An investigation of the effects of helminth worm infection on the capacity of HIV vaccines to boost vaccine-generated immune responses

By

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DECLARATION

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To My Loving Family, My Incredible Mentors and My Perfect Fiancé,

With Love

"***We set sail on this new sea because there is new knowledge to be gained***"

John F. Kennedy, 1962

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List of Abbreviations

ABSL	Animal biosafety level
Ad5	Adenovirus serotype 5
ADCC	Antibody-dependent cell-mediated cytotoxicity
AEC	Animal Ethics Committee
AIDS	Acquired immune deficiency syndrome
APC	Antigen-presenting cell
ART	Antiretroviral treatment
BALB	Bagg ALBino
BCG	Bacille Calmette Guérin
BFA	Brefaldin A
CBA	Cytometric bead array
CD	Cluster of differentiation
CO_2	Carbon dioxide
ConA	Concanavalin A
CTL	Cytotoxic T lymphocyte
DNA	Deoxyribonucleic acid
ELISA	Enzyme-linked immunosorbent assay
ELISPOT	Enzyme-linked immunospot
Env	Envelope
FACS	Fluorescence-activated cell-sorting
FPV	Fowlpoxvirus
Gag	Group specific antigen
GDP	Gross domestic product
GMP	Good manufacturing practices
gp	Glycoprotein
HA	Hemagglutinin
HAART	Highly active antiretroviral therapy
HIV	Human immunodeficiency virus
HLA	Human leukocyte antigen
ICS	Intracellular cytokine staining
IFN-γ	Interferon gamma

Ig	Immunoglobulin
IL	Interleukin
i.m.	Intramuscularly
i.p.	Intraperitoneal
LF	Lymphatic filariasis
MFI	Median fluorescence intensity
MHC	Major histocompatibility complex
MPER	Membrane-proximal external region
MVA	Modified vaccinia Ankara
Nef	Negative regulatory factor
OD	Optical density
PBMC	Peripheral-blood mononuclear cells
PBS	Phosphate-buffered saline
PE	Phycoerythrin
pfu	Plaque forming units
PMTCT	Prevention of mother-to-child transmission
Pol	Polymerase
PrEP	Pre-exposure prophylaxis
PVDF	Polyvinylidene difluoride
RAF	Research animal facility
Rev	Regulator of virion expression
RNA	Ribonucleic acid
RPMI	Roswell Park Memorial Institute media
RT	Reverse transcriptase
rVV	Recombinant vaccinia virus
SEA	Soluble egg antigen
sfu	Spot forming units
SIV	Simian immunodeficiency virus
SSA	Sub-Saharan Africa
Tat	Trans-activator of transcription
TB	Tuberculosis
TCM	T cell central memory
TEM	T cell effector memory

Th1/2	T helper cell type 1/type 2
TMB	Tetramethyl-benzidine
TNF	Tumour necrosis factor
TT	Tetanus toxoid
UNAIDS	The Joint United Nations Programme on HIV/AIDS
U.S.	United States of America
V1V2	First and second variable domains
Vif	Viral protein regulatory
VMMC	Voluntary medical male circumcision
Vpr	Viral protein regulatory
Vpu	Viral protein unknown
WHO	World Health Organisation

Abstract

To protect against sexual transmission, successful future HIV vaccines will likely be given to adolescents as a booster subsequent to primary immunization during infancy. In sub-Saharan Africa (SSA), a large proportion of children are chronically infected with a variety of helminths. These infections may suppress the ability of a host to elicit vaccine-induced Th1 responses that are considered important for a successful HIV vaccine. This study investigated the effect of chronic helminthic infection on the boosting capacity of a poxvirus-protein HIV vaccine regimen (SAAVI MVA-C and Env gp140 protein) in a mouse model.

Groups of mice were prime-vaccinated with SAAVI MVA-C through an intramuscular injection, and Env gp140 protein formulated in Alum adjuvant which was administered via an intraperitoneal injection. These vaccinations were given concurrently, two weeks prior to infection with *Schistosoma mansoni* (Sm) through a percutaneous route. Control mice were either left uninfected (Naïve) or infected in the same manner (Sm) without vaccination. A booster vaccination was given eight weeks post helminth infection. HIV-specific immune responses were analysed in the blood and spleens two weeks after booster vaccination.

The magnitudes of cumulative IFN-γ ELISPOT responses to HIV Gag, RT and Env peptides were significantly ($p<0.05$) lower in the vaccinated and helminth-infected (Vaccine+Sm) mice (948 sfu/10^6) than vaccinated and uninfected (Vaccine) mice (1733 sfu/10^6), with IFN-γ responses to RT (CD8) being the most dominant for both mouse groups (Vaccine+Sm: 734 ± 221 sfu/10^6, Vaccine: 521 ± 116 sfu/10^6). No significant difference was observed in the magnitudes of cumulative IL-2 ELISPOT responses to the vaccine peptides between the Vaccine+Sm and Vaccine groups, however IL-2-producing T cell responses to Env (CD4) dominated in both mouse groups. Vaccine+Sm and Sm groups had similar IFN-γ- and IL-2-producing T cell responses to SEA. Splenocytes from Vaccine+Sm mice secreted less Th1

(IFN-γ, IL-2, TNF-α) and Th2 (IL-4, IL-6, IL-10) cytokines than those from uninfected vaccinated mice in response to HIV vaccine peptides. The total number of activated CD4+ T cells responding to vaccine peptides was greater for Vaccine+ Sm mice than Vaccine mice ($p<0.05$), however, no such statistical significance was observed in the differences seen between these vaccinated mouse groups for the number of activated CD8+ T cells. The frequencies of central memory activated CD4+ T cells were seen to be greater in Vaccine group (Gag; 34.28 ± 8.35%, Pol; 33.53 ± 6.34%, Env (CD4); 33.92 ± 3.87%, Env (CD8); 38.76 ± 10.52%) as opposed to the Vaccine+Sm group (Gag; 28.09 ± 3.95%, Pol; 26.45 ± 4.66%, Env (CD4); 28.79 ± 6.95%, Env (CD8); 28.65 ± 3.29%). Furthermore, Vaccine+Sm mice had higher titres of HIV-1 gp140-specific IgG1 antibodies ($p<0.0001$) (a Th2 antibody marker) but significantly less gp140-specific IgG2a ($p<0.0001$) and IgG2b ($p<0.001$) (Th1 antibody markers) antibodies. This trend was also observed with total non-Env-specific antibody titres.

This study demonstrates that chronic helminthic infection is associated with an attenuated boosting capacity of a poxvirus-protein HIV vaccine in a mouse model, suppressing both T cell cytokine production and Th1-type antibody responses. Since HIV vaccine-induced Th1 responses are considered important for a successful HIV vaccine, these data suggest that chronic helminthiasis may impact negatively on future HIV vaccination outcomes in adolescents living in SSA where helminthic parasites are endemic.

1. Literature Review

1.1 HIV Virology

Human immunodeficiency virus (HIV) is a member of the *Retroviridae* family, in the Lentivirus genus, together with the simian, feline and bovine immunodeficiency viruses (Nye & Parkin, 2003). These enveloped viruses all cause characteristically slow and steadily progressive infections and are transmitted by sexual contact, injection of infected blood or blood products, and from mother-to-child during delivery or breastfeeding (CDC, 2009). The HIV genome consists of two identical copies of a single-stranded, positive sense RNA of 9.2 kb in size (Feinberg & Greene, 1992). As shown in Figure 1.1, it encodes for typical retrovirus proteins such as Gag, which is cleaved into matrix, capsid and nucleocapsid proteins, Pol, which is also cleaved further into protease, reverse transcriptase and integrase, and Env, a glycoprotein, again, cleaved into gp120 external subunit and a gp41 transmembrane subunit that, together, form trimeric prongs on the surface of the virion (Feinberg & Greene, 1992). In addition to Gag, Pol and Env, the RNA of HIV also encodes for regulatory proteins Tat and Rev, as well as accessory proteins Nef, Vif, Vpr and Vpu (Girard *et al.*, 2011).

