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An Investigation of Memory T cell Phenotypes in Peripheral Blood of Chacma Baboons after Immunisation with Candidate HIV-1 Vaccines.

by

Vedantha Singh

Division of Medical Virology
Department of Clinical Laboratory Sciences
Faculty of Health Sciences
UNIVERSITY OF CAPE TOWN

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Declaration

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Abbreviations and acronyms

| | |
|-----------------------|--|
| Ad5 | Adenovirus type 5 |
| Ad 26 | Adenovirus type 26 |
| Ad35 | Adenovirus 35 |
| ADCC | Antibody dependent cell-mediated cytotoxicity |
| Ag | Antigen |
| AIDS | Acquired immune deficiency syndrome |
| APC | Allophycocyanin |
| APC-Cy7 | Allophycocyanin-Cy7 |
| APOBEC | Apolipoprotein B mRNA-editing, enzyme-catalytic polypeptide like 3 |
| ART | Antiretroviral therapy |
| ARV(s) | Antiretroviral(s) |
| BCG | Bacille Calmette-Guérin |
| BFA | Brefeldin-A |
| BNAbs | Broadly neutralising antibodies |
| CD | Cluster of differentiation |
| cDNA | Complementary DNA |
| CMV | Cytomegalovirus |
| CO₂ | Carbon dioxide |
| CTL | Cytotoxic T lymphocyte |
| DC | Dendritic cell |
| DMEM | Dulbecco's Modified Eagle Medium |
| DMSO | Dimethyl sulfoxide |
| DNA | Deoxyribonucleic acid |
| ELISPOT | Enzyme-linked immunosorbent spot |
| Env | Envelope |
| FACS | Fluorescence-activated cell sorter |
| FBS | Foetal bovine serum |
| FITC | Fluorescein isothiocyanate |
| FMO | fluorescence minus one |
| FSC | Forward scatter |
| g | Gram |

| | |
|--------------------------------|--|
| gp | Glycoprotein |
| HAART | Highly active antiretroviral treatment |
| HEPES | 2-[4-(2-hydroxyethyl)piperazin-1-yl] ethanesulfonic acid |
| HIV/HIV-1 | Human immunodeficiency virus type 1 |
| HLA-DR | Human leukocyte antigen disease resistant |
| ICS | Intracellular cytokine staining |
| IFN-γ | Interferon- γ (gamma) |
| IL-2 | Interleukin 2 |
| mg | Milligram |
| MHC | Major histocompatibility complex |
| ml | Millilitre |
| mm | Millimeter |
| MVA | Modified Vaccinia Ankara virus |
| Nef | Negative regulatory factor |
| NHP | Nonhuman primate |
| nm | Nanometer |
| 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 |
| Qdot | Quantum dot |
| r | Recombinant |
| Rev | Regulator of virion |
| RNA | Ribonucleic acid |
| RPMI | Roswell Park Memorial Institute cell culture medium |
| RT | Room temperature |
| SAAVI | South African AIDS Vaccine Initiative |
| SEB | Staphylococcal enterotoxin B |
| SHIV | Simian human immunodeficiency virus |
| SIV | Simian immunodeficiency virus |
| SSC | Side scatter |

| | |
|--------------------------------|--------------------------------------|
| Tat | Transactivator of transcription |
| TB | Tuberculosis |
| Tcm | Central memory T cell |
| Tem | Effector memory T cell |
| TNF-α | Tumor necrosis factor alpha |
| UCT | University of Cape Town |
| ViViD | Violet-fluorescent reactive dye |
| VLP | Virus-like particle |
| x g | Times gravity (centrifugation speed) |
| μg | Microgram |
| μl | Microlitre |
| μm | Micrometer |
| % | Percent |
| $^{\circ}$C | Degrees Celsius |

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Abstract

Previous studies from the HIV Vaccine Development Group at the University of Cape Town have reported that immunisation with recombinant modified vaccinia Ankara (SAAVI MVA-C) or DNA prime and Pr55 Gag virus-like particle (VLP) boost based on HIV-1 subtype C are able to successfully induce vaccine specific responses in Chacma baboons. The aim of the current study was to characterise the T cell memory phenotype distribution in peripheral mononuclear cells (PBMCs) isolated from Chacma baboons vaccinated with SAAVI MVA-C/VLP and DNA/VLP prime-boost vaccination regimens by flow cytometry.

In order to achieve the above aim, cross-reactive antibodies were investigated in various combinations using 4-colour flow cytometry to select a combination of two antibodies to accurately delineate naïve and memory T cells. The selected combination was further investigated for functional capacity (IFN- γ and IL-2 production) in response to *staphylococcus enterotoxin B* (SEB) stimulation and expression of a marker of T cell maturation (CD45RA) in order to assess its suitability for use in this study. On confirming the validity of these markers in the Chacma baboon model, a seven colour flow cytometry panel was then developed and optimised to evaluate vaccine specific T cell memory recall responses in vaccinated baboons primed with SAAVI MVA-C and boosted with VLP (n=3) or DNA prime and boosted with VLP (n=5) or vaccinated with VLP only (n=2). The combined cytokine production (TNF- α , IFN- γ and IL-2) by T cells and memory phenotype were then determined using flow cytometry analysis.

The anti-CD28 and anti-CD95 combination showed the best delineation of central memory (Tcm; CD28+CD95+) and effector memory (Tem; CD28-CD95+) populations from the naïve T cells (CD28+CD95-) compared to the CD45RA/CD27 and CD95/CD27 combinations which were also tested. The memory subsets delineated by the CD28 and CD95 marker combination were further characterised. CD45RA expression was detected on a large percentage (>80%) of the naïve CD4 and CD8 cells and minimally (<10%) on CD4 Tem cells. However, 30-40% of Tcm and Tem CD8 cells expressed CD45RA. It was observed that the CD4+ and CD8+ Tcm cells expressed higher levels of IL-2 (10-15%) compared to Tem (2-6%) while the reverse was observed for IFN- γ expression (10-15% for Tem compared to 5-8% for Tcm). In contrast, less than 1% naïve T cells expressed any of the two cytokine.

Vaccine specific cytokine responses peaked four weeks after VLP boost in both MVA/VLP and DNA/VLP vaccinations. At this time, Gag-specific CD4+ cells from MVA/VLPs prime-boost animals were significantly skewed towards a Tcm phenotype (80-90%) of total cytokine responses compared to the Tem phenotype (10-20%). In these animals, Gag-specific CD8+ Tem comprised 30-40% of total cytokine responses. A similar memory distribution profile of Gag-specific CD4+ cells in DNA/VLPs was observed. Gag-specific CD8+ cells were distributed between Tcm (~60%) and Tem (~40%) phenotypes. The memory distribution profile of Gag-specific CD4+ cells was detectable in the MVA/VLPs and DNA/VLPs group 24 weeks and 8 weeks after the VLP boost respectively. The proportion of CD4+ T cells in the VLP only group was predominantly of the Tcm phenotype (70-80%). However, in CD8+ T cells the Tem phenotype comprised 50-70% of the total vaccine specific memory response.

The results demonstrate that anti- CD28 and -CD95 antibodies are suitable for the delineation of baboon Tem and Tcm cells since these subsets exhibited the expected functional characteristics observed in healthy rhesus macaques. The skewing of vaccine specific CD4+ T cell responses in both vaccine regimens towards the Tcm phenotype imply that these vaccines have the capacity to generate Tcm pools associated with rapid differentiation and replenishment of vaccine specific effector cells. In addition, the presence of functional CD8+ effector memory T cells observed here, have been previously associated with the control of infection at the portals of viral entry.

This study indicates the suitability of CD28 and CD95 for inclusion in flow cytometry panels to investigate T cell memory in baboon PBMCs. A multicolour flow cytometry panel for analysis of vaccine specific T cell memory was also developed. In addition, this study demonstrates the generation of vaccine specific, functional and long-lived Tcm and Tem in T lymphocytes of baboons vaccinated with SAAVI MVA-C/VLP and DNA/VLP prime-boost vaccination regimen. Taken together, this study demonstrates the potential inclusion of these candidate vaccines in future clinical trials.

Chapter 1

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Chapter 1. Literature Review

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1. LITERATURE REVIEW

Based on recent estimates, Sub-Saharan Africa remains the region most heavily affected by HIV and accounts for more than two thirds of infections worldwide (UNAIDS, 2012a). Unfortunately, it is projected that the number of infected people will continue to increase unless a novel and effective means of intervention is found. The search for an effective human immunodeficiency virus (HIV) vaccine capable of preventing the transmission and spread of infection was started almost 20 years ago. To date there have been no reports of a vaccine which offers total protection from HIV infection.

However, during this time, there have been numerous breakthroughs in the field of HIV vaccine research, and it is now widely accepted that in order for a protective vaccine to exist, there is a dire need to further understand the interactions between the virus and the host immune response. In addition, innovative and improved vaccine platforms need to be examined in terms of how to optimally deliver HIV immunogens to the host to generate robust, long-lived and safe immune responses.

This chapter will briefly review the virology, infection and immunopathogenesis of HIV type 1 (HIV-1) and discuss the ongoing research and challenges faced in the field of HIV-1 vaccine development. This chapter will also highlight candidate HIV-1 vaccines developed by the HIV-1 Vaccine Development Group based at the University of Cape Town (UCT). Finally, this chapter will address the rationale, aim and objectives of this study.

1.1. HIV virology

1.1.1. HIV epidemiology and disease burden in a South African context

Since the first recognised case of Acquired Immunodeficiency Disease Syndrome (AIDS) in 1981 and the discovery of HIV as the virus responsible for AIDS in 1983, more than 60 million people have contracted HIV and almost 30 million have died of HIV-related causes (Girard *et al.*, 2011). At the end of 2011, there were 34 million people living with HIV and 1.7 million AIDS related deaths were reported (UNAIDS, 2012a). An alarming 69% of the people living with HIV are from sub-Saharan Africa (UNAIDS, 2012a). In summary, the total number of HIV related deaths in adults and children in this region reportedly contributed 71% of the global HIV related deaths. It has also been well established that women in sub-Saharan Africa, where HIV-1

subtype C is the predominant pathogenic strain of the virus (UNAIDS, 2012b), are most at risk of infection and contribute to 59% of all people living with HIV. In South Africa, there are 5.6 million people living with HIV and a total of 270, 000 AIDS related deaths were reported at the end of 2011 (UNAIDS, 2012b). The improved treatment policies and increased roll of ARVs has helped to considerably reduce incidence rates from 2003, however the HIV prevalence rate in adults remains the highest in the world at 17.3% with reports of 380, 000 newly infected South Africans at the end of 2011 alone (UNAIDS, 2012b). Therefore, there is still a need to find an integrated approach to the treatment and prevention of HIV which comprise of an effective, safe and widely available prophylactic vaccine.

1.1.2. Viral diversity of HIV

HIV belongs to the genus *Lentivirus* in the family of enveloped ribonucleic acid (RNA) viruses called *Retroviridae*. The viruses classified in this family are usually associated with the ability to induce slow, progressive infections with long-term symptoms and latency periods. HIV is thought to have originated from the zoonotic transmission of Simian Immunodeficiency Virus (SIV) from infected nonhuman primates (NHPs) in Central or Western Africa (Gao *et al.*, 1999). The virus was transmitted to humans early in the twentieth century and has since evolved into an effective, virulent human pathogen (Korber *et al.*, 2000).

Two lineages of HIV namely HIV type 1 (HIV-1) which is made up of groups M, N, O and P and HIV type 2 (HIV-2) made up of groups A to H have been defined. These two lineages differ considerably in terms of both species of origin and relative virulence. HIV-1 is by far the more virulent of the two lineages and HIV-1 group M is responsible for the current global HIV pandemic. Infection with HIV-1 group O is most prevalent in Western and Central Africa (Simon *et al.*, 1998) and HIV-1 group N and P infection has been reported in Cameroon (Ayoub *et al.*, 2001, Vallari *et al.*, 2011). Both HIV-1 group M and group N are thought to have originated from SIV infection in chimpanzee subspecies *Pan troglodytes troglodytes* SIV (SIVcpz) endemic to central Africa (Gao *et al.*, 1999) while the HIV group P is suggested to have originated from the SIV infections (SIVgor) in gorillas (*Gorilla gorilla gorilla*) from West Africa (Vallari *et al.*, 2011).

There is also a high level of diversity within each group leading to a further division of the virus into subtypes (also called clades). In particular, group M, can be further subdivided into ten recognised phylogenetic subtypes, or clades (A to K) (Buonaguro *et al.*, 2007). The level of amino acid diversity between subtypes is estimated to be between 17-35% and intra-subtype

diversity is as high as 8-17% (Korber *et al.*, 2001). Furthermore, the immune response to HIV infection is the driving force of viral evolution and escape mutation. The formation of escape mutants (albeit at the cost of viral fitness) and high viral diversity is a major challenge for the development of an effective HIV vaccine.

1.1.3 Structural and genomic organization of HIV-1

Like most Lentiviruses, HIV is capable of converting its positive sense, single-stranded RNA (ssRNA) into double stranded deoxyribonucleic acid (dsDNA). This is achieved by the integration of the HIV particles to host cells and the action of reverse transcriptase encoded within the viral genome. The newly synthesised viral DNA is then integrated into the host cellular DNA by host co-factors and viral integrase which is encoded within the viral particle (Smith *et al.*, 2006). The integrated virus either becomes latent (Levy *et al.*, 1993) which ensure the virus and infected cells avoid detection by the immune system. Alternatively, the virus produces new RNA genomes through transcription and the viral proteins produced are released from the cell as new virus particles that go on to infect other cells.

1.1.3.1 Structural organisation and fundamental proteins of HIV-1

The spherical shaped HIV particle is 120nm in diameter and is composed of a bi-layer lipid envelope surrounding a matrix which contains viral proteins. The genome of HIV is made up of two copies of ssRNA comprising genomic information required for the replication, maturation and structure of HIV and is surrounded by a conical capsid. The capsid contains p24 protein which is enclosed within a nucleocapsid. Viral proteins enclosed within the capsid such as Reverse transcriptase (RT) and Integrase are closely associated to the RNA genome and are fundamental to the development and pathogenesis of both virions and mature virus (Bieniasz, 2009, Sundquist *et al.*, 2012).

In addition, other proteins such viral inhibitory factor (Vif) which disrupts the entry of the anti-viral host protein known as apolipoprotein B mRNA-editing, enzyme-catalytic polypeptide like 3 (APOBEC3) and enhances HIV-1 replication (Schrofelbauer *et al.*, 2004). Other proteins such as viral protein R (Vpr), negative fact (Nef) and protease are also present within the capsid. Vpr is a key regulator of maturation of the virion and is required for virus replication and infection of non-dividing host immune cells such as macrophages (Pandey *et al.*, 2009). Nef is expressed early in the life cycle of HIV and acts as an essential virulence factor which effectively induces

host T cell activation and drives chronic viral replication in these activated cells during infection (Das and Jameel, 2005), whilst HIV protease functions to cleave newly synthesised viral polyproteins to form appropriate structure and regulatory proteins in new virions (Kohl *et al.*, 1988). The lipid envelope surrounding the matrix contains glycoproteins (extracellular gp120 and trans-membrane gp41). Gp120 and gp41 glycoproteins are responsible for the fusion of the virus to host cells (Wyatt *et al.*, 1998) and have therefore been targeted for anti-viral vaccine development.

1.1.3.2 The genome of HIV-1 comprises structural and regulatory genes

Lentiviruses that infect primates have numerous accessory genes used to evade the host immune response (Kirchhoff, 2010). The genome of HIV is composed of three structural and six regulatory genes which encode nine open reading frames. The proviral form of HIV-1 is flanked by two long terminal repeat (LTR) sequences (Muesing *et al.*, 1985).

The structural genes namely *gag*, *pol*, and *env* encode structural proteins for new virus particles (Figure 1.1). The *gag* gene encodes the Gag polypeptide which is eventually processed into a range of structural proteins such as p17, p24, p6 and p7.

The viral enzymes reverse transcriptase, integrase and protease are encoded for by the *pol* gene and the *env* gene codes for the gp160 protein that is enzymatically processed during virion maturation into gp120 and gp41. These proteins are the structural components of the Env protein. The trans-activator of transcription (*tat*) gene is a regulatory gene encoding for Tat, which is in turn a critical viral protein that enhances both the transcription of viral RNA into dsDNA (Debaisieux *et al.*, 2012) and infection of host bystander CD4+T cells (Campbell *et al.*, 2004). The gene regulator of virion expression (*rev*) is responsible for the regulation of viral transcription. Additional regulatory genes such as viral protein R (*vpr*), viral infectivity factor (*vif*), viral protein U (*vpu*) and negative regulator protein (*nef*) encode for proteins responsible for viral replication in non-dividing host cells such as macrophages, evading host immune responses, viral budding and the establishment of chronic infection respectively.

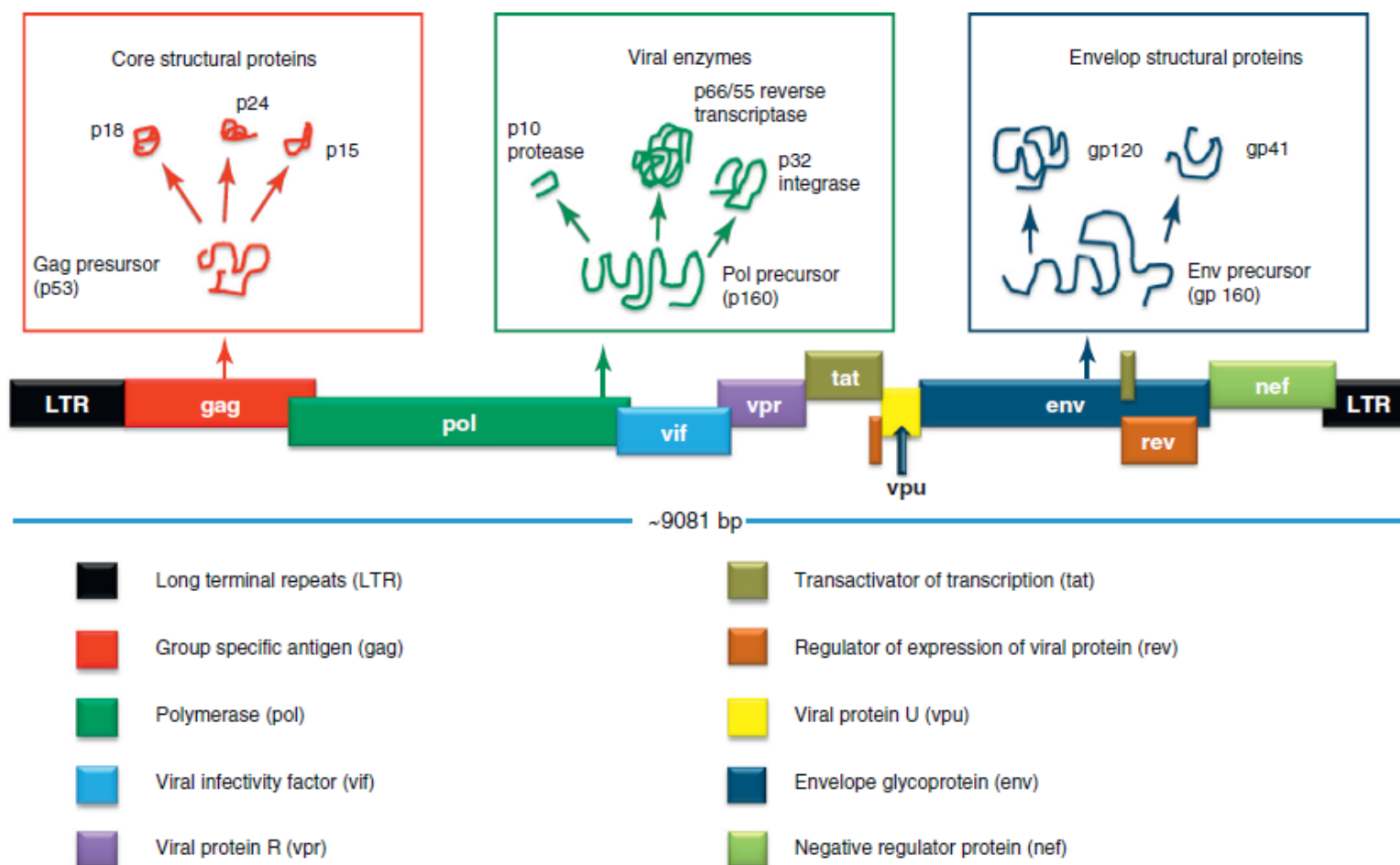


Figure 1.1 Structural organisation of HIV-1 genome. The genome of HIV-1 is approximately 9087 base pairs in length, contains structural and regulatory genes and is flanked by long terminal repeat (LTR) sequences on either end. Structural genes such as *gag* and *env* encode for core and envelope proteins respectively and the remaining genes (*pol*, *vif*, *vpr*, *tat*, *vpu*, *rev* and *nef*) are associated with the regulation of viral replication, transcription and integration. (Taken from Chhatbar *et al.*, 2011).

1.2 Immunopathogenesis of HIV-1

1.2.1 HIV-1 infection of target cells

Transmission of HIV-1 infection occurs through sexual intercourse; exposure to infectious body fluids; and vertical infection from mother-to-child during pregnancy, delivery or breast-feeding. In about 80% of infections, only a single virus (founder virus) is transmitted across the mucosa (Keele and Derdeyn 2009; Abrahams *et al.*, 2009). The virus preferentially infects gastrointestinal and genital mucosa tissues in which a high frequency of activated CD4⁺ T cells can be found (Brenchley and Douek 2008).

These T lymphocytes, express the surface molecule CD4, the receptor for HIV-1 binding and co-receptors for HIV-1 binding such as chemokine co-receptor 5 (CCR5) or the C-X-C chemokine receptor type 4 (CXCR4) (Douek *et al.*, 2002) and are thus the main target cells of HIV-1 infection. In addition, a number of other cellular proteins have been associated with HIV-1 binding and these molecules are found on B cells, dendritic cells and macrophages. These molecules include CD21 a surface molecule expressed on B cells (Moir *et al.*, 2000), the C type lectins such as DC-SIGN (Hioe *et al.* 1998), intracellular adhesion molecules (ICAMs) (Bounou *et al.*, 2002), leukocyte function-associated antigens (LFAs) and $\alpha 4\beta 7$ integrin (Arthos *et al.*, 2008). As such, HIV-1 is also able to infect B cells, dendritic cells (DCs) (Steinman *et al.*, 2003) and macrophages (Li *et al.*, 1999). The binding of HIV-1 to its target cells, particularly the CD4⁺ T cells, is reviewed in Lever and Jeang (2011).

In summary, the gp120 protein on the viral surface first binds to surface CD4 and co-receptors CCR5 or CXCR4. This binding releases gp41 and allows for the fusion of the viral envelope with the host cell membrane. The viral core is then released into the cytoplasm of the host cell. This is followed by the release of viral RNA from the viral core. The ssRNA is then reverse-transcribed into double stranded cDNA by the viral reverse transcriptase enzyme. The newly formed dsDNA is then translocated into the host nucleus and is integrated into the host cell genome to form a provirus with the assistance of viral integrase and Vpr. T cell activation then perpetuates proviral transcription (Stevenson *et al.*, 1990). The first RNA transcripts that are processed and spliced into messenger RNA (mRNA) encoding for regulatory viral proteins Tat and Rev which enhances transcription of viral RNA and the cytoplasmic transport of viral RNA respectively (Bieniasz, 2009). The processed mRNA transcripts encodes for structural proteins

Gag, Pol and Env which together with the viral genomic material are then assembled to form new viral particles (Sundquist and Krasslich, 2012). The newly formed viral particles bud from the host cells and are released to infect more target cells. This leads to the depletion of target cells and is followed by the subsequent dysfunction of the immune system. In the absence of antiretroviral therapy (ART), HIV-1 replication occurs at exponential rates, rapidly depleting both infected and uninfected (bystander) CD4⁺ T cells through HIV proteins such as Env, Tat and Nef that are secreted extracellularly (Finkel *et al.*, 1995).

1.2.2. Acute infection

The typical course of untreated HIV-1 infection is initiated with an acute phase of infection which commences 2 to 4 weeks after transmission (Figure 1.2). During this phase, most individuals develop acute HIV syndrome, characterised by fever, lymphadenopathy and high plasma viraemia (Gurunathan *et al.*, 2009). The acute phase of HIV infection is also associated with high rates of HIV replication with peak viraemia achieved approximately 4 weeks after infection (Piatak *et al.*, 1993, Little *et al.*, 1999) and a steady decline in CD4⁺ T cells. The host immune response to HIV-1 is present early during the initial infection when viral replication is still largely isolated in mucosal tissues.

1.2.2.1 Dendritic cells and natural killer cells during acute HIV-1 infection

During the acute phase of HIV-1 infection, CD4⁺ T cell depletion is amplified and this coincides with the release of acute phase proteins such as serum amyloid A and pro-inflammatory cytokines such as IL-15 and type I interferons (as reviewed by McMichael *et al.*, 2010) by dendritic cells. In addition C-X-C chemokine ligand 10 (CXCL10) and other soluble factors such as lipopolysaccharide (LPS) are also released.

The onset of these early cytokines and chemokines corresponds to an increase in viraemia (Figure 1.3). During this time, the number of circulating dendritic cells (DCs) are also reduced (Killian *et al.*, 2006) and natural killer (NK) cells become activated (Alter *et al.*, 2007). Human DCs, either in the form of plasmacytoid DCs (pDCs) or myeloid CD11c⁺ conventional DCs (mDCs) play a central role in virus detection, the initiation of adaptive immune responses and viral elimination. Specific pattern recognition receptors (PRRs) expressed on DCs such as the C-type lectins and toll-like receptors (TLRs) are able to detect free viruses and virally infected cells (including HIV-1 infected cells) in the periphery and become activated (Kawai and Akira, 2007). The pDCs that have been exposed to HIV (i.e. antigen experienced pDCs) co-ordinate

the early adaptive immune responses by the production and release of IFN α . Furthermore, type I IFNs released by pDC initiate the proliferation and cytotoxicity of NK cells (Gerosa *et al.*, 2005).

During acute HIV infection, NK cells induce antiviral response by the production of pro-inflammatory cytokines and lysis of infected cells (Biron *et al.*, 1999; Altfeld *et al.*, 2011). However, as mentioned above, NK cell activation requires the release of cytokines from DCs. Studies have shown that mDCs release IL-12 and IL-18 which promotes the production of IFN- γ by NK cells *in vitro* (O'Leary *et al.*, 2006; Borg *et al.*, 2004).

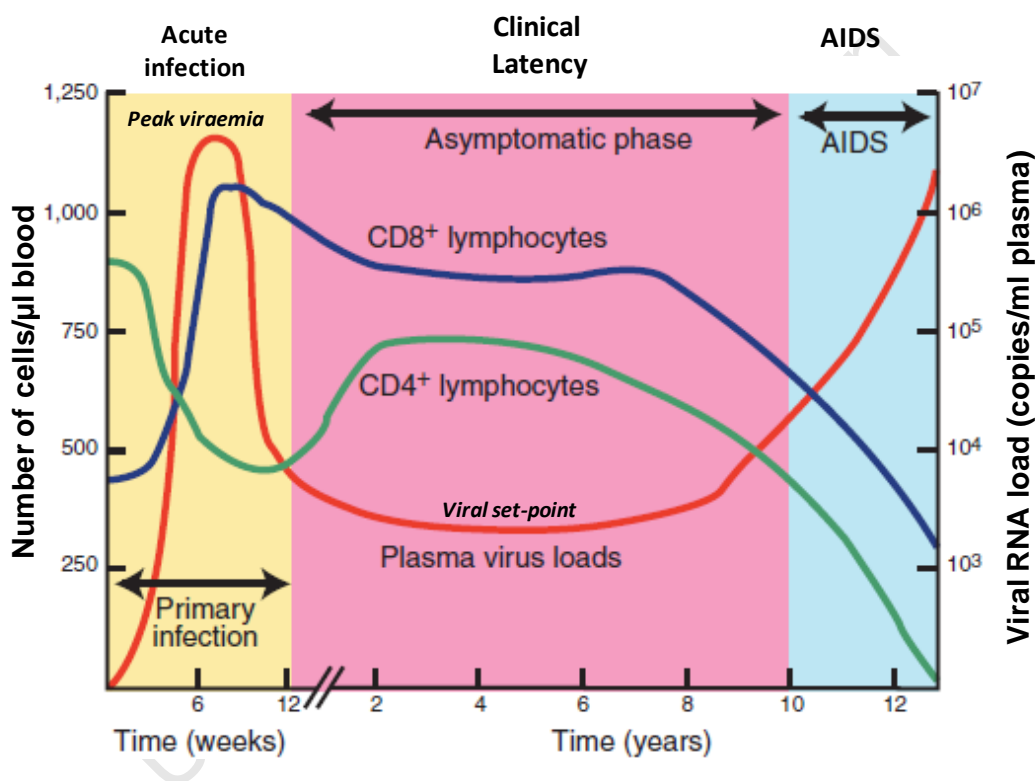


Figure 1.2. T cell dynamics during the typical phases of untreated HIV-1 infection. During acute HIV-1 infection, the number of CD4+ T cells (green line) decline as a result of increasing viral load (red line). Soon after infection, the number of CD8+ T cells increase (blue line) in an attempt to control viraemia. In effect, the viral load decreases and reaches a viral set point which is maintained during the clinical latency phase of infection. During this stage, CD4+ T cells are replenished, but only to a level lower than before infection. However, viral escape from CD8+ T cells and antibody responses (not shown) occurs and viral load increases followed by the depletion of CD4+ and CD8+ T cells. This phase of infection characterised by immune deterioration is clinically defined as AIDS. (Adapted from Sewell *et al.*, 2010).

1.2.2.2 Adaptive immune responses during acute infection

i. Antibody responses during the acute phase of HIV-1 infection

The initial production of HIV-1 specific antibodies (occurs around 5 days following the onset of cytokine release) is directed towards the non-neutralising sites of gp41 and does not select for viral escape (Moore *et al.*, 2006). The antibodies directed towards the founder virus are capable of non-neutralising activity such as antibody-dependent cell-mediated cytotoxicity (ADCC) and antibody-dependent cell-mediated viral inhibition (ADCVI), are only detectable 3 months after infection has occurred. However, by the time these antibodies are generated, the virus has already established infection and can mutate to avoid binding to these early antibodies altogether (Wei *et al.*, 2003; Moore *et al.*, 2006).

ii. T cell responses during acute infection

As the plasma viral load increases during acute infection, the ratio of CD4:CD8 T cells is inverted with CD8+ T cells expanding with viral load and CD4+ T cells declining. The first HIV-1 specific CD8+ T cell responses are detected just before peak viraemia is established. This response selects for escape mutant epitopes or regions of the virus that mutate at the cost of viral fitness (Goonetilleke *et al.*, 2009). Evidence suggests that CD8+ T cells exert direct cytotoxic effects on infected cells, suppress viral replication via cytokines, chemokines and other mediators or inhibit viral entry by soluble factors as reviewed by Freel *et al.* (2011).

The initial expansion of HIV specific CD8+ T cells during the acute phase of infection is followed by a decline in viral load over several weeks. This transient decline in viral load is due to a robust immune response driven by these activated CD8+ T cells. It has also been suggested that the decline in viral load could also result from the loss of susceptible CD4+ target cells at the site of HIV-1 infection and accounts for the limited CD4+ HIV-specific responses (Brenchley *et al.*, 2004). The immune pressure exerted by the host leads to an establishment of a stable level of viral load, (known as viral set point) (Piatak *et al.*, 1993) and is critical to the rate of disease progression.

1.2.3. Clinical latency and viral escape

Once viral set point is established, untreated HIV infected individuals almost always progress toward chronic infection known as clinical latency (Figure 1.2.) which can persist for around 2 to 20 years. This phase of infection occurs approximately 6 months following acute infection during which virus levels in the plasma reach a set point below 20 000 RNA copies/ml. Within 10 days following CD8 expansion, the virus has already generated new mutants capable of CD8 escape. At this stage viral reservoirs are established. It takes up to 80 days for the development of early autologous virus specific neutralising antibodies. The virus once again escapes just weeks after the antibodies are detectable in plasma samples.

