The development of novel HIV-1 vaccines using modified recombinant BCG

By

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“Somewhere, something incredible is waiting to be known”

~ Carl Sagan

“Science knows no country, because knowledge belongs to humanity, and is the torch which illuminates the world”

~ Louis Pasteur

“There is a single light of science, and to brighten it anywhere is to brighten it everywhere”

~ Isaac Asimov

GENERAL CONTENTS

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PLAGIARISM DECLARATION

This study was performed in the Division of Medical Virology, Department of Pathology of the University of Cape Town, under the supervision and guidance of Prof Anna-Lise Williamson and Dr Rosamund Chapman. I hereby declare this to be my own work compiled using my own words. Each significant contribution to this study as well as work of others has been referenced. Specialist assistance kindly provided by other people has also been acknowledged.

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ABSTRACT

Vaccination continues to represent the most effective means of providing immunological protection against infectious disease in human populations. With the World Health Organisation (WHO) reporting over 1.2 million AIDS related deaths in 2014, an efficacious HIV1 vaccine is urgently needed. The UCT Human Immunodeficiency Virus (HIV) Vaccine Development Group has focussed on understanding novel HIV-1 vaccine vectors, such as modified BCG, combined with modified vaccinia Ankara in the context of heterologous prime boost regimes. The tuberculosis vaccine Bacillus Calmette–Guérin (BCG) has a wellestablished safety profile as well as notable adjuvant activity with an estimated 3 billion doses administered globally since 1921. The development of modern molecular biology techniques in recent times has led to the creation of modified strains of BCG which have been shown in many cases to be safer and/or more immunogenic than the wild type strains in terms of delivering heterologous antigen.

This study focuses on exploiting and combining two particular strategies of rBCG modification. The first is the development of auxotrophic strains that contain deletions geared towards preventing the bacteria from synthesising essential growth compounds or amino acids. An example of this is the Δ*panCD* auxotroph of rBCG which does not synthesise pantothenic acid and thus has limited intracellular replication leading to less pathology. The UCT group has previously reported that HIV vaccines vectored by the Pasteur strain of rBCG Δ*panCD* induced less pathology but improved immunogenicity as compared to Pasteur WT in the murine model (when used a prime in conjunction with an MVA boost). A second notable BCG modification strategy is the inclusion of exogenous genes to improve the immunogenicity of rBCG as a vaccine vector. An example of this is the insertion of the detoxified *Clostridium perfringens* toxin, perfringolysin O (*pfo*), into the rBCG genome. Upon expression, *pfo* forms pores in the endosome which facilitates translocation of vaccine derived antigen into the cytoplasm. This modification has been shown to lead to cross priming of T cells and improve the induction of vaccine specific CD8+ T cell as compared to controls.

For this study, recombinant BCG (rBCG) based vaccines expressing codon optimized HIV-1 subtype C Gag, were constructed using three novel BCG strains and a standard strain (kindly supplied by AERAS): the Danish wild type strain of BCG (rBCG WT), a pantothenate auxotrophic strain of BCG Danish (rBCG Δ*panCD*), the Danish strain expressing perfringolysin O (rBCG *pfo*) and a pantothenate auxotrophic Danish strain expressing perfringolysin O (rBCG Δ*panCDpfo)*. We hypothesized that priming with either rBCG Δ*panCD* (*gag*) or rBCG Δ*panCDpfo* (*gag*) would lead to less pathology and enhanced T cell responses whilst priming with either rBCG *pfo* (*gag*) or rBCG Δ*panCDpfo* (*gag*) would lead to improved T cell responses (as compared the wild type strain following a SAAVI MVA-C boost).

A longitudinal study was firstly undertaken to assess the overall pathology which included; splenocyte phenotyping, assessment of granuloma formation, iNos activity in the liver and disseminated bacterial load in lymph nodes. Mice were vaccinated on day 0 with rBCG vaccines (expressing Gag or an empty vector control, 1 x 10^7cfu/200 µl, i.p injection) and sacrificed at multiple time points: days 2, 3, 7, 12, 21 and 28 post vaccination. Mice vaccinated with rBCG Δ*panCD* (*gag*/control) as well as rBCG Δ*panCDpfo* (*gag*/control) were able to clear the mycobacteria by day 7 post-infection. This clearance could have been due to immune responses or alternatively the inability of the mycobacteria to survive in an environment limited in pantothenate. In addition to clearance, mice vaccinated with Δ*panCD* and Δ*panCDpfo* (*gag*/control) presented with significantly fewer liver granulomas (as measured by CD3+ staining) and less active granulomas (as measured by iNos) as compared to mice vaccinated with the WT and pfo strains. The formation and maintenance of granulomas is indicative of the inability of the immune system to clear infection. This data demonstrates the superiority of Δ*panCD* well as Δ*panCDpfo* strains of rBCG in inducing less pathology than the WT Danish and the pfo strains.

Next, the magnitude and quality of vaccine induced HIV specific T cell immune responses following ex vivo stimulation of splenocytes was assessed in mice primed with rBCG and boosted with SAAVI MVA-C. Responses to stimulation with two HIV-specific CD4+ and one

CD8+ peptide were measured by IFNγ ELISPOT assay, cytokine bead array (CBA) and multi-parameter flow cytometry. All rBCG (gag) vaccines were able to prime for significant vaccine specific boost responses. The response to the two CD4+ peptides tested differed according to the rBCG used to prime the MVA response. Whilst rBCG *pfo* (*gag*) and rBCG Δ*panCD* (*gag*) primed for significant CD4+ (13) and CD4+ (17) boost responses respectively, rBCG Δ*panCDpfo* primed for a SAAVI MVA-C boost of both CD4+ epitopes. An increased breadth of CD4+ responses has been shown to mechanistically improve CD8+ T cell responses and lead to lower viral load in chronically infected HIV+ individuals. Multi-parameter flow cytometry indicated that the rBCG modifications can work synergistically to induce the greatest collective HIV specific bi- and multi- functional CD4+ and CD8+ cells as seen when priming with rBCG Δ*panCDpfo* (*gag*). Lastly, mice primed with rBCG *pfo* (*gag*) and rBCG Δ*panCDpfo* (*gag*) induced greater CD8+ T_{EM} and T_{CM} responses as compared to mice primed with rBCG WT (*gag*) and rBCG Δ*panCD* (*gag*).

The relative expression of 86 immunologically pertinent genes in *ex vivo* stimulated and unstimulated splenocytes were studied to determine which genes (common and variable) are induced in mice primed with the four rBCG (*gag*) as compared to the corresponding rBCG (control) 12 days after the SAAVI MVA-C boost. In a comparison of the different rBCG primes, the IL-17 pathway was found to be uniquely up regulated and the IL-18 pathway to be down regulated in mice primed with rBCG *pfo* (*gag*) and rBCG Δ*panCDpfo* (*gag*). After a six hour *ex vivo* stimulation with HIV-1 peptides, mice primed with rBCG Δ*panCD* (*gag*) were observed to have a 42 fold down regulation of the Th1 transcription factor STAT4 suggesting strong suppression of the Th1 immune response 6 hours after the recall response was induced. In contrast, the IFNγ pathway was up regulated in mice primed with rBCG *pfo* (*gag*) or rBCG Δ*panCDpfo* (*gag*) suggesting a persistent Th1 (inflammatory) response is present 6 hours after *ex vivo* stimulation.

To conclude, we propose systemic models of how the Δ*panCD*, *pfo* and Δ*panCDpfo* modifications influence host gene expression which translate to the improved safety and

immunogenicity associated with these rBCG Danish vaccines. This work further promotes the use of modified rBCG vaccine vectors as HIV-1 vaccines. Furthermore, the systems vaccinology approach provides a greater insight into the mechanism of these modifications and we suggest that other pre-clinical vaccine studies use such approach to maximise knowledge gained from pre-clinical vaccine work. Whilst we have demonstrated these principals in the murine model, future work will include assessment of rBCG Δ*panCDpfo* expressing Gag and combinations of different HIV-1 antigens in non-human primates, including and combining the rBCG prime with different heterologous boosts.

LIST OF ABBREVIATIONS

Ad5	Adenovirus type 5	MDR	Multi-drug resistant
AIDS	Acquired immune deficiency syndrome	ml	Millilitre
ART	Antiretroviral therapy	MSM	Men who have sex with men
AUC	Area under the curve	MVA	Modified vaccinia Ankara
BCG	Bacille Calmette-Guerin	NHP	Nonhuman primate
bNab	Broadly neutralising antibodies	PAMPs	Pathogen-associated molecular patterns
CBA	Cytokine bead array	PCR	Polymerase chain reaction
CFU	Colony forming unit	pfo	Perfringolysin
CMV	Cytomegalovirus	PPD	Purified protein derivative
CRF	Circulating recombinant form	RBC	Red blood cell
CRPV	Cottontail rabbit papilloma virus	rBCG	Recombinant bacille Calmette-Guerin
CTL	Cytotoxic T lymphocyte	RCF	Relative centrifugal force
CVA	Chorioallantois vaccinia virus Ankara	RhCMV	Rhesus Cytomegalovirus
DC	Dendritic cell	RNA	Ribonucleic acid
DNA	Deoxyribonucleic acid	RPMI	Roswell Park Memorial Institute medium
DTH	Delayed-type hypersensitivity	SCID	Severe combined immunodeficiency
ELISA	Enzyme-linked immunosorbent assay	SFU	Spot-forming units
ELISPOT	Enzyme-Linked ImmunoSpot	SIV	Simian immunodeficiency viruses
FACS	Fluorescence-activated cell sorting	SSA	Side scatter area
FSA	Forward scatter area	SV5	Simian Virus 5
H_2O_2	Hydrogen peroxide	TB	Tuberculosis
HESN	HIV-1-exposed seronegative	TIV	Trivalent influenza vaccine
HIV	Human immunodeficiency virus	TLR	Toll-like receptor
HLA	Human leukocyte antigen	UNAIDS	Joint United Nations Programme on HIV and AIDS

HVTN	HIV Vaccine Trials Network	USA	United States of America
iNos	Inducible nitric oxide synthase	VACV	Vaccinia virus
IRIS	Immune reconstitution inflammatory syndrome	WHO	World Health Organisation
kDa	Kilodalton	XDR	Extensively drug-resistant
LNTP	Long-term nonprogressors	µm	Micrometre
LTR	Long terminal repeats		

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CHAPTER 1: LITERATURE REVIEW

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1.1 The importance of vaccination in overcoming the global burden of HIV-1/AIDS

Following the identification of the first case of acquired immunodeficiency disease syndrome (AIDS) and its causative agent the human immunodeficiency virus (HIV), in 1981 and 1983 respectively, there have been in excess of 30 million AIDS related deaths (Barre-Sinoussi *et al*., 1983). There are currently an estimated 36.9 million people who are HIV positive (UNAIDS, 2015). Global data on HIV indicates that there were 2 million new infections as well as 1.2 million AIDS related deaths in 2014. Sub-Saharan Africa has borne the brunt of the pandemic and accounts for 66% of global HIV infections (UNAIDS, 2015). In South Africa, there were an estimated 6.8 million people living with HIV in 2015. Currently, a multifaceted approach to control South Africa's HIV epidemic using pre-exposure prophylaxis, improved anti-retroviral therapy (ART) and increased public awareness is responsible for decreasing infection rates as well as improving life expectancy of HIV infected individuals (Mayosi *et al*., 2012).

However, the challenges of access to ART as well non-compliance to drug regimes, further highlights the need for a vaccine (Derache *et al*., 2015, Bociaga-Jasik *et al*., 2014). An effective vaccine, in conjunction with prophylactic initiatives and ART, would dramatically enhance the decline of the epidemic (Fauci *et al*., 2014). Vaccination, particularly against viral infections, has been the mainstay of infection prevention since Edward Jenner's smallpox vaccine in 1796 (Nabel, 2013). The social and economic value of vaccines can be most appreciated with the drastic reduction in measles, mumps, influenza, hepatitis B and polio over the last 70 years (Esparza and Van Regenmortel, 2014, Patel *et al*., 2015). Whilst numerous advancements in HIV research have taken place over the past 30 years, no reports of a totally efficacious prophylactic or therapeutic vaccine exist (Dangeti, 2013). Currently, it is accepted that an efficacious HIV vaccine would consist of an optimised vector and/or immunogen capable of generating broad, long-lived and safe immune responses (Esparza and Van Regenmortel,

2014). An HIV vaccine which elicits both broadly neutralizing antibodies and cell-mediated immune responses would be ideal but due to the limited success of previous trials, two independent approaches are currently favoured for vaccine development (Mann and Ndung'u, 2015). The first is the elicitation of broadly neutralising antibodies (bNAb). This approach aims to prevent infection or minimise the chance of infection (Sadanand *et al*., 2015). The second approach, which is fundamental to the rationale of this thesis, is the development of an effective T cell based vaccine to either prevent initial infection or slow disease progression by limiting early viral replication and viral load set point during the acute phase of infection (Haynes, 2015).

This chapter will discuss the broad body of clinical and experimental data which currently informs the development of a successful T cell based HIV-1 vaccine. Furthermore, this chapter will focus on candidate HIV-1 vaccine vectors, particularly highlighting recombinant BCG (rBCG) based vaccines developed by HIV-1 Vaccine Development Group based at the University of Cape Town (UCT).

1.2 The global diversity of HIV

Belonging to the genus *Lentivirus* which lies in the family of *Retroviridae*, HIV is thought to have originated from zoonotic transmission of simian immunodeficiency virus (SIV) from nonhuman primates to humans in the early 20th century in West and Central Africa (Faria *et al*., 2014). Since introduction into man, HIV has evolved into several diverse and pathogenic strains classified in two lineages (HIV-1 and HIV-2) which have been found to be pathogenically and geographically distinctive (Beyrer and Abdool Karim, 2013). These lineages are further divided into groups (as shown in Figure 1.1.) of which HIV-1 group M contributes to the majority of the global burden of HIV with an estimated 33 million infections (Hemelaar, 2012). Based on genetic diversity, these groups are further divided into clades, also known as

subtypes. HIV-1 subtype C is dominant in both India and sub-Saharan Africa and is estimated to be responsible for 50% of global HIV infections (Neogi *et al*., 2012).

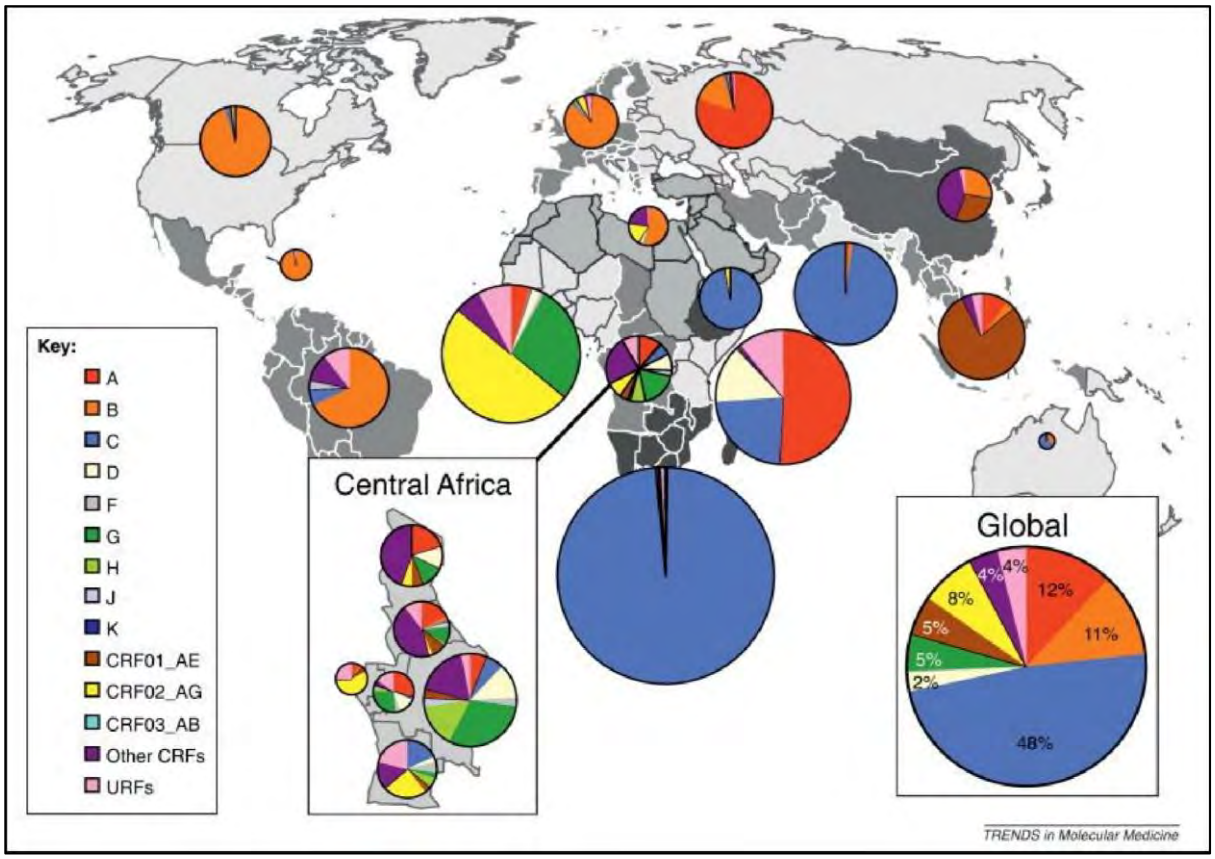


Figure 1. 1. The global diversity and burden of Human Immunodeficiency Virus (HIV) showing dominant and minor subtypes as well as circulating recombinant forms. HIV subtype C (shown in blue) affects the greatest number of people with the largest burdens in sub-Saharan Africa and the Indian subcontinent. Taken from (Hemelaar, 2012).

There is immense inter subtype-(17-35%) and intra-subtype genetic diversity (8-17%) (Hemelaar, 2012, Hemelaar *et al*., 2011). Diversity within regions is expected to grow with new recombinants entering regions where established subtypes are predominant (reviewed by (Vuilleumier and Bonhoeffer, 2015). This, in addition to viral mutation via immune escape further renders the development of HIV-1 vaccines exceptionally challenging (Korber *et al*., 2001, Barouch, 2008).

1.3 The structure and genomic organisation of HIV-1

HIV is a spherical virus approximately 120nm in diameter with an outer lipid bilayer containing HIV envelope (Env) proteins (Figure 1.2) (Engelman and Cherepanov, 2012). Contained within the virion is a capsid containing two copies of single stranded positive sense RNA. These 9.8 kilo bases (kb) RNA strands are comprised of 9 open reading frames that code for 15 proteins.

A

B

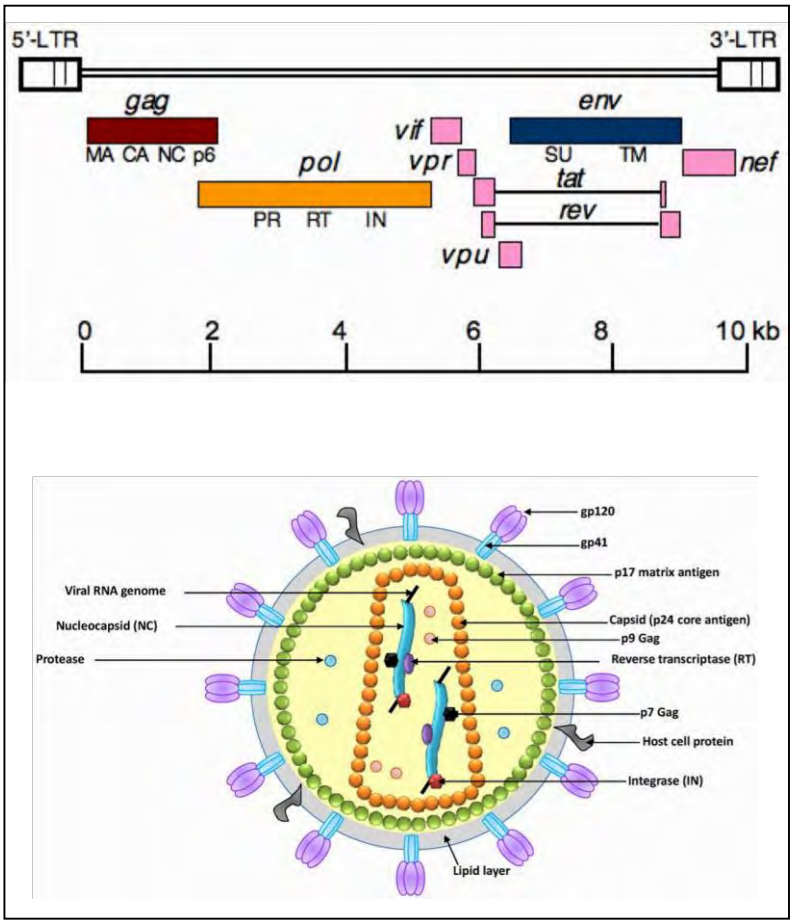


Figure 1. 2. Basic genomic and structural representation of HIV-1. (A) The HIV genome is composed of 9 open reading frames with a long terminal repeat on both the 5' and 3' ends. The 3 structural genes, *gag*, *pol* and *env*, are translated into the p53, p160 and gp160 polypeptide precursors respectively. The Gag precursor (p53) is cleaved into the matrix (MA, p18), capsid (CA, p24) and nuceloscapsid (NC, p15) proteins. The Pol polypeptide precursor (p160) is cleaved to yield Reverse Transcriptase (RT), Protease (PR), Integrase (IN) and Ribonuclease H (RNaseH). The Env precursor is cleaved into the extracellular membrane bound gp120 glycoprotein as well as the transmembrane gp41 glycoprotein. Taken from (Waheed and Freed, 2010). **(B)** Extracellular (gp120) and transmembrane (gp41) glycoproteins are arranged on the outer lipid layer of the virus. The p17 matrix protein assists in proving structural integrity to the virion. The capsid core, comprised of p24 core protein, encases two single stranded non-covalently linked copies of viral RNA. Taken from (Shum *et al*., 2013).

Functionally these 15 proteins are separated into structural, regulatory and accessory functions. The structural genes are group specific antigen (Gag) and envelope (Env) and they code for proteins that function collectively to promote virion structure and binding to host receptors (Shum *et al*., 2013). The Pol gene codes for 3 enzymes which include reverse transcriptase (facilitates the transcription of viral RNA to DNA), integrase (integrates viral DNA into the hosts genome) and protease (cleaves the Gag/Pol complex follow expression).

The regulatory genes are regulators of virion expression (Rev) and a trans-activator of transcription (Tat) which code for proteins that function to control the transcription of viral proteins. Lastly, the accessory proteins include viral protein unknown (Vpu), viral protein regulatory (Vpr), viral infectivity factor (Vif), and negative regulatory factor (Nef). In addition, the genome of HIV contains 2 long terminal repeats (LTR) which are found on 3' and 5' ends of the genome. These contain transcription initiation sites as well as transcriptional factor binding sites.

1.4 Molecular mechanisms of HIV infection and viral reservoir formation

HIV is a predominantly sexually transmitted pathogen via semen or vaginal mucosa. However, non-sexual routes of transmission may occur via contact with other body fluids. Examples of these include mother to child transmission, sharing of needles between intravenous drug users and exposure to HIV containing blood products (Moir *et al*., 2011, Patel *et al*., 2014). Once the virus enters the body, fusion of the viral envelope with the cell membrane of target host cells (CD4+ T cells and macrophages) occurs via receptor mediated absorption of the envelope glycoproteins (Wyatt and Sodroski, 1998). HIV infects CD4+ T cells via the CXCR4 and CCR5 chemokine receptors as well as macrophages via the CCR5 co-receptor (Naif, 2013). Once infection has occurred, typical or atypical disease progression may occur (discussed in section 1.6). During acute infection, HIV induces the nuclear translocation of potent host

transcription factors that are usually excluded from the nuclei of resting cells (such as NF-κB, NFAT Sp1 and AP-1) (Figure 1.3).

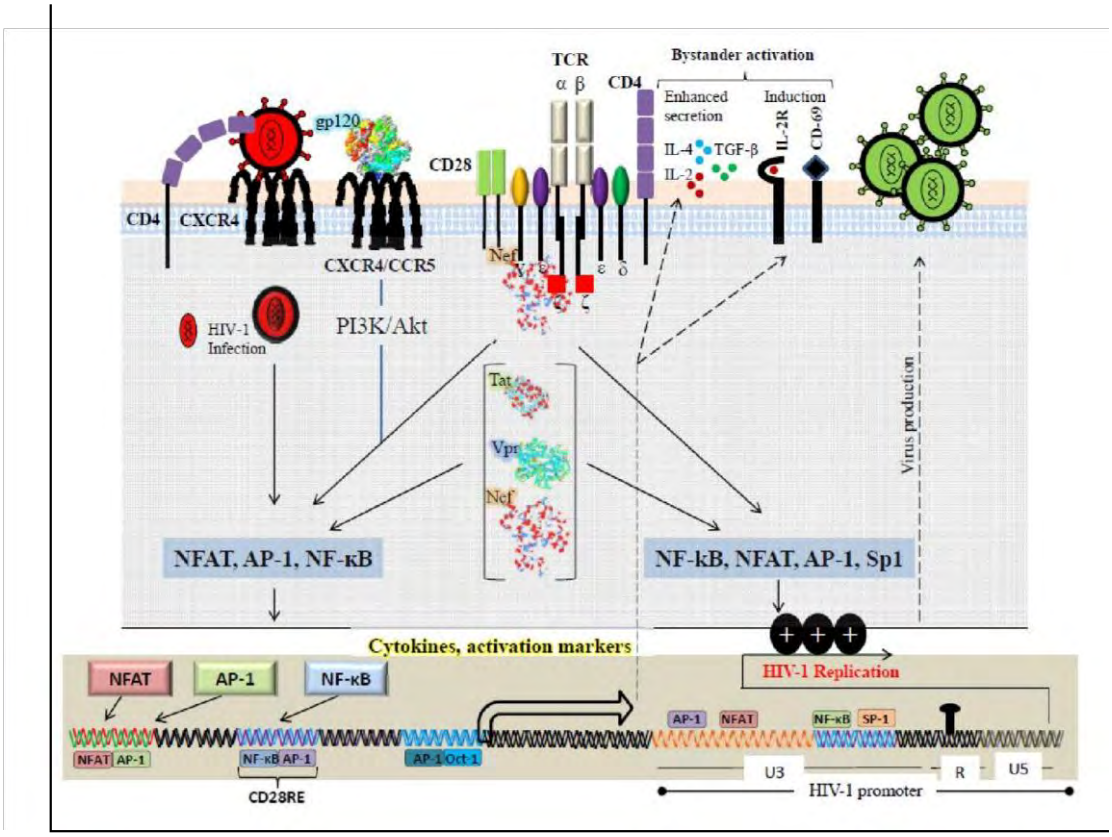


Figure 1. 3. Modulation of T-cell signalling pathways by HIV-1 proteins. Viral proteins both transcription factors and cell surface receptors of infected CD4+ T cells that support HIV-1 infection and pathogenesis.HIV-1 proteins (gp120, Nef, Tat and Vpr) induce the activation of several keys regulators of cytokine production and cellular activation (nuclear factor of activated T cells (NFAT), nuclear factor kappa B (NFκB), activator protein 1 (AP-1) and specificity protein 1 (Sp1)). Furthermore, HIV-1 viral proteins enhance the transcription of HIV-1 using host inducible promoters thereby promoting viral replication Taken from (Abbas and Herbein, 2013).

This promotes the production of non-specific inflammatory cytokines (such as TNF-α, IL-1, IL6) and T cell activation which thereby increases the number of potential target cells for HIV infection. This immune activation creates a highly inflammatory microenvironment which contributes to the decline of effective T cell immunity through lysis of HIV infected T cells, bystander T cell death and eventual immune exhaustion (Naif, 2013).

Although anti-retroviral therapy (ART) can lead to the effective restoration of immune function and control of HIV replication, persistent viral reservoirs make the clearance and cure of HIV infection difficult once established (Picker, 2014). Whilst HIV will preferentially infect, replicate and lead to the death of activated CD4+ T cells, HIV gene expression is silenced in infected CD4+ T cells that differentiate into central memory T cells (Hermankova *et al*., 2003). This leads to the formation of a transcriptionally inactive but stably integrated provirus which can produce viable virus when the T cell is activated by cytokines under the control of the inducible transcription factors NFκB and NFAT (Nabel and Baltimore, 1987). Interestingly, these cells remain impervious to the effects of ART (Abbas and Herbein, 2013). Therefore, an efficacious T cell based HIV-1 vaccine will have to limit excessive systemic inflammation, promote specific lysis of infected cells during early infection and induce a durable effector memory response to clear virus that may emerge from potential reservoirs.

1.5. The role of T cells in typical HIV disease progression

Typical chronic HIV disease progression to AIDS is characterised by a highly suppressed immune system, decreased numbers of CD4+ T cells and macrophages, excessive immune activation and uncontrolled vireamia (Naif, 2013, Moir *et al*., 2011). T cells play a central role in the immune response to viral, bacterial and parasitic infection by directly mediating the death of pathogen infected cells and indirectly by stimulating B cells to produce pathogen specific antibodies (Kapsenberg, 2003). Traditionally, CD4+ T cells mediate and assist the two major arms of intracellular pathogen elimination, B cell antibody production and cytotoxic CD8+ T cell function (CTL) (Masopust and Schenkel, 2013, Sun and Bevan, 2003). Following infection, HIV can bind to the CD4 receptor on CD4+ T cells and these cells become the major host for exponential HIV viral replication during the first 0-12 weeks. Subsequently, infected cells producing virus lyse or are killed by increasing levels of HIV specific cytotoxic CD8+ T cells therefore depleting CD4+ T cell levels (Klatzmann *et al*., 1986). During acute infection, peak vireamia typically coincides with an inversion of the CD4:CD8 T cell ratio (Figure 1.4).

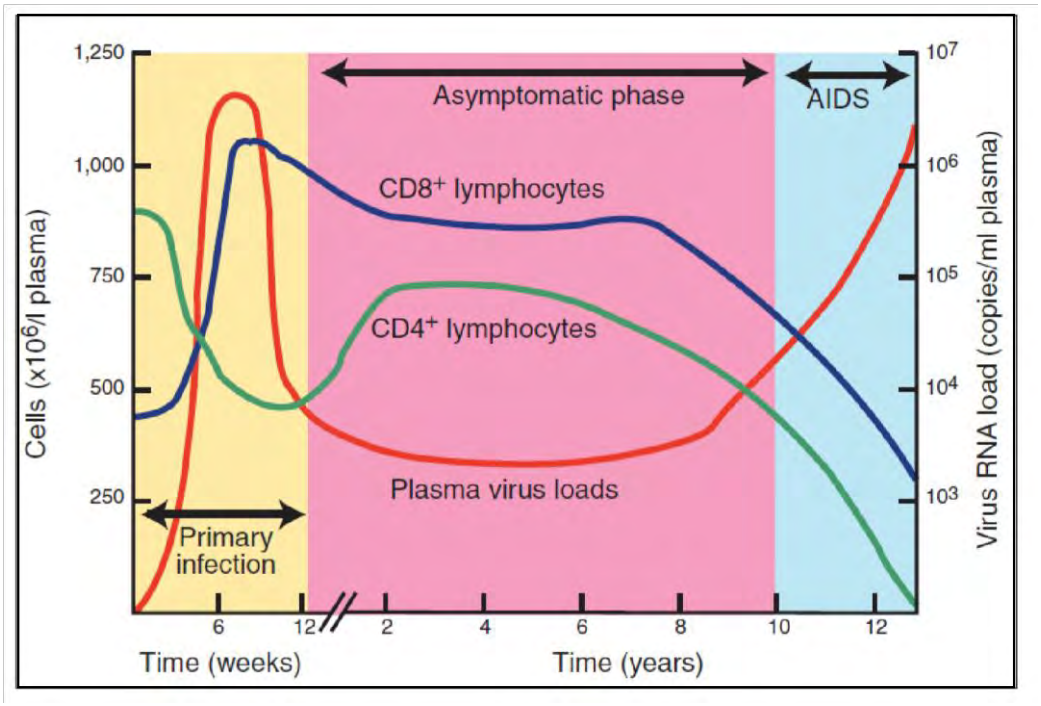


Figure 1.4. Longitudinal depiction of the typical fluctuation of T cell level and plasma viral load following HIV infection. An efficacious T cell based vaccine would aim to minimise viral set point during primary infection by promoting greater quantitative and qualitative T cell responses. Taken from Sewell *et al*., 2000.

This viral set point is important for the rate of disease progression and signals the start of the latent stage of HIV infection which can persist for 2-20 years. Whilst cytotoxic CD8+ T cells and antibody responses may keep viral load relatively constant during this latent phase, immune escape and prolonged immune activation eventually weakens immune efficacy leading to increased viral replication and further CD4+ depletion (Saez-Cirion *et al*., 2014).

This final immune depreciation is the defining characteristic of progressors who often succumb to opportunistic infections, the hallmark of HIV related AIDS which is characterised by a CD4+ count of <200 cells/mm^3. Incapable of mounting effective immune responses to pathogens, untreated chronic infection leads to death. Whilst revolutionary advancements in anti-retroviral therapy and patient care have been able to reduce disease progression and transmission, an effective HIV vaccine is still required to further counter the epidemic.

1.6. Atypical disease progression: Protective immune responses inform T cell vaccine design

Atypically, not all HIV infected subject's progress to AIDS. Understanding how the immune systems of these individuals that are able to control HIV and mount effective protection has been of massive interest to scientists and may be key to informing vaccine design (Gaardbo *et al*., 2012). Following the discovery of HIV being the causative virus of AIDS, infected patients who did not progress to AIDS were identified.

These patients fall into to three broad groups termed long-term non-progressors (LNTPs), viraemic controllers and elite controllers who all exhibit far lower viral loads and lack of CD4+ depletion (Gaardbo *et al*., 2012). Viraemic controllers (<2000 copies/ml) and LNTPs (<10 000 copies/ml) can be defined as HIV infected individuals that suppress HIV viral load without the aid of ARVs. Elite controllers, the group with the most robust infection control, are HIV infected individuals who maintain CD4+ counts without treatment and present with undetectable viral loads (<50 copies/ml). Lastly, highly exposed seronegative individuals (HESN) represent a model to assess natural resistance to HIV-1 (Rowland-Jones and McMichael, 1995, Fowke *et al*., 1996). These individuals include intravenous drug users (Saez Cirion *et al*., 2006), discordant couples (Bégaud *et al*., 2006), commercial sex workers (Fowke *et al*., 1996) and children born to seropositive mothers.

Understanding host gene regulation patterns that have been associated with protection in highly exposed-uninfected individuals (HESN) and HIV controller cohorts can greatly inform vaccine design (and interpretation of the effects of candidate vaccine vectors on host gene expression). Approximately 40 host genes have been shown to be associated with resistance to HIV in these cohorts (reviewed by Lama *et al*, 2007)(Lama and Planelles, 2007). Whilst infected, a major immunological hallmark of HIV-1 controllers is that they longitudinally maintain functional CD4+ and CD8+ T cell responses (Radebe *et al*., 2015, Saez-Cirion *et al*., 2014). Some major histocompatibility complex antigens (MHC), which present viral antigens

to CD8+ T cells which then attempt to lyse them, have been shown to correlate with HIV viral control (Goulder and Walker, 2012). These individual's ability to maintain persistent and functional T cell immunity represents a clinical model for the type of immunity researchers expect an efficacious T cell vaccine would elicit.

In addition to protective MHC molecules, preservation of T cell signalling has also been associated with control of HIV-1 vireamia.In a study done by Wu *et al*, (2011), gene-expression profiles in CD4+ and CD8+ T cells derived from LNTP, vireamic untreated and ART-treated avireamic patients were compared. They observed an up regulation of the MAPK T cell signalling pathway in LNTPs (particularly the JNK, ERK and p38 branches) suggesting that activation of these T cell signalling pathways are associated with improved T cell antiviral responses and survival (Wu *et al*., 2011).

HIV further promotes immune dysfunction by increasing the expression of inhibitory immunoregulatory markers such as cytotoxic T-lymphocyte-associated protein 4 (CTLA4) and Programmed Death-1 (PD-1) with elevated expression levels of these on T cells reported in individuals presenting with progressive infection but not controllers (Day *et al*., 2006, Trautmann *et al*., 2006). Binding of PD-1 to either of its ligands, PD-L1 or PD-L2 on CD8+ T cells leads to decreased proliferative capacity and cytokine secretion. Up regulation of CTLA4 on the other hand, is thought to decrease the production of IL-2, a cytokine which promotes recruitment of immune effector cells as well as stimulates T cell proliferation (Kaufmann *et al*., 2007, Zaunders *et al*., 2006). In addition to the above identified protective associations in humans, studies of atypical disease progression in NHPs have also lead to the elucidation of correlates of T cell mediated protection and these are discussed in conjunction with clinical correlates below.

1.6.1 Correlates of HIV/SIV specific T cell immune protection in animal models and the clinical setting inform HIV vaccine design

Traditionally, live attenuated viruses have been the used to prevent infection by viral pathogens such as mumps, measles, and smallpox (Nabel, 2013). Once HIV infection is established, eradication of the virus becomes exceedingly difficult due to escape mutations following immune pressure as well as the establishment of latent viral reservoirs. Therefore, an effective T cell vaccine should induce a rapid immune response to clear HIV infected cells prior to the establishment of infection (Haynes *et al*., 2016). T cells are recognised as the drivers of immunity towards a range of bacterial and viral pathogens. Several decades of investigation into trying to understand how the very cells that HIV targets can still remain functionally viable and immunologically competent have yielded interesting protective associations in both humans as well as animal models. Discussed below are several readouts of T cell function including; breadth and specificity of T cell response, preservation of T cell functionality, cytotoxic T cell function and the induction of HIV specific T cell memory.

1.6.1.1 Breadth and specificity of HIV+ T cell responses

Clinical cohort studies in untreated patients chronically infected with HIV subtype C virus indicate that an increased breadth of T cell response, particularly towards Gag epitopes correlated with decreased viraemia (Kiepiela *et al*., 2007a). Furthermore, the preferential targeting of certain viral epitopes has been reported to correlate with T cell mediated control of infection. Recent multivariate studies comparing the breadth and specificity of T cell immune responses in viraemic controllers and non-controllers indicate that Gag-specific CD8+ T cell responses are predictive of host control of the viraemia (Tomescu *et al*., 2014). Similarly for the CD4+ T cell response, Ransinghe *et al*, (2012) assessed the specificity of HIV-specific CD4+ T cells in a variety of controller and progressor cohorts (Ranasinghe *et al*., 2012). They were able to distinguish controllers from progressors by assessing the ratio of Gag/Env CD4+ T cell responses. A high frequency and magnitude of Gag responses combined with low proportion of Env responses were associated with immune control.

Immune control has also been linked to the breadth of Gag specific T cell response in early infection prior to the establishment of viral load set point. (Radebe *et al*., 2015) recently characterised the impact of CD8+ T-cell immunodominance patterns on viral escape and mutation during the first year of HIV-1 infection. At 26 weeks post-infection, they reported an inverse correlation between the breadth of Gag-specific responses and viral load set point in viraemic controllers. The protective associations favouring Gag-specific immune responses are not only limited to Gag CD8+ T cell epitopes. These clinical studies collectively demonstrate the protective importance of inducing a broad Gag specific T cell response early in infection. Consequently, this was one of the justifications for the utilisation of a full length HIV-1 subtype C Gag as the immunogen of choice for rBCG vectored vaccines used in this study.

1.6.1.2 The preservation of T cell functionality

The ability of T cells to produce cytokines is vital to an efficiently functioning immune system. HIV specific cytokine production has been widely reported to become diminished as HIV disease progresses and the quantification of cytokines in various states of HIV disease progression has become a vital marker of T cell protective function. HIV primarily elicits a type 1 immune response which includes the production of; IFNγ, IL-1, TNFα, IL-2 and IL-6 (primarily by T cells and macrophages) (Romagnani *et al*., 1994, Vandergeeten *et al*., 2012). Cytokines, an amalgamation of the Greek of words for 'cell' and 'movement', are a broad group of 520kDa proteins involved in cellular signalling primarily between cells of the immune system. These molecules are so synonymous with the immune response that they are routinely used as basic indicators of immune response, in assays such as ELISPOT and ELISA (Ahmed and Gottschalk, 2009). Two main arms of the cytokine network can be broadly classified into those that promote a type 1 (inflammatory) or type 2 (anti-inflammatory) immune responses with a high level of regulation between each type of response (Becker, 2004). Studies assessing the functionality of HIV specific T cells in elite controllers and LNTPs suggest that T cells from these

patients maintain a low activation state whilst simultaneously being able to produce multiple cytokines (termed T cell polyfunctionality) (Minton, 2014, Owen *et al*., 2010, Betts *et al*., 2006a). Furthermore, preservation of T cell polyfunctionality is present in elite controllers and LNTPs but not chronic progressors indicating that T cell polyfunctionality may be vital for immune control of HIV (Baker *et al*., 2009, Kiepiela *et al*., 2007b, Betts *et al*., 2006b). This observation is so profound that T cells from untreated elite controllers appear to maintain greater T cell polyfunctionality than those from LNTPs undergoing ART (Owen *et al*., 2010). T cell polyfunctionality has been shown to be regulated by the MAPK/ERK pathway which has been recently reported to be negatively regulated by the protein Sprouty-2 (SPRY2). Recently, Chiu *et al*, (2014) reported high levels of SPRY2 expression in donor derived HIV-specific T cells. They additionally found that they could enhance HIV Gag-specific CTL polyfunctional responses by *ex vivo* inhibition of SPRY2 (Chiu *et al*., 2014).

Whilst the production of these cytokines has been associated with immune responses to HIV, excessive immune activation and production of pro-inflammatory cytokines leads to increased viral replication and T cell exhaustion (Vandergeeten *et al*., 2012, Khaitan and Unutmaz, 2011). Therefore, it is vital that any candidate T cell vaccine induces a controlled and HIV specific cytokine response. A number of non-human primate and murine studies have indicated that HIV vaccines vectored by BCG can induce significant HIV specific T cell cytokine release (Chege *et al*., 2013, Chapman *et al*., 2012).

