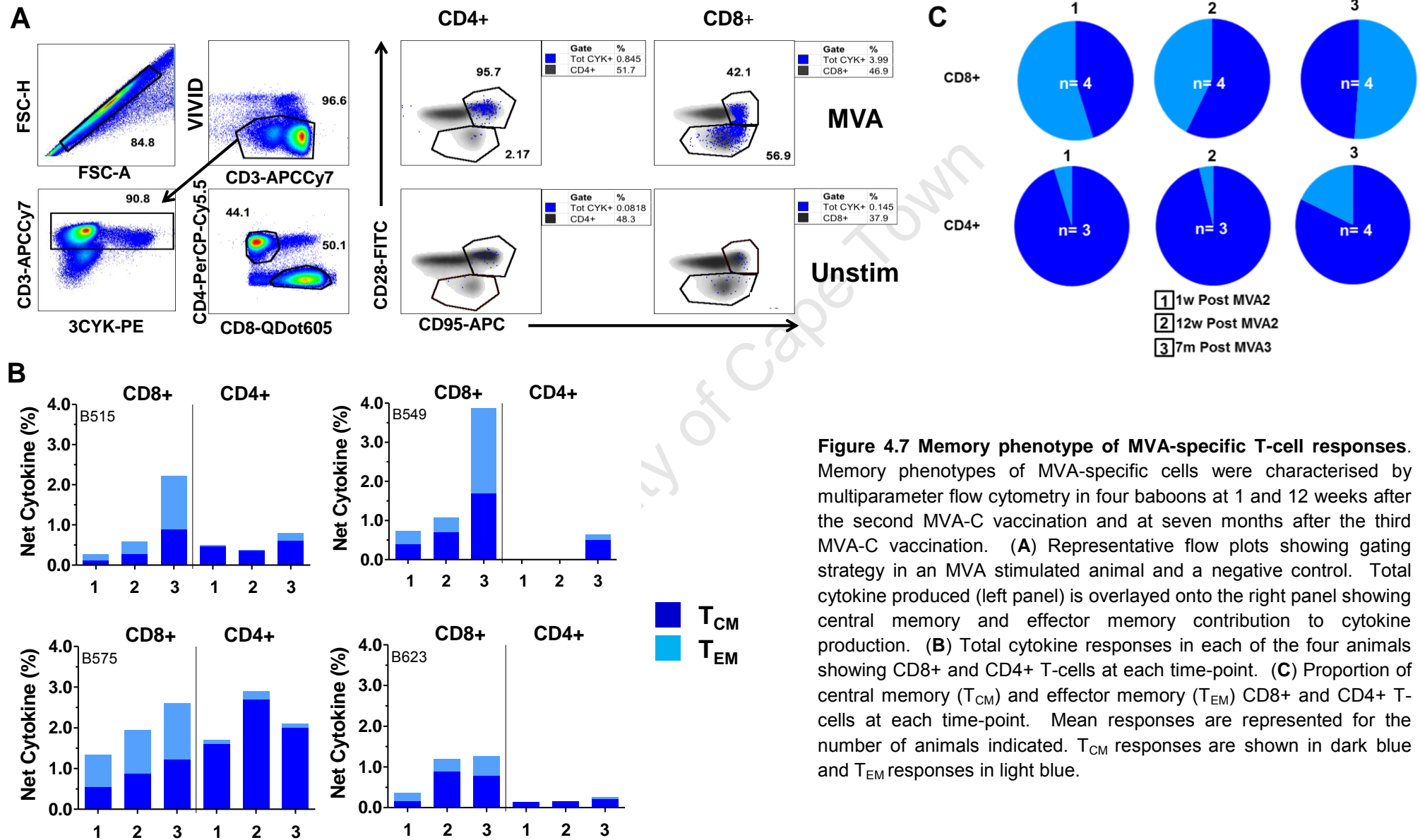


Figure 4.7 Memory phenotype of MVA-specific T-cell responses. Memory phenotypes of MVA-specific cells were characterised by multiparameter flow cytometry in four baboons at 1 and 12 weeks after the second MVA-C vaccination and at seven months after the third MVA-C vaccination. (A) Representative flow plots showing gating strategy in an MVA stimulated animal and a negative control. Total cytokine produced (left panel) is overlaid onto the right panel showing central memory and effector memory contribution to cytokine production. (B) Total cytokine responses in each of the four animals showing CD8+ and CD4+ T-cells at each time-point. (C) Proportion of central memory (T_{CM}) and effector memory (T_{EM}) CD8+ and CD4+ T-cells at each time-point. Mean responses are represented for the number of animals indicated. T_{CM} responses are shown in dark blue and T_{EM} responses in light blue.

One animal (B531) that was primed with DNA did not mount any response to the HIV antigens in the vectors. Four animals went on to receive a third MVA vaccination almost a year later. Here, it was interesting to note that responses were boosted to peak responses higher than those after the first or second MVA vaccination, ranging from 2340 to 7640 net SFU/10⁶ PBMC per animal (median 4459 SFU/10⁶ PBMC; Figure 4.8B). HIV-specific responses were significantly higher in animals receiving a DNA prime compared to animals that received MVA only ($p=0.0475$, Figure 4.8C), indicating clearly that although DNA-priming did not elicit measurable responses, a robust priming of HIV responses occurred.

Thus, MVA vaccinations given in close succession did not appear to boost responses to HIV antigens higher than previous peaks, whilst anti-HIV responses are boosted when MVA was given one year after the previous MVA vaccination. HIV-specific responses measured in macaques that were primed with DNA showed similar kinetics and magnitudes, reaching a median of 1399 SFU/10⁶ PBMC after the first MVA-C vaccination following DNA priming, waning and being boosted after the second MVA-C to levels not higher than those after the first (median 839 SFU/10⁶ PBMC; data not shown). MVA-only animals had low magnitude responses that were detected in two of the six animals 1 week after the first MVA.

4.4.2 The effect of vaccinia virus A33 and B5R binding antibody responses on HIV-specific responses

As described in section 4.2.1, antibody responses were generated to A33 and B5R capsid proteins of MVA in baboons. There were strong A33 and lower B5R responses that peaked after the second MVA vaccination and subsequently waned rapidly. These were boosted by the third MVA vaccination to levels not higher than those after the second MVA vaccination.

The magnitude and kinetics of MVA capsid binding antibodies were compared to HIV-specific cellular responses, in order to determine whether they might be inhibiting the latter responses. The kinetics of HIV cellular responses and MVA-specific A33 and B5R binding antibody responses were investigated in each animal (Figure 4.9). Apart from the single DNA-MVA HIV non-responder, cellular responses to HIV were present in all these animals prior to the development of A33 and B5R antibodies to MVA. In fact, the MVA binding antibodies reached their peak only after the second MVA vaccination. Thus, it is very likely that if MVA-specific antibody responses would have any effect on HIV-specific responses, this would come into play only at the second and third MVA vaccinations, when “pre-existing” antibodies as a result of the first MVA vaccination, that may clear or reduce the vaccine dose, would be present.

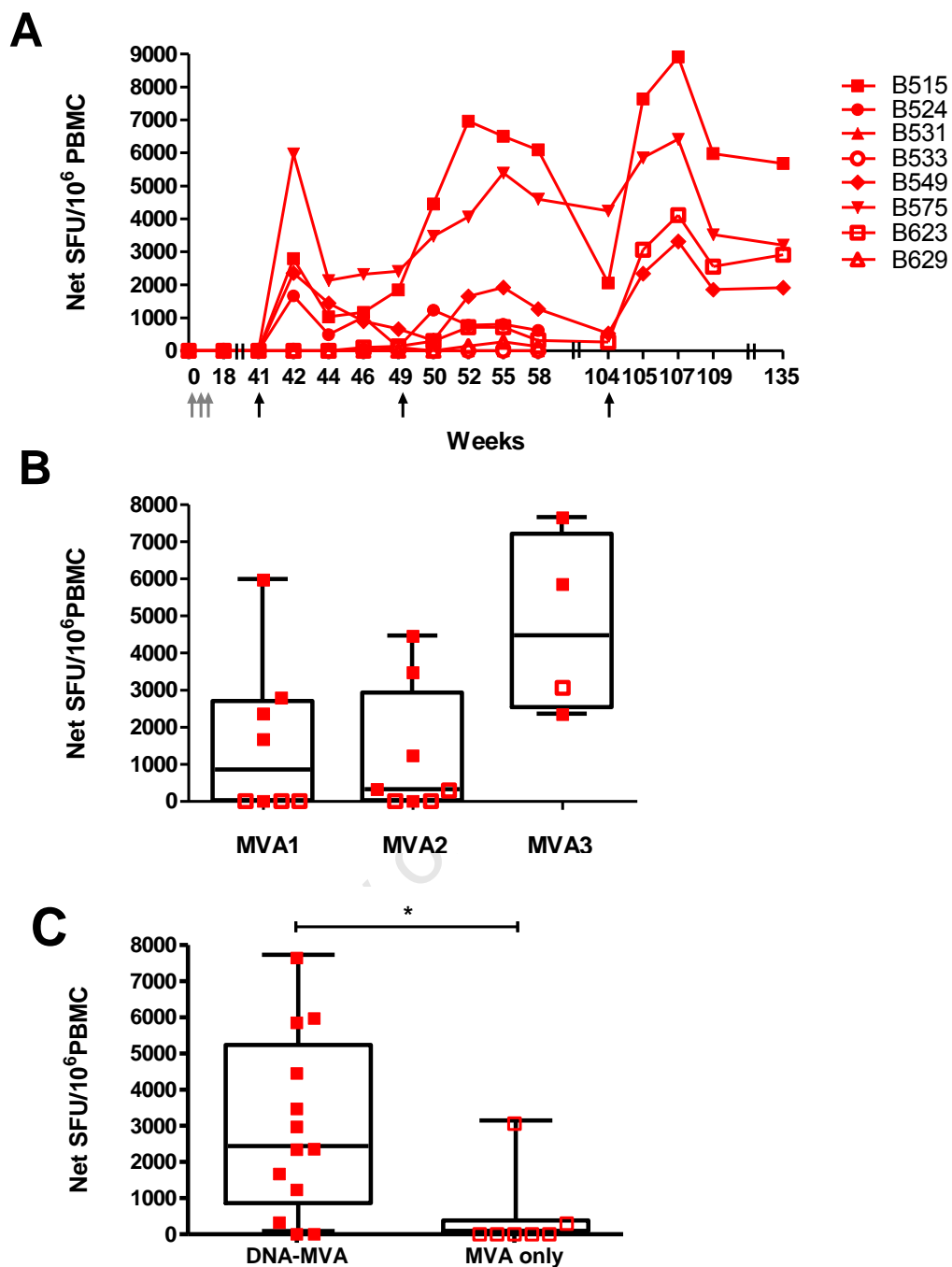


Figure 4.8 IFN- γ ELISPOT responses to HIV vaccine antigens. Eight baboons were immunised with SAAVI MVA-C. Five of the animals had previously received three vaccinations with DNA-C (B515, B524, B531, B549, B575). ELISPOT responses are expressed as net spot forming units (SFU)/10⁶ PBMC. The DNA-C prime vaccinations are represented by grey arrows and the MVA-C vaccinations by black arrows. Four animals (B515, B549, B575, and B623) received a third MVA-C vaccination. **(A)** Kinetics of total magnitude of IFN- γ ELISPOT responses. **(B)** Total magnitude of responses 1 week after one, two, or three MVA-C vaccinations. **(C)** Total magnitude of responses 1 week after one, two or three MVA-C vaccinations comparing animals that received a DNA prime with those that did not. Filled symbols represent DNA-MVA vaccinated animals and open symbols indicate animals that received MVA only. Box and whisker plots show medians and ranges.

The kinetics of HIV responses in non-primed MVA-only animals differed substantially. Here, responses to HIV were being generated at the same time as responses to MVA, as there was no “advantage” of previous priming. While it may be tempting to speculate that this gave rise to the lower HIV-specific responses observed in these three animals (B533, B623, B629), an equally likely explanation is that the HIV antigens expressed by the MVA construct are poorly immunogenic in the absence of DNA priming. HIV-specific responses were boosted in the presence of titres of MVA binding antibodies of 40 to 80 at the second or third MVA vaccination in animals B515 and B549; conversely, in animal B524, HIV-specific responses were boosted, but not to levels higher than after the first MVA vaccination, in the presence of an A33 titre of 320 on the day of vaccination.

The relationship between MVA-specific antibody and HIV-specific cellular responses was examined using Spearman’s rank correlation, 1 week after the second and third MVA-C vaccinations. When responses in DNA-MVA and MVA-only animals were examined together, no significant relationships were found ($p=0.27$, $r=-0.34$ and $p=0.22$, $r=-0.38$ for B5R and A33, respectively; data not shown). Since the kinetics of HIV-specific responses was different in these two groups of animals, relationships were investigated separately. Interestingly, there was a trend towards an inverse correlation between B5R antibody responses and HIV-specific cellular responses in those animals that had been primed with DNA ($p=0.06$, $r=-0.72$; Figure 4.10A), but not in the MVA-only animals ($p=0.33$, $r=0.83$; Figure 4.10B), although the few data points for the latter group may preclude robust statistical analysis. There was no correlation between A33 responses and HIV-specific cellular responses in either group (Figure 4.10C and D).

4.4.3 The relationship between neutralising antibody responses to MVA and HIV-specific responses

Neutralising antibody responses to MVA were measured 1 week after the second MVA boost in eight baboons and 22 macaques. The relationship between neutralising antibody end-point titres and HIV-specific cellular responses at the same time-point were examined. Interestingly, there was a trend toward an inverse correlation between neutralising antibodies to MVA and HIV-specific T cell responses in baboons primed with DNA ($p=0.08$, $r=-0.89$; Figure 4.11A), indicating that neutralising antibodies generated to MVA may be having an effect on HIV-specific cellular responses, although few data points were available for this analysis. No such relationship was evident for those that received MVA only ($p=0.33$, $r=1$; Figure 4.11B), although again, limited data points were available to conduct this analysis.

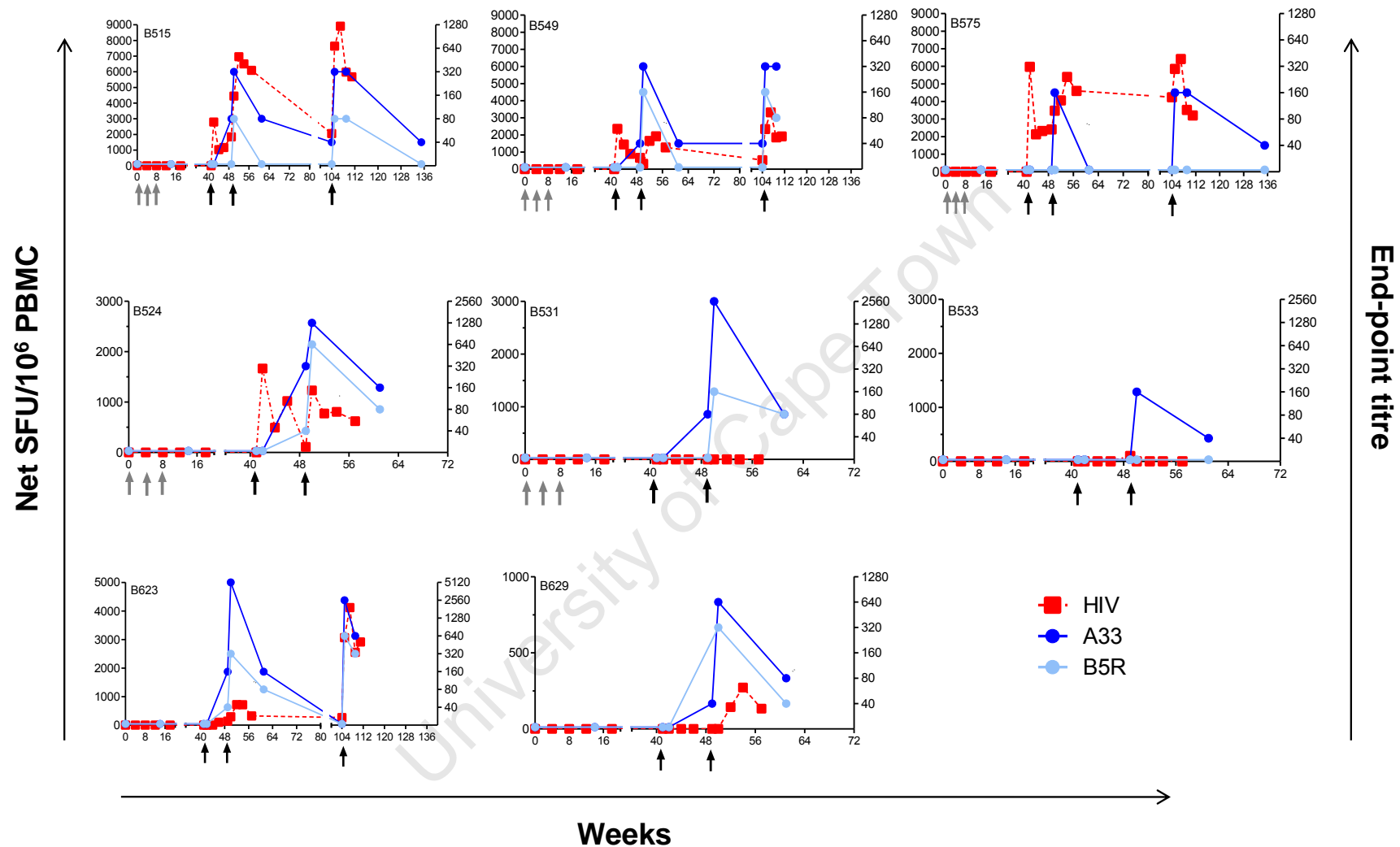


Figure 4.9 Binding antibody responses to vaccinia envelope proteins and IFN- γ ELISPOT responses to HIV. Eight baboons were immunised with SAAVI MVA-C. Five of the animals had previously received three vaccinations with DNA-C (B515, B524, B531, B549, B575). ELISPOT responses are expressed as net spot forming units (SFU)/ 10^6 PBMC, and binding antibody responses as end-point titres. The DNA-C prime vaccinations are represented by grey arrows and the MVA-C vaccinations by black arrows. A33 and B5R antibody responses are represented in dark blue and light blue respectively, and HIV responses in red. Four animals (B515, B549, B575, B623) received a third MVA vaccination.

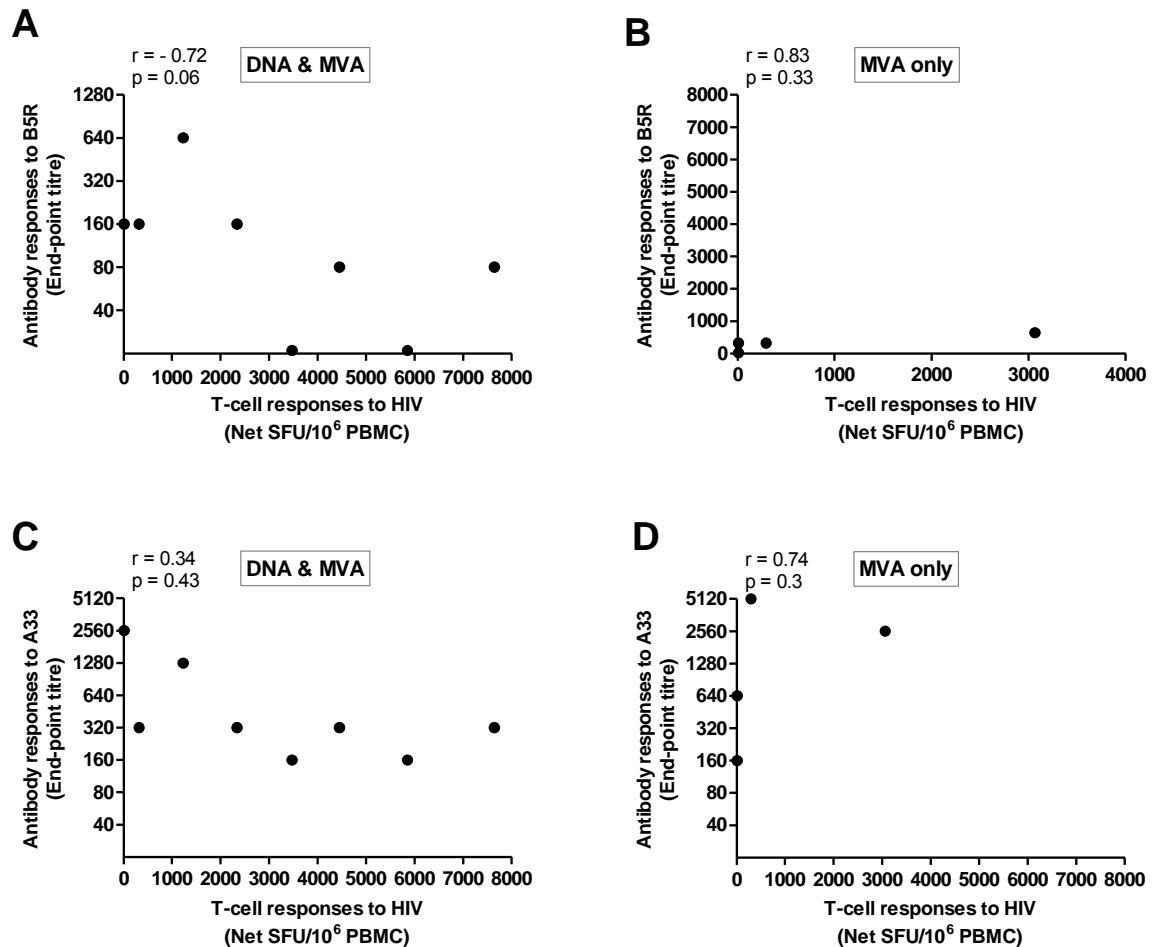


Figure 4.10 Effect of binding antibody responses to MVA on IFN- γ ELISPOT T-cell responses to HIV antigens expressed by the vector. Eight baboons were immunised with SAAVI MVA-C. Five of the animals had previously received three vaccinations with DNA-C (B515, B524, B531, B549 and B575). Four animals (B515, B549, B575, and B623) received a third MVA-C vaccination. Correlations between the total magnitude of HIV-specific T-cell responses 1 week after the second and third MVA-C vaccinations and matching end-point binding antibody titres to vaccinia capsid proteins B5R (**A** and **B**) and A33 (**C** and **D**) were examined. (**A**) and (**C**) show responses in DNA-MVA vaccinated animals, and (**B**) and (**D**) shows animals that received MVA only. Relationships were determined by Spearman's rank correlation.