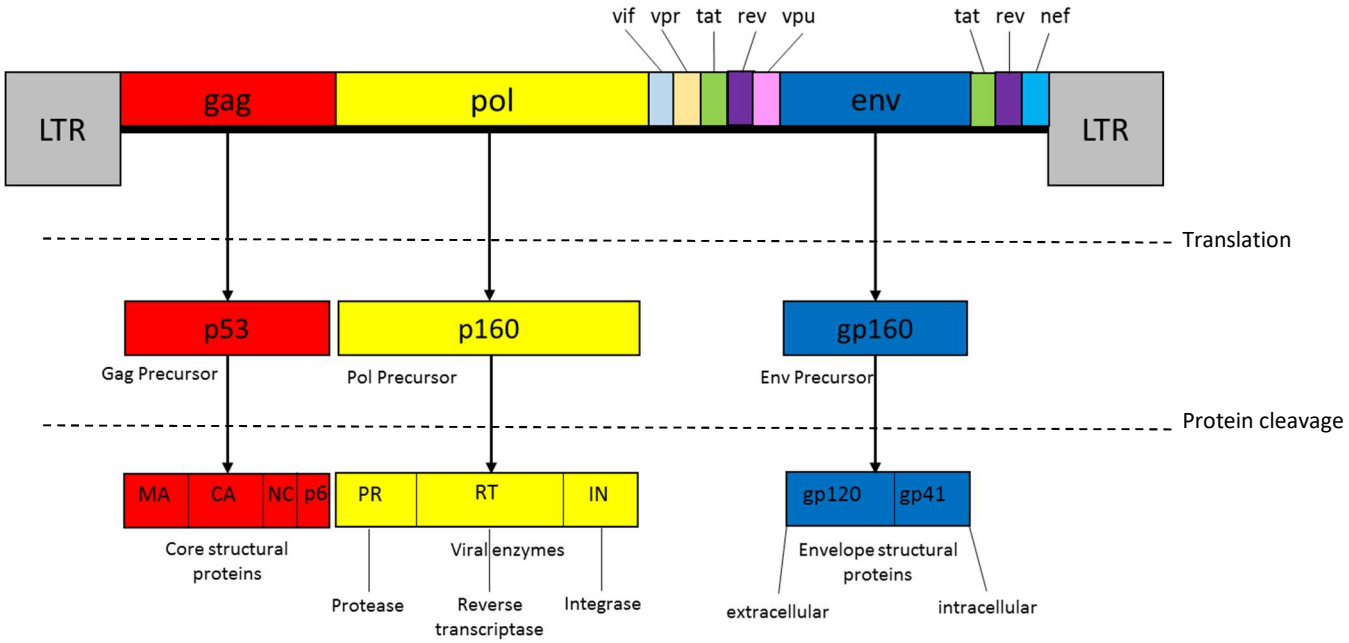

Figure 1.1 - The genome structure of HIV with protein precursors and final products. The *gag* gene is translated into the structural Gag precursor (p53) that is cleaved into matrix (MA), capsid (CA), nucleocapsid (NC) and p6. The *pol* gene is translated as a Pol precursor (p160) that is further cleaved into enzymes reverse transcriptase (RT), integrase (IN) and protease (PR). The *env* gene encodes for the structural precursor Env (gp160) which is cleaved into the extracellular (gp120) and intracellular (gp41) glycoproteins (adapted from Li & De Clercq, 2016).

1.2 HIV Epidemiology

1.2.1 Global epidemiology

Since its discovery in the early 1980s, HIV has infected over 60 million people, with the resultant acquired immunodeficiency syndrome (AIDS) developing into one of the leading causes of death worldwide, with an estimated 2 million deaths per year (Barré-Sinoussi *et al.*, 1983; Girard *et al.*, 2011; Koff *et al.*, 2013). It is estimated that 36.7 million people are currently living with HIV globally (UNAIDS, 2016a).

There are two types that affect humans; HIV-1 and the less virulent HIV-2 (Barré-Sinoussi *et al.*, 1983; Clavel *et al.*, 1986). It is hypothesised that HIV-1 in humans originated from cross-species transmission from chimpanzees, and HIV-2 from sooty mangabeys (Hahn *et al.*, 2000; Lemey *et al.*, 2003; Sharp, Shaw & Hahn, 2005). Groups of HIV-1 is further divided into 4

groups defined as M (major), O (outlier), N (non-M, non-O) and P (Charneau *et al.*, 1994; Simon *et al.*, 1998; Plantier *et al.*, 2009). Group M viruses are responsible for the majority of the world's infections and, within this group, there are several subtypes or clades (named A to K) that exist and dominate different regions of the globe (McCutchan, 2000; Korber *et al.*, 2001). Subtype B dominates in the Americas, Europe and Australia whereas subtype C is spread throughout southern Africa and India and subtype D strains are found in East and West Africa (McCutchan, 2006).

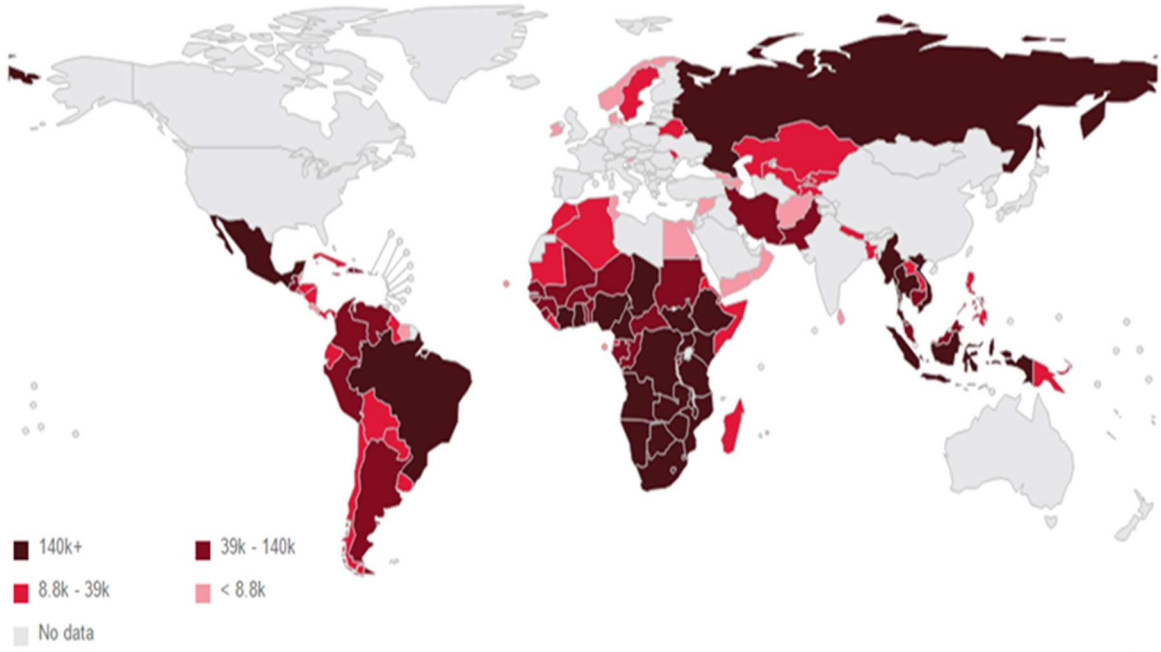

Figure 1.2 - Adults and children estimated to be living with HIV (Source: UNAIDS, 2014)

1.2.2 HIV in sub-Saharan Africa and South Africa

It is estimated that, of the 36.9 million people currently living with HIV globally, sub-Saharan Africa (SSA) accounts for estimated 25.8 million infected individuals as shown in Figure 1.2 (UNAIDS, 2015). With an estimated 1.4 million new HIV infections in sub-Saharan Africa in the last year, with 190 000 of those being new infections in children, while a decrease from previous years (1.5 million new HIV infections and 210 000 new infections among children in

2013), this epidemic is far from abating and remains a large concern (UNAIDS, 2015). Sub-Saharan Africa is estimated to account for 75% of the world's HIV-infected women and over 80% of the world's HIV-infected children and AIDS orphans. (Nye & Parkin, 2003). South Africa has the highest number of infected people, with an estimated 7 million people living with HIV by the end of 2015, which is an increase from 6.8 million in 2014 and 6.4 million in 2011 (UNAIDS, 2016a). Prevalence rates in South Africa in 2015 were 19.2%, only lower than Botswana (22.2%) (UNAIDS, 2016a). However, it was reported that prevalence varies between the provinces, ranging from 27.9% in KwaZulu Natal to 7.8% in the Western Cape in 15-49 year olds in 2012 (Shisana *et al.*, 2014).

1.2.3 HIV/AIDS as an economic burden in sub-Saharan Africa

The impact of this epidemic in Africa is evident in the increased mortality rates and decreased life expectancy. This has been demonstrated in rural parts of Kenya where 25% of adults were infected with HIV and the resulting life expectancy was 38 years (Buve, Bishikwabo-Nsarhaza & Mutangandura, 2002). In South Africa, UNAIDS has stated that the annual number of AIDS-related deaths was about 180,000 in 2015 (UNAIDS, 2016a). The U.S. Census Bureau has sited South Africa as the country with the lowest life expectancy at birth of 49.7 years in 2015 and cited HIV/AIDS-related deaths as a factor (He, Goodkind & Kowal, 2016). An indirect effect of high mortality rates would be an increase in orphans and thus increased family poverty and socioeconomic losses to the community (Buve, Bishikwabo-Nsarhaza & Mutangandura, 2002). South Africa alone is reported to have 2.1 million orphans between the ages of 0 and 17 due to AIDS as of 2015 (UNAIDS, 2016a).

A major strategy for managing HIV and the related mortality rates is the utilisation and distribution of highly active antiretroviral therapy (HAART). These medicinal interventions in the form of antiretroviral treatments (ART) come in various drug compositions and

combinations. These therapy regimens have been shown to significantly reduce the rates of morbidity and mortality associated with HIV/AIDS (UNAIDS, 2013a). However, HAART is costly and necessitates life-long adherence as well as regular hospital and doctor check-ups. Thus, HIV positive individuals would consume a high amount of resources for the duration of their lifetimes to utilise these life-saving therapies. An individual from a low-income household may have to depend on government or charitable assistance, although countries with the highest HIV positive populace are generally third world countries with a low GDP and resource constrained governments which may not be able to afford to provide for all the people who need these therapies (UNAIDS, 2016b). About 46% of all adults living with HIV, and 49% of all HIV positive children were able to get treatment in 2015. Thus more than half of HIV positive individuals did not receive treatment (UNAIDS, 2016b).