1.2.4. The development of AIDS

Eventually, infection spreads to the peripheral lymphoid organs and CD4+CCR5+ T cells are progressively depleted (Brenchley and Douek, 2008). Intervention from ARVs might impede HIV infection and disease progression but HIV-1 has the ability to rapidly establish a reservoir of latently infected long-lived resting CD4+ memory T cells (Finzi *et al.*, 1999; Alexaki *et al.*, 2008).

At this stage, the immune system begins to progressively deteriorate and infected individuals become highly susceptible to opportunistic infections (Figure 1.2) (Kitahata *et al.*, 2009). Infected individuals at this stage are usually diagnosed with AIDS with a CD4+ below 200 cells/mm³ and without treatment these people will eventually die as a result of AIDS related diseases.

1.2.5. HIV controllers

The vast majority of HIV infected individuals will succumb to AIDS without treatment as a result of immune deterioration and opportunistic infections. However, some groups of HIV infected individuals are able to control infection. One such group known as elite controllers, have the ability to spontaneously control viraemia without ARVs and are defined by virologic criteria as having low to undetectable viral loads despite being infected for more than two years (Okulicz *et al.*, 2009). The prevalence of this group is less than 1% in the population of infected individuals and elite controllers have a reduced risk of AIDS and death.

Another group of HIV infected individuals defined as long-term nonprogressors (LTNPs) on the basis of having elevated CD4+ T cell counts for prolonged periods of time in the absence of

ARVs (as reviewed by Okulicz, 2012). These individuals have low to moderate levels of viraemia but have ≥ 500 CD4+ cells/ μ l. The mechanism responsible for this level of control is unclear but both elite controllers and LTNPs are the focus of numerous research efforts to identify these mechanisms of spontaneous control (Okulicz, 2012).

Highly exposed persistently seronegative (HEPS) individuals are groups of individuals that are exposed to HIV and despite this, remain HIV-negative in the absence of ARVs. In Kenya and The Gambia, HEPS prostitutes have been identified (Fowke *et al.*, 1996; Rowland-Jones *et al.*, 1995). Although there is evidence of HIV-1 specific CD8+ cytotoxicity *in vitro* these individuals remain HIV-1 negative. It has been shown that in these individuals HIV specific CD8+ responses depends on persistent exposure to HIV and is acquired over time (Kaul *et al.*, 2001).

1.3. The development of an effective HIV-1 vaccine

1.3.1 The need for an HIV-1 vaccine

The implementation of HAART and improved ARV roll out has ensured a prolonged, relatively asymptomatic life for infected individuals and has decreased the incidence of HIV-1. The HIV Prevention Trial Network (HPTN) trial known as HPTN 52 concluded that the use of ARTs to reduce the viral load of the infected partner in HIV discordant couples effectively decreased the probability of HIV transmission to the uninfected partner (Cohen *et al.*, 2012). Improved ART coverage has also been reported to reduce the number of HIV related deaths and improved the life expectancy of adults in populations with high HIV prevalence (Bor *et al.*, 2013). Recent reports have also demonstrated that the risk of HIV acquisition decreases substantially as the coverage of ART increases which implies that HIV negative individuals that live in areas with high ART coverage are less at risk of HIV infection compared to those in areas with low or no ART coverage (Tanser *et al.*, 2013).

Currently, there are ongoing studies aimed at evaluating the effectiveness of post exposure prophylaxis (PEP) and pre-exposure prophylaxis (PrEP) as part of treatment and prevention policies. There has also been recent improvements and significant advancement in anti-viral microbicides particularly Tenofovir gel containing ARVs which has shown to reduce infection of HIV-1 by 38% in women in the CAPRISA -004 study (Abdool *et al.*, 2010). However, in regions such as South Africa, with high HIV prevalence, there are many social, cultural and economic challenges still associated with the use of ARTs (Ojikutu *et al.*, 2007) and PrEP. Non-

adherence to treatment is a major hindrance to the success of these treatment options. The phase 2B Vaginal and Oral Interventions to Control Epidemic (VOICE) trial, known as the Microbicide Trial Network (MTN)-003 trial, was designed to assess the safety and efficacy of oral and vaginal PrEP (including tenofovir). The results of this study showed no significant decline in HIV acquisition in participants (van der Straten *et al.*, 2012). These results were in contrast to what was found in terms of efficacy in the CAPRISA-004 study. It is believed that the lack of participant adherence to the daily dosage of the treatment is a major contributing factor to these conflicting results (van der Straten *et al.*, 2012).

Apart from PrEP treatment, there are promising results from studies investigating male circumcision as a method of HIV-1 prevention. To date, studies investigating the prevention of HIV infection by circumcision conducted in South Africa, Kenya and Uganda showed a significant decline in HIV incidence of 60%, 53%, and 51% respectively in circumcised versus uncircumcised participants (Auvert *et al.*, 2005; Bailey *et al.*, 2007; Gray *et al.*, 2007). The protective effects of circumcision were sustained for up to 66 months in circumcised men in Kenya and 5 years in Ugandan men (Bailey *et al.* 2010; Gray *et al.*, 2012) and it has been proposed that circumcision performed in infancy can offer further protection from HIV. However, there are many concerns arising from this including the cost, safety concerns during circumcision and the risk of circumcised men engaging in unprotected sex.

In light of these concerns, together with the challenges of non-adherence to PrEP and ARVs; the possibility of new drug resistance HIV strains surfacing and the safety concerns of using ARVs in a setting with a high prevalence of co-infections, the development of an effective prophylactic HIV-1 vaccine is still viewed as the most cost-effective means of preventing HIV-1 transmission. However, the development of an effective HIV-1 vaccine poses numerous challenges.

The correlates of protection for HIV vaccines remain unclear despite the advances in the understanding of the pathogenesis of the virus. In the past, the development of potent antibody responses has been the hallmark of successful vaccines against numerous diseases (Plotkin, 2010). However, one of the most challenging aspects of HIV-1 vaccine design is the development of an immunogen to successfully elicit broadly neutralizing antibodies against a broad spectrum of HIV-1 isolates. In addition, there are other challenges that oppose the progression of HIV vaccine development, such as the inherent ability of the virus to escape from host adaptive immune responses and the progressive degeneration and ultimately failure of the

host immune system to co-ordinate effective clearance of the infection (Walker and Burton, 2008; Barouch, 2008).

1.3.2. Characteristics of an effective HIV-1 vaccine

Ideally, an HIV vaccine should mediate both the production of antibodies and T cell immune responses (Mascola *et al.*, 2005; Watkins *et al.*, 2008). Studies regarding viral control in HIV-1 infected individuals, nonhuman primate models of disease and increasing evidence from clinical HIV vaccine trials have provided researchers with an understanding of some of the immunological responses that an effective HIV-1 vaccine should induce.

The development of vaccines to induce antibody responses capable of neutralisation usually includes envelope proteins as immunogens. These vaccines aim to prevent the binding of HIV-1 to target cells and inhibit infection in this manner. T cell based vaccines on the other hand, induce cell mediated immune responses to combinations of viral regulatory proteins such as Gag, RT or Nef which are included as vaccine immunogens. An ideal T cell based vaccine (Figure 1.3), should have the ability to reduce viral replication, limit the infection of CD4+ T cells and delay disease progression during the acute phase of infection in order to minimise the risk of transmission (Watkins *et al.*, 2008; Barouch, 2008).

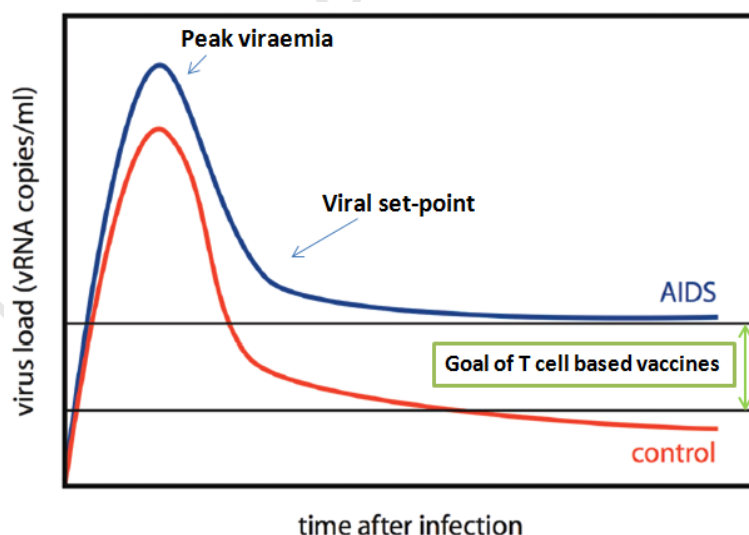


Figure 1.3. Goal of T cell based HIV-1 vaccines. Following untreated infection, HIV-1 replication occurs and the viral load (measured as number of viral RNA copies/ml) continues to increase until peak viraemia is reached. At this stage, the viral load declines to establish a viral set-point and if untreated, this infection progresses to AIDS (blue line). An ideal T cell based vaccine (red line) aims to reduce viral replication during the acute phase of infection. This in effect controls the infection and prevents transmission. (Adapted from Watkins *et al.*, 2008).

1.3.2.1. Insights from HIV infections and the control of viraemia

Studies of HIV-1 infection demonstrate that transmission from infected individuals to HIV-1 negative partners is significantly reduced when the viral plasma loads are below 1700 RNA/ml in the infected individual (Quinn *et al.*, 2000, Gray *et al.*, 2001). There is evidence from studies investigating the immune responses of elite controllers, LTNPs and seronegative individuals exposed to HIV infection which demonstrates that T cell responses are responsible for limiting viral replication and therefore have the ability to limit infection. These studies have led to the understanding of what is required in the design of HIV-1 vaccines.

As discussed above, the reduction of peak viraemia in acute HIV-1 infection coincides with an increase in CD8+ T cells which suggests that CD8+ T cell responses are involved in the control of viraemia (Koup *et al.*, 1994; Borrow *et al.*, 1994; Betts *et al.*, 2006). Studies investigating LTNPs have found that progression to disease is hindered in the presence of HIV-specific CD8+ T cell response and correlates to the reduction of viral replication (Betts *et al.*, 2006; Gea-Banacloche, 2000). In addition, the maintenance of proliferative, polyfunctional HIV-specific CD4+ T cell responses have been associated with long term non-progression (Boaz *et al.*, 2002). In addition, Duvall *et al.* (2008) compared the prevalence of polyfunctional CD4+ and CD8+ T cells in HIV-1 infected individuals to HIV-2 infected individuals. HIV-2 infected individuals exhibit delayed progression to disease and preserved CD4+ T-cell counts close to normal levels. The results of this study suggest that there is a higher frequency of polyfunctional T cells produced by HIV-2 infected individuals compared to that in HIV-1 infected individuals. Cytotoxic T cell mediated responses were also observed in studies investigating seronegative sex workers in Nairobi that are exposed to HIV infection and exposed uninfected children born to HIV infected mothers (Rowland-Jones *et al.*, 1998; Cheyner *et al.*, 1992).

The role of CD8+ T cells in the control of infection is not restricted to cytotoxic responses. Chronic asymptomatic HIV infected individuals have greater CD8+ non-cytotoxic antiviral response compared to symptomatic individuals (Walker *et al.*, 1989, Mackewicz *et al.*, 1994, Castelli *et al.*, 2002). Viral entry into CD4+ T cells and macrophages is in part mediated by soluble factors such as macrophage inhibitory protein (MIP)-1 α , MIP-1 β and regulated and normal T cell expressed and secreted (RANTES). These chemokines are ligands for the HIV-1 co-receptor, CCR5, and inhibit viral replication in CD4+ T cells and macrophages (Cocchi *et al.* 1995). Studies indicate that the ability of HIV specific CD8+ T cells to express perforin, proliferate and mediate the elimination of infected CD4+ T cells by granular exocytosis is

associated with control in HIV infected controllers (Hersperger *et al.*, 2010; Migueles *et al.*, 2002) The role of CD8+ T cells in the control of infection is therefore not restricted to one cellular function but rather to the ability of a cell to produce multiple functions (as defined by cellular marker such as, CD107a, MIP-1 α , IFN- γ , IL-2, TNF- α , and perforin) (Betts *et al.*, 2006; Almeida *et al.*, 2009; Ferre *et al.*, 2009).

As such, studies of HIV infection and controllers of infection have indicated that CD8+ T cell are a necessary component of anti- HIV-1 immune responses and vaccines that induce robust, functional HIV-1 specific CD8+ responses are highly sought. Although T cell based vaccines may not be able to provide protection from infection, there is evidence to suggest that these vaccines should ideally be able to control early viral replication after infection and possibly prevent dissemination of the virus into tissues and delay the progression to disease.

Early studies in LTNP also indicated that neutralising antibody responses were associated with viral control (Montefiori *et al.*, 1996). However it has been found in studies investigating HIV-1 infected individuals that at the time at which broadly neutralising antibody responses are present, viral load is higher (Deeks *et al.*, 2006; Pereyra *et al.*, 2008; Doria-Rose and Connors, 2009). It is suggested that these antibodies are specific to early viral clones. These studies have been useful in suggesting that constant antigenic stimulation is required for the production of broadly neutralising antibodies. Recently, broadly cross-reactive neutralising (BN Abs) were identified in two long-term HIV-1 infected individuals. These antibodies targeted the Asn 332 glycan on gp120 which evolved through viral escape mutations (Moore *et al.*, 2012). These BN Abs were able to neutralise other escape clones in which the Asn332 glycan was also present. This offers hope that BN Abs are still a viable option to reduce and eliminate HIV-1 infection.

1.3.2.2. Non-human primate models and HIV-1 vaccine development

i. The use of nonhuman primates as a model for HIV-1 vaccine studies

The lack of an ideal animal model to test candidate HIV-1 vaccines has been one of the many reasons cited to explain the slow progress in developing an effective vaccine. During NHP preclinical trials of candidate HIV-1 vaccines, it is possible to determine whether the vaccine has the ability to safely induce HIV-1 specific immune responses. These studies can also determine the presence of anti-vector immunity and long term protective adaptive and/or antibody responses. There is increasing evidence that suggests that a universal preclinical evaluation of HIV-1 vaccine candidates will be necessary to ensure accuracy and uniformity between trials. The NHP model is still considered relevant especially since there is a high level of genetic

similarity between nonhuman primates and humans. HIV-1 does not infect NHPs and several strains of SIV are used to predict the efficacy of HIV vaccines in this model. Both HIV and SIV have been theorised to originate from a common ancestor and therefore exhibit numerous nucleotide sequence homologies (Meyer *et al.*, 1999). However, SIV differs from HIV-1 in the structural and antigenic nature of the Env protein and has limited the reliability of NHP studies of vaccine efficacy using SIV challenge.

This has led to the development of the chimeric virus which is the *env* gene from HIV expressed on SIV backbone referred to as Simian/Human Immunodeficiency virus (SHIV) (Li *et al.*, 1995). This has allowed for the evaluation of vaccines containing Env immunogens. SIV infection leads to the depletion of CD4+T cells, rapid viral replication and progression to AIDS in most rhesus macaques in a similar manner to infection of humans with HIV-1. In addition, some rhesus macaques with the MHC class I haplotype profile of *Mamu-A*01*, *Mamu-B*17* and *MamuB*08* exhibit similar levels of control observed in elite controllers or LTNPs in which viral loads are almost undetectable in the absence of ARVs (Bontrop and Watkins, 2005). Based on this, the rhesus macaque model is considered useful in HIV-1 studies.

The NHP model serves as arguably the most useful predictive model of safety and immunogenicity of candidate HIV-1 vaccines. The NHP model can also be used to test the efficacy of vaccine candidates containing the Env using matched SHIV challenges (Amara *et al.*, 2002; Polacino *et al.*, 1999). However, the efficacy of candidate HIV vaccines can only be tested in Phase 3 clinical trials.

ii. The relevance of using Chacma baboons as models of HIV-1 vaccine evaluation

Baboon species have been established as important predictor models to measure candidate vaccine safety and immunogenicity prior to clinical studies. Most studies have used the olive baboon (*Papio anubis*) or the yellow baboon (*Papio cynocephalus*) for the evaluation of malaria and *Schistosoma mansoni* vaccine efficacy (Chowdhury *et al.*, 2009; Ahmad *et al.*, 2011). These species of baboons have also been used to measure the immunogenicity of HIV-1 vaccines (Casimiro *et al.*, 2003; Leung *et al.*, 2004). In South Africa, the Chacma baboon (*Papio ursinus*) has also been used to determine the safety and immunogenicity of HIV-1 vaccines (Chege *et al.*, 2009; Burgers *et al.*, 2009). Baboons are closely related phylogenetically to humans with DNA homology between baboons and humans estimated at 96% (Hixson *et al.*, 1988). Furthermore, the *Papio* species can be infected with some human pathogens and

therefore have the potential to induce similar immune responses and share a broad range of cross-reactive T lymphocyte CD antigen repertoires with humans (Perry *et al.*, 2012). In terms of preclinical evaluation of vaccines, baboons are widely available in South Africa making vaccine development and immunogenicity testing cost effective. To date, the baboon model has been used to evaluate candidate vaccines which are designed to generate neutralizing antibodies (Anderson *et al.*, 1989; Stephens *et al.*, 1992; Haigwood *et al.*, 1992), and the ability of vaccines to induce cellular immune responses in Chacma baboons (Casimiro *et al.*, 2003; Chege *et al.*, 2008a; Chege *et al.*, 2008b; Burgers *et al.*, 2009).

iii. Insights into HIV vaccine development from non-human primate models

To date, NHP models have led to the understanding and analysis of transmission of HIV/SIV across mucosal barriers, the establishment of viral reservoirs and latency in hosts, the effect of vaccination and challenge via different routes and viral dissemination (as reviewed in Lifson and Haigwood, 2012). In addition, NHP models have also allowed for the evaluation of innate and adaptive immune responses to vaccination as well as the production of antibodies and are invaluable for comparative assessment of candidate HIV-1 vaccines.

There are also lessons to be learnt for HIV vaccine development from investigating primate species with the ability to control SIV replication and pathogenesis. For instance, natural hosts of SIV, such as African green monkeys and sooty mangabeys do not develop chronic disease, despite viral loads similar to those detected in humans infected with HIV-1 and rhesus macaques infected with the SIV (SIVmac) (Liovat *et al.*, 2009). Investigating the molecular mechanisms that prevent the onset of chronic disease may be beneficial in determining the type of vaccine responses which could protect against disease progression.

The importance of HIV-1 specific CD8⁺ T cells responses has been supported by numerous studies in NHPs. Studies investigating the role of CD8⁺ T cell responses in SIV infected rhesus macaques showed that viremia and viral load increased significantly after CD8⁺ T cells were depleted compared to SIV infected macaques in which CD8⁺ T cell responses were present (Schmitz *et al.*, 1999; Watkins *et al.*, 2008). This confirmed that CD8⁺ T cells not only limited viral replication, but might also have the ability to control infection if they are primed early during acute infection before significant CD4⁺ T cells depletion. More recently, Wong *et al.* (2010), not only confirmed the association between CD8⁺ T cell loss and increased viral replication in

macaques, but they also confirmed findings that non cytotoxic CD8+ T cells functions, such as the production of cytokines and chemokines, are involved in viral control during infection.

To date, a number of vectors and vaccination regimens have been evaluated in NHPs. The use of T cell based HIV-1 vaccines (such as recombinant adenovirus serotype 5 (rAd5) vector-based vaccine) have proven to effectively reduce viral load in *Mamu-A 01* negative rhesus macaque-SIV challenged models of infection (Liu *et al.*, 2009). The adenovirus, serotype 5 (Ad5) vector developed by Merck and the NIH was first observed to induce high magnitude CD8+ responses and immune protection against SHIV89.6P in macaques (Shiver *et al.*, 2002) and went on to form part of the STEP study. In another study, priming with recombinant Bacille Calmette-Guerin (rBCG), the attenuated *Mycobacterium bovis* bacterium, containing SIV Gag, Pol and Env followed by a rAd5 boost expressing the same antigens successfully induced persistent cellular responses compared to animals that only received rAd5 vaccination alone (Cayabyab *et al.*, 2009). Studies conducted at UCT, using Chacma baboons indicate that rBCG expressing Gag can be used to effectively prime immune responses after boosting with virus (Chapman *et al.*, 2010).

Vaccine studies in NHPs have re-affirmed that in order to achieve a sustained level of protection, an effective vaccine should stimulate T cells that generate HIV-1 specific, protective and long lived effector memory and central memory responses (Robinson, 2007). T cell memory is defined as long lived responses that can be initiated long after the initial exposure to antigen to induce a robust, strong secondary immune response to the same antigen upon re-encounter (Dutton *et al.*, 1998). In a study in which rhesus macaques were primed with plasmid DNA containing SIV antigens and boosted with rAd5 indicated the increased survival and reduced viral loads after SIV challenge is associated with the presence of CD4+ central memory T cells (T_{cm}) (Letvin *et al.*, 2006). These results formed the basis of the ongoing phase 2b efficacy trial that investigate these vaccines in the HIV Trial Network (HVTN)-505 study.

Central memory CD8+ T cells have also been found to play a key role in the control of viral replication following challenge in rhesus macaques vaccinated with DNA prime and either recombinant viral vector boost (Vaccari *et al.*, 2005; Acierno *et al.*, 2006). Hansen *et al.*, (2011) demonstrated that viral control is associated with SIV specific CD8+ T cell responses in rhesus macaques vaccinated with cytomegalovirus vaccines and that CD8+ effector memory (T_{em}) was associated with control. A review of T cell memory is provided in Chapter 4 of this thesis.

In addition, studies involving rhesus macaques that were passively immunised with broadly neutralising antibodies showed that the vaccinated animals were protected against infection from virulent SHIV challenge (Baba *et al.*, 2000; Parren *et al.*, 2001; Veazey *et al.*, 2003; Ferrantelli *et al.*, 2004.) These studies provide evidence that vaccination designed to elicit broadly neutralising antibodies to provide protection against infection is possible.

Non-human primates have been used to identify routes of immunisation that induce the best possible immune responses and protection. In a recent study, SIV DNA prime and SIV rMVA boost vaccines administered via rectal immunisations in rhesus macaques, were found to induce SIV specific cellular immune responses both systemically and mucosally. Intranasal immunisation with the same vaccines elicited better cellular and humoral responses compared to animals vaccinated via the intra-muscular route and protected against SIV vaginal challenge (Manrique *et al.*, 2013).

Studies in NHPs have also been useful in determining the ideal immunogens that should be incorporated into candidate HIV-1 vaccines. It has been suggested that low viral load and higher levels of CD4+ T cells can be achieved by increasing the breadth of immunogens (particularly to a broad range of Gag epitopes) recognised by the immune system (Rolland *et al.*, 2008). In a study by Sun *et al.* (2010), it was found that a vaccine regime containing all genes of the SIVmac239 virus afforded a broader immune response in macaques compared to vaccination with only a single antigen. Candidate HIV-1 vaccines should be designed to present the immune system with a complement of relatively conserved and immunogenic viral epitopes such as Gag and Nef in order to initiate an early, rapid and effective response before the virus has produced escape mutants (Addo *et al.*, 2003). It has been demonstrated that SIV Gag, Pol, and Env reduced SIV infection in macaques and Env in particular was required for protection against SIV (Barouch *et al.*, 2012). Gag-specific cellular immune responses have also been correlated with control of viral infection in vaccinated rhesus monkeys following SIV (Shiver *et al.*, 2002; Iwamoto *et al.*, 2010; Barouch *et al.*, 2012; Hansen *et al.*, 2011; Liu *et al.*, 2009). In fact, it has been found that Gag-specific but not Pol- or Env-specific CD8+ responses were associated with viral inhibition (Stephenson *et al.*, 2012). These studies highlight the need to incorporate Gag into HIV vaccine candidates to optimise cellular responses and control infection.

1.3.2.3. Lessons from clinical trials

During the last 26 years of research, a total of 220 HIV vaccine trials have been conducted but only some have advanced to phase 2b proof-of-concept or phase 3 clinical efficacy trials (as reviewed by Saunders *et al.*, 2012). The approach of the vaccines tested in these trials was either to induce antibody responses, T cell responses or both. Despite the poor to moderate results observed, these trials have outlined the challenges and opportunities for the development of an effective HIV-1 vaccine. This review highlights the outcomes of four of these trials and how these findings enhanced the field of the HIV vaccine development.

i. VAX003 and VAX004

The first two vaccines tested in phase 3 clinical trials were comprised of the recombinant gp120 proteins from Env and were administered with the adjuvant alum manufactured by Vaxgen. These vaccines aimed to induce Env-specific antibodies. The VAX003 was comprised of one clade B and one clade E gp120 (AIDSVAX B/E) and was used to vaccinate high risk drug users in Thailand. The participants received seven immunisations over 30 months. The VAX004 was comprised of two clade B gp120s (AIDSVAX B/B) and was tested in a cohort of high risk individuals from Canada, The United States of America and The Netherlands (Harro *et al.*, 2004; Pitisuttihum *et al.*, 2006).

These early antibody based candidate vaccines induced gp 120 specific antibody responses but overall the results were disappointing. The vaccines induced no protection from infection, and were not able to delay the progression of disease or reduce viral load in phase III human trials (Flynn *et al.*, 2005; Pitisuttihum *et al.*, 2006). It is believed that the immunogens used in these trials were not able to neutralise circulating strains of HIV-1 despite being able to neutralise laboratory strains of the virus (Mascola *et al.*, 1996). However, these trials confirmed that B cell based vaccines require potent broadly neutralising antibodies. These trials also showed that candidate HIV vaccines aimed at eliciting humoral responses can be used safely in humans.

ii. STEP and Phambili study

The STEP study was a phase IIb proof of concept trial which commenced in 2004 and evaluated the non-replicating MRK adenovirus type 5 (MRKAd5) vaccine that expressed *gag*, *pol* and *nef*. Since this vaccine did not contain Env, it was designed to primarily induce T cell

responses to decrease viral load and prevent transmission. The STEP (HVTN 0052) study enrolled healthy, but at risk participants from the Americas, Caribbean and Australia whilst the Phambili trial (HVTN 0053) enrolled individuals from South Africa.

During preclinical evaluation, vaccinated rhesus macaques showed effective control of viral load after challenge with SHIV (Shiver *et al.*, 2002; Casimiro *et al.*, 2005). The phase 1 clinical trial also deemed this vaccine safe and the Ad5 vector proved to be effective in terms of inducing detectable immune responses. However, the phase 2b clinical trial was terminated in 2007 following a report from the Data and Safety monitoring Board that deemed the study incapable of determining the efficacy of the vaccines (Fauci *et al.*, 2008). Despite the detection of HIV-1 specific CD8+ T cell responses directed towards *gag*, *pol* and *nef*, there was no significant evidence of protection in vaccinated individuals compared to unvaccinated individuals that received placebos (Gray *et al.*, 2010). Further analysis of the failure of the trial indicates that vaccine specific T cells responses exerted weak selection pressure on the viruses that eventually infected vaccinated participants (Rolland *et al.*, 2011).

Further analysis of this study indicated that the rate of HIV-1 infection was increased in participants, particularly in uncircumcised men, that were Ad5 seropositive and hence possessed pre-existing anti-vector immunity (Buchbinder *et al.*, 2008; Gray *et al.*, 2010). The increased risk of HIV acquisition among vaccinated Ad5 seropositive participants compared to individuals that received the placebo could be a result of higher levels of Ad5-specific CD4+ T cell responses in the seropositive individuals (Gray *et al.*, 2010). It is hypothesised that the rapid expansion of Ad5-specific CD4+ T cells after vaccination gives rise to potential target cells for infection. As reviewed by Barouch (2010), other rare serotypes of adenoviruses which have lower prevalence in human populations such as Ad26 and Ad35 (Vogels *et al.*, 2003; Abbink *et al.*, 2007) are being investigated as potential HIV-1 vaccine vectors in phase 1 clinical trials.

From the STEP study, it is evident that pre-existing anti-vector immunity, particularly to Ad5, can negatively impact the success of vaccines. In addition, the use of Ad5 vaccine only and not the more potent prime-boost vaccination regimen induced responses with insufficient magnitude and breadth to induce protection from infection (McElrath *et al.*, 2010). This trial also highlighted the importance of investigating the impact of mucosal tissue responses during the evaluation of HIV vaccines as vaccinated, but uncircumcised men appeared to be infected at higher rates compared to vaccinated, circumcised men in this trial (Gray *et al.*, 2010).

iii. RV 144

The RV 144 study (Thai trial) conducted in Thailand investigated the efficacy of canarypox ALVAC prime expressing *gag*, *pol* and *env* followed by AIDSVAX CM-244 Env rgp120 protein boost was the first heterozygous prime-boost vaccine regimen in phase 3 clinical trials. In contrast to the other clinical trials discussed, the vaccines tested could induce both T cell and humoral immune responses. Infection was reduced in 31.2% of individuals in the vaccinated arm of the study compared to the placebo arm (Rerks-Ngram *et al.*, 2009).

However, this efficacy was insufficient for licensure, but further analysis of the trial revealed several key factors that will contribute to the development of future HIV-1 vaccines. This study was the first to demonstrate modest, but promising vaccination efficacy although no differences in viral load was observed in vaccine and unvaccinated participants. This vaccine regimen was found to induce strong Env-specific antibody responses which decreased in magnitude after the first year (McElrath, 2010).

In addition, Env-specific CD4+ T cell proliferative responses were observed in vaccinated individuals and there were no significant HIV-specific CD8+ T cell responses in vaccinated individuals (McElrath and Haynes, 2010; Rerks-Ngram *et al.*, 2009). This trial demonstrated that although no neutralising antibodies were present, V1/V2 binding antibodies in vaccinated individuals were associated with protection (Haynes *et al.*, 2012; Zolla-Pazner *et al.*, 2013) and Env specific IgA antibodies were associated with infection and identified as a correlate of risk (Haynes *et al.*, 2012).

A novel set of immunogens are being investigated for incorporation in next generation vaccines which induce non-neutralising, broadly neutralising antibodies, the possible induction of ADCC, ADCVI responses together with improved T cell responses (O'Connell *et al.*, 2012; Bianchi *et al.*, 2010 Koup *et al.*, 2011).

1.3.3. Delivery vector systems for HIV-1 vaccines used in the current study

In the past, live attenuated SIV vaccines have proven to be effective in rhesus monkey (Daniel *et al.*, 1992) but the safety concerns of using live HIV in humans are too great. As such, novel vaccine strategies based on vector systems have been developed over the past decade. These include systems to deliver antigenic proteins directly to the host or to deliver antigenic proteins

in plasmid DNA vector vaccines and recombinant viral vector vaccines. This review will highlight the vectors used as part of this study conducted at UCT.

1.3.3.1. MVA

The Modified vaccinia Ankara (MVA) is an Orthopoxvirus that has been attenuated by approximately 570 passages in primary chicken embryo cells and is replication defective in mammalian cells making it safe for use in humans (reviewed by Sutter and Staib, 2003). Like other pox-viruses, the genome of MVA is large enough to accommodate immunogens making it an effective gene delivery vector (Blanchard *et al.*, 1998). The production of MVA based vaccines is cost effective, and as vector, MVA is usually stable after freeze drying (Gomez *et al.*, 2008). In addition, MVA vectors are able to induce immune responses despite lacking replication mechanisms (Blanchard *et al.*, 1998).

MVA vectors containing SIV or HIV antigens as part of heterologous prime-boost strategies have been able to successfully elicit both antibody and cytotoxic T cell responses in rhesus macaques and effectively controlled SHIV 89.6P and SIVmac251 mucosal challenge (Ramsburg *et al.*, 2004; Horton *et al.*, 2002; Amara *et al.*, 2001). Evidence in studies conducted using rhesus macaques suggests that MVA based vaccines administered via the nasal mucosal route is associated with increased survival after challenge and stronger immunogenicity (Ourmanov *et al.*, 2009; Manrique *et al.*, 2011). There have also been reports of long-lived, CD8+ immune responses induced by MVA based vaccination in macaques (Nigam *et al.*, 2007).