The relationship was therefore examined in macaques, where sixteen animals received DNA-MVA and six received MVA only, and MVA-specific neutralising antibodies were of higher titres than in baboons. There was however no detectable influence of MVA-specific neutralising titres on HIV-specific cellular responses in those animals primed with DNA ($p=0.49$; $r=0.2$; Figure 4.11C) or not ($p=1$, $r=0$; Figure 4.11D). Thus, although there was a trend for lower HIV-specific responses the higher the MVA neutralising antibodies in 5 DNA-MVA vaccinated baboons, this was not observed in 16 DNA-MVA vaccinated macaques.

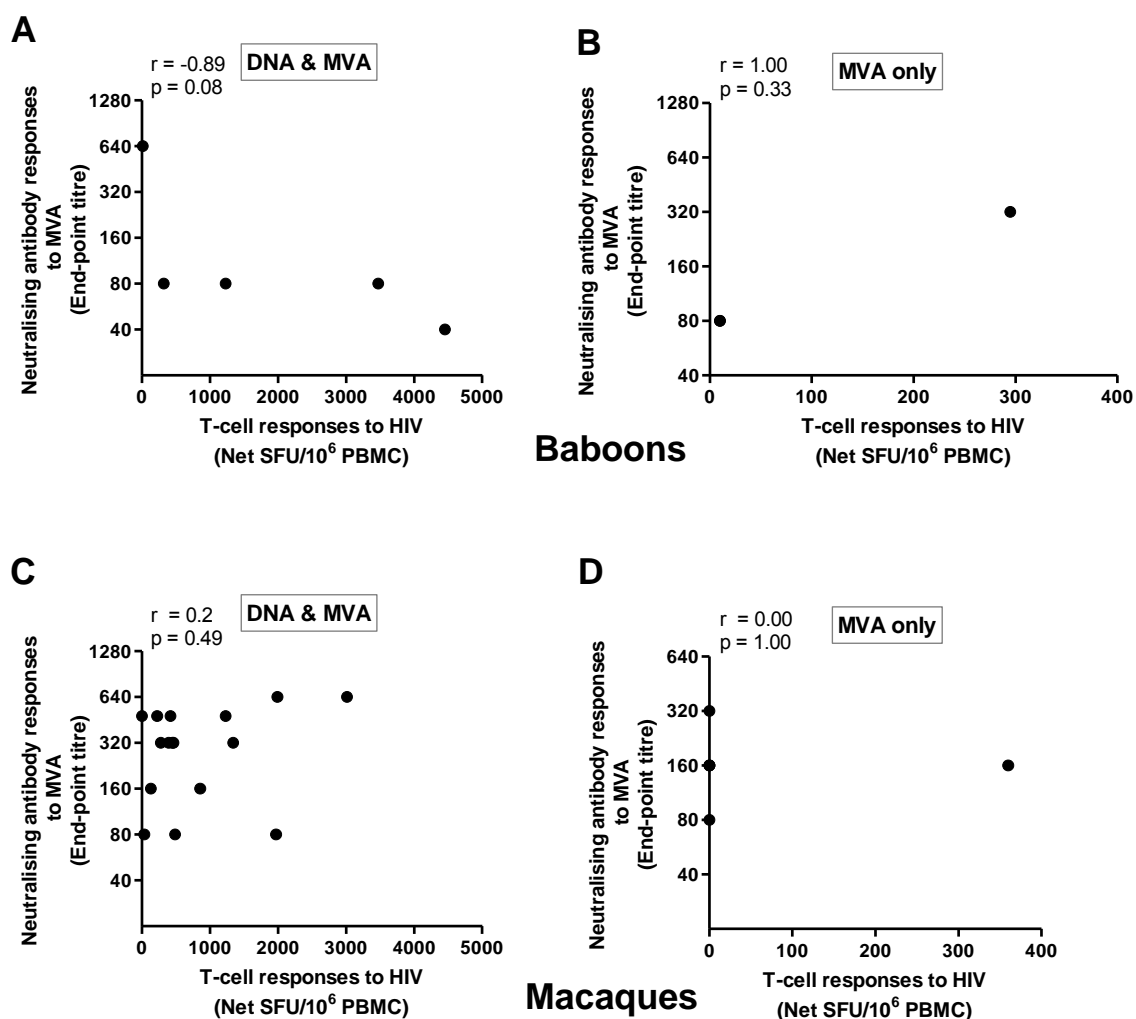


Figure 4.11 Effect of neutralising antibody responses to MVA on IFN- γ ELISPOT T-cell responses to HIV antigens expressed by the vector. Eight baboons and twenty two macaques were immunised with SAAVI MVA-C. Five of the baboons and sixteen of the macaques had previously received three vaccinations with DNA-C. Correlations between the total magnitude of HIV-specific T-cell responses 1 week after the second MVA-C vaccination and matching end-point neutralising antibody titres to MVA in baboons (A and B) and macaques (C and D) were examined. (A) and (C) show responses in DNA-MVA vaccinated animals, and (B) and (D) shows animals that received MVA only. Relationships were determined by Spearman's rank correlation.

4.4.4 MVA-specific cellular responses do not affect HIV cellular responses

Since no convincing relationship between MVA-specific antibody responses and HIV-specific responses was detected, I next investigated whether the MVA-specific cellular response may have had an influence on the HIV-specific response. Comparisons of the total IFN- γ ELISPOT response to HIV antigens and MVA are shown for individual animals in Figure 4.12. After the first MVA boost, four of five DNA primed animals generated T-cell responses to HIV, ranging from 1667-7268 SFU/10⁶ PBMC (Figure 4.12). Responses were generated to MVA in all of the

animals. Two of the four DNA-MVA responders had higher magnitude peak responses to HIV, whilst the other two had higher magnitude peak responses to MVA. In all three animals that received MVA only, the MVA-specific responses were of greater magnitude than the HIV-specific responses, at all time-points. It was interesting to observe that in animal B531, where no HIV-specific responses were detected, that there was a robust response to MVA, indicating that the vaccine did 'take'. The kinetics of the responses differed dramatically. Whilst the HIV responses contracted by an average of 64%, to 1230 SFU/10⁶ PBMC by 8 weeks following the first MVA, and in three of the four DNA-MVA responders were boosted to lower than peak after the second MVA, MVA-specific responses were boosted to higher levels. In fact, in all of the animals except B629, they did not contract and steadily increased over time. After the third MVA vaccination, both HIV and MVA responses expanded dramatically, to peak levels higher than previously generated. The MVA responses were highly stable over time, and in two of the four animals (B549 and B524) appeared to still be increasing 7 months after the third MVA.

Spearman's rank correlation was performed to determine whether the strong MVA-specific cellular responses had any effect on the HIV-specific responses. There was a weak but significant positive correlation between HIV and MVA-specific cellular responses ($p=0.03$, $r=0.5$; Figure 4.13), indicating that higher vector-specific cellular responses may result in higher HIV responses. In other words, immunogenic vectors may lead to immunogenic vector-encoded antigen responses.

4.4.5 Functional responses to MVA are dominated by single cytokine-producing cells, whereas responses to HIV show greater heterogeneity

It was of interest to compare the nature of the responses elicited to the vector, compared to the HIV antigens it encoded. Four animals were studied at three time-points to determine the polyfunctionality of responses elicited to MVA compared to HIV. The time-points tested were at 1 and 12 weeks following the second MVA vaccination, and at the final time-point of 7 months following the third MVA vaccination. T-cell responses to MVA were dominated by single cytokine-producing cells (discussed in section 4.3.2), whereas HIV-specific T-cell responses had a much larger proportion of cells producing two or three cytokines at each of the three time-points tested, irrespective of whether they received a DNA prime (Figure 4.14A) or not (Figure 4.14B). This observation was true for both CD8⁺ and CD4⁺ T-cell responses. As for MVA-specific responses, there were no changes in the functional repertoire of cytokines produced over time in HIV-specific T cells. Thus, HIV-specific responses were more polyfunctional than those produced by MVA-specific T-cells.

Chapter 4: Results

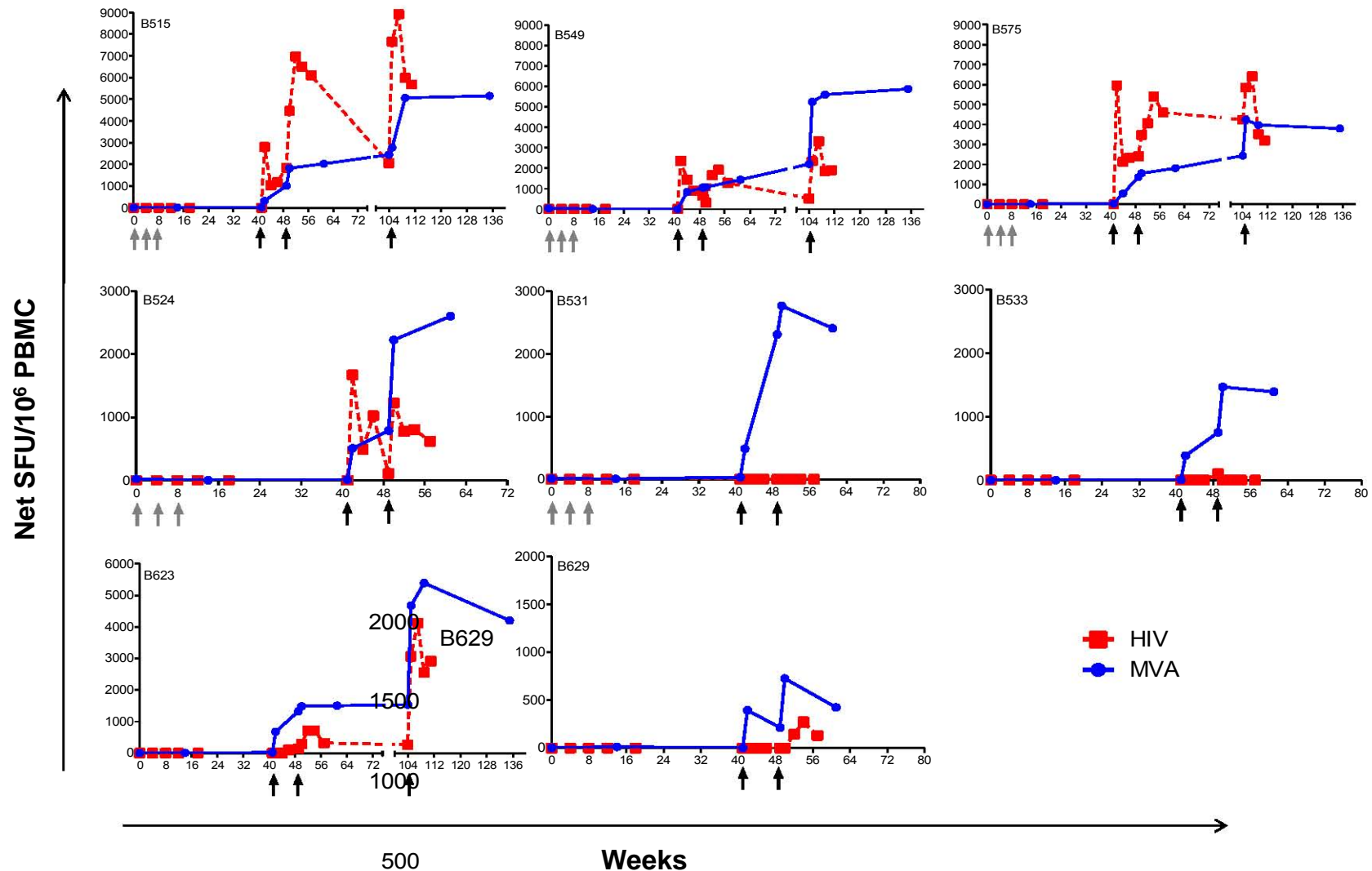


Figure 4.12 IFN- γ ELISPOT responses to MVA and HIV. Eight baboons were vaccinated with SAAVI MVA-C. Five of the animals had previously received three vaccinations with DNA-C (B515, B524, B531, B549, B575). ELISPOT responses are expressed as net spot forming units (SFU)/ 10^6 PBMC. The DNA-C prime vaccinations are represented by grey arrows and the MVA-C vaccinations by black arrows. MVA responses are represented in blue and HIV-specific responses in red. Four animals (B515, B549, B575, B623) received a third MVA-C vaccination.

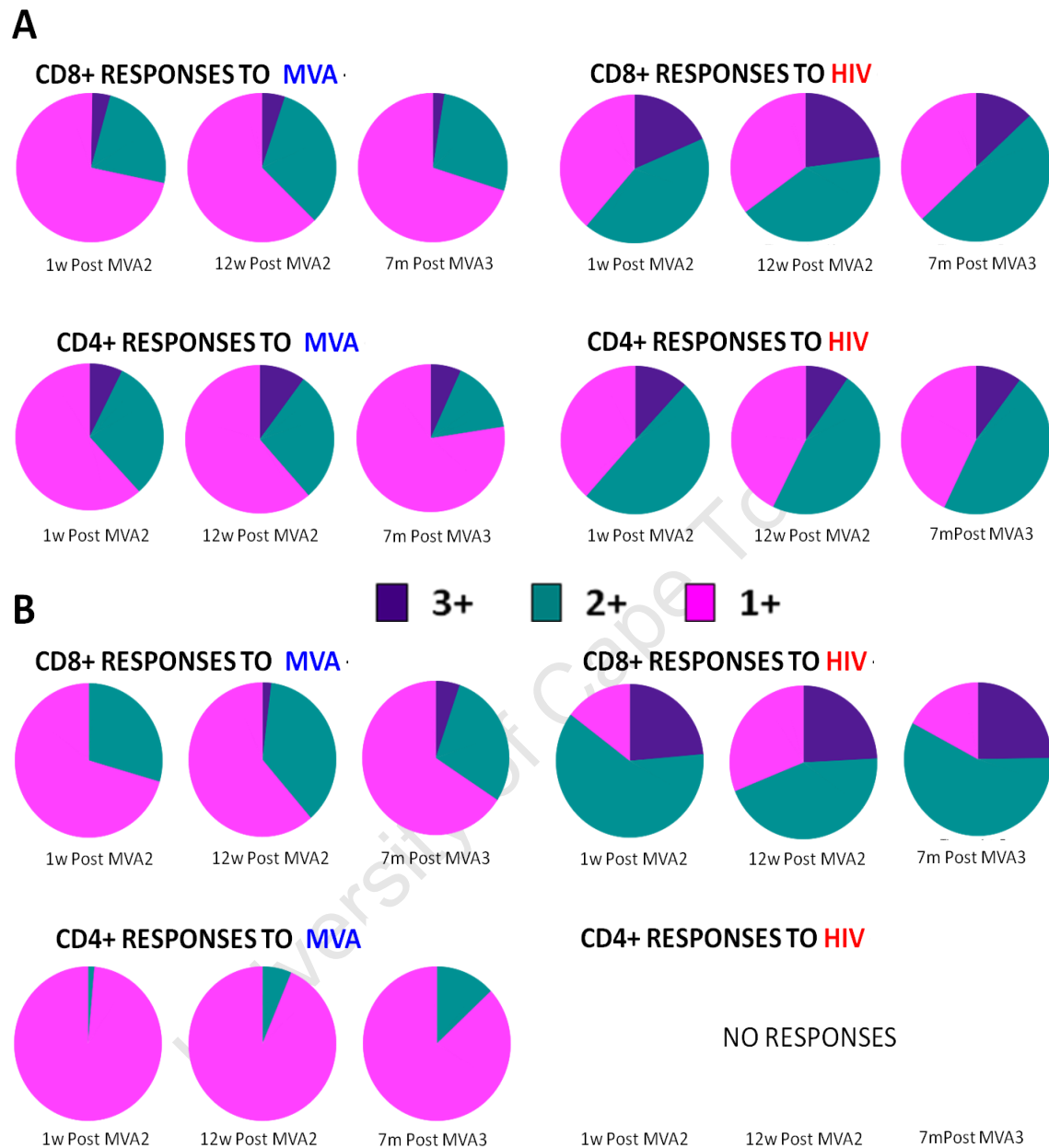


Figure 4.14 Polyfunctionality of cytokine responses to MVA and HIV. IFN- γ , IL-2 and TNF- α responses were measured by multiparameter flow cytometry at 1 week and 12 weeks after the second MVA-C vaccination and at 7 months after the third MVA vaccination in four animals. Proportions of one, two or three cytokine-producing cells were determined. (A) MVA-specific polyfunctional responses generated by CD8+ T-cells (top row, left panel) and CD4+ T-cells (bottom row, left panel) compared to HIV-specific responses in animals that received MVA and DNA primes (right panel, B515, B549, B575). (B) MVA-specific median IFN- γ , IL-2 and TNF- α cytokine responses generated by CD8+ T-cells (top row, left panel) and CD4+ T-cells (bottom row, left panel) compared to HIV-specific responses in the baboon that received MVA only (right panel, B623). One, two or three cytokine-producing cells are represented by pink, green and purple respectively.