The UNAIDS (2016b) estimates that funding needed by low- and middle-income countries will need to increase from $19 billion in 2015 to $26.2 billion in 2020 in order to curb the epidemic effectively. As sub-Saharan Africa is the region with the highest HIV burden, it accounts for the largest proportion of global HIV spending; 47% in 2012 (UNAIDS, 2013b). More developed countries have increased their funding and support for HIV and AIDS prevention strategies and research in sub-Saharan Africa recently, with most donations going through the Global Fund. By way of this fund, 2.3 million people in sub-Saharan received ART in 2010. Also, the Global Fund finances all of the ART programmes in numerous countries in sub-Saharan Africa including Ethiopia, Ghana, Guinea, Malawi, Namibia and Tanzania (Global Fund, 2011). As such, a total of 80% of finances for HIV programmes in sub-Saharan Africa was from donor funding (International AIDS Society, 2010). However, South Africa, the country with the most people living with HIV, funds most of its own HIV response and programs and only relies on 20% of its HIV funding from international funding opportunities (South African National AIDS Council, 2015). Based on South Africa's National Strategic

Plan 2012-2016 targets, the discrepancy between funding requirements and available funding for HIV is expected to increase (South African National AIDS Council, 2015). Also, in recent years, South Africa has been working to negotiate lower prices for ART, as it has been paying more than most other low and middle income countries regardless of having the world's largest procurement programme (South African National AIDS Council, 2015). Through a more competitive bidding process, South Africa managed to reduce the cost of buying ART to the lowest price anywhere in the world in 2013, a 53% reduction in spending on ART (UNAIDS, 2013c).

While funding is important for a country's HIV treatment and prevention programmes to succeed, the health and education infrastructure to be developed sufficiently in order for implementation of any large-scale program to thrive. Many countries in sub-Saharan Africa have come under increasing economic pressure as the epidemic has evolved, worsened further by the shortage of trained healthcare professionals in the region (World Health Organisation (WHO), 2007).

1.3 Development of HIV-1 Vaccines

1.3.1 The necessity for an HIV vaccine

A number of countries in sub-Saharan Africa have conducted large-scale prevention programmes in an effort to contain and reduce their HIV epidemics. One of the cost-effective strategies implemented to prevent the spread of HIV is the promotion of condom use. While UNAIDS (2016) estimates that, since 1990 to present day, 45 million HIV infections around the world have been averted through condom use, in sub-Saharan Africa, the degree of condom use remains low (Maticka-Tyndale, 2012). Another strategy has been to increase prevention of mother-to-child transmission (PMTCT) by ART (UNAIDS, 2014). But there is evidence that progress is slowing in the scaling up of ART for pregnant women; 37,000 additional

pregnant women were reached by PMTCT programmes in 2013 compared with 97,000 in previous years (UNAIDS, 2014). In many sub-Saharan countries, such as Chad, Lesotho, Uganda and Zimbabwe, there has been a decrease in number of pregnant women receiving ART. Similarly for promotion of voluntary medical male circumcision, following years of rapid increase, within 8 of the 14 sub-Saharan countries surveyed, circumcisions performed declined in 2015; from 3.2 million in 2014 to2.6 million in 2015 (UNAIDS, 2016a). Harm reduction and antiretroviral treatment (ART) have also had limited success (UNAIDS, 2016a,b).

As such, globally, the number of deaths associated with HIV infections have decreased in recent years due to the varying degrees of success of the numerous aforementioned prevention and treatment strategies, (UNAIDS, 2014). However, the unrelenting spread of the viral infections continues to surmount these efforts, especially in SSA (Girard *et al.*, 2011). South Africa alone had 380 000 new infections in 2015 (UNAIDS, 2017). In the past, vaccines have been our best weapon against the world's deadliest infectious diseases, for example smallpox, polio, measles, and yellow fever (Siegrist, 2008a). Therefore, candidate HIV vaccines have been designed with the aim of developing an effective, durable and affordable method for combating the HIV epidemic (Cohen & Dolin, 2013).

1.3.2 Vaccine strategies

Current strategies to develop novel vaccines can be divided into traditional and novel strategies. Traditional strategies include live attenuated viruses, whole killed viruses and protein subunits. Novel strategies include recombinant vaccines, DNA vaccines and novel methods of presenting vaccine antigens.

Live attenuated virus vaccines have proved successful in the yellow fever, mumps and measles vaccines (Minor, 2015). Non-human primate challenged with SIV represent an animal model

to test various vaccine strategies and to inform the design of HIV vaccine strategies (Koff *et al.*, 2006; Silvestri *et al.*, 2007; Morgan *et al.*, 2008). Attenuated SIV vaccines have shown some degree of success against SIV challenges in rhesus macaques (Daniel *et al.*, 1992; Wyand *et al.*, 1996; Siegrist, 2008b). Nevertheless, it is generally accepted that an attentuated version of HIV will not get to human trials due to safety concerns (Baba *et al.*, 1999). Whole killed virus has shown some promise, and was demonstrated to be capable of protecting 8 out of 9 rhesus macaques from SIV challenges in one study, but unable to produce high titres of broadly neutralising antibodies (Murphey-Corb *et al.*, 1989). Protein subunits have also been tested in various studies and showed no significant protection nor the ability of the vaccines to elicit observable protective humoral nor cellular immunity (Flynn *et al.*, 2005; Pitisuttithum *et al.*, 2006).

Live recombinant, or vectored, vaccines are designed to transport HIV antigens such as Gag, Pol, Env, or Nef within a live viral/bacterial vector. These antigens are then expressed in the cytoplasm of infected cells in order to be presented on the MHC class I molecules to prime celullar T cell responses.

1.3.3 Challenges in developing HIV Vaccines

An ideal HIV vaccine is considered to be capable of both induction of potent T cell mediated immune responses as well as a durable humoral immune response through antibody production (Johnston & Fauci, 2007, 2011; Fauci *et al.*, 2008; Ross *et al.*, 2010; Chhatbar *et al.*, 2011; Cohen & Dolin, 2013; Haynes & McElrath, 2013; McMichael & Koff, 2014). Cohen and Dolin (2013) postulate that a successful HIV vaccine would stimulate protective immunity similar to that induced during a natural infection but acknowledge that naturally acquired immunity might not exist. Therefore this would suggest that the mechanisms of an effective vaccine might need to induce an immune response completely different from that observed in natural infection (Johnston & Fauci, 2011).

Barouch (2008) holds that the goal of an HIV-1 vaccine would be to completely block and prevent infection or, alternatively, to reduce viral loads and slow clinical disease progression after infection has taken place. However, the task has proven challenging for numerous reasons, possibly the greatest being the extensive sequence and clade diversity of HIV-1 (Gaschen *et al.*, 2002). Other challenges include the early establishment of a latent viral reservoirs, the lack of understanding and observance of immune correlates, the evasion of humoral and cellular immune responses by the virus, antibody responses being fairly type specific, the inability of current techniques to elicit broadly reactive neutralising antibodies, the safety concerns surrounding attenuated viruses in humans as well as the declining pharmaceutical interest (Barouch, 2008).

Most successful viral vaccines that have been developed are against viruses that undergo some form of initial replication and distribution from the point of entry before the virus reaches its target organ (Johnston & Fauci, 2007). During this dispersal period, the virus is exposed to eradication by the immune system and, when prior immunization or exposure to the virus has provided virus-specific immunologic memory, the increased speed and intensity of the immune response can prevent disease (Johnston & Fauci, 2007). This is not unlike the initial stage of HIV infection. The HIV viremia burst is detected around day 7 of infection and peaks around 3 weeks after exposure. Increased levels of HIV-specific CD8+ and CD4+ T cells stand as the first indications of HIV-specific immune responses (Johnston & Fauci, 2007). A pool of latently infected, resting CD4+ T cells is established early in infection but the primary CD8+ T cell response seems to result in a decline of viral load, by a factor of 10 to 100, until a stable plateau or "set point" 2-6 months after infection (Koup *et al.*, 1994). Meanwhile, binding antibodies emerge 6-12 weeks after exposure but neutralizing antibodies only appear closer to the point of equilibrium (Johnston & Fauci, 2007). The effectiveness of the antibody responses are further retarded by the swift genetic changes in the viral envelope protein that allow the

virus to escape antibody recognition (Richman *et al.*, 2003). The CD8+ T cells continue to suppress the virus throughout, and the specificity of this T cell response evolves in response to changes in the envelope protein sequence (Koup *et al.*, 1994). However, HIV infection is established indefinitely in all patients and is regarded as relently progressive albeit a small fraction of cells are infected at any point in time and the HIV reservoir established early on is not eradicated (Chun *et al.*, 1998). Thus, the HIV window of vulnerability is only as long as it takes for the virus to infect a pool of latently infected cells, a strategy that almost all other viral infections do not utilize. As such, this permanent reservoire of the virus is a perceived obstacle of a successful vaccination.

Humoral Immunity

Various studies have shown the importance of broadly neutralising antibodies in protecting subjects from HIV infection (Baba *et al.*, 2000; Mascola *et al.*, 2000; Burton *et al.*, 2004; Barouch *et al.*, 2013; Buchbinder *et al.*, 2014; Goepfert *et al.*, 2014). As such, early efforts to develop an effective vaccine focused on the same strategy deployed in the vaccine against hepatitis B, whereby recombinant forms of the viral envelope were genetically engineered in order to target the production of neutralising antibodies in hopes that the antibodies would recognise HIV virons by their envelope proteins and clear them before infection became established (Johnston & Fauci, 2007). Soluble forms of gp120, all or pieces of the uncleavded gp160 precursor protein were tested for safety and immunogenicity in more than 20 phase 1 clinical trials (Spearman, 2006; Hoffenberg *et al.*, 2013). These subprotein vaccines failed to protect high risk populations (high risk women and MSM in North America and the Netherlands, and injection drug users in Thailand) from infection in two phase 3 trials (Flynn *et al.*, 2005; Pitisuttithum *et al.*, 2006). A possible explanation as to why induction of antibodies able to neutralise primary isolates was unsuccessful by gp120 revolves around the fact that the envelope on the virion exists as a trimer and the conserved, immunogenic regions

of the monomer are blocked in the native trimer (Sanders *et al.*, in press; Kwong *et al.*, 1998; Wyatt *et al.*, 1998; Merk & Subramaniam, 2013). The envelope peptide is covered with numerous N-linked glycans and it undergoes conformational changes when it binds to the cell-surface CD4 receptor (Sanders *et al.*, in press; Chen *et al.*, 2005; Merk & Subramaniam, 2013). These conformational changes then expose sequences that are highly variable, as mentioned before.