In the context of HIV vaccine development, the MVA based vaccines have proven to be safe yet highly immunogenic in both preclinical and phase 1 clinical trials (Mwau *et al.*, 2004; Cebere *et al.*, 2006; Harari *et al.*, 2008). Several clinical trials in healthy and HIV-1-infected human participants have been conducted using MVA-based vaccines (Goepfert *et al.*, 2011; Keefer *et al.*, 2011; Vasan *et al.*, 2010). These studies indicate that recombinant MVA vectors are safe and are able to induce HIV-1-specific immune responses when used in combination as part of a heterozygous, prime-boost vaccine strategy.

Safety, moderate antibody and T cell responses were detected in the phase 2 clinical trial performed by IAVI in which participants were vaccinated with DNA prime and MVA boost with both vectors containing HIV clade A immunogens (Paris *et al.*, 2010; Cebere *et al.*, 2006; Hanke *et al.*, 2007). In a recent phase 1 clinical trial conducted in uninfected volunteers that received DNA vaccines containing HIV-1 subtype C *env*, *gp160*, *gag*, *nef* and *tat* genes (ADVAX) and

MVA boost encoding the same genes and RT (TB-M4). The results of this study indicate that the vaccines were safe and Tier 1 neutralising antibodies were detected against viruses (Mehendale *et al.*, 2013). Investigation of vaccine specific cytokine production revealed that T cell responses were polyfunctional and Env specific responses were predominantly skewed towards the CD4+ phenotype (Hayes *et al.*, 2013).

1.3.3.4. DNA plasmids

Plasmid DNA vector delivery systems generate HIV proteins within the host, and allow for direct transfer of genes since this vector does not rely on pathogenic delivery of immunogens (Brave *et al.*, 2007). DNA plasmids are easy to manipulate and are often modified with specific promoters and RNA stabilisers. In addition, a variety of possible codon optimised HIV genes can be incorporated into the plasmid and are relatively cheap to produce, stable and safe (as reviewed by Hanke, 2001). The plasmid DNA is presented to host cells by antigen presenting cells and begins transcription and translation of mRNA into immunogenic proteins which are released into the cytoplasm. The antigenic proteins are then presented by major histocompatibility complex class I (MHC I) molecules to CD8+ T cells.

Pre-clinical evaluation of DNA plasmid based HIV vaccines have been shown to elicit HIV specific responses in mouse models (Giri *et al.*, 2004) and to confer protection against SHIV89.6P in macaques (Barouch *et al.*, 2000). These immune responses can be enhanced in vaccine regimens consisting of DNA plasmid vectors in conjunction with other vectors in a prime boost vaccination strategy.

Recent phase I clinical studies have shown that heterozygous vaccination regimens comprising of one or more DNA primes followed by boosting with either Ad5 (Jaoko *et al.*, 2010) or rMVA (Goepfert *et al.*, 2011) are well tolerated in seronegative participants. Furthermore, these studies suggest that the highest frequency and magnitude of T cell immune responses were detected in the individuals that received DNA priming compared to those without. These, and other ongoing clinical trials, including the HVTN 505 (HVTN website) have been initiated in the hope that DNA plasmids containing HIV antigens present in the boost vaccine induce a more effective and rapid cell mediated response upon infection. Several DNA plasmid based vaccines including the SAAVI DNA-C developed at UCT are currently in clinical trials.

1.3.3.5. Virus-like particles

HIV-1 virus like particles (VLPs) are a class of subunit proteins which have the same structure of the surface envelope found in HIV-1 virions but do not contain genetic material from the virus (Jennings and Bachmann, 2008). Therefore the VLPs are not infectious and non-replicating and can be used safely. Most VLPs are produced by expressing structural proteins which self assemble once inside the cell. It is possible that VLPs can be used in combination with other vaccines as part of prime boost regimens to induce both humoral and cell mediated responses against HIV-1 (Scotti and Rybicki, 2013).

Compared to vaccination with soluble Env protein, VLPs containing the Env of HIV-1 have shown to induce broader cellular and humoral immunity (McBurney and Ross, 2009). To date, there is evidence that VLPs designed to induce anti-CCR5 immune response to prevent HIV binding and entry have been able to successfully induce strong anti-CCR5 IgG and IgA responses in both serum and mucosal compartments in the rat model (Hunter *et al.*, 2009).

The advantages of using VLPs to induce HIV-1 specific immune responses are reviewed in Doan *et al.*, 2005. In summary, these particles are safe, can induce strong immune responses specific to HIV-1 epitopes and are required in smaller doses compared to other vaccines without compromising immune responses. Vaccination with p55gag VLPs in rhesus macaques induced long-lived, broad CD8+ cytotoxic responses and have thus been considered useful as part of a prime-boost regimen (Paliard *et al.*, 2000). Used in combination to boost DNA plasmid vaccination containing HIV-1 subtype C gag (pTHGag) and recombinant pantothenate auxotroph of BCG (rBCGpan-Gag), VLPs containing the matched gene (Pr55^{gag}) have been able to induce broad, polyfunctional responses in vaccinated Chacma baboons (Chege *et al.*, 2008b; Chege *et al.*, 2013).

1.4 HIV vaccine development efforts at UCT

For over a decade, the University Of Cape Town (UCT) HIV Vaccine Development Group has been engaged in the development of HIV-1 subtype C vaccine in South Africa. To date, candidate vaccines based on vectors such as BCG, poxviruses and include DNA and subunit vaccines have been developed and tested in various prime-boost combinations in mice and nonhuman primate models (Williamson *et al.*, 2012). Apart from the development and

optimisation of novel vaccines, the group has also documented the utility of the Chacma baboon as a model for HIV-1 vaccine evaluation (Chege *et al.*, 2005; Burgers *et al.*, 2009).

The group has already reported that prime-boost immunization of Chacma baboons with rBCG and Pr55Gag virus-like particle (VLP) based on HIV type 1 (HIV-1) subtype C induces Gag specific responses (Chege *et al.*, 2009). The Pr55gag virus-like particle (VLP) was previously shown to boost the immune response when mice are primed with a subtype C gag DNA vaccination (Jaffray *et al.*, 2004) and was subsequently used together with other candidate vaccines in heterozygous vaccination regimens in NHPs.

One of the promising vaccine candidates developed by the group is the SAAVI MVA-C comprised of a polyprotein including gag, reverse transcriptase, tat and nef (*grttn*) and truncated pg150 (*env*) (Burgers *et al.*, 2008) has also been used to prime Chacma baboons prior to VLP boost. In this case, Gag specific IFN- γ ELISPOT cellular immune responses and moderate humoral responses were induced in vaccinated animals (Chege *et al.*, 2008a). In another group of baboons, vaccinated with a prime-boost regimen of pTHgag DNA- Pr55GagVLP vaccine, both humoral and cellular responses were observed using ELISA and IFN- γ ELISPOT assays (Chege *et al.*, 2008b). In this case, the pTHgag DNA vaccine construct expressed the HIV-1 subtype C gag gene of Du422 (van Harmelen *et al.*, 2003; Williamson *et al.*, 2003).

To date, candidate vaccine developed by the group have been shown to induce successful cellular and moderate humoral responses in preclinical evaluation. In 2009, two HIV-1 vaccine candidates namely SAAVI DNA -C2 and SAAVI MVA-C have recently entered human clinical trials after being characterised in terms of potency and immunogenicity in both mice and NHP studies (Burgers *et al.*, 2008; Burgers *et al.*, 2009).

The ongoing trial (HVTN 073/SAAVI 102) aims to evaluate the safety and immunogenicity of SAAVI DNA-C2 vaccine boosted by SAAVI MVA-C vaccine in healthy volunteers in South Africa and USA. Immunogenicity testing revealed that the vaccines were safe and HIV-1 specific CD4+ and moderate HIV-specific CD8+ T-cell responses were detected (Williamson *et al.*, 2012). In addition, another phase 1 trial known as HVTN 086/SAAVI 103 has also been initiated to evaluate the safety of SAAVI DNA-C2, SAAVI MVA-CC and Norvartis subtype C gp140 with MF59 adjuvant (Williamson *et al.*, 2012). Due to this success, the efforts of the UCT HIV Vaccine Development Group to develop novel vaccine candidates and to improve on existing prime-boost vaccination regimens are ongoing.

2. PROBLEM IDENTIFICATION AND RATIONALE

The UCT HIV vaccine development group has already reported that immunisation with a recombinant modified vaccinia Ankara (MVA-C) prime and Pr55 Gag VLP boost based on HIV-1 subtype C has successfully induce Gag-specific responses in Chacma baboons (Chege *et al.*, 2008a) by IFN- γ ELISPOT assay measurements. In addition, both humoral and cellular responses (based on ELISA and IFN- γ ELISPOT assay respectively) have been observed in another group of baboons, primed with pTHgag DNA and boosted with Pr55 Gag VLPs (Chege *et al.*, 2008b). However, as discussed earlier, the evaluation and characterization of T cell immune responses induced by candidate vaccines cannot be limited to IFN- γ ELISPOT data. There is now a need to develop additional assays to further investigate immunological characteristics of these vaccines such as the development of durable vaccine specific memory T cells. To date, the baboon model has been used to evaluate candidate vaccines which are designed to generate neutralizing antibodies (Anderson *et al.*, 1989; Stephens *et al.*, 1992; Haigwood *et al.*, 1992), but only a limited number of studies have investigated the immunogenicity of vaccines that induce cellular immune responses (Chege *et al.*, 2008a; Chege *et al.*, 2008b; Burgers *et al.*, 2009).

Therefore, there is now a need to further characterise the quality of the vaccine induced T cell immune responses by assessing the Gag-specific cytokine production and memory recall responses generated by the SAAVI MVA-C/VLP and DNA/VLP prime-boost vaccination regimen in Chacma baboons by flow cytometry. This will involve the development of flow cytometry panels made up of T-cell memory markers and functional markers to determine the level of cytokine production and the distribution of vaccine specific memory T cell responses induced by these candidate vaccines. However, there is a need to identify cellular markers of T cell memory that have previously been used in human and rhesus macaque studies that can be used to delineate T cell memory populations in the Chacma baboon model. Furthermore, the selected markers need to be further assessed and characterised based on additional functional and phenotypic parameters to ensure that these markers accurately define memory populations in the Chacma baboon. These new multi-parameter flow cytometry panels could be used to evaluate future candidate HIV vaccines in preclinical development using the Chacma baboon as a NHP model of vaccine efficacy.

3. AIM AND OBJECTIVES

The aim of the current study was to characterise the T cell memory phenotype distribution in peripheral mononuclear cells (PBMCs) isolated from Chacma baboons vaccinated with SAAVI MVA-C/VLP and DNA/VLP prime-boost vaccination regimens by flow cytometry.

The following specific objectives were used to achieve the aim of this study:

- i. Identify and evaluate various anti-human antibodies to select a suitable combination of markers to detect T cell memory subsets in baboon PBMCs.
- ii. To determine the suitability of the selected markers by investigating the functional, activation and maturation characteristics of the baboon T cell memory phenotypes defined by these markers using a four-colour flow cytometry.
- iii. Investigate the vaccine specific T cell memory phenotype distribution in Chacma baboons vaccinated with SAAVI MVA-C/VLP and DNA/VLP prime/boost vaccine regimens, using a multicolour flow cytometry panel comprising the selected markers.

Chapter 2.

University of Cape Town

Chapter 2. Materials and Methods

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2.1. General laboratory equipment

Samples were thawed, stimulated and stained in the Primate Immunology Laboratory situated within the Division of Medical Virology, UCT. All experimental procedures and preparation of media and wash buffers were performed in a Bio-safety level II (BSL II) laminar flow. Cryopreserved material was stored in access restricted freezer rooms. Flow cytometry analysis was conducted using the FACS Calibur situated within the Division of Virology, UCT or the BD LSRII Fortessa situated within the Institute of Infectious Diseases and Molecular Medicine (IIDMM).

2.2. Thawing of cryopreserved PBMCs

Cryopreserved peripheral blood mononuclear cells (PBMCs) samples from Chacma baboons (*Papio ursinus*) were used in this study. All animals were part of previous studies aimed at investigating immune responses to candidate HIV-1 vaccines developed at the HIV vaccine discovery group, UCT. PBMCs were cryopreserved at 1×10^7 to 2×10^7 cells per ml in storage media containing foetal bovine serum (FBS, Gibco Invitrogen) containing 10% dimethyl sulfoxide (DMSO) (Sigma-Aldrich) and stored in a vapour phase liquid nitrogen cryotank.

During the thawing procedure, cryopreserved PBMCs were removed from liquid nitrogen, and thawed in a water bath set at 37°C for approximately 3 to 5 minutes. Care was taken not to disrupt the tubes during the thawing process so as to prevent cell damage by ice micro-crystals formed between cells and to maintain maximum cell viability. Thawed PBMCs were then quickly transferred into 50ml centrifuge tubes. To remove the freezing media which contained DMSO, 25ml of freshly prepared Roswell Park Memorial Institute (RPMI) 1640 media (Gibco, Invitrogen) containing 0.01% *Penicillin* and *Streptomycin* (PenStrep) (Invitrogen) and further supplemented with 1% FBS (R1) was warmed to room temperature and then added drop wise to protect the cells from osmotic and heat shock. The cells were centrifuged at room temperature at relative centrifugal force (RCF) of 230xg (1200rpm) for 10 minutes. The supernatant was discarded and the remaining cell pellet was re-suspended in 5ml of R1 containing DNase (Roche) at a final concentration of 0.02mg/ml and incubated at room temperature for 2 to 5 minutes. The cells were treated with DNase to hydrolyse the DNA extruded from lysed cells in order to prevent cell clumping. The DNase was then removed from the cell suspension by washing the cells with 25ml of R1 as above, the cell pellet was re-suspended in pre-warmed RPMI 1640 media supplemented with 20% FBS (R20) and incubated at 37°C to rest in a humidified atmosphere of 5% CO₂ for 4 to 6 hours.

The cells were rested to reduce the detection of non-specific responses or background responses during flow cytometry analysis (Horton *et al.*, 2007). The common reagents used during the thawing process are summarised in Table 2.1.

Table 2.1. Description of reagents used for thawing and culture of baboon PBMCs

| Reagent | Description | Source |
|--------------------------------|---|------------|
| RPMI 1640 | Media for cell culture containing L-glutamine and 25mM HEPES | Invitrogen |
| Penicillin Streptomycin | Antibiotic containing 5000 units/ml Penicillin and 5000 µg/ml Streptomycin to prevent contamination in cell culture | Invitrogen |
| FBS | Heat-Inactivated foetal bovine serum to supplement cell culture media | Invitrogen |
| R1 | RPMI supplemented with 1% FBS | * |
| R10 | RPMI supplemented with 10% FBS | * |
| R20 | RPMI supplemented with 20% FBS | * |
| DNase | Enzyme to digest DNA from dead cells to prevent cell clumping | Roche |

* Prepared in Primate Immunology Laboratory (UCT)

2.3. Viable cell counting

The number of viable cells recovered and the viability (calculated as a percentage) of each sample was determined after the cells were rested for 4-6 hours. The Trypan blue exclusion test was used. This test is based on the principle that viable cells with intact cell membranes will not allow Trypan blue dye to enter the cytoplasm and therefore live cells will not stain blue. In contrast, dead cells that do not have intact cell membranes will take up the dye and stain blue (Strober, 2001). Since red blood cells, like viable cells, will not take up the Trypan blue dye, contaminating red blood cells in the sample could be mistaken for live lymphocytes during counting. To minimise this error, Turk's solution was used in parallel with the Trypan Blue. Turk's solution contains acetic acid to lyse red blood cells and stains the nuclei which is absent in mature red blood cells. However, Turk's solution does not differentiate between live and dead cells and thus indicates the total number of cells. In summary, the cells in suspension were stained in a 1:1 ratio with either Turk's solution or Trypan Blue. The number of dead cells (determined by Trypan Blue exclusion test) were excluded from the total number of cells (determined by Turk's solution staining), to calculate the number of viable cells per sample. All cell counts were performed manually using the Improved

Neubauer haemocytometer cell counting chamber (Hausser Scientific) and light microscopy. The number of viable cells was then adjusted to $10\text{-}20 \times 10^6$ per ml by adding the appropriate volume of media.

2.4 Stimulation of PBMCs for the detection of functional and activation markers

For assays intended to detect the production of cytokines by intracellular staining (ICS), the cells were stimulated with Gag peptide pools or a polyclonal T cell stimulant such as the Staphylococcal enterotoxin B (SEB) (Sigma-Aldrich) as a positive control. In addition, cells to which no stimulant was added were used as negative (unstimulated) controls and were used to determine the level of background or non-specific responses for each experiment. The reagents used for stimulation are described in Table 2.2.

In general, the cells in suspension were transferred into V-bottomed 96 well plates and stimulated with SEB at a final concentration of $2\mu\text{g/ml}$ or Gag subtype C peptide pools (prepared in-house) at a final concentration of $2\mu\text{g/ml}$ at 37°C for a total of 16 hours in a humidified atmosphere of 5% CO_2 . For the detection of vaccine specific cytokine production by T cells, co-stimulants anti-CD28 and $-\text{CD}49\text{d}$ antibodies were also added at the final concentrations of $1\mu\text{g/ml}$ each to maximise antigen presentation to T cells via T cell receptors (TCRs). This induces T cell activation required for the *in vitro* production of cytokines (Waldrop *et al.*, 1998). The CD28 molecule, as reviewed in Lenschow *et al.*, (1996), binds to the CD80 and CD86 receptor expressed on antigen presenting cells and provides a second cross-linked co-stimulatory signal required for T cell activation. CD49d, an $\alpha 4\beta 1$ integrin, which is involved in the CD3-dependent $\text{CD}4^+$ T lymphocyte activation, provides further co-stimulatory signals to induce the production of IL-2, TNF- α and IFN- γ (Udagawa *et al.*, 1996).

For the detection of cytokine production by ICS, Brefeldin A (BFA) (Sigma-Aldrich), an antibiotic produced by *Penicillium brefeldianum* fungus, was added to the cells at a final concentration of $10\mu\text{g/ml}$ after the first 2 hours of stimulation. BFA causes the accumulation of cytokines produced within the cell by blocking the transport of cytokines from the endoplasmic reticulum (ER) to the Golgi complex (Klausner *et al.*, 1992; Nylander and Kalies, 1999). In this way, the cytokines remain within the cell and can be detected by intracellular staining (ICS). For the detection of the activation marker CD69, BD GolgiStop (BD Biosciences), a protein transport inhibitor containing monensin was used instead of BFA. The use of BFA has been reported to block CD69 expression in a large proportion of $\text{CD}3^+$ cells (O'Neil-Andersen and Lawrence 2002). Monensin is an antibiotic isolated from

Streptomyces cinnamomensis, used as a substitute for BFA and prevents the transport of proteins and cytokines out of the Golgi complex (Jung *et al.*, 1993). The cytokines or protein markers produced then accumulate within the cell and can be detected by ICS. After the addition of either BFA or monensin, the cells were incubated for the remainder of the 16 hours after which, they were removed from the incubator and placed at 4°C to stop the stimulation and stored for no longer than 6 hours or until staining.

Table 2.2. Description of reagents used to stimulate baboon PBMCs.

| Reagent | Description | Source |
|-----------------------------|--|----------------------------|
| SEB | Staphylococcal enterotoxin B from <i>Staphylococcus aureus</i> | Sigma-Aldrich |
| Gag-C peptide pools | Peptide pools comprising of 66 synthetic peptides which span the HIV-1 subtype C Gag protein sequence. These peptides were 15–18 amino acids in length and overlapped by 10 amino acids. The peptides were dissolved in 100% DMSO (Sigma) and have been previously described in Masemola <i>et al.</i> , 2004 and Chege <i>et al.</i> , 2008b. | NIH/AIDS Reagent Programme |
| anti-CD28 | Co-stimulatory molecule used to augment T cell receptor (TCR) signalling | Merck |
| anti-CD49d | Co-stimulatory molecule used to augment TCR signalling | BD Bioscience |
| BFA | Brefeldin A from <i>Penicillium brefeldianum</i> to inhibit protein transport between ER and the Golgi complex and allows for the accumulation of newly synthesised proteins within the cell | Sigma-Aldrich |
| GolgiStop (Monensin) | Isolated from <i>Streptomyces cinnamomensis</i> . Prevents transport of newly synthesised proteins in the Golgi complex and allows for the accumulation of newly synthesised proteins within the cell | BD Bioscience |

2.5. Antibodies and fluorochrome conjugates

Fluorochromes typically used for flow cytometry are fluorescent dyes that absorb light energy at low wavelengths (referred to as excitation) and re-emit this light energy at longer wavelengths within the visible spectrum (referred to as emission). Monoclonal antibodies are directly conjugated to fluorochromes that have distinct excitation and emission ranges. It is possible to detect these antibodies bound to a cellular marker of interest from cells by exciting the conjugated fluorochrome. In flow cytometry, the light emitted from the

fluorochrome then is channelled through a series of filters and mirrors to detectors assigned to specific wavelengths. The light signals are then converted to electronic data which can be depicted graphically. In this study, antibodies were conjugated to fluorochromes classified either as classic dyes, tandem dyes or inorganic Quantum Dot (Qdots) nanocrystals.

Classic dyes are stable, naturally occurring fluorescent molecules such as fluorescein isothiocyanate (FITC); phycoerythrin (PE); peridinin chlorophyll protein (PerCP) allophycocyanin (APC); Alexa Fluor (from Molecular Probes) and Pacific blue (based on the 6,8-difluoro-7-hydroxycoumarin fluorochrome). On the other hand, tandem dyes are less stable compared to the classic dyes and are light and temperature sensitive. However, tandem dyes offer a wider range of emission which increases the number of possible parameters (markers) that can be detected by a single laser (adapted from AbD Serotec website). Tandem dyes are usually made up of two covalently linked fluorescent dyes and emits fluorescence by the process of fluorescence resonance energy transfer (FRET). One of the dyes, usually a classic dye, is excited by the laser and the energy it emits is transferred to the second dye such as cyanine dye (Cy) 5, Cy5.5 or Cy7, which acts as an acceptor molecule and then produces a combined fluorescence emission. The Qdot semiconductor nanoparticles however, are made up of a cadmium selenide core coated with zinc sulfide and the wavelength of emitted light can be varied according to the size of the core (Chattopadhyay *et al.*, 2006). In addition, these nanocrystals are photostable and have narrow emission spectrum which reduces the spectral overlap between dyes.

The antibodies used in this study were directly conjugated to fluorochromes FITC, PE, PerCP, phycoerythrin-cyanine 5 (PECy5), phycoerythrin-cyanine 5.5 (PECy5.5), APC, allophycocyanin-cyanine 7 (APC.Cy7) and Pacific Blue fluorochromes. Some antibodies were conjugated to Quantum Dot Nanocrystals (Invitrogen) namely Qdot605. Table 2.3 highlights the properties of the fluorochromes used in this study.

Table 2.3 Properties of fluorochemicals used in this study for flow cytometry analysis by the FACS Calibur and/or LSR II Fortessa.

| Fluorochrome | Type of dye | Excitation _{max} (nm) | Emmision _{max} (nm) |
|--------------|------------------|--------------------------------|------------------------------|
| APC* | Classic | 650 | 660 |
| FITC* | Classic | 494 | 520 |
| PE* | Classic | 496 | 578 |
| PerCP* | Classic | 482 | 678 |
| PECy5* | Tandem | 496 | 667 |
| PECy5.5. | Tandem | 488 | 690 |
| APCCy7 | Tandem | 650 | 785 |
| Pacific Blue | Classic | 401 | 452 |
| Qdot 605 | Qdot nanocrystal | 350 | 605 |

*Used for flow cytometry analysis by FACS Calibur and LSR II Fortessa. Adapted from AbD Serotec website, <http://static.abdserotec.com>.

Several commercially available clones of anti-human monoclonal antibodies are known to detect CD3, CD4, CD8, CD28, CD95, CD27, CD45RA, CD69, IL-2, IFN- γ , TNF- α , CCR7 and CCR5, in PBMCs isolated from humans and/or rhesus macaques. Certain clones of anti-human CD3, CD4, CD8, IFN- γ and IL-2 antibodies have also previously been used to stain baboon PBMCs (Casimiro *et al.*, 2003; Chege *et al.*, 2005; Chege *et al.*, 2008b; Burgers *et al.*, 2009). In the current study, several clones of homing and maturation markers such as CD45RA, CD27, CD28, CD95, CCR5 and CCR7 were tested for cross-reactivity using baboon PBMCs. Table 2.4 provides a comprehensive list of antibodies and their conjugates investigated in the current study.

Table 2.4. Properties of antibody-fluorochrome conjugates used in this study.

| Antibody | Clone | Fluorochrome | Manufacturer | Function/role of marker * |
|--------------------------------|-----------|--------------|--------------|---|
| CD3 | SP34-2 | PerCP | BD | Phenotypic surface marker of T cells and can be expressed on NK-like T cells |
| | FN-18 | FITC | Invitrogen | |
| | SP34-2 | APC.Cy7 | BD | |
| CD4 | L200 | PerCP | BD | Phenotypic surface of helper T cells and can be expressed on monocytes, macrophages and dendritic cells. Acts as a co-receptor for MHC Class II peptides. |
| | L200 | APC | BD | |
| | S5 | PE.Cy5.5 | Invitrogen | |
| CD8 | SK1 | PerCP | BD | Phenotypic surface marker of cytotoxic T cells |
| | SK1 | APC | BD | |
| | 3B5 | QDot605 | Invitrogen | |
| CD28 | CD28.2 | PE | BD | Co-stimulatory molecule involved in TCR induced t cell activation. Memory marker for T cells capable of secreting IL-2 (eg. naive T cells and Tcm) |
| | CD28.2 | APC | BD | |
| | CD28.2 | FITC | BD | |
| CD95 | DX2 | PECy5 | BD | Memory marker to detect Fas expression on T cells (eg. Tem) |
| | DX2 | FITC | BD | |
| | DX2 | APC | BD | |
| CD45Ra | 5H9 | PE | BD | Isoform of CD 4 which is a marker of naive T cells |
| | 5H9 | PECy5 | BD | |
| CD27 | M-T271 | PE | BD | Co-stimulatory molecule involved in TCR induced T cell activation. Memory marker for T cells capable of secreting IL-2 (naive T cells and Tcm) |
| | M-T271 | APC | BD | |
| | M-T271 | FITC | BD | |
| CD69 | FN50 | PE | BD | Early marker of T cell activation. |
| TNF-α | mAB11 | PE | BD | Th1 cytokine |
| IL-2 | MQ1-17H12 | PE | BD | Th1 cytokine |
| IFN-γ | 4SB3 | PE | BD | Th1 cytokine |
| CCR5 | 3A9 | PE | BD | Regulates lymphocyte chemotaxis and activation. Co-receptor for HIV-1 infection. |
| CCR7 | 150503 | PerCP | R&D systems | Involved in the recruitment and retention of cells to lymphoid tissues |

* Adapted from eBioscience website (<http://www.ebioscience.com/resources/human-cd-chart.htm>)

2.6. Principles of flow cytometry

Flow cytometry allows for the measurement and analysis of the characteristics of a single particle (0.2 to 150 μm in size) as it passes through a beam of light from a laser source. Usually, flow cytometry is used to characterise cells by assessing the relative size, granularity and fluorescence of cells that have been stained with appropriate fluorescent molecules (fluorochromes) conjugated to antibody markers of specific surface or intracellular epitopes. The components of a flow cytometer include (i) a fluidics system which conveys the particles (cells) in a single stream to be interrogated by a laser beam; (ii) an optics and detection system comprising a number of laser at different wavelengths that can illuminate the particle, as well as optic filters and mirrors to direct the light emitted from the particle to the appropriate detectors and (iii) a signal processing component that converts light signals generated into electronic data (Figure 2.1).

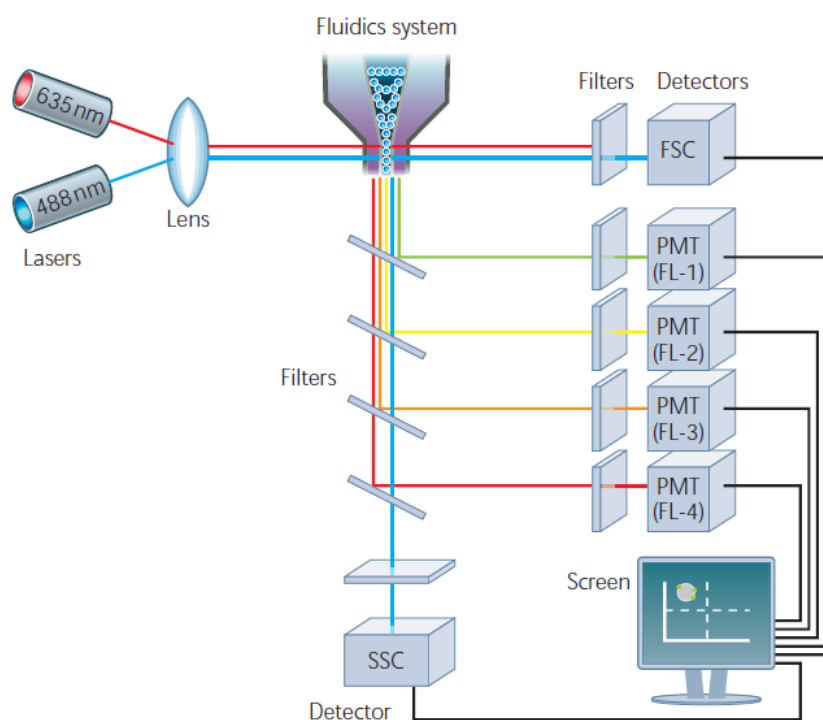


Figure 2.1. Schematic overview of a typical two laser flow cytometer (eg. FACS Calibur). The fluidics system draws sample into a stream of single cells/particles by hydrodynamic focusing. The single particles can then be interrogated by the red laser (635nm) and blue laser (488nm) in this case. Light in the form of side scatter (SSC) or fluorescence is emitted to photomultiplier tubes (PMTs) and forward scatter (FSC) is emitted to a photodiode detector. A series of filters and mirrors direct the light to specific PMTs. The light signal is then amplified and converted into digital signals to be processed by analysis software. (Taken from AbD Serotec website)

The light signals are channelled to a detector designated to a specific fluorescent dye by placing a filter and/or mirror in front of the photomultiplier tube (PMT), which allows only a narrow range of wavelengths to reach the detector. The filters used (Figure 2.2.) are either bandpass (BP) filters which allow the entry of light emission between a narrow range of wavelengths (eg. 500/50 BP will allow light in the range of 460 to 540nm to pass through to the PMT). Long pass (LP) filters allow light of longer or equal wavelength to pass through or short pass (SP) filters which allow light of shorter or equal wavelength to pass thorough to the PMT. Other components of this system include beam splitters such as dichroic mirrors that direct light of different wavelengths in different directions. For example, a 560 SP dichroic mirror transmits wavelengths of light 560 nm or shorter and reflects light at longer wavelengths at 45°.