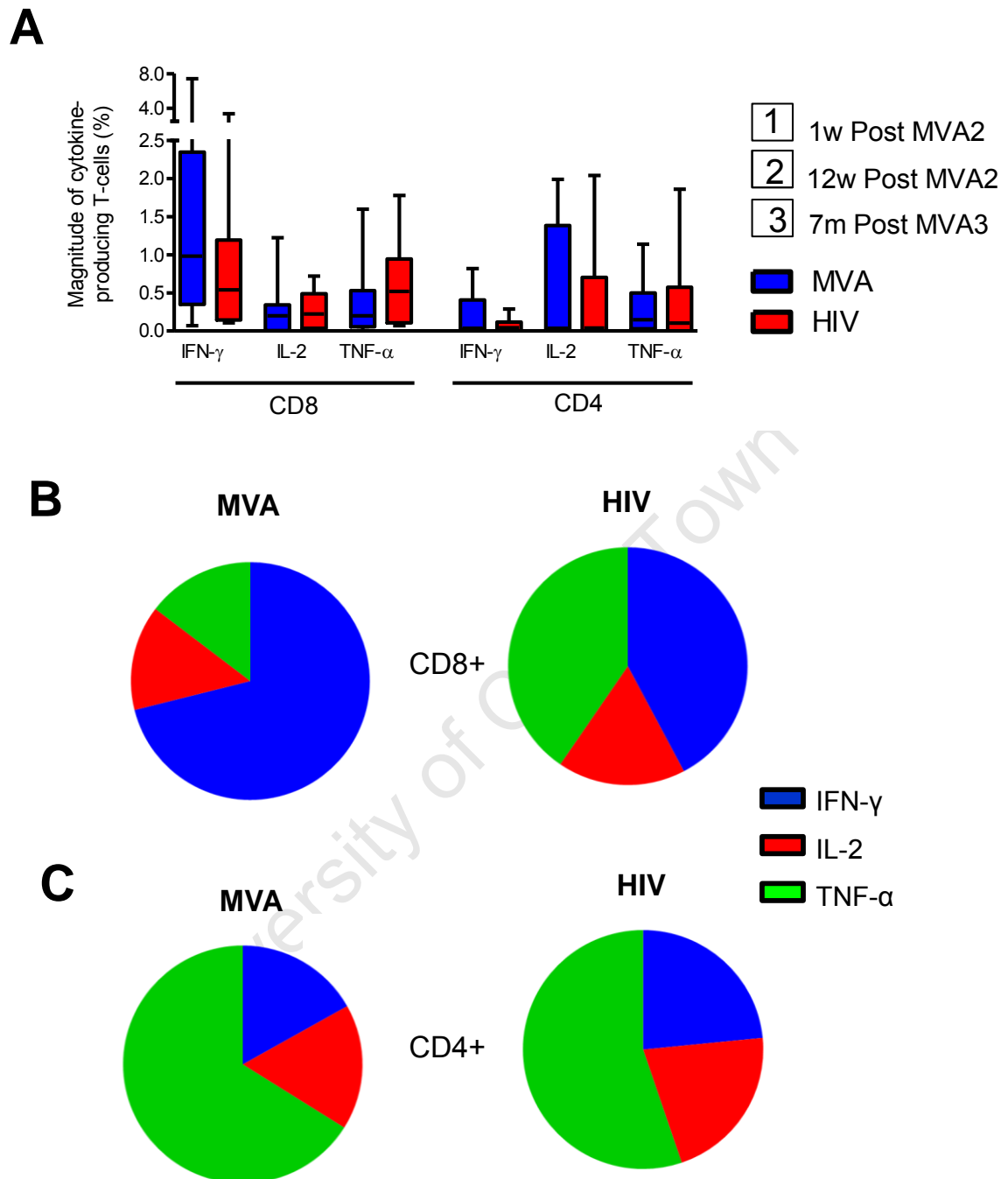


Figure 4.15 Frequency of cytokine responses to MVA and HIV. IFN- γ , IL-2 and TNF- α responses were characterised in four animals by multiparameter flow cytometry at 1 and 12 weeks after the second MVA-C vaccination and at 7 months after the third MVA vaccination. **(A)** MVA-specific median IFN- γ , IL-2 and TNF- α cytokine responses compared to HIV-specific responses elicited by T-cells. MVA responses are represented by blue bars, and HIV responses by red bars. Combined proportions of MVA-specific median IFN- γ , IL-2 and TNF- α cytokine responses compared to HIV-specific median responses elicited by CD8+ T-cells **(B)** and CD4+ T-cells **(C)** at all three time-points.

4.4.6 Phenotypic responses to MVA are identical to those of HIV

It was of interest to compare the memory profiles of HIV-specific and MVA-specific responses. Four animals were studied at three time-points to determine the phenotype of cytokine responses elicited to MVA compared to HIV. At 1 week after the second MVA vaccination, there was a significant difference between T_{CM} and T_{EM} proportions of CD8+ T-cells (Figure 4.16A), with T_{CM} making up a significantly higher ($p=0.03$) and T_{EM} a significantly lower ($p=0.03$) proportion of CD8+ T cells specific for HIV compared to MVA. At the later time-points, responses to HIV and MVA were similar, with balanced proportions of T_{CM} and T_{EM} cells. The proportions of CD4+ T-cell responses to MVA and HIV were similar (Figure 4.16B), and were dominated by central memory T-cells, with mean of 97% for the two time-points after the second MVA vaccination, and 70% at 7 months after the third MVA vaccination. Thus, the memory phenotypes of HIV-specific and MVA-specific T cells did not differ greatly.

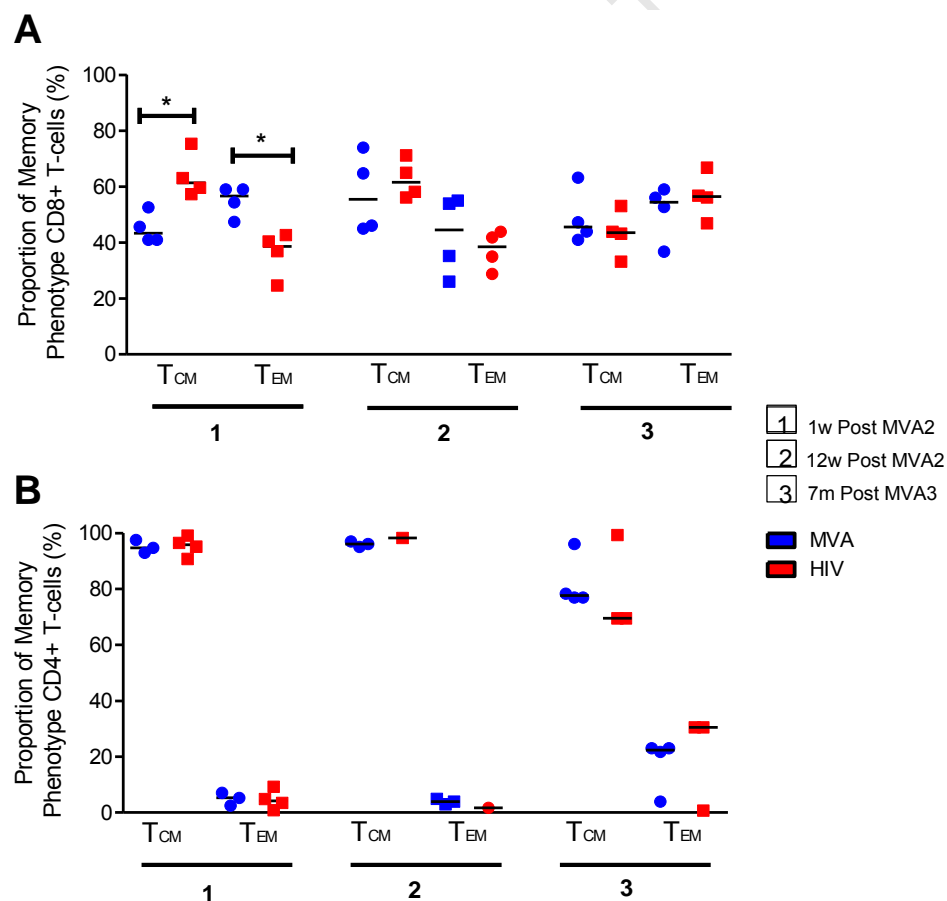


Figure 4.16 MVA-specific phenotype responses and their comparison to HIV-specific responses. Memory phenotypes of MVA-specific cells were characterised by multiparameter flow cytometry in four animals at 1 and 12 weeks after the second MVA-C vaccination and at 7 months after the third MVA-C vaccination. MVA-specific CD4+ T-cell responses (A) and CD8+ T-cell responses (B) showing proportions of central memory and effector memory at each time-point. MVA responses are represented by blue circles and bars, and HIV responses by red squares and bars.

4.5 Summary

MVA-specific responses generated in a small group of eight baboons that had been vaccinated with candidate DNA-MVA HIV vaccines were assessed, to determine whether anti-vector cellular and humoral immune responses could be detected, and what effect, if any, they had on the generation or boosting of responses to the HIV antigens expressed by the vectors. Limited analysis was also performed in 22 macaques that received a similar vaccine regimen.

Binding antibody responses to vaccinia virus envelope proteins A33 and B5R and MVA-specific neutralising antibodies were generated, however these had no clear effect on the generation or boosting of T-cell responses to the HIV antigens in the vaccine. Strong cellular responses to MVA were identified by IFN- γ ELISPOT, and although these responses were long-lasting and boosted to higher levels by each subsequent MVA vaccination, high magnitude cellular responses to the HIV antigens were elicited in most animals. Indeed, even in an environment of strong anti-vector cellular responses, HIV responses were boosted. Cellular responses to HIV showed greater polyfunctionality than MVA responses, and memory subsets generated were similar for HIV and MVA-specific T cells responses. Thus, it appears that anti-vector immunity did not impair the generation or boosting of HIV-specific cellular responses.

CHAPTER 5

Discussion

University of Cape Town

Chapter 5

Discussion

Viral vectors are proving to be important vehicles for recombinant vaccines against some of the most important pathogens, such as HIV and TB. A challenge with the use of these complex viruses is that anti-vector immune responses can potentially dampen the immune response to the vaccine antigens they express, by rapid clearance of the vaccine vector and thus shorter duration of antigen exposure to T cells (as reviewed by Duerr *et al.*, 2006). Indeed, the effect of anti-vector immunity has been recently highlighted by findings from the STEP trial, where uncircumcised men with pre-existing Ad5 immune responses showed increased acquisition of HIV after vaccination with an Ad5-vectored HIV vaccine (McElrath *et al.*, 2008). A further issue is that immunity to an HIV vaccine may wane over time, and may need to be boosted again at a later stage with the same vector, in the presence of immunity that developed at the first vaccination. Thus, both existing anti-vector immunity from natural infection, or from previous vaccination with the same recombinant vector, may be a challenge for vaccines.

This study aimed to determine whether vector responses in eight baboons vaccinated with an HIV candidate vaccine, consisting of a DNA prime and MVA boost vaccine regimen could be detected, if they persisted, and if after multiple vaccinations with MVA there was an effect on immunity to the HIV antigens they expressed. These vector-specific responses were compared to previously published HIV-specific cellular and humoral responses in the same animals (Burgers *et al.*, 2009). The major findings were as follows: (1) Both humoral and cellular vector-specific responses could indeed be detected in all vaccinated baboons; (2) Binding antibody responses to vaccinia virus membrane proteins A33 and B5R reached peak titres one week after the second MVA vaccination. They were of short duration, and even though they were boosted by the third MVA, waned rapidly. There was no correlation between these responses and HIV-specific cellular responses, or HIV envelope gp120 antibody responses; (3) Neutralising antibodies to MVA were detected in all animals at one week after the second MVA vaccination, but there was no correlation between their titres and the HIV-specific humoral or cellular responses; this analysis included 22 macaques that were immunised with a similar vaccine regimen, and similarly, no relationship was found between MVA-specific neutralising titres and HIV-specific cellular responses; (4) Robust IFN- γ responses to MVA were readily detectable by ELISPOT after the first MVA vaccination. These responses increased in magnitude following each MVA vaccination and were long-lasting.

There was no correlation between MVA-specific cellular responses and HIV-specific cellular responses; (5) Analysis in a subset of animals revealed that the cellular responses to MVA were dominated by CD8⁺ T-cells producing mainly IFN- γ alone, while HIV-specific responses showed greater heterogeneity, with more cells producing two or three cytokines simultaneously by both CD4⁺ and CD8⁺ T-cells; (6) Finally, there were no differences in the T cell memory phenotypes of responses generated to MVA and HIV, with CD4⁺ T cells falling predominantly within the central memory subset and CD8⁺ T cells distributed evenly between central and effector memory subsets.

Although not an issue that the present study dealt with, prior immunity to MVA may be as a result of prior vaccination against smallpox, with vaccinia virus. Smallpox vaccination was halted in 1971 in the UK, 1972 in the US and 1979 in South Africa, and smallpox was declared eradicated by the World Health Organisation in 1980 (Strassburg *et al.*, 1982). Thus, most people over the age of 32 in South Africa will have been vaccinated against smallpox. Thus, many older individuals (and also some laboratory and healthcare workers) will have pre-existing immunity to vaccinia virus that may influence the efficacy of an MVA-vectored HIV vaccine. Hammarlund *et al.* (2003) reported that over 90% of individuals that were vaccinated with vaccinia virus between 25 and 75 years before still had detectable humoral and cellular immune responses. In fact, humoral responses were very stable over time, detectable up to 75 years after vaccination. T-cells responses were not as long lasting with a half-life of 8 to 15 years. Interestingly, neutralising antibody titres > 1:32 were protective against smallpox. In the present study, neutralising titres of >1:40 were detectable in all animals studied; they did not, however, appear to dampen vector-induced responses.

Lower MOI were found to be better than higher MOI for detecting cellular responses, and therefore extensive optimisation was performed for all assays. MVA has been shown to result in necrosis and apoptosis of immature dendritic cells in humans at high MOI due to cytopathogenic effects, with lower MOI more efficiently infecting the cells (Kastenmuller *et al.*, 2006). This has also been shown recently in a murine model (Norder *et al.*, 2010) where an MOI of 0.5 was shown to be more infective than an MOI of 5. In studies using human PBMC, a range of MOI have been used, from as low as 0.1 (Cosma *et al.*, 2007), through an intermediate of 1.0 (Smith *et al.*, 2005; Howles *et al.*, 2010) and up to 10 (Speller and Warren, 2002). This highlights the need for optimisation to be carried out specific to the species and MVA that are being used. Relatively low MOIs may be required in baboons, and titrations resulted in an MOI of 0.1 of wild-type MVA being used-

Binding antibody responses to MVA were measured. Peak binding antibody responses of short duration were detected to the extracellular enveloped virion proteins (EEV) A33 and B5R after the second MVA vaccination, but not to the intracellular mature virion proteins (IMV), L1R and A27. Previous studies in monkeys have shown detectable MVA-specific binding antibodies after vaccination with MVA, however, the timing, specificity and duration of responses vary greatly. Earl *et al.* (2004) showed detection of durable A33, B5R and L1R responses after the first MVA vaccination, and subsequent boosting to higher levels after the second MVA. Binding antibody titres to MVA are generally lower than those generated by replication competent vaccinia virus (Sharpe *et al.*, 2001). Studies in humans have confirmed differing detection of antibody titres depending on the route and dose of MVA vaccination, with higher titres elicited when higher doses of MVA are delivered, irrespective of the route (Wilck *et al.*, 2010; Parrino *et al.*, 2006), although it does appear that higher doses delivered intramuscularly as opposed to intradermally elicit higher binding antibody responses (Currier *et al.*, 2010). Interestingly, in these studies it was found that anti-A27 responses are low or undetectable, which has also been described in macaques vaccinated with MVA (Grandpre *et al.*, 2009). This may mean that anti-A27 antibody responses are lacking in response to certain attenuated vaccinia strains, possibly due to lower levels of A27 expression (Wilck *et al.*, 2010). Anti-L1R responses were more widely described, but at least one study in humans found no responses to L1R (Parrino *et al.*, 2006). Poxvirus purification processes only treat the virus particles packaged within a cell (IMV) and EEV particles are shed into the culture medium and discarded (Malarme *et al.*, 2010). EEV and IMV forms of the virus are both infectious, with IMV located in the cytoplasm of infected cells, while EEV are IMV surrounded by an extra membrane (Earl *et al.*, 2004). MVA is infectious but cannot replicate in most primary mammalian cells as there is a block in late gene expression. A33 and B5R are expressed early and late in infection, while L1R and A27, which are required for cell entry and membrane fusion, are expressed only late in infection (Coulibaly *et al.*, 2005). The MVA used in our candidate vaccines was likely only IMV, which infected cells and early gene expression would have commenced, thus A33 and B5R responses were detected. It may be possible that the point at which the transcriptional block occurs in late expression varies between different recombinant MVA strains, and L1R and A27 expression was minimal or absent, which may explain why antibodies were not detected in this study.

This study showed no relationship between binding antibody responses and the HIV-specific cellular or humoral response, irrespective of DNA priming, although there was a trend towards an inverse correlation between B5R titres and HIV-specific cellular responses that did not reach significance ($p=0.06$). Perhaps a larger study would resolve this relationship. Interestingly, a recent study in macaques by Kannanganat *et al.* (2010) showed that pre-

existing vaccinia antibodies decreased SIV-specific cellular responses but not humoral responses, but this was in animals that were vaccinated with vaccinia 17 months prior to the first DNA prime. However, in humans it has been shown that pre-existing vaccinia antibodies can decrease both HIV-specific cellular and humoral responses (Gudmundsdotter *et al.*, 2009). It is important to consider there may be additional functions of antibodies other than neutralisation that could contribute to reducing the vaccine inoculum and therefore the responses to the transgenes. Evidence for this is suggested by the modest success of the RV-144 trial, where canarypox expressing HIV genes was tested (Rerks-Ngarm *et al.*, 2009). There was no association between the protection conferred by the vaccination regimen and the T-cell responses or production of neutralising antibodies, although antibodies to HIV Env were present. It may be possible that protection was mediated by other functions of antibody, such as antibody-dependent cellular cytotoxicity (ADCC) activity. ADCC-capable antibodies can bind virus and form immune complexes, resulting in clearance of the virus by natural killer cells before infection could take place. It would be of interest to examine the sera from animals in my study to determine whether any of the vector-specific antibodies have other functions, such as ADCC activity, that would clear (or reduce) the vaccine inoculum before an immune response could be elicited.

Neutralising antibodies to MVA were detected in all animals after the second MVA vaccination, however these titres did not correspond with capsid binding antibody titres detected. It is important to note the difference in assay antigens used, where the binding antibodies were determined in response to a few vaccinia membrane proteins, and the neutralising antibodies to whole MVA virus. Vaccinia has a range of capsid proteins such as H3L, D8L, and A28L which have been implicated in binding and entry of vaccinia virus strains into cells (Carter *et al.*, 2005). It is thus possible that capsid proteins other than the ones tested for led to the production of neutralising antibodies (Berhanu *et al.*, 2008; Davies *et al.*, 2005; Nelson *et al.*, 2008). It may be important to further characterise humoral responses elicited by MVA to a broader range of vaccinia virus proteins (Wilck *et al.*, 2010). Interestingly, the levels of neutralising antibodies in macaques were significantly higher than those in baboons, which is possibly as a result of the combination of vaccine dose and animal weight, as all the animals received 10^9 pfu/ml of vaccine, and the macaques weigh an average of between 5.34 kg (female) and 7.7 kg (male), whereas baboons weigh between 15 kg (female) and 33 kg (male). The presence of neutralising antibodies in the eight baboons and 22 macaques however had no effect on the HIV-specific cellular or humoral responses. In a recent macaque study, vaccinia-specific neutralising antibodies did show an inverse correlation with SIV-specific CD8 T-cells after the second MVA boost, but did not have an effect on SIV-specific antibody responses (Kannanganat *et al.*, 2010). The reasons for this different effect are unclear.

Robust MVA-specific cellular responses were elicited by the first MVA vaccination. They were boosted to significantly higher magnitudes after each MVA vaccination and were long lasting. Despite their high magnitudes there was no relationship between these responses and responses to the HIV inserts, even though by the third MVA vaccination, the MVA-specific cellular responses out-grew the HIV-specific cellular responses, in three of the five animals that received DNA and MVA. In other non-human primate studies, MVA also resulted in cellular responses after the first vaccination, however there were no differences between the first and second vaccinations (Earl *et al.*, 2004). This may possibly be due to a lower dose of MVA having been used (10^8). Human studies have reported strong and durable MVA-specific responses that are boosted by subsequent vaccinations (Smith *et al.*, 2005; Currier *et al.*, 2010; Precopio *et al.*, 2007). These studies suggest that vaccinia responses are always immunodominant when used as a vector, and that priming of responses, for example by DNA, is required. Interestingly a recent study in humans found that vaccinia-specific T-cells were significantly lower in those receiving the DNA prime compared to those receiving MVA only (Goepfert *et al.*, 2011). These authors suggested that DNA priming may limit anti-MVA responses, however this was not evident from my study. I found no difference between MVA-specific responses when comparing those baboons that were primed with DNA to those that were not. Further studies with greater animal numbers would shed more light on this issue. Few studies have directly compared vector-specific and HIV insert-specific responses to determine the impact, if any, of vector-specific cellular responses on HIV cellular responses. The focus tends to be on whether vector-specific antibodies impact on the production of insert-specific responses, as antibody responses are the most likely to limit the vector inoculum. However, consistent with the results of my study, Howles *et al.* (2010) showed that MVA-specific cellular responses generated by a DNA-MVA prime boost regimen in humans had no effect on HIV-specific cellular responses.

The MVA-specific cellular responses were dominated by CD8⁺ T-cells that produced mainly IFN- γ , in addition to a small number of polyfunctional cells producing combinations of IFN- γ , IL-2 and TNF- α . HIV-specific responses were evenly distributed with polyfunctional CD4⁺ and CD8⁺ T-cells producing two or more combinations of IFN- γ , IL-2 and TNF- α . Polyfunctional responses have been shown to mediate protection against some pathogens (Darrah *et al.*, 2007) and may be important in control of HIV replication (Betts *et al.*, 2006). It is possible that the largely monofunctional response to MVA in my study is the reason that there was no effect on the immune response to the HIV inserts. MVA vaccine has previously been shown to elicit high frequency CD8⁺ T-cells that are polyfunctional, with similar frequencies of CD8⁺ T-cells

elicited to vaccinia virus and MVA (Precopio *et al.*, 2007). Cells were capable of producing up to five cytokines (IFN- γ , IL-2, TNF- α , MIP-1 β and CD107a) simultaneously. Of course, I cannot exclude the possibility, having only measured three cytokines, that MVA-specific cells were not producing a range of additional cytokines, as well as cytotoxic molecules. A wide range of different cytokines are produced in response to other vectors (Pine *et al.*, 2011), and this may be true for MVA also.