1.6.1.3 The importance of HIV specific CD8+ cytotoxic T cells

Host mediated lysis of HIV-1 infected cells is vital to stop viral replication. This is largely accomplished by the actions of cytotoxic CD8+ T cells. Binding of antigen specific T cell receptors to MHC molecules expressing recognizable HIV epitopes elicits a cytokine and chemokine cascade that leads to clonal expansion of the CD8+ T cells as well as the production of a group of molecules called cytotoxins (Walker and McMichael, 2012). These cytotoxins, which include perforin, granzyme and granulysin, are released by the cytotoxic CD8+ T cells

into the cytoplasm of the infected target cell (Chowdhury and Lieberman, 2008). Here, these cytotoxins activate the caspase pathway which leads to programmed cell death, termed apoptosis. Chronic progressors have been characterised to have diminished cytotoxic functions and this is thought to contribute to unregulated viral replication and progression to AIDS (Appay *et al*., 2000, Goulder *et al*., 1997). Elite controllers and LNTPs have demonstrated that the quality and preservation of cytotoxic T cell function in HIV infection is vital for reducing viral load and slower disease progression (Hersperger *et al*., 2011, Yan *et al*., 2013). Findings from a longitudinal study, which assessed epitope immunodominance in a cohort of 527 recently infected individuals up until chronic infection, reported that preservation of CTL immunodominance patterns from the acute into the chronic phase of infection was significantly associated with slower $CD4^+$ T-cell decline (Streeck *et al*., 2009b). In addition to controllers of disease progression, other examples of potent CTL mediated responses have been reported in Kenyan seronegative sex workers, who are routinely exposed to HIV (Kaul *et al*., 2004) as well as in uninfected neonates born to HIV+ mothers (Cheynier *et al*., 1992).

NHP vaccine studies have been equally as comprehensive in describing the protective role of CTLs. Over the past 15 years, researchers have improved CTL inducing vaccines from being able to control viraemia to being able to eradicate virus to undetectable levels (Figure 1.5). Partial control of viral load using DNA vaccines expressing SIVmac239 Gag and HIV-1 89.6P Env was one of the first milestones reached (Barouch *et al*., 2000). This study demonstrated the prevention and progression of AIDS like disease in rhesus macaques challenged with SHIV89.6P. Analysis of protective correlates associated low viral load set point with potent CTL responses. Whilst early CTL responses can control viraemia, eradication of SIV requires the generation of an HIV specific effector memory compartment which can elicit HIV CTL function for a prolonged duration following infection. More recently, studies using a rhesus cytomegalovirus (RhCMV) vaccine vector have demonstrated immune control and in some cases complete eradication of highly pathogenic SIV mac239 strain (Hansen *et al*., 2009, Hansen *et al*., 2011, Hansen *et al*., 2013a). They associated the ability of the rhesus cytomegalovirus vector (clone 68-1) with the induction of a persistent T cell effector memory (Tem) compartment which was shown to lead to the eradication of virus from disseminated

viral reservoirs (RhCMV vector is discussed further in section 1.8.1). Furthermore whilst this clone fails to induce MHC-1a restricted CD8 T cells, MHC class II restricted CD8 T cells were present in both protected and non-protected monkeys. This is particularly interesting for BCG vaccine development, because in TB patients, *M. tuberculosis* induces CD8+ T cells, which recognize HLA restricted peptides in a CD3/αβ TCR mediated CD8 dependent manner and, therefore, BCG is likely to induce similar reponses. Peptide presentation by MHC may reveal an additional type of effector cells potentially playing a role in protection against *M.tuberculosis*, SIV and HIV. Whilst these results are promising, the use of live CMV vectors in humans is not safe but inducing similar potent CTL and Tem responses using safer vectors, such as BCG, may allow us to translate these observed protective correlates from the animal model to the clinical setting.

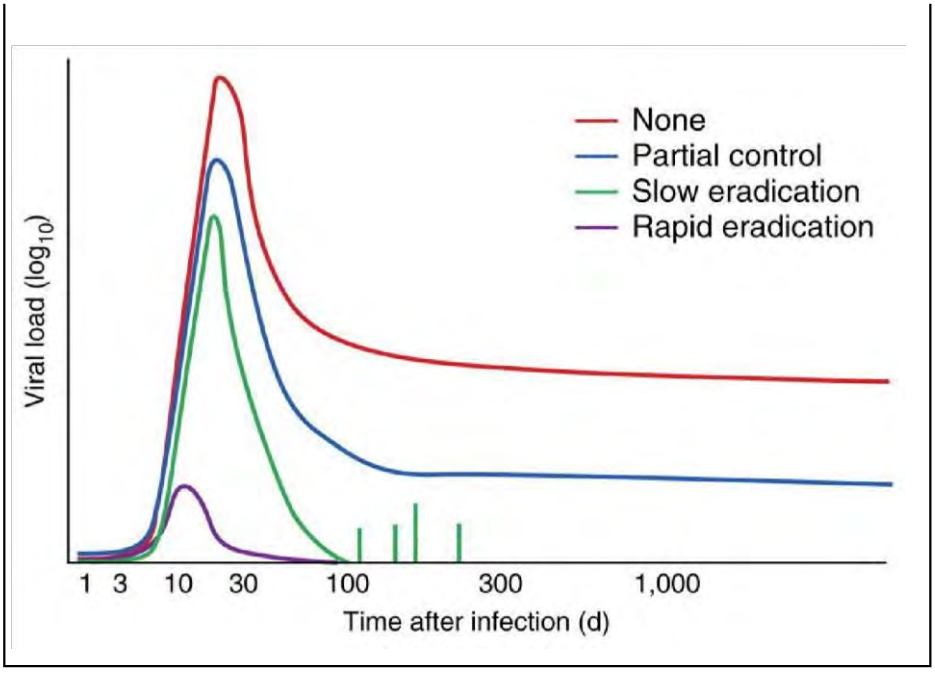


Figure 1.5. Variable control of SIV infected NHPs mediated by CTL stimulating vaccines. Typical disease progression in unvaccinated macaques leads to the maintenance of high viral loads following viral load set point (None). A partially effective T cell vaccine can lower viral load set point which in turn will lead to lower viral loads as disease progresses (partial control). Persistent vaccines, such as the rhesus cytomegalovirus (RhCMV) vaccine have been shown to induce persistent CTL responses which not only lower viral set point but continue to elicit a sufficient immune response to clear viral reservoirs and eradicate virus (slow eradication). The primary goal for a T cell vaccine is the rapid eradication of virus before the establishment of significant infection (rapid eradication).Taken from McMichael and Koff, 2014.

1.6.1.4 Preservation of T cell memory

A hallmark of the adaptive immune system is the generation of long-term T cell memory following initial exposure to a pathogen (Kaech *et al*., 2002). Following re-exposure to the same pathogen, the immune response is far more rapid and robust than the initial immune response. Therefore, T cell vaccines developed to combat intracellular pathogens often focus on the generation of long-term antigen specific T cell memory (Kaech *et al*., 2002). In both non-human primates as well as humans, typical SIV and HIV infections are characterised by severe depletion of memory CD4+ T cells (Okoye and Picker, 2013). Studies assessing elite controllers suggest that in conjunction with the maintenance of T cell polyfunctionality, potent antigen specific memory responses are the fundamental mechanism in limitation of viral replication and disease progression (Deeks and Walker, 2007, O'Connell *et al*., 2009). Elite controllers have also been reported to maintain more active effector memory CD8+ T cells than chronic progressors undergoing successful ART (Lopez *et al*., 2011).

NHP T cell based SIV vaccine studies using the persistent CMV vector (discussed further in section 1.8.1) indicate that protection from highly pathogenic SIV infection can be attributed to the high frequency of effector memory T cells (Hansen *et al*., 2009). However, having a persistent vector is no guarantee of eliciting a population of long lived memory phenotype. Recent TB studies investigating a safer but similarly persistent vaccine vector, BCG, have indicated that persistence of BCG leads to decreased central memory phenotype in the murine model (Nandakumar *et al*., 2014). Supporting this, other studies have reported improved central memory responses when BCG has been cleared (Andersen and Smedegaard, 2000, Kipnis *et al*., 2005, Jagannath *et al*., 2009). This variance indicates that new vaccines vectors, such as the ones used in this thesis, need to be understood both independently and in relation to the current correlates of HIV/SIV specific T cell immune protection in NHP and clinical models.

1.7 A review of HIV vaccine clinical efficacy trials

During the last 3 decades of HIV research, there have been over 200 HIV vaccine trials with only a small fraction showing enough promise to continue to phase 3 efficacy trials (Girard *et al*., Saunders *et al*., 2012, Esparza, 2013). Whilst these trials have been largely disappointing, they highlight the tremendous difficulty associated in developing effective HIV vaccines as well as providing vital information to assist pre-clinical development of novel vaccines. Reviewed below, are phase 2B and phase 3 HIV vaccine efficacy trials.

1.7.1 The VAX003 and VAX004 trials

The Vax003 and Vax004 trials by Vaxgen, were the first phase 3 HIV vaccine clinical trials conducted (1998-2003). Immunologically, these vaccines aimed to elicit protective Env specific antibodies by using a combination of recombinant gp120 proteins and alum adjuvant (Flynn *et al*., 2005). Vax003, containing a clade B and clade E gp120, was used to vaccinate 5100 men who have sex with men (MSM) as well as 300 high-risk women in Thailand. Vax004 (2x clade B gp120) was used to vaccinate 2500 high-risk intravenous drug users in North America and Europe (Flynn *et al*., 2005, Pitisuttithum *et al*., 2006). Whilst both vaccines elicited gp120 specific antibodies, neither protective efficacy nor delayed HIV disease progression was associated with either vaccine (Flynn *et al*., 2005, Pitisuttithum *et al*., 2006).

1.7.2 The HIV Vaccine Trials Network trials (HVTN 0052, 0053, 505)

Commonly referred to as the STEP and Phambili studies respectively, the HIV Vaccine Trials Network (HVTN) 0052 and 0053 trials (Phase 2B) were carried out between 2005 and 2007 by Merck in conjunction with HVTN. Unlike the Vaxgen vaccines, these vaccines aimed to primarily elicit T cell responses to kill HIV infected cells following infection which was anticipated to lead to partial or complete protection. The vaccine was comprised of a non-

replicating adenovirus type 5 (MRKAd5) vector expressing HIV subtype B *gag*, *pol* and *nef*. Preclinical rhesus macaque studies, demonstrated that this MRKAd5 vaccine could control viral load following SHIV challenge and phase 1 clinical trials confirmed safety and immunogenicity (Shiver *et al*., 2002, Casimiro *et al*., 2005). This paved the way for phase 2B clinical trials and 3000 healthy at risk individuals were enrolled in the Caribbean, Americas and Australia (for STEP) and in South Africa (for Phambili). Results from the STEP trial indicated that the vaccine was immunogenic with the induction of HIV specific IFNγ release in 77% of vaccinated study participants (McElrath *et al*., 2008). Breadth of the immune response was assessed by IFNγ release to Gag, Pol and Nef following *ex vivo* stimulation. Only a moderate 62% of vaccinated participants were able to induce a T cell response to 2 or 3 of these proteins. Assessment of specificity indicated that HIV specific CD4+ T cell responses were mostly to Gag whilst dominant CD8+ T cell responses were mainly Pol specific. The trial was stopped in 2007 following pre-planned analysis which suggested endpoint study futility (Sekaly, 2008). Unfortunately, a higher rate of HIV-1 infection was reported in vaccinated men who were uncircumcised who presented with pre-existing anti-vector immunity to adenovirus (McElrath *et al*., 2008). Following this, the Phambili study was also stopped and further analysis indicated a 7% greater chance of infection in the vaccinated group as compared to the placebo group (Gray *et al*., 2014).

While these results of the STEP and Phambili trial were disconcerting, an Ad5 vector was again utilised during the phase 2B HVTN 505 trial which was terminated in 2013.This trial exclusively used male and transgender women who were Ad5 seronegative and circumcised (Hammer *et al*., 2013). Participants were vaccinated with 3 doses of a DNA prime consisting of six plasmids individually expressing HIV-1 clade B (Gag, Pol, and Nef) and Env proteins (from clades A, B, and C). The rAd5 boost consisted of 4 Ad5 constructs expressing a HIV-1 clade B Gag-Pol fusion protein as well as 3 Env glycoproteins (from clades A, B, and C). This trial aimed to elicit both cellular and humoral responses. Due to the lack of efficacy, the trial was terminated 28 weeks in with more vaccine recipients found to be positive than individuals who received the placebo. Furthermore, gut biopsies of trial participants indicated a high proportion of

activated CD4+ T cells expressing the HIV-1 co-receptor CCR5. In order to understand the effect of anti-vector mechanisms behind the failure in efficacy of these Ad5 vectored vaccine trials, the STEP trial was mimicked in Rhesus macaques. They initially infected macaques with Ad5 and then vaccinated with replication defective Ad5 SIVmac239 Gag/Pol/Nef vaccine or placebo. Finally, groups were exposed to escalating doses of SIVmac 251. Interestingly, even at the lowest SIV exposure, vaccination resulted in 2/9 Ad5 seropositive animals becoming infected whilst 0/34 in control groups were infected.

These HVTN trials collectively highlight the need for vaccine vectors that do not induce significant anti-vector immunity. An example of a vector with a good clinical record of limited anti-vector immunity is BCG, utilised in our study (Korioth-Schmitz *et al*., 2015). Additionally, vaccines need to induce a greater breadth of T cell response than that exhibited in the HVTN trials. Whilst the immunogens utilised in these trials aimed to elicit responses for multiple HIV proteins (Gag, Pol, Nef and Env), recent data suggests that immunodominance towards Gag induces greater control (Turk *et al*., 2013, Radebe *et al*., 2015, Yang *et al.*, 2015b). Consequently, because of this, and other data, we have chosen to use a full length HIV-1 Gag as the immunogen of choice for designing our BCG vaccines (Stephenson *et al*., 2012).

1.7.3 The RV 144 efficacy trial

Whilst the vaccines used in the Vaxgen and STEP trials aimed to elicit antibody and T cell dominated immune responses respectively, the vaccines used in the RV144 trial aimed to do both. Beginning in Thailand in 2007, this trial assessed the efficacy of a heterologous prime boost regime. The ALVAC canarypox vaccine expressing Gag, Env and Pol was used as a prime followed by a recombinant protein boost (VAX003 gp120) (Rerks-Ngarm *et al*., 2009). Upon phase 3 completion, the trial reported a 31.2% infection reduction in subjects who received the vaccine as compared to those that received placebo. Coupled with this, high levels of Env specific IgG antibody titres were also observed but these decreased in magnitude after a year

from study endpoint (McElrath). However, the antibodies associated with reduced risk of infection were non-neutralising and were characterised to be V1/V2 binding antibodies (Haynes *et al*., 2012). Additionally, they reported an association between infection and the production of Env specific IgA antibodies.

Whilst the vaccine elicited minimal HIV specific CD8+ T cell responses, proliferative CD4+ Env responses were reported (Rerks-Ngarm *et al*., 2009). IFNγ ELISPOT responses on samples collected 6 months after immunization showed Gag or Env specific responses in 20% of vaccinated group as opposed to 7% of placebo recipients. Comprehensive assessment of T cell specificity and functionality were assessed by (de Souza *et al*., 2012). They reported that the RV144 T cell responses were modest as compared to humoral immune responses. Additionally, they found that most $CD4^+$ T cell responses were directed to the V2 region of Env. Recent studies assessing the impact of the T cell response on breakthrough virus suggests that immune pressure was mainly induced by the Env specific humoral response. A follow up study to the RV144 trial, The HVTN 097, is currently underway in South Africa.

RV144 therefore represented the first phase 3 clinical trial to show any protective efficacy, albeit moderate. Whilst protective correlates indicate that a humoral response was prominent for reducing risk of infection, the RV144 trial demonstrates the importance of poxvrial vectors. The poxviral SAAVI MVA-C vaccine, (reviewed in section 1.8.2), was incorporated into our heterologous prime boost vaccination regime as a boost to the rBCG prime.

1.8 HIV vaccine vectors to induce protective T cell immune responses

Classically, vaccination against viral and bacterial pathogens has been achieved by the use of attenuated or killed pathogen. However, this is not possible with HIV. Therefore, gene delivery vectors, which express carefully selected epitopes, are considered the best option to induce

protective T cell immune responses. Commonly touted vectors can be categorised into; replicating viral vectors, attenuated viral vectors, bacterial vectors and plasmid DNA. Furthermore, combinations of these vectors in prime-boost regimes have been suggested to elicit desired immune responses. As previously discussed both correlates of HIV/SIV specific T cell immune protection (section 1.6) as well as previous clinical efficacy trials (section 1.7) inform current vector selection and vaccine design. Highlighted below are two prominent vectors which have shown novel results in recent studies. Studies assessing cytomegalovirus (CMV) as a vaccine vector demonstrate the concept of a persistent vector being able to generate persistent CD8+ effector responses leading to eradication of SIV viral reservoirs in Rhesus macaques. The second, modified vaccinia Ankara (MVA), which has been a research focus of our group, demonstrates the ability of a safe non-replicating viral vector to boost for immunodominant Gag specific response.

1.8.1 Cytomegalovirus (CMV) as an HIV vaccine vector

Belonging to the *Herpesviridae* family Cytomegalovirus (CMV) has a global prevalence of greater than 90% (Manicklal *et al*., 2013, Lanzieri *et al*., 2014). Whilst infected individuals are often asymptomatic, CMV can be fatal especially to newborns and immunocompromised individuals (Manicklal *et al*., 2013). Whilst these global seroprevalence rates, as well as the pathogenicity of CMV in immunocompromised individuals is a concern, CMV is of high interest due to its ability to elicit a significantly large persistent T cell response (Barouch and Picker, 2014). Additionally, CMV can accommodate up to 6 kb of exogenous DNA allowing for multiple inserts as well as being able to be highly attenuated whilst not compromising immunogenicity (Barouch and Picker, 2014). As an SIV vaccine vector, CMV holds the distinction of being the first vaccine that has been able to clear SIV infection in rhesus macaques. A novel study using a replicating rhesus CMV vector encoding 6 SIV genes (*gag*, *rev*, *tat*, *nef*, *env* and *pol*) reported that 50% of vaccinated rhesus macaques could suppress viral load following repeated high dose rectal SIV challenge (Hansen *et al*., 2009). More recent studies have found this vaccine to be able to completely clear all traces of highly pathogenic SIV by maintaining relatively high

levels of CD8+ T cell effector memory (Hansen *et al*., 2013a, Hansen *et al*., 2011). Protection was correlated with the magnitude of peak SIV-specific effector memory CTL responses. Notably this protection was present in the absence of significant bNAbs response.

There are several challenges associated with translating these results from macaques to humans. Whilst live unattentuated CMV vectors are not considered acceptable for humans, attenuation of CMV to reduce replication to an optimal amount to longitudinally maintain a broad, high frequency effector CTL response is currently being pursued (Picker, 2014). Another challenge is that of T cell epitope MHC restriction discrepancies between humans and macaques. Protective MHC restricted CTL responses in macaques vaccinated with RhCMV and challenged with SIV mac291 were largely of the MHC class 2 antigen-E type with SIV infected cells expressing this MHC-E (Hansen *et al*., 2013b). This may explain to some degree the robust responses observed in these studies and CMV trials expressing human restricted HIV epitopes may further inform the translation of these CMV mediated persistent responses from macaques to humans. Currently, human trials are planned for attenuated CMV vectors as HIV vaccines (Barouch and Picker, 2014). Another option would be to use similarly persistent vectors with improved safety profiles such as BCG. Whilst BCG may not induce the massive T cell responses seen by CMV, modification of BCG by introducing exogenous immunomodulatory genes has been shown to increase the immunogenicity (discussed further in section 1.10.1).

1.8.2 Modified vaccinia Ankara (MVA) as an HIV vaccine vector

Modified vaccinia Ankara (MVA) is a replication deficient pox virus that has exhibited an extensive safety profile and immunogenicity as a vaccine vector (Esteban, 2009). One of the greatest vaccine intervention successes was the eradication of smallpox using vaccinia virus (VACV). However, this vaccine induced a number of side effects and a safer alternative was sought, which led to the development of MVA following 572 passages of chorioallantois

vaccinia virus Ankara (CVA) (Mayr and Munz, 1964). The safety profile of MVA was further demonstrated following the vaccination of 120 000 individuals against smallpox in Germany in 1979. As opposed to VACV, MVA did not induce any adverse reactions. The most notable safety feature of MVA is that it's unable to replicate past the point of forming immature virions. Several phase 2 HIV vaccine clinical trials have demonstrated immunogenicity using MVA as a vaccine vector on its own or in a heterologous prime boost strategy. Notably, HVTN 205 reported more durable antibody responses than RV144 (reviewed in 1.7.3) following a DNA/MVA prime boost vaccination regime. Additionally, assessment of T cell responses indicated this vaccine regime preferentially targeted Gag (Goepfert *et al*., 2014). Immunodominance of Gag specific T cell responses has been associated with improved clinical outcomes (Radebe *et al*., 2015). Our group, the HIV-1 Vaccine Development Group at UCT, has previously focussed on the development of MVA as a vaccine vector in the guise of the SAAVI MVA-C vaccine (Ncayiyana, 2009, Shephard *et al*., 2008). The SAAVI MVA-C immunogen was designed to include five HIV-1 subtype C genes to maximize epitope coverage. These included *gag*, reverse transcriptase, *tat*, and *nef* expressed as a polyprotein (grttn) as well as truncated envelope protein (gp150)(Burgers *et al*., 2008). This vaccine was utilised in the HVTN 086 phase 2a trial as part of a DNA-MVA heterologous prime boost regime in combination with a HIV-1 subtype C gp140 protein in a MF59 adjuvant (supplied by Novartis).

In animal models, this vaccine has been found to be particularly immunogenic as a boost vaccine in a heterologous prime boost regime with DNA, virus like particles and recently with *Mycobacterium bovis* bacillus Calmette–Guérin (BCG) (Chege *et al*., 2009, Chapman *et al*., Shephard *et al*., 2008).In this thesis, the use of novel strains of rBCG is investigated as a priming vaccine for a SAAVI-MVA-C boost in the murine model. The next section details the use of BCG as a vaccine vector and highlights how modification of BCG using modern molecular techniques may optimise BCG as an HIV-1 vaccine.

1.9. The use of BCG as a vaccine vector

Mycobacterium bovis BCG was developed in 1908 at the Pasteur Institute by Léon Charles Albert Calmette and Jean-Marie Camille Guérin by repeated subculturing of less virulent strains (Hawgood, 2007). Over a 13 year period they developed the first avirulent strain of *M.bovis* BCG by passaging the bacilli 198 times. Since its first use as a vaccine against *Mycobacterium tuberculosis* in 1921, BCG has become the most widely used vaccine in the world with an estimated 3 billion doses administered (Zheng *et al*., 2015). There are currently 6 major strains of BCG used worldwide due to gene deletions and mutations that have occurred or been induced through laboratory propagation. These stains include the original Pasteur strain (1173 P2), as well as; Danish (1331), Tokyo (172-1), Russian (BCG-1), Glaxo 1077 and Moreau (RDJ) (Behr *et al*., 1999). Differences in the persistence and pathogenicity of these strains should be carefully considered when attempting to use BCG as vaccine vector (Oettinger *et al*., 1999, Behr *et al*., 1999, Gordon *et al*., 2001). There are several advantages as well as limitations to using BCG as a vaccine vector. How these advantages contribute to efficacious HIV-1 vaccine design and how modification of BCG can overcome these limitations are discussed below.

1.9.1 Numerous advantages of BCG as a vaccine vector

There are a variety of characteristics that make BCG an attractive vaccine vector.First and foremost, BCG has a well-established safety record with a low incidence of vector based complications (Hanson *et al*., 1995). Logistically, BCG does not require a cold chain and is relatively cheap and easy to manufacture making it extremely attractive for use in resource limited settings (Gheorghiu *et al*., 1988, Roche *et al*., 1995). Furthermore, BCG can be administered orally or intramuscularly and it can be administered to neonates and the efficacy is unaffected by maternal antibodies. Long lived cellular and humoral responses to BCG have been well documented with responses to tuberculoproteins reported up to 50 years after initial vaccination (Hanson *et al*., 1995).

Due to these advantages, a number of heterologous pathogen and human antigens have been expressed in BCG and have resulted in protective immunity in animal models. Notably, Langermann *et al*, (2005) demonstrated that BCG expressing the pneumococcal surface protein A (PspA) could protect mice from lethal doses of *Streptococcus pneumoniae* (Langermann *et al*., 1994). Similarly, BCG expressing a detoxified S1 subunit of pertussis toxin was found to completely protect mice against *Bordetella pertussis* challenge (Nascimento *et al*., 2008, Nascimento *et al*., 2009). Additionally, in measles studies, Fennelly *et al,* (1995) reported that mice vaccinated with BCG expressing full-length measles virus nucleocapsid protein developed substantial measles specific T cell and humoral responses which were associated with decreased viral titer following intra-cranial measles infection(Fennelly *et al*., 1995). In addition to measles virus, BCG expressing papillomavirus antigens have been shown to protect rabbits from cottontail rabbit papillomavirus challenge (Govan *et al*., 2006, Govan and Williamson, 2007). Other notable examples of BCG based vaccines inducing protective immunity in animal models include vaccines against; listeria, malaria, leishmaniasis, rotavirus, tetanus, human metapneumovirus and Lyme disease (Grode *et al*., 2005, Matsumoto *et al*., 1998, Streit *et al*., 2000, Stover *et al*., 1993a, Stover *et al*., 1993b, Dennehy *et al*., 2007, Mazzantini *et al*., 2004, Palavecino *et al*., 2014).

1.9.2 Strategies to overcome the limitations of BCG as a vaccine vector

There is currently no perfect vector able to elicit the desired HIV specific protective responses. However, modification of vectors and antigen expressions systems can lead to stable, safer and more immunogenic vectors. Discussed below, are notable examples of how modern molecular techniques have been used to improve BCG to deliver heterologous antigens.

1.9.2.1 The use of auxotrophic complementation to improve vector stability

Primarily, instability of episomal vectors, which results in deletion or rearrangement of the heterologous gene/s, has been an extensively reported (Dennehy and Williamson, 2005,

Bastos *et al*., 2002). BCG with these deletions usually do not express the recombinant antigen and tend to outgrow stable BCG (Medeiros *et al*., 2002). Studies have shown improvement in the stability of episomal vectors by using auxotrophic complementation which utilises auxotrophic stains of BCG and includes complementary genes added to the vector (Borsuk *et al*., 2007). Successful use of this method was shown to increase rBCG stability and lead to protection against pertussis when using BCG expressing pertussis toxin (Nascimento *et al*., 2009).

1.9.2.2 The use of inducible promoters to control antigen expression

A vital factor for the expression of heterologous antigen in mycobacteria is the rate of transcription initiation. This is governed by the strength of the promoter. Whilst a high transcription rate may be desirable to maximise the production of antigen, the cost is often that high antigen levels lead to toxicity in the mycobacteria (Al Zarouni and Dale, 2002). Such is the case with traditional mycobacterial promoters such as the heat shock protein 60 (*hsp 60*) and heat shock protein 70 (*hsp 70*)(Al Zarouni and Dale, 2002, Kumar *et al*., 1998).

Inducible systems that can be used to down regulate antigen expression *in vitro* and upregulate expression in *vivo* have been shown to improve stability. A variety of regulated expression systems have been tested in mycobacteria (reviewed (Schnappinger and Ehrt, 2014). Our group has previously demonstrated the successful expression of HIV-1 RT in wildtype Pasteur BCG, as well as a pantothenate auxotroph, using a Tet repressor expression system (Mbele, 2012).

Another inducible promoter system that can limit toxicity is the *mtrA* promoter from *M. tuberculosis*, (Via *et al*., 1996, Dhandayuthapani *et al*., 1995). Our group has recently reported

effective use of the *mtrA* promoter, which is up regulated in the macrophage and down regulated *in vitro*, in immunogenic rBCG vaccines expressing HIV antigens in both the murine and non-human primate models (Chapman *et al*., 2012, Chege *et al*., 2013).

1.9.2.3 Exploiting antigen localization to increase immunogenicity

Localization of the antigen to the cell membrane or secretion (thereby limiting cytoplasmic accumulation of antigen) has also been reported to decrease toxicity in mycobacteria (Chapman *et al*., 2011b, Toussi and Massari, 2014). In addition to toxicity, the location of recombinant antigen expression and presentation enhances cell mediated immune responses when utilising BCG as a vaccine vector (Grode *et al*., 2002, Govan *et al*., 2006, Yu *et al*., 2007). Membrane bound antigens have been shown to be more immunogenic than antigens that are expressed in the cytoplasm (Bastos *et al*., 2002).

Several groups have assessed and compared the effect of antigen localization on immune response and protection. Firstly a murine study compared protective efficacy and cell mediated immunity in mice vaccinated with 1 of 3 BCG vaccines expressing the immunodominant Listeria p60 protein in different bacterial compartments (Grode *et al*., 2002). Notably, mice were protected by vaccines expressing cell membrane bound p60 and secreted p60 but vaccines that expressed p60 in the bacterial cytoplasm did not confer protection.

The *M. tuberculosis* 19kDa leader sequence in particular has been extensively shown to facilitate translocation of expressed antigens to the cell membrane (Stover *et al*., 1993b, Chege *et al*., 2005, Cayabyab *et al*., 2009, Im *et al*., 2007). The action of the 19kDa leader sequence is also exceptionally rapid with evidence suggesting export of the protein occurs within an hour of phagocytosis of the mycobacterium (Neyrolles *et al*., 2001). Improvements

in immunogenicity and protective efficacy of BCG based vaccines, when using the 19kDa leader sequence, have also been reported in pneumococcal and SIV studies (Langermann *et al*., 1994, Grode *et al*., 2002, Someya *et al*., 2005). Our group has previously demonstrated in the murine model that BCG expressing rotavirus VP6 with the 19kDa was more immunogenic and protective than when using an α antigen leader sequence (Dennehy *et al*., 2007). Furthermore, our group has demonstrated protective efficacy against cottontail rabbit papillomavirus (CRPV) challenge in rabbits vaccinated with rBCG expressing the L1 antigen. This was successfully achieved with the antigen fused to a 19kDa leader sequence under the control of an *mtrA* promoter (Govan *et al*., 2006). Therefore, careful selection of leader sequences, promoters and antigen can improve the stability and immunogenicity of BCG as a vaccine expressing heterologous antigens.

1.9.2.4 Overcoming the effects of environmental mycobacteria and helminth infections on immunity

The variable efficacy of BCG as a TB vaccine has been well documented (Trunz *et al*., 2006). Factors behind this variable efficacy may affect outcomes when attempting to use BCG as an HIV vaccine. Whilst BCG confers protection against disseminated forms of TB following administration at birth, protective efficacy against pulmonary TB ranges from 0-80% (Lugosi, 1992, Brandt *et al*., 2002a). Decreases in efficacy are most notable in tropical climates with factors such as environmental mycobacteria and exposure to extremely high levels of TB being postulated to affect vaccine efficacy (Trunz *et al*., 2006, Fine, 1995, Brandt *et al*., 2002b).

Studies assessing the antagonistic effects of *M. avium* on BCG efficacy in the small animal model have shown that prior sensitisation with *M. avium*decreases BCG mediatedprotection against MTB (de Lisle *et al*., 2005). The effects of prior environmental sensitization (*M. avium, M. vaccae* or *M. scrofulaceum*) on the protective efficacy of rBCG expressing the *Mycobacterium tuberculosis* specific region of difference antigens (RD-1) was reported

(control BCG not expressing RD-1 was used in parallel) (Demangel *et al*., 2005). Increased protection against TB following sensitization with *M. vaccae* or *M. scrofulaceum* was observed. Subsequently, they showed that T cell antigens between *M*. *avium* and BCG are conserved suggesting that antigen cross reactivity may be the driver of the antagonistic effects of environmental mycobacteria on BCG efficacy (Demangel *et al*., 2005).

In addition to environmental mycobacteria, there is considerable evidence that parasitic infection may affect the efficacy of BCG (Crampin *et al*., 2009). The association between helminth infection and pulmonary TB has been well documented (Elias *et al*., 2006, Diniz *et al*., 2001, Tristao-Sa *et al*., 2002). Murine studies have reported decreased protection against TB following BCG vaccination in mice presenting with chronic helminth infection (Elias *et al*., 2005). In this case, impaired anti-mycobacterial Th1 responses were reported which are most likely attributed to the dominant Th2 responses elicited by helminth infection (Elias *et al*., 2005, Elias *et al*., 2006).

In summary, for the advantages of BCG to be optimally exploited as an HIV vaccine vector, modern molecular techniques incorporating optimal expression systems and considerations towards overcoming antagonistic environmental effects need to be incorporated.

1.10 Increasing the safety and immunogenicity of BCG as vaccine vector

Whilst BCG has been considered an exceptionally safe vaccine for decades, there has been increased risk and incidence of BCG disease reported in immunocompromised individuals in the HIV era (Colditz *et al*., 1994, Trunz *et al*., 2006). Additionally, BCG may induce immune reconstitution inflammatory syndrome (IRIS) in children following ARV therapy (Nuttall *et al*., 2008). Currently, the WHO guidelines suggest that BCG not be given to HIV infected or

immunocomprimised individuals. With the increase in prevalence in MDR/XDR TB and the advancement of molecular techniques in the 1990s, the need and means to genetically enhance BCG has been present (Ottenhoff and Kaufmann, 2012). Modifying BCG strains to increase immunogenicity and decrease pathogenicity may be the key to overcoming the limitations of BCG and unlocking its full potential as an HIV vaccine vector (Ottenhoff and Kaufmann, 2012, Chapman *et al*., 2011a).

Several pre-clinical and Phase 1 TB vaccine trials involving novel BCG vaccines/vaccination regimes have been undertaken in the recent past (Hesseling *et al*., 2007a, Haile and Kallenius, McShane and Hill, 2005, Horwitz *et al*., 2005, Tullius *et al*., 2008, Hoft *et al*., 2008). Various successful strategies to further increase the safety and immunogenicity of BCG include the use of homologous and heterologous prime boost vaccination regimes (Hawkridge *et al*., 2008, McShane and Hill, 2005, Griffin *et al*., 2006, Rahman and Fernandez, 2009, Badell *et al*., 2009, Magalhaes *et al*., 2008b), as well as the use of adjuvants (Haile *et al*., 2005).

1.10.1 The development of auxotrophic strains to enhance safety and/or immunogenicity of BCG as a vaccine vector

One particularly promising strategy to enhance safety and/or immunogenicity of BCG is the development of auxotrophic strains of BCG and MTB (Tullius *et al*., 2008, McAdam *et al*., 1995). Auxotrophic strains contain mutations geared towards preventing the bacteria from synthesising essential growth compounds or amino acids. Auxotrophs are unable to grow in culture unless the culture is supplemented with the essential compound that the bacteria is unable to produce (McAdam *et al*., 1995, Sampson *et al*., 2004). Interestingly, these auxotrophs have been reported to be safer than wildtype strains of BCG in immune compromised mice which is promising in the context of using BCG as an HIV vaccine (Chambers *et al*., 2000, Sampson *et al*., 2004, Tullius *et al*., 2008). Several auxotrophs that have been shown to be equally, if not more, immunogenic and safer than wildtype strains of BCG include;

leucine auxotrophs, lysine auxotrophs and pantothenate auxotrophs amongst others (Sampson *et al*., 2004, Bange *et al*., 1996, Hondalus *et al*., 2000, Sambandamurthy *et al*., 2002).

The pantothenate auxotroph of BCG Pasteur has been well characterised and studied by our group. The Δ*panCD* auxotrophs of BCG and Mtb do not synthesise pantothenic acid and thus have limited intracellular replication, are safe in SCID mice and protect from challenge with *M. tuberculosis* (Sambandamurthy *et al*., 2002, Tullius *et al*., 2008). Mice vaccinated with this auxotroph are also characterised by the induction of better T cell responses and decreased pathology as compared to corresponding wildtype strains (Chapman *et al*., 2012). Additionally, our group has previously demonstrated that a Pasteur Δ*panCD* auxotrophic strain expressing HIV-1 Gag is immunogenic when used as a prime followed by a MVA-Gag boost (Chapman *et al*., 2012, Chege *et al*., 2009, Chege *et al*., 2013).

1.10.2 The development of recombinant strains to enhance safety and/or immunogenicity of BCG as a vaccine vector

An alternate strategy to increase immunogenicity, and possibly safety, is the addition of exogenous genes to BCG to create recombinant BCG strains (Chapman *et al*., 2011b, Ottenhoff and Kaufmann, 2012). One approach, aimed to promote antigen translocation from the endosome to the cytoplasm, is the addition of endosome perforating endolysin genes to BCG. The use of 2 such endolysins in BCG vaccine studies, listeriolysin (derived from *Listeria monocytogenes)* and perfringolysin-o (derived from *Clostridium perfringens*), are discussed below.

These molecules perforate the phagosomal membrane allowing for the formation of pores through which antigen translocation can occur. Antigen released into the cytoplasm elicits improved T cell responses (stimulating MHC I antigen presentation) and limits toxicity to the mycobacterial vector (as discussed in section 1.9.2.3). Listeriolysin has been suggested to

promote apoptosis of infected macrophages as well as lysis of the phagosome thereby allowing for antigens to move into the cytoplasm (Grode *et al*., 2005). This group first reported that a urease C-deficient BCG strain expressing listeriolysin (Hly) is more protective against aerosol MTB challenge in mice as compared to the parental BCG strain (Grode *et al*., 2005). The urease C deficient strain decreases the pH of the phagosome allowing for the optimal functionality of listeriolysin (pH 5.5). Furthermore, listeriolysin degrades upon entering the cytoplasm which enhances the safety of this strategy. Additionally they demonstrated that Hly promoted apoptosis of macrophages infected with BCG leading to cross priming by dendritic cells and the induction of stronger T cell responses than the parental BCG strain. This vaccine (VPM1002) successfully passed a phase 1 safety trial and a phase IIa neonate trial is currently under way (Kaufmann *et al*., 2014). A similar molecule to listeriolysin is perfringolysin-O *(pfo)* which is derived from *Clostridium perfringens* (Sun *et al*., 2009). A representation of the mechanism of perfringolysin-O action is shown below in Figure 1.6.

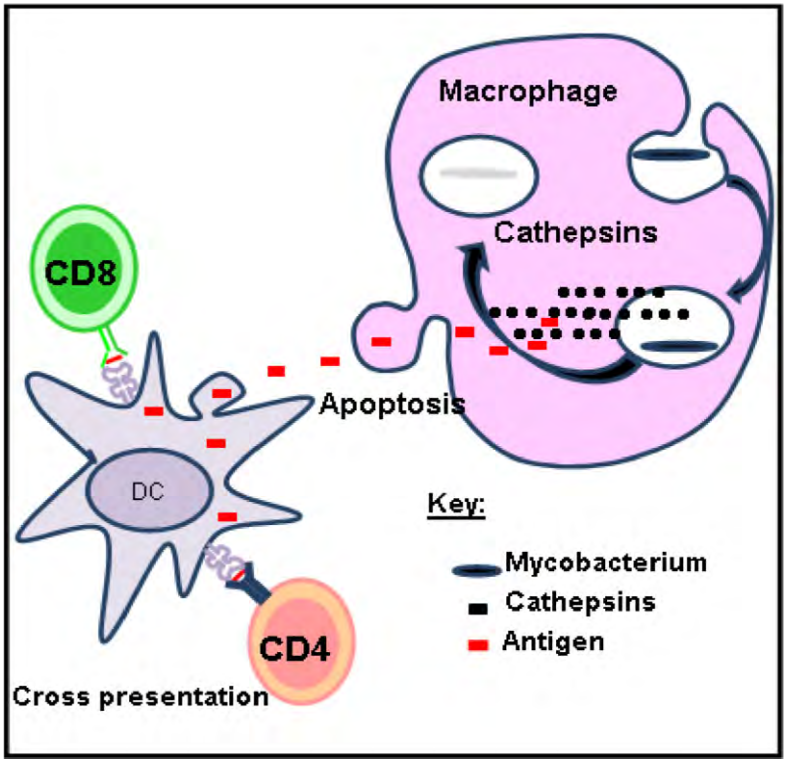


Figure 1.6. The action of perfringolysin on antigen presentation. Following the formation of the mycobacteria containing endosome by the macrophage, perfringolysin acts to perforate the endosome allowing for the release of apoptosis promoting cathepsins as well as the translocation of antigen through the cytoplasm to the cell membrane. Presentation of antigen via the MHC I pathway, allows for cross priming which leads to improved antigen specific T cell responses.

Murine and rhesus macaque studies have demonstrated that TB vaccines vectored by a perfringolysin knock in, urease C knock out BCG (Aeras 401) elicit enhanced protection and/or stronger CD8+ T cell responses to MTB as compared to the wild type strain (Rosario *et al*., 2010, Magalhaes *et al*., 2008b). The perfringolysin gene selected was a mutated gene that was picked as it was less toxic to the host cells than the wild type. It has a Gly 137 to Gln mutation which decreases the half-life of the protein in the cytosol. Unfortunately, the phase 1 TB vaccine safety trial using BCG Danish 1331 expressing pefringolysin O (Aeras-422) was terminated due to side effects which included the reactivation of shingles (Kupferschmidt, 2011, Ottenhoff and Kaufmann, 2012, Hoft *et al*., 2016). The effects of combining recombinant BCG strains containing lysins with auxotrophic strains, such as the pantothenate auxotroph, remain undetermined.

1.11. Using animal models to pre-clinically assess immunogenicity of rBCG vaccines expressing HIV-1/SIV Gag

In addition to clinical correlates of protection from elite controllers and LNTPs, studies have suggested that a full length HIV-1 Gag may be the most effectively expressed HIV immunogen in BCG based vaccines (Cayabyab *et al*., 2009, Mederle *et al*., 2002). Small animal and nonhuman primate studies using BCG to expresses HIV-1 Gag (exclusively or in conjunction with other HIV antigens) are reviewed below.

1.11.1 Small animal model studies suggest that rBCG is most effective as a priming vaccine

Small animal models used in pre-clinical vaccine testing are invaluable for cost effective assessment of vaccine immunogenicity. In addition to HIV-1 Gag, the small animal model has also been used to pre-clinically test rBCG expressing novel antigens such as Env gp120, Env V3 consensus envelope (CON6), Pol, Nef and the HIV-1 V3 loop (mV3) (Chapman *et al*., 2011a).

To date, several studies have reported testing rBCG vaccines expressing Gag (exclusively or in conjunction with other HIV antigens) in the small animal model. The first reported pre-clinical study to show the induction of Gag specific humoral and CTL responses using a BCG vector was performed in the early 1990s (Aldovini and Young, 1990, Aldovini and Young, 1991). This study, which assessed the induction of HIV specific responses in BALB/c mice following vaccination with rBCG expressing HIV-1 Gag, Pol or Env separately, demonstrated the induction of Gag specific IFNγ and IL-2 production (Aldovini and Young, 1991). Long lived DTH responses to SIV Gag and pure protein derivative (PPD) following intra-dermal vaccination with rBCG expressing a full length SIV *gag* have been reported in the guinea pig model. Additionally, they reported the induction of HIV specific humoral responses in the guise of IgG2 and IgG1 antibody titres (Kawahara *et al*., 2006, Kawahara, 2008). Recent reports indicate that rBCG expressing HIV-1 CRF01-AE Gag gene (HIV-1gagE) primes BALB/c mice fora matching recombinant vaccinia virus boost (Promkhatkaew *et al*., 2009). They compared a subcutaneous BCG prime and intravenous vaccinia boost to a corresponding intradermal prime boost regime. Whilst higher Gag specific CTL levels were initially observed following subcutaneous vaccination as compared to the intradermal route, similar HIV specific responses were observed at seven months post vaccination.