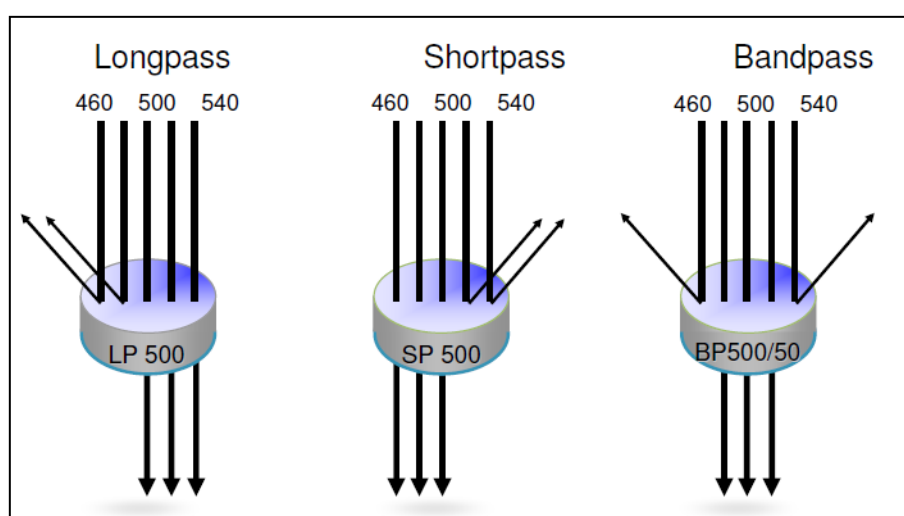


Figure 2.2. Light passage through typical longpass, shortpass and bandpass filters. These filters are placed in front of PMT detectors in a flow cytometer (Taken from BD Bioscience website)

iii. Signal processing

On receiving a light emission signal from the cell (or particle), the detector allocates each signal into a voltage pulse. The voltage pulse signal is amplified by a voltage that is assigned to each PMT by increasing the current or by increasing the amplification gain settings. The amplifier settings can be set at logarithmic or linear amplification which are used to separate negative from dim positive signals and to amplify fluorescent parameters respectively. The voltage pulse is then converted into a digital signal by an Analogue-to-Digital Converter (ADC). The digital signal is assigned a specific number on a channel and data can be stored in this format. During data analysis, software programs graphically represent these data in a variety of plots such as histograms, dot pots and saturation plots.

2.7. BD FACS Calibur and LSR II Fortessa

In this study, the BD FACS Calibur (Figure 2.1) was used to screen for cross-reactive antibody clones, determine optimal antibody staining conditions and optimal staining concentrations. In addition, the FACS Calibur, containing two lasers (488nm and 635nm), was also used to select and validate cellular markers to be included in memory, and function flow cytometry panels to analyse baboon PBMCs. The configuration of the optical and collection system of the FACS Calibur used in this study is detailed in Table 2.5. This study also made use of the BD LSR Fortessa which was used to investigate the memory and function phenotypes of vaccinated baboons. The machine used for this study had four lasers namely the blue (488nm), green (532nm), red (640nm) and the violet (407 nm) lasers, allowing for the detection of up to eighteen parameters simultaneously. The configuration and optical setup of the Fortessa used in this study is detailed in Table 2.6.

Table 2.5. Configuration of four colour BD FACS Calibur

| Laser | Detector | Fluorochromes | Filter |
|--------------|--------------|---------------|----------|
| Blue (488nm) | FL-1 (525nm) | FITC | 530/30nm |
| | FL-2 (580nm) | PE | 585/42nm |
| | FL-3 (675nm) | PECy5/ PerCP | 670nm LP |
| Red (635nm) | FL-4 (650nm) | APC | 661/16nm |

One of the most significant differences between these two machines is that the Fortessa could accommodate a larger number of markers and therefore more parameters of a cell could be investigated simultaneously. Conversely, the FACS Calibur was limited to the detection of four colours but was readily available for use. In addition, the limited parameters available in the FACS Calibur meant there were fewer problems caused by spectral overlap and thus limited the error introduced by compensation. However, it became necessary to evaluate more than four fluorochrome bound markers at a time in order to achieve the objectives of this study. The Fortessa allows for the simultaneous detection of markers of T cell lineage, viability, memory and cytokine production in one panel thus preserving reagents and irreplaceable samples from preclinical vaccine studies. The issue of spectral overlap and compensation was dealt with during the panel design by purposely assigning markers to fluorochromes which have previously been found to exhibit minimum to zero spectral overlap with each other. The use of compensation beads (described later in this chapter) was also necessary to facilitate compensation.

Both these flow cytometers share a common fluidics system and function to hydrodynamically focus the sample into a stream of single cells. The optics of the BD FACS Calibur include a SSC detectors with a 488nm band pass filter for clear signal detection and red-diode laser (635nm) signal rejection. A maximum of four fluorescence detectors and filters with band pass filters of 530 nm (FITC), 585 nm (PE), and >670 nm (PerCP or PECy5) and 661 nm (APC) can be used.

In contrast to the FACS Calibur, the BD Fortessa collection system is composed of a series of PMTs arranged in octagon (green laser and violet laser detectors) or trigon (red laser and blue laser detectors). The octagonal arrangement of PMTs (Figure 2.3) ensures that light emission which enters the system are methodological reflected between detectors by a dichroic mirror or transmitted through the band pass filters to be detected by a specific PMT (Perfetto *et al.*, 2004).

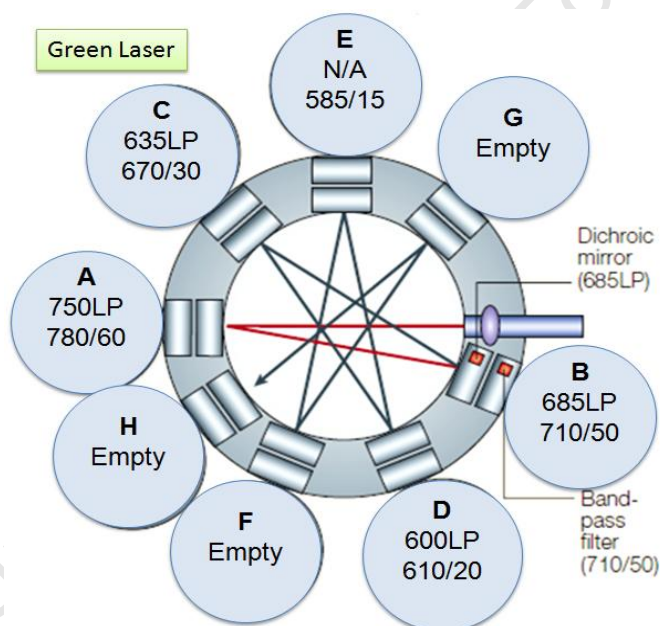


Figure 2.3. Graphical representation of the octagonal arrangement collection system (specific to the green laser in the BD Fortessa). Each octagon is composed of a series of photomultiplier tubes (PMT) (A-H) and optics. Dichroic long pass (LP) mirrors and band pass (BP) filters are placed in front of the PMTs. Light emitted from cell/particle enters the detector and engages with the LP dichroic mirror in front of the PMT labelled A first. If the wavelength of the light is in the range of 710 to 840nm, it will pass through the LP filter and the BP filters (780/60) and be detected at the PMT labelled A. If the wavelength of the light is shorter than 750nm, it is reflected by the dichroic mirror to the next set of mirrors and filters in front of the PMT labelled B and so on (adapted from Perfetto *et al.*, 2004).

Table 2.6. Configuration of BD LSR II Fortessa

| Laser | Detector (nm) | Fluorochrome/Parameter | Dichroic LP Filter | BP Filter |
|----------------------|---------------|------------------------|--------------------|-----------|
| Blue 488nm | SSC | Side scatter (SSC) | | 488/10 |
| Blue 488nm (50mW) | B710 | PerCP Cy5.5 | 670LP | 685/35 |
| Blue 488nm | B515 | FITC | 505LP | 515/20 |
| Green 532nm (150mW) | G780 | PECy7 | 750LP | 780/60 |
| Green 532nm | G710 | PECy5.5 | 685LP | 710/50 |
| Green 532nm | G660 | PECy5 | 635LP | 670/30 |
| Green 532nm | G610 | Texas Red PE | 600LP | 610/20 |
| Green 532nm | G560 | PE | EMPTY | 585/15 |
| Green 532nm | Empty | N/A | | |
| Green 532nm | Empty | N/A | | |
| Green 532nm | Empty | N/A | | |
| Red 640nm (40mW) | R 780 | APCCy7 | 740LP | 780/60 |
| Red 640nm | R 710 | Alexa680, Alexa700 | 685LP | 730/45 |
| Red 640nm | R 660 | APC, Alexa 647 | EMPTY | 670/30 |
| Violet 407nm (100mW) | V800 | QDot800 | 750LP | 780/60 |
| Violet 407nm | V705 | QDot705 | 685LP | 705/70 |
| Violet 407nm | V655 | QDot655 | 630LP | 670/30 |
| Violet 407nm | V605 | QDot605, 585 | 595LP | 605/40 |
| Violet 407nm | V585 | QDot565 | 556LP | 585/42 |
| Violet 407nm | V545 | QDot545 | 535LP | 560/40 |
| Violet 407nm | V525 | QDot525, Horizon V500 | 505LP | 525/50 |
| Violet 407nm | V450 | Pacific Blue, ViViD | EMPTY | 450/50 |

2.8. Selection of fluorochromes for multi-parameter flow cytometry panels

The allocation of antibody markers of memory and function (cytokine production) to conjugates for four-colour analysis was determined by considering the following aspects of flow cytometry:

i. Instrument configuration

The ability of the cytometer to excite and detect a specific fluorochrome influences the antigen expression levels (brightness) and spectral overlap of the fluorochrome (Maecker *et al.*, 2004). Therefore, in order to determine which fluorochrome conjugates to use, the number of lasers and detectors in the available BD FACS Calibur and LSR II Fortessa were first established.

ii. Antigen expression levels (Brightness)

The brightness of a fluorochrome conjugate is usually a measure of the cell-associated fluorescence intensity when it binds to its cellular antigen (Baumgarth and Roederer, 2000). Commercially available conjugate dyes were ranked in order of their relative brightness to each other. The brightest dyes such as PE, APC, PECy5.5 were reserved for antibodies to antigens that were known to be expressed at low levels and therefore stained dim, called tertiary antigens according to Mahnke and Roederer (2007) (eg. CCR5, CCR7). Primary antigens identify lineage markers of cells that have been well characterised and have distinct positive and negative populations (eg. CD3, CD4, CD8). Therefore dyes that are least bright such as FITC were used for these antibodies. The remaining dyes were selected for antibodies to CD28, CD95, CD27 and CD45RA for example, which bind to secondary antigens that are expressed by distinct subsets of cells. However, the separation between positive, negative and intermediate stained populations was less defined in this case and fluorescence-minus-one (FMO) controls were used to define gating amongst these populations.

iii. Amount of fluorochrome spectral overlap

One of the greatest challenges in flow cytometry is the overlap of emission spectra of individual conjugate or dyes (Baumgarth and Roederer, 2000). It is unlikely that emission filters measure signals from only one dye at a time. Instead, light from conjugates spill over into each other depending on the choice of fluorochrome and filters used. In the case of four colour flow cytometry, there are two lasers which reduces the amount of spectral overlap amongst most fluorochrome conjugates.

iv. Commercial availability of the antibody-conjugate pair

There are a limited amount of antibody-conjugate pairs that are cross-reactive to baboon and other nonhuman primates. As such, the choice of fluorochrome used was dependant on the availability the antibody of interest and whether or not it was manufactured from a cross-reactive clone. Financial resource restrictions were also taken to account with the least expensive conjugate pair taking preference.

2.9. Titration of antibody-fluorochrome conjugates

The optimal staining concentration is the minimum amount of antibody required to stain cells at the saturation point. Most manufacturers recommend that antibody fluorochrome conjugates should first be titrated to determine the optimal concentration needed to stain specific cell types. In addition, antibody-fluorochrome conjugates need to be titrated in order to optimise the separation of positive and negative populations.

To determine the optimal concentration of antibody required to stain cells, all antibody conjugates were individually titrated by two-fold serial dilution. The antibody conjugates were titrated starting from the initial volume of twice that recommended by the manufacturers and up to eight two-fold serial dilutions were setup for each antibody conjugate. The median fluorescence intensity (MFI) of the antibody-fluorochrome conjugate at each concentration was used to generate a saturation curve. From these data, the concentration of antibody that results in the highest positive population, the lowest background caused by non-specific binding and at saturation point was selected as the optimal staining concentration (Perfetto *et al.*, 2004).

2.10. Staining protocol to exclude dead cells

A marker of T cell viability was added to all panels involving multi-parameter (polychromatic) flow analysis using the BD LSR II Fortessa. The addition of a viability marker reduces false-positive events and increases sensitivity for cytokine detection (Mahnke and Roederer, 2007) since fluorochromes bound to markers may bind non-specifically to dead cells or cells with a damaged membrane. For this study, a commercially available amine-reactive dye known as violet viability dye (ViViD) (Invitrogen) was used to stain dead cells. This dye binds to free amines in the cytoplasm of dead cells which do not have intact cell membranes (Perfetto *et al.*, 2006; Perfetto *et al.*, 2010). Live cells however, have intact cell membranes and ViViD will not stain these cells (hence the viable cells will be ViViD negative). ViViD is excited by the violet laser (407nm) and has the same emission wavelength as Pacific Blue (425-475nm), making it possible to use Pacific Blue- conjugated antibodies as a compensation control for ViViD.

In brief, PBMC were dispensed into a sterile V-bottomed 96-well plate and washed twice with sterile 1x PBS (Gibco, Invitrogen) to remove any debris and proteins from the culture medium. This was achieved by spinning the plate in a centrifuge at RCF of 1000 xg (2100 rpm) for 3 minutes and discarding the supernatant from the wells. Cell pellets were then re-

suspended in 1x PBS (200 µl/well) and the plate was centrifuged as before. To stain, the cells were re-suspended in ViViD (50 µl/well) at a pre-determined optimal concentration and the plate was incubated for 20 minutes at room temperature in the dark. Next, the cells were washed twice in FACS wash and pelleted by centrifugation (2100 rpm for 3 minutes), to remove unbound ViViD before surface staining.

2.11. Surface marker staining protocol

To identify the markers expressed on the cell surface (using the BD FACS Calibur or BD LSR II Fortessa), cells were re-suspended in pre-titred surface marker antibodies (to a final volume of 50 µl per reaction and were incubated for 25-30 minutes at room temperature in the dark. Excess antibody was removed by washing twice with FACS wash as described before (2.10 above). For the assays not proceeding to ICS, the cell pellets were re-suspended in 1x CellFIX (BD Bioscience; 100 µl/well). CellFIX contains PBS with 4% paraformaldehyde which is converted to formaldehyde in this solution which preserves the molecular structure of the molecules in fixed positions within the cell matrix. The content of each well was then transferred into correspondingly labelled FACS tubes (BD BioScience). The FACS tubes were covered with aluminium foil to prevent light interference and kept in a refrigerator at 4°C for no longer than 48 hours or until acquisition.

2.12. Intracellular marker staining protocol

In order to stain for intracellular markers (e.g. IL-2, IFN- γ , TNF- α), cells were re-suspended with 100µl BD CytoPERM/CytoFIX (BD BioScience) per well for 20 minutes at room temperature. The cells were permeabilised to allow the antibody-fluorochrome conjugate markers to enter the cell and bind to intracellular markers within the cell. Since saponin-mediated cell permeabilisation by CytoPERM/CytoFIX is a reversible process, the cells were stained and washed in PBS buffer containing saponin (BD Perm wash, BD Bioscience) The cells were washed twice and pelleted, as before, by centrifugation using 1x BD Perm wash. Thereafter, intracellular antibody mix comprising either a cocktail of antibody-conjugate markers of interest or individual antibody-conjugate markers at optimal staining concentrations, made up to a final volume of 50µl per reaction with 1x BD Perm/Wash, was added to each well. A well containing the same number of cells was also included as an unstained control. To this well, 50µl of BD Perm wash was added.

Thereafter, the plate was placed away from light and the cells were stained for 25 to 30 minutes at the optimal staining temperature conditions, usually at room temperature. Excess antibody and debris was then removed by adding 100µl of BD Perm wash to each well prior

to centrifugation of the plate at 2100rpm for 3 minutes and this process was repeated. The supernatant was discarded before the addition of 100µl of 1x BD CellFIX (BD BioScience) per well. The contents of each well was mixed well by pipette and transferred into correspondingly labelled FACS tubes (BD BioScience). The FACS tubes were covered with aluminium foil to prevent light interference and kept in a refrigerator at 4°C for no longer than 48 hours or until acquisition. The common reagents used to stain cells for flow cytometry analysis are described in Table 2.7.

Table 2.7. Description of reagents used for staining of cells for flow cytometry

| Reagent | Description | Source |
|-------------------------|--|------------------|
| PBS | Phosphate buffered saline pH 7.2 without CaCl ₂ or MgCl ₂ | Invitrogen |
| FACS Wash buffer | Buffer comprised of PBS with 1% FBS and 0.01% sodium azide as a preservative to prevent bacterial growth in buffer | * |
| Perm wash buffer | Buffer comprised of PBS with FBS and saponin to maintain saponin-mediated cell permeabilisation of cells. Diluted to 1x solution with distilled water and used to wash cells during ICS. | BD Bioscience |
| CytoPERM/CytoFIX | Solution containing formaldehyde and saponin which allows for the simultaneous fixation and permeabilisation of cells prior to intracellular cytokine staining. | BD Bioscience |
| CellFIX | Fixation buffer containing PBS with 4% w/V paraformaldehyde | BD Bioscience |

* Prepared in the Primate Immunology Laboratory

2.13. FMO Controls

In order to gauge the sensitivity of the antibody conjugate in relation to each other and to distinguish amongst positive, intermediate and negative populations of certain antibodies, fluorescence-minus-one (FMO) controls were setup for each panel. An FMO control was setup such that PBMCs are stained with all but one antibody conjugates in the panel. A separate FMO that excluded each antibody conjugate present in the full panel was setup using baboon PBMCs which stained as per surface and intracellular staining protocols above.

2.14. Compensation controls

The process of compensation is a fundamental aspect of flow cytometry analysis involving more than two colours or parameters with different fluorochromes. The aim of compensation is to minimize overlap between spectral adjacent detectors such as that between FITC and PE or PE and PECy5 (Baumgarth and Roederer, 2000). This analysis requires the addition of compensation controls which are stained with individual antibody fluorochromes and should be included for every experiment conducted regardless of the panel used. For four-color flow cytometry analysis, PBMCs from the samples of interest were stained with each antibody according to either surface staining or intracellular staining protocols above. A compensation control was setup for each antibody used in a particular experiment and acquired separately. This data was then used to generate a compensation matrix using FlowJo (Tree Star Inc, Ashland, OR). The compensation matrix was used to compensate the spectral overlap of all samples stained with multiple antibody-fluorochrome conjugates.

2.15. The use of compensation beads as compensation controls

Compensation controls were prepared by labelling anti-mouse or, in the case of IL-2, anti-rat antibody capture beads (Compensation beads) with individual antibody conjugates used in the panel. Compensation beads were setup for every experiment by staining two drops of beads with the optimal volume of antibody conjugate. Beads were stained for 5 to 10 minutes at room temperature and 100µl of FACS wash was added to beads prior to fixation with 100µl 1x BD CellFIX (BD BioScience). The stained beads were analysed on the BD Fortessa and the data obtained was used to calculate a compensation matrix using FlowJo analysis. An unstained control which comprised of beads only was included and used to gate on negative populations during the setup of the compensation matrix.

2. 16. Data acquisition and analysis

During flow cytometry analysis using the four colour BD FACS Calibur, an average of 50 000 events based in the lymphocyte gate were analysed per sample using BD Cell Quest, version 5.1 (BD BioScience) and data analysis was performed using FlowJo (Tree Star Inc, Ashland, OR) analysis software. For four colour flow cytometry analysis, samples were analysed and compensated using FlowJo compensation matrices. For polychromatic flow cytometry analysis using the BD Fortessa an average of 250 000 events were acquired per sample using BD Diva (BD BioScience) and were analysed using FlowJo analysis software. For multi-colour flow cytometry analysis, compensation beads were also analysed (5000

events per compensation control) and were used to generate FlowJo compensation matrices.

2.17. Statistical analysis

Statistical analyses for non-parametric one way ANOVA (Kruskal-Wallis), mean and standard deviation calculations were performed using Graph Pad Prism version 6.0 for Windows (GraphPad Software).

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Chapter 3

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Chapter 3. Selection of markers to analyse T cell memory phenotypes in baboon PBMCs by flow cytometry.

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3.1 Introduction and background

Memory T cells differ from naïve T cells in their specific expression of surface markers, homing markers and functional capacity. As reviewed Sallusto *et al.*, 2004, naïve T cells express homing markers such as CD62L and CCR7 that limit them to lymphoid tissues, high levels of co-stimulatory molecules such as CD27 and CD28 and have limited (almost no) ability to produce cytokines. These cells are not antigen experienced and therefore do not express markers of T cell activation such as CD25, CD44, HLA-DR or CD69 (Akbar *et al.*, 1991, De Rosa *et al.*, 2001). Like naïve T cells, the central memory population has the ability to occupy lymphoid tissues and expresses co-stimulatory molecules. Unlike naïve T cells however, the central memory T cells secrete proliferation inducing cytokines such as IL-2. Effector memory T cells (Tem) constitute the first line of defence at the portals of pathogen entry and function to rapidly secrete significant amounts of anti-viral and anti-bacterial cytokines such as IFN- γ and TNF- α . These cells which have now differentiated to persist in the periphery and mucosal sites express adhesion molecules such as CD11a instead of lymphoid homing molecules (Dutton *et al.*, 1998). Effector memory T cells are usually considered as mature T cells which have the ability to undergo apoptosis and express CD95, but do not express co-stimulatory molecules.

3.1.1. Markers of memory T cell populations

The identification of naive and memory T cell populations are based on the relative expression of several surface and functional markers which convey the tissue homing status, level of differentiation, antigen experience and functional capacity of the cell. The characterisation of T cell memory subsets using a number of commercially available markers have been extensively studied in humans, rhesus macaques and mice.

Tissue homing markers such as CCR7 together with adhesion markers CD62L (L-selectin), CD44 (H-CAM) and lymphocyte fusion antigen 1 (LFA-1) mediates the entry of T cells via high endothelial venules (HEVs) into secondary lymphoid tissues (Campbell *et al.*, 2001). These markers have been used to differentiate between naive and memory T cells (Sallusto *et al.*, 1999). It has been previously demonstrated in mice that naive T cells have a CD62L^{hi}CD44^{lo} phenotype, whereas memory T cells exhibit a CD62L^{lo}CD44^{hi} phenotype (Wherry *et al.*, 2003). Other marker combinations previously used to define T cell phenotypes such as CD45RA and/or CD45RO and the lymphoid homing marker, CCR7, which together define naive T cells (CCR7⁺ CD45RA⁺CD45RO⁻), Tcm (CCR7⁺CD45RA-

CD45RO+) and Tem (CCR7-CD45RA-CD45RO+) (McLaughlin *et al.*, 2008; Sallusto *et al.*, 1999). The type of cytokines produced and the frequency of cytokine producing cells have also been used to characterise memory T cell phenotypes. It is accepted that naive T cells do not produce cytokines IL-2 and IFN- γ , whilst Tcm produce high levels of IL-2 and Tem produce high levels of IFN- γ and TNF- α (Sallusto *et al.*, 2004).

Co-stimulatory molecules expressed at the surface of T cells such as CD28 and CD27 have also been used to classify T cell memory populations (Weekes *et al.*, 1999, Tomiyama *et al.*, 2002). During antigen presentation, CD28 engages with molecules on the surface of APCs such as CD80 and this is a fundamental step in T cell activation (Lenschow *et al.*, 1996; Howland *et al.*, 2000). T cells which will be in direct contact with antigen presenting cells, such as naive and Tcm cells, express CD28. Unlike CD28, the function of CD27 is less well defined but it is known that the expression of this molecule is enhanced during antigen presentation and that it is probably involved in the expansion of memory cells (Hendricks *et al.*, 2005; Borst *et al.*, 2005). The results of previous studies highlight that CD45RA, CCR7 and CD27 can be used in combination to define T naive cells as CD45RA+CCR7+CD27+, Tcm as CD45RA-CCR7+CD27+ and Tem as CD45RA+CCR7-CD27- (Mercier *et al.*, 2008; Tilton *et al.*, 2007).

The Fas ligand receptor, CD95, is expressed on cells programmed to undergo apoptosis and has also been used in combination with either markers of maturation (CD45RA or CD45RO) (Mattapallil *et al.*, 2005) or co-stimulatory markers such as CD27 and CD28 to characterise T cell memory phenotypes in rhesus macaques (Pitcher *et al.*, 2002). It has been shown that CD95 regulates T cell homeostasis of peripheral memory T cells (Arens *et al.*, 2005, Mogil *et al.*, 1995). In the rhesus macaque model, a combination of CD28 and CD95 has been used to delineate naive T cells (CD28+ CD95-) from Tcm (CD28+CD95+) and Tem (CD28-CD95+) (Sun *et al.*, 2005; Letvin *et al.*, 2006; Sun *et al.*, 2009). To date, the use of CD28 and CD95 in combination has not been reported for the memory phenotype analysis of T cells from Chacma baboons.

The Chacma baboon is widely available in Southern Africa and could be used as a predictive model for preclinical studies investigating vaccine efficacy, immunity and pathogenesis. Despite this however, there are limited studies that have evaluated T cell memory phenotypes in the Chacma baboon model. As such, there are no widely accepted memory markers which can be used to accurately and effectively define T cell memory phenotypes in PBMCs from Chacma baboons.

In this study, T cell memory markers were investigated for cross-reactivity to select those that are suitable for inclusion in flow cytometry analysis of T cell memory phenotypes. The objectives of this chapter are to select a combination of memory markers that are suitable for inclusion in a flow cytometry panel of antibodies to investigate T cell memory phenotypes using baboon PBMC and to examine the characteristics of T cell memory subset delineated by the selected combination of markers to determine if they are similar to those of typical phenotypes.

3.2 Specific objectives

3.2.1. Identify and evaluate various anti-human antibodies to select a suitable combination of markers to detect T cell memory subsets in baboon PBMCs.

3.2.2. To determine the suitability of the selected markers by investigating the functional, activation and maturation characteristics of the baboon T cell memory phenotypes defined by these markers using a four-colour flow cytometry.

3.3. Material and methods

3.3.1. Samples

Cryopreserved PBMCs isolated from unvaccinated, healthy Chacma baboons were used in this study to select suitable markers to investigate T cell memory by flow cytometry analysis. The PBMCs were thawed according to the protocol outlined in Chapter 2. For assays used to detect activation markers or cytokine production, the PBMCs were rested for 4-6 hours and stimulated with SEB as described in Chapter 2.

3.3.2. Cross-reactivity of clones of anti-human antibodies with baboon PBMCs

Several commercially available clones of anti-human antibodies which are known to detect CD28, CD95, CD27, CD45RA, CD69, CCR7 and CCR5 in humans and/or rhesus macaques were tested for cross-reactivity with baboon PBMCs. For cross-reactivity testing, PBMCs isolated from baboons were stained with individual clones of antibodies using the manufacturer's recommended concentrations and staining conditions. Flow cytometry was then used to determine cross-reactivity. Lineage markers CD3, CD4 and CD8 were also included for gating purposes. Cross-reactivity of several clones of these markers, together with markers for the cytokines IL-2, IFN- γ and TNF- α have already been demonstrated in the baboon model (Casimiro *et al.*, 2003; Chege *et al.*, 2005; Chege *et al.*, 2008; Burgers *et al.*, 2009).

Antibody clones that provided a distinct signal for both the antibody negative and positive populations (bimodal expression of markers) in accordance to the manufacturers recommendations were considered cross-reactive. For antibody markers which had overlapping positive and negative population, such as CD45RA and CD95, isotype controls were used as a control to stain cells. Isotype controls are matched to the antibody flourochrome conjugate of interest and they are conjugated to the same fluochrome but bind to all cells that express the same fragment crystalizable (Fc) receptor of the antibody (Maecker and Trotter 2006). Isotype controls are used to determine the level of non-specific binding of fluorochromes (Maecker and Trotter 2006). In this way, positive population of cells can be determined and differentiated from the background staining and negative population.

3.3.3. Optimisation of staining temperature used for four-colour flow cytometry

To determine the optimal staining temperature that ensured optimal antibody -conjugate binding to its corresponding cellular marker, PBMCs from unvaccinated baboons were stained at either 37°C (37degC), room temperature (RT) or on ice with antibodies at manufacturer's recommended concentrations. Positive populations were gated according to the recommendations made by the manufacturer and by considering the unstained cells.

3.3.4 Titration of antibodies

The optimal staining concentration of each antibody-fluorochrome conjugate was determined by staining baboon PBMCs with two-fold serial dilutions starting from twice the manufacturers' recommendations. The titration of each antibody was performed according to protocols detailed in Chapter 2.

3.3.5. Selection of a suitable combination of T-cell memory markers using four-colour flow cytometry.

The principles used to assign antibodies to fluorochrome markers and to select combinations of antibody-fluorochrome conjugates to limit spectral overlap are detailed in Chapter 2. In this study, CD95 was combined with CD27, CD28 or CD45RA in a four colour antibody panel to select a combination that displayed the best separation of memory subsets for both CD4 and CD8 subsets. The combinations of memory markers tested in this study are detailed in Table 3.1.

Table 3.1. Flow cytometry panels designed for the selection of maturation markers to identify T cell memory phenotypes in PBMCs isolated from Chacma baboons.

| Panel | Antibody marker | Fluorochrome |
|---------|-----------------|--------------|
| Panel A | CD28 | PE |
| | CD95 | PECy5 |
| | CD3 | FITC |
| | CD4 or CD8 | APC |
| Panel B | CD45RA | PE |
| | CD95 | PECy5 |
| | CD3 | FITC |
| | CD4 or CD8 | APC |
| Panel C | CD27 | PE |
| | CD95 | PECy5 |
| | CD3 | FITC |
| | CD4 or CD8 | APC |

3.3.5. Characterisation of memory phenotypes defined by the CD28 and CD95 flow cytometry panel in baboon PBMCs.

At the time this study was started, the CD28 and CD95 antibody markers had not been used for the memory phenotype analysis of T cells from baboons. However, these markers have been used to define memory phenotypes in other nonhuman primate models such as the rhesus macaque (Sun *et al.*, 2005; Sun *et al.*, 2009). In order to determine the suitability of these markers, CD28 and CD95 were used to stain PBMCs from five unvaccinated animals. In addition, other markers of T cell maturation (CD45RA PE), activation status (CD69 PE) and cytokine production (IL-2 PE, IFN- γ PE) were also included in individual panels. These additional markers were used to further characterize the memory subsets of CD4+ and CD8+ T cells with the intention to confirm whether these subsets are accurately defined by CD28 and CD95. Table 3.2 details the validation panels used in this study.

For the flow cytometry panels which included functional markers (cytokines), cells were first stimulated with SEB for a total of 16 hours and after the first 2 hours, BFA was added. Since BFA has been reported to prevent the expression of surface CD69 (O'Neil-Andersen and Lawrence, 2002), the cells were first stimulated with SEB for 16 hours and after the first 2 hours, BD GolgiStop (BD Biosciences) containing monensin was added. The cells were then stained and fixed using the surface and intracellular staining protocols above. For all experiments, the cells were analysed using the FACS Calibur.

Table 3.2. Flow cytometry panels designed to determine the suitability of CD28 and CD95 to define T cell memory phenotypes in PBMCs isolated from Chacma baboons.

| Panel | Antibody marker | Fluorochrome |
|---------|---------------------------------|--------------|
| Panel A | CD95 | FITC |
| | *CD45RA | PE |
| | CD4/8 | PerCP |
| | CD28 | APC |
| Panel B | CD95 | FITC |
| | *CD69 | PE |
| | CD4/8 | PerCP |
| | CD28 | APC |
| Panel C | CD95 | FITC |
| | *IL-2 | PE |
| | CD4/8 | PerCP |
| | CD28 | APC |
| Panel D | CD95 | FITC |
| | *IFN-γ | PE |
| | CD4/8 | PerCP |
| | CD28 | APC |

* Marker used to further characterise the memory phenotypes defined by CD28 and CD95

3.3.5.1. Gating strategy and data analysis

On average, 50 000 events gated on the lymphocyte population for each experiment, and 5000 events from the compensation controls (Chapter 2) were analysed by flow cytometry (FACS Calibur) using BD Cell Quest, version 5.1 (BD BioScience). Data analysis and compensation was performed in FlowJo (Tree Star Inc, Ashland, OR). The overall gating strategy employed for the validation experiments in this chapter are illustrated in Figure 3.1. The frequency (%) of expression of the markers CD45RA, IL-2, IFN- γ and CD69 by naive and memory T cells were then determined.