This study found no differences in the memory phenotypes of MVA and HIV responses, with cytokine producing CD4⁺ T cells being predominantly central memory and CD8⁺ cells showing a balance between central and effector memory subsets. It has been previously described that MVA primed a predominantly central memory response to the HIV inserts it encoded (Pillai *et al.*, 2011). CD8⁺ T-cells to MVA in humans have been described as having an unusual phenotype, consisting of cells expressing both CD45RA and CD27, indicating a naive population of cells (Precopio *et al.*, 2007). CD27 expression was however dimmer than expected in a true naive population, and it seems MVA-specific T-cells are phenotypically unique. These were however long-lived, with responses detected 5 years later in one individual. In a recent study in mice, it was shown that MVA vaccination results in the rapid formation of central memory cells as early as two weeks after vaccination (Reyes-Sandoval *et al.*, 2011). The implications of a predominantly central-memory skewed response for HIV vaccine protection are not fully known.

This study observed strong HIV-specific immune responses despite the presence of vector-specific cellular or humoral responses. However, one issue that was not determined was whether the responses to HIV may have been even higher had there been no immunity to the MVA vector. In light of the fact that MVA elicits such strong immune responses that are often immunodominant (Smith *et al.*, 2005), genetic engineering of MVA may be one way to reduce anti-vector immunity. Garber *et al.* (2009) constructed a less antigenically complex MVA by deleting structural genes. They showed, using this construct as a vector for HIV Gag, that fewer vector-specific CD8⁺ T-cell responses and significantly higher Gag-specific responses were elicited in mice and macaques compared to the parental vector.

There were several limitations to this study. Firstly, it was difficult to draw conclusions from direct comparison between the magnitude of cellular responses to HIV and MVA, because antigen stimulation in the ELISPOT assays differed. Stimuli for HIV antigens were overlapping peptides, whilst MVA ELISPOT assays used whole virus as stimulation. Peptides do not need processing to be presented by antigen presenting cells, while whole MVA virus needs to infect cells and requires processing and presentation. This may influence the magnitude and

breadth of responses detected to MVA. Indeed, Smith *et al.* (2005) found higher MVA-specific responses when using single MVA epitopes, than when using whole MVA in ELISPOT assays. However, Howles *et al.* (2010) found a strong correlation between responses generated to whole MVA virus and vaccinia virus peptides by IFN- γ ELISPOT. Secondly, the animals in our study were not challenged, so even though we did not show a detectable effect of vector-specific immune responses on the HIV-specific responses, anti-vector responses could not be compared with protection from challenge, and it is thus impossible to know whether vaccine-induced responses could provide protection from disease or lead to control of viraemia. Indeed, even if there was an effect of vector responses on HIV-specific responses, there may have been no difference upon challenge, as observed by Kannanganat *et al.* (2010). Here, vaccinia-immune macaques with low SIV-specific cellular responses in the presence of strong anti-vector CD8⁺ T-cell responses and neutralising antibodies exhibited better control of an intra-rectal SIV challenge than those animals that had no pre-existing vaccinia virus immunity. Finally, it may be important to look at levels of CCR5 expression. In light of the presence of high level persisting MVA cellular responses, it may be important to determine their CCR5 expression to determine the infectability of the cells. CCR5 is one of the co-receptors that HIV targets for cell entry and its importance was highlighted by the failure of the STEP trial of an Ad5 HIV vaccination regimen. It was found that CCR5 expressing activated CD4⁺ T-cells were higher in those individuals with high Ad5 antibody titre (McElrath *et al.*, 2008) and that these cells could home to the genital mucosa where they presented an increased target for HIV entry (Benlahrech *et al.*, 2009). Furthermore, these vaccine candidates are in clinical trials in humans and it would be interesting to see if anti-vector responses are present, whether they are as long-lived as those seen in baboons, and if they have any effect on HIV-insert specific responses.

In summary, the cellular and humoral responses to HIV antigens expressed by a recombinant MVA vaccine vector were boosted even in an environment in which strong cellular and humoral responses to the MVA vector were present. The only successful HIV vaccine trial to date used a poxvirus vector (Rerks-Ngarm *et al.*, 2009), and thus, MVA remains a promising vaccine vector for HIV and other important infectious diseases.

REFERENCES

University of Cape Town

- Ahmad, R., S. T. Sindhu, E. Toma, R. Morisset, J. Vincelette, J. Menezes, and A. Ahmad. 2001. Evidence for a correlation between antibody-dependent cellular cytotoxicity-mediating anti-HIV-1 antibodies and prognostic predictors of HIV infection. *J. Clin. Immunol.* **21**:227-233.
- Aldaz-Carroll, L., J. C. Whitbeck, M. Ponce de Leon, H. Lou, L. Hirao, S. N. Isaacs, B. Moss, R. J. Eisenberg, and G. H. Cohen. 2005. Epitope-mapping studies define two major neutralization sites on the vaccinia virus extracellular enveloped virus glycoprotein B5R. *J. Virol.* **79**:6260-6271. doi: 10.1128/JVI.79.10.6260-6271.2005.
- Aldaz-Carroll, L., J. C. Whitbeck, M. Ponce de Leon, H. Lou, L. K. Pannell, J. Lebowitz, C. Fogg, C. L. White, B. Moss, G. H. Cohen, and R. J. Eisenberg. 2005. Physical and immunological characterization of a recombinant secreted form of the membrane protein encoded by the vaccinia virus L1R gene. *Virology.* **341**:59-71. doi: 10.1016/j.virol.2005.07.006.
- Allen, T. M., M. Altfeld, X. G. Yu, K. M. O'Sullivan, M. Lichterfeld, S. Le Gall, M. John, B. R. Mothe, P. K. Lee, E. T. Kalife, D. E. Cohen, K. A. Freedberg, D. A. Strick, M. N. Johnston, A. Sette, E. S. Rosenberg, S. A. Mallal, P. J. Goulder, C. Brander, and B. D. Walker. 2004. Selection, transmission, and reversion of an antigen-processing cytotoxic T-lymphocyte escape mutation in human immunodeficiency virus type 1 infection. *J. Virol.* **78**:7069-7078. doi: 10.1128/JVI.78.13.7069-7078.2004.
- Baba, T. W., V. Liska, R. Hofmann-Lehmann, J. Vlasak, W. Xu, S. Ayehunie, L. A. Cavacini, M. R. Posner, H. Katinger, G. Stiegler, B. J. Bernacky, T. A. Rizvi, R. Schmidt, L. R. Hill, M. E. Keeling, Y. Lu, J. E. Wright, T. C. Chou, and R. M. Ruprecht. 2000. Human neutralizing monoclonal antibodies of the IgG1 subtype protect against mucosal simian-human immunodeficiency virus infection. *Nat. Med.* **6**:200-206. doi: 10.1038/72309.
- Baden, L. R., R. Dolin, K. L. O'Brien, P. Abbrink, A. La Porte, M. S. Seaman, E. Choi, R. Tucker, M. Weitjens, M. G. Pau, J. Goudsmit, D. H. Barouch. 2009. OA05-06 LB. First-in-human Phase 1 safety and immunogenicity of an adenovirus serotype 26 HIV-1 vaccine vector. *Retrovirology.* **6**(Suppl3):036. doi: 10.1186/1742-4690-6-S3-O36
- Barouch, D. H. 2008. Challenges in the development of an HIV-1 vaccine. *Nature.* **455**:613-619. doi: 10.1038/nature07352.
- Barouch, D. H., Z. Y. Yang, W. P. Kong, B. Koriath-Schmitz, S. M. Sumida, D. M. Truitt, M. G. Kishko, J. C. Arthur, A. Miura, J. R. Mascola, N. L. Letvin, and G. J. Nabel. 2005. A human T-cell leukemia virus type 1 regulatory element enhances the immunogenicity of human immunodeficiency virus type 1 DNA vaccines in mice and nonhuman primates. *J. Virol.* **79**:8828-8834. doi: 10.1128/JVI.79.14.8828-8834.2005.
- Belshe, R. B., G. J. Gorse, M. J. Mulligan, T. G. Evans, M. C. Keefer, J. L. Excler, A. M. Duliege, J. Tartaglia, W. I. Cox, J. McNamara, K. L. Hwang, A. Bradney, D. Montefiori, and K. J. Weinhold. 1998. Induction of immune responses to HIV-1 by canarypox virus (ALVAC) HIV-1 and gp120 SF-2 recombinant vaccines in uninfected volunteers. NIAID AIDS Vaccine Evaluation Group. *AIDS.* **12**:2407-2415.
- Benlahrech, A., J. Harris, A. Meiser, T. Papagatsias, J. Hornig, P. Hayes, A. Lieber, T. Athanasopoulos, V. Bachy, E. Csomor, R. Daniels, K. Fisher, F. Gotch, L. Seymour, K. Logan, R. Barbagallo, L. Klavinskis, G. Dickson, and S. Patterson. 2009. Adenovirus vector vaccination induces expansion of memory CD4 T cells with a mucosal homing phenotype that are readily susceptible to HIV-1. *Proc. Natl. Acad. Sci. U. S. A.* **106**:19940-19945. doi: 10.1073/pnas.0907898106.
- Berhanu, A., R. L. Wilson, D. L. Kirkwood-Watts, D. S. King, T. K. Warren, S. A. Lund, L. L. Brown, A. K. Krupkin, E. Vandermay, W. Weimers, K. M. Honeychurch, D. W. Grosenbach, K. F. Jones, and D. E. Hruby. 2008. Vaccination of BALB/c mice with Escherichia coli-expressed vaccinia virus proteins A27L, B5R, and D8L protects mice from lethal vaccinia virus challenge. *J. Virol.* **82**:3517-3529. doi: 10.1128/JVI.01854-07.

- Betts, M. R., M. C. Nason, S. M. West, S. C. De Rosa, S. A. Migueles, J. Abraham, M. M. Lederman, J. M. Benito, P. A. Goepfert, M. Connors, M. Roederer, and R. A. Koup.** 2006. HIV nonprogressors preferentially maintain highly functional HIV-specific CD8+ T cells. *Blood*. **107**:4781-4789. doi: 10.1182/blood-2005-12-4818.
- Binley, J. M., E. A. Lybarger, E. T. Crooks, M. S. Seaman, E. Gray, K. L. Davis, J. M. Decker, D. Wycuff, L. Harris, N. Hawkins, B. Wood, C. Nathe, D. Richman, G. D. Tomaras, F. Bibollet-Ruche, J. E. Robinson, L. Morris, G. M. Shaw, D. C. Montefiori, and J. R. Mascola.** 2008. Profiling the specificity of neutralizing antibodies in a large panel of plasmas from patients chronically infected with human immunodeficiency virus type 1 subtypes B and C. *J. Virol.* **82**:11651-11668. doi: 10.1128/JVI.01762-08.
- Blanchard, T. J., A. Alcami, P. Andrea, and G. L. Smith.** 1998. Modified vaccinia virus Ankara undergoes limited replication in human cells and lacks several immunomodulatory proteins: implications for use as a human vaccine. *J. Gen. Virol.* **79 (Pt 5)**:1159-1167.
- Bonaldo, M. C., M. A. Martins, R. Rudersdorf, P. A. Mudd, J. B. Sacha, S. M. Piaskowski, P. C. Costa Neves, M. G. Veloso de Santana, L. Vojnov, S. Capuano 3rd, E. G. Rakasz, N. A. Wilson, J. Fulkerson, J. C. Sadoff, D. I. Watkins, and R. Galler.** 2010. Recombinant yellow fever vaccine virus 17D expressing simian immunodeficiency virus SIVmac239 gag induces SIV-specific CD8+ T-cell responses in rhesus macaques. *J. Virol.* **84**:3699-3706. doi: 10.1128/JVI.02255-09.
- Brave, A., K. Ljungberg, A. Boberg, E. Rollman, G. Engstrom, J. Hinkula, and B. Wahren.** 2006. Reduced cellular immune responses following immunization with a multi-gene HIV-1 vaccine. *Vaccine*. **24**:4524-4526. doi: 10.1016/j.vaccine.2005.08.018.
- Brave, A., K. Ljungberg, B. Wahren, and M. A. Liu.** 2007. Vaccine delivery methods using viral vectors. *Mol. Pharm.* **4**:18-32. doi: 10.1021/mp060098+.
- Brumme, Z. L., C. J. Brumme, J. Carlson, H. Streeck, M. John, Q. Eichbaum, B. L. Block, B. Baker, C. Kadie, M. Markowitz, H. Jessen, A. D. Kelleher, E. Rosenberg, J. Kaldor, Y. Yuki, M. Carrington, T. M. Allen, S. Mallal, M. Altfeld, D. Heckerman, and B. D. Walker.** 2008. Marked epitope- and allele-specific differences in rates of mutation in human immunodeficiency type 1 (HIV-1) Gag, Pol, and Nef cytotoxic T-lymphocyte epitopes in acute/early HIV-1 infection. *J. Virol.* **82**:9216-9227. doi: 10.1128/JVI.01041-08.
- Buchbinder, S. P., D. V. Mehrotra, A. Duerr, D. W. Fitzgerald, R. Mogg, D. Li, P. B. Gilbert, J. R. Lama, M. Marmor, C. Del Rio, M. J. McElrath, D. R. Casimiro, K. M. Gottesdiener, J. A. Chodakewitz, L. Corey, M. N. Robertson, and Step Study Protocol Team.** 2008. Efficacy assessment of a cell-mediated immunity HIV-1 vaccine (the Step Study): a double-blind, randomised, placebo-controlled, test-of-concept trial. *Lancet*. **372**:1881-1893. doi: 10.1016/S0140-6736(08)61591-3.
- Burgers, W. A., G. K. Chege, T. L. Muller, J. H. van Harmelen, G. Houry, E. G. Shephard, C. M. Gray, C. Williamson, and A. L. Williamson.** 2009. Broad, high-magnitude and multifunctional CD4+ and CD8+ T-cell responses elicited by a DNA and modified vaccinia Ankara vaccine containing human immunodeficiency virus type 1 subtype C genes in baboons. *J. Gen. Virol.* **90**:468-480. doi: 10.1099/vir.0.004614-0.
- Burgers, W. A., E. Shephard, J. E. Monroe, T. Greenhalgh, A. Binder, E. Hurter, J. H. Van Harmelen, C. Williamson, and A. L. Williamson.** 2008. Construction, characterization, and immunogenicity of a multigene modified vaccinia Ankara (MVA) vaccine based on HIV type 1 subtype C. *AIDS Res. Hum. Retroviruses*. **24**:195-206. doi: 10.1089/aid.2007.0205.
- Burgers, W. A., J. H. van Harmelen, E. Shephard, C. Adams, T. Mgwebi, W. Bourn, T. Hanke, A. L. Williamson, and C. Williamson.** 2006. Design and preclinical evaluation of a multigene human immunodeficiency virus type 1 subtype C DNA vaccine for clinical trial. *J. Gen. Virol.* **87**:399-410. doi: 10.1099/vir.0.81379-0.

- Burgers, W. A., and C. Williamson.** 2005. The challenges of HIV vaccine development and testing. *Best Pract. Res. Clin. Obstet. Gynaecol.* **19**:277-291. doi: 10.1016/j.bpobgyn.2004.11.001.
- Burton, D. R., A. J. Hessel, B. F. Keele, P. J. Klasse, T. A. Ketas, B. Moldt, D. C. Dunlop, P. Poignard, L. A. Doyle, L. Cavacini, R. S. Veazey, and J. P. Moore.** 2011. Limited or no protection by weakly or nonneutralizing antibodies against vaginal SHIV challenge of macaques compared with a strongly neutralizing antibody. *Proc. Natl. Acad. Sci. U. S. A.* **108**:11181-11186. doi: 10.1073/pnas.1103012108.
- Carter, G. C., M. Law, M. Hollinshead, and G. L. Smith.** 2005. Entry of the vaccinia virus intracellular mature virion and its interactions with glycosaminoglycans. *J. Gen. Virol.* **86**:1279-1290. doi: 10.1099/vir.0.80831-0.
- Casimiro, D. R., A. Tang, L. Chen, T. M. Fu, R. K. Evans, M. E. Davies, D. C. Freed, W. Hurni, J. M. Aste-Amezaga, L. Guan, R. Long, L. Huang, V. Harris, D. K. Nawrocki, H. Mach, R. D. Troutman, L. A. Isopi, K. K. Murthy, K. Rice, K. A. Wilson, D. B. Volkin, E. A. Emini, and J. W. Shiver.** 2003. Vaccine-induced immunity in baboons by using DNA and replication-incompetent adenovirus type 5 vectors expressing a human immunodeficiency virus type 1 gag gene. *J. Virol.* **77**:7663-7668.
- Casimiro, D. R., F. Wang, W. A. Schleif, X. Liang, Z. Q. Zhang, T. W. Tobery, M. E. Davies, A. B. McDermott, D. H. O'Connor, A. Fridman, A. Bagchi, L. G. Tussey, A. J. Bett, A. C. Finnefrock, T. M. Fu, A. Tang, K. A. Wilson, M. Chen, H. C. Perry, G. J. Heidecker, D. C. Freed, A. Carella, K. S. Punt, K. J. Sykes, L. Huang, V. I. Ausensi, M. Bachinsky, U. Sadasivan-Nair, D. I. Watkins, E. A. Emini, and J. W. Shiver.** 2005. Attenuation of simian immunodeficiency virus SIVmac239 infection by prophylactic immunization with dna and recombinant adenoviral vaccine vectors expressing Gag. *J. Virol.* **79**:15547-15555. doi: 10.1128/JVI.79.24.15547-15555.2005.
- Cayabyab, M. J., B. Koriath-Schmitz, Y. Sun, A. Carville, H. Balachandran, A. Miura, K. R. Carlson, A. P. Buzby, B. F. Haynes, W. R. Jacobs, and N. L. Letvin.** 2009. Recombinant Mycobacterium bovis BCG prime-recombinant adenovirus boost vaccination in rhesus monkeys elicits robust polyfunctional simian immunodeficiency virus-specific T-cell responses. *J. Virol.* **83**:5505-5513. doi: 10.1128/JVI.02544-08.
- Cebere, I., L. Dorrell, H. McShane, A. Simmons, S. McCormack, C. Schmidt, C. Smith, M. Brooks, J. E. Roberts, S. C. Darwin, P. E. Fast, C. Conlon, S. Rowland-Jones, A. J. McMichael, and T. Hanke.** 2006. Phase I clinical trial safety of DNA- and modified virus Ankara-vectored human immunodeficiency virus type 1 (HIV-1) vaccines administered alone and in a prime-boost regime to healthy HIV-1-uninfected volunteers. *Vaccine.* **24**:417-425. doi: 10.1016/j.vaccine.2005.08.041.
- Chattopadhyay, P. K., C. M. Hogerkorp, and M. Roederer.** 2008. A chromatic explosion: the development and future of multiparameter flow cytometry. *Immunology.* **125**:441-449.
- Chege, G. K., E. G. Shephard, A. Meyers, J. van Harmelen, C. Williamson, A. Lynch, C. M. Gray, E. P. Rybicki, and A. L. Williamson.** 2008. HIV-1 subtype C Pr55gag virus-like particle vaccine efficiently boosts baboons primed with a matched DNA vaccine. *J. Gen. Virol.* **89**:2214-2227. doi: 10.1099/vir.0.83501-0.
- Chege, G. K., R. Thomas, E. G. Shephard, A. Meyers, W. Bourn, C. Williamson, J. Maclean, C. M. Gray, E. P. Rybicki, and A. L. Williamson.** 2009. A prime-boost immunisation regimen using recombinant BCG and Pr55(gag) virus-like particle vaccines based on HIV type 1 subtype C successfully elicits Gag-specific responses in baboons. *Vaccine.* **27**:4857-4866. doi: 10.1016/j.vaccine.2009.05.064.
- Chege, G. K., A. L. Williamson, J. S. Passmore, W. Bourn, B. Ryffel, and E. G. Shephard.** 2005. The immune response of the Chacma baboon to Bacille Calmette Guerin: development of a primate model for BCG-based vaccine research. *Vaccine.* **23**:5783-5791. doi: 10.1016/j.vaccine.2005.07.106.