Recent studies have been conducted with the aim to induce neutralising antibodies based on the mutational changes of the virus (Sanders *et al.*, in press; Li *et al.*, 2007; Doria-Rose *et al.*, 2014). In a small group of HIV-1 infected subjects, the activity of broadly neutralising antibodies has been identified and shown to be directed against conserved regions of the Env glycoprotein (Li *et al.*, 2007). One such conserved region is membrane-proximal external region (MPER) on the gp41 protein (Haynes *et al.*, 2005). This region is the target of 2F5 and 4E10, MPER-specific broadly reactive monoclonal antibodies. These are difficult to elicit via vaccination due to immunoregulation and tolerance control, the acquisition of the epitope in the membrane, and transient exposure of the epitope during viral entry (Haynes *et al.*, 2005; Frey *et al.*, 2008; Sun *et al.*, 2008).

The Thai RV144 trial demonstrated vaccine-induced IgG antibody activity against the first and second variable domains (V1V2) of Env was associated with lower risk of HIV infection but IgA against Env was directly linked to greater risk of infection (Haynes *et al.*, 2012; Rolland *et al.*, 2012; Tomaras *et al.*, 2013; Tomaras & Plotkin, 2017). While V1V2 antibodies are not broadly neutralizing, they appear to mediate antibody-dependent cell-mediated cytotoxicity (ADCC) and when Env-specific IgA levels are low, these V1V2 antibodies were found to be inversely correlated with the risk of infection (Pollara *et al.*, 2011; Bonsignori *et al.*, 2012; Montefiori *et al.*, 2012; Liao *et al.*, 2013; Tomaras & Plotkin, 2017). Studies have

demonstrated that ADCC activity plays a role in SIV control and protection in nonhuman primate models (Gómez-Román *et al.*, 2005; Barouch *et al.*, 2012; Smalls-Mantey *et al.*, 2012). The importance of a protein immunogen, such as Env, in eliciting an ADCC response has been shown previously, whereby ADCC was identified in the sera of rhesus macaques after immunization with an Env protein (Chege *et al.*, 2017)

Cellular Immunity

CD8+ cytotoxic T lymphocytes are the key effectors of cellular immunity; these cells recognize the viral peptides presented by the major histocompatibility complex (MHC) on virally-infected cells (Janeway *et al.*, 1997). These CTLs proliferate in the blood in order to contain the viremia during natural infection with HIV-1, but they are unable to eliminate viremia (Hansen *et al.*, 2011, 2013; McMichael & Koff, 2014). This is due to the fact that vaccine-elicited T cells would not emerge and accumulate fast enough in the early stages of infection (Mattapallil *et al.*, 2006). The disease is accelerated and viremia increases without a plateau stage in monkeys depleted of CD8+ cells, but when CD8+ cells were reintroduced, the viremia was suppressed (Schmitz *et al.*, 1999). However, a study in SIV-challenged nonhuman primates has shown that some T cell vaccines may have the potential to provide protection or enable the immune system to clear an estabilished viral infection (Hansen *et al.*, 2013).

Another foreseeable obstacle is the high probability that the candidate HIV vaccine sequences are not correlated with that of the infecting. HIV-1 viral sequences can vary up to 20% and a 10% variance in viral sequence from the sequence used to prime the CTLs has a 30–50% chance that these lymphocytes will not recognise the virus (McMichael & Koff, 2014).

1.3.4 Vaccine-induced Th1 immunity

As demonstrated, effective HIV vaccine-induced immune responses would need to 1) include functional antibodies that are produced by B lymphocytes and are able to specifically bind to

HIV proteins, and 2) generate CD8+ T lymphocytes that would restrict the spread of HIV infection by being able to recognise and kill infected CD4+ T cells early on in the infection cycle, as well as secreting antiviral cytokines (Walker, Ahmed & Plotkin, 2011). However, both the generation of these responses is, in part, mediated by growth factors and signals given by CD4+ T helper (Th) cells (Bacchetta, Gregori & Roncarolo, 2005). These helper lymphocytes are generally divided into T helper 1 (Th1) and T helper 2 (Th2) types, effectors that are controlled by regulatory T cells (Treg) to maintain and differentiate immunity (Bacchetta, Gregori & Roncarolo, 2005).

T helper 1 (Th1) cells produce IFN-γ, TNF-α, IL-2, and IL-3 all the while supporting activation and differentiation of B cells, CD8+ T cells and macrophages. Vaccines able to elicit robust cell-mediated immunity are thought be the most promising for control of infections by many intracellular viruses, including HIV (Hansen *et al.*, 2011).

1.3.5 Vaccine trials

Vaccines undergo three phases of trials; phase 1 and 2 focus on vaccine safety and immunogenicity, and phase 3 delves into the efficacy of the vaccine. In the last two decades, four HIV vaccine efficacy trials (shown in Figure 1.3) have provided invaluable information about host immune protection desired and required.

<u>VaxGen</u>

The first two phase 3 clinical trials of a VaxGen Corp. HIV vaccine were undertaken in 1998. This vaccine was a gp120 subunit immunogen in an alum adjuvant suspension. In the phase 1 and 2 trials, it was found to generate only limited levels of neutralizing antibodies. In the phase 3 clinical trials, the vaccine had no protective efficacy in the men who have sex with men (MSM) population, nor in the injecting drug user population (Gilbert *et al.*, 2005; The rgp120 HIV Vaccine Study Group, 2005; Pitisuttithum *et al.*, 2006).

The STEP Trial

The STEP trial, initiated in 2005, was the next HIV vaccine trial to evaluate a vaccine engineered to activate a T cell-mediated immunity. As expected, this immunogen, in a recominant adenovirus (Ad5) vector, induced strong T cell responses to HIV proteins as determined by ELISPOT and flow cytometry assays but failed to reduce acquisition or long-term control of post-infection viremia (Buchbinder *et al.*, 2008). It was later shown in a study that human leukocyte antigen (HLA) alleles that were common in the STEP study cohort restricted the Gag amino acid 84 site that incorporated in several epitopes in the vaccine (Rolland *et al.*, 2011). The study subjects that had this specific HLA type, as well as those that had a robust CD8+ T cell response to Gag and Nef HIV epitopes were able to elicit an immune response against the virus *in vivo*. This study also demonstrated that subjects that were both uncircumsized and immune to Ad5 had an increased risk of HIV infection in the first 18 months after receiving the vaccine when compared to the placebo group. However, this risk decreased and equaled that of the placebo group after the 18 month mark. The reasons for this phenomenon remain unknown (Rolland *et al.*, 2011).

The RV144 Trial

The RV144 trial, initiated in 2003, set out to evaluate the protective efficacy of a prime-boost combination vaccine in Thailand. The vaccine is combination of envelope protein subtype B and E and a canary poxvirus vector ALVAC-HIV expressing HIV-1 subtype B protease and Gag, and a fusion subtype B and E envelope. When the results were reported in 2009, the trial demonstrated a 31.2% reduction in the acquisition of HIV infections in the heterosexual male and female populations that received the vaccine regimen as opposed to the placebo group after 3.5 years (Rerks-Ngarm *et al.*, 2009). Thus, this trial was proof that a correctly designed and administered vaccine could have the potential to prevent new HIV-1 infections.

Expanding on this, vaccine recipients from the RV144 trial that were still HIV-1 negative were reimmunized 6–8 years later with AIDSVAX B/E gp120 alone, ALVAC-HIV alone, or a combination of ALVAC-HIV and AIDSVAX B/E gp120 in the RV305 trial. This was found to boost memory B cells. Antibodies elicited were shown to be reactive to the gp140 Env trimer (Easterhoff *et al.*, 2017).

HVTN 505

The HIV Vaccine Trial Network (HVTN) 505 trial commenced participant enrolment in June 2009 and tested another variation of the rAd5 vaccine (Hammer *et al.*, 2013). The regimen consisted of three doses of a plasmid containing non-HIV DNA and certain HIV genes as a prime, followed by one boost of rAd5 expressing HIV Env and viral structural antigens (Hammer *et al.*, 2013). The study restricted enrollment to circumcised MSM who who were screened for Ad5 antibodies prior to the trial (Hammer *et al.*, 2013). This was due to the lack of risk to this demographic as shown by the STEP trial (Duerr *et al.*, 2012). Unfortunately, the HVTN 505 trial was stopped prematurely as it met futility criteria, whereby both the 95% confidence intervals for vaccine efficacy and the difference in viral load were below their respective design limit of reductions of 50% and 1.0 $\log_{10}$ copies per mL, respectively (Hammer *et al.*, 2013).