Im *et al*, (2007) assessed the induction of HIV specific immune responses in BALB/c mice following a rBCG lysine auxotroph prime and rMVA boost (both expressing consensus subtype A Gag)(Im *et al*., 2007). They reported a dose dependent increase in HIV specific CD8+ T cell responses. Using a heterologous prime boost vaccination regime, our group have recently reported increased immunogenicity and decreased BCG pathology in mice vaccinated with a rBCG Δ*panCD* /MVA expressing Gag as compared to mice vaccinated with rBCG wildtype /MVA expressing Gag (Chapman *et al*., 2012). Collectively, these small animal studies support the use of rBCG expressing *gag* as a priming vaccine. These studies also suggest that rBCG

auxotrophs, which have been shown to be safer and more immunogenic than wildtype BCG, may prime more effectively for heterologous MVA boost.

1.11.2 Non human primate studies suggest that rBCG is most effective as a priming vector

Non-human primates are immunologically and physiologically more similar to humans than mice models and therefore represent a better model for the assessment of vaccines. Furthermore, the similarity in response to mycobacterial infection between non-human primates and humans makes these models more biologically relevant than small animal models (Bontrop and Watkins, 2005, Doxiadis *et al*., 2001, Shinkai and Locksley, 2000). To date, several studies have reported testing rBCG vaccines expressing HIV/SIV Gag in the nonhuman primate model. Studies using rBCG to express SIV Gag (exclusively or in conjunction with other SIV antigens) as part of a homologous prime boost vaccination regime were reported to elicit SIV specific responses but not protect from SIV challenge (Yasutomi *et al*., 1993, Mederle *et al*., 2003).

However, when rBCG has been used as a priming vaccine in a heterologous prime boost regime, improved immunogenicity and in some cases protective efficacy has been reported (Cayabyab *et al*., 2009, Ami *et al*., 2005). Using an rBCG prime (expressing SIV Gag, Pol, Env) followed by a recombinant adenovirus 5 boost, Cayabyab *et al,* (2009) reported substantial vaccine induced CD8+ T cell polyfunctionality as well as a distinct central memory phenotype (Cayabyab *et al*., 2009). Both of these characteristics are thought to be required for protective efficacy of T cell based HIV vaccines (Dangeti, 2013, Barouch and Picker, 2014). Protective efficacy against mucosal SHIV challenge induced by an rBCG (SIV *gag*)/ vaccinia virus (SIV *gagpol*) heterologous prime boost regime has been previously reported (Ami *et al*., 2005). Interestingly, similar protective efficacy was not observed in control animals that received the inverse prime-boost combination or single vaccination with either vaccine therefore

suggesting that rBCG is most effective in preventing infection when used as a prime in a heterologous prime boost regime. Our group has previously shown that modified rBCG (pantothenate auxotroph) expressing Gag can prime successfully for a Gag virus-like-particle boost and that these responses are superior to a single vaccination with either vaccine (Chege *et al*., 2009). Collectively, these non-human primate studies promote the development of rBCG expressing HIV-1 Gag as a priming vaccine in a heterologous prime-boost vaccination strategy.

1.12 Study Aim and Hypotheses

Both non-replicating, safe, viral (poxviral) and persistent (RhCMV) vectors have recently be shown to induce the best correlates to protective efficacy in HIV/SIV research to date (Pitisuttithum *et al*., 2013, Picker, 2014). In the case of the latter, persistent effector memory CTL responses were correlated to the eradication of HIV viral reservoirs (Barouch and Picker, 2014). However application of CMV to humans is challenging and other persistent vector systems with established safety profiles, such as rBCG, represent interesting alternatives to study. Furthermore, the deletion of virulence genes as well as the inclusion of exogenous genes to promote improved immunogenicity merits the study of the four strains of BCG described in Table 1.1.

This study aimed to assess and compare the safety and immunogenicity profile of four BCG based candidate HIV-1 vaccines as priming vaccines in a heterologous prime boost regime. In this study recombinant BCG (rBCG) based vaccines expressing codon optimized HIV-1 subtype C *gag*, were constructed by the Anna-Lise Williamson group using varying strains of Danish BCG (kindly supplied by AERAS).

Hypothesis 1: We hypothesized that priming with either rBCG Δ*panCD* (*gag*) or rBCG Δ*panCDpfo* (*gag*) and boosting with SAAVI-MVA-C would lead to less pathology.

Hypothesis 2: We hypothesized that priming with either rBCG *pfo* (*gag*) or rBCG Δ*panCDpfo* (*gag*) and boosting with SAAVI MVA-C would lead to enhanced T cell responses as compared to the wild type strain.

Hypothesis 3: We hypothesized that assessment of murine gene expression following vaccination with modified rBCG would explain the molecular origins for vaccine induced pathology and immunogenicity.

Table 1. 2. Summary of rBCG vaccines used in this study

Strain of rBCG	#	Full name	Abbreviated name
Wild type strain	1	rBCG[pHS501]	rBCG WT (gag)
	2	rBCG[pConepi]	rBCG WT (control)
Pantothenate auxotrophic strain	3	rBCG Δ*panCD*[pHS501]	rBCG Δ*panCD* (gag)
	4	rBCG Δ*panCD*[pConepi]	rBCG Δ*panCD* (control)
Perfringolysin O expressing strain	5	rBCG *pfo*[pHS501]	rBCG *pfo* (gag)
	6	rBCG *pfo*[pConepi]	rBCG *pfo* (control)
Pantothenate auxotrophic strain expressing perfringolysin O	7	rBCG Δ*panCDpfo*[pHS501]	rBCG Δ*panCDpfo* (gag)
	8	rBCG Δ*panCDpfo*[pConepi]	rBCGΔ*panCDpfo* (control)

CHAPTER 2: Longitudinal comparison of the development of rBCG vaccine pathology

Contents

2.1 Introduction

There is an urgent need to develop candidate HIV vaccines which are both safe and immunogenic (Esparza and Van Regenmortel, 2014). BCG represents a safe and immunogenic priming vaccine vector and advances in developing novel strains of BCG have led to the opportunity to further assess BCG as an HIV vaccine vector. In order to characterise end point immunogenicity conferred by priming with these novel strains of rBCG and boosting with SAAVI MVA-C, we first have to understand the safety and immunological properties of each rBCG strain. Whilst the Pasteur rBCG Δ*panCD* has been well characterised by our group (Chapman *et al*., 2012, Chege *et al*., 2013), this study represents, to our knowledge, the first use of Danish Δ*panCDpfo* to express HIV-1 antigens. The Danish strain of BCG has been reported to be superior in safety and TB protective efficacy to the Pasteur strain (reviewed by Luca *et al*, 2013 (Luca and Mihaescu, 2013)). Whilst BCG is a safe vaccine vector in healthy individuals, it is virulent enough to cause pathology in immunocompromised individuals (Hesseling *et al*., 2003). Modified BCG vaccine vectors that induce lesser pathology than wild type strains would be ideal for use as an HIV vaccine in immunocompromised infants and adults.

Independent of heterologous antigen expression, BCG acts as an adjuvant by stimulating Toll-like receptor (TLR) pathways which lead to the recruitment and maturation of cells at the site of disease (Talat Iqbal and Hussain, 2014). Longitudinal assessment of the development of the immune response at a major lymphoid site, such as the spleen, would allow us the compare ability of different strains of BCG to promote the induction of a Th1 response (Talat Iqbal and Hussain, 2014, Hesseling *et al*., 2007b). Taken in context of the vaccine specific cellular (Chapter 3) and molecular (Chapter 4) immunogenicity results, this data would allow us to compare how different rBCG strains, with different properties, can influence the immune response as part of a heterologous prime boost vaccination strategy (Rao *et al*., 2013).

This chapter therefore aims to assess and compare the development of strain specific rBCG induced pathology/immune response between groups of mice vaccinated with the 4 strains of BCG used in this study. In order to accomplish this, we undertook a longitudinal study in the murine model to assess pathology readouts at multiple time points at the organ and cellular levels post vaccination. We longitudinally assessed typical markers of BCG infection in the murine model including; the enlargement of the spleen due to expansion of immune effector cells, granuloma formation in the liver, iNos activity in the liver and disseminated bacterial load in lymph nodes (North and Izzo, 1993, Nandakumar *et al*., 2014, Chapman *et al*., 2012). To further understand the cellular mechanisms of potential adjuvant properties and/or pathology development we longitudinally compared levels and activation of splenic T cell, B cells and dendritic cells between mice vaccinated with different strains of rBCG.

2.2 Materials and methods

2.2.1 Design of rBCG shuttle vector and preparation of rBCG vaccines

The following rBCG recombinants were generated by Dr Ros Chapman. The *E.coli*/ mycobacterial shuttle vector pHS501, expressing a full length BCG codon optimised HIV-1 subtype C *gag* gene was used to transform the four strains of BCG (Figure 2.1) ((Chapman *et al*., 2013). The 3' end of the *gag* gene is fused to an immunodominant BALB/C Env cytotoxic T lymphocyte epitope (RGPGRAFVTI) from the V3 region of an HIV-1 subtype B Env protein. Additionally, this was fused to a paramyxovirus Simian-Virus 5 monoclonal antibody tag (SV5). The 5' end of the *gag* gene was fused to the *M. tuberculosis* 19 kDa signal sequence with expression under the control of a *M. tuberculosis mtrA* promoter. An empty vector control not expressing *gag* (pConepi) was additionally created for each of the 4 strains of BCG.

Following transformation, BCG strains were grown on Middlebrook 7H10 agar supplemented with 10% oleic acid-albumin-dextrose-catalase (OADC) and 0.5% glycerol (7H10) or in

Middlebrook 7H9 broth supplemented with 10% OADC, 0.2% glycerol and 0.025% tyloxapol (7H9) on rollers (4 rpm) at 37^0C. Kanamycin (10 µg/ml) was included in the media for plasmid selection. Media was supplemented with pantothenate (48 µg/ml) for the growth of BCG containing the Δ*panCD* deletion.

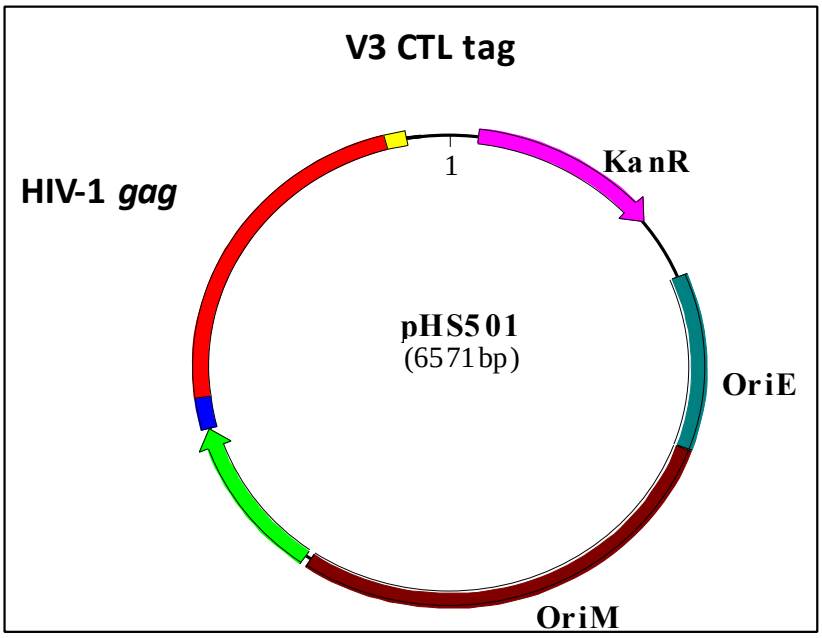


Figure 2.1: Schematic map of E.coli/mycobacterial shuttle vector expressing Gag.

2.2.3 Characteristics of the SAAVI-MVA-C vaccine

The SAAVI MVA-C vaccine has been designed and well characterised by the HIV Vaccine Development Group at UCT (Ncayiyana, 2009, Burgers *et al*., 2008, Burgers *et al*., 2009). This vaccine expresses an HIV-1 subtype C polyprotein containing Gag, reverse transcriptase, Tat, and Nef (Grttn) which is inserted into the del III region of MVA. Additionally, this vaccine contains a truncated HIV-1 subtype C Env gp150 which is inserted into the 49/50 region of MVA (Figure 2.2). The Grttn is under the control of a 40K promoter whilst the gp150 is under the control of the I3 promoter. The same V3 CTL tag is present on this gp150 as in the rBCG shuttle vector.

2.2.4 Details of mice used in this study

Female BALB/c mice (age: 4-6 weeks) were purchased from the South Africa Vaccine Producers Pty Ltd (Johannesburg, South Africa). Mice were housed at the University of Cape Town Animal Unit for 10 days in order to acclimatise prior to vaccination. Vaccination and handling were performed by a trained laboratory animal technologist, Mr Rodney Lucas. Ethics approval for the experiments was obtained from the University of Cape Town Animal Research Ethics Committee (UCT Protocol number: 012/001).

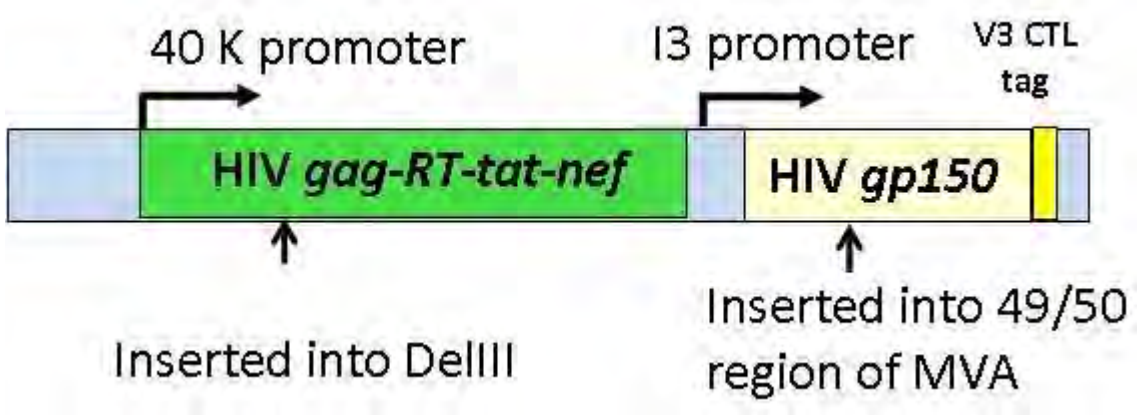


Figure 2.2: Schematic map of SAAVI MVA-C vaccine insert. This rMVA is designed to express a Grttn polyprotein under the control of a 40k promoter as well as a truncated gp150 under the control of an I3 promoter (Burgers *et al*., 2008).

2.2.5 Vaccine preparation and vaccination regime

Stocks of rBCG vaccines and SAAVI MVA-C were stored at -80 °C. Prior to vaccination, vaccines were allowed to thaw on ice for an hour. Resuspension buffer (200 µl) (Appendix 1) was used to dilute rBCG vaccines to a dose of of 1 x 10^7 cfu. This was given via intraperitoneal injection on Day 0 to 7 groups of 3 mice per rBCG vaccine (8 rBCG vaccines in total). One group of mice per each rBCG vaccine was sacrificed at days; 2, 3, 7, 12, 21 and 28 post vaccination (Figure 2.3). The final group of rBCG vaccinated mice were vaccinated with intramuscular injection with 10^4 pfu of SAAVI MVA-C in 100µl PBS on day 28. This group was sacrificed 12 days after the day 28 boost. Mice were killed via cervical dislocation. Spleens, livers and abdominal

lymph nodes were harvested from sacrificed mice at each time point in order to undertake comprehensive immunological, histological and pathology characterisation.

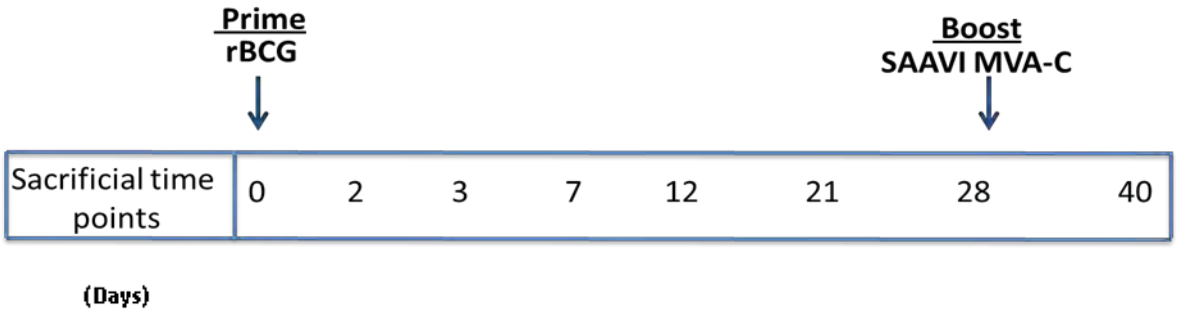


Figure 2.3. Vaccination schedule and sacrificial time points for longitudinal assessment of the development of BCG pathology. Groups of mice (n=3) were vaccinated with 1 of 8 rBCG vaccines (dosage: 10^7cfu) on day 0. Groups were sacrificed at days 2, 3, 7, 12, 21 and 28 post rBCG vaccinations. One group of day 0 BCG vaccinated mice (8 groups of 3 mice) were vaccinated on day 28 with SAAVI MVA-C (10^4 pfu) and sacrificed 12 days after this boost on day 40 in concordance with our prime boost vaccination strategy used in chapters 3 and 4.

2.2.6 Preparation of single cell suspension of murine splenocytes following sacrifice

Spleens from each vaccinated group of 3 mice per time point were pooled in 30 ml of sterile Roswell Park Memorial Institute cell culture medium (RPMI) (Life Technologies, Waltham, USA) following every sacrifice (1 spleen per/10 ml RPMI). In a bio-safety cabinet, spleens were poured onto a steel cell strainer (70 µm pores) placed in a petri dish and homogenised using a 2 ml syringe plunger (Sigma Aldrich, St Louis, USA). The homogenate was transferred to a 50 ml Falcon centrifuge tube (Becton Dickinson, Franklin Lakes, USA) and the petri dish was washed with 10 ml RPMI and this was added to the 50 ml Falcon tube. The homogenate was made up to 50 ml using RPMI and this was centrifuged for 5 minutes at relative centrifugal force (RCF) of 230 g (1400 rpm) in order to pellet the cells. The supernatant was discarded and the pellet was resuspended in 50 ml RPMI and washed twice more. A Pasteur pipette was used to remove extracellular fibrin clots. Following the final wash, the pellet was resuspended in 1 ml of RPMI. Red blood cells (RBCs) were lysed by adding 1 ml of RBC lysis buffer (Sigma,St Louis, USA) to the Falcon tube. This mixture was gently agitated for 1 minute exactly and topped up to 50 ml with RPMI. Following centrifugation for 5 minutes at 230 g (1400 rpm), the supernatant was discarded and the cells were resuspended in 30 ml of R10 complete media (Appendix 1). A Neubauer counting chamber (Marienfeld Superior, Lauda-Königshofen Germany) was used to count cells using a 1:10 dilution

in Trypan Blue (Sigma, St Louis, USA). The cell concentration was adjusted to the concentration of 1 million/ml for each assay by adjusting the volume of R10 media.

2.2.7 Confirmation of the Δ*panCD* deletion and *pfo* insertion in vaccine stocks

The deletion of the *panCD* genes in the rBCG Δ*panCD* (*gag*) and rBCG Δ*panCDpfo* (*gag*) vaccines was confirmed by culturing vaccine stocks in the absence of pantothenate (Vitamin B5) in 7H9 media. Following a 3 week culture at 37° C, the optical density of the cultures was measured at 600nm. Data was plotted using Graphpad Prism Version 5 (Graphpad Software, San Diego, USA). PCR was used to assess the presence of the *pfo* gene in all rBCG vaccine stocks. Primers to amplify the perfringolysin gene were synthesised based on the primer sequences from (Sun *et al*., 2009). Primers A (5'- ACGGCTACCGTCTGGACAT- 3') and B (5'- CGATGGCTTCTTCGATGC- 3') were used to amplify a 2085 base pair segment containing the *pfo* gene. The following PCR conditions were used: 1: A single 95° C denaturing cycle for 4 min, 2: 95 ˚C for 30 seconds denaturing, 60 ˚C 1 min annealing followed by a 72 ˚C 2 min for extension (30 cycles), 3: 72 ˚C 10 min with one cycle, 4: 4 ˚C storage. All PCR reactions were carried out on a GeneAmp PCR machine (Applied Biosystems, Waltham, USA) and PCR products were separated on a 0.8% agrose gel.

2.2.8 Histopathology of murine liver using CD3 staining and iNos

Preparation of histology samples was performed by the Histology Core Facility (Division of Immunology, University of Cape Town). Following sacrifice, murine liver lobes from individual mice (n=3 per group) were fixed overnight in 4% phosphate buffered formalin. Sections (5 µm) were cut from embedded blocks and mounted on glass slides. Sections were incubated overnight at 56° C and rehydrated using Zylol and water. These sections were then blocked for 15 min in 3% hydrogen peroxide (H_2O_2) which was then rinsed off with water. Antigen retrieval was performed by incubating the slides in 10mM citrate buffer (pH6) in a pressure cooker for 2 minutes. These slides were then cooled in water before being blocked for 20 minutes with normal goat serum (Dako X0907). Following blocking, slides were rinsed in sterile

PBS. Slides were stained with rabbit anti-human CD3 (Dako pab A0452) (1:100) or iNos (K4003) (1:100) for 90 minutes at room temperature. Staining with CD11b proved unsuccessful because of high background whilst B220 staining enumerated exceptionally few activated B cells. Following staining, slides were rinsed in sterile PBS. Slides were stained with anti-rabbit Envision secondary antibody (Dako K4003) for 30 minutes which was then rinsed with sterile PBS. DAB (1,4-diamino-2-butanone) substrate (Dako K3468) was applied to the slides and washed off with sterile PBS after 10 minutes. These sections were then counterstained with Mayer's haematoxylin for 4 minutes and washed underwater for 5 minutes. Following this rinse, sections were dehydrated by increasing concentrations of Zylol, covered with a cover slip and mounted in a synthetic resin based mount, Entellan (Merck, USA). Sections were photographed and analysed under the guidance of an experienced pathologist (Dr Irene Garcia-Gabay, University of Geneva). Further imaging support was kindly provided by Susan Cooper (University of Cape Town). A Zeiss AxioVert200M fluorescence microscope with a Zeiss AxioCam High resolution monochrome camera was used to analyse and capture magnified photographs of sections.

2.2.9 *Ex vivo* growth of rBCG from abdominal lymph nodes of vaccinated mice to determine disseminated bacterial burden

Following sacrifice, 6 abdominal lymph nodes were collected from each group of mice and placed in a 2 ml eppendorf tube containing 1 ml of resuspension buffer (Appendix 1). A Qiagen homogeniser was used to homogenise the sample into a smooth homogenate with no clumps. Two aliquots of 100 µl per sample were stored in sterile Eppendorf tubes. The sample was diluted 1 in 10 by adding 900 µl of resuspension buffer to 100 µl of the homogenate. 100 ul of sample was plated in duplicate on 7H10 Agar plates. For mice vaccinated with Δ*panCD* containing strains, 48 µg/ml pantothenate was used to supplement the agar. Resuspension buffer alone was plated separately as a negative control. The plates were incubated at 37 °C for 21 days. Colonies were then counted using a Stuart Plus touch sensitive colony counter equipped with a magnifying glass (Bibby Scientific, Staffordshire, UK).

2.2.10 Longitudinal assessment via flow cytometry of dendritic cells, T cells and B cells in vaccinated murine splenocytes

Three individual panels of cytometry antibodies were used to longitudinally assess dendritic, T and B cell levels and activation status. For T and B cells, optimised antibody cocktails were used according to manufacturer's instructions (Becton Dickinson, Franklin Lakes, USA) (Table 2.1). For the dendritic cell panel, individual antibodies and isotype controls were sourced (Becton Dickinson and Biolegend) and titrated. Following the preparation of single cell suspension of splenocytes, 1.5 x 10^6 splenocytes per group were added to a FACS tube in a final volume of 1 ml R10 media. Three tubes per group were used so that separate T, B and dendritic cell staining could be done. The supernatant was discarded and 25 µl blocking solution (Appendix 1) was added to each tube and mixed gently with a pipette. This was incubated on ice for 20 minutes and washed with cold sterile FACS buffer (Appendix 1). Cocktails of flourochrome conjugated antibodies were added to the appropriate tubes. These tubes were incubated on ice in the dark for 30 minutes and then washed twice with 2 ml sterile cold FACS buffer (Appendix 1). Samples were acquired on a BD LSRII flow cytometer. Flow cytometry data was analysed using FlowJo Version 7.1 (Treestar) based on the gating strategies suggested by the antibody manufacturer (Figure 2.4). We observed that >80% of DC subsets were of myeloid origin and focussed on assessing total DC activation (CD11c+ cells). Additionally, using the pre-optimised B cell cocktail, we were able to easily identify activated B cells as opposed to using the B220 marker in the dendritic cell panel. In order to quantify cellular phenotype and activation at the organ level, positively stained cells were adjusted by multiplying by the total count per spleen (Calculated by the Neubauer counting chamber count done when preparing single cell suspensions of splenocytes for this assay). Longitudinal data for these adjusted estimated cell numbers was plotted using GraphPad Prism Version 5 (GraphPad Software, San Diego, USA). Area under the curve values (AUC) were calculated using the trapezoid rule following statistical assistance from the Clinical Research Centre (CRC).

Table 2.1: Antibody panels to assess cellular activation with optimised assay volumes

T cell panel (10 µl)	B cell panel (20 µl)	DC panel	
CD3 APC-Cy7	CD19 APC	CD11c PE-Cy7(1 µl)	CD11b PerCP (0.5 µl)
CD4-PerCP Cy5.5	CD25 PE-Cy7	CD80 PE (0.5 µl)	B220 APC-Cy7 (0.5 µl)
CD44 FITC	CD69 PE	CD86 FITC (0.5 µl)	Gr-1 APC (0.5 µl)

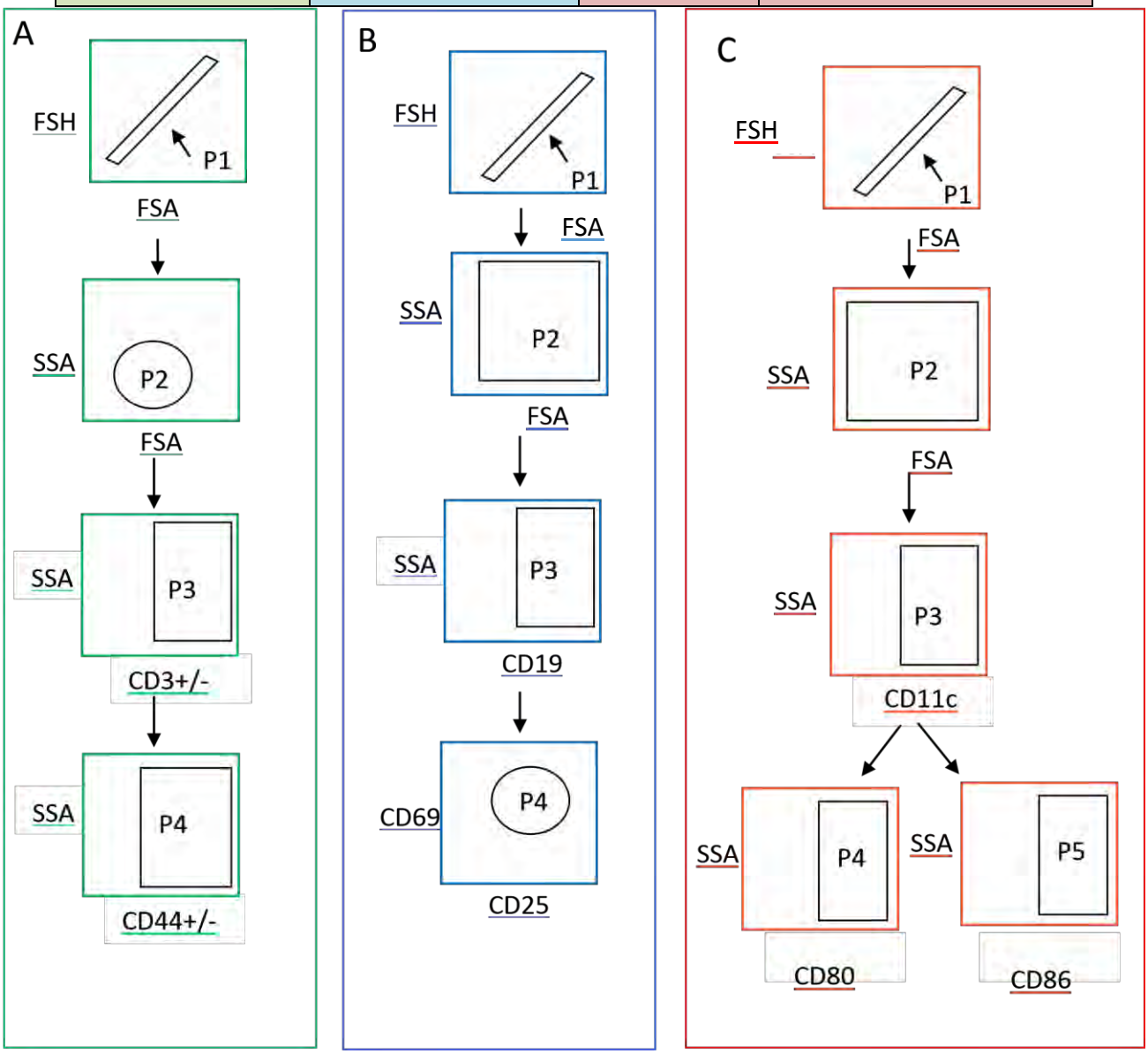


Figure 2.4. Gating strategy to determine cellular subset phenotype and activation in rBCG vaccinated mice. (A) In order to quantify T cells and T cell activation we first gated on single cells (P1) followed by lymphocytes (P2). CD3+ cells (P3) were used as a parent population to enumerate the frequency of activated (CD44+) T cells (P4). **(B)** In order to quantify B cells and B cell activation (P4) we first gated on single cells (P1) followed by a keeper gate (P2) and CD19+ cells (P3). **(C)** DCs were enumerated by defining a population for CD11c+ cells (P3). Activation markers (CD86) (P4) and (CD80) (P5) were assessed.

2.3 Results

2.3.1 Functional confirmation of the Δ*panCD* deletion

In order to confirm the Δ*panCD* deletion phenotype in rBCG Δ*panCD* (*gag*/control) vaccine stocks, we cultured vaccine stocks in 7H9 Middlebrook broth for 21 days in the presence and absence of pantothenate (vitamin B5) supplementation (Figure 2.5). Growth was measured 21 days after culture began using spectrophotometry. Detectable optical density, and hence bacterial growth of Δ*panCD* (gag/control) and Δ*panCDpfo* (*gag*/control) from vaccinated mice was only observed in the presence of pantothenate supplementation indicating that the Δ*panCD* deletion was present in rBCG Δ*panCD* (*gag*/control) and rBCG Δ*panCDpfo* (*gag*/control) vaccines used in this study.

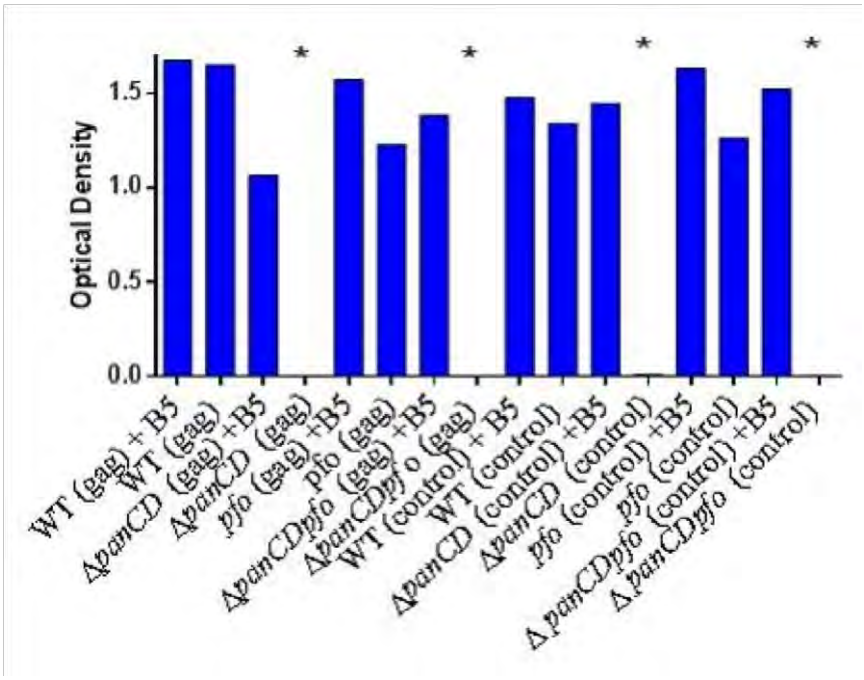
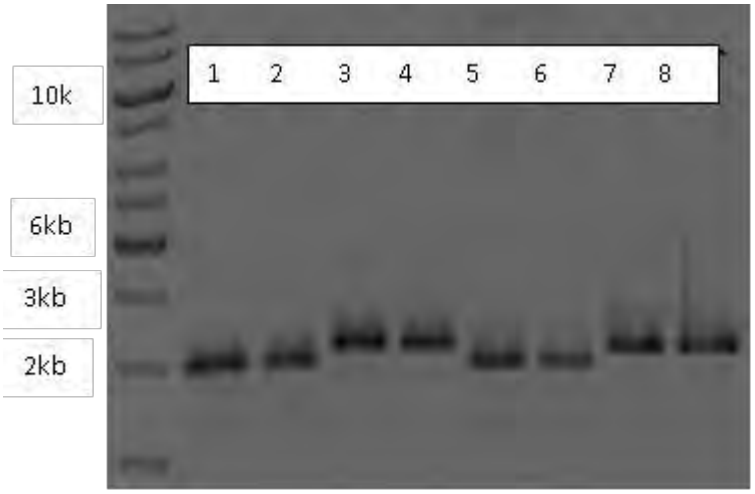


Figure 2.5: Functional confirmation of the Δ*panCD* deletion in vaccine stocks by 21 day culture with and without pantothenate (Vitamin B5) supplementation. Only Δ*panCD* containing strains demonstrated no growth in the absence of pantothenate supplementation (as indicated by astricies).

2.3.2 PCR confirmation of insertion of *pfo* gene into *UreC* locus using PCR

In addition to confirming the deletion of the Δ*panCD* deletion in vaccine stocks, we validated the presence of the *pfo* gene in *pfo* containing rBCG strains (Figure 2.6). Confirmation of the insertion of *pfo* into the *UreC* locus of BCG was performed using PCR. PCR products were run on an agrose gel parallel to a molecular weight marker. Products from rBCG *pfo* (*gag*/control) and Δ*panCDpfo* (*gag*/control) were observed to be larger (approximately 2083 bp) as compared to products from Δ*panCD* and WT strains of rBCG (approximately, 1967 bp). This was found to be in line with the validation of the original mutants reported by (Sun *et al*., 2009).



1: WT (*gag*) 2: Δ*panCD* (*gag*) 3: *pfo* (*gag*) 4: Δ*panCDpfo* (*gag*)
5: WT (control) 6: Δ*panCD* (control) 7: *pfo* (control) 8: Δ*panCDpfo* (control)

Figure 2.6: Confirmation of the presence of the *pfo*-specific insertion allele. Presence was verified by PCR using primer pairs targeted to amplify a 2083bp fragment of the *pfo* gene in the case of the *pfo* containing-BCG strains (lanes 3,4,7,8) or a 1967bp *ureC* fragment for the WT and Δ*panCD* strains (lanes 1,2,5,6). A no DNA (blank control, lane 9) was also used to confirm specificity of product.

2.3.3 Longitudinal assessment of disseminated bacterial burden in rBCG vaccinated mice

Following validation of vaccine stocks and vaccination of mice, we aimed to characterise bacterial burden at a peripheral site (Figure 2.7). In order to determine the longitudinal kinetics of disseminated bacterial load in mice vaccinated with rBCG vaccines, we grew colonies of rBCG from the abdominal lymph nodes of sacrificed mice at each time point using *ex vivo* mycobacterial culture. We observed that mice vaccinated with rBCG Δ*panCD* (*gag*/control) and Δ*panCDpfo* (*gag*/control) did not have detectable mycobacteria from 7 days post rBCG vaccination. However, detectable and persistent bacterial loads (peaking between days 7-12) were observed throughout the time course for mice vaccinated with WT (*gag/control*) and *pfo* (*gag/*control). In persistent strains, there was an increase in bacterial load following SAAVI MVA-C boost at day 28 suggesting that boosting persistent BCG can increase endpoint bacterial load.

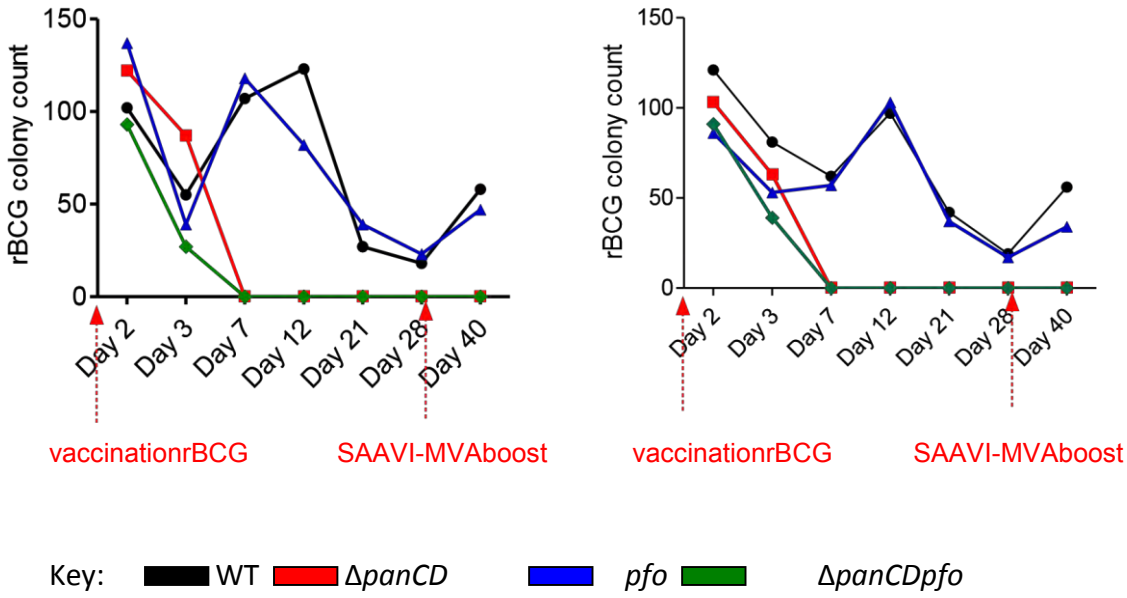


Figure 2.7: Colony counts from homogenized abdominal lymph nodes of mice vaccinated with rBCG (*gag*) vaccines (A) and rBCG (control) vaccines (B) following culture for 21 days on 7H10 agar. Following Day 0 prime with rBCG, mice cleared Δ*panCD* containing strains by day 7. Wildtype and *pfo* strains were found to persist until day 40 and endpoint.

2.3.4 Longitudinal assessment of murine liver histopathology

Anti-vector immunity is often detrimental when using vector based antigen delivery systems. Developing safer vaccines is a priority whilst simultaneously attempting to maximise immunogenicity. Mycobacterial granulomas form in multiple organs as a result of immune effector cells surrounding infected cells to limit the dissemination of the pathogen following failure to kill the pathogen (Gideon *et al*., 2015, Ufimtseva, 2015). In order to longitudinally assess and compare the pathology associated with each strain of rBCG used in this study, we stained liver sections from sacrificed mice with anti-CD3 specific antibody at each time point to assess BCG granuloma formation (n=3). Representative figures of positive control staining in rBCG vaccinated mouse and negative control CD3+ staining are shown in Figure 2.8. Longitudinally, we observed that mice vaccinated with rBCG Δ*panCD* (*gag*/control), *pfo* (*gag*/control) and Δ*panCDpfo* (*gag*/control) presented with consistently fewer granulomas than the WT strain. We observed peak granuloma counts for most groups at day 21 which coincides with peak T cell count. However, peak granuloma counts for groups vaccinated with the Δ*panCDpfo* strain of rBCG (*gag/*control) were observed on day 12 indicating an earlier T cell response. Representative plots for time points 2, 21 and 40 are shown in Figure 2.8 C. Additionally, we stained liver sections for the oxidative response marker iNos (peak responses shown in Figure 2.9). iNos activity was markedly less intensive in rBCG Δ*panCD* (*gag*/control) and rBCG Δ*panCDpfo* (*gag*/control) strains as compared to mice vaccinated with rBCG WT (*gag*/control) and rBCG *pfo* (*gag*/control). To summarise, murine liver histological analysis of granulomas associated less pathology to Δ*panCD*, Δ*panCDpfo* and to some degree *pfo* strains of rBCG vaccinated mice as compared to Danish WT.

2.3.4 Longitudinal comparison of splenocyte numbers in rBCG vaccinated mice

Following the vaccination and sacrifice regime described in Figure 2.3, total splenocytes numbers were assessed and compared (Figure 2.10). Similar patterns of increasing splenocytes count were observed in all vaccinated groups with maximum splenocyte numbers peaking on Day 21 post rBCG vaccination. Across the duration of the experimental time course, mice vaccinated with modified rBCG (*gag*) vaccines presented with lower splenocyte

counts, as determined by the area under the curve value (AUC), as compared to WT (*gag*). Mice vaccinated with the rBCG Δ*panCD* (*gag*) and rBCG Δ*panCDpfo* (*gag*) strains presented with lower splenocytes counts than the WT and *pfo* strains. Mice vaccinated with rBCG (*gag*) vaccines consistently presented with slightly higher splenocyte counts as compared to mice vaccinated with the corresponding rBCG (control) vaccine.