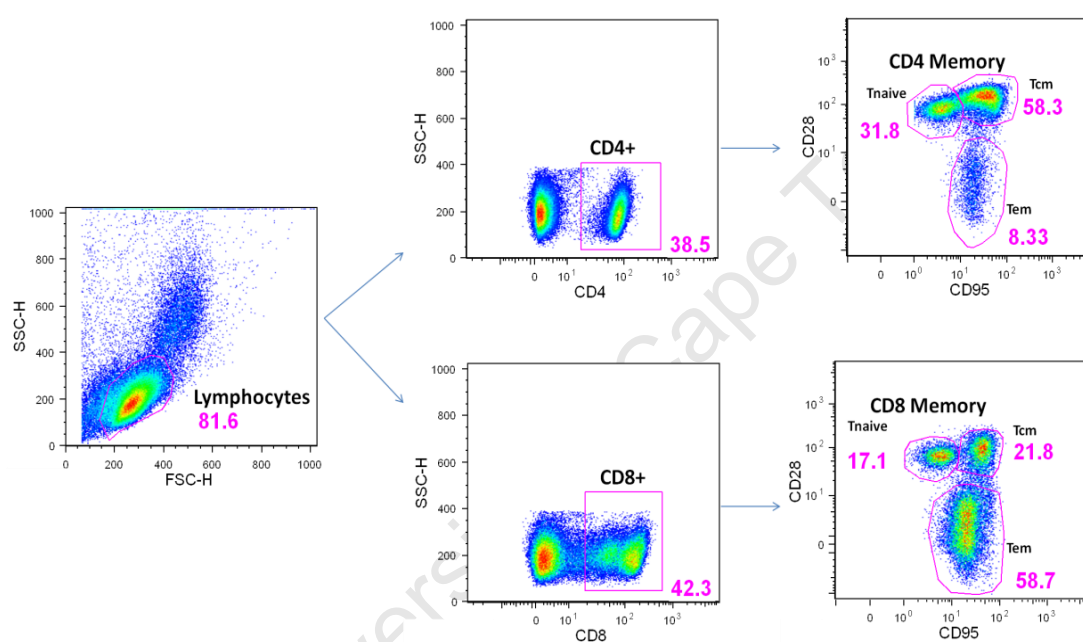


Figure 3.1. Gating strategy used for FACS Calibur analysis of PBMCs isolated from a representative healthy baboon (sample B363). Firstly, the lymphocyte population was isolated by the relative size and density of PBMCs gated by analysing forward Scatter (FSC-H) and side scatter (SSC-H). Then, CD4⁺ or CD8⁺ T-cells were gated by using either SSC-H versus CD4 or CD8 respectively. The memory phenotype of both CD4 and CD8 positive T-cells were then gated using CD28 vs CD95 (memory markers). For the validation of the panel, each subpopulation (Tnaive, Tem and Tcm) as defined by the CD28 vs. CD95 panel, were further characterised in terms of their relative expression of CD45RA, IL-2, IFN- γ and the activation marker CD69.

3.3.5.2. Statistical analysis

The expression of CD45RA, IL-2, IFN- γ and C69 were compared amongst T naive, Tcm and Tem using non-parametric one way ANOVA (Kruskal Wallis) followed by Dunn's multiple comparison using Graph Pad Prism version 6.00 for Windows (GraphPad Software).

3.4. Results

3.4.1. Cross-reactivity of clones of anti-human antibodies with baboon PBMCs

Distinct populations of positive staining cells were determined by analysis of histograms representing the stained baboon PBMCs (Figure 3.2). The unstained control was used to define positive versus negative cell populations. For cytokine bound conjugates, the positive population was distinguished from the negative population by using unstimulated controls. In this study, antibody markers of CD28 (clone CD28.2), CD95 (DX2), CD27 (clone M-T271), CD45RA (clone 5H9), and CD69 (clone FN50) were found to be cross-reactive in PBMCs isolated from healthy, unvaccinated Chacma baboons (Table 3.3). In addition, HLA-DR (clone G46-6) was also found to be cross-reactive but was not used in the remainder of the study. The cross-reactivity of several clones of CD3, CD4, CD8, TNF- α , IL-2 and IFN- γ were also confirmed (Table 3.3). However, cross-reactivity of CCR7 (clone 150503) and CCR5 (clone 3A9) to Chacma baboon PBMCs were not observed and these markers were therefore not used for further analysis in this study.

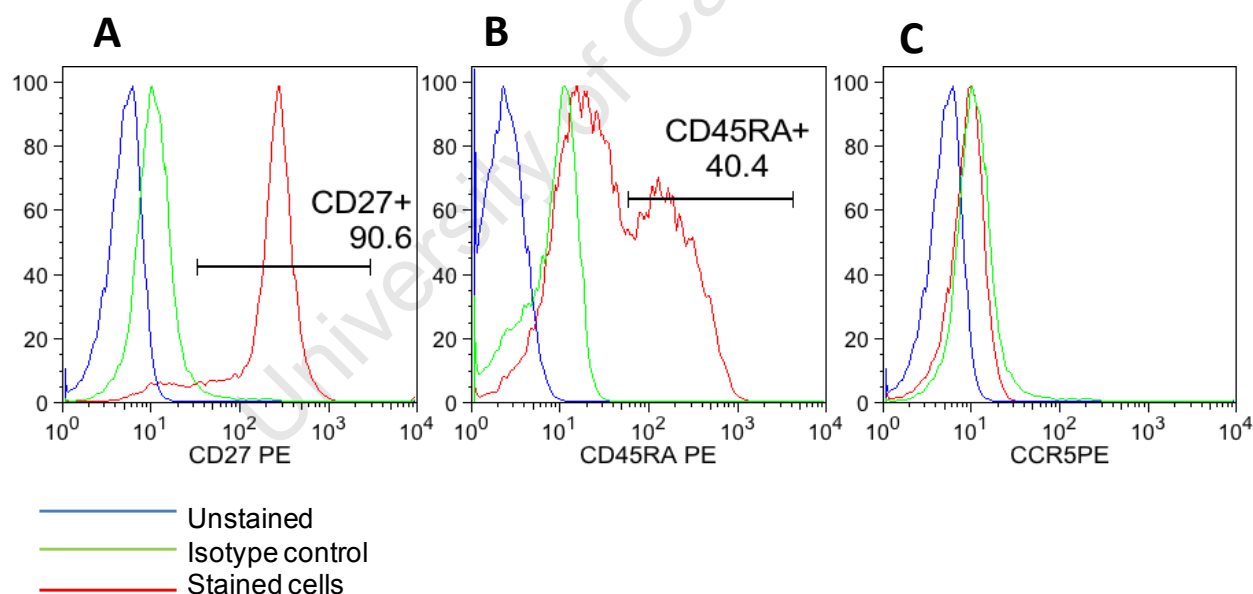


Figure 3.2. Cross-reactivity of antibody markers with PBMCs isolated from Chacma baboons. PBMCs were stained with antibody markers in accordance with the manufacturers' recommendations. Representative antibody-conjugates found to be cross-reactive in PBMCs isolated from unvaccinated healthy baboons A) CD27 PE clone M-T271 and B) CD45RA PE clone 5H9. In addition, an example non-cross reactivity of antibody-conjugate C) CCR5PE, clone 3A9 is represented.

Table 3.3. Optimal staining concentrations (per 1×10^6 cells in 50ul) and conditions, cross-reactivity and clone of antibody-conjugates for FACS Calibur flow cytometry analysis using lymphocytes from PBMCs isolated from baboons.

| Marker | Clone | Flouochrome | Source | Manufacturer's recommended concentration (μ l/100ul) | Optimal conc. |
|--------------------------------|-----------|-------------|-----------|--|---------------|
| CD3 | SP34-2 | PerCP | BD | 20 μ l | 3 μ l |
| | FN-18 | FITC | BioSource | 20-50 μ l | 1 μ l |
| CD4 | L200 | PerCP | BD | 20 μ l | 3 μ l |
| | L200 | APC | BD | 20 μ l | 1 μ l |
| CD8 | SK1 | PerCP | BD | 20 μ l | 3 μ l |
| | SK1 | APC | BD | 5 μ l | 1ul |
| CD28 | CD28.2 | PE | BD | 20 μ l | 1 μ l |
| | CD28.2 | APC | BD | 20 μ l | 1 μ l |
| | CD28.2 | FITC | BD | 20 μ l | 1 μ l |
| CD95 | DX2 | PECy5 | BD | 20 μ l | 1 μ l |
| | DX2 | FITC | BD | 20 μ l | 1 μ l |
| | DX2 | APC | BD | 20 μ l | 1 μ l |
| CD45RA | 5H9 | PE | BD | 20 μ l | 5 μ l |
| | 5H9 | PECy5 | BD | 20 μ l | 2.5ul |
| CD27 | M-T271 | PE | BD | 20 μ l | 1.25 μ l |
| | M-T271 | APC | BD | 20 μ l | 1 μ l |
| | M-T271 | FITC | BD | 20 μ l | 1.25 μ l |
| CD69 | FN50 | PE | BD | 20 μ l | 2.5 μ l |
| IL-2 | MQ1-17H12 | PE | BD | 20 μ l | 2 μ l |
| IFN-γ | 4SB3 | PE | BD | 20 μ l | 1 μ l |

3.4.2. Optimisation of staining temperature used for four-colour flow cytometry

To determine which staining temperature would allow for optimal antibody binding, minimal background and maximum detection of rare event populations, the relative frequency of lymphocytes that were positive and negative for the antibody of interest were determined by four colour flow cytometry analysis. The PBMCs from healthy, unvaccinated baboons were stained at either 37°C (37degC), room temperature (RT) or on ice (Figure 3.3). The optimal staining condition was determined by comparing the relative frequency of the positive population taking into account the lowest background (non-specific fluorescence) represented by the unstained control.

Most of the markers tested, optimally stained baboon PBMCs at both room temperature and on ice but not at 37°C with the exception of CD95 PEcy5. Specifically, CD3-PerCP,-FITC and CD45RA-PE were optimally stained on ice, whilst CD4-APC and CD27-PE stained optimally at room temperature. Since a higher level of non-specific fluorescence was observed at 37°C compared to that at room temperature and the lowest level of background staining was observed at room temperature (+/- 23 to 27 °C) these results suggest that baboon PBMCs could be stained with these markers at room temperature.

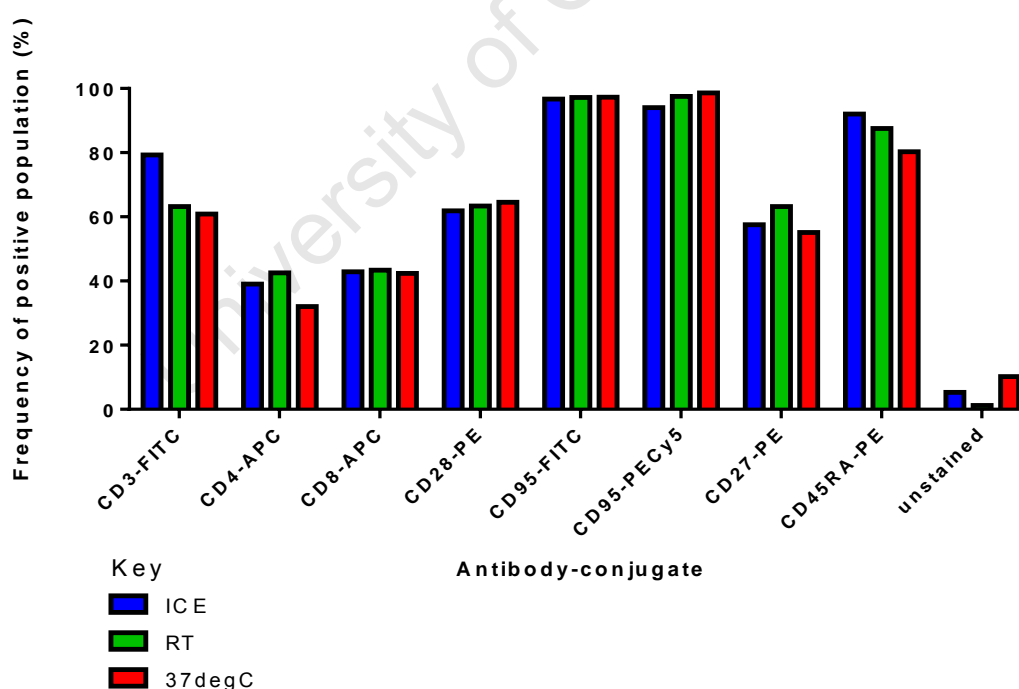


Figure 3.3. Optimisation of staining conditions for PBMCs isolated from Chacma baboons. Cryopreserved PBMCs isolated from unvaccinated, healthy baboons were stained with antibody markers on ice (blue bars), at room temperature (green bars) or 37°C (red bars). Bar graphs show the frequency (%) of positive populations after cells were gated according to SSC and FSC gates. An unstained reaction was also included at each staining condition as a negative control to account for background or non-specific fluorescence.

3.4.2. Titration of antibodies for four colour flow cytometry

It is widely accepted that the optimal concentration (and hence volume) of antibody conjugate used for flow cytometry analysis affects the overall quality of the data in terms of spectral overlap, compensation and accuracy. Therefore all commercial antibody conjugates were individually titrated by two-fold serial dilution to determine the optimal antibody concentration required for staining baboon PBMCs (Figure 3.4). The MFI of the positive staining population, as determined by flow cytometry analysis was plotted against the concentration of antibody conjugate used in a two-fold serial dilution as described in (Figure 3.4 B) Chapter 2.

A saturation curve of antibody-specific isotype controls indicate the MFI of the level of background or non-specific staining observed at particular antibody concentrations for some antibody-conjugates. The optimal antibody conjugate volume and concentration was typically found at the saturation point or just below saturation point depending on the level of background at that titre. A summary of the optimal staining volume of the antibody conjugates tested can be seen in Table 3.3.

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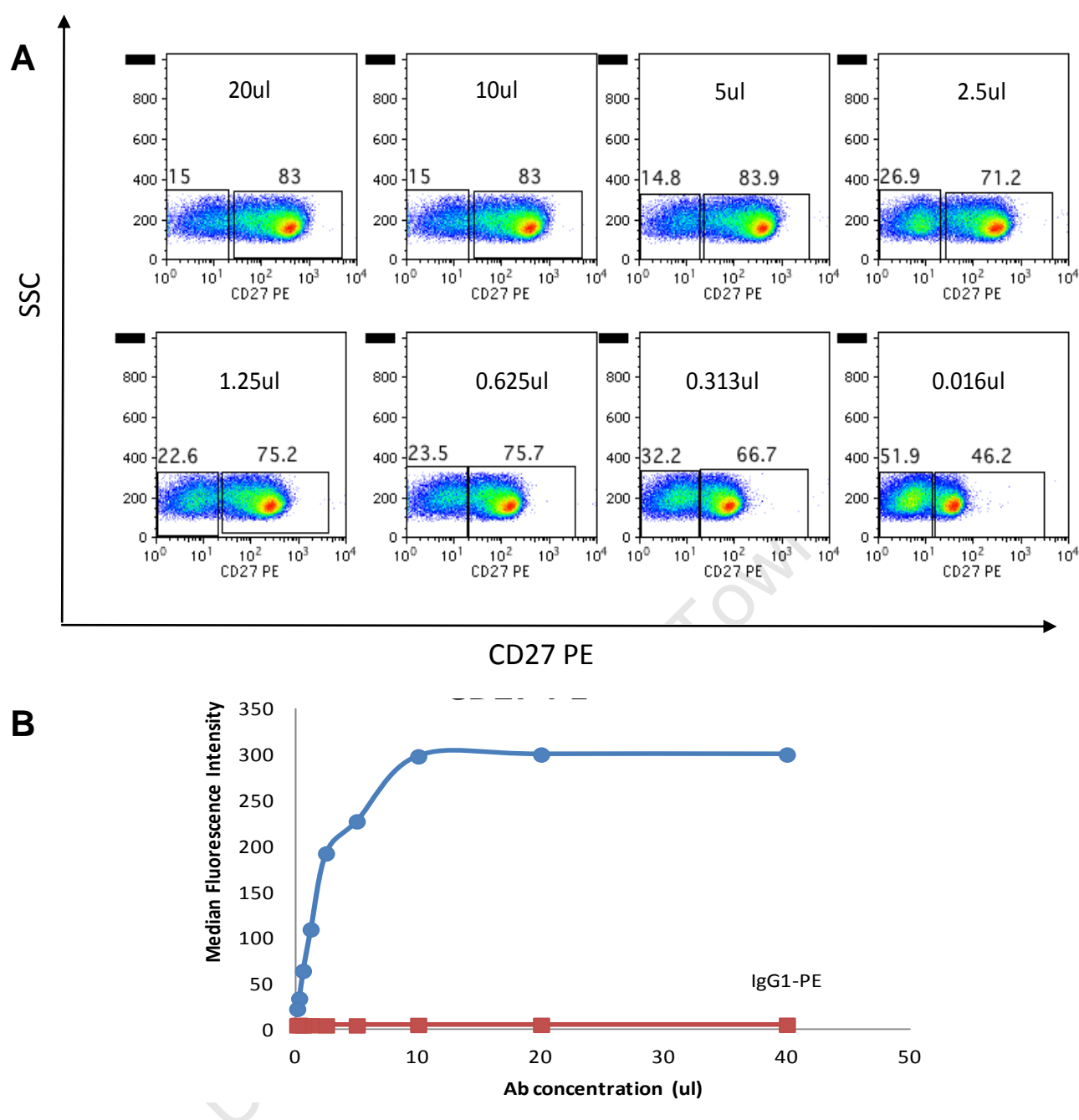


Figure 3.4. Representative antibody titration and saturation curve. Cryopreserved PBMCs isolated from healthy Chacma baboons were stained with CD27 PE in a two-fold serial dilution. **(A)** Flow plots show the two-fold serial dilution series with the concentration of antibody used for surface staining. Cells were first gated on lymphocytes based on their side scatter and forward scatter properties. **(B)** CD27 PE titration saturation curve (blue line) constructed by comparing the median fluorescence intensity (MFI) of the positive populations of lymphocytes (Ab concentration). Saturation curves of antibody-specific isotype controls (red line) indicate the MFI of the level of background or non-specific staining observed at particular antibody concentrations. For each antibody pair, optimal staining concentrations were determined at or just below the level of saturation.

3.4.3. Selection of a suitable combination of T-cell memory markers using four-colour flow cytometry.

Three 4-colour flow cytometry antibody panels that included a combination of anti-CD95 with anti- CD27, -CD28 or -CD45RA were tested to determine which combination of memory markers was most suitable to include in a final panel for the analysis of memory responses in PBMCs isolated from baboons. As shown in Figure 3.5, CD45RA and CD95 allowed for the separation of CD4+ and CD8+ naïve T cells (Tnaive: CD4RA+CD95-) and Tem (CD45RA-CD95+) but a clear Tcm population was not distinguishable. The CD27 and CD95 markers separated naïve T cells (CD27+ CD95-) from Tcm (CD27+CD95+) and Tem (CD27-CD95+) but the separation was not as clear as the CD28 and CD95 combination. Using this panel, the memory phenotypes were defined as central memory (Tcm; CD28+CD95+) and effector memory (Tem; CD28-CD95+) populations with the naïve T cells being defined as (CD28+CD95-). The CD28 and CD95 marker combination was therefore selected for inclusion in a flow cytometry panel to further investigate T cell memory phenotypes in PBMCs from Chacma baboons.

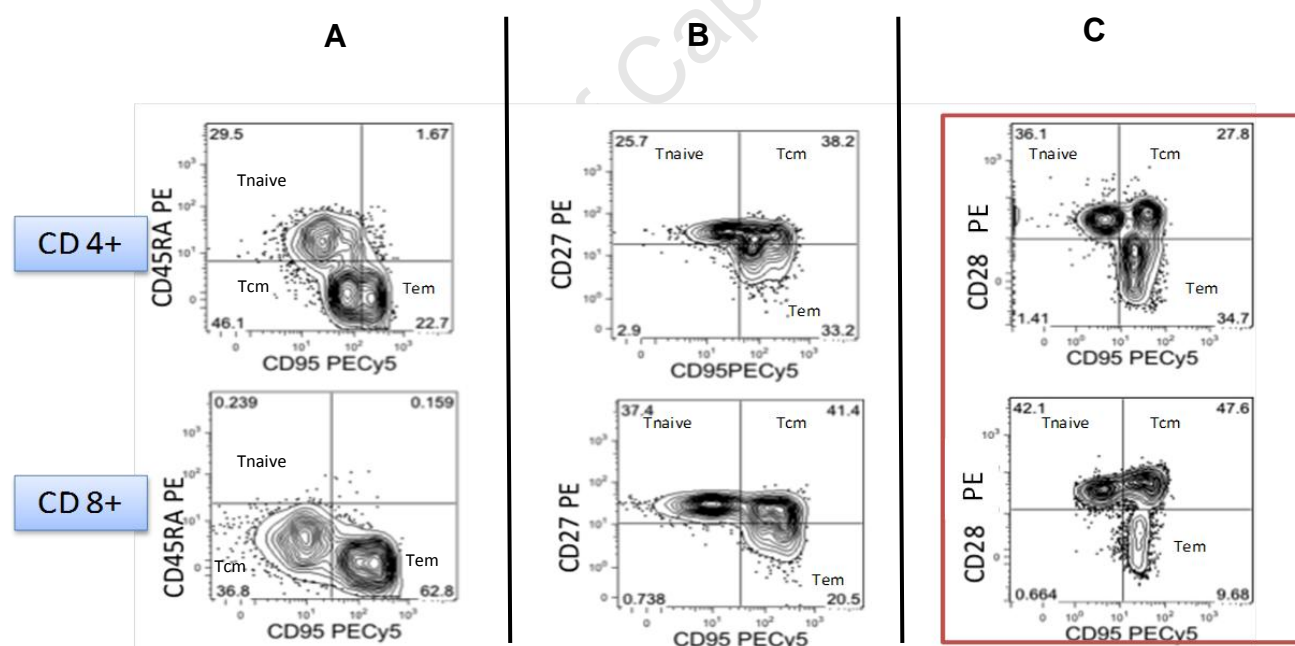


Figure 3.5 T cell memory marker combinations used for flow cytometry analysis of PBMCs isolated from a healthy unvaccinated baboon. Cryopreserved PBMCs were stained with several markers used together with CD95 to identify distinct subpopulation separation amongst T cell memory phenotypes. These combinations included A) CD45RA and CD95, B) CD27 and CD95 and C) CD28 and CD95. Cells were first gated on lymphocyte populations based on FSC and SSC properties, then CD4+ or CD8+ populations.

3.4.4. Characterisation of memory phenotypes defined by the CD28 and CD95 flow cytometry panel in baboon PBMCs.

Having shown that the CD28 and CD95 combination was the most suitable for inclusion in a multi-colour flow cytometry panel, the distribution of memory phenotypes in CD4⁺ and CD8⁺ T cell memory populations were determined for n=5 healthy, unvaccinated Chacma baboons by comparing the relative expression of CD28 and CD95.

Using this antibody combination, the mean frequencies (\pm SD, n=5) of CD4 naïve, Tcm and Tem were 49.2 \pm 12.3%, 27.3 \pm 9.8%, 16.8 \pm 2.4%, respectively. The mean frequencies (\pm SD, n=5) of CD8 naïve, Tcm and Tem were 37.0 \pm 16.2%, 17.6 \pm 6.4%, 42.5 \pm 8.3%, respectively (Figure 3.6). There was no significant variation in the frequencies of memory subpopulations in both CD4⁺ and CD8⁺ T-cells. However, there were a higher number of Tem cells in the CD8⁺ compartment compared to the CD4⁺ T-cells while the opposite was true for Tcm cells.

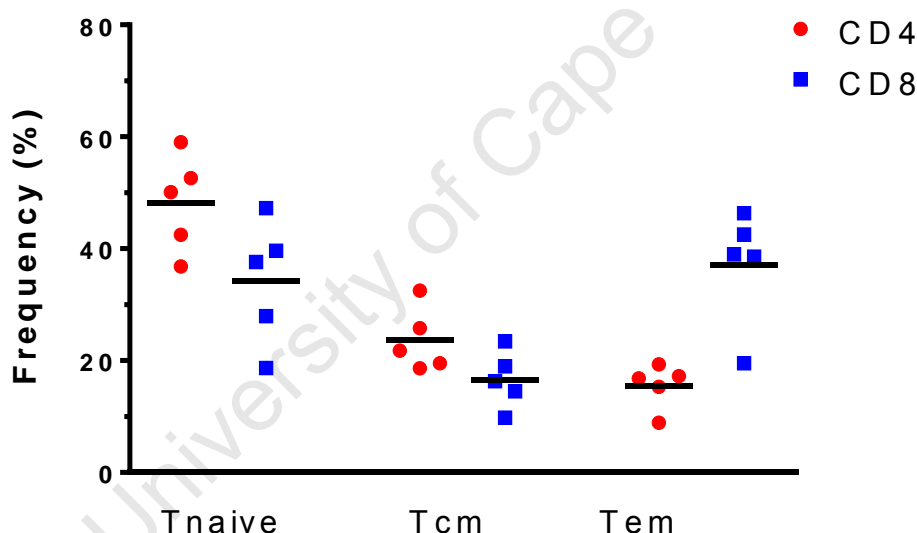


Figure 3.6. Mean frequency (n=5) of naïve, central and effector memory T-cell populations defined by CD28 and CD95 memory markers. Cryopreserved PBMCs isolated from healthy baboons were defined by the CD28 and CD95 memory panel as naïve (Tnaive: CD28+CD95-), central (Tcm: CD28+CD95+) and effector (Tem: CD28-CD95+) memory T-cell populations. Cells were first gated on lymphocyte populations based on their SSC and FSC staining properties. Then CD4⁺ and CD8⁺ cells were identified and further gated into memory populations using CD28 and CD95. The graph shows the mean frequency of CD4⁺ Tnaive, Tcm and Tem (red) and CD8⁺ Tnaive, Tcm and Tem (blue). A larger ratio of CD4⁺ T cells were defined as Tcm compared to that of CD8⁺ whilst a larger proportion of CD8⁺ T cells were defined as Tem.

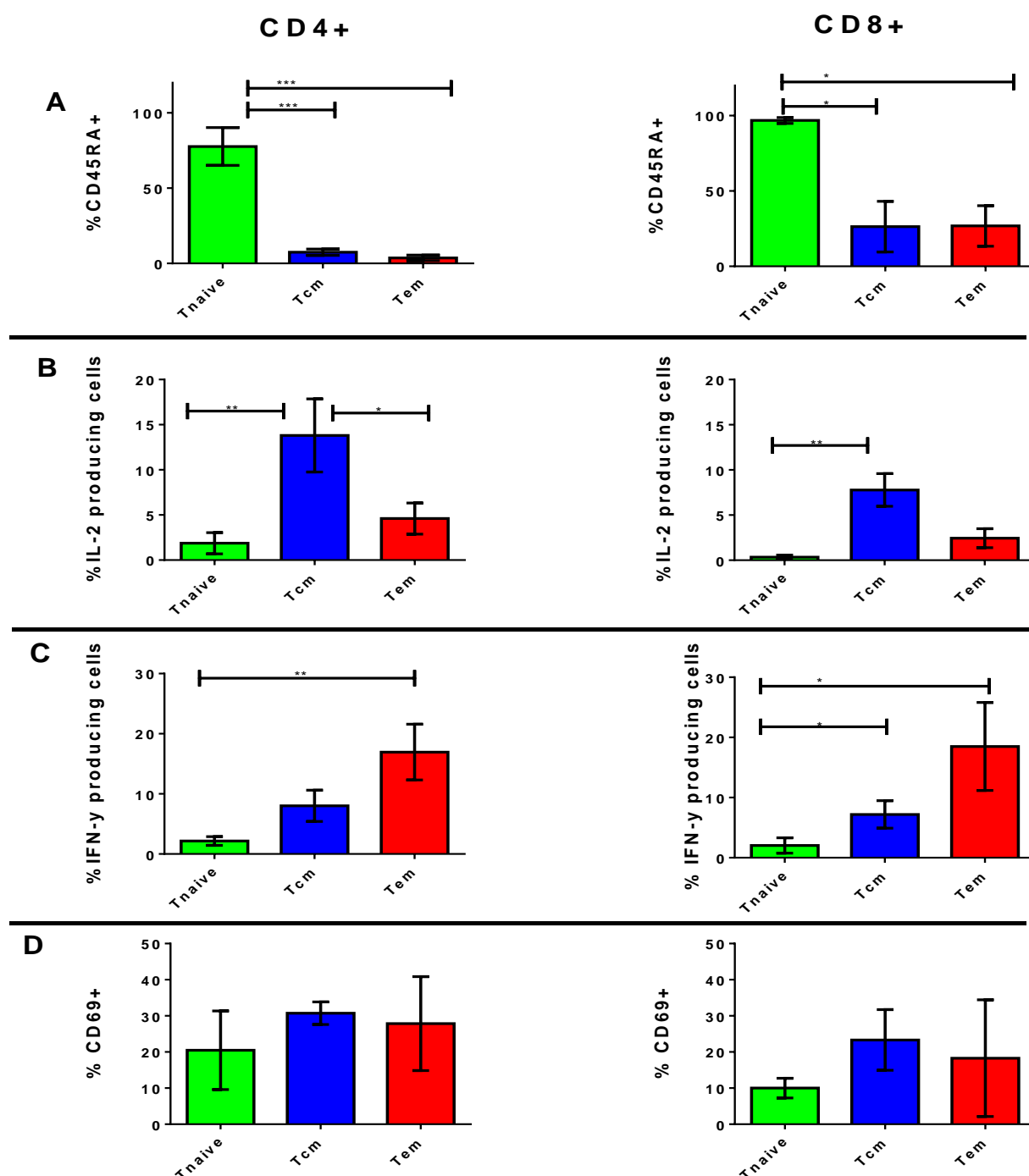


Figure 3.7. Characterisation of memory phenotypes defined by the CD28 and CD95 marker combination in baboon PBMCs. PBMCs from baboons (n=5) were stained with CD28, CD95, CD4 or CD8 and either CD45RA, IL-2, IFN- γ or CD69. The bar graphs represent the relative proportion (%) of **A**) CD45RA, **B**) IL-2, **C**) IFN- γ and **D**) CD69 expression on both CD4+ and CD8+ naive (Tnaive, green bars), central memory (Tcm, blue bars) and effector memory (Tem, red bars) phenotypes defined by CD28 and CD95. Statistically significant differences in expression are indicated as asterisks (* $p \leq 0.05$; ** $p \leq 0.01$; *** $p \leq 0.001$).

Further characterisation sought to determine if the T cell memory subsets based on CD28 and CD95 exhibited the expected functional, maturation and activation characteristics. The memory subsets delineated using the CD28 and CD95 memory markers were therefore investigated for functional capacity (expression of intracellular IFN- γ and IL-2) and activation status (expression of CD69) in response to staphylococcus enterotoxin B (SEB) stimulation. In addition, the expression of CD45RA, a marker for naïve T cells, was also investigated (Figure 3.7).