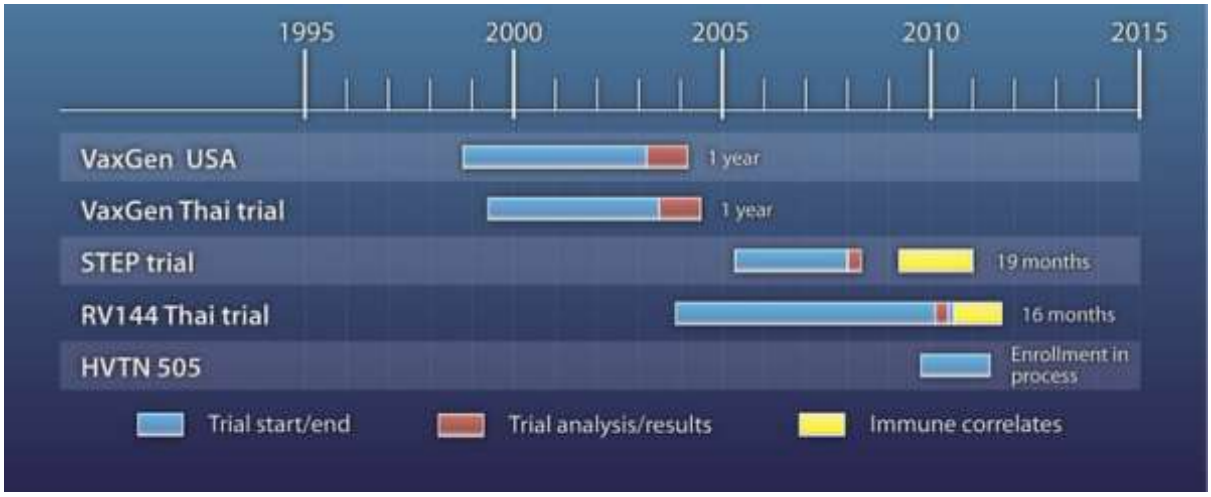

Figure 1.3 - Vaccine trials over the past decade. The past decade has seen 5 highly publicised HIV vaccine trials; VaxGen USA, VaxGen Thai trial, STEP trial, RV144 Thai trial and HVTN 505 trial. These trials have had varying levels of success, with the RV144 trial having the greatest efficacy and thus receiving the most attention from the scientific community (Source: Corey *et al.*, 2011)**.**

Trials in South Africa

In South Africa, there have been 18 trials conducted (table 1.1). Included in these are the clincal trials of two vaccines developed at the University of Cape Town; SAAVI DNA-C2 and SAAVI MVA-C. A phase 1 clinical study (HVTN 073E/SAAVI 102E) was conducted in South Africa and the United States using the SAAVI DNA-C2 and SAAVI MVA-C vaccines in a DNA/MVA prime-boost regimen. An additional boost of a V2-deleted envelope subunit HIV-1C protein vaccine adjuvanted with MF59 was administered to improve HIV Env-specific antibody responses. As well as being judged to be safe and well tolerated, the DNA-MVA regimen elicited CD8+ T cell responses in 32% of the participants and CD4+ T cell responses in 74% of the participants, which was further increased to 87% by the protein boost (Gray *et al.*, 2016). Tier 1 HIV-1C neutralizing antibody responses as well as durable Env binding antibodies were elicited by 100% of the participants (Churchyard *et al.*, 2016; Gray *et al.*, 2016) The SAAVI DNA-C2 vaccine is an equimolar mixture of 2 DNA plasmids, one of which expressed a polyprotein designated Grttn, consisting of translational fusions of HIV-1 subtype C Gag, reverse transcriptase (RT), Tat and Nef. The other plasmid expressed a

truncated envelope protein (Env gp150). The genes included were based on the closeness to a South African HIV consensus sequence and were inserted for optimal expression as well as to safety by restricting some functionality of the virus (Burgers *et al.*, 2006). The other vaccine, SAAVI MVA-C was based on a modified smallpox virus vaccine; modified vaccinia Ankara (MVA) and contained the same HIV genes as SAAVI DNA-C2. Both vaccines have shown to induce robust T cell immune responses in mice, as well as nonhuman primates, when coupled in prime-boost immunizations in pre-clinical testing (Burgers *et al.*, 2006, 2008; Shephard *et al.*, 2008; Chege *et al.*, 2017). A study conducted by Chege and colleagues (2017) showed that a single vaccination with SAAAVI MVA-C concurrently with HIV gp140 Env protein in rhesus macaques elicited both T cell-mediated and Env-specific antibody responses (Chege *et al.*, 2017).

Table 1. 1 Details of HIV vaccine trials conducted in South Africa (Source: International AIDS Vaccine Initiative, 2016)**.**

Phase	Trial ID	Status	Prime	Boost 1	Boost 2	Strategy
I	Extension HVTN 073E/SAAVI 102	Completed	Sub C gp140			Protein
I	HVTN 040	Completed	AVX101			Viral vector-Alphavirus
I	HVTN 050/Merck 018	Terminated	MRKAd5 HIV-1 gag			Viral vector-Adeno
I	HVTN 059	Completed	AVX101			Viral vector-Alphavirus
I	HVTN 073	Completed	SAAVI DNA-C2	SAAVI MVA-C		DNA, viral vector- Pox
I	HVTN 083, SAAVI 103	Ongoing	SAAVI MVA-C	SAAVI DNA-C2	Oligomeric gp140/MF59	Viral vector-Pox, DNA, protein
I	HVTN 097	Ongoing	ALVAC-HIV vCP1521	AIDSVAX B/E		Viral vector-Pox, Protein
I/II	HVTN 100	Ongoing	ALVAC-HIV-C (vCP2438)	Bivalent Subtype C gp120/MF59		Viral vector-Pox, protein
I	HTVN 116	Ongoing	VCR-HIVMAB0 60-00-AB	VRCHIV/MAB08 0-00-AB		Passive immunization, passive immunization
II	HVTN 204	Closed to follow-up	VRC-HIVDNA0 16-00-VP	VRC-HIVADV0 14-00-VP		DNA, viral vector- Adeno
IIb	HVTN 503 (Phambili)	Terminated	MRKAd5 HIV-1 gag/pol/nef			Viral vector-Adeno
IIb	HVTN 702	Scheduled	ALVAC-HIV-C (vCP2438)	Bivalent Subtype C gp120/MF59		Viral vector-Pox Protein
IIb	HVTN 703 AMP	Ongoing	VRC-HIVMAB0 60-00-AB			Passive immunization
I	IAVI 011	Completed	MVA.HIVA			Viral vector-Pox
II	IAVI A002	Completed	TgAAC09			Viral vector-Adeno-associated virus
I	IAVI B003	Completed	Ad26.EnvA-01	Ad35-ENV		Viral vector-Adeno, zviral vector- Adeno
I	IMPAACT P1112	Ongoing	VRC-HIVMAB0 60-00-AB			Passive immunization
I/II	IPCAVD009	Ongoing	Ad26.Mos.HIV Trivalent	Gp140 DP	MVA mosaic	Viral vector-Adeno, protein, viral vector-Pox

1.4 Helminth infections

1.4.1 General background of parasitic helminths

Parasitic helminths are parasitic worms that reside within a host for nourishment and protection, while causing poor nutrient absorption and illness in the host (Pullan & Brooker, 2008). The groups of worms that are clinically relevant are classified by their internal and external morphology as eggs, larvae and adults. There are two major phyla of helminths;

1) Nematodes, or phylum Nematoda (roundworms), which include the major intestinal worms (soil-transmitted helminths, such as hookworms and ascarids) and the filarial worms that cause lymphatic filariasis (LF) and onchocerciasis. Nematodes are dioecious, with cylindrical bodies that have a cuticle after their larval stage, and a complete digestive system throughout their life cycle.
2) Platyhelminths, phylum Platyhelminthes (flatworms), which include the flukes (trematodes), such as the schistosomes, and the tape-worms (cestodes). These worms have a soft body with tegument and an incomplete digestive system but without coelom. Platyhelminths are hermaphroditic except for schistosomes.

1.4.2 Prevalence and global distribution

It is estimated that more than two million people are infected with a helminth globally, and almost half live in developing regions of sub-Saharan Africa, Asia, and the Americas and are often chronically infected with more than one helminth (shown in Figure 1.4) (Elliott *et al.*, 2007; Hotez *et al.*, 2008; Means *et al.*, 2016). The most common human helminth infections are the intestinal helminths, such as ascariasis, trichuriasis, and hook-worm, followed by schistosomiasis and LF (Hotez *et al.*, 2008). This great burden of helminth infection is further compounded by the fact that in sub-Saharan Africa and elsewhere, helminthiases are frequently co-endemic with HIV (Borkow & Bentwich, 2008; Hotez *et al.*, 2008; Secor, 2012; Means *et*

al., 2016). Thus, it is likely that an individual infected with HIV is also infected with one or more parasitic worm and *vis a vis*. Helminths cause an immunomodulatory response in an HIV infected host, which increases the survival rates of the parasite and thus the subsequent establishment of chronic helminth infections (Bethony *et al.*, 2006; Downs & Fitzgerald, 2016; Storey *et al.*, 2017). A prospective study by Kroidl *et al.* (2016) demonstrated a significantly increased risk (almost double) of acquiring HIV for helminth-infected adults and adolescents in rural Tanzania (Downs & Fitzgerald, 2016; Kroidl *et al.*, 2016).