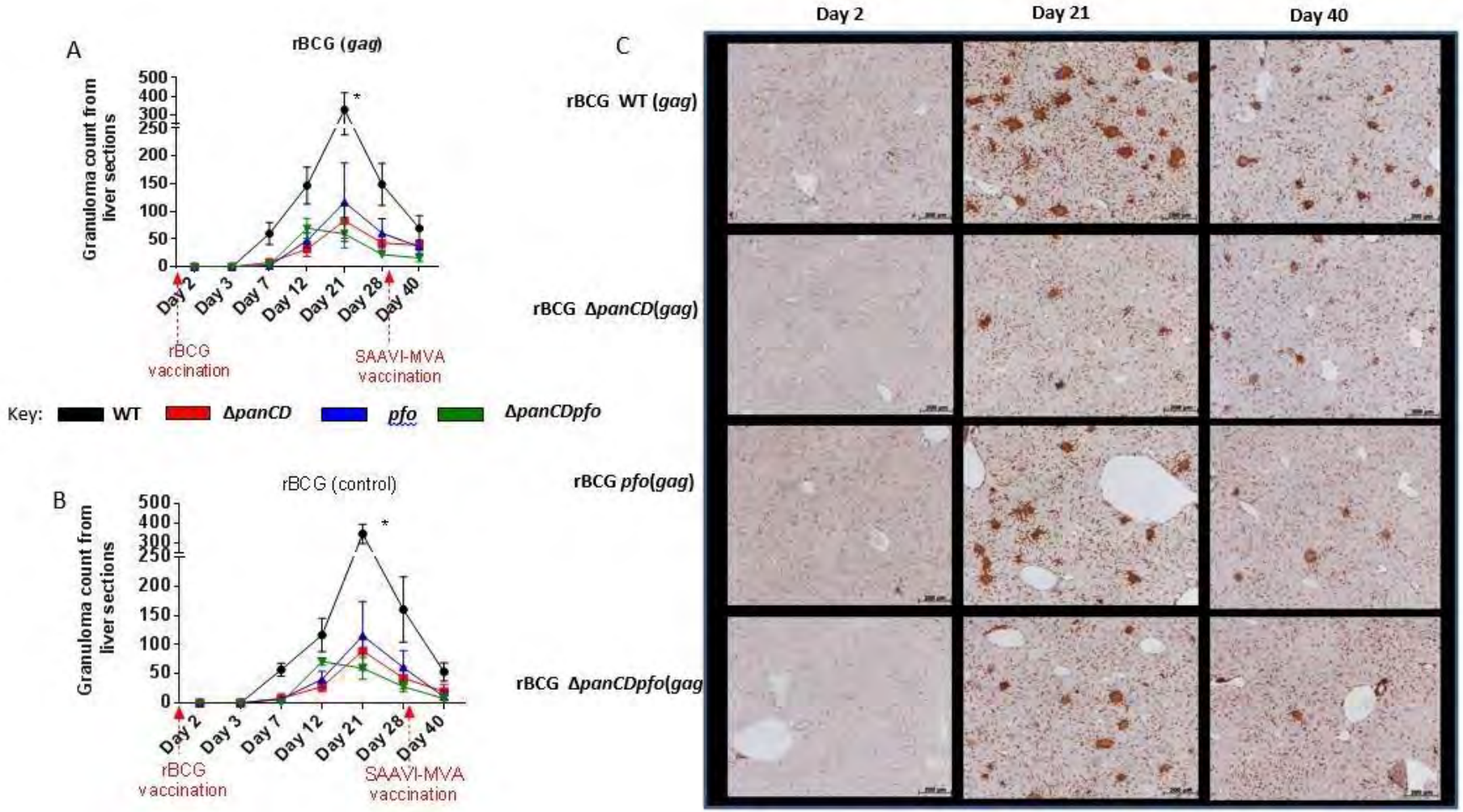


Figure 2.8. Longitudinal assessment of granuloma formation by immunohistochemistry in murine liver following vaccination with rBCG vaccines. Following sacrifice at days post vaccination (x-axis), liver sections were stained with anti-CD3 to quantify granulomas in rBCG (Gag) **(A)** and rBCG (control) **(B)** vaccinated mice. (C) Representative figures showing CD3 staining for rBCG (Gag) vaccines at days 2, 21 and 40 indicate decreased granuloma formation following vaccination with rBCG Δ*panCD* (*gag*), rBCG Δ*panCDpfo* (*gag*) and surprisingly rBCG *pfo* (*gag*) as compared to rBCG WT (*gag*).

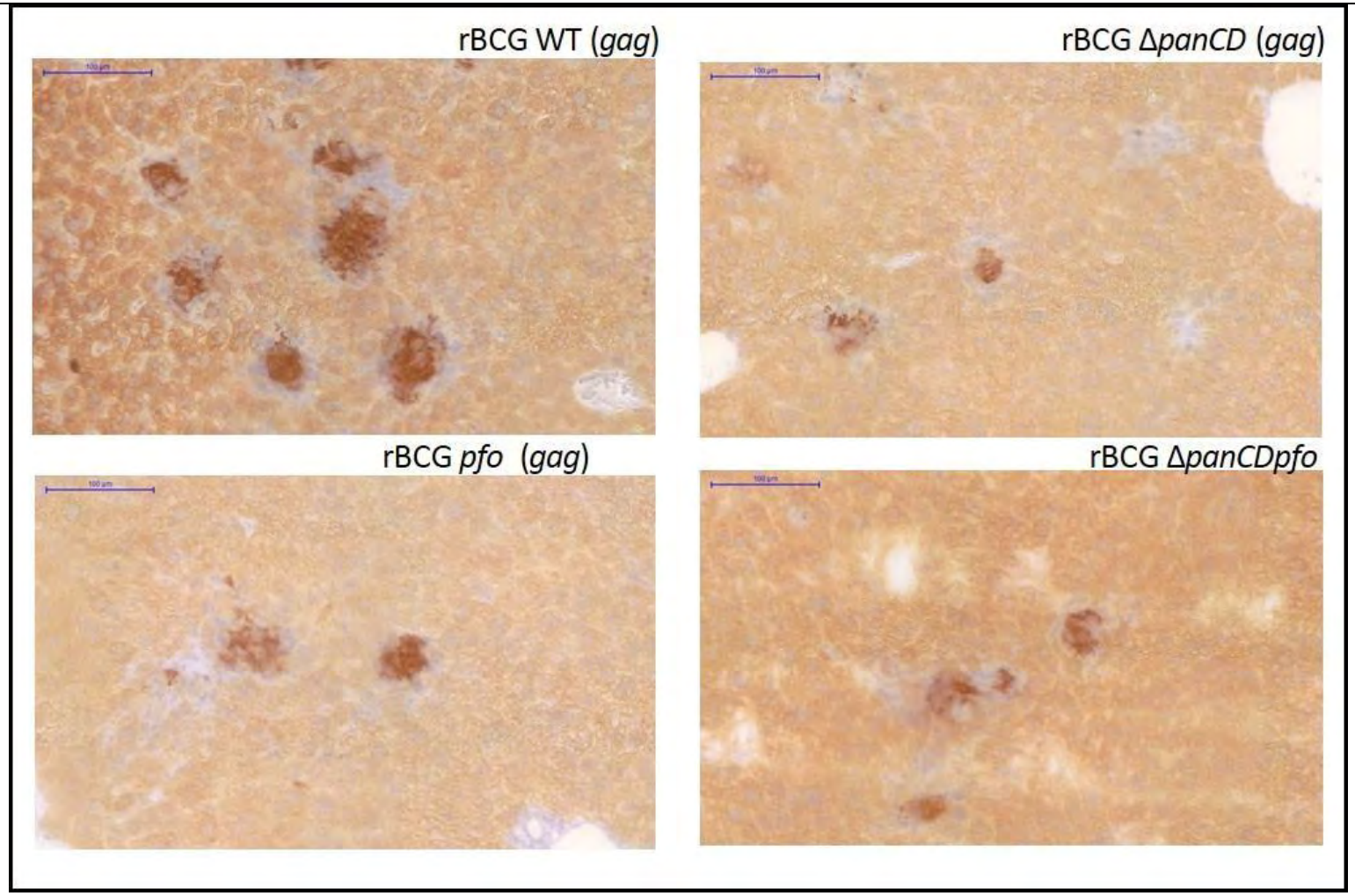


Figure 2. 9: Assessment of iNos production by immunohistochemistry in murine liver 21 days after vaccination with rBCG vaccines. Following sacrifice at 21 days post vaccination, liver sections were stained with anti-iNos to quantify granulomas in rBCG (*gag*) and rBCG (*control*) vaccinated mice.

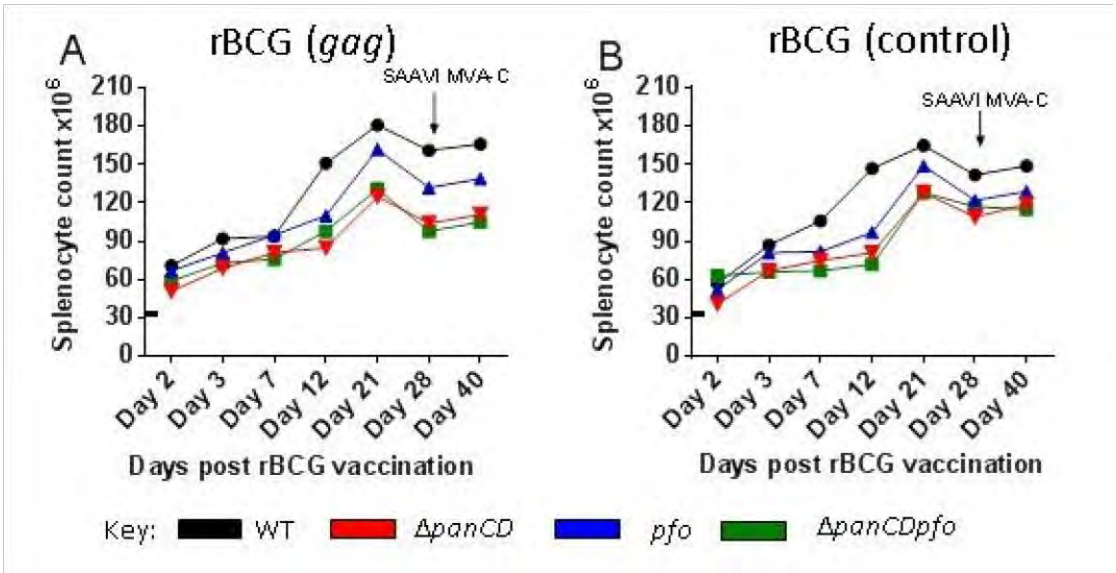


Figure 2.10: Longitudinal splenocytes counts of mice vaccinated with rBCG vaccines. Total splenocyte count for rBCG (*gag*) vaccines **(A)** and rBCG (control) vaccines **(B)** were measured at each time point following sacrifice (n=3 per group). Indentation of the y-axis represents splenocyte counts of unvaccinated control group.

Table 2.2: Area under the curve (AUC) values for longitudinal splenocyte counts

	WT	Δ*panCD*	*pfo*	Δ*panCDpfo*
(*gag*)	5.7 E+09	3.8 E+09	4.8 E+09	3.8 E+09
(control)	5.3 E+09	3.5 E+09	4.4 E+09	3.7 E+09

2.3.5 Longitudinal comparison of T cell count and activation in rBCG vaccinated mice

A T cell stimulating HIV vaccine vector should be able to deliver antigen and support the induction of a specific and controlled immune response. Excessive nonspecific or vector specific T cell activation has been shown to increase the chances of infection and has been linked to faster HIV disease progression. In order to longitudinally assess and compare the effects of vaccination with different strains of rBCG (*gag* and control) on T cell count and activation in the murine model we stained splenocytes of vaccinated mice with an optimised antibody cocktail comprising of CD3, CD4 and CD44 as per manufacturers instruction. T cell counts in mice vaccinated with recombinant strains of rBCG (*gag*) and rBCG (control) induced similar sigmoid profiles up until peak T cell count at day 21 (Figure 2.11). T cell levels decreased for all groups from day 21 to day 28 for all groups. Following a SAAVI MVA-C boost

on day 28, mice vaccinated at day 40 displayed higher T cell counts than mice sacrificed on day 28 that only received rBCG (gag or control) vaccination. Quantification of T cell count across the experimental time course (Figure 2.11A, Table 2.3) revealed that mice vaccinated with rBCG *ΔpanCD* (*gag*) (1.65E+08) and rBCG Δ*panCDpfo* (*gag*) (1.64E+08) presented with fewer T cells as compared to groups vaccinated with rBCG WT (*gag*) (2.84E+08) and *pfo* (*gag*) *(*2.2E+08). Similar profiles were observed for rBCG (control) groups (Figure 2.11B). Analysis of T cell activation across the experimental time course (AUC, table 2.4) revealed that mice vaccinated with rBCG *ΔpanCD*(*gag*) (5.46E+08) and rBCG Δ*panCDpfo*(*gag*)(5.14E+07) activated fewer T cells as compared to rBCG WT (*gag*) (1.20E+08) and rBCG *pfo* (*gag*) (8.73E+07) (Figure 2.11 C and D). Similar profiles were observed for rBCG (control) groups (Figure 2.11D).

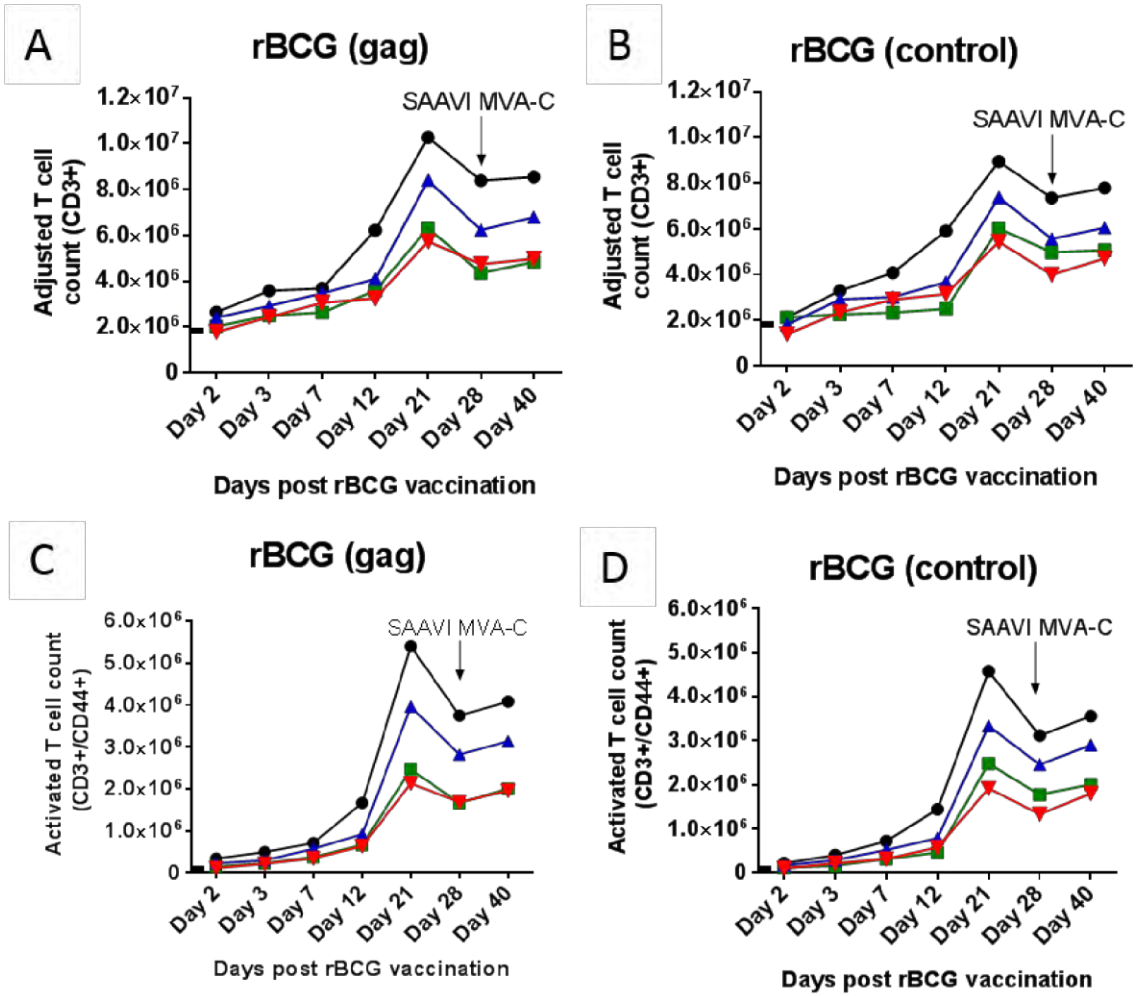


Table 2.3: Area under the curve (AUC) values for T cell counts

	WT	Δ*panCD*	*pfo*	Δ*panCDpfo*
(*gag*)	2.84E+08	1.65E+08	2.2E+08	1.64E+08
(control)	2.57E+08	1.61E+08	1.96E+08	1.51E+08

Key: WT Δ*panCD* *pfo* Δ*panCDpfo*

Table 2.4: Area under the curve (AUC) values for T cell activation

	WT	Δ*panCD*	pfo	Δ*panCDpfo*
(*gag*)	1.20E+08	5.46E+07	8.73E+07	5.14E+07
(control)	1.01E+08	5.38E+07	7.61E+07	4.48E+07

Figure 2.11. Longitudinal splenic T cell count and activation in mice vaccinated with rBCG vaccines. T cell count in mice vaccinated with rBCG (*gag*) **(A)** and rBCG (control) **(B)** (*n*=3 per group). T cell activation in rBCG (*gag*) (**C**) and rBCG (control) (**D**) vaccinated mice. Indentation of the y-axis represents T cell count/activation of unvaccinated control group.

2.3.5 Longitudinal comparison of dendritic cell (DC) counts and activation

Dendritic cells are key mediators of early immune response and function to present antigen as well as release soluble cytokine and chemokines to attract T cells to the site of infection. In order to longitudinally assess and compare the effects of vaccination with different strains of rBCG (*gag*) on dendritic cell count, we stained splenocytes of vaccinated mice with CD11c, (Figure 2.12). Area under the curve (AUC) analysis revealed that mice vaccinated with WT(*gag*)(9.89E+07) presented with a greater quantity of splenic DCs over the experimental time course as compared to rBCG *ΔpanCD* (gag*)* (8.12E+07), rBCG *pfo* (*gag*) (8.83E+07) and rBCG Δ*panCDpfo* (*gag*) (7.91E+07) . Interestingly, AUC values indicated that rBCG (control) vaccines recruited more dendritic cells to the spleen as compared to their corresponding rBCG (*gag*) vaccines. Another notable trend was the observed decrease in dendritic cell count from the day 28 time point to the day 40 time point in mice vaccinated with the rBCG Δ*panCD* strains (*gag* and control) of rBCG.

Antigen presenting cell activation markers, CD80 and CD86 are integral to modulating a T cell response during antigen presentation (Sansom *et al*., 2003). Area under the curve (AUC) analysis revealed that mice vaccinated with WT (gag)(2.33E+06) presented with a greater quantity of CD80+ DCs over the experimental time course as compared to rBCG *ΔpanCD* (gag) (1.82E+06), rBCG *pfo* (*gag*) (1.99E+06) and rBCG Δ*panCDpfo* (*gag*) (1.69E+06). Similarly, mice vaccinated with rBCG WT (*gag*) (1.75E+06) presented with a greater quantity of CD86+ DCs over the experimental time course as compared to rBCG *ΔpanCD* (*gag*) (1.40E+06), rBCG *pfo* (*gag*) (1.57E+06) and rBCG Δ*panCDpfo* (*gag*) (1.35E+06.). As observed with dendritic cell counts, AUC values indicated that rBCG (control) vaccines recruited more activated dendritic cells to the spleen as compared to their corresponding rBCG (*gag*) vaccines.

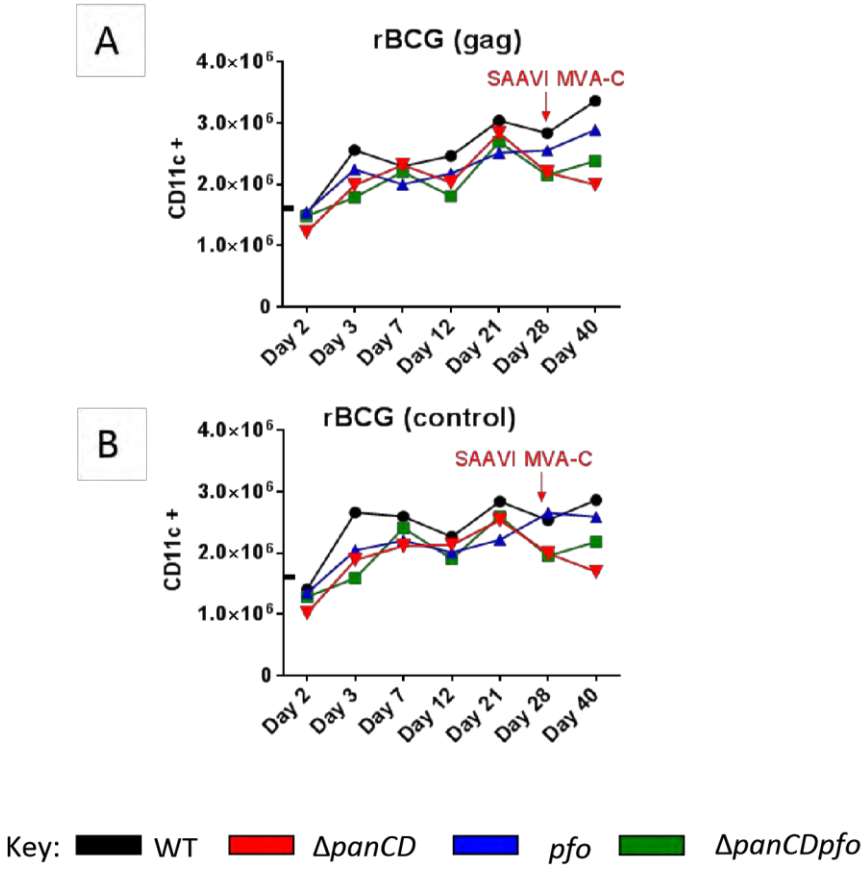


Table 2.5: Area under the curve (AUC) values for DC cell counts

	WT	ΔpanCD	pfo	ΔpanCDpfo
(*gag*)	9.89E+07	8.12E+07	8.83E+07	7.91E+07
(control)	1.06E+08	8.41E+07	9.24E+07	8.58E+07

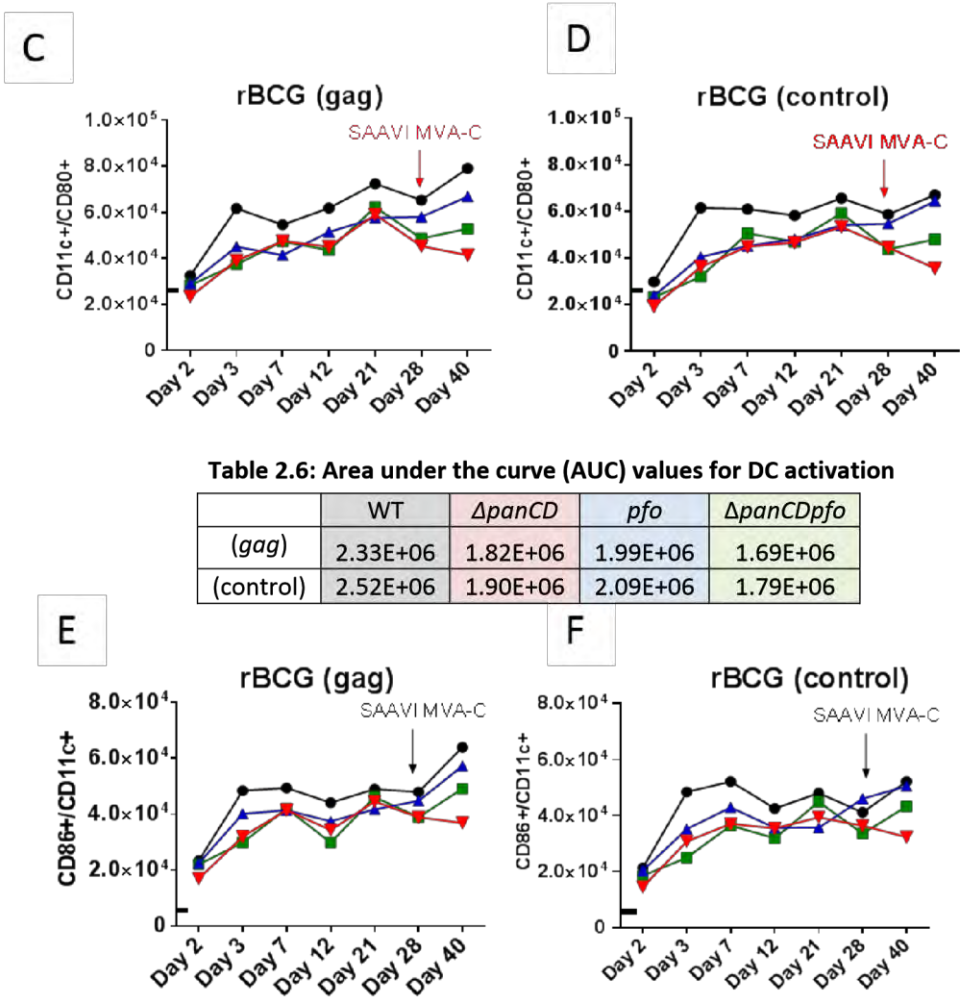


Table 2.6: Area under the curve (AUC) values for DC activation

	WT	ΔpanCD	pfo	ΔpanCDpfo
(*gag*)	2.33E+06	1.82E+06	1.99E+06	1.69E+06
(control)	2.52E+06	1.90E+06	2.09E+06	1.79E+06

Table 2.7: Area under the curve (AUC) values for DC activation

	WT	ΔpanCD	pfo	ΔpanCDpfo
(*gag*)	1.75E+06	1.40E+06	1.57E+06	1.35E+06
(control)	1.89E+06	1.52E+06	1.66E+06	1.46E+06

Figure 2.12: Longitudinal splenic dendritic cell (DC) count and activation in mice vaccinated with rBCG vaccines. DC count in mice vaccinated with rBCG (*gag*) (**A**) and rBCG (control) (**B**) vaccines (*n*=3 per group). Activation and maturation (**C-F**) of dendritic cells were rapid and superior following vaccination of WT (*gag*/control) as compared to modified strains. Indentation of the y-axis represents T cell count/activation of unvaccinated control group.

2.3.5 B cell counts and activation levels

Whilst our vaccines did not contain B cell specific epitopes, activation of B cells and the function of B cells as antigen presenting cells are vital for broad based effective T cell responses. Following the infiltration of BCG into organs, B cells, together with dendritic cells are key in orchestrating the presentation of antigen to stimulate the development and expansion of a T cell response. Notably, the B cells activation is most dominant 1-4 days after infection. In order to longitudinally assess and compare the effects of vaccination with different strains of rBCG (*gag* and control) on B cell count and activation status we stained splenocytes of vaccinated mice with an optimised antibody cocktail comprising of CD19, CD25 and CD69. Total B cell numbers were observed to increase to peak levels at 12 days post rBCG vaccination. Quantification of B cell count across the experimental time course (Figure 2.13A, Table 2.8) revealed that mice vaccinated with rBCG *pfo* (*gag*) (2.0E+09) presented with more B cells as compared to groups vaccinated with rBCG WT (*gag*) (1.87E+09), rBCG *ΔpanCD* (*gag*) *(*1.72E+09) and rBCG *ΔpanCDpfo* (*gag*) (1.66E+09). Similar profiles were observed for rBCG (control) groups (Figure 2.13B).

Analysis of B cell activation across the experimental time course (Figure 2.13 C, Table 2.9) revealed that mice vaccinated with rBCG *pfo* (*gag*) (2.15E+07) presented with more activated B cells as compared to groups vaccinated with rBCG WT (*gag*) (1.697E+07), rBCG Δ*panCD* (*gag*) (1.66E+07) and rBCG Δ*panCDpfo* (*gag*) (1.83E+07). Similar profiles were observed for rBCG (control) groups (Figure 2.13D). rBCG (*gag*) vaccines appeared to induce greater activated B cell frequencies in the spleens as compared to their respective controls. Furthermore, boosting with SAAVI MVA-C appeared to increase activated endpoint B cell level.

Figure 2.8. Area under the curve (AUC) values for B cell count

	WT	ΔpanCD	pfo	ΔpanCDpfo
rBCG (*gag*)	1.69E+07	1.66E+07	2.15E+07	1.83E+07
rBCG (control)	1.32E+07	1.58E+07	1.72E+07	1.61E+07

Key: WT ΔpanCD pfo ΔpanCDpfo

Figure 2.9. Area under the curve (AUC) values for B cell

	WT	ΔpanCD	pfo	ΔpanCDpfo
rBCG (*gag*)	1.87E+09	1.72E+09	2.0E+09	1.66E+09
rBCG (control)	1.75E+09	1.59E+09	1.85E+09	1.52E+09

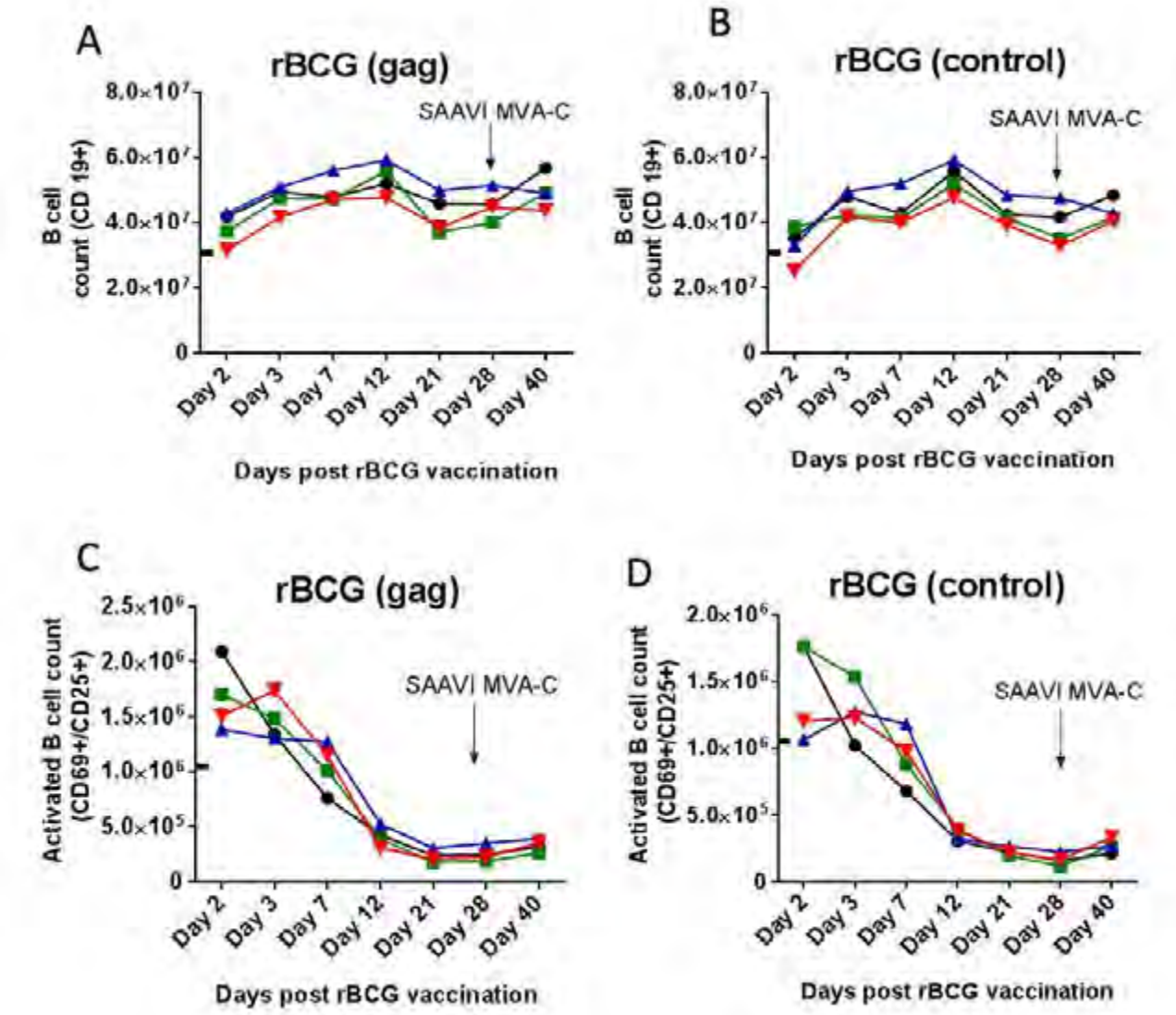


Figure 2.13. Longitudinal splenic B cell count and activation in mice vaccinated with rBCG vaccines. B cell count in mice vaccinated with rBCG (*gag*) **(A)** and rBCG (control) **(B)** vaccines (*n*=3 per group). B cell activation peaked on day 2 in spleens of rBCG (*gag*) **(C)** and rBCG (control) **(D)** vaccinated mice. Indentation of the y-axis represents B cell count/activation of unvaccinated control grou

2.4 Discussion

The data in this chapter provides a comprehensive longitudinal characterisation of the immune response to 4 rBCG vectored HIV-1 vaccines in the murine model. Additionally, this data highlights the differences that genetic modifications in the mycobacterial vector genome can have on pathology and immune response at the systemic, organ and cellular levels.

Following successful validation of vaccine strain phenotype, we longitudinally compared disseminated bacterial load in the abdominal lymph nodes, in order to assess systemic persistence of BCG in vaccinated mice. Mice vaccinated with Δ*panCD* well as Δ*panCDpfo* (*gag*/control) were able to clear the mycobacteria by Day 7 post-infection. This clearance could have been due to immune function or the inability of the mycobacteria to survive in an environment limited in pantothenate. Studies of other attenuated mycobacterial auxotrophs, such as the lysine auxotroph, indicate that this clearance is not due to immune function, but rather the inability of the BCG to survive in the absence of essential factors. Pavelka *et al*, (2003) demonstrated this by assessing clearance of the *M. tuberculosis* lysine auxotroph, following vaccination with 10^6 CFU in normal immunocompetent C57BL/6 and immunocompromised SCID mice (Pavelka *et al*., 2003). Immunocompetent mice were found to clear the auxotroph at the same rate as SCID mice indicating that clearance was due to the inability of the auxotroph to survive (Pavelka *et al*., 2003).

Whilst an HIV vaccine vector which is attenuated to such a degree is desired in a landscape where anti-vector immunity has been associated with the failure of clinical trials, current T cell HIV vaccine paradigms indicate that persistent vectors can induce prolonged CD8+ T cell effector memory responses. However, there is considerable debate particularly with BCG as a vaccine vector with some reports indicating improved memory responses upon clearing of BCG and others reporting that persistent BCG bacilli perpetuate CD4+ T effector memory (Nandakumar *et al*., 2014). The finding of a dominant yet synergistic effect of Δ*panCD* in combination with *pfo*, notably demonstrates a mechanism to improve the safety of rBCG *pfo* based vaccines. This is particularly pertinent in the context of the Aeras 422 TB vaccine trial

where despite impressive preclinical immunogenicity results, a *pfo* containing vaccine was deemed clinically unsafe (Sun *et al.*, 2009, Yang *et al.*, 2015a, da Costa *et al*., 2014).

In addition to clearance, mice vaccinated with Δ*panCD* well as Δ*panCDpfo* (gag/control) present with significantly fewer (as measured by CD3+ staining) and less active (as measured by iNos) liver granulomas as compared to mice *WT* and *pfo* strains. The formation and maintenance of granulomas is indicative of the inability of the immune system to clear infection. Whilst these findings for the Danish Δ*panCD* containing strains are consistent with our groups previous study into Pasteur rBCG Δ*panCD* (*gag*) (day 21 peak granuloma formation approximately 100 per lobe), these results represents the first indication, from an immunological readout, that rBCG *pfo* may be a less pathogenic strain than WT (Chapman *et al*., 2012). This data demonstrates the superiority of these modified strains of rBCG in inducing less pathology than the WT Danish strain.

In addition to granuloma number, iNos activity has been widely reported to control mycobacterial growth (Bekker *et al*., 2001). Our results indicated greater iNos activity in rBCG WT (gag/control) and to some degree rBCG *pfo* (*gag*) control as compared to Δ*panCD* containing strains. Specific mycobacterial components, termed mannose-capped lipoarabinomannan (ManLAM) have been shown to induce a significant proportion of iNos in macrophages in response to mycobacteria (Afonso-Barroso *et al*., 2013). Deletion of the *panC* and *panD* genes has been reported to decrease mycobacteria resistance to oxidative stress which suggests that this attenuation of BCG may responsible for the decreased iNos activity observed in mice vaccinated with rBCG Δ*panCD* (gag/control) Importantly in the context of an HIV vaccine, iNos has been shown to enhance HIV viral replication in primary macrophages so an excessive iNos response to BCG vectors is not desirable (Blond *et al*., 2000).

Following the assessment of rBCG induced pathology at the organ level we compared cellular phenotypes and activation in the spleens of vaccinated mice. Firstly, the longitudinal pattern of immune response to all strains of BCG was found to be in concordance with classical studies into dynamics to BCG (Khalil *et al*., 1975, Kaufmann *et al*., 1995). Namely, an increase in activated dendritic cells from days 2-3 post infection in conjunction with peak B cell activation.

This was followed by the slow accumulation of T cells in lymphoid organs (the spleen) to a peak activated T cell response 21 days post infection which then decreased progressively, until a SAAVI MVA-C boost. Optimally, an effective T cell priming vaccine would have to initiate a T cell specific immune response whilst moderating T cell recruitment and activation at the site of disease in order to limit potential target cells for HIV infection (Haynes, 2015). We firstly assessed levels of total splenocytes in mice vaccinated with each regime. Similarly to the liver histology, rBCG WT (*gag*/control) had the most profound effect recruiting/expanding 1.5x more cells in the spleen than *ΔpanCD* and *ΔpanCDpfo* strains further supporting *ΔpanCD* strains as being less inflammatory than WT. The activation and proliferation of DCs and T cells following mycobacterial infection is the major driver of inflammation and therefore anti-vector immunity but can additionally be vital to the establishment of a vaccine specific memory response (Cooper, 2009). Arama *et al*, (2012) showed that mice vaccinated with the immunogenic BCG-CS (BCG expressing *Plasmodium falciparum* circumsporozoite protein) up regulated CD80 and CD86 on DCs with the priming of circumsporozoite-specific memory cells (Arama *et al*., 2012). Our data indicated that rBCG WT (*gag*/control) conferred greater activation of DCs and T cells than modified strains of rBCG which may at a cellular level explain why we observe less inflammation in modified strains.

HIV vaccines need to be safe and immunogenic. Whilst the data in this chapter suggests the superiority of rBCG *ΔpanCD,* rBCG *ΔpanCDpfo* and to some degree BCG *pfo* in creating less inflammation and pathology than rBCG, next chapter of this thesis compares endpoint recall HIV specific T cell immune responses generated following vaccination with either of these strains as part of rBCG/SAAVI MVA-C regime. Chapter 4 primarily investigates the genetic differences associated with the characterised endpoint immunogenicity but additionally addresses the potential host genetic mechanisms involved in the differences in systemic immune response to vector discussed in this chapter.

CHAPTER 3: Comparison of vaccine induced immunogenicity in modified rBCG (*gag*)/SAAVI MVA-C prime-boost vaccination

Contents

3.0. Introduction

Following the characterisation of vector induced pathology and immune response (Chapter 2) for rBCG Danish WT (*gag*/control), rBCG Δ*panCD* (gag/control), rBCG *pfo* (*gag*/control) and rBCG Δ*panCDpfo* (*gag*/control), we assessed the ability of these newly characterised candidate HIV-1 vaccines to prime the murine immune system for a heterologous boost with the established poxviral SAAVI MVA-C vaccine. Modified strains of rBCG used in this study have shown impressive results in terms of limiting pathology. Readouts on the magnitude and quality of the HIV specific T cell response would inform on the suitability of these strains as candidate HIV-1 vaccine vectors.

Both persistent vectors (RhCMV) as well as non-replicating poxviral vectors (RV144 canarypox) have recently been shown to be associated with protective HIV/SIV specific responses (Hansen *et al.*, 2013a, Pitisuttithum *et al*., 2013) (reviewed in section 1.8). Historically, the UCT group has focused on the development of both persistent as well poxviral vectors in the guise of rBCG and SAAVI MVA-C respectively. Due to BCG being the imperfect standard of vaccination against TB, constant efforts have been made to improve the safety and immunogenicity of BCG by the genetic modification (da Costa *et al*., 2014). An additional benefit of this research is that these modified rBCG strains can be used to express heterologous antigen to maximise the potential of BCG as a vaccine vector for other diseases (Chapman *et al*., 2011b). A more comprehensive review detailing the advantages of BCG as a vaccine vector can be found in Chapter 1.

In this study, we hypothesized that combining antigen translocation enhancing lysins, such as perfringolysin O, with a Δ*panCD* knockout would increase the immunogenicity of Danish rBCG. We further hypothesised that combining the Danish perfringolysin O knock in with the Δ*panCD* knockout could increase the immunogenicity of rBCG as a vaccine vector capable of

generating CTL response to kill HIV infected cells during early infection in line with current HIV T cell vaccination models(McMichael and Koff, 2014).

The process for evaluating the immunogenicity of candidate HIV vaccines designed to induce T cell responses in the murine model are well established and desirable endpoint readouts are informed by both clinical correlates of protection (as seen in controllers, discussed in section 1.6) as well as non-human primate studies (Mann and Ndung'u, 2015, Barouch and Picker, 2014, Dangeti, 2013). Once HIV infection is established, eradication of the virus becomes exceedingly difficult due to escape mutations following immune pressure as well as the establishment of latent viral reservoirs (Picker, 2014). Therefore, an effective T cell vaccine should induce a rapid CD8+ CTL immune response assisted by CD4+ T cells to clear HIV infected cells prior to the establishment of infection (Figure 3.1). Additionally, the generation of persistent antigen specific T cell memory compartments, which would continue to lyse infected cells following acute infection, has been shown to clear SIV infection to undetectable levels in the NHP model (Hansen *et al*., 2011).

Assays that measure the quality and magnitude of vaccine induced HIV specific T cell immune responses, following *ex vivo* stimulation with HIV-specific CD4+ and CD8+ peptides, can be used to compare immunogenicity between candidate vaccines and regimes. *Ex vivo* quantification of IFNγ production by T cells, in response to HIV specific peptide stimulation, represents the most basic and cost effective preliminary comparison of the magnitude of vaccine induced T cell responses (Streeck *et al*., 2009a). The secretion of IFNγ (a Th1 cytokine) has been associated with the control of HIV/SIV viral replication (Roff *et al*., 2013). The IFNγ ELISPOT assay is routinely used in HIV vaccine research (Streeck *et al*., 2009a) to measure the quantity of CD4+ and CD8+ T cells producing IFNγ which are visualised as spot forming units (SFUs).