- Chirmule, N., K. Propert, S. Magosin, Y. Qian, R. Qian, and J. Wilson.** 1999. Immune responses to adenovirus and adeno-associated virus in humans. *Gene Ther.* **6**:1574-1583. doi: 10.1038/sj.gt.3300994.
- Chun, T. W., L. Stuyver, S. B. Mizell, L. A. Ehler, J. A. Mican, M. Baseler, A. L. Lloyd, M. A. Nowak, and A. S. Fauci.** 1997. Presence of an inducible HIV-1 latent reservoir during highly active antiretroviral therapy. *Proc. Natl. Acad. Sci. U. S. A.* **94**:13193-13197.
- Chung, A., E. Rollman, S. Johansson, S. J. Kent, and I. Stratov.** 2008. The utility of ADCC responses in HIV infection. *Curr. HIV. Res.* **6**:515-519.
- Chung, A. W., M. Navis, G. Isitman, R. Centre, R. Finlayson, M. Bloch, L. Gelgor, A. Kelleher, S. J. Kent, and I. Stratov.** 2011. Activation of NK cells by ADCC responses during early HIV infection. *Viral Immunol.* **24**:171-175. doi: 10.1089/vim.2010.0108.
- Corey, L., M. J. McElrath, and J. G. Kublin.** 2009. Post-step modifications for research on HIV vaccines. *AIDS.* **23**:3-8. doi: 10.1097/QAD.0b013e32830e6d6d.
- Corey, L., G. J. Nabel, C. Dieffenbach, P. Gilbert, B. F. Haynes, M. Johnston, J. Kublin, H. C. Lane, G. Pantaleo, L. J. Picker, and A. S. Fauci.** 2011. HIV-1 Vaccines and Adaptive Trial Designs. *Sci. Transl. Med.* **3**:79ps13. doi: 10.1126/scitranslmed.3001863.
- Corti, D., J. P. Langedijk, A. Hinz, M. S. Seaman, F. Vanzetta, B. M. Fernandez-Rodriguez, C. Silacci, D. Pinna, D. Jarrossay, S. Balla-Jhagjhoorsingh, B. Willems, M. J. Zekveld, H. Dreja, E. O'Sullivan, C. Pade, C. Orkin, S. A. Jeffs, D. C. Montefiori, D. Davis, W. Weissenhorn, A. McKnight, J. L. Heeney, F. Sallusto, Q. J. Sattentau, R. A. Weiss, and A. Lanzavecchia.** 2010. Analysis of memory B cell responses and isolation of novel monoclonal antibodies with neutralizing breadth from HIV-1-infected individuals. *PLoS One.* **5**:e8805. doi: 10.1371/journal.pone.0008805.
- Cosma, A., S. Buhler, R. Nagaraj, C. Staib, A. L. Hammarin, B. Wahren, F. D. Goebel, V. Erfle, and G. Sutter.** 2004. Neutralization assay using a modified vaccinia virus Ankara vector expressing the green fluorescent protein is a high-throughput method to monitor the humoral immune response against vaccinia virus. *Clin. Diagn. Lab. Immunol.* **11**:406-410.
- Cosma, A., R. Nagaraj, C. Staib, C. Diemer, F. Wopfner, H. Schatzl, D. H. Busch, G. Sutter, F. D. Goebel, and V. Erfle.** 2007. Evaluation of modified vaccinia virus Ankara as an alternative vaccine against smallpox in chronically HIV type 1-infected individuals undergoing HAART. *AIDS Res. Hum. Retroviruses.* **23**:782-793. doi: 10.1089/aid.2006.0226.
- Coulibaly, S., P. Bruhl, J. Mayrhofer, K. Schmid, M. Gerencer, and F. G. Falkner.** 2005. The nonreplicating smallpox candidate vaccines defective vaccinia Lister (dVVL) and modified vaccinia Ankara (MVA) elicit robust long-term protection. *Virology.* **341**:91-101. doi: 10.1016/j.virol.2005.06.043.
- Council of the Global HIV Vaccine Enterprise.** 2010. The 2010 scientific strategic plan of the Global HIV Vaccine Enterprise. *Nat. Med.* **16**:981-989. doi: 10.1038/nm0910-981.
- Currier, J. R., V. Ngaoy, M. S. de Souza, S. Ratto-Kim, J. H. Cox, V. R. Polonis, P. Earl, B. Moss, S. Peel, B. Slike, S. Sriplienchan, P. Thongcharoen, R. M. Paris, M. L. Robb, J. Kim, N. L. Michael, and M. A. Marovich.** 2010. Phase I safety and immunogenicity evaluation of MVA-CMDR, a multigenic, recombinant modified vaccinia Ankara-HIV-1 vaccine candidate. *PLoS One.* **5**:e13983. doi: 10.1371/journal.pone.0013983.
- Czerkinsky, C. C., L. A. Nilsson, H. Nygren, O. Ouchterlony, and A. Tarkowski.** 1983. A solid-phase enzyme-linked immunospot (ELISPOT) assay for enumeration of specific antibody-secreting cells. *J. Immunol. Methods.* **65**:109-121.

- Dahirel, V., K. Shekhar, F. Pereyra, T. Miura, M. Artyomov, S. Talsania, T. M. Allen, M. Altfeld, M. Carrington, D. J. Irvine, B. D. Walker, and A. K. Chakraborty.** 2011. From the Cover: Coordinate linkage of HIV evolution reveals regions of immunological vulnerability. *Proc. Natl. Acad. Sci. U. S. A.* **108**:11530-11535. doi: 10.1073/pnas.1105315108.
- Damian, R. T., N. D. Greene, and S. S. Kalter.** 1971. IgG subclasses in the baboon (*Papio cynocephalus*). *J. Immunol.* **106**:246-257.
- Darrah, P. A., D. T. Patel, P. M. De Luca, R. W. Lindsay, D. F. Davey, B. J. Flynn, S. T. Hoff, P. Andersen, S. G. Reed, S. L. Morris, M. Roederer, and R. A. Seder.** 2007. Multifunctional TH1 cells define a correlate of vaccine-mediated protection against *Leishmania major*. *Nat. Med.* **13**:843-850. doi: 10.1038/nm1592.
- Davenport, M. P., L. Loh, J. Petracic, and S. J. Kent.** 2008. Rates of HIV immune escape and reversion: implications for vaccination. *Trends Microbiol.* **16**:561-566. doi: 10.1016/j.tim.2008.09.001.
- Davies, D. H., M. M. McCausland, C. Valdez, D. Huynh, J. E. Hernandez, Y. Mu, S. Hirst, L. Villarreal, P. L. Felgner, and S. Crotty.** 2005. Vaccinia virus H3L envelope protein is a major target of neutralizing antibodies in humans and elicits protection against lethal challenge in mice. *J. Virol.* **79**:11724-11733. doi: 10.1128/JVI.79.18.11724-11733.2005.
- Day, C. L., P. Kiepiela, A. J. Leslie, M. van der Stok, K. Nair, N. Ismail, I. Honeyborne, H. Crawford, H. M. Coovadia, P. J. R. Goulder, B. D. Walker, and P. Klenerman.** 2007. Proliferative Capacity of Epitope-Specific CD8 T-Cell Responses Is Inversely Related to Viral Load in Chronic Human Immunodeficiency Virus Type 1 Infection. *J. Virol.* **81**:434-438. doi: 10.1128/JVI.01754-06.
- Detrick, B., R. G. Hamilton, I. D. Folds.** 2006. *Manual of Molecular and Clinical Laboratory Immunology.* ASM Press. ISBN 978-1-5581-364-2
- Dorrell, L., P. Williams, A. Suttill, D. Brown, J. Roberts, C. Conlon, T. Hanke, and A. McMichael.** 2007. Safety and tolerability of recombinant modified vaccinia virus Ankara expressing an HIV-1 gag/multiepitope immunogen (MVA.HIVA) in HIV-1-infected persons receiving combination antiretroviral therapy. *Vaccine.* **25**:3277-3283. doi: 10.1016/j.vaccine.2007.01.005.
- Dorrell, L., H. Yang, B. Ondondo, T. Dong, K. di Gleria, A. Suttill, C. Conlon, D. Brown, P. Williams, P. Bowness, N. Goonetilleke, T. Rostron, S. Rowland-Jones, T. Hanke, and A. McMichael.** 2006. Expansion and diversification of virus-specific T cells following immunization of human immunodeficiency virus type 1 (HIV-1)-infected individuals with a recombinant modified vaccinia virus Ankara/HIV-1 Gag vaccine. *J. Virol.* **80**:4705-4716. doi: 10.1128/JVI.80.10.4705-4716.2006.
- Duerr, A., J. N. Wasserheit, and L. Corey.** 2006. HIV vaccines: new frontiers in vaccine development. *Clin. Infect. Dis.* **43**:500-511. doi: 10.1086/505979.
- Earl, P. L., J. L. Americo, and B. Moss.** 2003. Development and use of a vaccinia virus neutralization assay based on flow cytometric detection of green fluorescent protein. *J. Virol.* **77**:10684-10688.
- Earl, P. L., J. L. Americo, L. S. Wyatt, L. A. Eller, D. C. Montefiori, R. Byrum, M. Piatak, J. D. Lifson, R. R. Amara, H. L. Robinson, J. W. Huggins, and B. Moss.** 2007. Recombinant modified vaccinia virus Ankara provides durable protection against disease caused by an immunodeficiency virus as well as long-term immunity to an orthopoxvirus in a non-human primate. *Virology.* **366**:84-97. doi: 10.1016/j.virol.2007.02.041.
- Earl, P. L., J. L. Americo, L. S. Wyatt, L. A. Eller, J. C. Whitbeck, G. H. Cohen, R. J. Eisenberg, C. J. Hartmann, D. L. Jackson, D. A. Kulesh, M. J. Martinez, D. M. Miller, E. M. Mucker, J. D. Shamblin, S. H. Zwiers, J. W. Huggins, P. B. Jahrling, and B. Moss.** 2004. Immunogenicity of a highly attenuated MVA smallpox vaccine and protection against monkeypox. *Nature.* **428**:182-185. doi: 10.1038/nature02331.

- Ennis, F. A., J. Cruz, W. E. Demkowicz Jr, A. L. Rothman, and D. J. McClain.** 2002. Primary induction of human CD8+ cytotoxic T lymphocytes and interferon-gamma-producing T cells after smallpox vaccination. *J. Infect. Dis.* **185**:1657-1659. doi: 10.1086/340517.
- Euler, Z., M. J. van Gils, E. M. Bunnik, P. Phung, B. Schweighardt, T. Wrin, and H. Schuitemaker.** 2010. Cross-reactive neutralizing humoral immunity does not protect from HIV type 1 disease progression. *J. Infect. Dis.* **201**:1045-1053. doi: 10.1086/651144.
- Fauci, A. S., M. I. Johnston, C. W. Dieffenbach, D. R. Burton, S. M. Hammer, J. A. Hoxie, M. Martin, J. Overbaugh, D. I. Watkins, A. Mahmoud, and W. C. Greene.** 2008. HIV vaccine research: the way forward. *Science.* **321**:530-532. doi: 10.1126/science.1161000.
- Finzi, D., M. Hermankova, T. Pierson, L. M. Carruth, C. Buck, R. E. Chaisson, T. C. Quinn, K. Chadwick, J. Margolick, R. Brookmeyer, J. Gallant, M. Markowitz, D. D. Ho, D. D. Richman, and R. F. Siliciano.** 1997. Identification of a reservoir for HIV-1 in patients on highly active antiretroviral therapy. *Science.* **278**:1295-1300.
- Flynn, N. M., D. N. Forthal, C. D. Harro, F. N. Judson, K. H. Mayer, M. F. Para, and rgp120 HIV Vaccine Study Group.** 2005. Placebo-controlled phase 3 trial of a recombinant glycoprotein 120 vaccine to prevent HIV-1 infection. *J. Infect. Dis.* **191**:654-665. doi: 10.1086/428404.
- Franchini, G.** 2009. Choosing the right memory T cell for HIV. *Nat. Med.* **15**:244-246. doi: 10.1038/nm0309-244.
- Frey, S. E., R. B. Couch, C. O. Tacket, J. J. Treanor, M. Wolff, F. K. Newman, R. L. Atmar, R. Edelman, C. M. Nolan, R. B. Belshe, and National Institute of Allergy and Infectious Diseases Smallpox Vaccine Study Group.** 2002. Clinical responses to undiluted and diluted smallpox vaccine. *N. Engl. J. Med.* **346**:1265-1274. doi: 10.1056/NEJMoa020534.
- Friedrich, T. C., A. B. McDermott, M. R. Reynolds, S. Piaskowski, S. Fuenger, I. P. De Souza, R. Rudersdorf, C. Cullen, L. J. Yant, L. Vojnov, J. Stephany, S. Martin, D. H. O'Connor, N. Wilson, and D. I. Watkins.** 2004. Consequences of cytotoxic T-lymphocyte escape: common escape mutations in simian immunodeficiency virus are poorly recognized in naive hosts. *J. Virol.* **78**:10064-10073. doi: 10.1128/JVI.78.18.10064-10073.2004.
- Gallarda, J. L., D. R. Henrard, D. Liu, S. Harrington, S. L. Stramer, J. E. Valinsky, and P. Wu.** 1992. Early detection of antibody to human immunodeficiency virus type 1 by using an antigen conjugate immunoassay correlates with the presence of immunoglobulin M antibody. *J. Clin. Microbiol.* **30**:2379-2384.
- Gamble, L. J., and Q. L. Matthews.** 2010. Current progress in the development of a prophylactic vaccine for HIV-1. *Drug Des. Devel. Ther.* **5**:9-26. doi: 10.2147/DDDT.S6959.
- Garber, D. A., L. A. O'Mara, J. Zhao, S. Gangadhara, I. An, and M. B. Feinberg.** 2009. Expanding the repertoire of Modified Vaccinia Ankara-based vaccine vectors via genetic complementation strategies. *PLoS One.* **4**:e5445. doi: 10.1371/journal.pone.0005445.
- Geldmacher, C., J. R. Currier, E. Herrmann, A. Haule, E. Kuta, F. McCutchan, L. Njovu, S. Geis, O. Hoffmann, L. Maboko, C. Williamson, D. Birx, A. Meyerhans, J. Cox, and M. Hoelscher.** 2007. CD8 T-Cell Recognition of Multiple Epitopes within Specific Gag Regions Is Associated with Maintenance of a Low Steady-State Viremia in Human Immunodeficiency Virus Type 1-Seropositive Patients. *J. Virol.* **81**:2440-2448. doi: 10.1128/JVI.01847-06.
- Gilbert, P., M. Wang, T. Wrin, C. Petropoulos, M. Gurwith, F. Sinangil, P. D'Souza, I. R. Rodriguez-Chavez, A. DeCamp, M. Giganti, P. W. Berman, S. G. Self, and D. C. Montefiori.** 2010. Magnitude and breadth of a nonprotective neutralizing antibody response in an efficacy trial of a candidate HIV-1 gp120 vaccine. *J. Infect. Dis.* **202**:595-605. doi: 10.1086/654816.

- Gilbert, P. B., M. L. Peterson, D. Follmann, M. G. Hudgens, D. P. Francis, M. Gurwith, W. L. Heyward, D. V. Jobes, V. Popovic, S. G. Self, F. Sinangil, D. Burke, and P. W. Berman.** 2005. Correlation between immunologic responses to a recombinant glycoprotein 120 vaccine and incidence of HIV-1 infection in a phase 3 HIV-1 preventive vaccine trial. *J. Infect. Dis.* **191**:666-677. doi: 10.1086/428405.
- Girard, M. P., S. K. Osmanov, and M. P. Kieny.** 2006. A review of vaccine research and development: the human immunodeficiency virus (HIV). *Vaccine.* **24**:4062-4081. doi: 10.1016/j.vaccine.2006.02.031.
- Goepfert, P. A., M. L. Elizaga, A. Sato, L. Qin, M. Cardinali, C. M. Hay, J. Hural, S. C. DeRosa, O. D. DeFawe, G. D. Tomaras, D. C. Montefiori, Y. Xu, L. Lai, S. A. Kalams, L. R. Baden, S. E. Frey, W. A. Blattner, L. S. Wyatt, B. Moss, H. L. Robinson, and National Institute of Allergy and Infectious Diseases HIV Vaccine Trials Network.** 2011. Phase 1 safety and immunogenicity testing of DNA and recombinant modified vaccinia Ankara vaccines expressing HIV-1 virus-like particles. *J. Infect. Dis.* **203**:610-619. doi: 10.1093/infdis/jiq105.
- Gomez-Roman, V. R., L. J. Patterson, D. Venzon, D. Liewehr, K. Aldrich, R. Florese, and M. Robert-Guroff.** 2005. Vaccine-elicited antibodies mediate antibody-dependent cellular cytotoxicity correlated with significantly reduced acute viremia in rhesus macaques challenged with SIVmac251. *J. Immunol.* **174**:2185-2189.
- Goonetilleke, N., M. K. Liu, J. F. Salazar-Gonzalez, G. Ferrari, E. Giorgi, V. V. Ganusov, B. F. Keele, G. H. Learn, E. L. Turnbull, M. G. Salazar, K. J. Weinhold, S. Moore, CHAVI Clinical Core B, N. Letvin, B. F. Haynes, M. S. Cohen, P. Hraber, T. Bhattacharya, P. Borrow, A. S. Perelson, B. H. Hahn, G. M. Shaw, B. T. Korber, and A. J. McMichael.** 2009. The first T cell response to transmitted/founder virus contributes to the control of acute viremia in HIV-1 infection. *J. Exp. Med.* **206**:1253-1272. doi: 10.1084/jem.20090365.
- Goonetilleke, N., S. Moore, L. Dally, N. Winstone, I. Cebere, A. Mahmoud, S. Pinheiro, G. Gillespie, D. Brown, V. Loach, J. Roberts, A. Guimaraes-Walker, P. Hayes, K. Loughran, C. Smith, J. De Bont, C. Verlinde, D. Vooijs, C. Schmidt, M. Boaz, J. Gilmour, P. Fast, L. Dorrell, T. Hanke, and A. J. McMichael.** 2006. Induction of multifunctional human immunodeficiency virus type 1 (HIV-1)-specific T cells capable of proliferation in healthy subjects by using a prime-boost regimen of DNA- and modified vaccinia virus Ankara-vectored vaccines expressing HIV-1 Gag coupled to CD8+ T-cell epitopes. *J. Virol.* **80**:4717-4728. doi: 10.1128/JVI.80.10.4717-4728.2006.
- Goulder, P. J., and D. I. Watkins.** 2004. HIV and SIV CTL escape: implications for vaccine design. *Nat. Rev. Immunol.* **4**:630-640. doi: 10.1038/nri1417.
- Graham, B. S., R. A. Koup, M. Roederer, R. T. Bailer, M. E. Enama, Z. Moodie, J. E. Martin, M. M. McCluskey, B. K. Chakrabarti, L. Lamoreaux, C. A. Andrews, P. L. Gomez, J. R. Mascola, G. J. Nabel, and Vaccine Research Center 004 Study Team.** 2006. Phase 1 safety and immunogenicity evaluation of a multiclade HIV-1 DNA candidate vaccine. *J. Infect. Dis.* **194**:1650-1660. doi: 10.1086/509259.
- Grandpre, L. E., J. S. Duke-Cohan, B. A. Ewald, C. Devoy, D. H. Barouch, N. L. Letvin, E. L. Reinherz, L. R. Baden, R. Dolin, and M. S. Seaman.** 2009. Immunogenicity of recombinant Modified Vaccinia Ankara following a single or multi-dose vaccine regimen in rhesus monkeys. *Vaccine.* **27**:1549-1556. doi: 10.1016/j.vaccine.2009.01.010.
- Gray, E. S., M. C. Madiga, P. L. Moore, K. Mlisana, S. S. Abdool Karim, J. M. Binley, G. M. Shaw, J. R. Mascola, and L. Morris.** 2009. Broad neutralization of human immunodeficiency virus type 1 mediated by plasma antibodies against the gp41 membrane proximal external region. *J. Virol.* **83**:11265-11274. doi: 10.1128/JVI.01359-09.
- Gray, G. E., M. Allen, Z. Moodie, G. Churchyard, L. G. Bekker, M. Nchabeleng, K. Mlisana, B. Metch, G. de Bruyn, M. H. Latka, S. Roux, M. Mathebula, N. Naicker, C. Ducar, D. K. Carter, A. Puren, N. Eaton, M. J. McElrath, M. Robertson, L. Corey, J. G. Kublin, and on behalf of the HVTN**