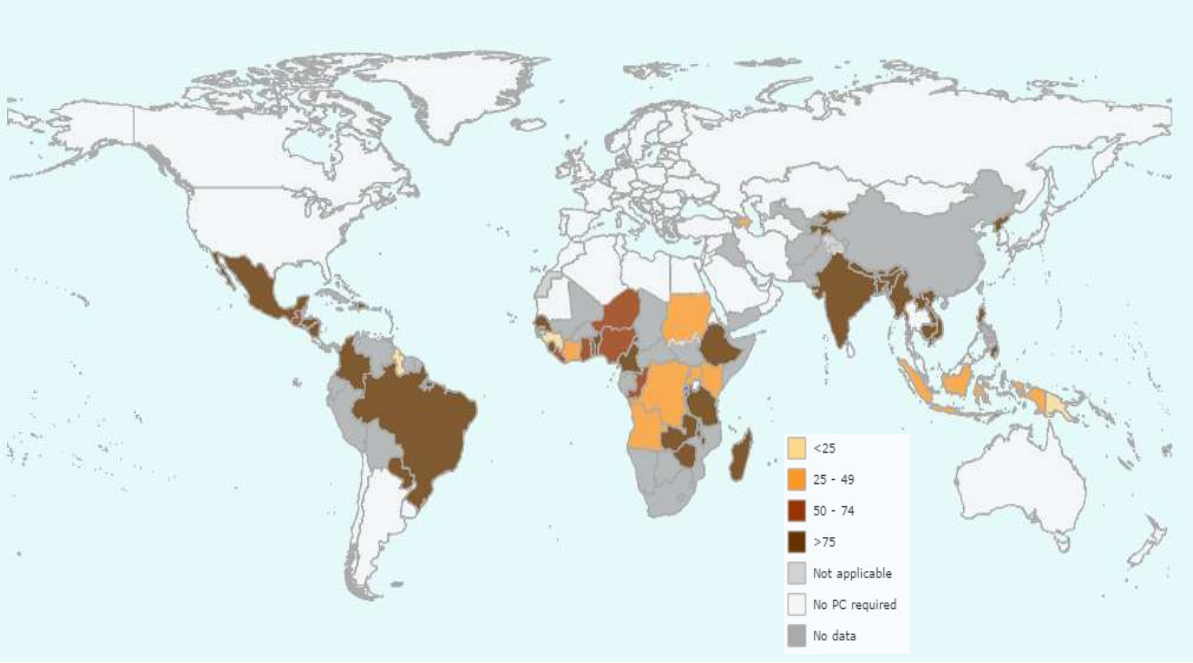

Figure 1.4 - Global prevalence and distribution of helminths in 2015 (Source: WHO, 2016 http://apps.who.int/neglected_diseases/ntddata/sth/sth.html)

More than 85% of infections with schistosomes specifically are in Africa. Schistosomes are predicted to have infected 50% of the population in high-risk rural communities (Bustinduy *et al.*, 2014). Schistosomiasis is conservatively reported to infect more than 230 million people globally, with more than 66.5 million people receiving treatment in 2015 (Colley *et al.*, 2014; WHO, 2017).

1.4.3 Prevalence of helminth infections in children

Children have been found to have the highest prevalence of helminth infections of other age group (Davis *et al.*, 2014; Shumbej *et al.*, 2015). A study conducted by Adedoja and colleagues (2015) assessed the parasitic burden amongst children between the ages of 4 and 15 in rural Nigeria. As shown in table 1.2. they reported that more than 25% of children surveyed within this age group had more than one parasitic infection with *S. haematobium*, a hookworm and/or a tapeworm (Adedoja *et al.*, 2015).

Table 1. 2 Prevalence of single and double parasitic infection among children (Source: Adedoja *et al.*, 2015)

		Prevalence of single infection (%)		
Age Group	**No.**	**SH**	**HW**	**HN**
4-9	445	182 (40.9)	86 (19.3)	45 (10.1)
10-15	572	270 (47.2)	143 (35)	55 (9.6)
Total	1017	452 (44.4)	229 (22.5)	100 (9.8)
p-value		**0.049**	**0.034**	0.83
		Prevalence of double infection (%)		
Age Group	**No.**	**SH + HW**	**SH + HN**	**HW + HN**
4-9	445	60 (13.5)	17 (3.8)	11 (2.5)
10-15	572	104 (18.2)	32 (5.6)	20 (3.5)
Total	1017	164 (16.1)	60 (5.9)	32 (3.1)

SH = *S. haematobium*
HW = Hookworm
HN = *Hymenolepis* sp.

Reflecting this trend, *Schistosoma* has higher reported infection rates in children than in adults (Gryseels *et al.*, 2006). The disease burden amongst children in high risk areas has been historically difficult due to the fact that areas of high prevalence of schistosomiasis transmission are usually where healthcare facilities are inadequate, so surveillance of the disease is mostly defunct and has thus far failed to monitor this age group. Regardless, routine parasitological screens miss some low-level infections as the parasite detection levels have been shown to be too high (Berhe *et al.*, 2004; Wilson *et al.*, 2006). Some studies that have been able to quantify the prevalence and intensity of infections in younger children demonstrate that schistosomiasis, either urinary (caused by *Schistosoma haematobium*) or intestinal (caused

by Schistosoma mansoni), can be common (Bosompem *et al.*, 2004; Odogwu *et al.*, 2006; Verani *et al.*, 2011). In the later study, 90% of children under the age of 10 and 14% under the age of 1 year old surveyed in a rural Kenyan town were infected with *S. mansoni* (Verani *et al.*, 2011).

The age distribution of infection rates and intensity has been attributed to increased frequency of contact with contaminated water among school-aged children and adolescents, whereas older adolescents and adults generally have less contact with the same water and have developed an acquired protective immunity against infection (Verani *et al.*, 2011) The intensity of *S. mansoni* infection in the different age groups is shown in table 1.3.

Table 1. 3 Estimated number of individuals with morbidity or pathology due to *S. mansoni* infection by age group in sub-Saharan Africa in millions (90% confidence interval) (Source: van der Werf *et al.*, 2003)

Category	**Pre-school children**	**School children**	**Adults**	**Total**
At risk of infection	65	152	177	393
Infected	4.7	25	23	54
Diarrhoea in last 2 weeks	0.034 (0.00–0.72)	0.42 (0.0–3.6)	0.32 (0.0–3.5)	0.78 (0.0–7.8)
Blood in stool in last 2 weeks	0.24 (0.16–0.69)	2.3 (1.6–4.1)	1.9 (1.3–3.7)	4.4 (3.0–8.3)
Hepatomegaly (MSL)	0.076	4.0	3.8	8.5
Splenomegaly	[0.61]	[2.9]	[2.8]	[6.3]
Ascitis	[0]	[0]	[0.29]	[0.29]
Haematemesis ever	[0]	[0]	[0.93]	[0.93]

[ ] use with caution, risk for confounding (splenomegaly) or estimations derived from limited literature data (ascitis and haematemesis). Intervals could be calculated for diarrhoea and blood in stool.

1.4.4 Schistosoma mansoni – a prototype parasitic helminth

Infection of humans with helminths of the Schistosoma genus causes schistosomiasis (also termed bilharzia), which displays many characteristics of a classical helminthic worm infection. The main species that cause diseases in humans *are S. haematobium, S. mansoni* and *S. japonicum*. Adult schistosome worms are grey, 7–20 mm long and cylindrical with two terminal suckers, a complex tegument, a blind digestive tract and a reproductive system. However, schistosomes, unlike other trematodes, are dioecious (Gryseels *et al.*, 2006). To reproduce, the longer, thinner female permanently embeds herself within the groove of the

male worm. These schistosomes live within the perivesical (only *S. haematobium*) or mesenteric (all other species) venous plexus. Once there, the females will produce hundreds to thousands of eggs per day. Each ovum contains a ciliated miracidium larva, which helps the eggs to migrate into the lumen of the bladder (only *S. haematobium*) or the intestine (all other species). The eggs are then excreted in the urine (only *S. haematobium*) or faeces (all other species) (Centers for Disease Control, 2012). When the eggs make contact with water, the egg can hatch and rid itself of the miracidium and is then able to infect the intermediate host, freshwater snails such as *Biomphalaria glabrata,* as miracidia (Gryseels *et al.*, 2006). Thereafter, the miracidia replicate asexually into sporocysts and develop into cercarial larvae with embryonic suckers and a bifurcated tail to aid in swimming once the cercariae are shed from the snail host. This shedding process is initiated by light. The released cercariae are then able to penetrate human skin and gain access to circulatory system and eventually end up in the portal vein where they mature into adults and begin the cycle again, as shown by Figure 1.5. The lifespan of an adult worm is around 3–5 years but it can be as long as 30 years (Gryseels *et al.*, 2006).

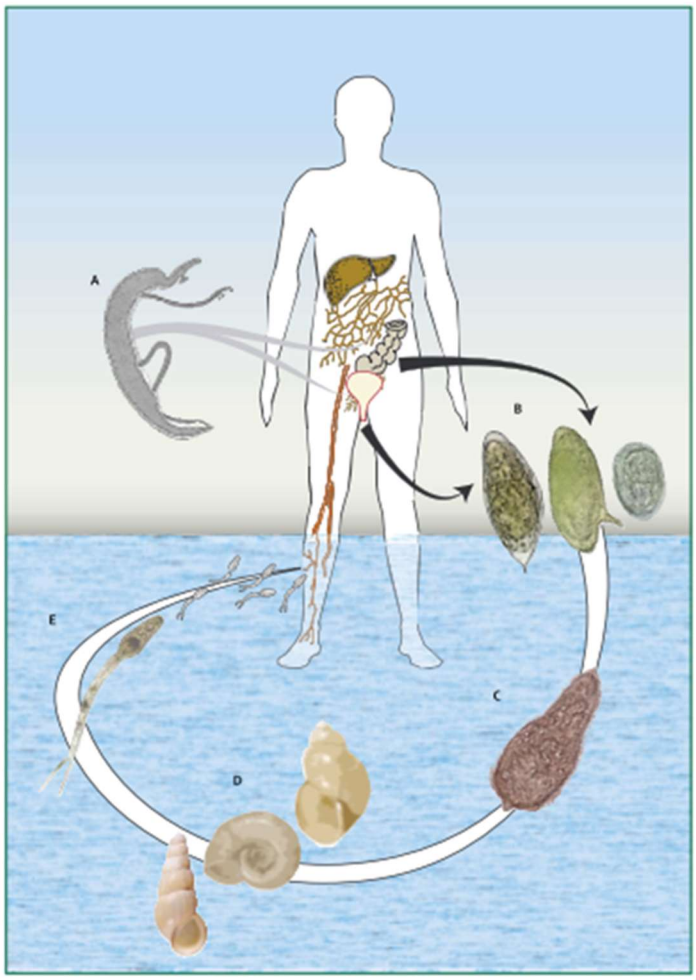

Figure 1.5 – Life cycle of *Schistosoma* species that commonly infect humans; *S. mansoni*, *S. japonicum* and *S. haematobium* A: paired adult worms (sturdy male holding slender female). B: eggs (left to right, *S haematobium, S mansoni*, *S japonicum*). C: ciliated miracidium. D: intermediate host snails (left to right, *Oncomelania, Biomphalaria, Bulinus*). E: cercariae (Source: Colley *et al.*, 2014)**.**