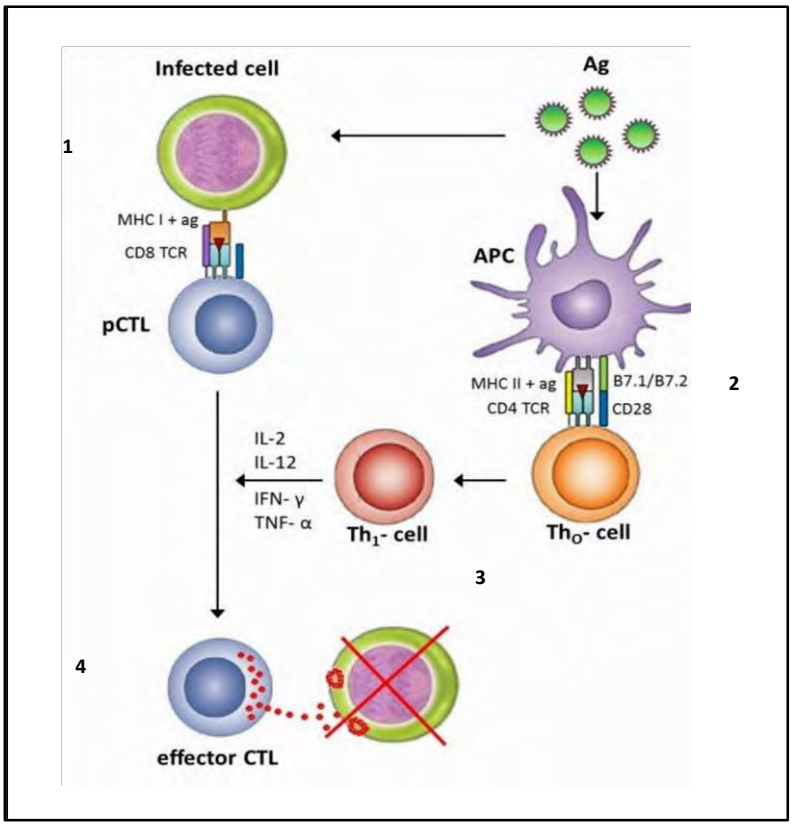


Figure 3. 1. The role of the T cell immune response in recognising antigen and lysing HIV infected cells. Presentation of antigen bound to MHC receptors trigger the adaptive immune response **(1)** Viral epitopes are presented by MHC I molecules on infected cells to T cells expressing the CD8+ T cell receptor (CD8+ TCR). **(2)** Viral epitopes are additionally presented by MHC II molecules on antigen presenting cells to T cells expressing the CD4+ T cell receptor (CD4+ TCR). **(3**) CD4 T cells mature into Th1 cells which produce the Th1 cytokines IL-2, IFNγ, and TNF-α which promotes the activation and maturation of effector CD8+ T cells **(4).** Effector CTL responses lyse infected cells and can thereby inhibit early viral replication. (Taken from *Immunodeficiency* (Winni De Haes, 2012).

The desired immune response for an HIV stimulating T cell vaccine is coordinated by a complex regulated network of Th1 and Th2 cytokines (as discussed in section 1.6). The soluble cytokine milieu induced by a vaccine has notable implications for protective efficacy and potentially for disease pathogenesis (Stacey *et al*., 2009). The desired immune response for an HIV vaccine designed to induce T cell responses would be coordinated by a complex regulated network of Th1 and Th2 cytokines (as discussed in section 1.6). Bead based quantification arrays, can detect the levels (pg/ml) of multiple soluble cytokines from single samples. The cytokine bead array (CBA), provides a sensitive and specific method to quantify

and compared the induction of Th1 cytokines (IFNγ, TNFα, IL-2) as well as Th2 cytokines (IL10, IL-4 and IL-6)(Chen *et al*., 1999).

In addition to the induced cytokine milieu, the quality of the immune response at the single cell level merits investigation. Depending on the markers expressed as well as maturation phenotype, T cells can plastically change function depending on the immune environment (Kolber, 2004). Central memory T cells (T_{CM}) serve as a persistent reservoir to generate rapid effector memory T cell (T_{EM}) populations which mature into effector T cells (Dutton *et al*., 1998). From a T cell vaccine perspective, the induction of (T_{CM}) and (T_{EM}) phenotypes are important in order to provide a prolonged immune control of viral replication (Hansen *et al*., 2011).

The simultaneous production of multiple cytokines by T cells, particularly IFNγ, IL-2, and TNFα (discussed in section 1.6) has been reported to be associated with delayed disease progression and control of viral replication (Minton, 2014, Owen *et al*., 2010). Flow cytometery allows the separation and quantification of specific cellular phenotypes based on physical size, granularity and staining with panels of flourochrome conjugated antibodies. The flow cytomtery based intracellular cytokine staining assay (ICS), uses the action of Golgi inhibitors to stain CD4+ and CD8+ T cells for multiple cytokines (such as IFNγ, IL-2, and TNF-

α) following *ex vivo* stimulation with peptide (Trigona *et al*., 2003). The addition of memory and activation markers, (such as CD62L and CDD44) (Gerberick *et al*., 1997, Kipnis *et al*., 2005) to this cytometry panel, can allow for the simultaneous quantification of vaccine induced T cell memory phenotypes. A Th1 dominant cytokine response is associated with the generation of long term vaccine specific memory T cell phenotypes. This chapter therefore aimed to assess the quality and magnitude of vaccine induced CD4+ and CD8+ T cell responses, in *ex vivo* stimulated splenocytes, from rBCG/SAAVI MVA-C vaccinated mice using the IFNγ ELISPOT assay, cytokine bead array (CBA) and multi-parameter flow cytometry to generate comparative readouts.

3.1 Materials and methods

3.1.1 Mice and Vaccination Strategy

Details of mice used in this study as well as preparation of rBCG vaccines is described in Chapter 2. rBCG vaccines were given via intraperitoneal injection on Day 0 to Groups of 3-5 BALB/c per rBCG vaccine (8 rBCG vaccines in total) (Table 3.1). All mice were boosted with 10^4 pfu of SAAVI MVA-C in 100µl PBS on day 28 by intramuscular injection. Protocol design, experimental procedures and data interpretation were conducted under the advice of an experienced murine immunologists, Professor Enid Shephard. Both the full length HIV-1 *gag* in the rBCG vaccines as well as the gp150 in SAAVI MVA-C were tagged with a immunodominant HIV-1 class I (H-2D^d)-restricted gp120 peptide (RGPGRAFVTI)(V3CTL peptide, also know if some publications as 'peptide H') for the detection of immune responses in BALB/c mice as used in pre-clinical testing of several rBCG vaccination regimes (Cayabyab *et al*., 2006, Chapman *et al*., 2012, Saubi *et al*., 2011). Mice were sacrificed by cervical dislocation 12 days after the day 28 boost. Spleens were harvested from sacrificed mice in order to evaluate vaccine induced T cell responses. Experiments were repeated 3-5 times.

Table 3.1: Summary of rBCG vaccines used in this study

Strain of rBCG	#	Full name	Abbreviated name
Wild type strain	1	rBCG[pHS501]	rBCG WT (gag)
	2	rBCG[pConepi]	rBCG WT (control)
Pantothenate auxotrophic strain	3	rBCG Δ*panCD*[pHS501]	rBCG Δ*panCD* (gag)
	4	rBCG Δ*panCD*[pConepi]	rBCG Δ*panCD* (control)
Perfringolysin O expressing strain	5	rBCG *pfo*[pHS501]	rBCG *pfo* (gag)
	6	rBCG *pfo*[pConepi]	rBCG *pfo* (control)
Pantothenate auxotrophic strain expressing perfringolysin O	7	rBCG Δ*panCDpfo*[pHS501]	rBCG Δ*panCDpfo* (gag)
	8	rBCG Δ*panCDpfo*[pConepi]	rBCGΔ*panCDpfo* (control)

3.1.2 Detection of HIV specific IFNγ secretion using the ELISpot assay

Single cell suspension of suspension of splenocytes were prepared as described in Chapter 2, section 2.2.6 and utilised in the below assays. The Enzyme-Linked ImmunoSpot (ELISPOT) assay was used to determine the frequency of IFNγ producing CD4+ and CD8+ T cells in response to stimulation with individual HIV peptides which have been shown to be specific for HIV CD4+ and CD8+ responses (Table 3.2). Whilst overlapping peptides are routinely used in human studies to assess HIV specific immune response, only a limited number of peptides are recognised in the murine model. The BD Biosciences IFNγ mouse ELISpot kit (Becton Dickinson, Franklin Lakes, and USA) was used as per manufacturer's instructions. When dry, plates were analysed using an ImmunoSpot Analyzer (Series 3B) and automatically enumerated using Immunospot Image Analyzer running ImmunoSpot Version 3.2 software (Cellular Technology Ltd, Shaker Heights, USA). For each condition, the mean spot forming unit triplicate and standard deviation was adjusted to spot forming units (SFU/10^6 splenocytes). Background responses were determined by irrelevant peptide and responses were considered positive if they were greater than twice background response. Data was plotted using GraphPad prism Version 5 (GraphPad, San Diego, USA).

Table 3.2: Stimulation peptides used in this study

Peptide	Final Concentration	MHC class of H-2Kd binding peptides	Sequence
VC3CTL	2 µg/ml	MHC I restricted	RGPGRAFVTI
Gag CD8+	2 µg/ml	MHC I restricted	AMQMLKETI
Gag CD4+ (13)	2 µg/ml	MHC class II-restricted	NPPIPVGDIYKRWIILGLNK
Gag CD4+ (17)	2 µg/ml	MHC class II-restricted	FRDYVDRFFKTLRAEQATQE
Irrelevant (NC)	2 µg/ml	(negative control)	TYSTVASSL
ConA (+)	1 µg/ml	Assay positive control	

3.1.3 Cytokine bead array

The mouse Th1/Th2 Cytokine Bead Array (CBA) kit (Becton Dickinson, Franklin Lakes, and USA) was used to quantify HIV–specific secreted IFNγ, TNF-α, IL-2, IL-4, IL-6, and IL-10. Following the isolation of single cell suspension of splenocytes from vaccinated mice, 1.5 x 10^6 cells were incubated for 48 hours in the presence of the immunodominant V3CTL CD8+ peptide or pooled HIV-1 specific CD4+ and CD8+ peptides (Table 3.2) in a 96 well plate. Following stimulation, the plates were centrifuged at 230 g (1400 rpm) for 5 min to pellet the cells. Supernatant was removed using a pipette and stored at -80°C in sterile Eppendorf tubes until all samples were collected. Samples were thawed slowly on ice on the day of the assay.

The assay was carried out as per manufacturer's instructions. Fluorescence was measured by flow cytometry using a FACS calibur cytometer (Beckton Dickinson, USA) with the assistance of an experienced technician, Desiree Bowers. The determined cut off for positivity was 8 pg/ml following subtraction of background responses from HIV specific responses. Data was plotted using GraphPad prism (Version 5, GraphPad, San Diego). The Students t test was used to assess significant differences between cytokine levels in mice under different vaccine regimes.

3.1.4 Intracellular cytokine staining (ICS)

In order to determine intracellular cytokine production and hence T cell polyfunctional profiles, we used the ICS assay. Following the procedure of splenocyte preparation (described in section 2.2.6) one and a half million splenocytes per condition were stimulated in a FACS tube, in triplicate, with either pooled HIV peptides (V3CTL CD8, *Gag* CD8, *Gag* CD4 (13 &17)) (Table 3.2), an irrelevant peptide negative control or Con A as a positive control. In order to prevent secretion of cytokine following stimulation, 0.02µg/µl brefaldin A (BFA; SigmaAldrich, USA) was added to each tube and splenocytes were incubated for 6 hours at 37°C/ 5% CO_2.

Cells were washed in 1 ml of wash buffer (Appendix 1) and blocked in 50µl blocking solution (Appendix 1) for 15 minutes at 4˚C. Cells were stained in 99.75µl stain buffer (BD Biosciences, USA) with 0.05µg of each fluorochrome-conjugated surface antibody (CD4-V500, CD8-PerCPCy5.5, CD3-Alexa 700, CD62L-APC and CD44-FITC). An APC isotype control only stain was used to ensure optimal separation of CD62L. Excess unbound antibodies were removed by a washing with 1 ml of wash buffer (Appendix 1).

Following 20 minute incubation at 4°C, cells were washed with 1 ml FACS wash buffer. Cytofix/Cytoperm (Becton Dickinson, Franklin Lakes, USA) (100 µl) was used to stain cells with IFN-γ-PE-Cy7 (0.1 µg), IL-2-PE (0.1 µg) and TNF-α-V450 (0.2 µg) for 20 minutes at 4°C. Cells were washed with FACS buffer, fixed with 4% paraformaldehyde (Merck, Frankfurt, Germany) and data was acquired on BD Fortessa cytometer. Data was analysed using FlowJo (Treestar) and GraphPad Prism Version 5.0 (Version 5, GraphPad, San Diego). Representative gating strategy is shown in Figure 3.2.

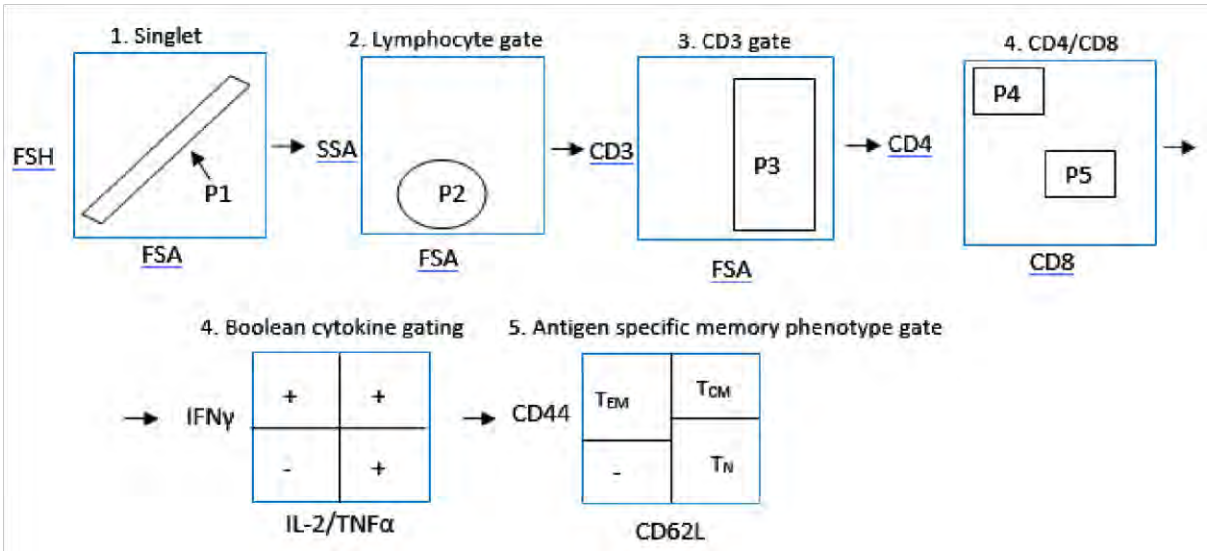


Figure 3.2. Gating strategy for flow cytometry analysis of HIV specific T cell functionality and memory phenotype. A singlet gate **(P1),** was used to exclude double events. A lymphocytes gate **(P2)** was used as a parent population for a CD3+ gate **(P3)** to identify T cells. CD3+ cells were further separated by CD4+/CD8+ gating **(P4** and **P5).** Separate cytokine gating was performed for CD4+ and CD8+ subsets and Boolean gating was used to measure the production of IFNγ, IL-2 and TNFα. HIV specific CD4+ and CD8+ cytokine positive cells were then phenotyped using CD44+ and CD62L+ separation to identify central memory (T_{CM}),effector memory (T_{EM}) memory and naïve (T_N).

3.2 Results

3.2.1 Induction HIV-1 specific IFNγ release by rBCG (*gag*)/SAAVI MVA-C prime-boost vaccination regime

We assessed four different strains of rBCG to prime groups of mice for HIV specific CD4+ and CD8+ T cell IFNγ release following a rBCG/SAAVI MVA-C prime boost regime. (Figure 3.3) (mean SFU±SD values shown in Table 3.3). All rBCG vaccines expressing V3CTL tagged Gag primed for a significant boost of the immunodominant V3CTL CD8+ peptide ($p<0.01$) (Figure 3.3A) (max SFU: rBCG *pfo* (*gag*) 773±19). This was not observed for the Gag (CD8+) peptide (Figure. 3.3B). Both rBCG *pfo* (*gag*) and rBCG Δ*panCDpfo* (*gag*) significantly primed for a boost for the CD4+ (13) peptide ($p<0.01$) (Figure 3.3C) (max SFU: rBCG Δ*panCDpfo* (*gag*) 654±39). Only the rBCG Δ*panCD* (*gag*) ($p<0.01$) and the rBCG Δ*panCDpfo* (*gag*) ($p<0.001$) vaccine primed for a boost of the *Gag* CD4+ (17) peptide (Figure 3.3D) (max SFU: rBCG Δ*panCDpfo* (*gag*) 537±25).

We next compared the magnitude of response between groups primed with rBCG vaccines expressing Gag. We found that priming with rBCG Δ*panCD* (*gag*) vaccine induced a greater

IFNγ response to Gag CD8+ ($p<0.01$) as compared to priming with rBCG Δ*panCDpfo* (*gag*) (Figure 3.3**.** B). Additionally, the rBCG *pfo* (*gag*) ($p<0.01$) and the rBCG Δ*panCDpfo* (*gag*) ($p<0.01$) vaccines induced a greater IFNγ response to *Gag* CD4+ (13) peptide as compared to the WT (*gag*) (Figure 3.3 C). The rBCG Δ*panCDpfo* (*gag*) vaccine also induced significantly higher *Gag* CD4+ (17) responses than rBCG WT (*gag*) ($p<0.05$) and rBCG *pfo* (*gag*) ($p<0.001$). Summarily, modified rBCG vaccines generally induced improved induction of IFNγ as compared to rBCG WT (*gag*).

rBCG prime	
1:	rBCG WT (*gag*)
2:	rBCG WT (control)
3:	rBCG Δ*panCD* (*gag*)
4:	rBCG Δ*panCD* (control)
5:	rBCG *pfo* (*gag*)
6:	rBCG *pfo* (control)
7:	rBCG Δ*panCDpfo* (*gag*)
8:	rBCG Δ*panCDpfo* (control)

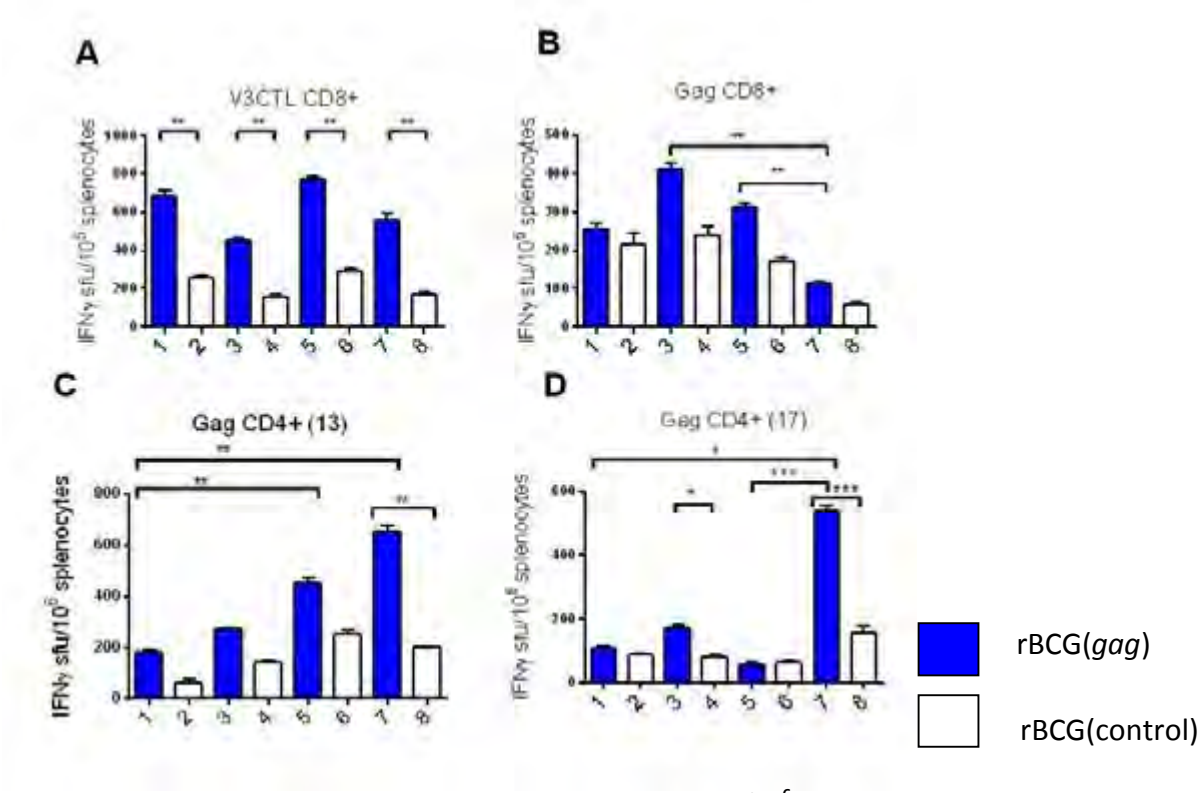


Table 3.3: Table of peptide specific IFNγ spot forming units (SFU)/10^6 splenocytes (mean SFU±SD)

rBCG	1: WT	2: WT	3: Δ*panCD*	4: Δ*panCD*	5: *pfo*	6: *pfo*	7: Δ*panCDpfo*	8: Δ*panCDpfo*
	(*gag*)	(control)	(*gag*)	(control)	(*gag*)	(control)	(*gag*)	(control)
V3CTL	688±41	259±21	449±25	158±14	451±19	293±18	561±56	173±17
Gag CD8	256±20	215±50	413±25	239±38	313±13	171±17	113±11	56±12
Gag CD4 (13)	180±23	62±29	270±16	141±11	684±25	252±25	654±39	201±5
Gag CD4 (17)	107±12	87±8	174±10	82±12	57±9	62±8	537±25	156±32

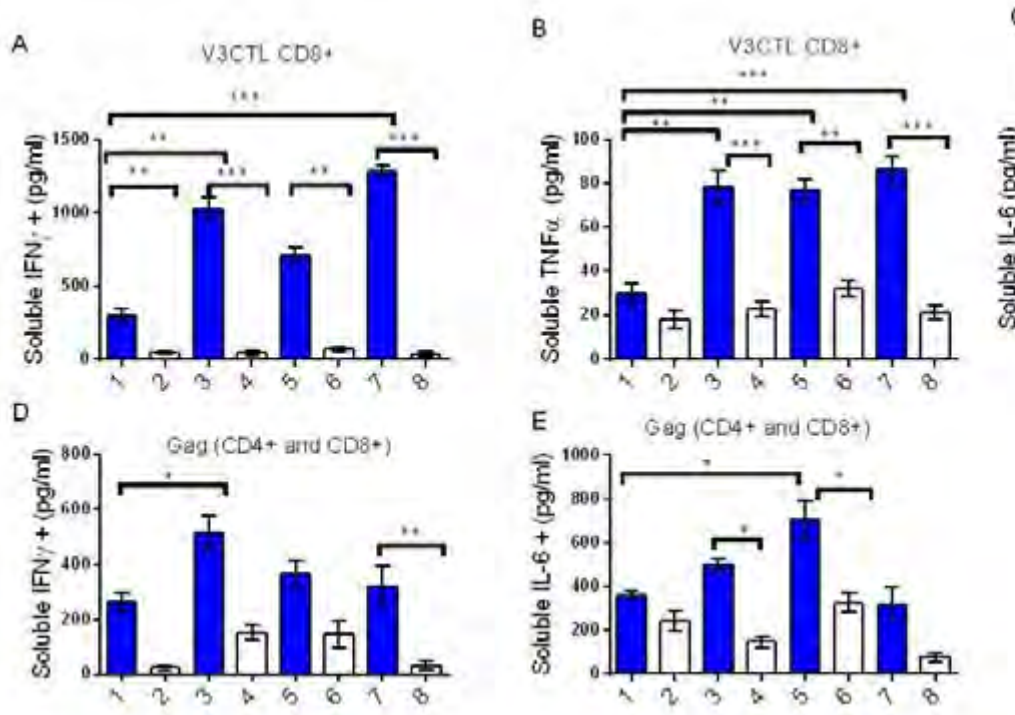
Figure 3. 3. Induction of CD4+ and CD8+ HIV specific T cell responses in rBCG/SAAVI-MVA-C prime boost vaccinated mice. Following vaccination and sacrifice, splenocytes IFNγ production was assessed following *ex vivo* stimulation with V3CTL CD8+ (**A**), Gag CD8+ (**B**), Gag CD4+ (13)(**C**) or Gag CD4+ (17) (**D**) peptides. (Responses were determined following subtraction of background responses which were consistently < 20 SFU/10^6 splenocytes. Students unpaired t test was used to compare means between groups, ***p≤0.001, **p≤0.01,*p≤0.05. Data is representative of 4 experiments with 5 mice per vaccine group.

3.2.2 Induction HIV-1 specific Th1 and Th2 cytokine release by rBCG (*gag*)/ SAAVI MVA-C prime-boost vaccination regime

As discussed in section 3.0, the systemic cytokine profile induced following vaccination has significant implications for the establishment of an effective persisting T cell response. In order to determine the cytokine milieu induced by rBCG/SAAVI MVA-C vaccination regimes, we measured cytokine secretion following splenocyte stimulation with either the V3CTL immundominant peptide or Gag (pooled CD4+/CD8+) peptides. Preliminary experiments indicated that individual Gag responses were not as robust as V3CTL responses following a 48 hour stimulation so we chose to pool Gag antigens. Splenocytes from vaccinated mice were found to produce detectable levels of IFNγ, TNFα and IL-6 (IL-2, IL-4 and IL-10 were undetectable) (Figure 3.4, pg/ml±SD values are shown in Table 3.4).

Following stimulation with the V3CTL peptides, all rBCG vaccines expressing *Gag* appeared to prime for the induction of significant IFNγ boost responses as compared to the strain specific empty vector controls ($p<0.01$-$p<0.001$) (Figure 3.4A)(max value rBCG Δ*panCDpfo* (*gag*): 1289±32). All rBCG vaccines expressing Gag (apart from rBCG WT) appeared to prime for significant boost responses when TNFα was measured as compared to the strain specific controls ($p<0.01$-$p<0.001$) (Figure 3.4B) (max value rBCG Δ*panCDpfo* (*gag*) : 355±35 pg/ml). Only the rBCG *ΔpanCD* (*gag*) ($p<0.01$) and rBCG Δ*panCDpfo* (*gag*) ($p<0.01$) strains primed for significant boost responses when IL-6 was measured (Figure 3.3 C) (max value: *pfo* (*gag*) 402±33).

In addition to V3CTL CD8+, we measured IFNγ and IL-6 for pooled *Gag* peptides (CD4+/CD8+). Only rBCG Δ*panCDpfo* (*gag*) appeared to prime for significant boost responses when IFNγ was measured as compared to the strain specific controls ($p<0.01$) (Figure 3.4 D).



rBCG prime	
1:	rBCG WT (*gag*)
2:	rBCG WT (control)
3:	rBCG Δ*panCD* (*gag*)
4:	rBCG Δ*panCD* (control)
5:	rBCG *pfo* (*gag*)
6:	rBCG *pfo* (control)
7:	rBCG Δ*panCDpfo* (gag)
8:	rBCG Δ*panCDpfo* (control)

Highest count for specific peptide

Table 3.4: Table of peptide specific cytokine release (mean pg/ml±SD)

	1: WT (*gag*)	**2: WT (control)**	**3: Δ*panCD* (*gag*)**	**4: Δ*panCD* (control)**	**5: *pfo* (*gag*)**	**6: *pfo* (control)**	**7:Δ*panCDpfo* (*gag*)**	**8: Δ*panCDpfo* (control)**
A	299±76	40±8	1027±73	42±16	706±51	65±10	1289±32	36±17
B	29±7	18±4	77±8	22±3	76±5	32±3	86±6	20±3
C	17±11	25±12	355±35	54±10	402±33	166±108	264±28	35±16
D	265±56	23±8	517±62	152±27	366±45	145±50	319±73	32±16
E	360±30	244±46	499±25	147±28	704±85	327±42	320±73	77±21

Figure 3.4 Induction of CD4+ and CD8+ HIV specific cytokine secretion. Production of IFNγ **(A)**, TNFα **(B)** and IL-6 **(C)** were observed following V3CTL CD8+ stimulation. Production of IFNγ **(D)** and IL-6 **(E)** was observed following stimulation with pooled Gag CD4+ and CD8+ peptides. (Following subtraction of non-specific background, a cytokine level of 8pg/ml was considered as a cut off for a positive response. Students unpaired t test was used to compare means between groups ***p≤0.001, **p≤0.01,* p≤0.05). Data is representative of 5 experiments with 3-5 mice per vaccine group.

Both rBCG Δ*panCD* (*gag*) and rBCG *pfo* (*gag*) appeared to prime for significant boost responses when IL-6 was measured as compared to the strain specific controls ($p<0.05$) (Figure 3.4E) (max value: rBCG (*gag*) Δ*panCDpfo* (*gag*) 704±85 pg/ml).

We next compared the magnitude of response between groups primed with rBCG (*Gag*) vaccines. Following V3CTL CD8+ stimulation, groups primed with rBCG Δ*panCD (gag*) ($p<0.01$), rBCG *pfo* (*gag*) ($p<0.01$) and rBCG Δ*panCDpfo* (*gag*) ($p<0.001$) produced more soluble TNFα than WT (*gag*) (Figure 3.4B.) This was also observed for IL-6 (rBCG Δ*panCD (gag*) ($p<0.001$), rBCG *pfo* (*gag*) ($p<0.001$) and rBCG Δ*panCDpfo* (*gag*) ($p<0.001$)) and (Figure 3.4C). Following stimulation only with pooled Gag peptides mice vaccinated with rBCG Δ*panCD (gag*) ($p<0.01$) produced more soluble IFNγ than rBCG WT (*gag*) (Figure 3.4D) and the rBCG *pfo (gag*) ($p<0.05$), group produced more soluble IL-6 than rBCG WT (*gag*) (Figure 3.4E).

3.3.3 Induction of multifunctional HIV specific CD4+ and CD8+ T cells following rBCG/SAAVI MVA-C prime boost vaccination

Clinical studies focussing on elite controllers have cited the preservation of multi-functional T cells as a correlate of protection (Owen *et al*., 2010). We aimed to compare the induction of CD4+ and CD8+ specific T cell functionality following the described heterologous prime boost regime. rBCG Δ*panCD (gag*) and rBCG Δ*panCDpfo* (*gag*) induced a greater percentage of tri-functional cytokine producing cells (IFNγ, TNFα, IL-2) than rBCG WT (*gag*) ($p< 0.05$) (Figure 3.5A) (max % of CD4 cells: rBCG Δ*panCD (gag*) 0.23±0.08). Furthermore rBCG *pfo* (*gag*) and rBCG Δ*panCDpfo* (*gag*) induced a greater percentage of $TNF\alpha^{+}IL\text{-}2^{+}$ cells than rBCG WT (*gag*) ($p< 0.01$) (Figure 3.5A) (max % of CD4 cells: *pfo* (*gag*) 0.61±0.17). We found that rBCG Δ*panCDpfo* (*gag*) induced a greater percentage of IFNγ mono-functional cells than rBCG WT (*gag*) ($p<0.01$) and rBCG *pfo* (*gag*) ($p<0.01$) (max % of CD4+ T cells: rBCG Δ*panCDpfo* (*gag*) 1.03±0.17).

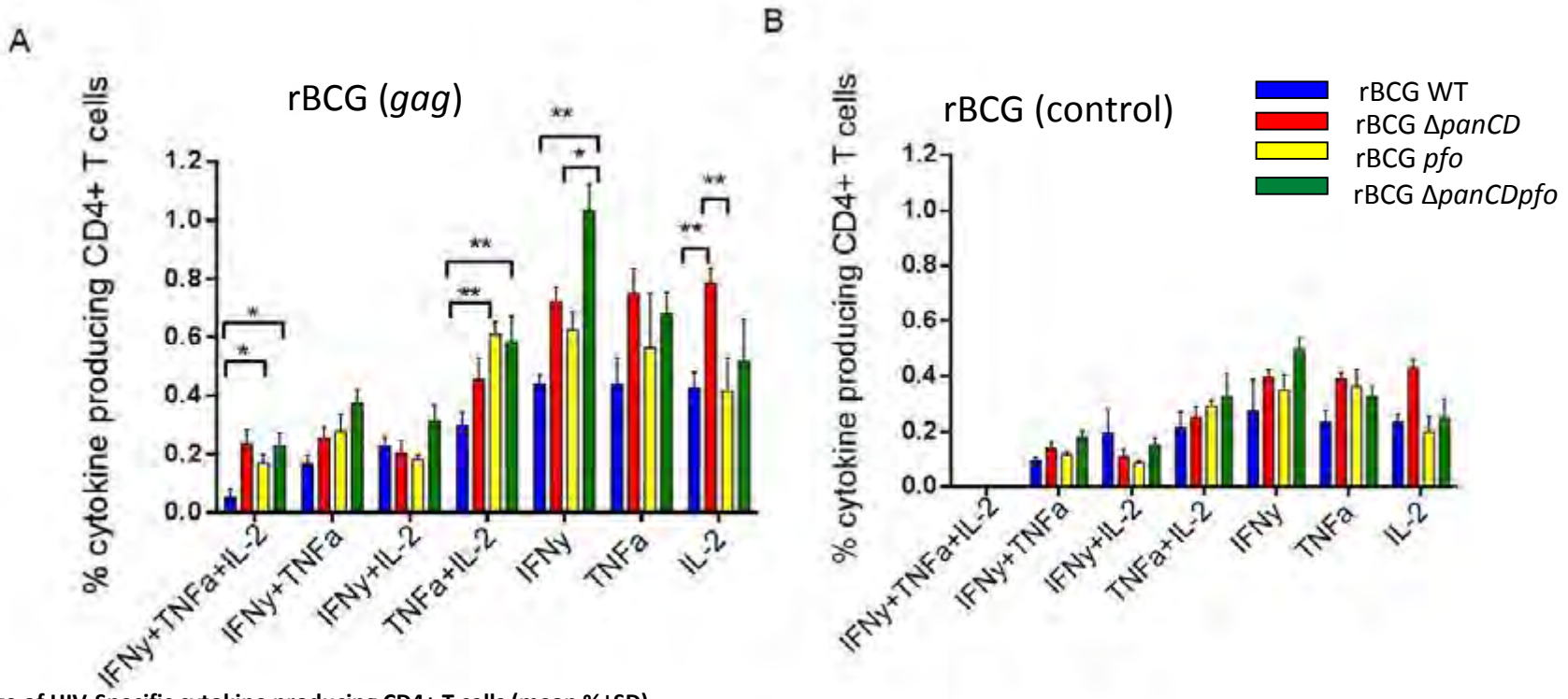


Table 3.5: Percentage of HIV-Specific cytokine producing CD4+ T cells (mean %±SD)

	IFNγ+ TNFα+IL-2	IFNγ+ TNFα	IFNγ+IL-2	TNFa+IL-2	IFNγ	TNFα	IL-2
WT	0.05±0.04	0.17±0.04	0.22±0.05	0.3±0.07	0.43±0.06	0.43±0.15	0.42±0.09
Δ*panCD*	0.23±0.08	0.25±0.07	0.2±0.07	0.45±0.13	0.72±0.09	0.75±0.14	0.78±0.09
pfo	0.18±0.04	0.37±0.080	0.18±0.03	0.61±0.17	0.62±0.13	0.57±0.17	0.39±0.21
Δ*panCDpfo*	0.22±0.07	0.45±0.15	0.31±0.09	0.58±0.15	1.03±0.15	0.68±0.2	0.51±0.25

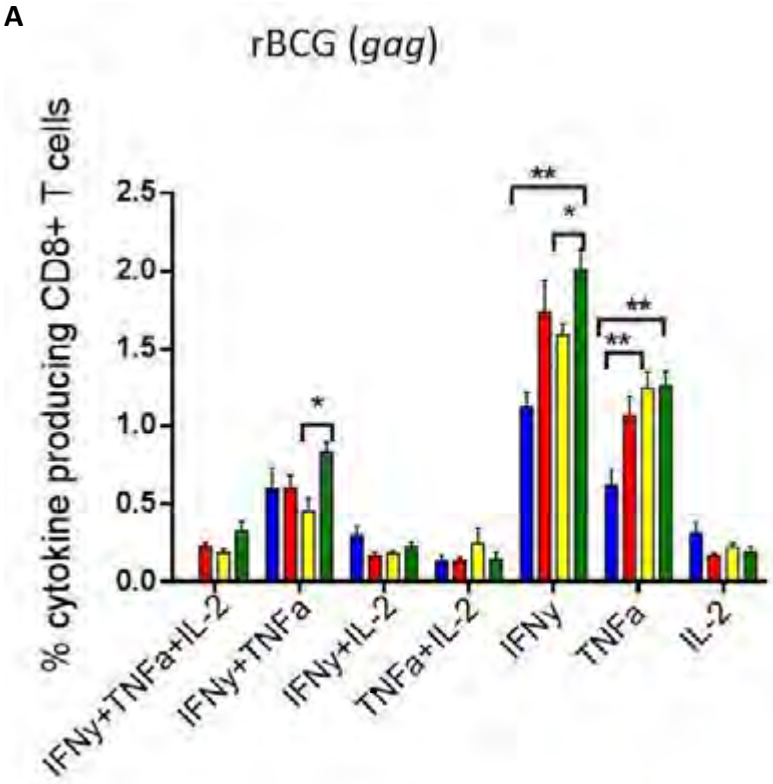
Greatest % per cytokine combination

Figure 3.5 Assessment of vaccine induced HIV specific CD4+ T cell functionality following *ex vivo* stimulation with pooled HIV-1 peptides. (A) Cytokine production in splenocytes of mice primed with rBCG (*gag*) and boosted with SAAVI MVA-C boosted **(B)** Cytokine production in splenocytes of mice primed with rBCG (control) vaccines and boosted with SAAVI MVA-C. Percentage cytokine producing cells for each cytokine combination are shown. Data is representative of 4 independent experiments containing 3 groups of 3-5 mice per vaccine. A cut-off criterion for positivity of cytokine response was 2x unstimulated background + minimum of 0.05% response + minimum of 20 cells for positivity. Negative control/unstimulated values have been subtracted and unpaired students t tests were used for statistical analysis. (***p≤0.001,**p≤0.01,*p≤0.05).

Lastly, rBCG Δ*panCD* (*gag*) induced a greater percentage of IL-2 mono-functional cells than rBCG WT (*gag*) ($p<0.01$) and rBCG *pfo* (*gag*) ($p<0.01$). We next assessed HIV specific CD8+ T cell functionality (Figure 3.6). The rBCG Δ*panCDpfo* (*gag*) vaccine induced a greater percentage of cells simultaneously producing three cytokines (0.32±0.11) but this was not significantly different from other groups. The rBCG Δ*panCDpfo* (*gag*) vaccine induced a greater percentage of $IFN\gamma^+$ $TNF\alpha^+$ double positive cells than rBCG *pfo* (*gag*) ($p<0.05$) (max % of CD8+ cells: rBCG Δ*panCDpfo* (*gag*) 0.83±0.11). Additionally, the rBCG Δ*panCDpfo* (*gag*) vaccine induced greater levels of IFNγ (max %: 2.01±0.21) than rBCG WT (*gag*) and rBCG *pfo* (*gag*). No significant differences were observed when comparing groups primed with any of the rBCG (control) vaccines.

3.3.4 Induction of HIV specific effector (T_{EM}) and central memory (T_{CM}) T cells following rBCG/SAAVI-MVA C prime boost vaccination

Recent RhCMV vectored SIV vaccine studies have associated prolonged control of SIV with persistent effector memory T cells (Hansen *et al*., 2011). The establishment of vaccine specific T cell memory phenotypes is a key protective correlate that T cell vaccines aim to elicit. By measuring the expression of CD62L and CD44, we identified and compared levels of effector memory (T_{EM} CD62L-, CD44high) and central memory (T_{CM} CD62L+, CD44+high) T cells between vaccine groups. For CD4+ cells, rBCG Δ*panCD* (*gag*) ($p< 0.01$) and rBCG Δ*panCDpfo* (*gag*) ($p< 0.01$) induced a greater percentage of T_{EM} than rBCG WT (*gag*) (Figure 3.7A) (max: rBCG Δ*panCD* (*gag*) 1.28±0.2). Additionally, rBCG Δ*panCD* (*gag*) ($p<0.05$) induced a greater percentage of T_{EM} than rBCG *pfo* (*gag*). No significant differences were observed between T_{CM} levels for CD4+ cytokine producing cells (max value: rBCG *pfo* (*gag*) 0.65±0.26.) For CD8+ cells, rBCG Δ*panCD (gag*) ($p< 0.05$), rBCG *pfo* (*gag*) ($p<0.01$) and rBCG Δ*panCDpfo* (*gag*) ($p<0.001$) induced a greater percentage of T_{EM} than WT (*gag*) (Figure 3.7B) (max value: max value: *pfo* (*gag*) 2.06±0.16). No significant differences were observed between T_{CM} levels for CD8+ cytokine producing cells (max value: rBCG *pfo* (*gag*) 0.92±0.26.).