- 503/Phambili study team.** 2011. Safety and efficacy of the HVTN 503/Phambili Study of a clade-B-based HIV-1 vaccine in South Africa: a double-blind, randomised, placebo-controlled test-of-concept phase 2b study. *Lancet Infect. Dis.* **11**:507-515. doi: 10.1016/S1473-3099(11)70098-6.
- Gudmundsdotter, L., C. Nilsson, A. Brave, B. Hejdeman, P. Earl, B. Moss, M. Robb, J. Cox, N. Michael, M. Marovich, G. Biberfeld, E. Sandstrom, and B. Wahren.** 2009. Recombinant Modified Vaccinia Ankara (MVA) effectively boosts DNA-primed HIV-specific immune responses in humans despite pre-existing vaccinia immunity. *Vaccine.* **27**:4468-4474. doi: 10.1016/j.vaccine.2009.05.018.
- Guimaraes-Walker, A., N. Mackie, S. McCormack, T. Hanke, C. Schmidt, J. Gilmour, B. Barin, A. McMichael, J. Weber, K. Legg, A. Babiker, P. Hayes, F. Gotch, C. Smith, L. Dally, L. Dorrell, I. Ceber, R. Kay, N. Winstone, S. Moore, N. Goonetilleke, P. Fast, and IAVI-006 Study Group.** 2008. Lessons from IAVI-006, a phase I clinical trial to evaluate the safety and immunogenicity of the pTHr.HIVA DNA and MVA.HIVA vaccines in a prime-boost strategy to induce HIV-1 specific T-cell responses in healthy volunteers. *Vaccine.* **26**:6671-6677. doi: 10.1016/j.vaccine.2008.09.016.
- Haigwood, N. L.** 2009. Update on animal models for HIV research. *Eur. J. Immunol.* **39**:1994-1999. doi: 10.1002/eji.200939576.
- Hammarlund, E., M. W. Lewis, S. G. Hansen, L. I. Strelow, J. A. Nelson, G. J. Sexton, J. M. Hanifin, and M. K. Slifka.** 2003. Duration of antiviral immunity after smallpox vaccination. *Nat. Med.* **9**:1131-1137. doi: 10.1038/nm917.
- Hanke, T.** 2001. Vehicles for genetic vaccines against human immunodeficiency virus: induction of T cell-mediated immune responses. *Curr. Mol. Med.* **1**:123-135.
- Hanke, T., N. Goonetilleke, A. J. McMichael, and L. Dorrell.** 2007. Clinical experience with plasmid DNA- and modified vaccinia virus Ankara-vectored human immunodeficiency virus type 1 clade A vaccine focusing on T-cell induction. *J. Gen. Virol.* **88**:1-12. doi: 10.1099/vir.0.82493-0.
- Hansen, S. G., J. C. Ford, M. S. Lewis, A. B. Ventura, C. M. Hughes, L. Coyne-Johnson, N. Whizin, K. Oswald, R. Shoemaker, T. Swanson, A. W. Legasse, M. J. Chiuchiolo, C. L. Parks, M. K. Axthelm, J. A. Nelson, M. A. Jarvis, M. Piatak Jr, J. D. Lifson, and L. J. Picker.** 2011. Profound early control of highly pathogenic SIV by an effector memory T-cell vaccine. *Nature.* **473**:523-527. doi: 10.1038/nature10003.
- Hansen, S. G., C. Vieville, N. Whizin, L. Coyne-Johnson, D. C. Siess, D. D. Drummond, A. W. Legasse, M. K. Axthelm, K. Oswald, C. M. Trubey, M. Piatak Jr, J. D. Lifson, J. A. Nelson, M. A. Jarvis, and L. J. Picker.** 2009. Effector memory T cell responses are associated with protection of rhesus monkeys from mucosal simian immunodeficiency virus challenge. *Nat. Med.* **15**:293-299. doi: 10.1038/nm.1935.
- Harari, A., P. A. Bart, W. Stohr, G. Tapia, M. Garcia, E. Medjitna-Rais, S. Burnet, C. Cellera, O. Erlwein, T. Barber, C. Moog, P. Liljestrom, R. Wagner, H. Wolf, J. P. Kraehenbuhl, M. Esteban, J. Heeney, M. J. Frchette, J. Tartaglia, S. McCormack, A. Babiker, J. Weber, and G. Pantaleo.** 2008. An HIV-1 clade C DNA prime, NYVAC boost vaccine regimen induces reliable, polyfunctional, and long-lasting T cell responses. *J. Exp. Med.* **205**:63-77. doi: 10.1084/jem.20071331.
- Harro, C., X. Sun, J. E. Stek, R. Y. Leavitt, D. V. Mehrotra, F. Wang, A. J. Bett, D. R. Casimiro, J. W. Shiver, M. J. DiNubile, E. Quirk, and Merck V526-001 Study Group.** 2009. Safety and immunogenicity of the Merck adenovirus serotype 5 (MRKAd5) and MRKAd6 human immunodeficiency virus type 1 trigenic vaccines alone and in combination in healthy adults. *Clin. Vaccine Immunol.* **16**:1285-1292. doi: 10.1128/CVI.00144-09.
- Hersperger, A. R., J. N. Martin, L. Y. Shin, P. M. Sheth, C. M. Kovacs, G. L. Cosma, G. Makedonas, F. Pereyra, B. D. Walker, R. Kaul, S. G. Deeks, and M. R. Betts.** 2011. Increased HIV-specific CD8+ T-cell cytotoxic potential in HIV elite controllers is associated with T-bet expression. *Blood.* **117**:3799-3808. doi: 10.1182/blood-2010-12-322727.

- Hersperger, A. R., F. Pereyra, M. Nason, K. Demers, P. Sheth, L. Y. Shin, C. M. Kovacs, B. Rodriguez, S. F. Sieg, L. Teixeira-Johnson, D. Gudonis, P. A. Goepfert, M. M. Lederman, I. Frank, G. Makedonas, R. Kaul, B. D. Walker, and M. R. Betts. 2010. Perforin expression directly ex vivo by HIV-specific CD8 T-cells is a correlate of HIV elite control. *PLoS Pathog.* **6**:e1000917. doi: 10.1371/journal.ppat.1000917.
- Hessell, A. J., L. Hangartner, M. Hunter, C. E. Havenith, F. J. Beurskens, J. M. Bakker, C. M. Lanigan, G. Landucci, D. N. Forthal, P. W. Parren, P. A. Marx, and D. R. Burton. 2007. Fc receptor but not complement binding is important in antibody protection against HIV. *Nature.* **449**:101-104. doi: 10.1038/nature06106.
- Hornbeck, P., S. E. Winston, and S. A. Fuller. 2001. Enzyme-linked immunosorbent assays (ELISA). *Curr. Protoc. Mol. Biol.* **Chapter 11**:Unit11.2. doi: 10.1002/0471142727.mb1102s15.
- Horton, H., E. P. Thomas, J. A. Stucky, I. Frank, Z. Moodie, Y. Huang, Y. L. Chiu, M. J. McElrath, and S. C. De Rosa. 2007. Optimization and validation of an 8-color intracellular cytokine staining (ICS) assay to quantify antigen-specific T cells induced by vaccination. *J. Immunol. Methods.* **323**:39-54. doi: 10.1016/j.jim.2007.03.002.
- Horton, H., T. U. Vogel, D. K. Carter, K. Vielhuber, D. H. Fuller, T. Shipley, J. T. Fuller, K. J. Kunstman, G. Sutter, D. C. Montefiori, V. Erfle, R. C. Desrosiers, N. Wilson, L. J. Picker, S. M. Wolinsky, C. Wang, D. B. Allison, and D. I. Watkins. 2002. Immunization of rhesus macaques with a DNA prime/modified vaccinia virus Ankara boost regimen induces broad simian immunodeficiency virus (SIV)-specific T-cell responses and reduces initial viral replication but does not prevent disease progression following challenge with pathogenic SIVmac239. *J. Virol.* **76**:7187-7202.
- Howles, S., A. Guimaraes-Walker, H. Yang, G. Hancock, K. di Gleria, T. Tarragona-Fiol, P. Hayes, J. Gilmour, A. Bridgeman, T. Hanke, A. McMichael, and L. Dorrell. 2010. Vaccination with a modified vaccinia virus Ankara (MVA)-vectored HIV-1 immunogen induces modest vector-specific T cell responses in human subjects. *Vaccine.* **28**:7306-7312. doi: 10.1016/j.vaccine.2010.08.077.
- Hutnick, N. A., D. G. Carnathan, S. A. Dubey, G. Makedonas, K. S. Cox, L. Kierstead, S. J. Ratcliffe, M. N. Robertson, D. R. Casimiro, H. C. Ertl, and M. R. Betts. 2009. Baseline Ad5 serostatus does not predict Ad5 HIV vaccine-induced expansion of adenovirus-specific CD4+ T cells. *Nat. Med.* **15**:876-878. doi: 10.1038/nm.1989.
- Im, E. J., J. P. Nkolola, K. di Gleria, A. J. McMichael, and T. Hanke. 2006. Induction of long-lasting multi-specific CD8+ T cells by a four-component DNA-MVA/HIVA-RENTA candidate HIV-1 vaccine in rhesus macaques. *Eur. J. Immunol.* **36**:2574-2584. doi: 10.1002/eji.200636482.
- Kannanganat, S., P. Nigam, V. Velu, P. L. Earl, L. Lai, L. Chennareddi, B. Lawson, R. L. Wilson, D. C. Montefiori, P. A. Kozlowski, B. Moss, H. L. Robinson, and R. R. Amara. 2010. Preexisting vaccinia virus immunity decreases SIV-specific cellular immunity but does not diminish humoral immunity and efficacy of a DNA/MVA vaccine. *J. Immunol.* **185**:7262-7273. doi: 10.4049/jimmunol.1000751.
- Karnasuta, C., R. M. Paris, J. H. Cox, S. Nitayaphan, P. Pitisuttithum, P. Thongcharoen, A. E. Brown, S. Gurunathan, J. Tartaglia, W. L. Heyward, J. G. McNeil, D. L. Bix, M. S. de Souza, and Thai AIDS Vaccine Evaluation Group, Thailand. 2005. Antibody-dependent cell-mediated cytotoxic responses in participants enrolled in a phase I/II ALVAC-HIV/AIDS VAX B/E prime-boost HIV-1 vaccine trial in Thailand. *Vaccine.* **23**:2522-2529. doi: 10.1016/j.vaccine.2004.10.028.
- Kastenmuller, W., I. Drexler, H. Ludwig, V. Erfle, C. Peschel, H. Bernhard, and G. Sutter. 2006. Infection of human dendritic cells with recombinant vaccinia virus MVA reveals general persistence of viral early transcription but distinct maturation-dependent cytopathogenicity. *Virology.* **350**:276-288. doi: 10.1016/j.virol.2006.02.039.

- Keckler, S.** MicrobeLibrary - Poxvirus Replication Cycle **2005**:
<http://archive.microbelibrary.org/ASMOOnly/Details.asp?ID=1944>
- Keefer, M. C., S. E. Frey, M. Elizaga, B. Metch, S. C. De Rosa, P. F. Barroso, G. Tomaras, M. Cardinali, P. Goepfert, A. Kalichman, V. Philippon, M. J. McElrath, X. Jin, G. Ferrari, O. D. Defawe, G. P. Mazzara, D. Montefiori, M. Pensiero, D. L. Panicali, L. Corey, and NIAID HIV Vaccine Trials Network.** 2011. A phase I trial of preventive HIV vaccination with heterologous poxviral-vectors containing matching HIV-1 inserts in healthy HIV-uninfected subjects. *Vaccine*. **29**:1948-1958. doi: 10.1016/j.vaccine.2010.12.104.
- Keele, B. F., H. Li, G. H. Learn, P. Hraber, E. E. Giorgi, T. Grayson, C. Sun, Y. Chen, W. W. Yeh, N. L. Letvin, J. R. Mascola, G. J. Nabel, B. F. Haynes, T. Bhattacharya, A. S. Perelson, B. T. Korber, B. H. Hahn, and G. M. Shaw.** 2009. Low-dose rectal inoculation of rhesus macaques by SIVsmE660 or SIVmac251 recapitulates human mucosal infection by HIV-1. *J. Exp. Med.* **206**:1117-1134. doi: 10.1084/jem.20082831.
- Kennedy, R. C., M. H. Shearer, and W. Hildebrand.** 1997. Nonhuman primate models to evaluate vaccine safety and immunogenicity. *Vaccine*. **15**:903-908.
- Kiepiela, P., K. Ngumbela, C. Thobakgale, D. Ramduth, I. Honeyborne, E. Moodley, S. Reddy, C. de Pierres, Z. Mncube, N. Mkhwanazi, K. Bishop, M. van der Stok, K. Nair, N. Khan, H. Crawford, R. Payne, A. Leslie, J. Prado, A. Prendergast, J. Frater, N. McCarthy, C. Brander, G. H. Learn, D. Nickle, C. Rousseau, H. Coovadia, J. I. Mullins, D. Heckerman, B. D. Walker, and P. Goulder.** 2007. CD8+ T-cell responses to different HIV proteins have discordant associations with viral load. *Nat. Med.* **13**:46-53. doi: 10.1038/nm1520.
- Kim, S. H., S. J. Choi, W. B. Park, H. B. Kim, N. J. Kim, M. D. Oh, and K. W. Choe.** 2007. Detailed kinetics of immune responses to a new cell culture-derived smallpox vaccine in vaccinia-naïve adults. *Vaccine*. **25**:6287-6291. doi: 10.1016/j.vaccine.2007.05.044.
- Kim, S. H., S. G. Yeo, J. H. Cho, H. B. Kim, N. J. Kim, M. D. Oh, K. W. Choe, Y. Jee, and H. Cho.** 2006. Cell-mediated immune responses to smallpox vaccination. *Clin. Vaccine Immunol.* **13**:1172-1174. doi: 10.1128/CVI.00181-06.
- Kim, S. H., S. G. Yeo, H. C. Jang, W. B. Park, C. S. Lee, K. D. Lee, H. B. Kim, N. J. Kim, Y. T. Kim, Y. Jee, H. Cho, M. D. Oh, and K. W. Choe.** 2005. Clinical responses to smallpox vaccine in vaccinia-naïve and previously vaccinated populations: undiluted and diluted Lancy-Vaxina vaccine in a single-blind, randomized, prospective trial. *J. Infect. Dis.* **192**:1066-1070. doi: 10.1086/432765.
- Klausner, R. D., J. G. Donaldson, and J. Lippincott-Schwartz.** 1992. Brefeldin A: insights into the control of membrane traffic and organelle structure. *J. Cell Biol.* **116**:1071-1080.
- Knipe, D. M., and P. M. Howley.** 2007. *Fields' Virology*. 5th edition. Wolters Kluwer, Lippincott Williams and Wilkins. ISBN-10: 0781760607.
- Korber, B. T., N. L. Letvin, and B. F. Haynes.** 2009. T-cell vaccine strategies for human immunodeficiency virus, the virus with a thousand faces. *J. Virol.* **83**:8300-8314. doi: 10.1128/JVI.00114-09.
- Koup, R. A., J. T. Safrit, Y. Cao, C. A. Andrews, G. McLeod, W. Borkowsky, C. Farthing, and D. D. Ho.** 1994. Temporal association of cellular immune responses with the initial control of viremia in primary human immunodeficiency virus type 1 syndrome. *J. Virol.* **68**:4650-4655.
- Krensky, A. M., and C. Clayberger.** 2005. Granulysin: a novel host defense molecule. *Am. J. Transplant.* **5**:1789-1792. doi: 10.1111/j.1600-6143.2005.00970.x.

- Kuroda, M. J., J. E. Schmitz, W. A. Charini, C. E. Nickerson, M. A. Lifton, C. I. Lord, M. A. Forman, and N. L. Letvin.** 1999. Emergence of CTL coincides with clearance of virus during primary simian immunodeficiency virus infection in rhesus monkeys. *J. Immunol.* **162**:5127-5133.
- Ladell, K., M. K. Hellerstein, D. Cesar, R. Busch, D. Boban, and J. M. McCune.** 2008. Central Memory CD8+ T Cells Appear to Have a Shorter Lifespan and Reduced Abundance as a Function of HIV Disease Progression. *The Journal of Immunology.* **180**:7907-7918.
- Lai, L., S. Kwa, P. A. Kozlowski, D. C. Montefiori, G. Ferrari, W. E. Johnson, V. Hirsch, F. Villinger, L. Chennareddi, P. L. Earl, B. Moss, R. R. Amara, and H. L. Robinson.** 2011. Prevention of infection by a granulocyte-macrophage colony-stimulating factor co-expressing DNA/modified vaccinia Ankara simian immunodeficiency virus vaccine. *J. Infect. Dis.* **204**:164-173. doi: 10.1093/infdis/jir199.
- Lan, K., S. C. Verma, M. Murakami, B. Bajaj, and E. S. Robertson.** 2007. Isolation of human peripheral blood mononuclear cells (PBMCs). *Curr. Protoc. Microbiol.* **Appendix 4**:Appendix 4C. doi: 10.1002/9780471729259.mca04cs6.
- Lawrence, S. J., K. R. Lottenbach, F. K. Newman, R. M. Buller, C. J. Bellone, J. J. Chen, G. H. Cohen, R. J. Eisenberg, R. B. Belshe, S. L. Stanley Jr, and S. E. Frey.** 2007. Antibody responses to vaccinia membrane proteins after smallpox vaccination. *J. Infect. Dis.* **196**:220-229. doi: 10.1086/518793.
- Letvin, N. L.** 2006. Progress and obstacles in the development of an AIDS vaccine. *Nat. Rev. Immunol.* **6**:930-939. doi: 10.1038/nri1959.
- Levy, J. A.** 2009. HIV pathogenesis: 25 years of progress and persistent challenges. *AIDS.* **23**:147-160. doi: 10.1097/QAD.0b013e3283217f9f.
- Liu, J., M. Hellerstein, M. McDonnell, R. R. Amara, L. S. Wyatt, B. Moss, and H. L. Robinson.** 2007. Dose-response studies for the elicitation of CD8 T cells by a DNA vaccine, used alone or as the prime for a modified vaccinia Ankara boost. *Vaccine.* **25**:2951-2958. doi: 10.1016/j.vaccine.2006.05.081.
- Liu, J., K. L. O'Brien, D. M. Lynch, N. L. Simmons, A. La Porte, A. M. Riggs, P. Abbink, R. T. Coffey, L. E. Grandpre, M. S. Seaman, G. Landucci, D. N. Forthal, D. C. Montefiori, A. Carville, K. G. Mansfield, M. J. Havenga, M. G. Pau, J. Goudsmit, and D. H. Barouch.** 2009. Immune control of an SIV challenge by a T-cell-based vaccine in rhesus monkeys. *Nature.* **457**:87-91. doi: 10.1038/nature07469.
- Liu, Y., J. McNevin, M. Rolland, H. Zhao, W. Deng, J. Maenza, C. E. Stevens, A. C. Collier, M. J. McElrath, and J. I. Mullins.** 2009. Conserved HIV-1 epitopes continuously elicit subdominant cytotoxic T-lymphocyte responses. *J. Infect. Dis.* **200**:1825-1833. doi: 10.1086/648401.
- Locher, C. P., S. Fujimura, K. K. Murthy, K. Brasky, M. Leland, and J. A. Levy.** 2003. Expression patterns of phenotypic markers on lymphocytes from human immunodeficiency virus type 2-infected baboons. *AIDS Res. Hum. Retroviruses.* **19**:31-40. doi: 10.1089/08892220360473943.
- Lu, S.** 1998. Developing DNA vaccines against immunodeficiency viruses. *Curr. Top. Microbiol. Immunol.* **226**:161-173.
- MacGregor, R. R., J. D. Boyer, K. E. Ugen, K. E. Lacy, S. J. Gluckman, M. L. Bagarazzi, M. A. Chattergoon, Y. Baine, T. J. Higgins, R. B. Ciccarelli, L. R. Coney, R. S. Ginsberg, and D. B. Weiner.** 1998. First human trial of a DNA-based vaccine for treatment of human immunodeficiency virus type 1 infection: safety and host response. *J. Infect. Dis.* **178**:92-100.
- Makedonas, G., and M. R. Betts.** 2011. Living in a house of cards: re-evaluating CD8+ T-cell immune correlates against HIV. *Immunol. Rev.* **239**:109-124. doi: 10.1111/j.1600-065X.2010.00968.x; 10.1111/j.1600-065X.2010.00968.x.