Acute schistosomiasis, or Katayama fever, is a feverish syndrome and a hypersensitivity reaction raised against mobilized schistosome parasites in the blood (Bottieau *et al.*, 2006). Whereas chronic schistosomal disease affects individuals with long-standing infections, and a reaction to the eggs released by the parasite, rather than to the parasite themselves (Gryseels *et al.*, 2006). These eggs get trapped in the liver, spleen, lungs or cerebrospinal system (Gryseels *et al.,* 2006). The resulting immunopathological reactions against schistosome eggs trapped in the tissues lead to inflammatory and obstructive disease in the urinary system (*S. haematobium*) or intestinal disease, hepatosplenic inflammation, and liver fibrosis (*S. mansoni, S. japonicum*).

The cytokine IL-17, secreted by T helper 17 (Th17) cells, has been described to have a role in the production of granulocytes as part of the inflammatory response against *Schistosoma* soluble egg antigen (SEA), the development of liver granulomas as well as affect liver fibrosis (Matsuzaki & Umemura, 2007; Sharma *et al.*, 2014; Nady *et al.*, 2016). Another cytokine secreted by Th17, IL-22, has been reported to protect against liver fibrosis and cirrhosis in chronic infections as well as repress inflammation in autoimmune diseases (Matsuzaki & Umemura, 2007; Wilson *et al.*, 2010; Sertorio *et al.*, 2015). However, the role of IL-22 in a schistosomal infection is unclear (Nakae *et al.*, 2007). The roles of Th17 cytokines on *S. haematobium* infections in humans and *S. mansoni* infections in mice have been investigated in some studies (Mbow *et al.*, 2013; Nady *et al.*, 2016). The immunological role of Th17 cytokines in mice infected with *S. mansoni* has been shown to be mediated by dendritic cells (not basophils, as previously thought) and the expression of CD209a by these cells (Ponichtera *et al.*, 2014; Sharma *et al.*, 2014). Thus, a study by Nady and colleagues (2016) showed that IL-17 can accelerate SEA-induced granuloma formation and as well as attenuate granulocyte functions in *S. mansoni* infected humans, while IL-22 conversely can attenuate formation of granulomas but enhanced granulocyte functions (Nady *et al.*, 2016).

1.4.5 Helminth immunomodulation

Since 1989, T cells have been differentiated into two major subsets, T helper 1 (Th1) and T helper 2 (Th2) cells. Detecting the different subsets can be done by measuring the production of specific cytokines (IFN-γ for Th1 and IL-4 for Th2) (Mosmann & Coffman, 1989; Hirahara & Nakayama, 2016). Signalling the induction of either Th1 or Th2 differentiation is done by IL-12 and IL-4 respectively. The Th1 response has been shown to be imperative for host defence against intracellular pathogens such as *Leishmania major* (Reiner & Locksley, 1995; Hirahara & Nakayama, 2016). Conversely, the Th2 response has been demonstrated to be vital for the clearance of helminthic parasites such as *Nippostrongylus brasiliensis* (Pulendran &

Artis, 2012; Hirahara & Nakayama, 2016). Thus, classification of the immune responses that occur in the elimination of microbial pathogens can be done by detecting either Th1 or Th2 differentiation (Hirahara & Nakayama, 2016).

As shown in Figure 1.6, chronic helminth infections can be characterized by an early Th1 response that switches to robust Th2 response when eggs are produced, as well as an overall down-regulated immune system (Pearce & MacDonald, 2002; Colley *et al.*, 2014). Type 2 immunity has evolved specifically to deal with helminths and similar types of pathogens (Pearce *et al.*, 1991; Lundie *et al.*, 2016). Helminths are known to skew the immune response towards Th2, characterized by Th2 related cytokines, that typically include IL-4, IL-5 and IL-13 that induce B lymphocytes to switch to IgE antibody production (Pearce & MacDonald, 2002; Mulu *et al.*, 2015). This immunity involves the rapid stimulation and engagement of cells of both the innate (eosinophils and basophils) and adaptive (CD4+ T cells that commit to the Th2 pathway) immune systems (Voehringer, Shinkai & Locksley, 2004). Despite the broad variation of characteristics between different helminth groups, most helminths induce similar adaptive immune responses in their human host. Cells of the innate and adaptive immune systems that are involved in type 2 immunity are able to synthesize type 2 cytokine IL-4, which directly and indirectly mediates the reactions that are considered to be indicative of helminth infection such as IgE production (Voehringer, Shinkai & Locksley, 2004; Mulu *et al.*, 2015). Chronic helminth infection has been shown to induce Th2 biasing, needed for immune defence against parasitic worm infections, as well as IL-4- with IL-10-mediated immune suppression, which compromises cell-mediated Th1 immune responses (Nutman, Kumaraswami & Ottesen, 1987; Grzych *et al.*, 1991; Pearce *et al.*, 1991, 2004, Araujo *et al.*, 1994, 1996; Sartono *et al.*, 1995; Maizels & McSorley, 2016). Many studies have postulated that Th1 and Th2 responses are mutually exclusive (Randolph *et al.*, 1999; Maizels & Yazdanbakhsh, 2003; Borkow & Bentwich, 2008; Mcsorley & Maizels, 2012).

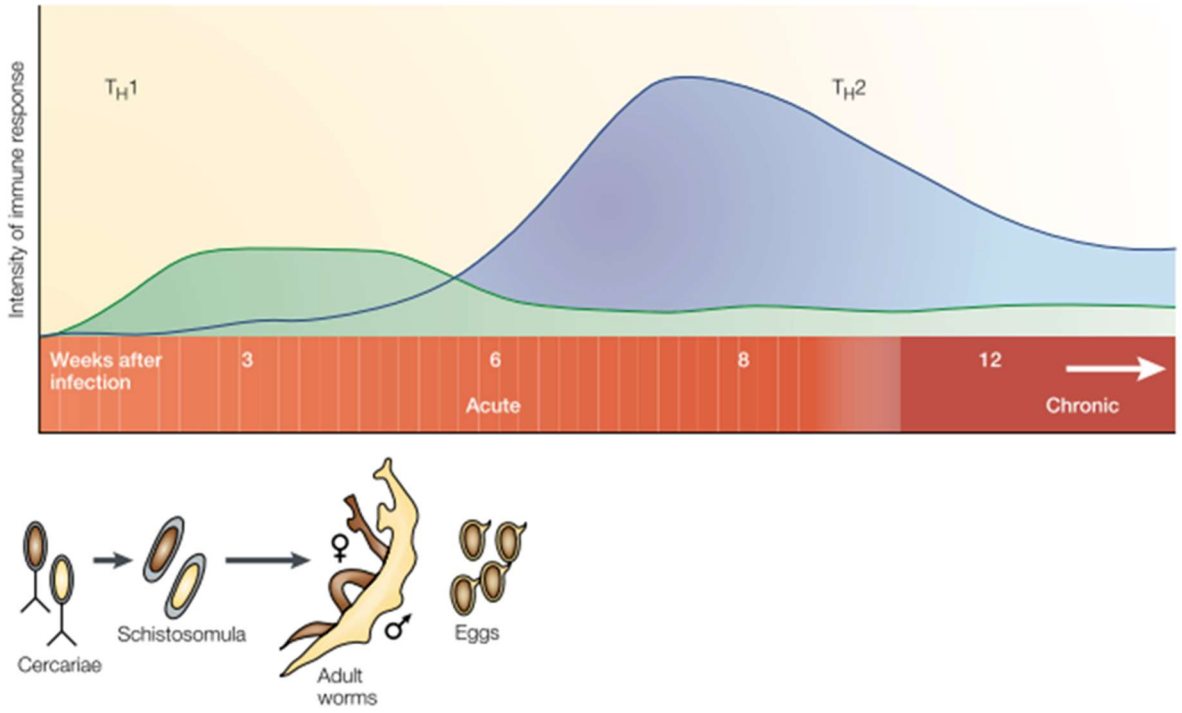

Figure 1.4 - Progression of the immune response during schistosomal infection. During the course of infection, immunity progresses through at least three phases. In the first 3–5 weeks, during host exposure to immature parasites, the dominant response is T helper 1 (Th1) type. The parasites mature, mate and begin to produce eggs at weeks 5–6, and the response morphs; the Th1 decreases and is replaced with a robust Th2 response, induced primarily by parasitic egg antigens. During the progression of the chronic stage, the Th2 response is regulated and controlled. (Source: Pearce & MacDonald, 2002)

The Th2-type responses appear to have a critical role in moderating the severity of chronic disease. The main Th2 cytokine that is responsible for hepatic fibrosis is IL-13. This has been proven in schistosome-infected mice whereby IL-13 is either absent (Il13−/−)(Fallon *et al.*, 2000), ineffective (IL-4 receptor α-chain-knockouts; IL4rα −/−)(Jankovic *et al.*, 1999) or neutralized (by treatment with soluble IL-13Rα2–Fc (Chiaramonte *et al.*, 1999)) were unable to develop the severe fibrosis that is normally associated with this stage of infection and as such, these mice were generally able to survive longer (Fallon *et al.*, 2000). Alternatively, mediators that are generally associated with Th1 immunity, such as IFN-γ, IL-12 and TNF-α have been shown to prevent IL-13-mediated fibrosis in the mouse model (Hesse *et al.*, 2001). Expanding on this, a paper by Yin and colleagues (2012) reported that schistosome infection induced high expression of IL-4 and IL-13 in mice, but after vaccination with their candidate

vaccine developed by their colleagues, the expression of the Th2 cytokine, IL-13, was decreased and replaced by Th1 cytokines such as TNF-α. However, the authors also showed that there was no change in expression of Th2-like IL-4 and Th1-like IFN-γ (Yin *et al.*, 2012).