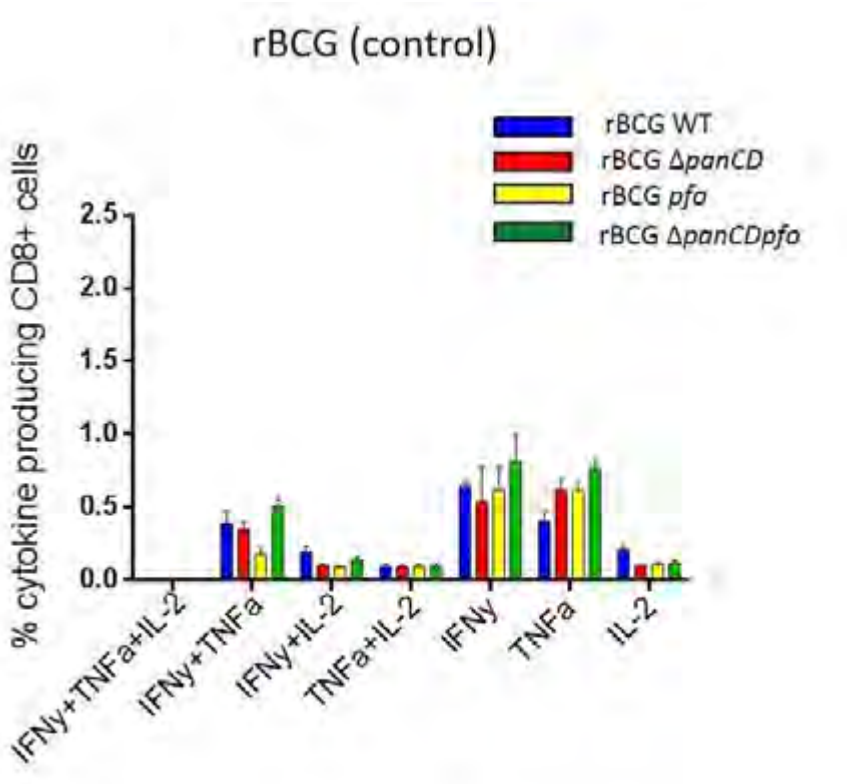


Table 3.6: Percentage of HIV Specific cytokine producing CD8+ T cells (mean %±SD)

	IFNγ+ TNFα+IL-2	IFNγ+ TNFα	IFNγ+IL-2	TNFα+IL-2	IFNγ	TNFα	IL-2
WT	0±0	0.59±0.23	0.29±0.1	0.13±0.06	1.12±0.16	0.62±0.18	0.31±0.1
Δ*panCD*	0.22±0.04	0.6±0.14	0.17±0.04	0.13±0.03	1.73±0.35	1.06±0.22	0.17±0.02
pfo	0.186±0.04	0.453±0.15	0.18±0.03	0.24±0.17	1.58±0.13	1.24±0.17	0.22±0.05
Δ*panCDpfo*	0.32±0.11	0.83±0.11	0.22±0.05	0.14±0.08	2.01±0.21	1.26±0.11	0.19±0.05

Greatest % per cytokine combination

Figure 3. 6 Assessment of vaccine induced HIV specific CD8+ T cell functionality following *ex vivo* stimulation with pooled HIV-1 peptides. (A) Cytokine production in splenocytes of mice primed with rBCG (*gag*) and boosted with SAAVI MVA-C boosted **(B)** Cytokine production in splenocytes of mice primed with rBCG (control) vaccines and boosted with SAAVI MVA-C. Percentage cytokine producing cells for each cytokine combination are shown. Data is representative of 4 independent experiments containing 3 groups of 3-5 mice per vaccine. A cut-off criterion for positivity of cytokine response was 2x unstimulated background + minimum of 0.05% response + minimum of 20 cells for positivity. Negative control/unstimulated values have been subtracted and unpaired students t tests were used for statistical analysis. (***p≤0.001,**p≤0.01,*p≤0.05).

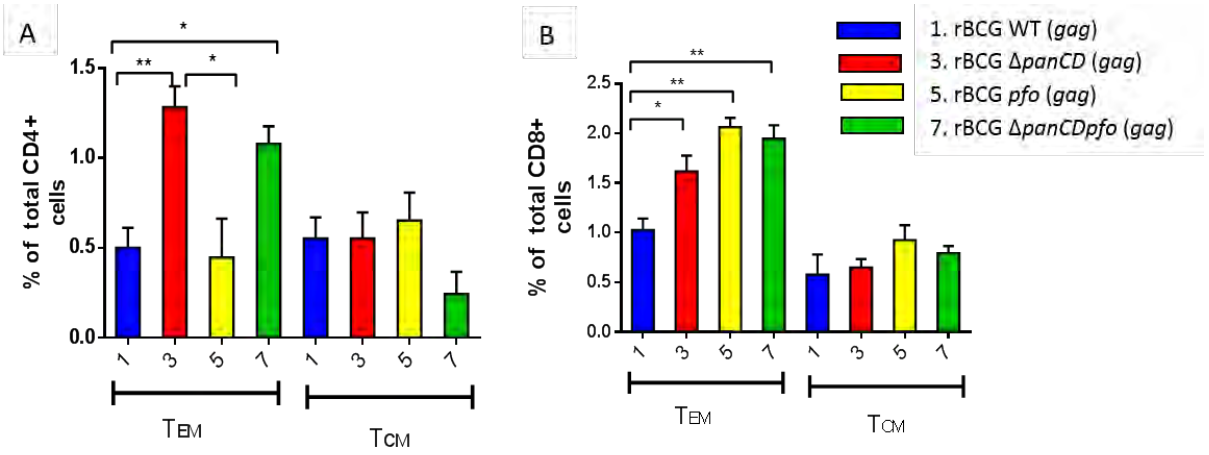


Figure 3. 7 Memory phenotype of HIV-1 specific CD4+ and CD8+ T cells. Effector memory (T_{EM} CD62L-, CD44high) and central memory (T_{CM} CD62L+, CD44high) HIV-1 specific CD4 **(A)** and CD8 **(B)** T cells were assessed by flow cytometry. Data is representative of 4 independent experiments containing 3 groups of 3-5 mice per vaccine. A cut-off criterion for positivity of cytokine response was 2x unstimulated background + minimum of 0.05% response + minimum of 20 cells for positivity. Unpaired students t tests were used for statistical analysis. (***p≤0.001,**p≤0.01,*p≤0.05).

Table 3.7: Percentage of HIV -specific CD4+ memory T cells (mean %±SD)

	1: WT	3:Δ*panCD*	5: *pfo*	7:Δ*panCDpfo*
T_{EM}	0.50±0.19	1.28±0.2	0.44±0.27	1.08±0.16
T_{CM}	0.55±0.2	0.55±0.25	0.65±0.26	0.24±0.11

Table 3.8: Percentage of HIV-specific CD8+ memory T cells

	1: WT	3: Δ*panCD*	5: *pfo*	7: Δ*panCDpfo*
T_{EM}	1.02 ±0.2	1.61±0.27	2.06±0.16	1.94±0.23
T_{CM}	0.57±0.35	0.65±0.14	0.92±0.26	0.79±0.12

3. 4 Discussion

Heterologous prime-boost regimens attempt to enhance and broaden immune responses by combining different vaccine strategies (Woodland, 2004). Notably, the only HIV vaccine clinical trial to demonstrate partial protective efficacy (RV144, reviewed in section 1.7.3) incorporated a heterologous prime-boost regimen consisting of an ALVAC canarypox prime with a protein boost. rBCG expressing HIV-1 Gag has been shown to prime the murine immune system for T cell specific responses as part of heterologous prime boost vaccination strategies with poxviral vectors (Chapman *et al*., 2011a). The UCT group has worked extensively with rBCG Pasteur as an HIV-1 vaccine vector in combination with heterologous boost (SAAVI MVA-C and others) and have optimised vector stability, vaccination regimes and dosage using these vaccines (Chapman *et al*., 2012, Chapman *et al*., 2013, Chege *et al*., 2013). This study

represents the first use of rBCG Danish as an HIV-1 vaccine vector. The Danish strain of BCG has been reported to be superior in safety and protective efficacy to the Pasteur strain (reviewed by Luca *et al*, 2013 (Luca and Mihaescu, 2013)).

Furthermore, modification of BCG through the individual use of the *panCD* deletion and perfringolysin O knock in has been shown to improve safety and immunogenicity in MTB and HIV-1 murine model systems (Sambandamurthy *et al*., 2002, Sun *et al*., 2009). We hypothesised that combining the Danish rBCG with Δ*panCD* and *pfo* modifications could enhance the safety and immunogenicity of rBCG as a candidate HIV-1 vaccine vector. Here, we assessed the ability of modified strains of Danish rBCG Δ*panCD* (*gag*), rBCG *pfo* (*gag*) as well as the combination rBCG Δ*panCDpfo* (*gag*) to prime for a SAAVI MVA-C boost as compared to WT Danish rBCG (*gag*).

Discussed below are comparative interpretations of our results in relation to clinical and experimental correlates of HIV-1 protection, suggested mechanisms of rBCG Danish strain specific vector effects on heterologous prime boost regime as well as suggested future work to build on the findings and address the short comings of this initial pre-clinical vaccine assessment.

3.4.1 Summary of comparative immunogenicity data

The above results of comparative readouts following *ex vivo* stimulation of splenocytes from vaccinated mice represent the basic tests to inform candidate vaccine development. However, in this model system both inter- and intra- assay variation and discrepancies in findings exist and this section summarises all results in a concise and comparative manner to extract maximum information from our murine model system of immunogenicity evaluation.

We summarised data from assays measuring peptide specific CD4+ and CD8+ responses (Table 3.9). For CD4+ T cells, we found that rBCG Δ*panCDpfo* (*gag*) primed for the greatest cumulative induction of IFNγ (following stimulation with Gag CD4 (13) and (17) peptides) (1191 SFU) as well priming for the greatest cumulative induction of multi- and bi-functional cells (1.56%).

Table 3.9: Summary of CD4+ and CD8+ induction following priming with 4 rBCG (gag) vaccines

		WT	Δ*panCD*	*pfo*	Δ*panCDpfo*
CD4+	IFNγ SFU/10^6 splenocytes	287±35	444±26	741±34	1191±47
	Multi/Bi-functional CD4+ T cells	0.74%±0.05	1.13%±0.08	1.34%±0.08	1.56%±0.11
	CD4+T_{EM}	0.50±0.19	1.28±0.2	0.44±0.27	1.08±0.16
	CD4+T_{CM}	0.55±0.2	0.55±0.25	0.65±0.26	0.24±0.11
CD8+	IFNγ SFU 10^6 splenocytes	944±61	862±198	764±33	674±39
	Multi/Bi-functional CD8+ T cells	1.01%±0.08	1.12%±0.07	1.05%±0.11	1.51%±0.09
	CD8+T_{EM}	1.02 ±0.2	1.61±0.27	2.06±0.16	1.94±0.23
	CD8+T_{CM}	0.57±0.35	0.65±0.14	0.92±0.26	0.79±0.12

Additionally, the rBCG Δ*panCD* (*gag*) vaccine primed for the greatest antigen specific CD4+ effector memory responses (1.28%). Conversely, comparison of vaccine specific CD8+ T cell induction indicated that rBCG *pfo* (*gag*) primed for the greatest IFNγ production (following stimulation with V3CTL and Gag CD8+ peptides) (764 SFU/10^6). Furthermore priming with rBCG *pfo* (*gag*) induced the greatest levels of HIV specific effector (2.06%) as well as central (0.92%) memory T cells. The variance within the ELISPOT was greater than expected with a number of factors possibly responsible for this (Smith *et al*., 2009). Nonetheless, the ICS assay confirmed antigen specific T cell induction. Priming with rBCG Δ*panCDpfo* (*gag*) induced the highest percentage of multi- and bi-functional vaccine specific CD8+ T cells (1.51%). Vaccination with the combination vaccine, rBCG Δ*panCDpfo* (*gag*), demonstrated that these

modifications can synergistically act to improve both HIV specific CD4+ and CD8+ responses. Lastly, mice primed with rBCG *pfo* (*gag*) and rBCG Δ*panCDpfo* (*gag*) induced greater CD8+ T_{EM} and T_{CM} responses as compare to mice primed with rBCG WT (*gag*) and rBCG Δ*panCD* (*gag*).

3.4.1 Induction of vaccine specific IFNγ producing CD4+ and CD8+ T cells

The generation and maintenance of HIV specific CD4+ and CD8+ T cell response has been associated with control of HIV vireamia in the clinical setting (Radebe *et al*., 2015, Ranasinghe *et al*., 2012)(Discussed in section 1.6.1.2). In this study, we assessed the induction of HIV specific CD4+ and CD8+ T cells following the described vaccination regime by firstly measuring IFNγ secretion in splenocytes following stimulation with HIV-1 specific peptides (Table 3.2). In order to facilitate detection of prime boost responses in the murine model, we utilised an immunodominant gp120 peptide, V3CTL (also known in some studies as 'peptide' H) (Cayabyab *et al*., 2006, Chapman *et al*., 2012, Saubi *et al*., 2011). This peptide was fused to the full length Gag immunogen in the rBCG vaccines and the gp150 sequence expressed by the SAAVI MVA-C boost.

All rBCG (*gag*) vaccines were able to prime for significant vaccine specific boost responses of the immunodominant V3CTL CD8+ epitope. Priming with the combination rBCG Δ*panCDpfo* (*gag*) vaccine significantly priming for boost of 3 vaccine specific epitopes (V3CTL, CD4+ (13) and CD4+ (17)). Notable differences were observed in the ability to different strains of rBCG to prime for a CD4+ Gag response. Whilst rBCG *pfo* (*gag*) and rBCG Δ*panCD* (*gag*) primed for either significant CD4+ (13) and CD4+ (17) boost responses respectively, rBCG Δ*panCDpfo* primed for a SAAVI MVA-C boost of both CD4+ epitopes. This suggests that priming with rBCG combining the *panCD* deletion and *pfo* insertion may synergistically improve CD4+ T cell responses following heterologous boost, possibly through differences in antigen presentation (As reviewed in Chapter 1).

3.4.2 Priming with rBCG Δ*panCDpfo* (*gag*) is associated with dominant Gag CD4+ responses

We also noted that mice primed with rBCG Δ*panCDpfo* (*gag*) induced Gag dominant CD4+ (14 and 17) responses as compared to V3CTL. This is an interesting result as dominance and preservation of Gag specific responses in chronically infected HIV-1 individuals has been associated with slower disease progression (Mann and Ndung'u, 2015, Ranasinghe *et al*., 2012). Gag has extensively been suggested as the preferred immunogen for T cell inducing HIV-1 vaccines (Williamson and Rybicki, 2015).

These results were comparable, and in some cases improved when compared to other studies that primed with rBCG expressing HIV-1 antigens as part of heterologous boost regimes (Hopkins *et al*., Chapman *et al*., 2013, Saubi *et al*., 2011). However, since these studies utilised different vector systems, HIV antigens and vaccine doses, we cannot empirically compare the magnitude of responses.

3.4.3 Effect of rBCG on SAAVI MVA-C boost responses

Previous studies done by the UCT group (Chapman *et al*., 2013), as well as preliminary experiments done in this study (data not shown) suggest that BCG prime did not affect SAAVI MVA-C responses. This indicated that differences in responses observed following rBCG (*gag*)/SAAVI MVA-C vaccination were due to the immunogenicity of the priming vaccine rather than differential suppression of the SAAVI MVA-C response. Furthermore, with this being the first study done by the UCT group using rBCG Danish, we noted that whilst this strain of rBCG is safer than Pasteur, it did not reduce immunogenicity and the ability to prime for a heterologous boost.

3.4.4 Combining *panCD and pfo* modifications can increase vaccine specific T cell functionality

In addition to investigating the success of the prime boost regime and quantifying the induction of antigen specific T cells, we aimed to assess the induction of polyfunctional CD4+ and CD8+ T cells responses following rBCG/SAAVI MVA-C vaccination regimes. Robust HIV specific CD4+ T cell responses have been associated with control of vireamia in elite controllers (Porichis and Kaufmann, 2011) and an efficacious vaccine would assist in the generation of such responses to assist cytotoxic T cell effector function (Figure 3.1). Antigen specific CD4+ T cells have previously been shown to assist in generating functional CD8+ T cell memory (Shedlock and Shen, 2003). In our study, mice primed with rBCG Δ*panCD* (*gag*) and rBCG Δ*panCDpfo* (*gag*) 4.4-4.6 fold increase in triple cytokine producing CD4+ T cells than WT (*gag*). Collective analysis of CD4+ T cell functionality (Table 3.9) indicate that the rBCG modifications can work synergistically to induce the greatest collective HIV specific bi- and multi- functional CD4+ when combined as in rBCG Δ*panCDpfo* (*gag*).

Multifunctional CD8+ T cells have also been shown to be associated with control of HIV vireamia in elite controllers (Betts *et al*., 2006b) and candidate HIV T cell vaccines should aim to elicit such responses. Assessment of CD8+ T cell polyfunctionality revealed that priming with modified strains of BCG induced triple cytokine producing CD8+ T (0.18-0.32%) cells whilst priming with rBCG WT (*gag*) did not (0%). Collective analysis of CD8+ T cell functionality (Table 3.10) indicates that priming with rBCG Δ*panCDpfo* (*gag*) induces the greatest collective bi- and multi-functional CD8+ T cells as compared to the other 3 rBCG priming vaccines used in this study. This suggests that priming with rBCG combining the *panCD* deletion and *pfo* insertion may synergistically increase the CD8+ T cell polyfunctionality following heterologous boost.

3.4.5 Effect or rBCG modifications on the induction of vaccine specific T cell memory

In addition to the magnitude and quality of the vaccine induced antigen specific T cell response, we were interested in the induction of HIV specific central (T_{CM}) and effector (T_{EM}) memory T cells in vaccinated mice. Both CD4+ and CD8+ T_{EM} cells have been shown to be associated with control of vireamia in clinical cohorts as well as in SIV challenge models that have demonstrated vaccine induced protection (Picker, 2014). Importantly, the induction of CD4+ and CD8+ T_{EM} responses have been shown to be the most promising cellular correlate of vaccine induced protection in SIV infected macaques (Picker, 2014). Hansen *et al*, (2011) vaccinated macaques with RhCMV (clone 68-1) vaccines expressing SIV antigens and challenged these macaques with highly pathogenic SIV_{239} (Hansen *et al*., 2011). They attributed early control of SIV viral replication as well as long term clearance of viral reservoirs to vaccine induced CD4+ and CD8+ T_{EM} responses.

3.4.6 The effects of bacilli clearance and antigen processing on immunogenicity

In our study individual modifications of rBCG Danish appear to synergistically support the induction of significant HIV specific CD4+ and CD8+ T_{EM} as compared to rBCG WT. Some reports have indicated that improved memory responses are associated with clearing of BCG whilst others show that persisting BCG bacilli perpetuate CD4+ T effector memory

(Nandakumar *et al*., 2014). In this study, we show that rBCG Δ*panCD* (*gag*) and rBCG Δ*panCDpfo* (*gag*) can be cleared from peripheral sites after 7 days post infection, induce less pathology than WT rBCG (*gag*) whilst still priming for significant heterologous boost responses associated with improved CD4+ and CD8+ $T_{EM.}$ Mechanistically, the *panCD* deletion (in rBCG Δ*panCD* (*gag*) and rBCG Δ*panCDpfo* (*gag*)) has been associated with decreased inflammation by rendering the bacilli more susceptible to oxidative stress as well as decreasing polyketide biosynthesis.

The induction of HIV specific CD4+ and CD8+ T cell responses following priming with modified rBCG expressing Gag indicates that both MHC class I and II presentation of the vaccine antigen occurs. Alternatively, rBCG expressing perfringolysin O (found in rBCG *pfo* (*gag*) and rBCG Δ*panCDpfo* (*gag)* has been shown to lead to the induction of improved CD8+ T cell responses as compared to rBCG WT by enhancing antigen translocation into the cytoplasm and stimulating MHC I antigen presentation (Sun *et al*., 2009).

NHP studies assessing the persistence of rBCG Δ*panCDpfo* (*gag*) vectored T_{EM} responses as well as possible *in vivo* challenge would provide an improved model system as compared to mice to assess immunogenicity of this vaccine in particular. Our study did not assess CTL cytotoxity or measure levels of cytolytic markers which would allow us to compare the potency and longevity of the CTL response (Koup and Douek, 2011) although we do look at the expression of genes involved in the CTL response in the next chapter.

3.4.7 The deletion of *panCD* can attenuate rBCG *pfo* without reducing immunogenicity

Whilst TB vaccine trials in NHP models of rBCG *pfo* (AERAS 401) indicated that this vaccine is highly immunogenic, a clinical trial (AERAS-422) was terminated due to side effects which included the reactivation of shingles (Kupferschmidt, 2011, Ottenhoff and Kaufmann, 2012). The contribution of the perfringolysin O in mediating these adverse effects is undetermined, but our results indicate that modification by deletion of *panCD* can improve the safety and immunogenicity of Danish rBCG *pfo* as an HIV-1 vaccine vector.

To further understand the molecular mechanisms that are associated with the improved vectored responses as well as to understand how these modifications to rBCG vectors contribute to the decreased pathology observed in chapter 2, we next assessed gene expression profiles in mice vaccinated with these rBCG/SAAVI MVA-C prime boost regimes.

Chapter 4: The effects of priming with different rBCG (*gag*) vaccines on host gene expression following SAAVI MVA-C boost

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4.0. Introduction

Modified Danish rBCG (*gag*) vaccines have been shown to induce less pathology (Chapter 2) and be more immunogenic (Chapter 3) as compared to rBCG WT (*gag*) when used as a priming vaccine for a SAAVI MVA-C boost. Notably, rBCG Δ*panCDpfo* (*gag*) has been shown to collectively prime for improved vaccine specific T cell responses following a SAAVI MVA-C boost as compared to priming with rBCG Δ*panCD* (*gag*) and rBCG *pfo* (gag). In this chapter, we comparatively assessed host Th1/Th2 gene expression profiles in the splenocytes of rBCG/SAAVI MVA-C vaccinated mice to characterise biomarkers associated with the described improved safety and immunogenicity in modified strains. This chapter introduction highlights the developing field of systems vaccinology, notable examples of gene expression profiling used in vaccine development, as well as discussing beneficial host gene networks regulated by rBCG as a vaccine vector.

4.0.1 Gene expression profiling informs vaccine development

The major aim of a vaccine is to establish protective immunity and/or immunological memory against the pathogen. The induction of an efficacious vaccine specific T cell immune response may affect the expression and activation of a number of host factors including; Th1 transcription factors, cytokines, chemokines, T cell regulatory markers and cell surface receptors. The advent of assays that measure a broad spectrum of host gene expression has led to the development of the field of systems vaccinology. Systems vaccinology aims to better understand host gene expressions (using 'omics' based technologies) that are associated with protective and non-protective responses in order to create safer and more immunogenic vaccines (Poland *et al*., 2013).

A number of technologies are available to assess post-vaccination gene expression in both animal models as well as humans. These include; high-throughput DNA and RNA sequencing (Han, 2008), microarrays, CyTOF mass cytometry (O'Gorman *et al*., 2014), RNAi screening as well as Real Time PCR based arrays (Suschak *et al*., 2016). Whilst these assays allow us to perceive the immune response at a greater depth than ever before, there are several considerations to be taken into account when interpreting this data. Firstly, gene expression profiling provides a measurement of gene expression at a particular time point. With the relatively short half-life of many RNAs, positive and negative feedback regulation as well as post translational modification, the timing of when to assess gene expression after vaccination/*ex vivo* stimulation is crucial (Nakaya and Pulendran, 2012). Secondly, these large data sets can generate many significant gene expression changes. In order to correctly make valid biological conclusions, data needs to be assessed in conjunction with conventional immunological assays such as pathology assays, ELISPOT and FACS (Sekaly and Pulendran, 2012, Poland *et al*., 2013).

Discussed below are several examples of how gene expression profiling has contributed to vaccine development against a variety of diseases as well as the implications and challenges in using these methods in HIV vaccine development.

4.0.1.1 Notable examples of informative gene profiling in human vaccine initiatives

The first validation of the systems vaccinology method was reported by studies assessing the yellow fever vaccine YF-17D (Querec *et al*., 2009a). This vaccine represents one of the most effective live attenuated vaccines with more than 600 million doses administered globally. Immunologically, this vaccine induces both cell-mediated and humoral responses and is exceptionally efficacious, conferring protection to more than 90% of vaccine recipients.

In a study performed by Querec *et al*, (2009) gene expression in PBMCs of vaccinated individuals were assessed at 1 week post vaccination. Early unique gene signatures (including B cell growth factor TNFRS17) could be used to predict the magnitude of neutralizing antibody responses with up to 100% accuracy several weeks later (Querec *et al*., 2009b). Independently, Gaucher *et al*, (2008) used whole genome transcriptional profiling as well as flow cytometry to assess genes associated with the induction of protective polyfunctional T cell responses (Gaucher *et al*., 2008). Both of these studies concluded that early gene expression signatures of type I interferon, complement genes, as well inflammasome related genes could predict protective efficacy of the vaccine. These independent proof of concept studies clearly demonstrate the benefits of incorporating systems based approaches to predict vaccine immunogenicity.

Another prominent example of a successful systems vaccinology approaches to predict human vaccine immunogenicity is that of the seasonal trivalent inactivated influenza virus (TIV) which induces predominantly antibody responses. In contrast to the yellow fever vaccine, individuals vaccinated with TIV have usually previously been exposed to either seasonal influenza or received previous TIV vaccination. A study done by Nakaya *et al*, (2011) therefore aimed to test if systems vaccinology based approaches could be used to predict recall response immunogenicity (Nakaya *et al*., 2011). Using individuals from 3 different influenza seasons, they reported that expression of the kinase CaMKIV at day 3 inversely correlated with antibody titres measured later on in the study. They were also able to stratify 90% of study participants as high responders (a >4 fold titre increase 30 days post vaccination) and low responders (< 4 fold antibody titre increase 30 days post vaccination) based on the patterns of gene expression suggesting that systems vaccinology based approaches can be used to predict vaccine specific recall responses.

4.0.2.1 The use of gene profiling in HIV vaccine development

Collectively, the above studies using systems vaccinology based approaches to predict immunogenicity suggest that systems vaccinology may represent a key methodology in the process to assess new HIV vaccines. The modestly successful RV144 trial (reviewed in Chapter 1) showed a protective efficacy of 31.2% against the acquisition of HIV with analysis revealing that IgG antibodies against the V1V2 regions of HIV-1 envelope 26 weeks post-vaccination was inversely correlated to infection (Pitisuttithum *et al*., 2013). Several reports have suggested that in cases where the correlates of protection remain unclear, retrospective analysis of gene signatures following vaccination/challenge may predict the quality of endpoint immunogenicity (Nakaya *et al*., 2011, Derrick *et al*., 2014). Therefore it is suggested, for vaccine development in the absence of clear correlates of protection (such as the rBCG based HIV-1 vaccine vectors used in our study), systems vaccinology based approaches incorporating gene expression profiling be used in the development phase in animal models as well as early stage human trials (Zak and Aderem, 2012, Andersen-Nissen *et al*., 2012).

Gene expression itself does not guarantee translation of encoded proteins because a variety of regulatory processes following gene expression could prevent or modify levels of protein translation. These processes include amongst others; methylation of mRNA (Fu *et al*., 2014), decreased translation due to micro RNA (Cai *et al*., 2009) as well as post-translational protein modification (Filtz *et al*., 2014). The assessment of relative gene expression in animal and human vaccine studies provides a snap shot of the inherent effects of vaccination on the host immune system as well as the host immune response to encountering antigen again (recall response upon stimulation).

4.0.3 The molecular mechanisms of BCG adjuvant activity

With a well-established safety profile, induction of both adaptive and humoral immune responses and well documented adjuvant activity, BCG represents a well characterised vaccine vector (Chapman *et al*., 2011a). The inherent effects of rBCG on mammalian host gene expression have been well documented and its adjuvant activity has been shown to have therapeutic effects against a range of diseases including disseminated forms of TB, leprosy and cancer (Talat Iqbal and Hussain, 2014). A comprehensive review of the advantages and recent improvements of rBCG as a vaccine vector is provided in Chapter 1.

Once of the main reasons cited for BCG displaying such broad nonspecific protection is that BCG activates antigen presenting cells (APCs) through the interaction of BCG pathogenassociated molecular patterns (PAMPs) with Toll-Like receptors (TLRs) 2, 4 and 8. This initiates inflammatory pathways which recruit dendritic cells, neutrophils and macrophages to the site of infection (Talat Iqbal and Hussain, 2014). Activation of these TLRs additionally leads to the downstream activation of the transcription factor NFκB which in turn induces proinflammatory cytokines such as IL-12 and TNFα (Kumar *et al*., 2014).

In addition to activating the innate arm, internalisation of BCG by APCs leads to the activation of T helper cells which are thought to assist in the formation of antigen specific memory phenotypes (Talat Iqbal and Hussain, 2014). The predominant Th1 cytokines secreted by these BCG induced CD4+ T cells include IFNγ, IL-2 and TNFα (Li *et al*., 2011). The preservation of T cells that simultaneously produce these cytokines has been shown to correlate with longitudinal control of HIV viral load in controller cohorts (Porichis and Kaufmann, 2011, Owen *et al*., 2010). BCG has also been found to counterbalance inflammation (a concept pertinent in the context of HIV-1 vaccination) by inducing BCG activated T regulatory cells (Tregs) which secrete the potent anti-inflammatory cytokine IL-10 (Coleman *et al*., 2010). Discussed below

are BCG induced gene signatures that have been associated with protection in Tuberculosis vaccine trials as well as BCG induced gene signatures associated with protection in vaccine trials where rBCG has been used to express heterologous antigen.

4.0.3.1 rBCG induced gene expression in TB vaccine studies

As discussed in Chapter 1, the relative success and need for improvement of rBCG as a TB vaccine has unwittingly led to improvements of rBCG as a vaccine vector for other pathogens. The field of TB vaccine research therefore provides insight into protective genes regulated by BCG that are associated with protective immune responses. Discussed below are several TB vaccine studies that highlight vaccine induction of protective host pathways. In conjunction with knowledge of protective HIV immune networks (discussed in in Chapter 1), these studies assisted us in developing specific research questions about rBCG regulated protective gene networks involved in the immune response to rBCG vectored HIV-1 vaccines.

Several studies have used real-time PCR based arrays to assess protective gene associations following vaccination with BCG in the murine model. Lim *et al*, (2009) evaluated pulmonary cytokine and chemokine responses in the lungs of mice immunized with BCG or a Δ*secA2* mutant of *Mycobacterium tuberculosis* (Lim *et al., 2009)*. They found that that these vaccines induced comparable levels of vaccine specific cellular responses in the lung 10 days after vaccination and these protective responses were associated with the differential regulation of 12 genes. This included IFNγ, IL-21, IL-27, IL-17f, CXCL9, CXCL10, and CXCL11 as compared to naïve unvaccinated mice. In addition to correlates of protection, systems vaccinology approaches have also been used to characterise protective responses associated with varying routes of vaccination. In a recent study, gene expression related to BCG induced protective anti-TB responses were assessed using real time PCR arrays (Derrick *et al*., 2014). Mice were vaccinated with 5×10^5 CFU of BCG Pasteur either via the intranasal or subcutaneous route. They found that intranasal immunization induced elevated splenic immune responses as

compared to subcutaneous vaccination. They also reported significantly elevated expression levels of Th1 and T- helper genes IFNγ, interleukin-9 (IL-9), IL-11, and IL21 up to 8 months after vaccination in mice receiving intranasal BCG immunization as compared to naïve mice.

A microarray based study aimed to identify biomarkers that could predict the success of BCG mediated protection against virulent *M.bovis* infection (Aranday Cortes *et al*., 2010). Mice were vaccinated with Danish BCG via intradermal injection ($2x10^5$ cfu) and then intranasally infected with 600 cfu *M. bovis* 6 weeks later. Mice were sacrificed at days 3 and 14 post infection and microarray was used to assess the transcriptome profile in the lungs and spleen. Data was normalised to naive uninfected mice. In addition to the presence of IFNγ related genes, they also found the induction of Th17 genes to be associated with BCG protection. The T helper cell Th17 gene network functions to assist in the establishment and regulation of effective anti-microbial Th1 functions.

In addition to murine models, the systems vaccinology approach to understanding how BCG mediates protection by regulating host genes has also recently been studied in the human model of BCG (Matsumiya *et al*., 2015). Using samples from a phase 1 BCG clinical trial in the United Kingdom (clinical trials registration: NCT01194180) (Harris *et al*., 2014), in which BCG vaccinated or BCG naïve individuals were challenged with BCG, Matsumiya *et al*, (2015) correlated intradermal BCG growth following BCG challenge to gene expression (DNA microarray) and T cell function (flow cytometry) at 2 weeks post challenge (Matsumiya *et al*., 2015). They found that the IFNγ and IL-17 pathways are strongly induced in BCG vaccinated individuals and expression of these gene subsets was found to correlate to reduced mycobacterial growth. They furthermore observed a negative correlation between BCG growth and the frequencies of multi-functional T cells.

The above studies collectively demonstrate the host gene expression mechanisms associated with BCG mediated protection as a TB vaccine. In the context of our study, we were interested in how modifying BCG (through deletion of the *panCD* genes, insertion of perfringolysin (*pfo*) or combining these two modifications) leads to the improved safety and immunogenicity of rBCG as a priming vaccine for HIV specific responses following a SAAVI MVA-C boost.

4.1 Study Aims

The immunotherapeutic characteristics of BCG, as well as the reported induction of tightly regulated protective gene networks by BCG contribute to the body of evidence which indicates that rBCG represents a potentially safe and immunogenic HIV-1 vaccine vector. Based on both the identified protective gene/immunology associations in HIV studies as well as the protective associations linked to BCG mediated protection against mycobacterium, in this study we were primarily interested in addressing the following 2 aims with provided rationale.

1) We aimed to assess the induction of Th1 genes following priming with different rBCG (*gag*) vaccines and boosting with SAAVI MVA-C: Key markers of the Th1 immune response includes; IFNγ inducible genes (Taborda-Vanegas *et al*., 2011), T-helper related genes which assist in the downstream generation of vaccine specific multifunctional T cell phenotypes/T cell memory and genes related to cytotoxic T cell activity. In order to further understand the pathology and immunogenicity data of this study, we aimed to characterise Th1 gene networks and pathways differentially regulated in murine splenocytes following rBCG/SAAVI MVA-C prime boost regimes and subsequent ex vivo stimulation with HIV-1 peptides.

2) We aimed to assess the regulation of the Th1 immune response following priming with different rBCG (*gag*) vaccines and boosting with SAAVI MVA-C: Whilst the induction of potent T cell responses and inflammatory cytokines is vital for anti-viral immune function, excessive inflammation can lead to increased BCG pathology and HIV viral replication (Hesseling *et al*., 2009, Appay *et al*., 2014). As HIV vaccines have to be immunogenic as well as safe, we aimed to compare the relative expression levels of Th1 regulatory genes following vaccination and ex vivo stimulation with HIV peptides following the proposed vaccination regime.

4.2.1 Experimental design to assess inherent and recall gene expression

Gene expression was assessed in the unstimulated splenocytes of mice primed with rBCG (*gag*) compared to the corresponding rBCG (control) at 12 days after the SAAVI MVA-C boost (Vaccinated unstimulated group-VU). The following combinations of vaccinated unstimulated (VU) groups were included: rBCG (*gag*) prime/SAAVI MVA-C boost, rBCG (control) prime/SAAVI MVA-C boost and a control group receiving SAAVI MVA-C alone. We additionally aimed to characterise recall gene expression induced by rBCG/SAAVI MVA-C vaccination by assessing gene regulation in the splenocytes of vaccinated mice that were stimulated *ex vivo* for 6 hours with HIV-1 peptides (Vaccinated stimulated group-VS). The following combinations of vaccinated stimulated (VS) groups were included: rBCG (*gag*) prime/SAAVI MVA-C boost and rBCG (control) prime/SAAVI MVA-C boost. Since gene expression is most commonly assessed in relation to the baseline responses of unvaccinated mice, we included a group of unstimulated splenocytes from unvaccinated mice (Unstimulated unvaccinated –

UU) to be used to normalise relative gene expression of VU and VS groups before comparing.

Comparison Summary

Research Question	Comparison
Which genes (common and variable) are induced in mice primed with rBCG (*gag*) compared to the corresponding rBCG (control) at 12 days after the SAAVI MVA-C boost?	Unstimulated (VU): rBCG (*gag*) compared to rBCG controls
Which genes (common and variable) are induced 6 hours after *ex vivo* stimulation with HIV-1 peptides in mice primed with rBCG (*gag*) compared to the corresponding rBCG (control)?	Stimulated (VS): Comparison of rBCG (*gag*) to rBCG control

4.2 Materials and methods

As demonstrated in the TB vaccine studies discussed above, real-time PCR provides a standardised method for profiling gene expression in BCG vaccine models. The mouse Th1/Th2 RT² Profiler™ PCR Array (Qiagen, Hilden, Germany), provided a platform to simultaneously assess the relative expression of 86 immunologically pertinent genes involved in the Th1/Th2 immune response. Additional to the standardised array provided by SABiosciences, it was possible to add 4 genes to this array that were not present in the defined array. The genes we chose to additionally include coded for IL-21, programmed death-1 (PD1), granzyme B (Gzmb) and perforin (Prf1). The TB vaccine studies discussed above demonstrated the protective association of IL-21 in BCG mediated protection in the murine model. HIV pathogenesis studies have showed PD-1 to play a role in limiting HIV T cell responses and be associated with immune exhaustion (Porichis and Kaufmann, 2012). Lastly, both granzyme and perforin are cytolytic degranulation markers and are an essential part of CTL effector function (Voskoboinik *et al*., 2015). All genes contained in this array are shown in Table 4.1. Genes were grouped into several gene network categories based on their consensus function as part of anti-microbial/anti-viral immune responses.

Table 4.1: List of genes and functional groupings for RT2-Th1/Th2 PCR array

Gene Symbol	Official Full Name	Gene Symbol	Official Full Name
1. Th1 a ssociated cytokine, transcription factors and associated genes		**3. T-hel per associated genes and innate functions**	
		Il17a	Interleukin 17A
Il23a	Interleukin 23, alpha subunit p19	*Il15*	Interleukin 15
Nfkb1	Nuclear factor of kappa light polypeptide gene enhancer in B-cells 1, p105	*Il2*	Interleukin 2
Il12b	Interleukin 12B	*Il7*	Interleukin 7
Il12rb2	Interleukin 12 receptor, beta 2	*Il9*	Interleukin 9
Stat1	Signal transducer and activator of transcription 1	*Il2ra*	Interleukin 2 receptor, alpha chain
Stat4	Signal transducer and activator of transcription 4	*Il5*	Interleukin 5
Tbx21	T-box 21	*Tmed1*	Transmembrane emp24 domain containing 1
Il18bp	Interleukin 18 binding protein	*Tlr4*	Toll-like receptor 4
Il18r1	Interleukin 18 receptor 1	*Tlr6*	Toll-like receptor 6
Nfatc1	Nuclear factor of activated T-cells	*Il21*	Interleukin 21
Nfatc2	Nuclear factor of activated T-cells	**4. T cell antig en presentation and co-stimulation**	
Nfatc2ip	Nuclear factor of activated T-cells	*Cd80*	CD80 antigen
Il1r1	Interleukin 1 receptor, type I	*Cd86*	CD86 antigen
Tnf	Tumor necrosis factor	*Cd28*	CD28 antigen
Tnfrsf8	Tumor necrosis factor receptor superfamily, member 8	*Cd4*	CD4 antigen
Tnfrsf4	Tumor necrosis factor (ligand) superfamily, member 4	*Cd40*	CD40 antigen
Spp1	Secreted phosphoprotein 1	*Cd27*	CD27 antigen
Il27	Interleukin 27	*Pdcd1*	Programmed cell death 1
Il27ra	Interleukin 27 receptor, alpha	*Icos*	Inducible T-cell co-stimulator
Il6	Interleukin 6	*Ctla4*	Cytotoxic T-lymphocyte-associated protein 4
***2.* Cytolyti c degranulation markers**		*Cd40lg*	CD40 ligand
Prf1	Perforin 1 (pore forming protein)	*Ccr5*	Chemokine (C-C motif) receptor 5
Gzmb	Granzyme B	*Igsf6*	Immunoglobulin superfamily, member 6

5. Chemokines and chemokine receptors for mucosal/tissue migration and T cell recruitment		**7. JAK-STAT signal transduction**	
Ccl11	Chemokine (C-C motif) ligand 11	*Irf1*	Interferon regulatory factor 1
Ccl5	Chemokine (C-C motif) ligand 5	*Irf4*	Interferon regulatory factor 4
Ccl7	Chemokine (C-C motif) ligand 7	*Jak1*	Janus kinase 1
Ccr2	Chemokine (C-C motif) receptor 2	*Jak2*	Janus kinase 2
Ccr3	Chemokine (C-C motif) receptor 3	*Jak3*	Janus kinase 3
Ccr4	Chemokine (C-C motif) receptor 4	*Junb*	Jun-B oncogene
Ccr10	Chemokine (C-C motif) receptor 10	*Tyk2*	*Tyrosine kinase 2*
Cxcr3	Chemokine (C-X-C motif) receptor 3	*Mapk9*	Mitogen-activated protein kinase 9
6. Th2 Respons e		*Socs1*	*Suppressor of cytokine signaling 1*
Gata3	GATA binding protein 3	*Socs3*	Suppressor of cytokine signaling 3
Gfi1	Growth factor independent 1	*Socs5*	Suppressor of cytokine signaling 5
Il10	Interleukin 10	**House keeping genes and controls**	
Bcl6	B-cell leukaemia/lymphoma 6	*Actb*	Actin, beta
Maf	Avian musculoaponeurotic fibrosarcoma	*RTC*	Reverse transcription control
Inha	Inhibin alpha	*Hsp90ab1*	Heat shock protein 90 alpha b1
Tcfcp2	Transcription factor CP2	*Gapdh*	Glyceraldehyde-3-phosphate dehydrogenase
Il13ra1	Interleukin 13 receptor, alpha 1		
Cebpb	CCAAT/enhancer binding protein (C/EBP), beta		
Crebbp	CREB binding protein		
Yy1	YY1 transcription factor		

4.2.2 Animals used and vaccine strategy

Similarly to other experiments in this study, female BALB/c mice (age: 4-6 weeks) were purchased from the South Africa Vaccine Producers Pty Ltd (Johannesburg, South Africa). Mice were housed at the University of Cape Town Animal Unit for 10 days in order to acclimatise prior to vaccination. Vaccination and handling were performed by a trained laboratory animal technologist, Mr Rodney Lucas. rBCG Vaccines (Table 3.1) were prepared before vaccination as described in Chapter 2. Mice were vaccinated on day 0 via intraperitoneal injection with 1 x 10^7cfu/200 µl of rBCG and boosted on day 28 with an intramuscular injection of 10^4 pfu SAAVI MVA-C.

Ethics approval for the experiments was obtained from the University of Cape Town Animal Research Ethics Committee (UCT Protocol number: 012/001). Protocol design and experimental procedures were conducted under the advice of experienced murine immunologist, Professor Enid Shephard. Mice were sacrificed by cervical dislocation 12 days after the day 28 boost. Spleens were harvested from sacrificed mice in order to evaluate vaccine induced gene expression. Experiments were repeated 3 times with 5 mice per group.

4.2.3 Preparation of splenocytes and stimulation

Single cell suspension of splenocytes was prepared as described in Chapter 2. Unstimulated splenocytes (1.5 million in 1 ml R10) for each vaccinated group were centrifuged at 1400 rpm (using an 18-place Cryovial rotor, Eppendorf, Hamburg, Germany) for 5 minutes in Eppendorf tubes and the supernatant discarded. The cell pellet was resuspended in 2 ml RNA later (Sigma, USA) and stored at -80°C. For HIV-1 stimulation, pooled peptides (Chapter 3, Table 3.2) were used to stimulate 1.5 million splenocytes per vaccine group and incubated for 6 hours at 37°C with 5% CO_2. Following stimulation, samples were centrifuged at 1400rpm for

5 minutes. Cell pellets were resuspended in 2 ml RNA later (Sigma, St Louis, USA) and samples were stored at -80°C.