The maturation marker, CD45RA, was detected on a large percentage with a mean \pm standard deviation of 78.82% \pm 11.17 on naïve CD4+ and 96.84% \pm 1.94 on naïve CD8+ cells. This marker was expressed in a significantly ($p \leq 0.001$) smaller proportion of CD4+ (7.07% \pm 2.05) and CD8+ (26.36% \pm 16.76) Tcm cells. In Tem, CD45RA expression was significantly lower compared to naïve T cells ($p \leq 0.05$) with a mean expression of 3.58% \pm 1.62 and 26.84% \pm 13.45 in CD4+ and CD8+ Tem respectively. Upon SEB stimulation, a significantly higher proportion of CD4+ Tcm (13.8% \pm 4.1) compared to Tem (4.6% \pm 1.7) ($p \leq 0.05$) and naïve T cells (1.9% \pm 1.2) ($p \leq 0.01$) expressed IL-2. In the CD4+ compartment, Tem produced significantly more ($p \leq 0.01$) IFN- γ (mean of 17% \pm 4.7) compared to naïve T cells which expressed this marker at a mean of 2.2% \pm 0.7. CD4+ Tem expressed more IFN- γ compared to CD4+ Tcm (mean 8.0% \pm 2.6) although this was not statistically significant. In the CD8+ compartment Tem produced significantly more ($p \leq 0.05$) IFN- γ (mean of 18.5% \pm 7.3) compared to naïve T cells which expressed this marker at a mean of 2.0% \pm 1.3. CD8+ Tem also expressed more IFN- γ compared to CD8+ Tcm (mean 7.2% \pm 2.3). In the CD4+ compartment, CD69 was expressed on 20.5% \pm 11, 30.8% \pm 3.1 and 27.9% \pm 13 of naïve, Tcm and Tem populations respectively (Figure 3.7).

3.5 Summary of key findings

Several commercially available anti-human antibodies were tested for cross-reactivity using PBMCs isolated from healthy, unvaccinated Chacma baboons. Antibody markers of CD28 (clone CD28.2), CD95 (DX2), CD27 (clone M-T271), CD45RA (clone 5H9), HLA-DR (clone G46-6) and CD69 (clone FN50) were found to be cross-reactive, however cross-reactivity of CCR7 (clone 150503) and CCR5 (clone 3A9) was not observed. These markers were removed from further analysis.

It was found that antibody markers stained baboon PBMCs optimally at room temperature. Staining cells at room temperature also had the added advantage of limited background (non-specific) staining. In addition, antibody conjugate markers were titrated using baboon PBMCs to determine the optimal staining concentrations.

Several combinations of memory markers including CD45RA/CD95, CD27/CD95 and CD28/CD95 were evaluated to determine the most suitable combination to delineate memory T cell phenotypes in PBMCs isolated from Chacma baboons. It was found that the CD28/CD95 combination provided the most distinct separation of naive, central memory and effector memory T cells in both CD4+ and CD8+ compartments.

The CD28 and CD95 marker combination was then further characterised by investigating whether the phenotypes delineated by these markers displayed expected levels of maturation, activation and functional capacity. The expression of the maturation marker CD45RA, cytokines IL-2 and IFN- γ and the early activation marker CD69, by each memory phenotype delineated by the CD28/CD95 markers was determined using four-colour flow cytometry. CD45RA expression was significantly higher in both CD4+ and CD8+ naive T cells compared to Tcm and Tem. The production of IL-2 by CD4+ Tcm was significantly higher compared to naive T cells and Tem cells. In the CD8+ compartment, IL-2 production was higher in the Tcm population compared to the naive T cell population ($p \leq 0.01$) and Tem. CD4+ and CD8+ Tem cells produced more IFN- γ than the naive T cell population ($p \leq 0.01$ and $p \leq 0.05$ respectively) and Tcm populations. The expression of CD69 was higher in CD4+ and CD8+ memory T cell populations compared to naive T cells although this was not statistically significant.

In conclusion, the selected CD28 and CD95 markers used in combination was characterised and these markers were found to be suitable for inclusion in a multi-parameter flow cytometry panel to investigate the distribution of memory T cell phenotypes using PBMCs isolated from Chacma baboons.

Chapter 4

University of Cape Town

Chapter 4. Investigation of vaccine specific T cell memory responses in Chacma baboons vaccinated with SAAVI MVA-C/VLP and DNA/VLP prime boost regimen

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4.1. Introduction and background

In the absence of sterilising immunity, HIV-1 vaccines should aim to have the ability to induce vaccine specific immune responses to limit infection, prevent the replication of viral particles so as to reduce escape mutant formation and to achieve a sustained level of protection (Robinson and Amara, 2005). In order to accomplish this, HIV-1 vaccines should induce adaptive T cell immune responses with the aim to generate long lived, HIV-1 specific memory recall responses (Robinson, 2007). These memory responses whether in the form of central memory T cells (T_{cm}) or effector memory T cells (T_{em}) should work in synergy to generate a robust, effective and HIV-1 specific immune response upon infection (Sallusto *et al.*, 2004). The distribution of memory T cell phenotypes and the maintenance of memory T cells depend on the type and amount of vector and HIV-1 immunogen delivered and is heralded as a crucial measure of vaccine efficacy (Pillai *et al.*, 2011). In this study, the presence, persistence and distribution of memory T cell phenotypes in Chacma baboons vaccinated with either SAAVI MVA-C prime followed by VLP boost or DNA prime followed by VLP boost regimens was investigated using flow cytometry analysis.

4.1.1. T cell memory

T cell memory is defined as long lived responses that can be initiated long after the initial exposure to antigen to induce a robust, strong secondary immune response to the same antigen upon re-encounter (Dutton *et al.*, 1998). In the naive host, there is a large proportion of naive T cells with the potential to commit to apoptosis or differentiation into effector and memory populations once antigenic and co-stimulatory signals are received by either immunisation or infection (Matloubian *et al.*, 1994; Kaech *et al.* 2002) The differentiation of the naive T cells depends on the strength of the signal received and the milieu of cytokines generated in response to antigenic stimulation (Lanzavecchia and Sallusto, 2005). Naive T cells that are committed to differentiation into effector or memory T cells change the profile of their gene expression and in turn, the expression of molecules on their surfaces.

Naive T cells and some terminally differentiated T cells express the surface marker CD45RA (CD45RA⁺) in contrast to memory T cells which express a different isoform of CD45 known as CD45RO (CD45RO⁺) (reviewed in Esser *et al.*, 2003). In addition, naive T cells are housed in the lymphoid tissues and therefore express L-selectin (CD62L) and CCR7 (Picker, 1993). These cells have the ability to readily proliferate upon encountering antigens or during depletion of T cell pools and therefore produce low levels of IL-2 during *in vitro* stimulation (De Rosa *et al.*, 2001).

Effector and memory T cell subsets can be defined by the phenotypic and functional changes that arise from their previous interactions with antigens. Two distinct memory T cell populations namely effector memory (Tem) and central memory (Tcm) have been defined on the basis of the expression of lymphoid homing markers, CD62L and CCR7 (Sallusto *et al.*, 1999), but it is now clear that the definition of memory phenotypes is more complex than this dogmatic definition (Wherry and Ahmed, 2004).

The classification of another subset of CD8⁺ memory T cells known as terminally differentiated cells with the phenotype of CD45RA⁺CD62L⁻CCR7⁻ where initially referred to as RA⁺ effector memory T cells because these cells produce perforin, IFN- γ and TNF- α , are cytotoxic, and are capable of rapid effector function after stimulation (Sallusto *et al.*, 1999; Tussey *et al.*, 2000). Recently, a new long lived T cell memory phenotype similar to naive T cells in terms of phenotypic expression of CD45RO⁻, CCR7⁺, CD45RA⁺, CD62L⁺, CD27⁺, CD28⁺ and IL-7R⁺, self renewal capacity, multi-potent ability to differentiate into effectors, and other memory phenotypes has been identified in mice (Gattinoni *et al.*, 2011). Despite being similar to naive T cells, these stem cell-like memory T cells however, also express CD95 and have many functional attributes synonymous to memory T cells (Gattinoni *et al.*, 2011). It is therefore becoming increasingly clear that even T cell differentiation may not occur as linearly as expected.

4.1.1.2. Models of memory T cell differentiation

Numerous models of T cell differentiation have also been proposed and it remains unclear as to which model best explains the process of T cell differentiation. Regardless of the most acceptable model of differentiation, it is now clear that the level of memory cell differentiation is dependent on the initial T cell frequency, clonal competition of T cell repertoires and the strength and persistence of the initial antigen stimulus (Bouneaud *et al.*, 2005; Jabbari and Harty, 2006; Badovinac *et al.*, 2007).

According to the linear model of T cell differentiation (Figure 4.1 A), after exposure to an antigen (and in the presence of co-stimulatory and cytokine signals), naïve T-cells undergo differentiation and proliferation which results in the development of effector functions; alter their ability to proliferate and change their homing tissue profiles (Oehen and Brduscha-Reim 1998). These changes usually result in the expression of subset specific cell surface markers that are used to monitor and define the production of memory T cell subsets as well as effector T cells. The majority (>90%) of antigen experienced effector T cells will undergo apoptosis during the contraction phase of the T cell response. It is theorised that the proportion of remaining effector cells will differentiate into antigen specific effector memory T

cells (CCR7⁻ and CD62L^{low}), which have the ability to induce a robust and highly focused response to the antigen. These effector memory T cells can give rise to long-lived central memory T cells that are CCR7⁺ and CD62L⁺, and proliferate in lymphoid tissues. Upon encountering the same antigen (either in the form of challenge or infection), memory T cells, rapidly proliferate and further differentiate generating a large population of antigen specific T cells.

Another possible model of memory T cell differentiation known as the bifurcative differentiation model (Figure 4.1 B), describes the formation of cells with multiple differentiation fates from one naive T cell. This occurs through asymmetric cell division following antigen stimulation (Gerlach *et al.*, 2010). These cells can either yield effector cells that undergo apoptosis after antigen clearance, long-lived T_{cm} or T_{em} cells. The self-renewing effector model of memory T cell differentiation (Figure 4.1 C) or decreasing potential hypothesis, proposes that naive T cells can either differentiate into T_{cm} cells or self-renewing effector T cells which express lymphoid homing markers and proliferation. This model suggests that these cells then can further differentiate into non-self renewing T_{em} cells which migrate to sites of infection or become senescent terminally differentiated effector T cells (Fearon *et al.*, 2001).

4.1.1.3. Factors that influence the distribution of memory T cell populations

The differentiation and maintenance of memory T cells is a crucial measure of vaccine immunogenicity. The expansion, contraction and differentiation of antiviral memory T cells following antigen exposure is strongly influenced by several factors such as antigen presenting cell driven signalling and the cytokine production which is in turn are dependent on the PRRs (Schluns and Lefrancois, 2003). The particular PRR which is engaged is influenced by the type of viral, bacterial or antigenic material presented to antigen presenting cells (Pulendran, 2006; Querec *et al.*, 2006).

In terms of vaccine development, the functional quality and quantity of CD4 and CD8 memory populations differ according to which type of vectors (recombinant viral or bacterial, or DNA) delivers antigen to the host (Pillai *et al.*, 2011). It is also evident that the type of immunogen encountered, the age of the host and the persistence of the antigen also determines the predominant memory phenotype. Studies in mice, NHPs and humans have clearly shown that the distribution of memory phenotypes change with age. The number of naive T cells eventually decrease from childhood to adulthood whereas the establishment of memory T cells increase (Linton and Dorshkind, 2004; Nikolich-Zugich, 2008). This is presumably due to the increase immunological capacity driven by antigen experience over

time as the host ages. The distribution of memory populations differ in the CD4⁺ and CD8⁺ T cell compartments as well as in specific tissues. CD4⁺ T cells in human peripheral blood are skewed towards T_{cm} and CD8⁺ skewed towards T_{em} (Sallusto *et al.*, 1999). Furthermore, whilst a large proportion of T cells from lymphoid organs and tissues are enriched with cells from the T_{cm} phenotype, gut, lung, liver and reproductive tissues have more T_{em} cells (Campbell and Butcher, 2002).

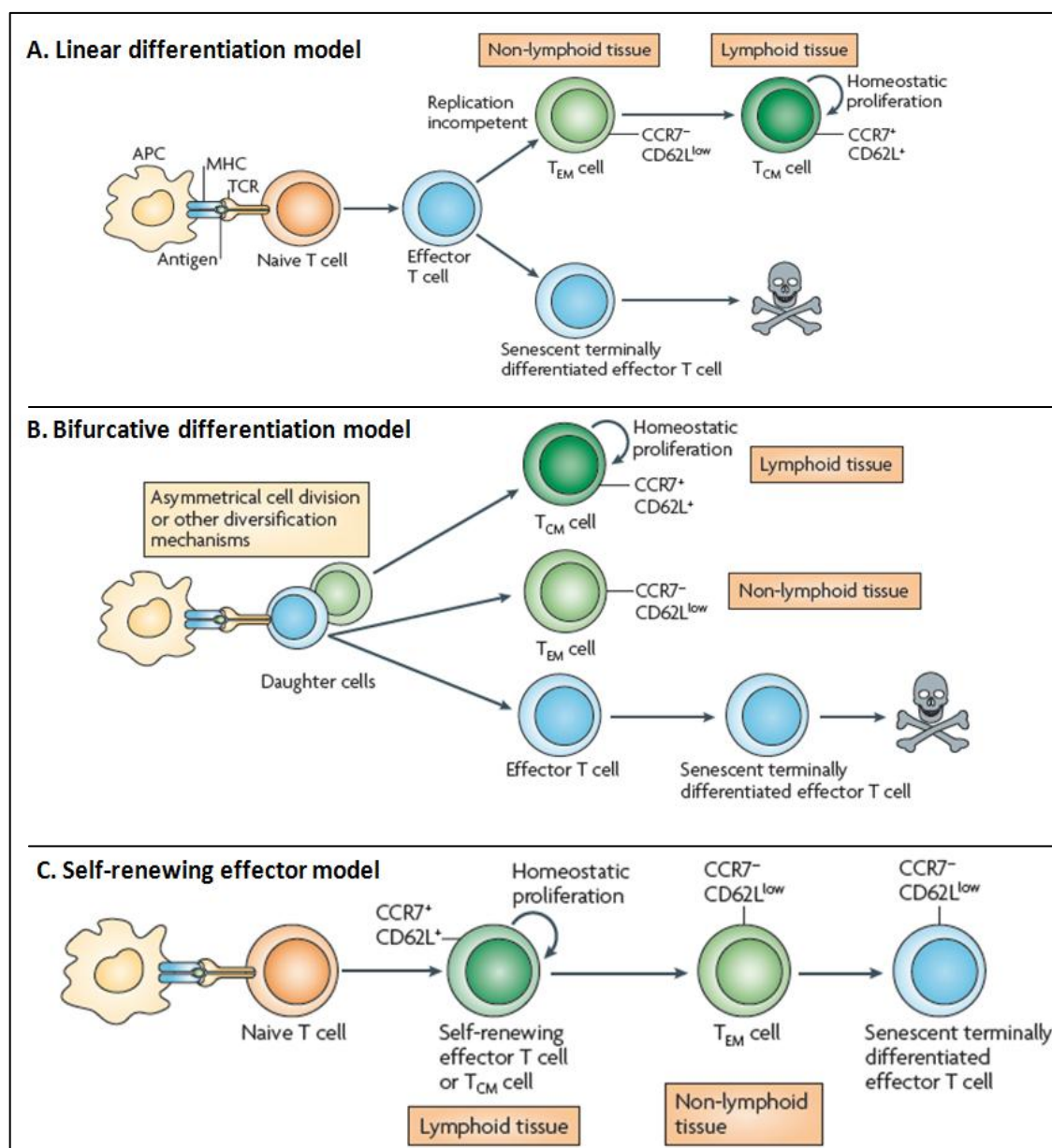


Figure 4.1 Proposed models of memory T cell differentiation. **A)** The linear differentiation model of memory T cell differentiation, **B)** The bifurcative differentiation model of memory T cell differentiation and **C)** The self-renewing effector model of memory T cell differentiation. (Taken from Ahmed *et al.*, 2009)

4.1.1.4. T cell memory populations and HIV vaccine efficacy

Investigation of the T cell responses induced by successful vaccines against other human diseases such as tetanus, yellow fever and small pox demonstrate that stable long-lived CD4+ and CD8+ memory T cells are associated with long-term protection (Cellerai *et al.*, 2007; Miller *et al.*, 2008). The same hypothesis can be applied to vaccines against HIV-1.

The characterisation of T cell memory subsets is therefore an essential measure of the immunogenicity of candidate HIV-1 vaccine. Studies have shown that HIV controllers were able to preserve the number of Tcm cells which were able to produce interleukin-2 (IL-2) (Potter *et al.*, 2007). In addition, Tem cells of these controllers were polyfunctional and reportedly secreted as much as three times more cytokines that expected in HIV-1 infected individuals (Potter *et al.*, 2007). The induction and preservation of SIV-specific CD4+ Tcm cells in vaccinated rhesus macaques have been associated with better survival and slow disease progression following challenge from either SIV or SHIV (Sun *et al.*, 2005; Letvin *et al.*, 2006, Manrique *et al.*, 2008). Furthermore, the presence of SIV specific CD8+Tcm has been shown to also correlate with delayed SIV disease progression (Vaccari *et al.*, 2005; Sun *et al.*, 2006).

An effective vaccine should therefore induce the differentiation of long-lived Tcm cells which are housed in lymphoid organs. These cells are involved in the replenishment of effector T cells and Tem cells. These cells produce high levels IL-2 and the ability to divide and differentiate rapidly into effector cells (Kaech *et al.*, 2002; Esser *et al.*, 2003). Similar to naive T cells, Tcm cells constitutively express CCR7 and CD62L due to their ability to enter and exit secondary lymphoid tissues (Sallusto *et al.*, 2002). However unlike naive T cells, Tcm cells are antigen experienced and have been activated by specific antigenic epitopes via TCRs. Therefore, Tcm have the ability to rapidly identify specific previously encountered antigens and upon re-emergence of the antigen these cells produce IL-2 which enables rapid proliferation. Tcm cells have the ability to rapidly differentiate and replenish existing populations of effector and Tem cells according to widely accepted models of T cell differentiation.

In addition, the vaccine should also generate effector Tem cells which are found surveying the peripheral blood and produce antiviral cytokines such as IFN- γ and TNF- α at the sites of infection (Kaech *et al.*, 2002). These cells lack the ability to re-enter lymphoid tissues (CCR7-) but instead express chemokines such as CCR5 and CXCR3 required for homing to mucosal and inflamed tissues at the site of infection where they are involved in robust effector functions (Moser and Loetscher, 2001) . It is also known that Tem cells lack the

capacity to proliferate and therefore produce relatively small levels of IL-2 but instead express high levels of Th1 and Th2 anti-viral cytokines (IFN- γ , TNF- α) and cytolytic enzymes (granzymes and perforin) (Sallusto *et al.*, 2002)

4.1.2. Aim of study

It has previously been demonstrated that SAAVI MVA-C and Pr55 Gag virus-like particle (VLP) based on HIV-1 subtype C, induced Gag-specific responses in Chacma baboons by IFN- γ ELISPOT assay measurements (Chege *et al.*, 2008a). Chacma baboons, primed with pTHgag DNA and boosted with Pr55 Gag VLPs (Chege *et al.*, 2008b) have also been shown to induce humoral and cellular responses specific to HIV-1 Gag. Collectively, the results highlight that both these vaccination strategies designed by the HIV Vaccine Development group at UCT are indeed capable of inducing specific T cell responses (and some humoral response) to HIV-1. However, there was a need to further investigate the ability of these vaccines to induce durable HIV-1 specific memory T cell responses. In addition, the relative distribution of vaccine specific T cell memory phenotypes induced by these vaccines needed to be determined as this is a measure of the level of protection induced by the vaccines in question.

The availability of flow cytometry facilities and expertise at UCT have allowed for the further investigation of these T cell responses measured in PBMCs of vaccinated Chacma baboons. The aim of this chapter was to investigate the distribution and durability of vaccine specific memory T cell responses induced by the SAAVI MVA-C/VLP and DNA/VLP prime boost vaccine modalities in the peripheral blood of Chacma baboons by flow cytometry analysis.

4.2. Specific objectives

4.2.1. Investigate the vaccine specific T cell memory phenotype distribution in Chacma baboons vaccinated with SAAVI MVA-C/VLP and DNA/VLP prime/boost vaccine regimens, using a multicolour flow cytometry panel comprising the selected markers.

4.3. Materials and methods

4.3.1 Samples and vaccination schedule

Cryopreserved PBMCs isolated from Chacma baboons that received two different vaccination regimens were used in this study. Baboons were vaccinated with either SAAVI MVA-C/VLP or DNA/VLP in prime/boost. Aliquots of cryopreserved PBMCs from vaccinated baboons at various time-points throughout both vaccination schedule (Figure 4.2) were used to investigate the vaccine-specific memory phenotypes.

Animals vaccinated with SAAVI MVA-C/VLP vaccination regimen (Group A) received SAAVI MVA-C prime at week 0 and the VLP boost at week 12. Animals that were vaccinated with the DNA/VLP vaccine regimen (Group B) received three DNA prime immunisations at weeks 0, 4 and 8. These animals were then boosted with two VLP immunisations at weeks 51 and 53. Samples from a third control group of animals that were not primed by any vaccinations and only received VLP boosts (Group C) were also included for comparative analysis.

Samples from Group A were selected for analysis just before the animals received the prime vaccination with SAAVI-MVA-C (baseline), one week following vaccination (week 1), twelve weeks following prime and before VLP boost was administered (week 12), four weeks following boost (week 16) and finally 20 weeks following the VLP boost (week 36).

Samples Group B were selected for analysis just before the animals received the first DNA prime (baseline) and at eight weeks following this (week 8) were used in this study. Samples from twelve weeks after the second DNA prime and before the first VLP boost was administered (week 12) were also used. In addition, samples from the day of the first VLP boost (week 51), three weeks after the first VLP boost (week 55), eight weeks after the first VLP boost (week 63), two weeks after the second VLP boost (week 65) and finally 6 weeks following the second VLP boost (week 71). Samples from Group C were selected for analysis from the same time-points after the VLP boosts (i.e. weeks 51,55,63,65 and 71)

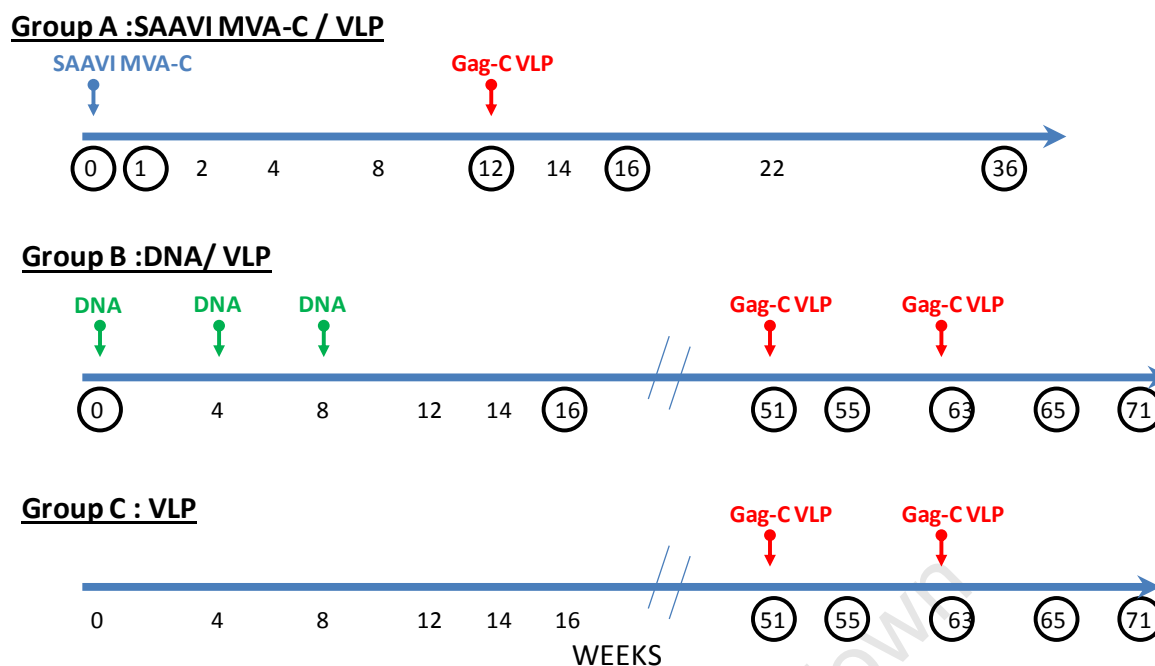


Figure 4.2. Immunisation schedule and samples selected for analysis from various time-points during vaccination with SAAVI MVA-C/VLP and DNA/VLP in prime-boost and VLP only. Samples from Group A were selected at the following time points, Wk0 (just before the animal received the first prime, blue arrow), Week 1, Week 12 (just before the animal received the VLP boost, red arrow), Week 16 (4 weeks after VLP boost) and Week 36 (20 weeks after VLP boost). Samples from Group B were selected at Week 0 (just before the animals received the first of three DNA prime vaccinations, green arrows), Week 16 (8 weeks after the last DNA vaccination), Week 51 (just before the first VLP boost, red arrow), Week 55, Week 63 (just before the second VLP, red arrow), Week 65 and Week 71. Samples from matched time-points were selected from Group C at Week 51 (just before the first VLP boost, red arrow), Week 55, Week 63 (just before the second VLP, red arrow), Week 65 and Week 71.

4.3.2. Selection of co-stimulatory antibodies

The detection of cytokine producing cells is essential to achieve the aim of quantifying the extent of vaccine induced memory immune responses. It has been well established that cytokine production to vaccine specific antigens can be enhanced by including co-stimulatory molecules to overcome activation thresholds *in vitro* (Waldrop *et al.*, 1998). As such, it was necessary in this study to determine which combination of co-stimulatory molecules was required to ensure optimal vaccine-specific cytokine detection in this study.

Co-stimulatory antibodies such as purified anti- CD28 and CD49d have been found to enhance the level of cytokine production when PBMCs are stimulated with peptides such as vaccine specific Gag pools. In this case, the CD28 marker is integral in delineating memory in T-cells as part of the CD28/CD95 memory panel. T cells have limited CD28 receptors and

when the co-stimulatory antibody purified anti-CD28 is added to cells (as part of stimulation) there are no longer sufficient receptors for the binding of CD28-fluorochrome (mAb) during the staining process.

4.3.2.1. The effect of purified anti-CD28 co-stimulatory antibodies on CD28 surface receptors

It was necessary to determine if the manufacturer's recommended concentrations of purified anti-human CD28 Abs had the potential to block detection of surface CD28 on baboon PBMCs. Three clones of anti-human CD28 co-stimulatory antibodies from three different sources were available and the aim of this experiment was to determine which one would have the least effect in blocking the detection of CD28 molecules on the surface of cells. To determine which clone and the optimal concentration of anti-CD28 co-stimulation antibodies to be used to augment the production of vaccine specific (Gag) cytokines, one aliquot of thawed PBMCs from an unvaccinated animal (B664) was incubated in the presence of the following purified anti-human CD28 antibodies (pCD28):

- pCD28 (clone CD28.2-BD) in two-fold serial dilutions starting from 1ug/ml (manufacturer's recommended concentration)
- pCD28 (clone CD28A-Mabtech) in two-fold serial dilutions starting from 0.1ug/ml (manufacturer's recommended concentration)
- pCD28 (clone E59- Merck) in two-fold serial dilutions starting from 1ug/ml (manufacturer's recommended concentration)

On average 0.5×10^6 cells per each reaction were incubated in the presence of the above for 30-40 minutes before surface staining for ViViD, CD3 APCCy7, CD4PECy5.5, CD8 QDot605, CD95 APC and CD28 FITC as per protocol outlined in chapter 2. The relative frequency of the respective cell populations were determined using FlowJo analysis.

4.3.2.2. The selection of purified anti-CD28 clones to augment cytokine production and to minimize blockade of surface CD28 detection

The effect of the various clones of purified anti- CD28 co-stimulatory antibodies were examined by stimulating PBMCs from a post vaccination time-points for 16 hours with the following stimulants and co-stimulants described in Table 4.1.

Table 4.1 Setup of stimulation and co-stimulation of PBMCs to investigate the selection of anti-CD28 clones

| | Stimulant | Co-stimulant(s) |
|--------------------------|------------------|-----------------------------------|
| SEB only | SEB | NONE |
| Gag only | Gag-C peptides | NONE |
| Gag+CD28 | Gag-C peptides | purified anti-CD28 |
| Gag + co-stim Abs | Gag-C peptides | purified anti-CD28 and anti-CD49d |

All stimulations were set in a humidified 37°C incubator with 5% CO₂ atmosphere. After the first 2 hour of incubation, BFA was added to all cells. The cells were then stained for viability (ViViD), surface markers (CD3 APCCy7, CD4 PECy5.5, CD8 QDot605, CD28 FITC and CD95 APC) and intracellular cytokines (IFN- γ , TNF- α and IL-2 multiplexed on PE) as per protocols outlined above. The relative frequencies of cytokines expression and CD28FITC expression was determined using FlowJo analysis.

4.3.3 Evaluation of vaccine specific memory responses

Revived and rested PBMCs were stimulated for 16 hour with a pool of Gag peptides and subsequently stained according to the staining protocols outlined in chapter 2. The cells were stained with a panel of flow cytometry antibodies (Table 4.1) to evaluate vaccine specific T cell memory phenotypes. In summary, cells were stained with the following antibody markers: ViViD to limit the analysis to viable cells prior to staining for the surface markers CD3 APCCy7, CD4PECy5.5, CD8 Qdot605, CD28 FITC and CD95 APC. The cells were permeabilised for intracellular staining and then stained for intracellular markers of cytokines (IL-2, TNF- α and IFN- γ which were multiplexed on PE). The stained cells were then fixed using 1x BD CellFIX (BD BioScience). The cells were then transferred to FACS tubes which were wrapped with aluminium foil stored at 4°C until data acquisition.

Table 4.2 Design of the seven colour flow cytometry panel used to investigate the vaccine specific cytokine production and memory distribution of T cells isolated from the PBMCs of Chacma baboons.

| Laser | Detector(nm) | Fluorochrome | Marker | Rationale |
|--------------|--------------|--------------|---------------------------------------|------------------------|
| Blue 488nm | B515 | FITC | CD28 | Memory marker |
| Green 532nm | G710 | PECy5.5 | CD4 | T cell lineage marker |
| Green 532nm | G560 | PE | IL-2, IFN- γ and TNF- α | Cytokine production |
| Red 640nm | R 780 | APCCy7 | CD3 | T cell lineage marker |
| Red 640nm | R 660 | APC | CD95 | Memory marker |
| Violet 407nm | V605 | QDot605 | CD8 | T cell lineage marker |
| Violet 407nm | V450 | ViViD | | T cell viability maker |

4.3.3.1. Data Acquisition and storage

The cells were analysed using the BD Fortessa. Voltages were setup using previous panels and adjusted accordingly during the acquisition of compensation beads and initial baboon PBMC samples. The data was acquired using BD Cell Diva acquisition software (BD Bioscience) and was set to analyse 250 000 events per sample. The data was stored on portal USB memory drives and saved on compact discs.

4.3.3.2. Data analysis and gating strategy

The data was analysed using FlowJo prior to the generation of a compensation matrix. The gating strategy used is represented in Figure 4.3. In summary, the data was first validated by comparing the analysis of the cells over time (SSC vs. Time). Only cells that were analysed by the flow cytometer as part of a continuum were gated for further analysis. To minimize false-positives, the forward scatter height (FSC-H) was then compared to forward scatter-area (FSC-A) to identify cells that moved through the laser as singlet cells. Lymphocytes were then identified by analyzing the relative granularity and size of the singlet cells by comparing the side scatter (SSC) and FSC. Viable, CD3+ T-cells were then gated on by comparing the viability maker on the pacific blue channel and CD3 marker on the APC.Cy7 channel. These cells were then separated into CD4+ and CD8+ cells by comparing the PECy5.5 and Qdot605 channels respectively. The total number of CD4+ and CD8+ cytokine producing was then determined. The net vaccine specific (Gag) cytokine response was considered a positive response if it was at least twice the background and greater than the cut-off value of 0.01. The background was deducted for the Gag responses to generate a net CD4+ and CD8+ cytokine response. These cytokine producing cells were then further

analysed such that the memory markers CD28 and CD95 were used to determine the relative frequencies of each memory phenotype.

4.3.3.3. Statistical analysis

The CD4+ and CD8+ cytokine responses were compared amongst groups of animals vaccinated with MVA/VLP, DNA/VLP and VLP only using non-parametric one way ANOVA (Kruskal Wallis) followed by Dunn's multiple comparison. The mean and standard deviations of cytokine responses and memory T cell phenotype distribution were also assessed. All statistic analysis was performed using Graph Pad Prism version 6.00 for Windows (GraphPad Software).