- Malarme, D., Y. Cordier, C. Sene.** 2010. Process for producing poxviruses and poxvirys compositions. <http://www.freepatentsonline.com/y2010/0008953.html>
- Manrique, M., P. A. Kozlowski, A. Cobo-Molinos, S. W. Wang, R. L. Wilson, D. C. Montefiori, K. G. Mansfield, A. Carville, and A. Aldovini.** 2011. Long-term control of simian immunodeficiency virus mac251 viremia to undetectable levels in half of infected female rhesus macaques nasally vaccinated with simian immunodeficiency virus DNA/recombinant modified vaccinia virus Ankara. *J. Immunol.* **186**:3581-3593. doi: 10.4049/jimmunol.1002594.
- Mascola, J. R., M. G. Lewis, G. Stiegler, D. Harris, T. C. VanCott, D. Hayes, M. K. Louder, C. R. Brown, C. V. Sapan, S. S. Frankel, Y. Lu, M. L. Robb, H. Katinger, and D. L. Birx.** 1999. Protection of Macaques against pathogenic simian/human immunodeficiency virus 89.6PD by passive transfer of neutralizing antibodies. *J. Virol.* **73**:4009-4018.
- Masemola, A., T. Mashishi, G. Khoury, P. Mohube, P. Mokgotho, E. Vardas, M. Colvin, L. Zijenah, D. Katzenstein, R. Musonda, S. Allen, N. Kumwenda, T. Taha, G. Gray, J. McIntyre, S. A. Karim, H. W. Sheppard, C. M. Gray, and HIVNET 028 Study Team.** 2004. Hierarchical targeting of subtype C human immunodeficiency virus type 1 proteins by CD8+ T cells: correlation with viral load. *J. Virol.* **78**:3233-3243.
- Mast, T. C., L. Kierstead, S. B. Gupta, A. A. Nikas, E. G. Kallas, V. Novitsky, B. Mbewe, P. Pitisuttithum, M. Schechter, E. Vardas, N. D. Wolfe, M. Aste-Amezaga, D. R. Casimiro, P. Coplan, W. L. Straus, and J. W. Shiver.** 2010. International epidemiology of human pre-existing adenovirus (Ad) type-5, type-6, type-26 and type-36 neutralizing antibodies: correlates of high Ad5 titers and implications for potential HIV vaccine trials. *Vaccine.* **28**:950-957. doi: 10.1016/j.vaccine.2009.10.145.
- Mattapallil, J. J., D. C. Douek, A. Buckler-White, D. Montefiori, N. L. Letvin, G. J. Nabel, and M. Roederer.** 2006. Vaccination preserves CD4 memory T cells during acute simian immunodeficiency virus challenge. *J. Exp. Med.* **203**:1533-1541. doi: 10.1084/jem.20060657.
- Mattapallil, J. J., D. C. Douek, B. Hill, Y. Nishimura, M. Martin, and M. Roederer.** 2005. Massive infection and loss of memory CD4+ T cells in multiple tissues during acute SIV infection. *Nature.* **434**:1093-1097. doi: 10.1038/nature03501.
- McCoy, K., N. Tatsis, B. Koriath-Schmitz, M. O. Lasaro, S. E. Hensley, S. W. Lin, Y. Li, W. Giles-Davis, A. Cun, D. Zhou, Z. Xiang, N. L. Letvin, and H. C. Ertl.** 2007. Effect of preexisting immunity to adenovirus human serotype 5 antigens on the immune responses of nonhuman primates to vaccine regimens based on human- or chimpanzee-derived adenovirus vectors. *J. Virol.* **81**:6594-6604. doi: 10.1128/JVI.02497-06.
- McCurdy, L. H., B. D. Larkin, J. E. Martin, and B. S. Graham.** 2004. Modified vaccinia Ankara: potential as an alternative smallpox vaccine. *Clin. Infect. Dis.* **38**:1749-1753. doi: 10.1086/421266.
- McElrath, M. J., S. C. De Rosa, Z. Moodie, S. Dubey, L. Kierstead, H. Janes, O. D. Defawe, D. K. Carter, J. Hural, R. Akondy, S. P. Buchbinder, M. N. Robertson, D. V. Mehrotra, S. G. Self, L. Corey, J. W. Shiver, D. R. Casimiro, and Step Study Protocol Team.** 2008. HIV-1 vaccine-induced immunity in the test-of-concept Step Study: a case-cohort analysis. *Lancet.* **372**:1894-1905. doi: 10.1016/S0140-6736(08)61592-5.
- McElrath, M. J., S. C. De Rosa, Z. Moodie, S. Dubey, L. Kierstead, H. Janes, O. D. Defawe, D. K. Carter, J. Hural, R. Akondy, S. P. Buchbinder, M. N. Robertson, D. V. Mehrotra, S. G. Self, L. Corey, J. W. Shiver, D. R. Casimiro, and Step Study Protocol Team.** 2008. HIV-1 vaccine-induced immunity in the test-of-concept Step Study: a case-cohort analysis. *Lancet.* **372**:1894-1905. doi: 10.1016/S0140-6736(08)61592-5.
- Meseda, C. A., A. D. Garcia, A. Kumar, A. E. Mayer, J. Manischewitz, L. R. King, H. Golding, M. Merchlinsky, and J. P. Weir.** 2005. Enhanced immunogenicity and protective effect conferred by

vaccination with combinations of modified vaccinia virus Ankara and licensed smallpox vaccine Dryvax in a mouse model. *Virology*. **339**:164-175. doi: 10.1016/j.virol.2005.06.002.

Montefiori, D. C., L. Morris, G. Ferrari, and J. R. Mascola. 2007. Neutralizing and other antiviral antibodies in HIV-1 infection and vaccination. *Curr. Opin. HIV. AIDS*. **2**:169-176. doi: 10.1097/COH.0b013e3280ef691e.

Mooij, P., S. S. Balla-Jhagjhoorsingh, G. Koopman, N. Beenhakker, P. van Haften, I. Baak, I. G. Nieuwenhuis, I. Kondova, R. Wagner, H. Wolf, C. E. Gomez, J. L. Najera, V. Jimenez, M. Esteban, and J. L. Heeney. 2008. Differential CD4+ versus CD8+ T-cell responses elicited by different poxvirus-based human immunodeficiency virus type 1 vaccine candidates provide comparable efficacies in primates. *J. Virol.* **82**:2975-2988. doi: 10.1128/JVI.02216-07.

Moore, C. B., M. John, I. R. James, F. T. Christiansen, C. S. Witt, and S. A. Mallal. 2002. Evidence of HIV-1 adaptation to HLA-restricted immune responses at a population level. *Science*. **296**:1439-1443. doi: 10.1126/science.1069660.

Moore, P. L., E. S. Gray, and L. Morris. 2009. Specificity of the autologous neutralizing antibody response. *Curr. Opin. HIV. AIDS*. **4**:358-363. doi: 10.1097/COH.0b013e32832ea7e8.

Morgan, C., M. Marthas, C. Miller, A. Duerr, C. Cheng-Mayer, R. Desrosiers, J. Flores, N. Haigwood, S. L. Hu, R. P. Johnson, J. Lifson, D. Montefiori, J. Moore, M. Robert-Guroff, H. Robinson, S. Self, and L. Corey. 2008. The use of nonhuman primate models in HIV vaccine development. *PLoS Med.* **5**:e173. doi: 10.1371/journal.pmed.0050173.

Murthy, K. K., M. T. Salas, K. D. Carey, and J. L. Patterson. 2006. Baboon as a nonhuman primate model for vaccine studies. *Vaccine*. **24**:4622-4624. doi: 10.1016/j.vaccine.2005.08.047.

Nag, P., J. Kim, V. Sapiega, A. L. Landay, J. W. Bremer, J. Mestecky, P. Reichelderfer, A. Kovacs, J. Cohn, B. Weiser, and L. L. Baum. 2004. Women with cervicovaginal antibody-dependent cell-mediated cytotoxicity have lower genital HIV-1 RNA loads. *J. Infect. Dis.* **190**:1970-1978. doi: 10.1086/425582.

Naito, T., Y. Kaneko, and D. Kozbor. 2007. Oral vaccination with modified vaccinia virus Ankara attached covalently to TMPEG-modified cationic liposomes overcomes pre-existing poxvirus immunity from recombinant vaccinia immunization. *J. Gen. Virol.* **88**:61-70. doi: 10.1099/vir.0.82216-0.

Nelson, G. E., J. R. Sisler, D. Chandran, and B. Moss. 2008. Vaccinia virus entry/fusion complex subunit A28 is a target of neutralizing and protective antibodies. *Virology*. **380**:394-401. doi: 10.1016/j.virol.2008.08.009.

Nickle, D. C., M. A. Jensen, G. S. Gottlieb, D. Shriner, G. H. Learn, A. G. Rodrigo, and J. I. Mullins. 2003. Consensus and ancestral state HIV vaccines. *Science*. **299**:1515-8; author reply 1515-8. doi: 10.1126/science.299.5612.1515c.

Nigam, P., P. L. Earl, J. L. Americo, S. Sharma, L. S. Wyatt, Y. Edghill-Spano, L. S. Chennareddi, P. Silvera, B. Moss, H. L. Robinson, and R. R. Amara. 2007. DNA/MVA HIV-1/AIDS vaccine elicits long-lived vaccinia virus-specific immunity and confers protection against a lethal monkeypox challenge. *Virology*. **366**:73-83. doi: 10.1016/j.virol.2007.04.010.

Norder, M., P. D. Becker, I. Drexler, C. Link, V. Erfle, and C. A. Guzman. 2010. Modified vaccinia virus Ankara exerts potent immune modulatory activities in a murine model. *PLoS One*. **5**:e11400. doi: 10.1371/journal.pone.0011400.

O'Brien, K. L., J. Liu, S. L. King, Y. H. Sun, J. E. Schmitz, M. A. Lifton, N. A. Hutnick, M. R. Betts, S. A. Dubey, J. Goudsmit, J. W. Shiver, M. N. Robertson, D. R. Casimiro, and D. H. Barouch. 2009.

Adenovirus-specific immunity after immunization with an Ad5 HIV-1 vaccine candidate in humans. *Nat. Med.* **15**:873-875. doi: 10.1038/nm.1991.

Okoye, A., H. Park, M. Rohankhedkar, L. Coyne-Johnson, R. Lum, J. M. Walker, S. L. Planer, A. W. Legasse, A. W. Sylwester, M. Piatak Jr, J. D. Lifson, D. L. Sodora, F. Villinger, M. K. Axthelm, J. E. Schmitz, and L. J. Picker. 2009. Profound CD4+/CCR5+ T cell expansion is induced by CD8+ lymphocyte depletion but does not account for accelerated SIV pathogenesis. *J. Exp. Med.* **206**:1575-1588. doi: 10.1084/jem.20090356.

Otten, G., M. Schaefer, B. Doe, H. Liu, I. Srivastava, J. zur Megede, D. O'Hagan, J. Donnelly, G. Widera, D. Rabussay, M. G. Lewis, S. Barnett, and J. B. Ulmer. 2004. Enhancement of DNA vaccine potency in rhesus macaques by electroporation. *Vaccine.* **22**:2489-2493. doi: 10.1016/j.vaccine.2003.11.073.

Ourmanov, I., T. Kuwata, R. Goeken, S. Goldstein, R. Iyengar, A. Buckler-White, B. Lafont, and V. M. Hirsch. 2009. Improved survival in rhesus macaques immunized with modified vaccinia virus Ankara recombinants expressing simian immunodeficiency virus envelope correlates with reduction in memory CD4+ T-cell loss and higher titers of neutralizing antibody. *J. Virol.* **83**:5388-5400. doi: 10.1128/JVI.02598-08.

Pancera, M., J. S. McLellan, X. Wu, J. Zhu, A. Changela, S. D. Schmidt, Y. Yang, T. Zhou, S. Phogat, J. R. Mascola, and P. D. Kwong. 2010. Crystal structure of PG16 and chimeric dissection with somatically related PG9: structure-function analysis of two quaternary-specific antibodies that effectively neutralize HIV-1. *J. Virol.* **84**:8098-8110. doi: 10.1128/JVI.00966-10.

Pantaleo, G., M. Esteban, B. Jacobs, and J. Tartaglia. 2010. Poxvirus vector-based HIV vaccines. *Curr. Opin. HIV. AIDS.* **5**:391-396. doi: 10.1097/COH.0b013e32833d1e87.

Pantaleo, G., and A. S. Fauci. 1996. Immunopathogenesis of HIV infection. *Annu. Rev. Microbiol.* **50**:825-854. doi: 10.1146/annurev.micro.50.1.825.

Paris, R. M., J. H. Kim, M. L. Robb, and N. L. Michael. 2010. Prime-boost immunization with poxvirus or adenovirus vectors as a strategy to develop a protective vaccine for HIV-1. *Expert Rev. Vaccines.* **9**:1055-1069. doi: 10.1586/erv.10.106.

Parren, P. W., P. A. Marx, A. J. Hessel, A. Luckay, J. Harouse, C. Cheng-Mayer, J. P. Moore, and D. R. Burton. 2001. Antibody protects macaques against vaginal challenge with a pathogenic R5 simian/human immunodeficiency virus at serum levels giving complete neutralization in vitro. *J. Virol.* **75**:8340-8347.

Parrino, J., L. H. McCurdy, B. D. Larkin, I. J. Gordon, S. E. Rucker, M. E. Enama, R. A. Koup, M. Roederer, R. T. Bailer, Z. Moodie, L. Gu, L. Yan, B. S. Graham, and VRC 201/203 Study Team. 2007. Safety, immunogenicity and efficacy of modified vaccinia Ankara (MVA) against Dryvax challenge in vaccinia-naïve and vaccinia-immune individuals. *Vaccine.* **25**:1513-1525. doi: 10.1016/j.vaccine.2006.10.047.

Pejchal, R., L. M. Walker, R. L. Stanfield, S. K. Phogat, W. C. Koff, P. Poignard, D. R. Burton, and I. A. Wilson. 2010. Structure and function of broadly reactive antibody PG16 reveal an H3 subdomain that mediates potent neutralization of HIV-1. *Proc. Natl. Acad. Sci. U. S. A.* **107**:11483-11488. doi: 10.1073/pnas.1004600107.

Perfetto, S. P., P. K. Chattopadhyay, L. Lamoreaux, R. Nguyen, D. Ambrozak, R. A. Koup, and M. Roederer. 2006. Amine reactive dyes: an effective tool to discriminate live and dead cells in polychromatic flow cytometry. *J. Immunol. Methods.* **313**:199-208. doi: 10.1016/j.jim.2006.04.007.

Perreau, M., G. Pantaleo, and E. J. Kremer. 2008. Activation of a dendritic cell-T cell axis by Ad5 immune complexes creates an improved environment for replication of HIV in T cells. *J. Exp. Med.* **205**:2717-2725. doi: 10.1084/jem.20081786.

- Pillai, V. K., S. Kannanganat, P. Penaloza-Macmaster, L. Chennareddi, H. L. Robinson, J. Blackwell, and R. R. Amara.** 2011. Different patterns of expansion, contraction and memory differentiation of HIV-1 Gag-specific CD8 T cells elicited by adenovirus type 5 and modified vaccinia Ankara vaccines. *Vaccine*. **29**:5399-5406. doi: 10.1016/j.vaccine.2011.05.083.
- Pine, S. O., J. G. Kublin, S. M. Hammer, J. Borgerding, Y. Huang, D. R. Casimiro, and M. J. McElrath.** 2011. Pre-existing adenovirus immunity modifies a complex mixed Th1 and Th2 cytokine response to an Ad5/HIV-1 vaccine candidate in humans. *PLoS One*. **6**:e18526. doi: 10.1371/journal.pone.0018526.
- Pitisuttithum, P., P. Gilbert, M. Gurwith, W. Heyward, M. Martin, F. van Griensven, D. Hu, J. . Tappero, and Bangkok Vaccine Evaluation Group.** 2006. Randomized, Double-Blind, Placebo-Controlled Efficacy Trial of a Bivalent Recombinant Glycoprotein 120 HIV-1 Vaccine among Injection Drug Users in Bangkok, Thailand. *Journal of Infectious Diseases*. **194**:1661-1671. doi: 10.1086/508748.
- Plantier, J. C., M. Leoz, J. E. Dickerson, F. De Oliveira, F. Cordonnier, V. Lemeé, F. Damond, D. L. Robertson, and F. Simon.** 2009. A new human immunodeficiency virus derived from gorillas. *Nat. Med.* **15**:871-872. doi: 10.1038/nm.2016.
- Poonia, B., M. S. Salvato, H. Yagita, T. Maeda, K. Okumura, and C. D. Pauza.** 2009. Treatment with anti-FasL antibody preserves memory lymphocytes and virus-specific cellular immunity in macaques challenged with simian immunodeficiency virus. *Blood*. **114**:1196-1204. doi: 10.1182/blood-2009-02-202655.
- Poropatich, K., and D. J. Sullivan Jr.** 2011. Human immunodeficiency virus type 1 long-term non-progressors: the viral, genetic and immunological basis for disease non-progression. *J. Gen. Virol.* **92**:247-268. doi: 10.1099/vir.0.027102-0.
- Potter, S. J., C. Lacabaratz, O. Lambotte, S. Perez-Patrigeon, B. Vingert, M. Sinet, J. Colle, A. Urrutia, D. Scott-Algara, F. Boufassa, J. Delfraissy, J. Theze, A. Venet, and L. A. Chakrabarti.** 2007. Preserved Central Memory and Activated Effector Memory CD4+ T-Cell Subsets in Human Immunodeficiency Virus Controllers: an ANRS EP36 Study. *J. Virol.* **81**:13904-13915. doi: 10.1128/JVI.01401-07.
- Precopio, M. L., M. R. Betts, J. Parrino, D. A. Price, E. Gostick, D. R. Ambrozak, T. E. Asher, D. C. Douek, A. Harari, G. Pantaleo, R. Bailer, B. S. Graham, M. Roederer, and R. A. Koup.** 2007. Immunization with vaccinia virus induces polyfunctional and phenotypically distinctive CD8(+) T cell responses. *J. Exp. Med.* **204**:1405-1416. doi: 10.1084/jem.20062363.
- Priddy, F. H., D. Brown, J. Kublin, K. Monahan, D. P. Wright, J. Lalezari, S. Santiago, M. Marmor, M. Lally, R. M. Novak, S. J. Brown, P. Kulkarni, S. A. Dubey, L. S. Kierstead, D. R. Casimiro, R. Mogg, M. J. DiNubile, J. W. Shiver, R. Y. Leavitt, M. N. Robertson, D. V. Mehrotra, E. Quirk, and Merck V520-016 Study Group.** 2008. Safety and immunogenicity of a replication-incompetent adenovirus type 5 HIV-1 clade B gag/pol/nef vaccine in healthy adults. *Clin. Infect. Dis.* **46**:1769-1781. doi: 10.1086/587993.
- Reece, J. C., L. Loh, S. Alcantara, C. S. Fernandez, J. Stambas, A. Sexton, R. De Rose, J. Petravic, M. P. Davenport, and S. J. Kent.** 2010. Timing of immune escape linked to success or failure of vaccination. *PLoS One*. **5**:e12774. doi: 10.1371/journal.pone.0012774.
- Rerks-Ngarm, S., P. Pitisuttithum, S. Nitayaphan, J. Kaewkungwal, J. Chiu, R. Paris, N. Prensri, C. Namwat, M. de Souza, E. Adams, M. Benenson, S. Gurunathan, J. Tartaglia, J. G. McNeil, D. P. Francis, D. Stablein, D. L. Birx, S. Chunsuttiwat, C. Khamboonruang, P. Thongcharoen, M. L. Robb, N. L. Michael, P. Kulasol, J. H. Kim, and MOPH-TAVEG Investigators.** 2009. Vaccination with ALVAC and AIDSVAX to prevent HIV-1 infection in Thailand. *N. Engl. J. Med.* **361**:2209-2220. doi: 10.1056/NEJMoa0908492.