4.2.4 RNA extraction

The RNeasy kit from Qiagen was used to extract RNA from slowly thawed splenocytes stored in RNA later (Sigma). Samples were centrifuged at 10 000 rpm (using an 18-place Cryovial rotor, Eppendorf, Germany) for 5 minutes to pellet RNA cells. The pellet was suspended in 350µl RLT buffer. This solution was transferred to a Qiashredder homogenising spin column (Qiagen). These columns were centrifuged at 10 000rpm (Cryovial rotor, Eppendorf) for 2 minutes. One volume of 70% ethanol was added to the lysate and gently mixed with a pipette. The sample was transferred to an RNeasy spin column placed in 2 ml collection tube. This was centrifuged at 10000rpm for 15 seconds. The flow through was discarded and 700µl of RW1 buffer was added to the RNeasy spin column. Tubes were centrifuged at 10000rpm for 15 seconds. The flow through was discarded and 500µl of RPE buffer was added to the RNeasy spin column. Tubes were centrifuged at 10000rpm for 15 seconds. The flow through was discarded and a further 500µl of RPE buffer was added to the RNeasy spin column. Tubes were centrifuged at 10000rpm for 2 minutes. The collection tube was discarded and the RNeasy spin tube placed in a new sterile collection tube. RNA was eluted by adding 50µl RNase free water to the RNeasy tube and centrifuging at 10000 rpm for 1 minute. An aliquot of 2µl was stored in a PCR tube for assessment of the RNA quality and concentration. The remaining 48µl of RNA was stored at -80°C to be used in real time PCR array.

4.2.5 RNA quality assessment

RNA quality and concentration was assessed using a bioanalyzer (Agilent, SA). Quality was assessed independently by experienced technicians at the Centre for Proteonomic and Genomic Research (CPRG, Mowbray, Cape Town). RNA with a RNA Integrity Number (RIN)

number ≥ 7 and a concentration higher than 5ng/µl was suitable to be used in RT-PCR arrays according to manufacturer's instructions.

4.2.6 cDNA synthesis

Synthesis of cDNA was performed in a PCR sterile room using the RT^2 First Strand Kit (Qiagen). All reagents supplied in the kit were vortexed briefly for 30 seconds. In a sterile PCR tube, 100ng of RNA was added to 2 µl of 5x genomic elimination buffer and made up to 10 µl using RNase free water. Following mixing with a sterile pipette, the genomic elimination mixture was incubated at 42°C for 5 minutes. The reaction was stopped by chilling the samples on ice.

The reverse transcription cocktail was prepared whilst the first strand mixture was on ice. To constitute this; 16 µl of BC3 RT buffer, 4 µl of P2 primer control, 8 µl of RT enzyme mix and 12 µl of RNase free water was added to a sterile PCR tube and gently mixed using a pipette. A volume of 10µl of the first strand mix was added to the RT cocktail to render a final volume of 50µl and this was mixed gently. The sample was then incubated for 15 minutes at 42°C and the reaction immediately stopped by heating for 5 minutes at 95°C. To stop the reaction and dilute the cDNA, 91 µl of RNase free water was added to the synthesised cDNA. These products were stored on ice and the RT^2-PCR array was set up immediately.

4.2.7 RT-PCR array

In order to make the master mix required for each sample, 550 µl of 2x RT^2 qPCR Master Mix was added to 448 µl RNase free water and 102 µl of diluted cDNA. This created a master mix of volume 1100 µl which was stored on ice. Four samples per 384 well RT^2-PCR array (Th1/Th2) were loaded using a repeating pipette and 384EZLoad™ Covers (Qiagen) with 10 µl

of master mix added to each sample well containing optimised printed primers pairs. Once all master mix samples were added, the plate was sealed using the supplied adhesive cellophane cover and centrifuged for 5 minutes at 230g. Plates were run on an ABI 7900HT PCR machine (Applied Biosytems, Foster City, USA) by staff at the Centre for Proteomic and Genomic Research (CPGR, Mowbray, Cape Town). The manufacture's recommended PCR cycle for the 7900HT PCR machine was used.

4.2.8 Data Analysis

Data was analysed using the Sabiosciences RT^2-PCR array software with support from Dr Judith Hornby from CPGR and Whitehead scientific. A list of genes assessed by this assay is shown in Table 4.1. Cut-off for significance was $p \leq 0.05$ and a fold change >2.0 when using $2^{\Delta\Delta Ct}$ Method (Livak and Schmittgen, 2001). Glyceraldehyde-3-Phosphate Dehydrogenase (*GAPDH*) was used as the housekeeping gene and all PCR array plates passed manufacturers as well as PCR machine quality control. Radial plots were generated using Microsoft Excel. A combination of pathway analysis tools (http://www.pathwaycommons.org/about/) and analysis resources provided by Qiagen (http://www.sabiosciences.com/rt_pcr_product/) were used to infer gene network groupings and pathway activity presented in the discussion of this chapter.

4.3 Results

We compared the differential host gene expression in splenocytes of BALB/c mice induced by priming with either 4 strains of rBCG (*gag*) (or empty vector control) and boosting with SAAVI MVA-C. Using RNA with RNA integrity number (RIN) >7, all RT-PCR array plate runs passed assay and RT-PCR machine quality controls. Data was normalised to naïve unstimulated unvaccinated murine splenocytes (UU) and is presented as normalised mean relative fold change ± standard deviation in tables as well as the range being provided in text. In order to visually compare patterns of gene expression, radial plots are presented with both significant

and corresponding non-significant differential fold changes across compared groups whilst significant fold changes are highlighted in the accompanying data tables. For each of the two comparisons we do (endpoint gene expression (VU) and gene expression during the recall response(VS)), we discuss; adjuvant activity of rBCG, the effect of priming with different rBCG strains on host gene expression, genes regulated by the SAAVI MVA-C vaccine and lastly, the effect of rBCG strain on Gag specific gene expression.

4.3.1 The impact of priming with different rBCG strains following SAAVI MVA-C boost on endpoint gene expression

In order to determine the effects of priming with rBCG (*gag*) vaccines on Th1/Th2 gene expression, we firstly normalised gene expression profiles from all rBCG (*gag*) vaccinated unstimulated samples (VU) to naive (UU) mice (Table 4.2 and Figure 4.1).

4.3.1.1 Genes associated to the adjuvant activity of rBCG

In order to identify genes associated to the adjuvant activity of each rBCG, we first looked at genes that were significantly regulated and common to all groups receiving an rBCG prime. Toll-like receptor 6 (TLR6), an innate receptor for microbial antigen, was found to be consistently down regulated in the splenocytes of all rBCG primed mice (-2.14 to -9.18). Similar results for the Th1 chemokine receptor CXCR3 were observed (-2.81 to -11.11). CXCR3 has been found to have multiple functional associations depending on the T cell that it is expressed on (reviewed by Groom and Luster, 2011)(Groom and Luster, 2011). In TB animal models, CXCR3 has been associated with T regulatory cells (Tregs) which infiltrate peripheral organs to limit inflammation.

Assessing gene expression in mice primed with rBCG (control) and boosted with SAAVI MVAC would suggest more genes associated to the strain specific adjuvant functions of rBCG. We found 2 genes to be down regulated in all mice primed with rBCG (control) but not in the

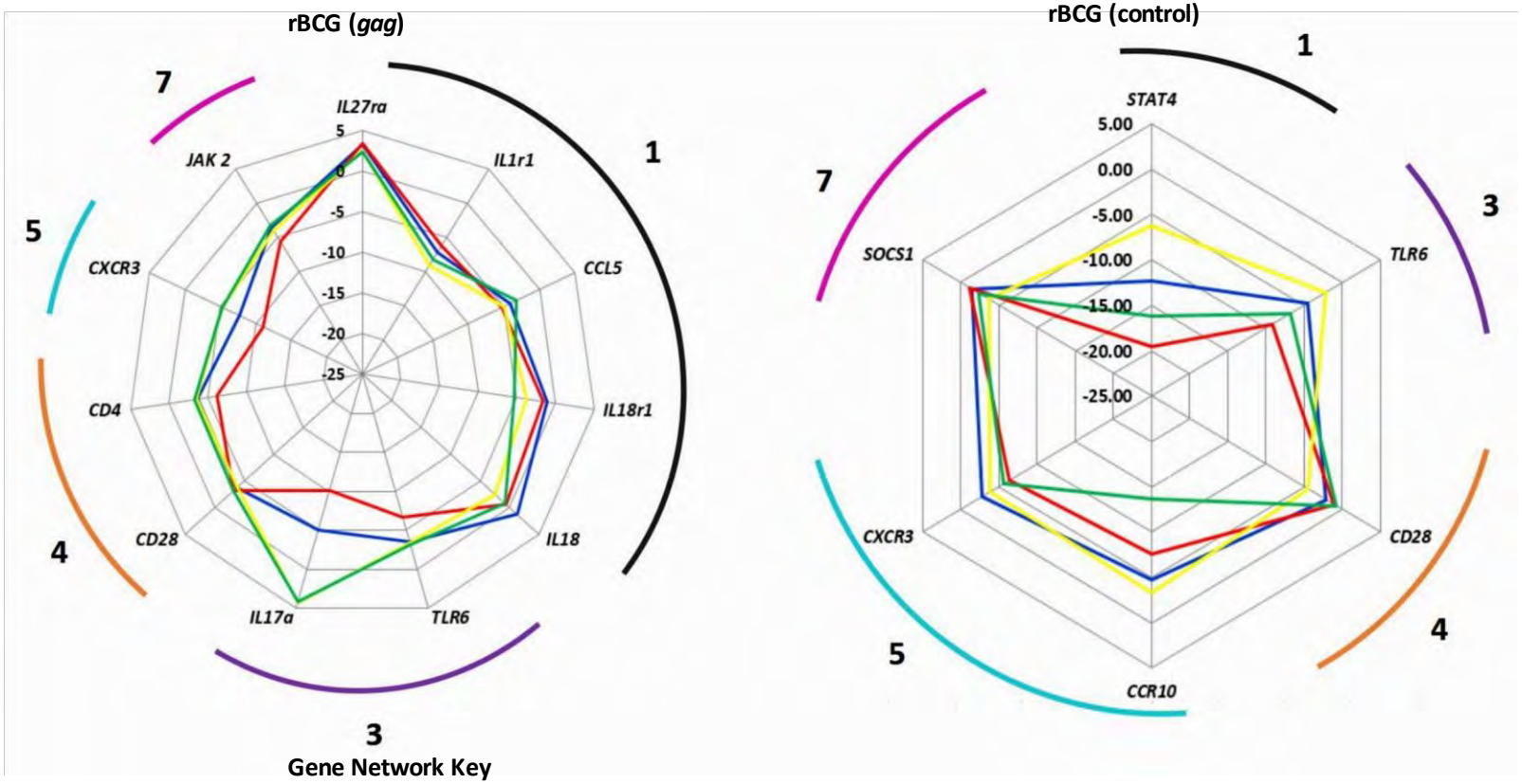
SAAVI MVA-C alone group. This included signal transducer and activator of transcription 4 (STAT4), which functions as a transcription factor aiding the development of CD4 T cells (-6.18 to -19.52), as well as CCR10 (-3.35 to -13.68) which has been reported to be involved in the chemotaxis of T cells between peripheral sites and central lymphoid organs.

Table 4.2: Vaccinated unstimulated (VU) normalised to unvaccinated unstimulated (UU) for all groups

#	Gene Symbol	rBCG WT (*gag*)	rBCG Δ*panCD* (*gag*)	rBCG *pfo* (*gag*)	rBCG Δ*panCDpfo* (*gag*)	WT (control)	Δ*panCD* (control)	*pfo* (control)	Δ*panCDpfo* (control)	SAAVI MVA-C alone
1	Il27ra	3.22± 0.47	3.03±0.51	2.43±0.06	2.32±0.36	-0.81±0.1	-0.60±0.01	-0.44±0.11	-0.53±0.03	3.22±0.45
	Il1r1	-7.14± 0.16	-6.25±0.71	-9.09±0.35	-8.52±0.72	-0.22±0.05	-0.47±0.01	-0.64±0.17	-0.30±0.01	-0.51±0.20
	Ccl5	-4.16± 0.87	-5.26±0.74	-5.04±0.14	-3.24±0.27	-1.02±0.03	-0.96±0.06	-1.09±0.01	-1.17±0.33	-0.83±0.1
	Il18r1	-1.04±0.21	-1.54±0.30	-3.71±0.46	-5.36±0.28	-0.29±0.01	-0.34±0.01	-0.91±0.02	-0.48±0.36	0.42±0.01
	Il18	1.34± 0.23	-0.54±0.61	-2.44±0.28	-0.78±0.40	-0.32±0.12		-1.15±0.37	-1.08±0.04	2.17±0.17
	Stat4	0.26±0.01	0.87±0.17	1.06±0.21	0.47±0.19	-12.34±1.06	-0.72±0.04 -19.52±0.98	-6.18±0.23	-16.13±0.74	-0.21±0.01
3	Tlr6	-3.57±0.63	-6.62±0.76	-3.41±0.31	-3.12±0.24	-4.53±0.31	-9.18±0.63 -1.37±0.14	-2.14±0.29	-6.82±0.13	0.84±0.26
	Il17a	-4.29±0.34	-10.04±0.97	4.34±0.64	4.16±0.35	-1.25±0.25		-1.09±0.34	-1.48±0.04	0.92±0.05
4	Cd28	-3.70±0.86	-3.13±0.62	-3.84±0.49	-3.22±0.48	-2.17±0.23	-1.41±0.31	-4.47±0.45	-0.74±0.31	0.35±0.12
	Cd4	-3.70±0.09	-6.25±0.31	-3.57±0.36	-3.73±0.15	-1.67±0.15	-1.84±0.01	-1.27±0.63	-1.06±0.17	-0.59±0.02
5	Cxcr3	-7.69±0.80	-11.11±1.07	-5.56±0.02	-5.26±0.74	-2.81±0.16	-6.41±0.51	-3.78±0.37	-5.56±0.42	0.40±0.06
	CCR2	-1.19±0.23	-0.92±0.14	-0.31±0.01	-1.54±0.04	0.84±0.05	0.79±0.2	1.01±27	1.67±0.24	2.23±0.13
	CCR10	-1.36±0.05	-1.40±0.43	-0.84±0.03	-1.07±0.01	-4.76±0.34	-7.62±0.20	-3.35±0.73	-13.68±0.27	0.95±0.47
7	Jak 2	-3.37±0.45	-5.35±0.12	-4.00±0.54	-3.17±0.08	0.97±0.01	1.34±0.13	0.21±0.01	1.06±0.17	0.08±0.01
	JunB	-0.57±0.20	-0.64±0.04	-0.21±0.06	-0.69±0.04	-1.18±0.47	-0.36±0.1	-1.05±0.2	-1.41±0.01	3.78±0.30
	SOCS1	-0.24±0.01	-0.93±0.11	-1.43±0.18	-0.87±0.14	-3.48±0.27	-2.01±0.13	-3.71±0.34	-2.51±0.24	2.87±0.18
	MAPK8	-0.66±0.01	-0.35±0.04	-0.16±0.07	-0.49±0.01	-0.09±0.03	-0.87±0.31	-0.92±	-1.11±	2.43±0.07

**Data is representative of 3 separate experiments (n=5 mice per experiment). Cut-off for significance fold change ≥ 2, p≤ 0.05 using*

Significant fold change up regulation ±SD
Significant fold change down regulation ± SD
No significant change ±SD



Gene Network Key

Colour	Network	Gene network group name
	1	Th1 associated genes
	2	Cytolytic degranulation markers
	3	T-helper and innate functions
	4	T cell antigen presentation and co-stimulation
	5	Chemokines and chemokine receptors
	6	Th2 Response
	7	JAK-STAT signal transduction

Priming Vaccine Key

rBCG WT (*gag*)	rBCG WT (control)
rBCG Δ*panCD* (*gag*)	rBCG Δ*panCD* (control)
rBCG *pfo* (*gag*)	rBCG *pfo* (control)
rBCG Δ*panCDpfo* (*gag*)	rBCG Δ*panCDpfo* (control)

Figure 4.1 Expression pattern of genes from unstimulated splenocytes of rBCG (gag)/SAAVI-MVA-C vaccinated mice assessed using real-time PCR. Relative gene expression in unstimulated splenocytes of rBCG (*gag*) and rBCG (control) vaccinated mice (VU) is shown. All samples were normalized to naive (unvaccinated mice). Data is representative of 3 individual experiments. The 2-ΔΔCt method was used to assess relative gene regulation changes.

We also observed the down regulation of suppressor of cytokine signalling 1 (SOCS1) (-2.01 to -3.71) in all groups primed with rBCG (control) as compared to naïve mice. SOCS1 was however up regulated in mice receiving SAAVI MVA-C alone (fold change: 2.87). SOCS1 functions in the JAK-STAT signal transduction pathway and has been reported to compete with STATs for binding of Th1 cytokine receptors and thereby assist in the regulation of the Th1 response (Linossi and Nicholson, 2015). Lastly, CD28, which is a co stimulatory marker involved in T cells binding to antigen presenting cells, was down regulated in mice primed with rBCG WT (control) and rBCG *pfo* (control) but not in mice primed with rBCG Δ*panCD* (control) and rBCG Δ*panCDpfo* (control).

4.3.1.2 Effect of rBCG modification on gene expression following rBCG (gag)/SAAVI MVA-C prime boost regime

Next, to assess the effect of rBCG modification on priming with rBCG (*gag*) and boosting with SAAVI MVA-C, we compared gene expression in groups primed with the 4 rBCG (*gag*) vaccines. We found 5 genes to be commonly down regulated across all four groups. This includes; Interleukin 1 receptor 1 (IL-1R1), Chemokine (C-C motif) ligand 5 (CCL5), CD28, CD4 and Janus kinase 2 (Jak 2). IL-27 receptor alpha subunit (IL-27Ra), which functions both to promote IFNγ release as well as reduce excessive pathology in established infection (Liu *et al*., 2014), was found to be consistently up regulated (2.32 to 3.22) in all groups primed with rBCG (*gag*) vaccines but also the SAAVI MVA-C alone group suggesting that this up regulation is due to the HIV specific boost response.

Next we investigated differentially regulated genes across these same rBCG (*gag*) primed groups. Notably interleukin 17 (IL-17A), which has been associated with the establishment of rBCG induced memory T cell populations, was down regulated in mince primed with rBCG WT (*gag*)(-4.29) and rBCG Δ*panCD* (*gag*)(-10.04) and up regulated in mice primed with rBCG *pfo* (*gag*)(4.34) and rBCG Δ*panCDpfo* (*gag*)(4.16). The Th1 cytokine IL-18 was down regulated

only in mice primed with rBCG *pfo* (*gag*) (-2.44) whilst its receptor IL-18R1 was down regulated in mice primed with rBCG *pfo* (*gag*) (-3.7) and rBCG Δ*panCDpfo* (*gag*) (-5.36).

4.3.1.3 Gene regulations associated to the SAAVI MVA-C boost

Understanding genes induced by the SAAVI MVA-C is important because regardless of the improvements in immunogenicity through modification, priming rBCG is best used in conjunction with a more immunogenic heterologous boost. In this study, groups of mice given SAAVI MVA-C only presented with up regulated Th1 cytokines including IL-18 (3.22) and IL27a (2.17)(also up regulated in rBCG (*gag*) primed mice). Additionally, chemokine (C-C Motif) receptor type 2 (CCR2) (2.23) as well as Junb (3.78) and Mapk8 (2.43) were up regulated. Lastly, whilst SOCS1 was down regulated in all rBCG (control) primed groups, it was up regulated in mice primed with SAAVI MVA-C (2.87). Up regulation of SOCS-1 is associated with shutting down Th-1 responses but since gene array data provides a single time point of gene expression data we cannot correlate this with the Th1 responses observed in Chapter 3. A reason for this could possibly be that Th1 gene expression peaked earlier than the measured time point.

4.3.1.4 Gene regulations changes associated to rBCG modification when priming for a Gag specific response

To assess the influence of different rBCG modifications on host gene expression following priming with rBCG containing Gag, we compared significant gene regulation changes between groups primed with rBCG (*gag*) vaccines and the matching strain specific rBCG (control) groups. Since this comparison overlaps with the above results on rBCG adjuvant activity we presented these rBCG (*gag*)/rBCG (control) comparisons as Venn diagrams to delineate genes that are involved in the response to Gag and are associated with modified rBCG strains.

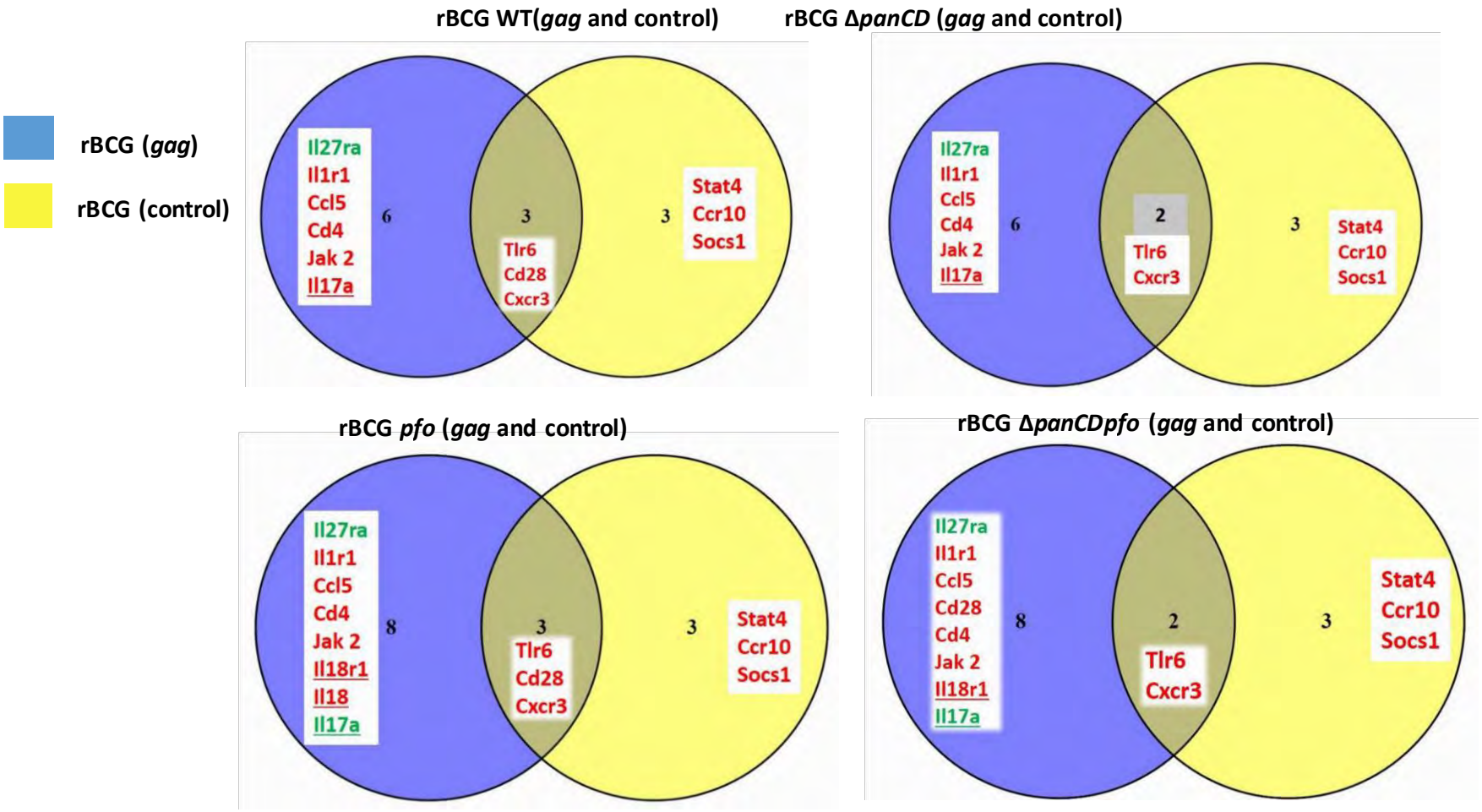


Figure 4.2 Venn diagrams showing the overlap between the differentially regulated genes induced by priming with rBCG (*gag*) or rBCG (control). For each diagram, the circles represent the number of differently expressed genes induced for rBCG (*gag*) (blue) and corresponding rBCG (control) (yellow). The intersections of each circle represents the number of genes commonly expressed by both rBCG (*gag*) and rBCG (control).

4.3.2 The impact of priming with different rBCG strains on the recall response following SAAVI MVA-C boost

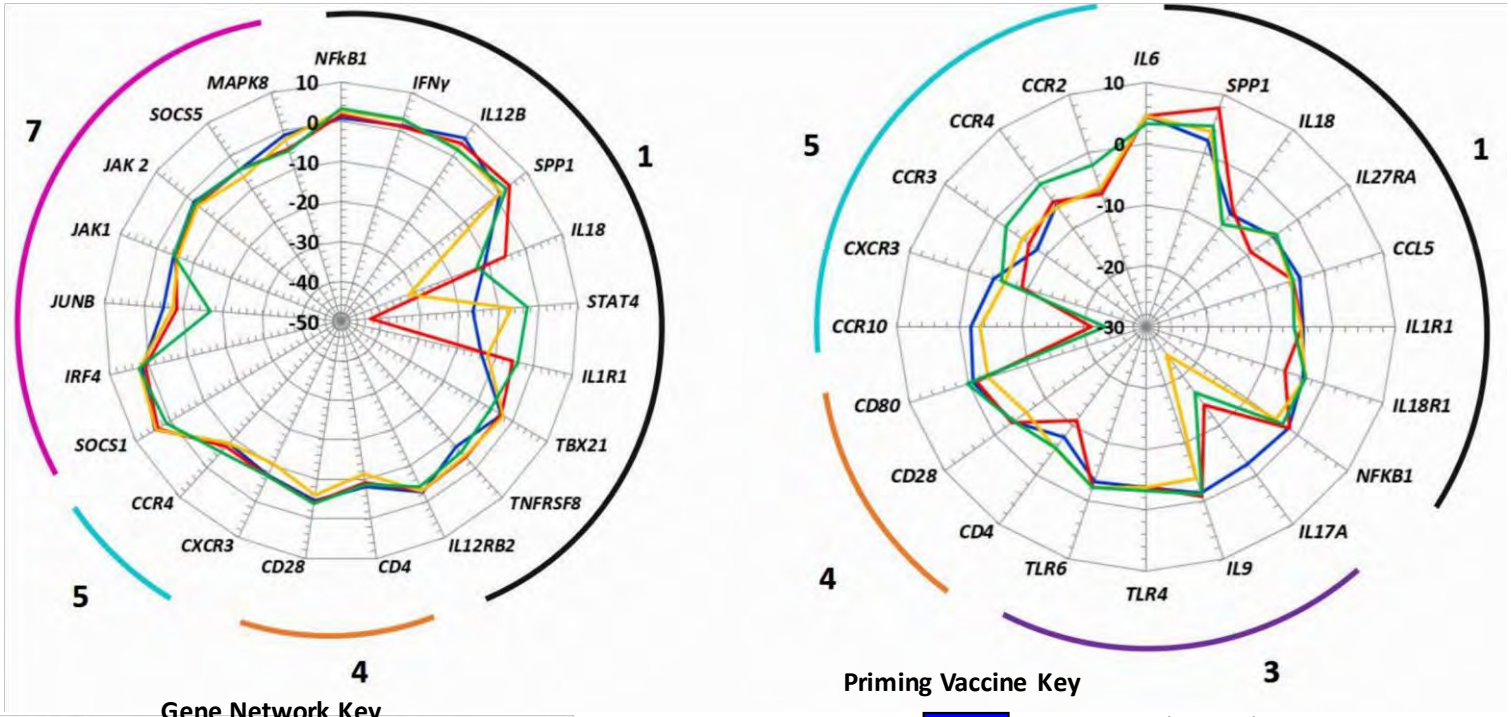
To assess and compare gene regulation changes associated with the recall response following rBCG/SAAVI MVA-C prime boost regimens, gene expression data from splenocytes that were stimulated *ex vivo* with HIV-1 peptides, normalised to data from unvaccinated mice (naïve) (UU) and compared (Table 4.3 and Figure 4.3).

4.3.2.1 Common genes associated with the adjuvant activity of rBCG regardless of modification

To identify genes associated to the adjuvant activity of each rBCG in relation to the recall response, we looked at genes that were significantly regulated and common to all groups receiving an rBCG prime. We found 6 common gene down regulations in these groups. These genes were; the pro-inflammatory cytokine Interleukin 18 (IL-18) (-5.34 to -31.47), the proinflammatory cytokine receptor Interleukin 1 receptor 1 (IL1R1) (-3.12 to -13.75), the T cell receptor CD4 (-5.26 to -11.35), the T cell co-stimulatory marker CD28 (-3.25 to -6.23), the chemokine receptor CXCR3 (-4.17 to -10.39) and the chemokine (C-C motif) receptor 4 CCR4 (-2.03 to -8.73). Assessing gene expression in mice primed with rBCG (control) and boosted with SAAVI MVA-C would further suggest genes associated to the adjuvant activity of rBCG that influence the recall response. We found 7 genes to be down regulated in all mice primed with rBCG (control) but not in the SAAVI MVA-C alone group. This included; the proinflammatory cytokine receptors IL-27Ra (-4.16 to -9.21) and IL-18R1 (-3.12 to -6.16), the proinflammatory chemokine/cytokine CCL5 (-4.17 to -5.63), the innate toll like receptors TLR4 (3.22 to-3.8) and TLR6 (-2.32 to -3.33) and lastly 2 chemokines CCR2 (-2.13 to -7.14) and CCR3 (-2.11 to -8.33).

#	Gene Symbol	WT (*gag*)	Δ*panCD* (*gag*)	*pfo* (*gag*)	Δ*panCDpfo* (*gag*)	WT (control)	Δ*panCD* (control)	*pfo* (control)	Δ*panCDpfo* (control)	SAAVI MVA-C
1	**NFkB1**	0.98±0.06	1.71±0.21	2.71±0.08	3.31±0.37	-1.56±0.26	-0.98±0.10	-4.16±0.44	-2.88± 0.02	0.26±0.13
	IFNγ	1.32±0.18	1.14±0.54	2.82±0.13	3.02±0.19	0.21±0.03	0.39±0.15	0.46±0.14	1.10±0.01	3.61±0.02
	IL12B	5.5±0.45	3.86±0.14	2.41±0.12	1.96±0.08	1.32±0.36	1.85±0.13	1.21±0.03	1.40±0.17	1.17±0.37
	IL6	1.26±0.06	1.05±0.04	1.39±0.09	1.45±0.12	4.6±0.11	4.57±0.27	4.66±0.25	3.24±0.07	4.66±0.14
	IL-9	1.07±0.01	0.63±0.2	0.15±0.02	1.26±0.03	-1.39±0.02	-1.04±0.31	-4.00 ±0.38	-0.65±0.19	1.82±0.03
	IL27Ra	0.86±0.21	0.31±0.07	0.44±0.13	0.29±0.11	-4.81±0.74	-9.21±0.37	-4.34±0.29	-4.16±0.22	0.48±0.05
	SPP1	-1.43±0.38	-4.47±0.22	-1.85±0.41	-3.41±0.13	1.87±0.07	7.76±0.21	3.6± 0.16	4.61± 0.52	3.15±0.31
	IL18	-11.21±0.87	-5.34±0.31	-31.47±1.08	-13.13±0.31	-7.14±0.46	-6.25±0.13	-9.12±0.87	-9.18±0.14	-3.02±0.04
	IL18R1	1.12±0.04	1.31±0.01	1.25±0.01	1.64±0.03	-3.97±0.31	-6.16±0.20	-3.12±0.34	-3.26±0.13	0.65±0.16
	STAT4	-16.6±0.86	-42.49±1.26	-6.67±0.15	-2.7±0.11	1.24±0.03	1.62±0.11	1.33±0.37	1.02±0.01	1.00±0.02
	IL1R1	-13.75±0.17	-5.41±0.51	-12.14±0.28	-4.21±0.71	-3.97±0.31	-6.47±0.23	-3.12±0.34	-3.26±0.13	0.34±0.05
	TBX21	-3.18±0.35	-3.23±0.15	-2.82±0.64	-6.51±0.37	1.37±0.04	1.66±0.19	1.79±0.05	1.20±0.01	0.75±0.19
	TNFRSF8	-7.29±0.12	-3.74±0.52	-3.98±0.31	-5.31±0.04	1.16±0.01	1.05±0.14	1.38±0.01	1.09±0.22	0.27±0.01
	IL12RB2	-2.56±0.44	-2.15±0.07	-2.92±0.10	-4.11±0.15	0.79±0.16	1.49±0.36	1.69±0.01	1.77±0.50	9.62±0.01
	CCL5	1.87±0.21	1.50±0.17	1.35±0.13	1.04±0.09	-4.17± 0.23	-5.06 ±0.53	-5.63 ±0.07	-5.16 ±0.31	1.14±0.14
3	**TLR 4**	0.36±0.40	0.98±0.13	0.74±0.18	0.57±0.01	-3.72±0.33	-3.76±0.15	-3.8±0.19	-3.22±0.04	1.29±0.01
	TLR6	0.94±0.24	1.27±0.08	0.19±0.26	1.36±0.24	-3.33±0.46	-2.43±0.08	-2.32±0.17	-2.39±0.09	0.63±0.20
4	**CD4**	-8.2±0.11	-9.29±0.47	-11.35±0.23	-8.64±0.48	-7.69±1.02	-11.11±0.96	-5.26±0.40	-5.37±0.43	0.24±0.06
	CD28	-4.55±0.74	-4.11±0.37	-6.06±0.48	-3.85±0.33	-3.57±0.15	-3.25±0.29	-6.23±0.06	-3.71±0.20	1.08±0.017
	CD80	0.29±0.01	0.31±0.01	0.86±0.22	0.94±0.02	-0.49±0.013	-0.78±0.30	-3.32± 0.03	-0.21±0.04	1.31±0.01
5	**CXCR3**	-7.37±0.23	-6.89±0.22	-10.39±0.65	-6.94±0.26	-4.17±0.20	-9.09±.0.41	-6.25±0.23	-5.55±0.15	0.15±0.01
	CCR2	1.16±0.01	1.24±0.01	1.40±0.01	1.27±0.12	-6.25 ±0.49	-7.14 ±0.12	-6.12 ±0.18	-2.13 ±0.01	1.96±0.1
	CCR3	0.80±0.05	0.76±0.12	1.17±0.02	1.03±0.17	-8.33 ± 0.24	-6.81 ± 0.26	-5.41± 0.41	-2.11±0.06	0.34±0.03
	CCR4	-8.73±0.29	-7.14±0.24	-8.45±0.21	-5.26±0.39	-5.26± 0.18	-4.55± 0.23	-5.58 ± 0.18	-2.03±0.64	0.62±0.21
	CCR10	1.69±0.04	1.52±0.01	1.34±0.37	1.58±0.18	1.51±0.16	-21.22 ± 1.13	-0.79±0.29	-23.32 ± 0.62	0.05±0.01
6	**SOCS1**	4.51±0.31	3.53±0.37	4.26±0.87	1.21±0.31	0.30±0.13	1.17±0.01	1.62±0.17	1.74±0.25	1.37±0.04
	IRF4	1.51±0.22	0.72±0.05	2.5±0.31	2.56±0.55	1.47±0.01	1.08±0.01	1.15±0.11	1.60±0.16	4.76±0.06
	JUNB	-4.76±0.20	-8.33±0.17	-7.14±0.16	-16.67±0.37	1.63±0.14	0.64±0.03	0.31±0.16	1.18±0.18	1.36±0.17
	JAK1	-4.55±0.47	-5.00±0.32	-5.56±0.07	-4.15±0.64	1.58±0.02	0.93±0.04	0.11±0.09	0.64±0.023	1.16±0.12
	JAK 2	-2.33±0.16	-3.23±0.64	-3.70±0.39	-2.56±0.20	1.19±0.07	1.01±0.13	0.62±0.08	0.14±0.01	0.36±0.16
	SOCS5	-4.15±42	-4.17±0.07	-6.25±0.19	-4.01±23	0.35±0.06	0.27±0.06	1.05±0.02	0.47±0.04	0.87±0.13
	MAPK8	-1.02±0.31	-4.85±0.17	-1.07±0.69	-5.32±0.65	1.31±0.06	1.07±0.14	1.16±0.15	1.27±0.15	0.18±0.03

Table 4.3: Table of relative gene expression: Vaccinated stimulated (VS) normalised to unvaccinated (UU)



Gene Network Key

Colour	Network	Gene network group name
	1	Th1 associated genes
	2	Cytolytic degranulation markers
	3	T-helper and innate functions
	4	T cell antigen presentation and co-stimulation
	5	Chemokines and chemokine receptors
	6	Th2 Response
	7	JAK-STAT signal transduction

Priming Vaccine Key

rBCG WT (*gag*)	rBCG WT (control)
rBCG Δ*panCD* (*gag*)	rBCG Δ*panCD* (control)
rBCG *pfo* (*gag*)	rBCG *pfo* (control)
rBCG Δ*panCDpfo* (*gag*)	rBCG Δ*panCDpfo* (control)

Figure 4.3 Expression pattern of genes from *ex vivo* stimulated splenocytes of rBCG (*gag*)/SAAVI-MVA-C vaccinated mice assessed using real-time PCR. Relative gene expression in stimulated splenocytes of vaccinated mice (VS) is shown. All samples were normalized to naive (unvaccinated mice). Data is representative of 3 individual experiments. The $2^{-\Delta\Delta Ct}$ method was used to assess relative gene regulation changes.

4.3.2.2 Variable gene regulations associated with strain specific adjuvant activity of rBCG associated with recall response

In order to determine strain specific rBCG adjuvant activity, we looked at genes that were differentially regulated in mice prime with rBCG (control) and boosted with SAAVI MVA-C but not significantly regulated in the SAAVI MVA-C alone groups. We observed that he Th1 transcription factor nuclear factor kappa-light-chain-enhancer of activated B cells (Nfκb1) was down regulated in mice primed with rBCG *pfo* (control) (-4.16) and rBCG Δ*panCDpfo* (control)(-2.88). In mice primed with rBCG *pfo* (*gag*), we additionally observed a down regulation of the cytokine interleukin 9 (IL-9) (-4.00) (which has been described to promote cell proliferation and limit apoptosis) as well as the T cell co-stimulatory molecule CD80 (-3.32). Lastly, the C-C chemokine receptor type 10 (CCR10) was found to be uniquely down regulated in mice primed with rBCG Δ*panCD* (control) (-21.22) and rBCG Δ*panCDpfo* (control) (-23.32) suggesting this gene regulation is associated to the Δ*panCD* modification.

4.3.2.3 Common gene regulations associated with the recall response following rBCG (gag)/SAAVI MVA-C prime boost regime

Next, to assess the effect of rBCG modification on the recall response following priming with rBCG (*gag*) and boosting with SAAVI MVA-C, we compared gene expression in groups primed with the 4 rBCG (*gag*) vaccines. We found 8 genes to be commonly down regulated across all four groups primed with rBCG (*gag*) vaccines. These included 4 Th1 related genes which were; the transcription factors TBX-21 (-2.82 to -6.51) and STAT4 (-2.7- to -42.49), the cytokine and cytokine receptor subunit IL12RB2 (-2.15 to -4.11) and the TNF receptor TNFRSF8 (-3.74 to 7.29). Additionally, 4 genes that are part of the JAK-STAT pathway were down regulated including JUNB (-4.76 to -16.6), JAK 1(-4.15 to -5.56), JAK 2(-2.33 to-3.70) and SOCS5 (-4.01 to-6.25).

4.3.2.4 Variable gene regulations associated with rBCG modification specific effects on recall response

In order to assess the effect of rBCG modifications on the recall response to HIV antigens after an rBCG (*gag*)/SAAVI MVA-C prime boost, we looked at variably regulated genes in the 4 rBCG

(*gag*) primed groups. Unlike in groups primed with rBCG *pfo* (control) and rBCG Δ*panCDpfo* (control) (discussed above), mice primed with rBCG *pfo* (*gag*) and rBCG Δ*panCDpfo* (*gag*) we found to have Nfkb1 up regulated (2.71 to 3.31). This was insignificant in groups primed with rBCG WT (*gag*) and rBCG Δ*panCD* (*gag*). Similarly the potent Th1 cytokine interferon-gamma (IFNγ) was up regulated only in groups primed with rBCG *pfo* (*gag*) (2.82) and rBCG Δ*panCDpfo* (*gag*) (3.02). So was the interferon regulatory factor 4 which belongs to a group of transcription factors that regulate interferon expression (2.5 to 2.56). Interleukin 12 beta subunit (IL12B) and suppression of cytokine secretion 1 (SOCS1) were up regulated in mice primed with rBCG (*gag*), rBCG Δ*panCD* (*gag*), rBCG *pfo* (*gag*) but not in mice primed with rBCG Δ*panCDpfo* (*gag*). Lastly, 2 genes were only significantly down regulated in mice primed with rBCG Δ*panCD* (*gag*) and rBCG Δ*panCDpfo* (*gag*). These were secreted phosphoprotein 1 (SPP1) (-3.41 to -4.47), which has been reported to promote cell survival by regulating apoptosis and Map Kinase 8 (MAPK8) (-4.85 to-5.32).

4.3.2.5 Gene regulations associated with SAAVI MVA-C

In the group receiving only the SAAVI MVA-C vaccination, we observed up regulation of the inflammatory cytokines IFNγ (3.61) and IL-6 (4.66). We additionally found SPP1 to be up regulated (3.15). These results suggest peak Th1 response at 12 days after SAAVI MVA-C vaccination confirming the timing of boost and sacrifice times for the prime boost regimes. Lastly, IL-18 was down regulated in mice receiving SAAVI MVA-C alone (-3.02).

4.2.3.6 Gene regulations changes associated to rBCG modification when priming for a Gag specific response

To assess the influence of different rBCG modifications on host gene expression following priming with rBCG containing Gag and recall response, we compared significant gene regulation changes between groups primed with rBCG (*gag*) vaccines and the matching strain specific rBCG (control) groups. Common genes associated to the effect of rBCG strains (regardless of *gag*/control insert) suggest pathways involved in rBCG adjuvant activity.