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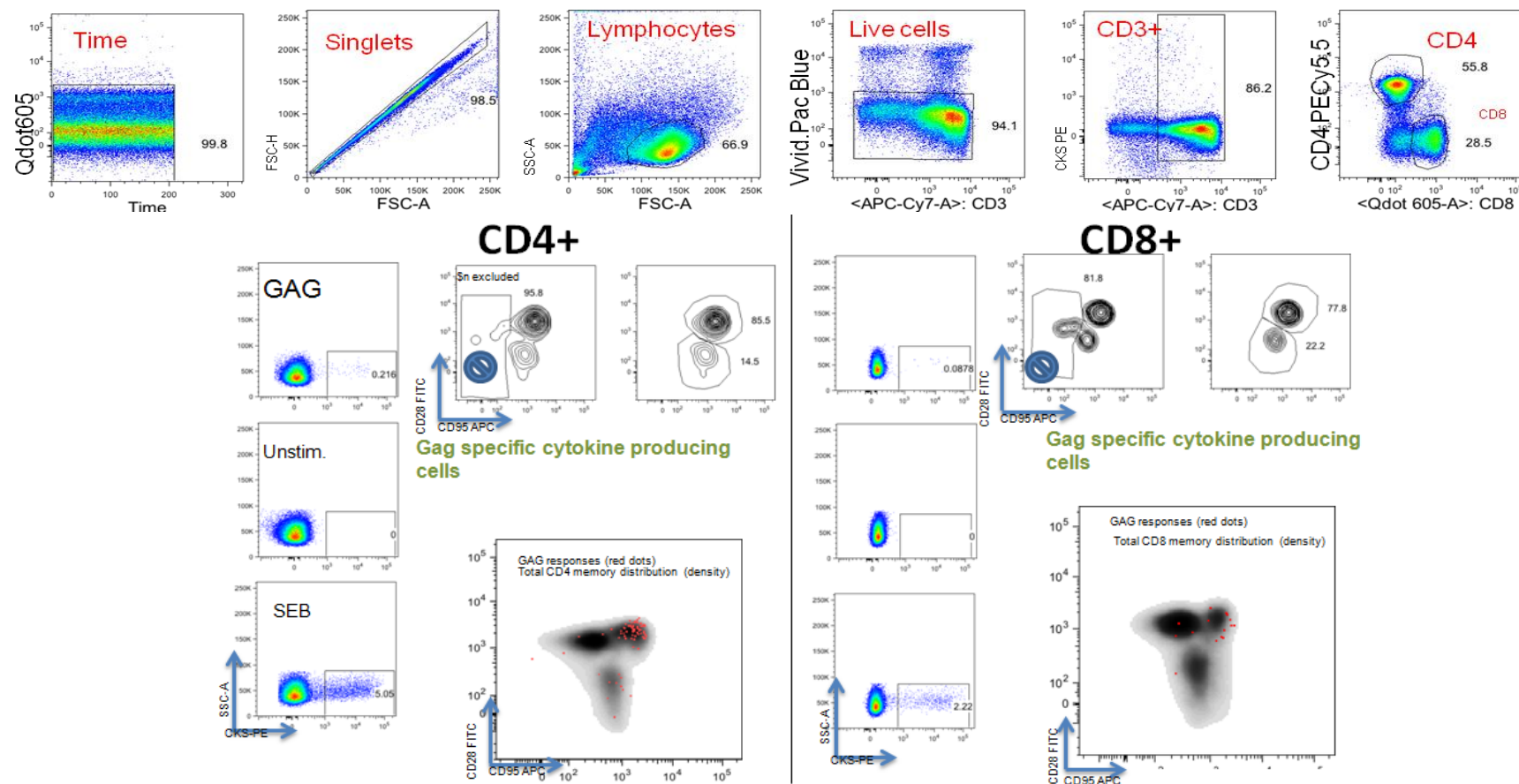


Figure 4.3 Gating strategy used to determine the vaccine specific cytokine response and T cell memory phenotypes. Cells from baboons were stimulated with Gag, SEB and R10 media only (unstim.). Time gates were plotted for each laser. In the case of CD8 QDot605 shown here, time gates were also used to exclude artefacts or aggregates (if any). Singlet gates based on forward scatter height (FSC-H) and forward scatter area (FSC-A) were used to exclude doublet cells. Lymphocytes were gated based on the relative size and granularity of the cells and plotted as FSC versus side scatter (SSC). Live cells are gated on ViViD negative populations. Then, CD3⁺ cells were gated using cytokine expression to confirm the CD3⁺ population. CD4⁺ and CD8⁺ T cells were identified. The expression of cytokines were then determined. The net vaccine specific (Gag) cytokine response was considered a positive response if it was at least twice the background and greater than the cut-off value of 0.01% and contained >25 events in total. The background was deducted for the Gag responses to generate a net cytokine response. The memory phenotypes of the cytokine positive cells were determined by their relative expression of CD28 and CD95.

4.4 Results

4.4.1. Selection of co-stimulatory antibodies

4.4.1.1. The effect of purified anti-CD28 co-stimulatory antibodies on CD28 surface receptors

To determine which of the three commercially available clones of purified anti-CD28 (pCD28) had the potential to block the detection of surface CD28 on PBMCs isolated from Chacma baboons, PBMCs were incubated with individual clones of pCD28 prior to staining for the flow cytometry analysis.

The frequency of CD4⁺ CD28⁺ T cells were maintained at 90-95% in the presence of pCD28 clones CD28.2 (BD Bioscience) and at 50-60% when in the presence of pCD28 clone 5E9 (Merck) at concentrations below 1ug/ml and 0.1ug/ml respectively (Figure 4.4). The frequency of CD8⁺ CD28⁺ T cells were also maintained at 90-95% in the presence of clones CD28.2 (BD Bioscience) and at 50-60% when in the presence of pCD28 clone 5E9 (Merck) at concentrations below 1ug/ml and 0.1ug/ml respectively (Figure 4.4). These data suggest that at these concentrations pCD28 clones did not compromise the detection of surface CD28 receptors on CD4⁺ and CD8⁺ T cells during flow cytometry analysis and therefore these optimal concentrations which were recommended by the manufacturer were used throughout this study.

4.4.1.2. The selection of purified anti-CD28 clones to augment cytokine production and to minimize blockade of surface CD28 detection

A suitable co-stimulatory molecule should induce T cell specific activation *in vitro* to enhance the expression and hence detection of rare cytokine events by flow cytometry analysis. In addition, the co-stimulatory molecule should be present at optimal concentrations without blocking the detection of CD28 molecules. The aim of this experiment was to determine which clone of pCD28 induced the highest proportion of cytokines for flow cytometry analysis of vaccine specific T cell memory. The frequency of conjugate bound CD28 FITC for each reaction was also determined by flow cytometry analysis.

Stimulation of PBMCs in the presence of purified anti- CD28 (clone CD28.2) purchased from BD and pCD49d induced 0.34% cytokine production in CD4⁺ T cells and 0.31% of cytokines in the CD8 T cell compartment (Figure 4.5A). The frequency of surface conjugate bound CD28 decreased by about 20-25% in both CD4 and CD8 T cells (Figure 4.5B). Stimulation of PBMCs in the presence of CD28 from Merck, showed no significant blockage

of the CD28 receptor (Figure 4.5B), and 0.3% of CD4+ T cells and 0.26% of CD8+ T cells produced cytokines (Figure 4.5A).

The purified anti-CD28 antibody purchased from Mabtech only induced 0.27% and 0.18% of cytokine production from CD4 and CD8 T cells respectively (Figure 4.5A). The frequency of CD28+ cells diminish to 49.3% and 39.3% in CD4+ and CD8+ T cells respectively that have been stimulated in the presence of pCD28 purchased from Mabtech (Figure 4.5B). The purified anti-CD28 purchased from Merck was therefore used to augment cytokine production in this study. Stimulation in the presence of this antibody did not block the detection of surface CD28FITC.

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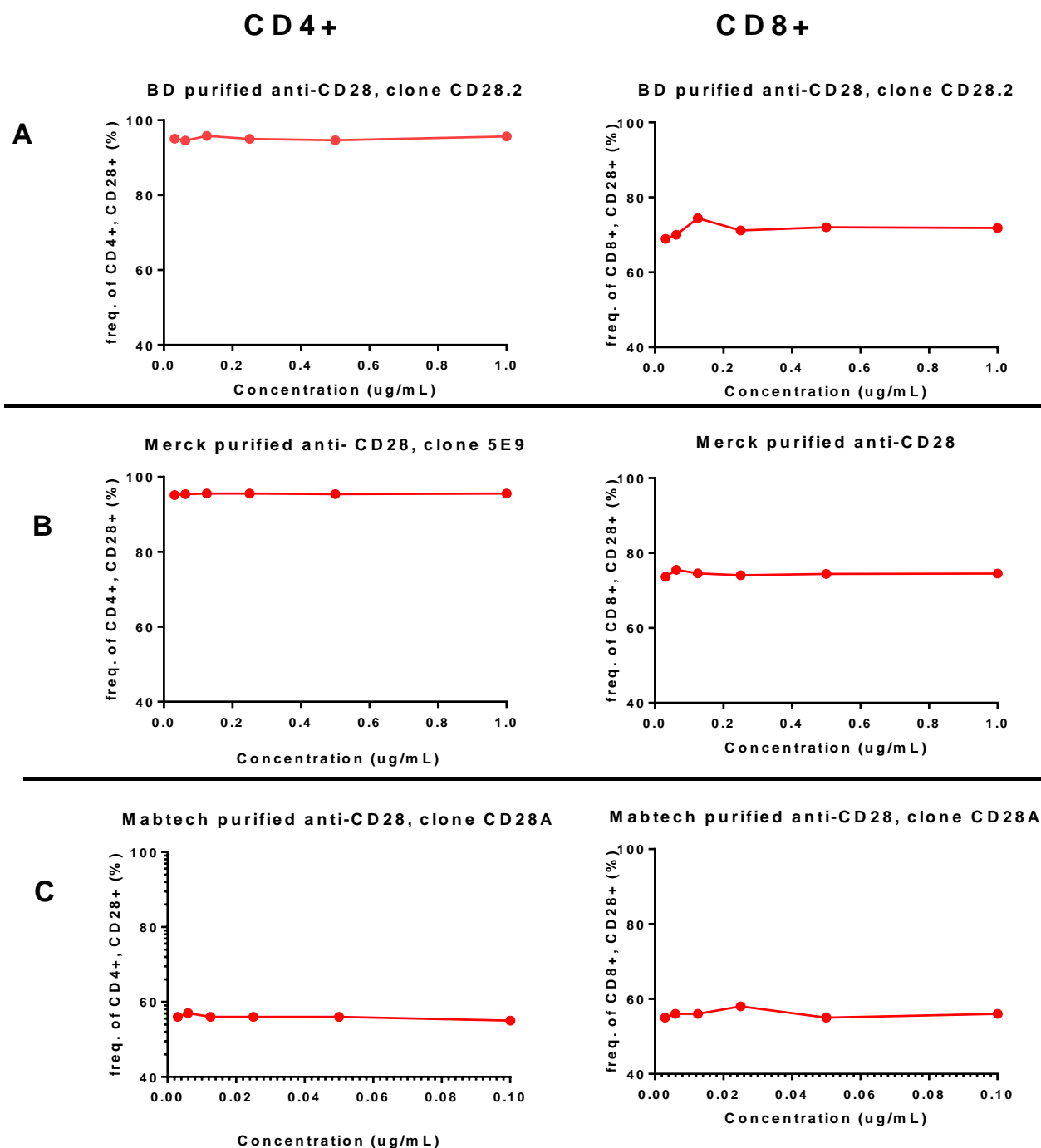


Figure 4.4 Frequency of surface CD28+ populations after pre-incubation with various clones of purified anti-CD28 during stimulation. Cryopreserved PBMCs from an unvaccinated animal was pre-incubated with **A**) pCD28 clone CD28.2, **B**) p28 clone 5E9 and **C**) pCD28 clone CD28A at serial dilutions (concentration). The cells were gated on lymphocyte populations based on SSC and FSC properties and further gated on viable (ViViD-) CD3+ populations. Graphs show the frequency of CD28 in CD4+ T cells (left panel) and CD8+ T cells (right panel).

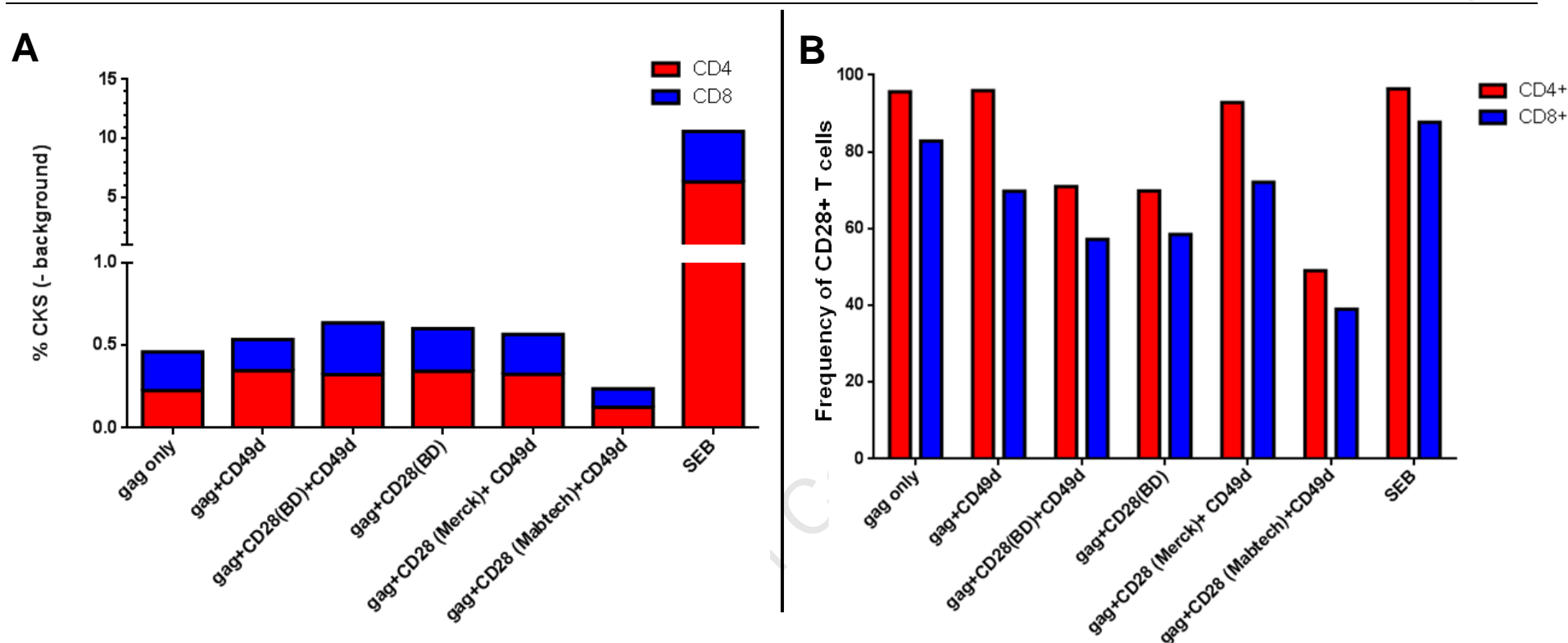


Figure 4.5. The net cytokine expression and frequency of surface CD28 FITC after stimulation of PBMCs in the presence of various clones of purified anti-CD28. Cryopreserved PBMCs from one week after vaccination with SAAVI-MVA C (animal B646) were stimulated with Gag peptide pools in the presence of purified anti-CD28 from BD, Merck and Mabtech together with purified anti-CD49. PBMCs stimulated with Gag peptide pools only and SEB only were also included. **A**) The bar graphs represent the net expression (background of 0.01% and 0.07% excluded from CD4+ and CD8+ responses respectively) of cytokines (multiplex of IL-2, IFN- γ and TNF- α on PE conjugate) when PBMCs were stimulated with Gag and various clones of the co-stimulants CD28 clone CD28.2 (CD28 BD) purchased from BD BioScience, CD28 clone E59 (CD28 Merck) purchased from Merck and CD28 clone CD28A (CD28 Mabtech), purchased from Mabtech together with CD49d. In the case of CD28 clone CD28.2 (CD28 BD), a control without CD49d (Gag only) was included. **B**) The bar graphs represent the frequency (%) of CD28 FITC CD4+ (red bars) and CD8+ (blue bars) cell populations after stimulation with a variety of purified CD28 clones.

4.4.2. Evaluation of vaccine specific memory responses

4.4.2.1. Comparison of vaccine (Gag) specific cytokine production assessed in PBMCs from baboons vaccinated with MVA-VLP, DNA-VLP prime-boost regimens or VLPs only.

Flow cytometry analysis was used to quantify the vaccine specific (Gag specific) cytokine responses induced by the MVA/VLP, DNA/VLP and VLP only groups of animals. The peak CD4⁺ and CD8⁺ cytokine responses for n=3 animals in the MVA/VLP vaccine regimen was observed 4 weeks after the VLP boost was administered. The mean of the net cytokine response at peak response were 0.21%± 0.01 and 0.24% ± 0.05 (mean± standard deviation) in the CD4⁺ and CD8⁺ T cells respectively. The cytokine response to Gag in these animals decreased after the peak at week 36 (0.13% ±0.03 and 0.15±0.04 in CD4⁺ and CD8⁺ respectively), but these responses were higher than those observed before the peak response. The peak CD4⁺ and CD8⁺ cytokine response for the DNA/VLP group n=5 was observed at week 55 (0.17%± 0.04 and 0.07%± 0.01 in CD4⁺ and CD8⁺ T cells respectively) which was also 4 weeks after receiving the first VLP boost (Figure 4.7). The cytokine responses also decreased in the CD4⁺ compartment of animals at week 63, 8 weeks after the peak response, but appeared to be maintained at a higher level (0.07%± 0.01) than prior to VLP boosting. In the CD8⁺ population, the peak response was not high compared to the CD4⁺ population, but it appears that the second VLP boost received at week 63 helped maintain a cytokine response at an average of 0.05% ± 0.009 (Figure 4.7).

The peak CD4⁺ and CD8⁺ responses for both vaccine regimens were observed 4 weeks after the first VLP was administered. The peak CD4⁺ cytokine response in the MVA/VLP group (n=3) was significantly higher (Adjusted *P* value= 0.037) compared to the VLP only group (n=2) and higher (although not statistically significant) compared to that of the DNA/VLP group (n=5). The peak CD8⁺ cytokine response in the MVA/VLP was significantly higher (Adjusted *P* value= 0.038) compared to the DNA/VLP group. However, the peak CD8⁺ cytokine responses in both vaccinated groups were higher compared to the VLP only group (although not statistically significant).

At the final time-point (Week 36) analysed in the MVA/VLP group, the mean CD4⁺ and CD8⁺ cytokine responses were significantly higher (Adjusted *P* value= 0.006 and 0.007 respectively) compared to the final time-point assessed in the DNA/VLP group (Week 71). However, the final CD4⁺ CD8⁺ cytokine responses in both vaccinated groups were higher compared to the final time-point assessed in the VLP only group (Week 20) (although not statistically significant).

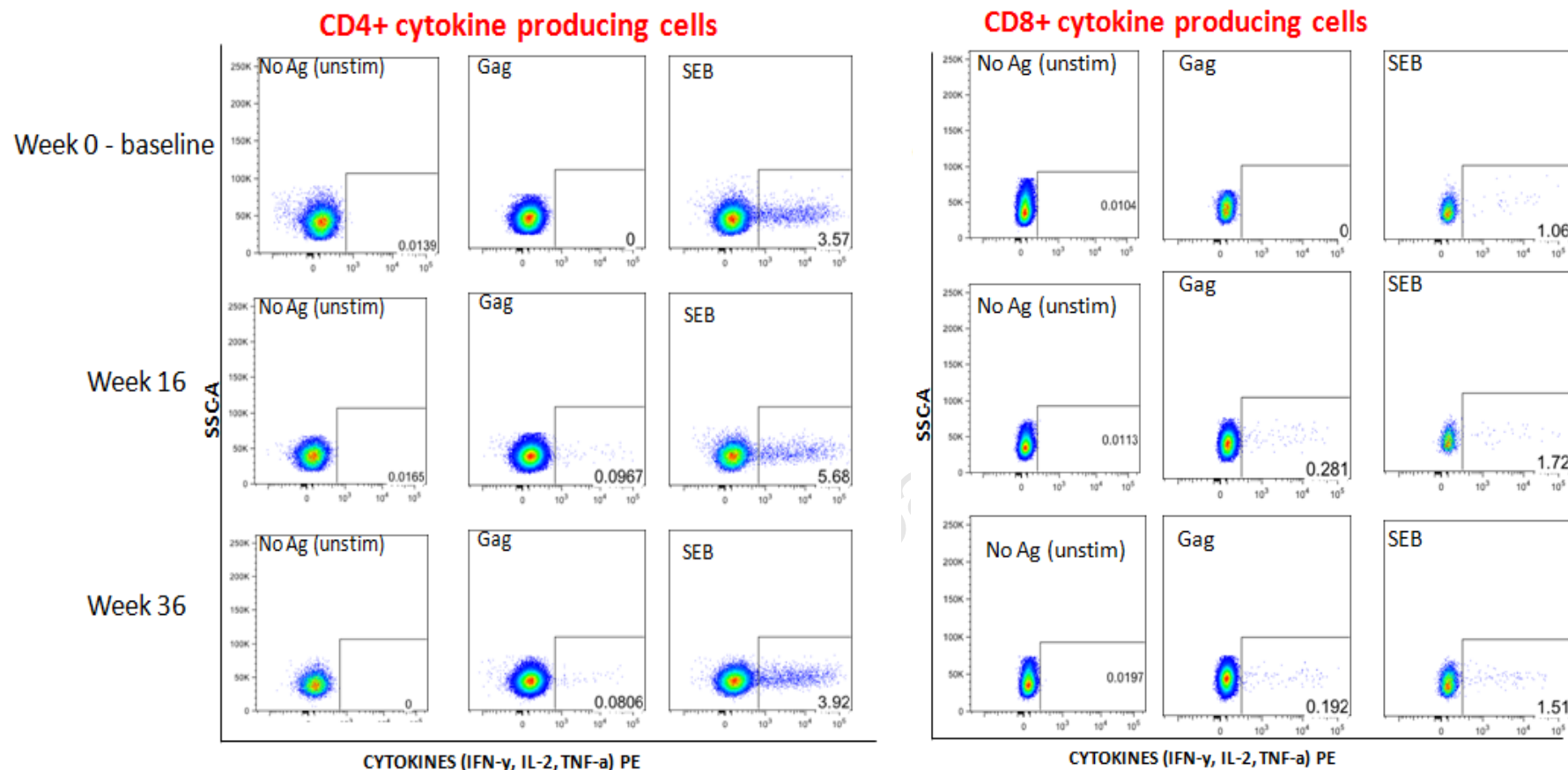
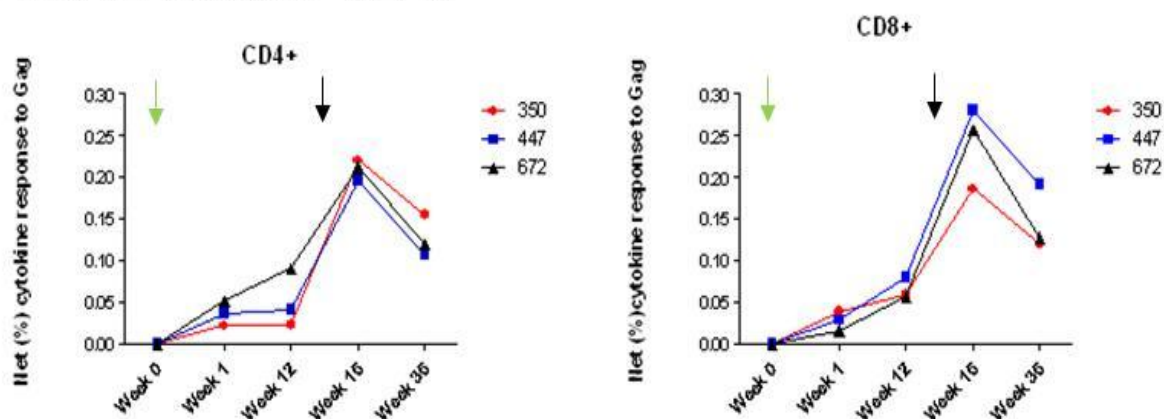
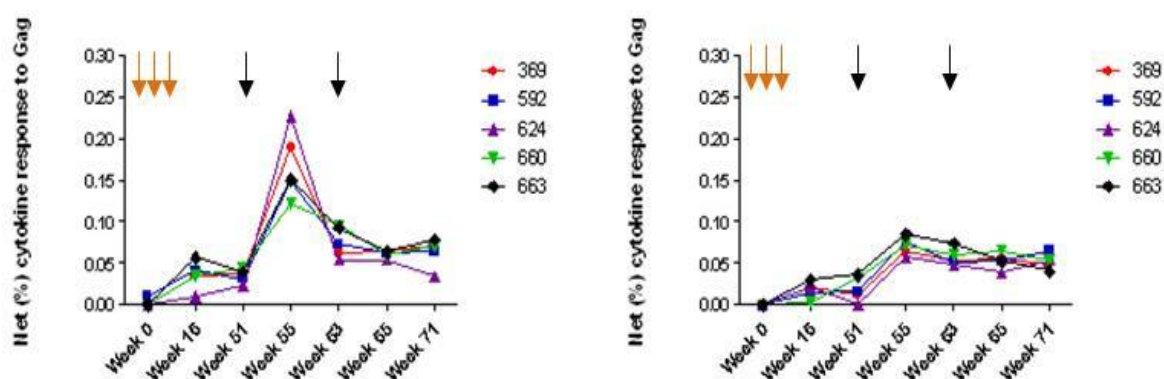


Figure 4.6. Representative pseudo-colour dot plots showing the flow cytometry analysis of vaccine specific cytokine expression (Gag) in CD4+ and CD8+ T cells in baboon 447 (SAAVI MVA-C/ VLP group). Cryopreserved PBMCs from various time-points during the vaccination schedule were stimulated with either vaccine specific Gag peptide pools (Gag), SEB or no antigen. The cells were gated on lymphocyte populations based on their SSC and FSC properties. Viable (ViViD negative) CD3+ T cells were then gated into either CD4+ or CD8+. The expression of cytokines IL-2, IFN- γ and TNF- α multiplexed onto PE were determined for both CD4+ and CD8+ T cells. The dot plots show cytokine expression relative to the negative controls in which cells were incubated in R10 media only (No Ag, unstim.) and positive control in which cells were stimulated with SEB. The net vaccine specific (Gag) cytokine response at each time-point was considered a positive response if it was at least twice the background (No Ag, unstim) and greater than the cut-off frequency value of 0.01% and greater than 25 events. Representative time-points shown include baseline (week 0), peak response (week 16, four weeks after VLP boost) and the final time-point (Week 36).

A: SAAVI MVA-C/ VLPs



B: pTH gag DNA / VLPs



C: VLPs only

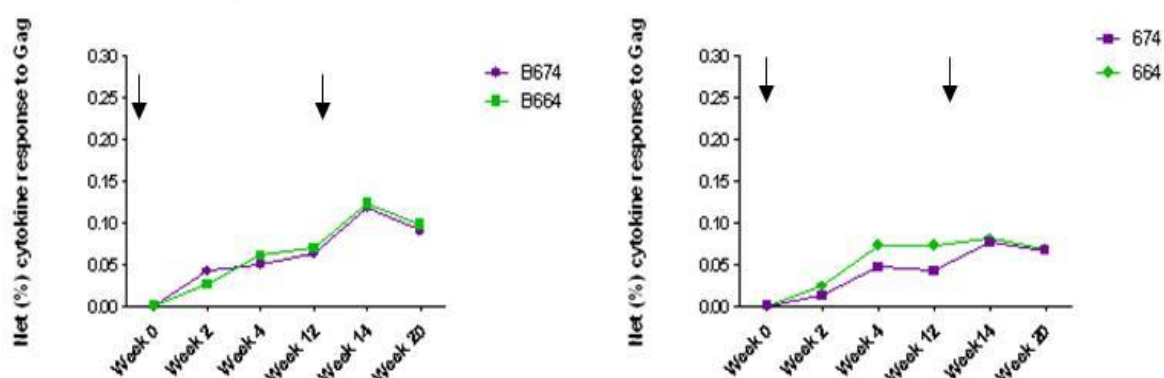


Figure 4.7. Vaccine specific (Gag) cytokine response in CD4+ and CD8+ T cells during vaccination regimens. Graphs depict the net cytokine expression in T cells for baboons that received either A) SAAVI MVA-C (green arrows) and VLP (black arrows), B) DNA (orange arrows) and C) VLP (black arrows) or VLP (black arrows) only. Net cytokine responses at each time-point were determined by subtracting the background from the unstimulated control. The net vaccine specific cytokine response was considered positive if it was at least twice the background (No Ag, unstim) and greater than the cut-off frequency value of 0.01% and greater than 25 events.

4.4.2.2. Distribution of vaccine (Gag) specific T cell memory phenotypes in PBMCs from baboons vaccinated with MVA-VLP or DNA-VLP prime-boost regimens or VLPs only.

The vaccine specific (cytokine producing cells in response to Gag) were then further analysed using CD28 and CD95 markers to determine the distribution of memory phenotypes in these vaccine specific cells.

The naïve CD4+ and CD8+ T cell populations were excluded from the statistical analysis. As a result, the distribution of effector and central memory phenotypes were determined in animals from the MVA/VLP, DNA/VLP and VLP only vaccine regimen.

Vaccination with MVA/ VLP appears to skew Gag specific CD4+ T cell responses towards the central memory phenotype (mean distribution \pm standard deviation = $84.23\% \pm 12.42$) at peak response (week 16) compared to the effector memory phenotype ($25.47\% \pm 7.33$) (Figure 4.8). A similar distribution of the memory phenotypes was maintained 20 weeks after the vaccination with a predominately Tcm phenotype ($80.4\% \pm 13.30$) compared to the Tem phenotype ($28.47\% \pm 7.33$). In these animals, Gag specific CD8+ T cell responses were more evenly distributed between Tcm and Tem phenotypes at peak response ($58.9\% \pm 21.01$ and $41.03\% \pm 20.90$ respectively) and at the final time point (20 weeks after vaccination), the mean distribution of Tcm was $55.6\% \pm 13.58$ compared to Tem which contributed to $44.40\% \pm 13.58$ of the Gag-specific CD8+ T cell responses.

A similar memory distribution profile of Gag-specific CD4+ cells in DNA/VLP vaccinated animals was observed at peak response during which responses were skewed towards Tcm phenotype ($91.17\% \pm 21.01$) and only a small percentage of responsive cells were characterised as the Tem phenotype ($6.6\% \pm 0.87$) (Figure 4.9). This skewing of CD4+ T cells towards a Tcm phenotype was maintained 16 weeks post vaccination with a mean of $88.17\% \pm 3.62$ of the responsive cells having a Tcm phenotype and $11.83\% \pm 3.62$ having a Tem phenotype. However, Gag-specific CD8+ cells were distributed in a different manner between Tcm ($44.43\% \pm 15.65$) and Tem ($55.57\% \pm 15.65$) phenotypes at peak response with Tem phenotype contributing to almost 10% more of the responsive cells whereas the distribution of CD8+ cytokine producing cells 16 weeks post vaccination were distributed between Tcm ($51.40\% \pm 17.29$) and Tem ($48.60\% \pm 17.29$).