- Reyes-Sandoval, A., D. H. Wyllie, K. Bauza, A. Milicic, E. K. Forbes, C. S. Rollier, and A. V. Hill.** 2011. CD8+ T Effector Memory Cells Protect against Liver-Stage Malaria. *J. Immunol.* **187**:1347-1357. doi: 10.4049/jimmunol.1100302.
- Richman, D. D., T. Wrin, S. J. Little, and C. J. Petropoulos.** 2003. Rapid evolution of the neutralizing antibody response to HIV type 1 infection. *Proc. Natl. Acad. Sci. U. S. A.* **100**:4144-4149. doi: 10.1073/pnas.0630530100.
- Roberts, D. M., A. Nanda, M. J. Havenga, P. Abbink, D. M. Lynch, B. A. Ewald, J. Liu, A. R. Thorner, P. E. Swanson, D. A. Gorgone, M. A. Lifton, A. A. Lemckert, L. Holterman, B. Chen, A. Dilraj, A. Carville, K. G. Mansfield, J. Goudsmit, and D. H. Barouch.** 2006. Hexon-chimaeric adenovirus serotype 5 vectors circumvent pre-existing anti-vector immunity. *Nature.* **441**:239-243. doi: 10.1038/nature04721.
- Robinson, H. L., S. Sharma, J. Zhao, S. Kannanganat, L. Lai, L. Chennareddi, T. Yu, D. C. Montefiori, R. R. Amara, L. S. Wyatt, and B. Moss.** 2007. Immunogenicity in macaques of the clinical product for a clade B DNA/MVA HIV vaccine: elicitation of IFN-gamma, IL-2, and TNF-alpha coproducing CD4 and CD8 T cells. *AIDS Res. Hum. Retroviruses.* **23**:1555-1562. doi: 10.1089/aid.2007.0165.
- Roederer, M., J. M. Brenchley, M. R. Betts, and S. C. De Rosa.** 2004. Flow cytometric analysis of vaccine responses: how many colors are enough? *Clin. Immunol.* **110**:199-205. doi: 10.1016/j.clim.2003.11.015.
- Roederer, M., J. L. Nozzi, and M. C. Nason.** 2011. SPICE: exploration and analysis of post-cytometric complex multivariate datasets. *Cytometry A.* **79**:167-174. doi: 10.1002/cyto.a.21015; 10.1002/cyto.a.21015.
- Rolland, M., J. M. Carlson, S. Manochewa, J. V. Swain, E. Lanxon-Cookson, W. Deng, C. M. Rousseau, D. N. Raugi, G. H. Learn, B. S. Maust, H. Coovadia, T. Ndung'u, P. J. Goulder, B. D. Walker, C. Brander, D. E. Heckerman, and J. I. Mullins.** 2010. Amino-acid co-variation in HIV-1 Gag subtype C: HLA-mediated selection pressure and compensatory dynamics. *PLoS One.* **5**:e12463. doi: 10.1371/journal.pone.0012463.
- Rolland, M., D. Heckerman, W. Deng, C. M. Rousseau, H. Coovadia, K. Bishop, P. J. Goulder, B. D. Walker, C. Brander, and J. I. Mullins.** 2008. Broad and Gag-biased HIV-1 epitope repertoires are associated with lower viral loads. *PLoS One.* **3**:e1424. doi: 10.1371/journal.pone.0001424.
- Rolland, M., M. A. Jensen, D. C. Nickle, J. Yan, G. H. Learn, L. Heath, D. Weiner, and J. I. Mullins.** 2007. Reconstruction and function of ancestral center-of-tree human immunodeficiency virus type 1 proteins. *J. Virol.* **81**:8507-8514. doi: 10.1128/JVI.02683-06.
- Rosario, M., R. Hopkins, J. Fulkerson, N. Borthwick, M. F. Quigley, J. Joseph, D. C. Douek, H. Y. Greenaway, V. Venturi, E. Gostick, D. A. Price, G. W. Both, J. C. Sadoff, and T. Hanke.** 2010. Novel recombinant Mycobacterium bovis BCG, ovine atadenovirus, and modified vaccinia virus Ankara vaccines combine to induce robust human immunodeficiency virus-specific CD4 and CD8 T-cell responses in rhesus macaques. *J. Virol.* **84**:5898-5908. doi: 10.1128/JVI.02607-09.
- Rosenberg, E. S., J. M. Billingsley, A. M. Caliendo, S. L. Boswell, P. E. Sax, S. A. Kalams, and B. D. Walker.** 1997. Vigorous HIV-1-specific CD4+ T cell responses associated with control of viremia. *Science.* **278**:1447-1450.
- Sancho, M. C., S. Schleich, G. Griffiths, and J. Krijnse-Locker.** 2002. The block in assembly of modified vaccinia virus Ankara in HeLa cells reveals new insights into vaccinia virus morphogenesis. *J. Virol.* **76**:8318-8334.
- Sandstrom, E., C. Nilsson, B. Hejdeman, A. Brave, G. Bratt, M. Robb, J. Cox, T. Vancott, M. Marovich, R. Stout, S. Aboud, M. Bakari, K. Pallangyo, K. Ljungberg, B. Moss, P. Earl, N. Michael,**

- D. Birx, F. Mhalu, B. Wahren, G. Biberfeld, and HIV Immunogenicity Study 01/02 Team.** 2008. Broad immunogenicity of a multigene, multiclade HIV-1 DNA vaccine boosted with heterologous HIV-1 recombinant modified vaccinia virus Ankara. *J. Infect. Dis.* **198**:1482-1490. doi: 10.1086/592507.
- Santra, S., H. X. Liao, R. Zhang, M. Muldoon, S. Watson, W. Fischer, J. Theiler, J. Szinger, H. Balachandran, A. Buzby, D. Quinn, R. J. Parks, C. Y. Tsao, A. Carville, K. G. Mansfield, G. N. Pavlakakis, B. K. Felber, B. F. Haynes, B. T. Korber, and N. L. Letvin.** 2010. Mosaic vaccines elicit CD8+ T lymphocyte responses that confer enhanced immune coverage of diverse HIV strains in monkeys. *Nat. Med.* **16**:324-328. doi: 10.1038/nm.2108.
- Sauter, S. L., A. Rahman, and G. Muralidhar.** 2005. Non-replicating viral vector-based AIDS vaccines: interplay between viral vectors and the immune system. *Curr. HIV. Res.* **3**:157-181.
- Schell, J. B., N. F. Rose, K. Bahl, K. Diller, L. Buonocore, M. Hunter, P. A. Marx, R. Gambhira, H. Tang, D. C. Montefiori, W. E. Johnson, and J. K. Rose.** 2011. Significant Protection against High-Dose Simian Immunodeficiency Virus Challenge Conferred by a New Prime-Boost Vaccine Regimen. *J. Virol.* **85**:5764-5772. doi: 10.1128/JVI.00342-11.
- Schmitz, J. E., M. J. Kuroda, S. Santra, V. G. Sasseville, M. A. Simon, M. A. Lifton, P. Racz, K. Tenner-Racz, M. Dalesandro, B. J. Scallon, J. Ghayeb, M. A. Forman, D. C. Montefiori, E. P. Rieber, N. L. Letvin, and K. A. Reimann.** 1999. Control of Viremia in Simian Immunodeficiency Virus Infection by CD8+ Lymphocytes. *Science.* **283**:857-860. doi: 10.1126/science.283.5403.857.
- Scriba, T. J., M. Tameris, N. Mansoor, E. Smit, L. van der Merwe, F. Isaacs, A. Keyser, S. Moyo, N. Brittain, A. Lawrie, S. Gelderbloem, A. Veldsman, M. Hatherill, A. Hawkrige, A. V. Hill, G. D. Hussey, H. Mahomed, H. McShane, and W. A. Hanekom.** 2010. Modified vaccinia Ankara-expressing Ag85A, a novel tuberculosis vaccine, is safe in adolescents and children, and induces polyfunctional CD4+ T cells. *Eur. J. Immunol.* **40**:279-290. doi: 10.1002/eji.200939754.
- Sekaly, R. P.** 2008. The failed HIV Merck vaccine study: a step back or a launching point for future vaccine development? *J. Exp. Med.* **205**:7-12. doi: 10.1084/jem.20072681.
- Shacklett, B. L.** 2008. Mucosal immunity to HIV: a review of recent literature. *Curr. Opin. HIV. AIDS.* **3**:541-547. doi: 10.1097/COH.0b013e32830ab9ee.
- Sharpe, S., N. Polyanskaya, M. Dennis, G. Sutter, T. Hanke, V. Erfle, V. Hirsch, and M. Cranage.** 2001. Induction of simian immunodeficiency virus (SIV)-specific CTL in rhesus macaques by vaccination with modified vaccinia virus Ankara expressing SIV transgenes: influence of pre-existing anti-vector immunity. *J. Gen. Virol.* **82**:2215-2223.
- Shibata, R., T. Igarashi, N. Haigwood, A. Buckler-White, R. Ogert, W. Ross, R. Willey, M. W. Cho, and M. A. Martin.** 1999. Neutralizing antibody directed against the HIV-1 envelope glycoprotein can completely block HIV-1/SIV chimeric virus infections of macaque monkeys. *Nat. Med.* **5**:204-210. doi: 10.1038/5568.
- Shiver, J. W., T. M. Fu, L. Chen, D. R. Casimiro, M. E. Davies, R. K. Evans, Z. Q. Zhang, A. J. Simon, W. L. Triglia, S. A. Dubey, L. Huang, V. A. Harris, R. S. Long, X. Liang, L. Handt, W. A. Schleif, L. Zhu, D. C. Freed, N. V. Persaud, L. Guan, K. S. Punt, A. Tang, M. Chen, K. A. Wilson, K. B. Collins, G. J. Heidecker, V. R. Fernandez, H. C. Perry, J. G. Joyce, K. M. Grimm, J. C. Cook, P. M. Keller, D. S. Kresock, H. Mach, R. D. Troutman, L. A. Isopi, D. M. Williams, Z. Xu, K. E. Bohannon, D. B. Volkin, D. C. Montefiori, A. Miura, G. R. Krivulka, M. A. Lifton, M. J. Kuroda, J. E. Schmitz, N. L. Letvin, M. J. Caulfield, A. J. Bett, R. Youil, D. C. Kaslow, and E. A. Emini.** 2002. Replication-incompetent adenoviral vaccine vector elicits effective anti-immunodeficiency-virus immunity. *Nature.* **415**:331-335. doi: 10.1038/415331a.
- Smith, C. L., F. Mirza, V. Pasquetto, D. C. Tscharke, M. J. Palmowski, P. R. Dunbar, A. Sette, A. L. Harris, and V. Cerundolo.** 2005. Immunodominance of poxviral-specific CTL in a human trial of recombinant-modified vaccinia Ankara. *J. Immunol.* **175**:8431-8437.

- Smith, G. L., A. Vanderplasschen, and M. Law.** 2002. The formation and function of extracellular enveloped vaccinia virus. *J. Gen. Virol.* **83**:2915-2931.
- Smith, G. L., A. Vanderplasschen, and M. Law.** 2002. The formation and function of extracellular enveloped vaccinia virus. *J. Gen. Virol.* **83**:2915-2931.
- Smith, J. M., R. R. Amara, L. S. Wyatt, D. L. Ellenberger, B. Li, J. G. Herndon, M. Patel, S. Sharma, L. Chennareddi, S. Butera, J. McNicholl, H. M. McClure, B. Moss, and H. L. Robinson.** 2005. Studies in macaques on cross-clade T cell responses elicited by a DNA/MVA AIDS vaccine, better conservation of CD8 than CD4 T cell responses. *AIDS Res. Hum. Retroviruses.* **21**:140-144. doi: 10.1089/aid.2005.21.140.
- Speller, S. A., and A. P. Warren.** 2002. Ex vivo detection and enumeration of human antigen-specific CD8+ T lymphocytes using antigen delivery by a recombinant vaccinia expression vector and intracellular cytokine staining. *J. Immunol. Methods.* **262**:167-180.
- Strassburg, M. A.** 1982. The global eradication of smallpox. *Am. J. Infect. Control.* **10**:53-59.
- Streeck, H., N. Frahm, and B. D. Walker.** 2009. The role of IFN-gamma Elispot assay in HIV vaccine research. *Nat. Protoc.* **4**:461-469. doi: 10.1038/nprot.2009.7.
- Studentsov, Y. Y., M. Schiffman, H. D. Strickler, G. Y. Ho, Y. Y. Pang, J. Schiller, R. Herrero, and R. D. Burk.** 2002. Enhanced enzyme-linked immunosorbent assay for detection of antibodies to virus-like particles of human papillomavirus. *J. Clin. Microbiol.* **40**:1755-1760.
- Suni, M. A., V. C. Maino, and H. T. Maecker.** 2005. Ex vivo analysis of T-cell function. *Curr. Opin. Immunol.* **17**:434-440. doi: 10.1016/j.coi.2005.05.002.
- Sutter, G., and C. Staib.** 2003. Vaccinia vectors as candidate vaccines: the development of modified vaccinia virus Ankara for antigen delivery. *Curr. Drug Targets Infect. Disord.* **3**:263-271.
- Trkola, A., H. Kuster, P. Rusert, B. Joos, M. Fischer, C. Leemann, A. Manrique, M. Huber, M. Rehr, A. Oxenius, R. Weber, G. Stiegler, B. Vcelar, H. Katinger, L. Aceto, and H. F. Gunthard.** 2005. Delay of HIV-1 rebound after cessation of antiretroviral therapy through passive transfer of human neutralizing antibodies. *Nat. Med.* **11**:615-622. doi: 10.1038/nm1244.
- UNAIDS Global report 2010 | UNAIDS Report on the global AIDS epidemic 2010 **2011**:
www.unaids.org/globalreport/global_report.htm
- Vaccari, M., P. Poonam, and G. Franchini.** 2010. Phase III HIV vaccine trial in Thailand: a step toward a protective vaccine for HIV. *Expert Rev. Vaccines.* **9**:997-1005. doi: 10.1586/erv.10.104.
- Vallari, A., V. Holzmayer, B. Harris, J. Yamaguchi, C. Ngansop, F. Makamche, D. Mbanya, L. Kaptue, N. Ndembi, L. Gurtler, S. Devare, and C. A. Brennan.** 2011. Confirmation of putative HIV-1 group P in Cameroon. *J. Virol.* **85**:1403-1407. doi: 10.1128/JVI.02005-10.
- Verkoczy, L., G. Kelsoe, M. A. Moody, and B. F. Haynes.** 2011. Role of immune mechanisms in induction of HIV-1 broadly neutralizing antibodies. *Curr. Opin. Immunol.* . doi: 10.1016/j.coi.2011.04.003.
- Waldrop, S. L., C. J. Pitcher, D. M. Peterson, V. C. Maino, and L. J. Picker.** 1997. Determination of antigen-specific memory/effector CD4+ T cell frequencies by flow cytometry: evidence for a novel, antigen-specific homeostatic mechanism in HIV-associated immunodeficiency. *J. Clin. Invest.* **99**:1739-1750. doi: 10.1172/JCI119338.
- Walker, L. M., S. K. Phogat, P. Y. Chan-Hui, D. Wagner, P. Phung, J. L. Goss, T. Wrin, M. D. Simek, S. Fling, J. L. Mitcham, J. K. Lehrman, F. H. Priddy, O. A. Olsen, S. M. Frey, P. W. Hammond, Protocol G Principal Investigators, S. Kaminsky, T. Zamb, M. Moyle, W. C. Koff, P. Poignard, and**

- D. R. Burton.** 2009. Broad and potent neutralizing antibodies from an African donor reveal a new HIV-1 vaccine target. *Science*. **326**:285-289. doi: 10.1126/science.1178746.
- Watkins, D. I., D. R. Burton, E. G. Kallas, J. P. Moore, and W. C. Koff.** 2008. Nonhuman primate models and the failure of the Merck HIV-1 vaccine in humans. **14**:621.
- Wei, X., J. M. Decker, S. Wang, H. Hui, J. C. Kappes, X. Wu, J. F. Salazar-Gonzalez, M. G. Salazar, J. M. Kilby, M. S. Saag, N. L. Komarova, M. A. Nowak, B. H. Hahn, P. D. Kwong, and G. M. Shaw.** 2003. Antibody neutralization and escape by HIV-1. *Nature*. **422**:307-312. doi: 10.1038/nature01470.
- Weinberg, A., D. A. Wohl, D. G. Brown, G. B. Pott, L. Zhang, M. G. Ray, and C. van der Horst.** 2000. Effect of cryopreservation on measurement of cytomegalovirus-specific cellular immune responses in HIV-infected patients. *J. Acquir. Immune Defic. Syndr.* **25**:109-114.
- Westwood, J. C., P. H. Phipps, and E. A. Boulter.** 1957. The titration of vaccinia virus on the chorioallantoic membrane of the developing chick embryo. *J. Hyg. (Lond)*. **55**:123-139.
- Whitney, J. B., C. Luedemann, P. Hraber, S. S. Rao, J. R. Mascola, G. J. Nabel, and N. L. Letvin.** 2009. T-cell vaccination reduces simian immunodeficiency virus levels in semen. *J. Virol.* **83**:10840-10843. doi: 10.1128/JVI.01202-09.
- Wilck, M. B., M. S. Seaman, L. R. Baden, S. R. Walsh, L. E. Grandpre, C. Devoy, A. Giri, J. A. Kleinjan, L. C. Noble, K. E. Stevenson, H. T. Kim, and R. Dolin.** 2010. Safety and immunogenicity of modified vaccinia Ankara (ACAM3000): effect of dose and route of administration. *J. Infect. Dis.* **201**:1361-1370. doi: 10.1086/651561.
- Williamson, C., L. Morris, M. F. Maughan, L. H. Ping, S. A. Dryga, R. Thomas, E. A. Reap, T. Cilliers, J. van Harmelen, A. Pascual, G. Ramjee, G. Gray, R. Johnston, S. A. Karim, and R. Swanstrom.** 2003. Characterization and selection of HIV-1 subtype C isolates for use in vaccine development. *AIDS Res. Hum. Retroviruses.* **19**:133-144. doi: 10.1089/088922203762688649.
- Wilson, N. A., J. Reed, G. S. Napoe, S. Piaskowski, A. Szymanski, J. Furlott, E. J. Gonzalez, L. J. Yant, N. J. Maness, G. E. May, T. Soma, M. R. Reynolds, E. Rakasz, R. Rudersdorf, A. B. McDermott, D. H. O'Connor, T. C. Friedrich, D. B. Allison, A. Patki, L. J. Picker, D. R. Burton, J. Lin, L. Huang, D. Patel, G. Heindecker, J. Fan, M. Citron, M. Horton, F. Wang, X. Liang, J. W. Shiver, D. R. Casimiro, and D. I. Watkins.** 2006. Vaccine-induced cellular immune responses reduce plasma viral concentrations after repeated low-dose challenge with pathogenic simian immunodeficiency virus SIVmac239. *J. Virol.* **80**:5875-5885. doi: 10.1128/JVI.00171-06.
- Wilson, N. A., and D. I. Watkins.** 2009. Is an HIV vaccine possible? *Braz. J. Infect. Dis.* **13**:304-310.
- Wu, X., Z. Y. Yang, Y. Li, C. M. Hogerkorp, W. R. Schief, M. S. Seaman, T. Zhou, S. D. Schmidt, L. Wu, L. Xu, N. S. Longo, K. McKee, S. O'Dell, M. K. Louder, D. L. Wycuff, Y. Feng, M. Nason, N. Doria-Rose, M. Connors, P. D. Kwong, M. Roederer, R. T. Wyatt, G. J. Nabel, and J. R. Mascola.** 2010. Rational design of envelope identifies broadly neutralizing human monoclonal antibodies to HIV-1. *Science*. **329**:856-861. doi: 10.1126/science.1187659.
- Wyand, M. S., K. Manson, D. C. Montefiori, J. D. Lifson, R. P. Johnson, and R. C. Desrosiers.** 1999. Protection by live, attenuated simian immunodeficiency virus against heterologous challenge. *J. Virol.* **73**:8356-8363.
- Wyatt, R., P. D. Kwong, E. Desjardins, R. W. Sweet, J. Robinson, W. A. Hendrickson, and J. G. Sodroski.** 1998. The antigenic structure of the HIV gp120 envelope glycoprotein. *Nature*. **393**:705-711. doi: 10.1038/31514.
- Yang, Z. Y., L. S. Wyatt, W. P. Kong, Z. Moodie, B. Moss, and G. J. Nabel.** 2003. Overcoming immunity to a viral vaccine by DNA priming before vector boosting. *J. Virol.* **77**:799-803.

Zhao, J., L. Lai, R. R. Amara, D. C. Montefiori, F. Villinger, L. Chennareddi, L. S. Wyatt, B. Moss, and H. L. Robinson. 2009. Preclinical studies of human immunodeficiency virus/AIDS vaccines: inverse correlation between avidity of anti-Env antibodies and peak postchallenge viremia. *J. Virol.* **83**:4102-4111. doi: 10.1128/JVI.02173-08.

Zhou, T., I. Georgiev, X. Wu, Z. Y. Yang, K. Dai, A. Finzi, Y. D. Kwon, J. F. Scheid, W. Shi, L. Xu, Y. Yang, J. Zhu, M. C. Nussenzweig, J. Sodroski, L. Shapiro, G. J. Nabel, J. R. Mascola, and P. D. Kwong. 2010. Structural basis for broad and potent neutralization of HIV-1 by antibody VRC01. *Science.* **329**:811-817. doi: 10.1126/science.1192819.

Zuniga, R., A. Lucchetti, P. Galvan, S. Sanchez, C. Sanchez, A. Hernandez, H. Sanchez, N. Frahm, C. H. Linde, H. S. Hewitt, W. Hildebrand, M. Altfeld, T. M. Allen, B. D. Walker, B. T. Korber, T. Leitner, J. Sanchez, and C. Brander. 2006. Relative Dominance of Gag p24-Specific Cytotoxic T Lymphocytes Is Associated with Human Immunodeficiency Virus Control. *J. Virol.* **80**:3122-3125. doi: 10.1128/JVI.80.6.3122-3125.2006.

Zwick, M. B., and D. R. Burton. 2007. HIV-1 neutralization: mechanisms and relevance to vaccine design. *Curr. HIV. Res.* **5**:608-624.

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