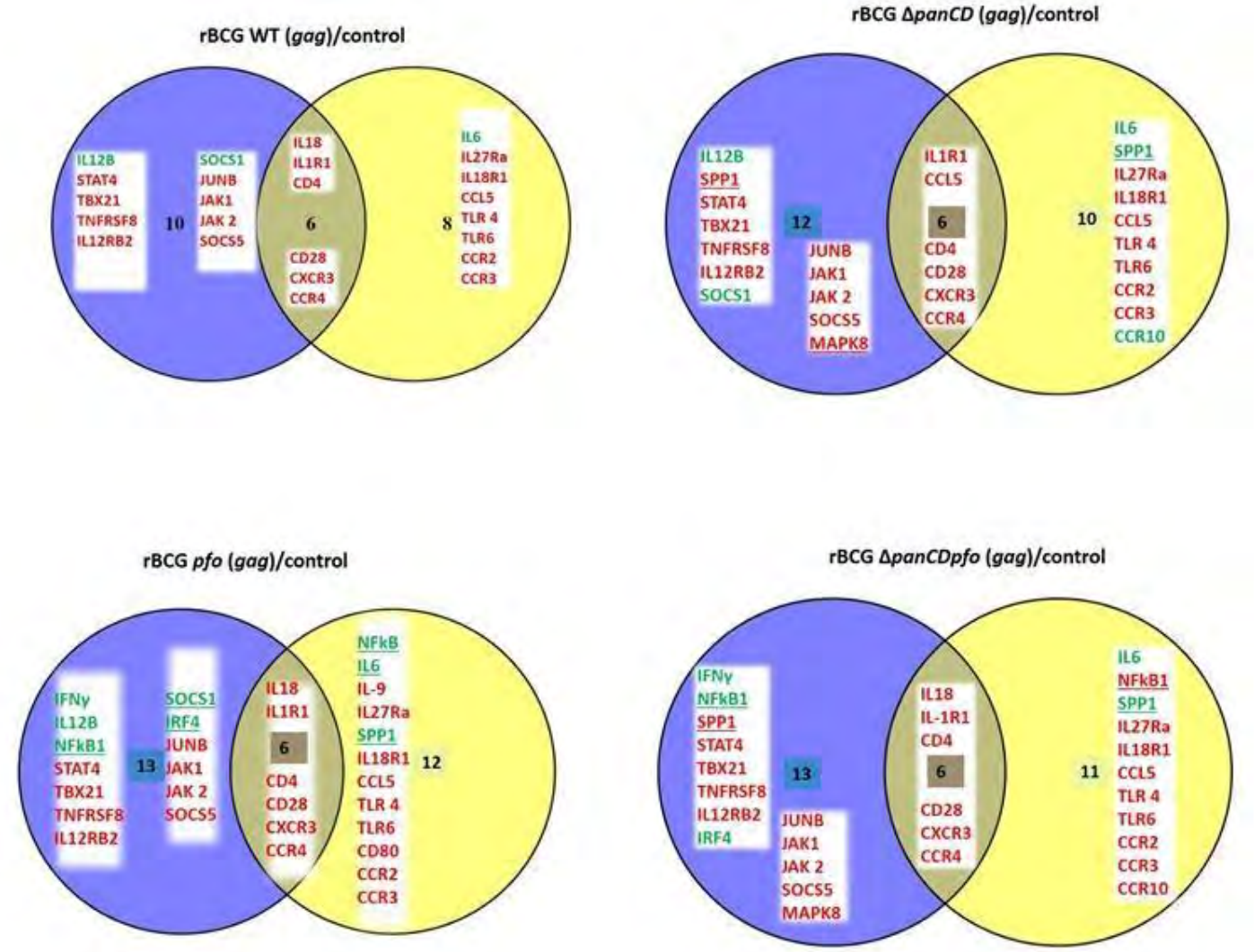


Figure 4.4 Venn diagrams showing the overlap between the differentially regulated genes induced by *ex vivo* stimulation after rBCG/SAAVI MVA-C prime boost regimen. For each diagram, the circles represent the number of differently expressed genes induced for rBCG (*gag*)(blue) and corresponding rBCG (control)(yellow) . The intersections of each circle represents the number of genes commonly expressed by both rBCG (*gag*) and rBCG (control). Gene changes of interest are underlined.

4.4. Discussion

We have shown in preceding chapters that modified rBCG (expressing HIV-1 Gag) induces less pathology (Chapter 2) and improved antigen specific CD4+ and CD8+ T cell responses (Chapter 3) as compared to rBCG WT (*gag*)(as part of a heterologous boost). In this chapter, we compared the expression of 86 immunologically pertinent Th1 and Th2 genes in the spleens of mice primed with either rBCG WT (*gag*), rBCG Δ*panCD* (*gag*), rBCG *pfo* (*gag*) or rBCG would elucidate the molecular mechanisms of reduced pathology and improved HIV specific T cell responses (induced by modified rBCG as compared to priming with WT Danish). Whilst a number of genes were consistently regulated across all groups as part of the immune response to vaccination *ex vivo* peptide stimulation, we analysed the unique gene associations to find associations with the pathology and immunogenicity data.

4.4.1 Effect of priming with different rBCG (*gag*) vaccines on host gene expression following SAAVI MVA-C boost

In order to determine molecular signatures associated with rBCG induced pathology/immunogenicity, we assessed gene expression in unstimulated splenocytes from groups of mice primed with rBCG (*gag*) vaccines and boosted with SAAVI MVA-C (28 days post rBCG vaccination). We noted that down regulation of genes involved in the IL-18 pathway, was associated with priming with rBCG *pfo* (*gag*) and rBCG Δ*panCDpfo* (*gag*). An up regulation of IL-17A, which is produced mostly by T helper cells was also associated with rBCG vaccines expressing perfringolysin. Inherent activation and regulation of the IL-17/IL-18 pathway (Figure 4.5), has varying implications for both BCG induced protection as well as HIV pathogenesis.

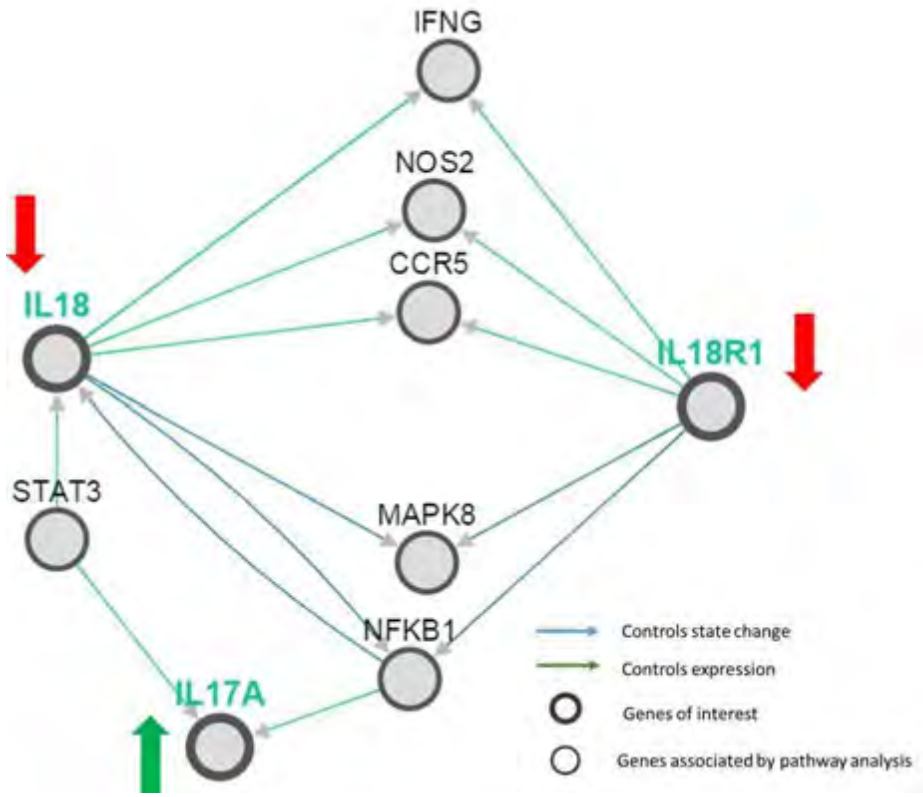


Figure 4. 5 Priming with rBCG expressing perfringolysin and HIV-1 Gag leads to induction of the IL-17/IL-18 pathway. IL-18/IL18R1 interaction can lead to the expression of anti-viral and anti-bacterial gene products IFN and NOS2 but also the HIV-1 co-receptor CCR5.

A TB vaccine study assessing gene expression in BCG vaccinated humans, reported that the IL-17 pathways are strongly induced in BCG vaccinated individuals and expression of these gene subsets was found to correlate to reduced mycobacterial growth (Matsumiya *et al*., 2015). They furthermore observed a negative correlation between BCG growth and the frequencies of IL-17 multi-functional T cells. Lastly, IL-17 production has been associated with protection against MTB in mice vaccinated with the recombinant rBCG Δ*ureC* hly which expresses an endolysin similar to perfringolysin called listeriolysin (Desel *et al*., 2011). As compared to the WT strain this rBCG Δ*ureC* hly, induced improved protective responses that were highly characterised by IL-17 production. This, together with our data, led us to believe that endolysins can increase the production of rBCG specific IL-17 following vaccination. IL-17 has been reported to be associated with both pathogenesis and protection in both HIV and

SIV. A NHP study reported that IL-17 producing CD4 T cells are infected at a greater rate *in vivo* than other T cells in macaques infected with SIV mac_{251} (Cecchinato *et al*., 2008). They also demonstrated that in highly vireamic animals, Th1 responses dominated over Th17 responses suggesting that the Th1/Th17 balance is important for protection. Human HIV studies have suggested that preferential infection of IL-17 CD4+ T cell subsets is irreversible and may lead to the lack of immune control of HIV pathogenesis (Mavigner *et al*., 2012).

4.4.2 Gene expression associations involved in the recall response to HIV-1 peptides

A major aim of T cell based HIV-1 vaccine approaches is to induce vaccine specific Th1 cytokine release, multi-functional T cells as well as vaccine specific memory T cells (Mann and Ndung'u, 2015). Whilst all 3 of the modified rBCG (*gag*) vaccines were deemed to induce improved T cell responses than rBCG WT (*gag*) when used in combination with a SAAVI MVA-C boost, we attempted to determine if we find gene regulations associated with differences in immunogenicity. To compare gene regulation changes associated with the recall response following rBCG/SAAVI MVA-C prime boost regimens, gene expression data from splenocytes that were stimulated *ex vivo* with HIV-1 peptides was normalised to data from unvaccinated mice (naïve). The recall response in mice primed with rBCG *pfo* (*gag*) and rBCG Δ*panCDpfo* (*gag*) was associated with up regulation of the major inflammatory cytokine and transcription factor IFNγ and NFκB as well as interferon regulating factor IRF4 (Figure 4.6).

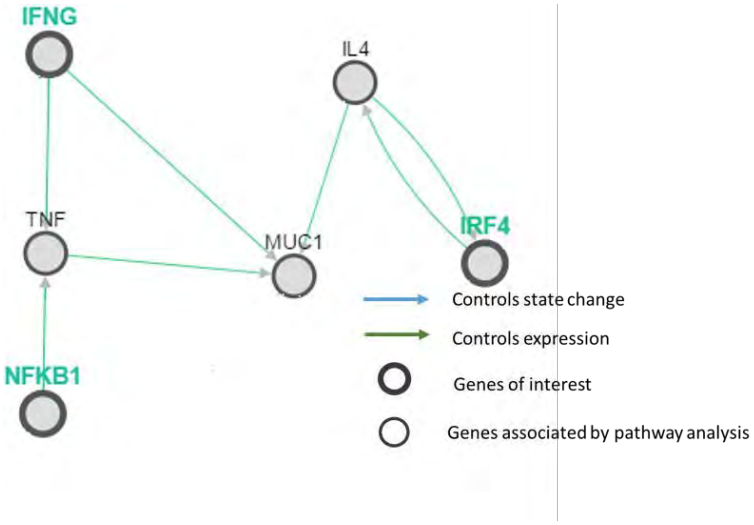


Figure 4.6: The recall response following *ex vivo* stimulation in mice primed with rBCG expressing perfringolysin and HIV-1 Gag. NFKB1, IFNγ and IRF4 commonly regulate Mucin 1, cell surface associated protein (MUC1). MUC1 is associated to reduced apoptosis as well as binding cytoplasmic antigen.

These differences indicate that the early aspects of Th1 gene induction (NFkB and IFNγ) persist even after 6 hours of *ex vivo* stimulation is associated with mice primed with rBCG expressing perfringolysin (rBCG *pfo* (*gag*) and rBCG Δ*panCDpfo* (*gag*). Whilst the induction of NFkB and IFNγ are essential for the establishment of effector T cell responses to HIV, consistent up regulation of these inflammatory transcription factors and cytokines have been associated with increase viral replication. Persistence of effector T cell function has been associated to highly inflammatory vaccine vectors such as CMV. We postulate that this persistent up regulation of inflammatory markers is additionally associated with the generation of improved T_{EM} responses in mice primed with rBCG *pfo* (*gag*) and rBCG Δ*panCDpfo* (*gag*) as seen in Chapter 3. In addition to the mechanisms of how perfringolysin influences the HIV-1 recall response, we were interested in the effects of the *panCD* deletion on the recall response. Uniquely, the recall response of mice primed with rBCG Δ*panCD* (*gag*) was associated with a 42 fold down regulation in STAT4 which acts to promotes CD4+ T cell development and IFNγ production following IL-12 stimulation. The 42 fold down regulation of STAT4 in mice primed with rBCG Δ*panCD* (*gag*), indicates a decrease in the activity of the JAK-STAT signal transduction pathway. STAT 4 is a vital Th1 transcription factor for the expression of IL-2 and IFNγ (Figure 4.7) which promotes the clonal expansion of antigen specific cells and migration to the site of disease. This interaction may explain the molecular basis of decreased

inflammation induced by the rBCG Δ*panCD* (*gag*) of rBCG. Whilst HIV specific IL-2 was found to be produced by splenocytes following *ex vivo* peptide stimulation in Chapter 3, gene expression data contradicted this with no IL-2 up regulation observed. This is probably due to cytokine secretion kinetics and assay sensitivity.

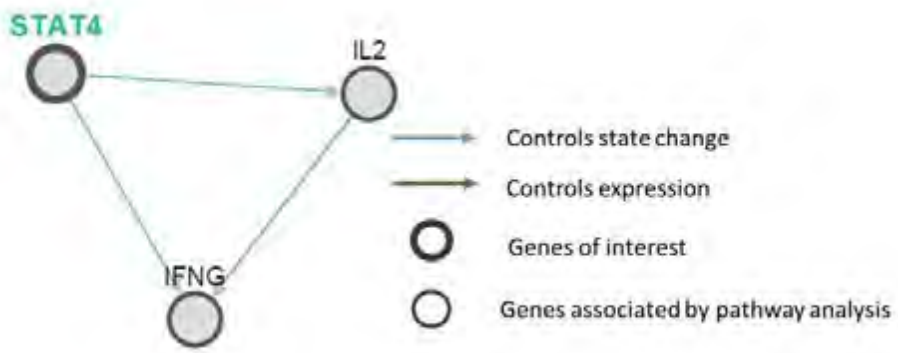


Figure 4.7 : Down regulation of STAT4 is associated with the less pathogenic rBCG Δ*panCD*(*gag*) vaccine. A large down regulation in the Th1 transcription factor STAT4 would lead to decreased IL2 and IFNγ production. Physiologically, this would present as decreased clonal expansion of T cells, decreased inflammation and therefore decreased pathology

In addition to STAT4, we observed chemokine and chemokine receptors (CCR2, CCR3, CCR4, and CXCR3) to be down regulated in rBCG (control) primed mice. Notably, CCR10, which is involved in tracking of cells to areas of inflammation, was highly down regulated in mice primed with rBCG Δ*panCD* (control) and rBCG Δ*panCDpfo* (control). Pathway analysis (Figure 4.8) of these chemokine/receptor interactions suggests that G protein-coupled receptor kinase 6 (GRK6) controls the expression of these cytokines (not included on the RT-PCR array). Decreased expression of chemokines and chemokine receptors following *ex vivo* stimulation in rBCG Δ*panCD* (control) and rBCG Δ*panCDpf*o (control) could explain decreased splenic inflammation in the spleens of these mice as compared to rBCG WT (control) and rBCG *pfo* (control).

In the next Chapter, we propose dynamic models for each priming vaccine which mechanistically link these observed host gene expression differences to the improved safety and immunogenicity associated with modified rBCG as compared to WT Danish.

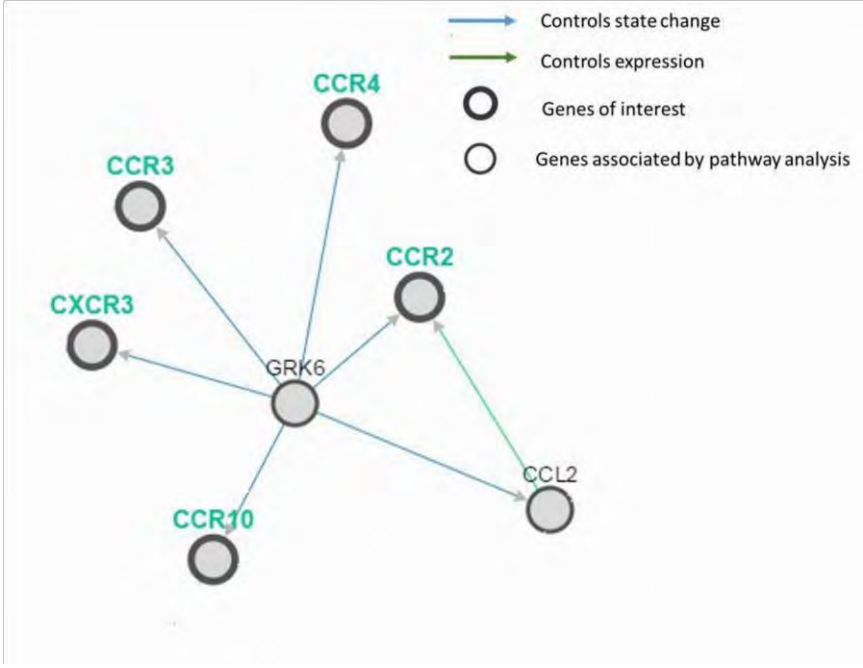


Figure 4.8 Down regulation of chemotactic function is associated with priming with rBCG with the Δ*panCD* deletion. By the process of phosphorylation, GRK6 can deactivate the expression of a number of chemokines and chemokine receptors including CCR10 which was highly down regulated in mice primed with rBCG Δ*panCD* (control) and rBCG Δ*panCDpfo* (control).

Chapter 5: Final Conclusions

5.1 Introduction

The UCT group has a long history of developing both persistent HIV-1 vectors (in the guise of rBCG) as well as HIV-1 poxviral vectors (such as MVA-Gag, SAAVI MVA-C). The development of improved modified strains of rBCG as candidate TB vaccines has furthered the potential of rBCG as a vaccine vector for heterologous antigens such as HIV-1 (da Costa *et al*., 2014, Chapman *et al*., 2011a, Chege *et al*., 2013, Hopkins *et al*., 2011). In this study, we assessed the safety and immunogenicity of 3 modified strains of Danish rBCG expressing HIV-1 Gag, (as well as a WT control) as priming vectors for a SAAVI MVA-C boost in the murine model. We have shown in preceding chapters that modified rBCG (expressing HIV-1 Gag) induces less pathology (Chapter 2) and improved antigen specific CD4+ and CD8+ T cell responses (Chapter 3) as compared to rBCG WT (*gag*)(as part of a heterologous boost). In Chapter 4, we compared the expression of 86 immunologically pertinent Th1 and Th2 genes in the spleens of mice primed with either rBCG WT (*gag*), rBCG Δ*panCD* (*gag*) rBCG *pfo* (*gag*) or rBCG Δ*panCDpfo* (*gag*) and boosted with SAAVI MVA-C. We hypothesised that identification of host gene expression changes in unstimulated splenocytes (VU) and stimulated splenocytes (VS) would elucidate the molecular mechanisms of reduced pathology and improved HIV specific T cell responses (induced by modified rBCG as compared to priming with WT Danish).The assessment of relative gene expression in the murine spleen provides a snap shot of the effects of vaccination on the host immune system (VU) as well as the host immune response to antigen (VS) (recall response upon stimulation). It should be noted that gene expression itself does not guarantee translation of encoded proteins. Therefore, a systems vaccinology based approach dictates that we interpret our relative gene expression data in relation to the pathology and immunogenicity data reported in Chapters 2 and 3 of this study.

5.1.1 Improved bacilli clearance and decreased pathology associated with priming with rBCG containing the panCD deletion

The attenuation of rBCG through deletion of the *panCD* genes has been shown to be a promising strategy to improve the safety of rBCG as a vaccine vector and has been shown to be associated to limited survival of the bacteria and hence pathology in mice (Sambandamurthy *et al*., 2002, Sampson *et al*., 2004, Chapman *et al*., 2012). The first of the 3 modified rBCG priming vaccines discussed is rBCG Δ*panCD* (*gag*) (Figure 5.1).

We observed fewer rBCG granulomas and CD3+ T cell levels in the livers of mice vaccinated with rBCG Δ*panCD* (*gag*) as compared to the other 3 strains. This decreased T cell tracking to the site of infection, which we hypothesise is in part caused by the reduced virulence and is favourable for an efficacious HIV vaccine that aims to limit the spread of infected T cells (Esparza and Van Regenmortel, 2014, McMichael and Haynes). Decreased T cell tracking to central organs could also be the result of the attenuated rBCG Δ*panCD* (*gag*) and rBCG Δ*panCDpfo* (*gag*) being cleared earlier (shown in Chapter 2 in the abdominal lymph nodes).

Both clearance of BCG and conversely persistence of the bacilli have been reported to be associated with improved memory T cell responses. In this study, we show that mice primed with rBCG Δ*panCD* (*gag*) (strains that are cleared from the periphery at day 7 post vaccination) present with improved CD4+ T cell effector memory following *ex vivo* stimulation with HIV peptide (as compared to rBCG WT (*gag*) and rBCG *pfo* (*gag*)).

Persistence of BCG has also been associated with impaired T cell responses. In a recent study by Nandkumar *et al*, (2014), waning murine BCG responses 78 weeks post infection were characterised by reduced T cell functionality and up regulation of the T-cell inhibitory receptors CTLA 4 and killer cell lectin-like receptor subfamily G member 1 (KLRG-1) (>97% of CTLA-4+ or KLRG-1+ T-cell subsets did not produce IFN-γ, IL-2 or TNF-α). In this study, we demonstrate that priming with an attenuated Danish rBCG with limited persistence (rBCG Δ*panCD* (*gag*)) induces improved bi- and multi-functional T cell responses as compared to

rBCG WT (*gag*) and rBCG *pfo* (*gag*). Multifunctional CD4+ and CD8+ T cells have been associated with control of HIV vireamia in non-progressors and rBCG HIV-1 vaccine vectors should support the generation of such T cell functionality (Baker *et al*., 2009). In terms of clearance of the bacillus, a recent study by Gideon *et al*, (2015) indicated that a balanced Th1/Th2 response is more favourable for sterilisation of tuberculosis granulomas and this could hold true for rBCG as well (Gideon *et al*., 2015).

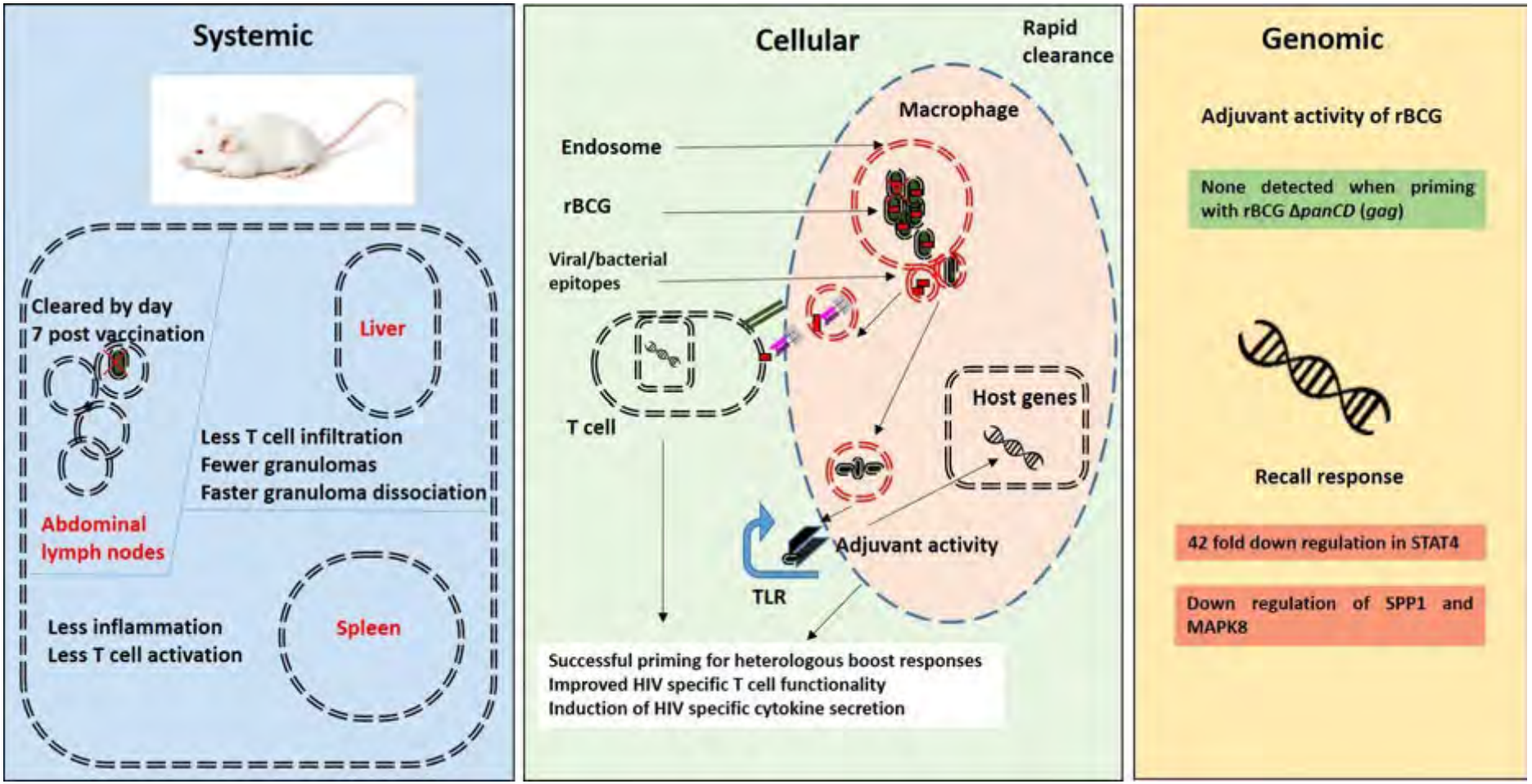


Figure 5.1: Systemic, cellular and genomic characteristics of priming with rBCG Δ*panCD* (*gag*). At the systemic level, mice primed with this vaccine cleared rBCG by day 7 post-vaccination in abdominal lymph nodes. CD3 staining of liver sections revealed less T cell infiltration, granuloma formation and a more rapid granuloma dissociation than rBCG WT (*gag*). Phenotyping of splenocytes revealed lower levels of T cell activation and T cell numbers than mice primed with rBCG WT (*gag*). At the cellular level we assessed, the induction of HIV-1 specific T cells following SAAVI MVA-C boost and subsequent *ex vivo* peptide stimulation. rBCG Δ*panCD* (*gag*) primed significantly for a boost of T cell responses and was associated with improved CD4+ T cell memory and T cell functionality as compared to groups primed with rBCG WT (*gag*). Regulation of *MAPK8* as well as *SPP1* indicated that the rBCG Δ*panCD* strain is associated with reduced apoptosis.

Regardless of the anti-inflammatory response, the induction of improved CD4+ and CD8+ T cell responses, as compared to WT (*gag*), indicate that Danish rBCG Δ*panCD* (*gag*) can be far less inflammatory whilst priming effectively for HIV specific T cell responses in combination with a SAAVI MVA-C boost.

A hallmark of the recall response in rBCG Δ*panCD* (*gag*) primed mice was the 42 fold down regulation of STAT4, a potent Th1 transcription factor. Additionally, down regulation of MAPK8 and SPP1 indicates potentially decreased rates of apoptosis in these mice. Preclinically, for both adjuvant activity and influencing the recall response, Danish rBCG Δ*panCD* (*gag*) appears to be a safe and immunogenic priming vaccines for a heterologous poxviral boost.

5.1.2 Prolonged inflammation following recall response and improved TEM frequency associated with priming with rBCG expressing perfringolysin (pfo)

We next investigated host biomarkers associated with priming with rBCG *pfo* (*gag*), a persistent BCG vector. Concurrent analysis of systemic, cellular and genomic data in this study revealed a great deal about the impact of priming with rBCG *pfo* (gag) on the murine model system (Figure 5.2). Following priming with rBCG *pfo* (*gag*), we observed persistence of bacteria in secondary lymphoid organs such as the abdominal lymph nodes until experimental endpoint unlike in mice primed with rBCG Δ*panCD* (*gag*). However, rBCG *pfo* (*gag*) was also able to prime for comparable boost responses to priming with rBCG Δ*panCD* (*gag*). A hallmark of rBCG *pfo* (*gag*) induced immunogenicity as part of a SAAVI MVA-C boost was the induction of improved CD8+ T cell effector memory responses. Clearance of SIV in macaques vaccinated with RhCMV vectored SIV vaccines has been associated with persistent CD8+ T_{EM} responses (Hansen *et al*., 2013a) and induction of such responses are favoured as an endpoint when testing T cell inducing HIV vaccines.

Gene expression analysis revealed a number of Th1 (inflammatory) genes were up regulated (*IFNy, NFkB*). Whilst a Th1 dominant response favours anti-viral function, uncontrolled and persistent inflammation leads to dysfunctional T cell responses and increased pathology.

Murine and rhesus macaque studies have demonstrated that TB vaccines vectored by perfringolysin knock in/urease C knock out BCG (Aeras 401) elicit enhanced protection and/or stronger CD8+ T cell responses to MTB as compared to the wild type strain (Sun *et al*., 2009, Rosario *et al*., 2010, Magalhaes *et al*., 2008a). Unfortunately during the duration of this study, the phase 1 TB vaccine safety trial using BCG Danish 1331 expressing pefringolysin O (Aeras422) was terminated due to side effects which included the reactivation of shingles (Kupferschmidt, 2011, Ottenhoff and Kaufmann, 2012). We hypothesised that the lysis of endosomes induced by perfringolysin in conjunction with increased apoptosis and a persistent vector could lead to an intracellular environment with a high level of non-specific inflammation and immune activation.

Whilst perfringolysin has been attenuated by the Gly 137 to Gln mutation which decreases the half-life of the protein in the cytosol, it still remains a toxin and antigen to rBCG as well as the host. Since an efficacious HIV-1 vaccine should be safe as well as immunogenic, our results, as well that of the AERAS 422 suggest that rBCG *pfo* (gag) should not be used exclusively as an HIV-1 vaccine. However, the perfringolysin molecule has enormous biological potential as an immunotherapeutic to induce translocation from the endosome and promote CD8+ T cell responses. Recent advances include the production of anti-perfringolysin antibodies which may assist with improving the safety of these therapies by limiting the longevity of *pfo* expression and function (Yang *et al*., 2015a). Conversely, it can be argued that the non-specific inflammation induced by lysing the endosome is too unregulated in the context of an HIV vaccine which has to tightly influence the immune response to the specific correlates of protection discussed in Chapter 1.

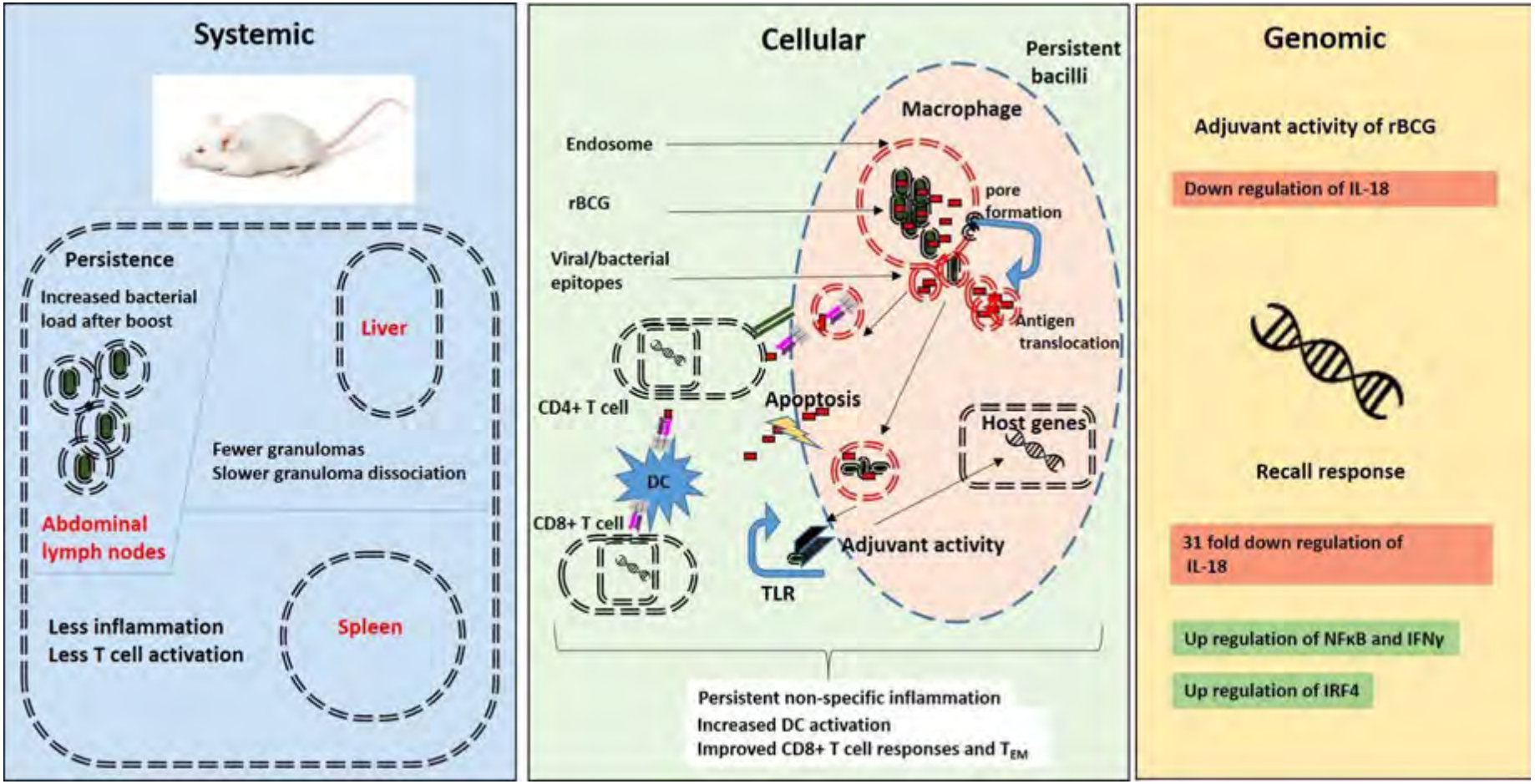


Figure 5.2: Systemic, cellular and genomic characteristics of priming with rBCG *pfo* (*gag*). At the systemic level, mice primed with this vaccine showed persistent rBCG in lymph nodes at experimental endpoint. At the cellular level we assessed, the induction of HIV-1 specific T cells following SAAVI MVA-C boost and subsequent *ex vivo* peptide stimulation. Phenotyping of splenocytes revealed greater dendritic cell activation as compared to rBCG WT (*gag*). We hypothesised this to be due to cross priming initiated by the pore forming action of *pfo* on the endosome. rBCG *pfo* (*gag*) primed significantly for a boost of T cell responses and was associated with improved CD8+ T cell effector memory with rBCG WT (*gag*) and rBCG Δ*panCD* (*gag*).

5.1.3 Synergistic action of panCD deletion and pfo to increase safety and immunogenicity of rBCG Danish

Individually, rBCG Δ*panCD* (*gag*) and rBCG *pfo* (*gag*) have been shown to induce improved CD4+ and CD8+ T cell responses as compared to rBCG WT (*gag*). One of the major aims of this study was to assess the immunological characteristics of priming with a combination of the two modifications in the guise of rBCG Δ*panCDpfo* (*gag*) (Figure 5.3). Concurrent analysis of systemic, cellular and genomic data for mice primed with this vaccine and boosted with SAAVI MVA-C showed host immunological characteristics to be in part a combination of that observed in mice primed with either rBCG Δ*panCD* (*gag*) or rBCG *pfo* (*gag*).

In terms of the development of liver pathology and peripheral bacterial clearance, mice primed with rBCG Δ*panCDpfo* (gag) were more similar to those primed with rBCG Δ*panCD* (*gag*) (reduced granuloma formation and clearance of bacterial load from abdominal lymph nodes by day 7). However, this did not diminish the induction of HIV specific CD8+ T cell effector memory function suggesting that whilst the *panCD* deletion confers improved safety, it does not abrogate the benefit of *pfo* mediated immunogenicity enhancement.

Preservation of similar anti-apoptotic pathways as seen in mice primed with rBCG Δ*panCD* (*gag*) was observed (*MAPK8, SPP1*) suggesting that less *pfo* driven apoptosis may occur. Taken together this study demonstrates unique safety and protective correlates that rBCG Danish can elicit as a HIV-1 vaccine vector when modified. Additionally, we demonstrate that combining the Δ*panCD* modification with *pfo* can synergistically improve the immunogenicity of rBCG as a vaccine vector whilst not compromising its safety profile.

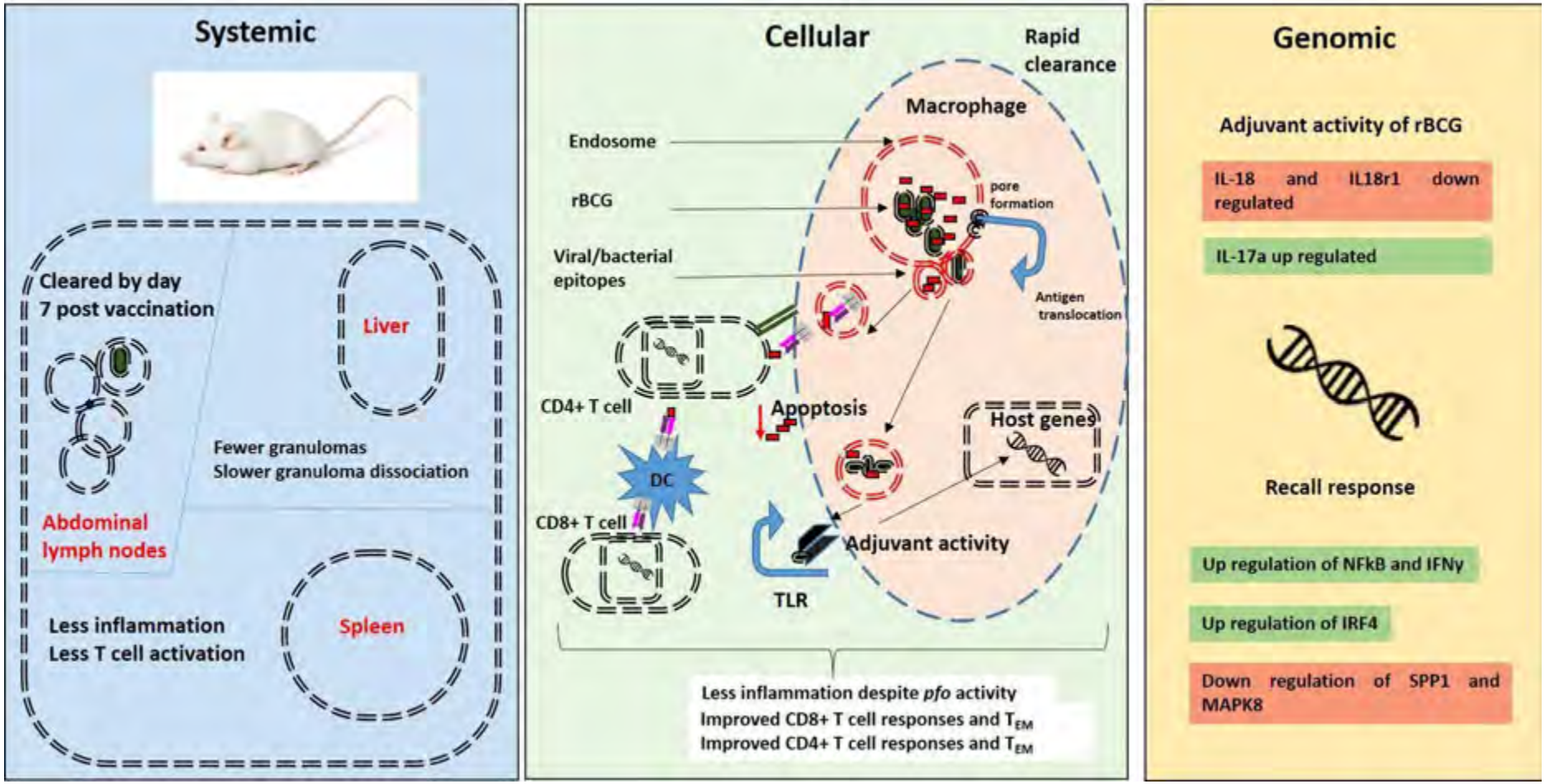


Figure 5.3: Systemic, cellular and genomic characteristics of priming with rBCG Δ*panCDpfo* (*gag*). At the systemic level, mice primed with this vaccine showed persistent rBCG in lymph nodes at experimental endpoint. CD3 staining of liver sections revealed moderately less T cell infiltration and granuloma formation than rBCG WT (*gag*). At the cellular level we assessed, the induction of HIV-1 specific T cells following SAAVI MVA-C boost and subsequent *ex vivo* peptide stimulation. rBCG *ΔpanCDpfo* (*gag*) primed significantly for a boost of T cell responses and was associated with improved CD4+ and CD8+ T cell effector memory compared to rBCG WT (*gag*). At the genomic level, persistent up regulation of inflammatory mediators NFkB and IFNγ were observed.

Appendix 1

Formulations of rBCG buffering and growth solutions

1. BCG resuspension buffer:

8.5% w/v NaCl, 10%glycerol, 10% Tyloxapol

2. MB-7H9 media:

4.7 g MB-7H9 broth; 100 ml OADC, 0.025% v/v tyloxapol, 0.25% w/v glycerol). Made up to 1litre of sterile H_2O

3. MB-7H10 agar:

19 g MB-7H9 agar; 100 ml OADC; 0.63% w/v glycerol. Made up to 1 litre of sterile H_2O

Appendix 2

Formulations of murine splenocyte suspension solutions for immunology

1. R10 complete media

RPMI medium 1640, 10% fetal bovine serum (FBS), 100 U/mL penicillin, 100 µg/mL streptomycin

2. Blocking solution for antibody binding

0.12µl normal mouse serum, 0.12µl normal rat serum, 0.16µg CD16/32

3. FACS buffer

PBS with 1% normal mouse serum

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