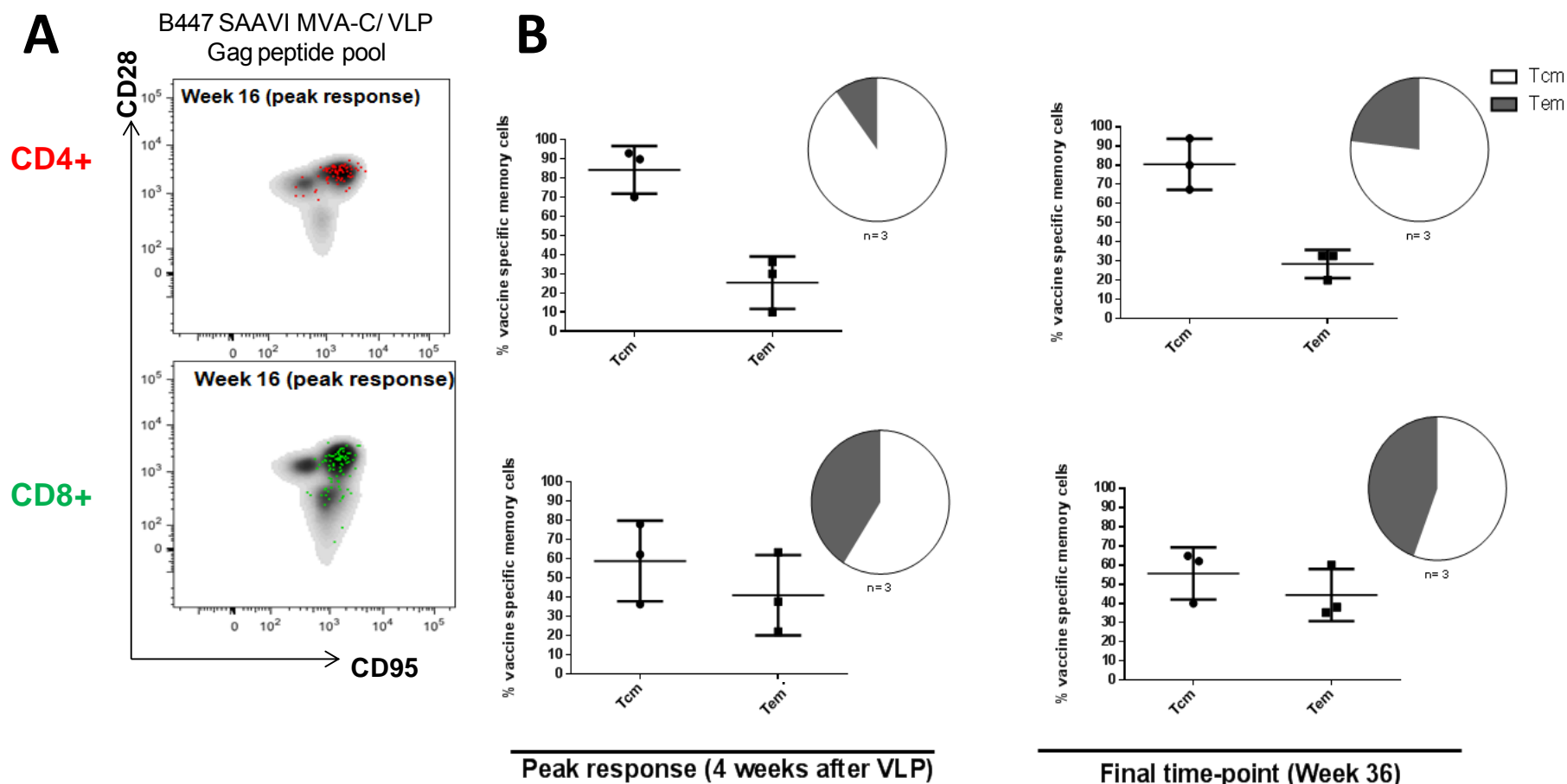


Figure 4.8. Memory phenotype distribution of Gag-specific T cells following vaccination with SAAVI MVA-C/VLP in prime-boost. Cryopreserved PBMCs from vaccinated Chacma baboons were examined for Gag-specific memory T cells. Cytokine producing CD4+ and CD8+ T cells were delineated into central (Tcm) and effector (Tem) memory cells based on CD28 and CD95 expression. A) Representative flow cytometry plots of the memory profile of total CD4+ (upper panel) and CD8+ (lower panel) T cells shown as density plots with overlaid Gag-specific total cytokine producing cells (red and green dots for CD4+ and CD8+ T cells respectively) in a vaccinated animal (B447) at peak response (Week 16). B) Proportion of Gag-specific CD4+ (upper panel) and CD8+ (lower panel) T cells at peak response (Week 16, 4 weeks after VLP vaccination) and the final time-point assessed (Week 36). Data are depicted as mean \pm standard deviation and summarised in adjacent pie charts. A total of three vaccinated animals were evaluated at both time-points.

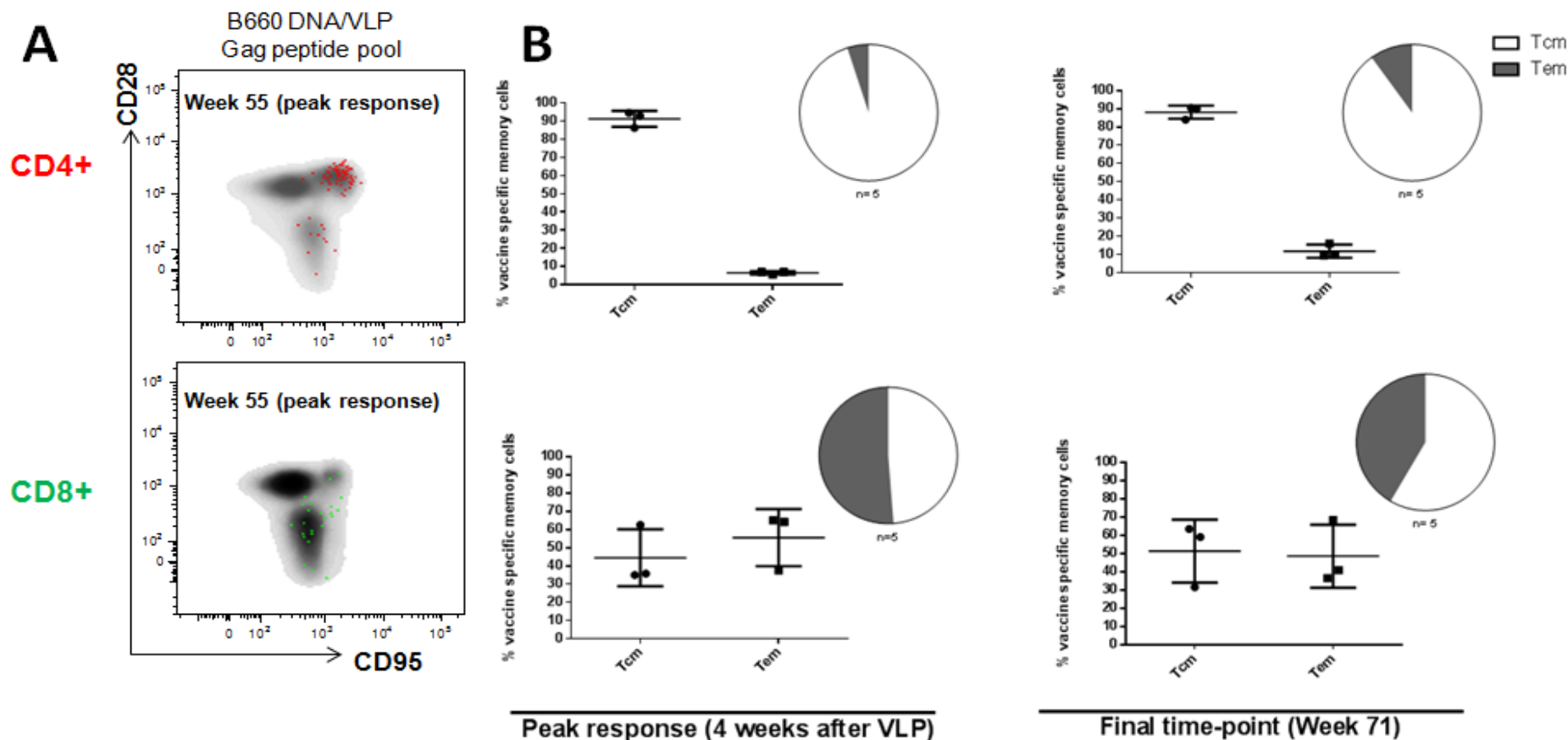


Figure 4.9. Memory phenotype distribution of Gag-specific T cells following vaccination with DNA/VLP in prime-boost. Cryopreserved PBMCs from vaccinated Chacma baboons were examined for Gag-specific memory T cells. Cytokine producing CD4+ and CD8+ T cells were delineated into central (Tcm) and effector (Tem) memory cells based on CD28 and CD95 expression. A) Representative flow cytometry plots of the memory profile of total CD4+ (upper panel) and CD8+ (lower panel) T cells shown as density plots with overlaid Gag-specific total cytokine producing cells (red and green dots for CD4+ and CD8+ T cells respectively) in a vaccinated animal (B660) at peak response (Week 55). B) Proportion of Gag-specific CD4+ (upper panel) and CD8+ (lower panel) T cells at peak response (Week 55, 4 weeks after VLP vaccination) and the final time-point assessed (Week 71). Data are depicted as mean \pm standard deviation and summarised in adjacent pie charts. A total of five vaccinated animals were evaluated at both time-points.

4.5 Summary of key findings

This study shows that two out of three available clones of co-stimulatory pCD28 antibodies can be used in combination with pCD49d antibodies to augment the production of Gag-specific cytokine production by CD4⁺ and CD8⁺ T cells. These clones include CD28.2 and CD28A from BD Bioscience and Merck respectively, do not block the detection of surface CD28 by surface staining for flow cytometry analysis. However the E59 clone of pCD28 purchased from Mabtech, reduces the detection of CD28 and was therefore not used in this study.

The major findings of this study demonstrate that vaccination with SAAVI MVA-C and VLP prime boost induces the production of Gag-specific cytokines in CD4⁺ and CD8⁺ T cells from the peripheral blood of Chacma baboons. The peak response was observed at week 16, four weeks following the VLP boost vaccination and the CD4⁺ peak response was significantly higher compared to the VLP only group. At peak response, the CD8⁺ responses were significantly higher in the MVA/VLP group compared to the DNA/VLP group. This response was maintained at levels lower than the peak at week 36. In addition, vaccination with DNA/VLP prime boost induced cytokine producing CD4⁺ and CD8⁺ T cells in response to Gag stimulation in peripheral blood from Chacma baboons. The peak cytokine responses were observed at week 55, four weeks after the animals received the first VLP boost vaccination. This response was still detectable at week 71. However, CD4⁺ and CD8⁺ were significantly lower compared to the responses observed at the final timepoint (Week 36) in animals vaccinated with MVA/VLP.

Vaccination with SAAVI MVA-C/VLP induced Gag-specific CD4⁺ and CD8⁺ T cell responses that were skewed towards the T_{cm} phenotype at peak response. Gag-specific CD4⁺ cells persisted for 20 weeks post vaccination and were predominantly central memory T cells. Vaccine specific CD8⁺ T cells were also observed at this stage and comprised around 40% of total vaccine specific memory. Further, the DNA/VLPs prime boost regimen induced quantifiable Gag-specific CD4⁺ and CD8⁺ responses which persisted for 16 weeks after the peak response. These responses were comprised predominately of CD4⁺ T_{cm} and both CD8⁺ T_{cm} and T_{em} which was maintained at week 71.

Chapter 5.

University of Cape Town

Chapter 5. Discussion

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5.1 Introduction

The ability of candidate HIV-1 vaccines to induce the differentiation of long-lived, functional and vaccine specific T cell memory recall responses is a measure of vaccine immune response and durability. Both central memory and effector memory T cells work in synergy to induce secondary responses to antigen (Sprent and Surh, 2002). In general, Tcm cells provide a pool of antigen specific precursor cells capable of rapid proliferation in lymphoid tissues to generate and replenish effector or effector memory cells (Sallusto *et al.*, 2004) whilst Tem cells are associated with a robust effector response at the site of infection or pathogen entry (Lefrancois and Masopust, 2002).

One of the aims of this study was to investigate various anti-human antibody markers of T cell function and maturation to select a suitable combination of markers which can be used to detect T cell memory phenotypes in peripheral blood mononuclear cells (PBMCs) from Chacma baboons. In addition, this study aimed to determine the suitability of the selected combination for use in multicolour flow cytometry. The key aim of this study was to develop a multi-parameter flow cytometry panel to assess vaccine specific T cell memory response in PBMCs from Chacma baboons. These panels were then used to assess the vaccine specific memory recall responses induced by the candidate SAAVI MVA-C/VLP and DNA/VLP prime-boost vaccination regimen in Chacma baboons by flow cytometry.

5.2. Evaluation of commercially available clones of anti-human T cell memory markers for cross-reactivity with baboon PBMCs

Several clones of anti-human antibody markers were evaluated for cross-reactivity using cryopreserved PBMCs isolated from healthy, unvaccinated Chacma baboons. Cross-reactivity was confirmed for various clones of T cell phenotype markers (CD3, CD4, and CD8), maturation markers (CD28, CD27, CD95, CD45RA) and functional capacity (IL-2, TNF- α , IFN- γ). However, anti-human marker of CCR7 (clone 150503), the chemokine receptor commonly used in combination with CD45RA in human and rhesus macaque (Mooij *et al.*, 2009) studies to delineate T cell memory phenotypes did not cross-reactively stain PBMCs isolated from Chacma baboons despite staining cells at the recommended temperature (37°C). This marker was therefore excluded from memory marker panels to investigate T cell memory phenotype distribution in Chacma baboons. Furthermore, CCR5 (clone 3A9) did not appear to be cross-reactive in Chacma baboon PBMCs. This marker is commonly used to investigate the level of T cell activation and HIV-1 target cells (CD4+CCR5+) in rhesus macaques (Liu *et al.*, 2009) and humans. The expression of CCR5 on CD4+ T cells could therefore not be evaluated in this study.

Chemokine receptor detection sensitivity is reduced during the freeze-thawing of cryopreserved PBMCs. Other findings indicate that the detection of surface CCR5 is reduced in cryopreserved PBMCs compared to freshly isolated samples in human studies (Costantini *et al.*, 2003). It is hypothesised that this reduction in the surface expression of CCR5 is to be due to molecular shedding of the CCR5 receptors during the cryopreservation process itself (Costantini *et al.*, 2003). The length of cryopreservation and liquid nitrogen storage also impacts negatively on the detection of chemokine receptor markers (Tollerud *et al.*, 1991). It is recommended by the manufacturers that staining with these chemokine receptors should be performed on freshly isolated T cells instead. In addition, it is also possible that genetic differences between rhesus macaques and Chacma baboons could also account for the limited recognition of the CCR5 molecule in this study.

In this study, the optimal staining temperature was also determined by staining cryopreserved PBMCs from healthy Chacma baboons at either room temperature, 37 degrees Celsius or on ice. Since a higher level of non-specific fluorescence was observed at 37°C compared to that at room temperature and the lowest level of background staining was observed at room temperature (+/- 23 to 27 °C) this study suggests that baboon PBMCs should be stained at room temperature. This was in keeping with previous flow cytometry analysis performed using baboon PBMCs by other groups within the Division of Virology, IIDMM, and UCT.

5.3. Selection of suitable T cell memory markers for inclusion in a flow cytometry panel to investigate memory phenotypes in baboon PBMCs.

Four colour flow cytometry antibody panels were used to investigate which combination of memory markers was most suitable to include in a final panel for the analysis of memory responses in PBMCs isolated from baboons. In this study, CD45RA and CD95 allowed for the separation of CD4+ and CD8+ naive T cells (T_{naive}: CD45RA+CD95-) and memory T cells (CD45RA-CD95+) but clear T_{cm} and T_{em} populations were not distinguishable. The addition of either a co-stimulatory molecules (such as CD28 or CD27) or CCR7 would be useful in this regard (Sun *et al.*, 2005). However, CCR7 was not found to be cross-reactive with cryopreserved PBMCs isolated from Chacma baboons and a minimum of two markers could be accommodated in this panel. The CD27 and CD95 markers separated naive T cells (CD27+ CD95-) from T_{cm} (CD27+CD95+) and T_{em} (CD27-CD95+) but the separation was not as clear compared to the CD28 and CD95 combination. Using the combination of CD28 and CD95, the memory phenotypes were defined as central memory (T_{cm}; CD28+CD95+) and effector memory (T_{em}; CD28-CD95+) populations with the naïve T cells being defined as (CD28+CD95-). The CD28 and CD95 marker combination was therefore selected for

inclusion in a flow cytometry panel to further investigate T cell memory phenotypes in PBMCs from Chacma baboons.

5.4. The characteristics of T cell memory phenotypes delineated by the selected combination of markers to determine the suitability of these markers for inclusion in a flow cytometry panel using baboon PBMCs.

The results of this study show that in combination, anti- CD28 and -CD95 antibodies provide a distinct separation of total effector and central memory T cells from the naïve T-cell subset. Consistent with the findings of other studies conducted in adult rhesus macaques (Jankovic *et al.*, 2003; Pitcher *et al.*, 2002) and cynomolgus monkeys (Nadazdin *et al.*, 2010) the relative proportions of CD28+CD95+ central memory and CD28-CD95+ effector memory cells in peripheral blood of Chacma baboons varied in the CD4+ and CD8+ compartments. A larger proportion of Tcm cells were observed in the CD4+ compartment compared to the CD8+ T cells while the Tem were the primary CD8+ memory phenotype. There was no significant variation in the frequencies of memory subpopulations in both CD4+ and CD8+ T-cells. However, there were a higher proportion of Tem cells in the CD8+ compartment compared to the CD4+ T-cells while the opposite was true for Tcm cells. This observation was also found in other pilot studies using baboon PBMCs conducted within the Division of Medical Virology.

Table 5.1. Comparison of the frequency (%) of CD4+ and CD8+ memory T cell populations amongst non human primates.

| Non human primate | CD4+ (%) | | | CD8+ (%) | | |
|--------------------------------|----------------|------------|------------|----------------|------------|------------|
| | <i>T naive</i> | <i>Tcm</i> | <i>Tem</i> | <i>T naive</i> | <i>Tcm</i> | <i>Tem</i> |
| Rhesus macaque ^A | 25-30 | 30-60 | 15-20 | 25-30 | 15-20 | 50-70 |
| Cynomolgus monkey ^B | 30-50 | 30-50 | 10-15 | 30-40 | 20 | 50-60 |
| Chacma baboon ^C | 49.2 | 27.3 | 16.8 | 37 | 27 | 42 |

A. Sun *et al.* (2005); Jankovic *et al.* (2003); Pitcher *et al.* (2002); **B.** Nadazdin *et al.*, 2010; **C.** Findings of current study (Chapter 3)

The Tcm and Tem phenotypes delineated by the selected anti- CD28 and -CD95 antibody markers were then further characterised in terms of level of maturation (measured as expression of CD45RA). These subsets were also characterised further by investigating the expression of the early activation marker (CD69) and the production of cytokines (IL-2 and IFN- γ) in response to SEB stimulation. The Tcm and Tem subsets exhibited the expected functional and phenotypic characteristics, similar to those previously observed for T cells

from normal rhesus macaques (Pitcher *et al.*, 2002, Jankovic *et al.*, 2003; Sun *et al.*, 2005). In particular, CD45RA expression was investigated in the T cell memory phenotypes defined by CD28 and CD95. This isoform of CD45 was used because it is available in numerous clones that have been found to cross-react with non-human primate species. In contrast clones of CD45RO which is commonly used to delineate T cell memory populations in human studies, do not cross react with rhesus macaques (of Chinese origin) (Wang *et al.*, 2008) and cross-reactivity with Chacma baboons have not been determined. In this study, the expression of CD45RA was limited to CD4+ and CD8+ naive T cells and IL-2 production was observed primarily in CD28+CD95+ central memory CD4+ and CD8+ T cells. This phenotype of memory T cell has the ability to rapidly proliferate and differentiate further into effector or effector memory T cells upon antigen exposure, a characteristic influenced by the release of IL-2, a cytokine involved in the homeostatic maintenance of memory T cells (Sallusto *et al.*, 2004).

On the other hand, IFN- γ production was higher in Tem cells compared to naive and Tcm in the CD4+ and CD8+ compartments but was also produced by CD8+ Tcm cells. The production of IFN- γ is associated with the ability of CD8+ Tcm and Tem to induce a robust, sustained antiviral response to antigen at the level of the lymphoid tissues and mucosal portals of viral entry respectively. CD69 expression was not significantly different amongst populations of memory and naive T cells in the peripheral blood CD4+ and CD8+ compartments. However, both CD4+ and CD8+ memory phenotypes appeared to express more CD69 compared to naive T cells in response to SEB stimulation. This indicates that the memory populations defined by CD28 and CD95 markers are more responsive to antigen stimulation compared to naive T cells, an expected outcome since naive T cells have a lower activation threshold compared to memory T cells (Kimachi *et al.*, 2003) These results therefore indicate that the combination of anti- CD28 and -CD95 antibody markers effectively separate naive T cells , Tem and Tcm from each other and are suitable for inclusion in a multicolour flow cytometry panel for evaluation of candidate HIV vaccines in the baboon model.

5.5. Optimisation of a flow cytometry panel for the investigation of vaccine specific memory T cell responses in PBMCs isolated from Chacma baboons.

The measurement for cytokine production in response to vaccine specific antigens is a method employed to identify vaccine responsive cells (Jung *et al.*, 1993). However, the addition of co-stimulatory molecules such as purified CD28 (Waldrop *et al.*, 1998) and CD49d (Udagawa *et al.*, 1996) is required to augment the *in vitro* stimulation of cytokine

production. In this study, CD28 was also used as a surface T cell memory marker and the addition of purified CD28 during stimulation could block the CD28 surface receptors and reduce the detection of this marker by flow cytometry analysis. Three clones of purified CD28 was used as co-stimulatory antibodies in PBMCs isolated from Chacma baboons to determine which clone adequately augmented cytokine production without reducing the detection of surface CD28 markers by flow cytometry analysis.

Clones CD28.2 (BD Bioscience) and 5E9 (Merck) of purified CD28 did not compromise the detection and frequency of surface CD28 receptors on CD4⁺ and CD8⁺ T cells during flow cytometry analysis when used at 1 μ g/ml and 0.1 μ g/ml respectively. Co-stimulation with Gag peptide pools and purified anti- CD28 (clones CD28.2 and 5E9) together with pCD49d improved cytokine production in both CD4⁺ and CD8⁺ compared to cells which were only stimulated with peptide pools. However, co-stimulation of Gag-peptide pools with CD28 (clone CD28A, from Mabtech) and CD49d failed to augment cytokine production in CD4⁺ and CD8⁺ T cells compared to when cells were stimulated with Gag peptides only. Further, co-stimulation with this clone reduced the frequency of surface CD28 detection by flow cytometry analysis. This clone was therefore not used in this study. It is possible that this clone blocked the CD28 receptors during stimulation and fluorochrome bound anti-CD28 antibodies could not attach to the cell surface during staining. This could explain the decrease in the frequency of CD28 expression during flow cytometry analysis.

5.6. Vaccine specific T cell memory phenotype distribution in Chacma baboons vaccinated with SAAVI MVA-C/VLP and DNA/VLP prime/boost vaccine regimens

The memory markers, anti- CD28 and -CD95 were then used to develop and optimise flow cytometry panels to investigate the distribution of memory T-cell phenotypes in Chacma baboons vaccinated with either SAAVI MVA-C prime and VLP boost, DNA prime and VLP boost or VLP only vaccine regimens. This panel developed also comprised of cytokines (IL-2, IFN- γ and TNF- α) multiplexed onto one fluorochrome (PE) for the detection of Gag-specific cytokine producing T cells in response to *in vitro* stimulation of PBMCs from vaccinated Chacma baboons.

This study demonstrate that vaccination with SAAVI MVA-C and VLP prime boost induces the production of Gag-specific cytokines in CD4⁺ and CD8⁺ T cells from the peripheral blood of Chacma baboons. The peak response was observed at week 16, four week following the VLP boost vaccination and the CD4⁺ peak response was significantly higher compared to the VLP only group. At peak response, the CD8⁺ responses were significantly higher in the

MVA/VLP group compared to the DNA/VLP group. This response was maintained at levels lower than the peak at week 36 suggesting that vaccination with MVA/VLP induces improved Gag-specific CD8+ responses in Chacma baboons

In addition, vaccination with DNA/VLP prime boost induced cytokine producing CD4+ and CD8+ T cells in response to Gag stimulation in peripheral blood from Chacma baboons. The peak cytokine responses were observed at week 55, four weeks after the animals received the first VLP boost vaccination. This response was still detectable at week 71. However, CD4+ and CD8+ were significantly lower compared to the responses observed at the final time point (Week 36) in animals vaccinated with MVA/VLP but higher than those observed in animals in the VLP only group.

The findings of this study indicate that vaccination with the SAAVI MVA-C/ VLP prime boost regimen generated Gag-specific cytokine producing CD4+ cells which persisted for 20 weeks post vaccination and were predominantly central memory T cells. Similar distribution patterns of T cell memory phenotypes following vaccination were observed in the DNA/VLP prime boost regimen in which CD4+ T cell responses persisted for 16 weeks after vaccination and were skewed towards a Tcm phenotype. The skewing of vaccine specific CD4+ T cell responses in both vaccine regimens towards the Tcm phenotype imply that these vaccines have the capacity to generate CD4+ Tcm pools associated with rapid differentiation and replenishment of vaccine specific effector cells. Previous studies provide further evidence that the establishment and preservation of HIV/SIV/SHIV specific CD4+ Tcm pools by vaccination are important in preventing AIDS pathogenesis. Immunization with plasmid DNA and recombinant adenovirus SIV vaccines induced prolonged survival following SIV or SHIV 89.6P challenge and was attributed to the preservation of CD4+ Tcm cells in vaccinated rhesus macaques (Letvin *et al.*, 2006; Sun *et al.*, 2005, Liu *et al.*, 2008). HIV-1 infected individuals on ART that were vaccinated with HIV-1 rgp160 demonstrated an increase in HIV-1 specific CD4+ Tcm population and improved level of survival compared to unvaccinated HIV-1+ patients (Gudmundsdotter *et al.*, 2008). The importance of a vaccine induced pool of antigen specific CD4+ Tcm has been highlighted in previous studies investigating the turn-over of T cell memory populations in healthy humans. These data shows that CD4+ Tem cells are replaced at a faster rate than CD4+ Tcm cells and imply that a continuous supply of new cells is required to maintain the CD4+ Tem population to provide a sustained rapid effector response at the sites of infection (Macallan *et al.*, 2004). The same phenomenon has been observed in SIV challenged rhesus macaques in which the gradual depletion and infection of CD4+ Tcm by continual immune activation limits and disrupts the

homeostatic generation of CD4⁺ Tem at sites of infection and correlates to disease progression (Okoye *et al.* 2007).

In this study, vaccination with MVA/VLP also induced vaccine specific CD8⁺ T cells which were almost evenly distributed between Tcm and Tem at the peak response. Gag-specific, cytokine producing CD8⁺ cells persisted for 20 weeks post vaccination and were comprised of both Tcm and Tem phenotypes. Similarly, in the DNA/VLP regimen, quantifiable Gag-specific CD8⁺ responses were detected and persisted for 16 weeks after the peak response. These responses were both CD8⁺ Tcm and Tem cells. This result indicates that both vaccination regimens induced quantifiable CD8⁺ Tcm which have been associated with rapid proliferation in response to antigen (Wherry *et al.*, 2003) and the maintenance of sustained CD8⁺ T cell secondary memory response which are important in controlling prolonged infections (Butler *et al.*, 2011). The presence of CD8⁺ Tcm has previously been inversely correlated to SIVmac251 replication in challenged rhesus macaques following prime/boost vaccination with DNA/NYVAC, suggesting that these cells are associated with the control of viral replication (Vaccari *et al.*, 2005). Acierno *et al.* (2006) also demonstrated a loss in Gag-specific CD8⁺ Tcm in unvaccinated rhesus macaques which progressed rapidly to disease compared to rhesus macaques vaccinated with DNA/recombinant pox- and adeno-virus vectors following SHIV challenge.

In addition, the presence of Gag specific CD8⁺ effector memory T cells observed in the current study has also been previously associated with the control of infection by inducing immediate effector functions at the portals of mucosal HIV-1 entry without having to undergo further proliferation. In fact, Hansen *et al.* 2009 demonstrated that SIV challenge in rhesus macaques vaccinated with rhesus macaque cytomegalovirus (RhCMV) vectors, effectively controlled infection by generating Gag-specific CD8⁺ Tem biased response, particularly in the mucosal portals of entry. Since most HIV-1 infections are acquired via the mucosal route, vaccination regimens, which induce a population of available CD8⁺ Tem at these sites will be invaluable for the control of viral replication early on during infection. In the current study we have identified Gag-specific CD8⁺ Tem in the peripheral blood of Chacma baboons vaccinated with either DNA/VLP or MVA/VLP and this implies that both vaccine regimens are capable of inducing a population of vaccine specific Tem cells. However, the functional quality and the distribution of these vaccine specific CD8⁺ Tem cells in mucosal tissues are yet to be determined.

In summary, this study demonstrated that both MVA/VLP and DNA/VLP vaccination effectively induced long-lived Gag-specific memory T cells compared to the VLP only control group.

5.7. Future research

In this study, only cryopreserved peripheral lymphocyte samples were available for analysis. Identifying cellular immune responses in blood alone does not adequately summarise the full milieu of the vaccine specific immune responses. Further assessment of lymphoid tissues and mucosal tissues to determine the distribution of memory T cells in response to vaccination was therefore not possible in this study, but can and should be done in future work. In addition, vaccine induced T cell activation and inflammation were not investigated in this study and need to be assessed in order to further characterise the safety of these candidate HIV-1 vaccines. In addition, the pre-existing anti-vector immune responses to the candidate vaccine regimens, particularly to SAAVI MVA-C need to be investigated in future studies. In addition, the vaccine specific cytokine responses in this study were only monitored for up to 20 weeks and 16 weeks post vaccination in the MVA/VLP and DNA/VLP groups respectively due to the limited availability of cryopreserved samples. It is therefore not clear whether the vaccine responses plateaued or if they waned over time. Future experiments should therefore also include time-points beyond those examined in this study. Although this study was based on T cell memory responses, an ideal vaccine should be able to induce both T cell and B cell immune responses. It has been demonstrated in the RV144 study that vaccines with the ability to induce non-neutralising antibodies can be protective (Haynes *et al.*, 2012; Zolla-Pazner *et al.*, 2013). Therefore, more experiments should be performed to further characterise the B cell immune responses induced by these vaccine regimens.

5.8. Conclusion

In this study, anti-human antibodies which are markers of maturation and that were cross-reactive with baboon PBMCs were identified. A suitable combination of two antibodies was identified and shown to be suitable for delineating T cell memory phenotypes. Used in combination, CD28 and CD95 were confirmed as suitable markers to delineate T cell memory phenotypes using PBMCs isolated from Chacma baboons. The memory T cell phenotypes defined by these markers had the expected maturation, functional and activation characteristics in terms of CD45RA, IL-2, IFN- γ and CD69 expression respectively. These markers were then used together with markers of viability (ViViD), T cell phenotype (CD3, CD4, CD8) and cytokines multiplexed onto one colour (IL-2, TNF- α , IFN- γ) to develop a

multiparamter flow cytometry panel . This panel was then used to further investigate the distribution of vaccine specific T cell memory phenotypes in PBMCs isolated from Chacma baboons vaccinated with prime-boost regimens of either SAAVV MVA-C/VLP orDNA/VLP.

This study also demonstrates that vaccination with SAAVI MVA-C/VLP and DNA/VLP prime-boost vaccination regimen successfully induced functional, long-lived vaccine specific, Tcm and Tem in the peripheral blood of Chacma baboons and further support the use of Chacma baboons, a nonhuman primate model readily available in southern Africa, in HIV vaccine research. In addition, this study suggests that South African developed HIV-1 vaccines, capable of inducing long-lived, functional memory T cell responses are not far from realisation.

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University of Cape Town