Recent studies have shown that tuft cells are an important source of IL-25, a cytokine also necessary to mount a type 2 immune response (von Moltke *et al.*, 2015; Gronke & Diefenbach, 2016; Howitt *et al.*, 2016). A new regulatory circuit involving IL-25 production by tuft cells and group 2 innate lymphoid cells (ILC2) has been described, which affects intestinal stem cell differentiation favouring the generation tuft cells as well as goblet cell hyperplasia which has been shown to be effective in the expulsion of helminth worms such as *N. brasiliensis* (von Moltke *et al.*, 2015; Gronke & Diefenbach, 2016).

1.4.6 Helminthiasis and interaction with vaccines and other pathogens

Due to high HIV prevalence in Africa, as stated previously, successful future HIV vaccines will be most needed in Africa. (Barouch, 2008). However, there is a large prevalence of helminth infections in SSA (Chan, 1997; de Silva *et al.*, 2003; Van Der Werf *et al.*, 2003; Hotez *et al.*, 2004; Utzinger & Keiser, 2004; Obuku *et al.*, 2016). As stated before, helminth infections have been shown to skew the immune system of human as well as animal hosts to a Th2 type and to suppress the Th1 immune responses (Nutman, Kumaraswami & Ottesen, 1987; Grzych *et al.*, 1991; Pearce *et al.*, 1991, 2004, Araujo *et al.*, 1994, 1996; Sartono *et al.*, 1995; McKee & Pearce, 2004; Lundie *et al.*, 2016). Helminth-infected people may not be able to generate the required immune responses

As such, in the presence of chronic helminthic infections, Th1 responses and cytotoxic T cell responses elicited by vaccines may be attenuated, hence vaccine efficiency would be reduced (Bentwich *et al.*, 1999; Borkow & Bentwich, 2000; Thomas & Harn, 2004; Obuku *et al.*, 2016). For example, a recent study published in 2014 that investigated the effect of *Nippostrogylus*

brasiliensis on the natural and vaccine-mediated immunity to *Salmonella* (Bobat *et al.*, 2014). These authors found that mice infected with *N. brasiliensis* prior to immunization with a subunit vaccine of *Salmonella* porins developed a higher bacterial load of *Salmonella* after *Salmonella* challenge, when compared to the helminth-uninfected and vaccinated group (Bobat *et al.*, 2014). A similar mouse study by Haben, Hartmann and Breloer (2014) investigated the impact of an infection with *Litomosoides sigmodontis* at different life stages on the efficacy of an experimental vaccine against *Plasmodium berghei*. This study demonstrated that the helminth infection was able to attenuate accessory T helper cells, thus suppressing the antibody-producing B cells and ultimately completely suppressing humoral antibody responses to the vaccine. This antibody suppression was more evident in mice that had been previously infected with the helminth, or had a current chronic stage infection (Haben, Hartmann & Breloer, 2014).

Clinical and pre-clinical research has suggested that the negative impact of the immune deviation of reduced Th1-type reduces responses to BCG and tetanus toxoid vaccine on populations living in schistosome predominant areas (Sabin *et al.*, 1996; Elias *et al.,* 2005). In a pre-clinical study by Elias and colleagues (2005), mice vaccinated with BCG prior to *S. mansoni* infection significantly expressed the TB virus, suggesting the protective efficacy of the BCG vaccine was reduced by *S. mansoni* infection possibly via polarization of the general immune responses to the Th2 profile.

These pre-clinical findings are supported by an earlier clinical study conducted on human subjects in Brazil, whereby subjects infected with *S. mansoni* were evaluated for their ability to mount Th1 IFN-γ responses to a tetanus toxoid (TT) vaccine as compared to an uninfected control group (Sabin *et al.*, 1996). The resulting data reported that the IFN-γ produced in response to TT was significantly diminished in the helminth-infected group (Sabin *et al.*, 1996). A similar study found that patients infected with *Ascaris lumbricoides* mounted lower Th1

(IFN-γ and IL-2) responses to cholera toxin B subunit vaccine as opposed to uninfected patients (Cooper *et al.*, 2001). This study also showed that if parasitic treatment with albendazole was administered before the vaccination, IL-2 production to the vaccine was significantly enhanced compared with the placebo group, whereas IFN-γ production was not restored (Cooper *et al.*, 2001).

Continuing along the treatment of helminth infection, Da'dara and Harn (2010) investigated the immunogenicity of a HIV-1 DNA vaccine in mice that were infected with *S. mansoni* and then treated with praziquantel prior to vaccination. In this study, it was found that the antiparasitic treatment completely restored the ability of mice to induce robust vaccine-specific T cell responses (Da'dara & Harn, 2010). Surprisingly, this study also showed that IL-10 (an immunosuppressive cytokine) in treated mice was higher than the mice that were infected with the helminth (Da'dara & Harn, 2010). This suggests that the IL-10 cytokine should not be used as a predictive marker of immune status for T cell based vaccines (Da'dara & Harn, 2010).

Further evidence to show the negative impact of helminthiasis on Th1 immune responses comes from a coinfection study with malaria in a mouse model. In the study, the murine immune responses to malaria, *Plasmodium chabaudi*, showed that coinfection with *Heligmosomoides polygyrus*, a gastrointestinal nematode, resulted in diminished production of malaria-specific IFN-γ and IgG2a (Su *et al.*, 2005). The mice that were coinfected were also impaired in their ability to control the malaria infection when compared to the mice that were not infected with the helminth (Su *et al.*, 2005). Actor and colleagues (1993) demonstrated that schistosome-infected mice elicited reduced Th1 cytokines, as expected, but that these mice also had impaired CTL responses against HIV-1 Env epitopes. It was also shown that these helminth-infected mice had a diminished capacity to clear vaccinia in an experimental viral challenge (Actor *et al.*, 1993). These findings were supported by a later study conducted on humans whereby *S. mansoni*-infected HIV positive patients had decreased HIV-specific CD8+

CTL responses as well as an increased number of HIV-specific IL-10 positive CD8+ T cells, when compared with HIV positive patients that were not infected with a helminth (McElroy *et al.*, 2005). In contrast, in a study conducted on HIV infected Ugandan fishermen, the frequencies of HIV-specific IFN-γ+ IL-2– TNF-α− CD8+ T cells and IFN-γ+ IL-2– TNF-α+ CD4+ T cells were significantly increased in HIV and *S. mansoni*-coinfected fishermen when compared with helminth-free HIV positive individuals (Obuku *et al.*, 2016). The findings in this study appear to contradict the theory that *S. mansoni* downregulated HIV-specific Th1 responses in HIV and *S. mansoni*-coinfected human hosts (Obuku *et al.*, 2016).

In addition to the above, another study by Shollenberger and colleagues (2013) in a mouse model showed that on-going helminthiasis does not inhibit an HIV-1 vaccine (Shollenberger *et al.*, 2013). The study demonstrated that the immunogenicity if a HIV-1 Gag vaccine that they had developed using *Listeria monocytogenes* as a vector was not hindered by a *S. mansoni* infection experimentally acquired between initial vaccination and a subsequent boost. These studies demonstrate the feasibility of the theory that helminth infections could devastate the immunity elicited by a Th1/CTL-based vaccine, and thus reduce the efficacy of the vaccine administered, however the contrasting findings validate the need to conduct further and more detailed investigations.

1.5 Research justification

1.5.1 Problem identification

HIV and helminth parasite infection are two of the most widespread infections that are endemic to most parts of SSA (World Health Organisation (WHO), 2016). The development of effective affordable HIV vaccines is one of the most cost-effective strategy of controlling the HIV epidemic (UNAIDS, 2016b). This is proposed to be achieved through the development of an HIV vaccination regimen that can elicit the induction of potent T cell mediated immune

responses to control viral replication in the tissues and disease progression as well as a durable humoral immune response (Johnston & Fauci, 2007; Fauci *et al.*, 2008; Ross *et al.*, 2010; Cohen & Dolin, 2013). These types of immune responses are influenced by a T helper cell type 1 (Th1) immune response. However, several studies show that chronic helminth infection induces a Th1-Th2 immune imbalance with a predominant Th2 immune response (Sabin *et al.*, 1996; Elias *et al.*, 2005; Da'Dara *et al.*, 2006; Da'dara & Harn, 2010).

The majority of individuals living in SSA and other developing countries, especially school-aged children, are infected with one or more helminth parasites, which result in predominant Th2 immune settings (de Silva *et al.*, 2003; Hotez *et al.*, 2004, 2008; Verani *et al.*, 2011; Colley *et al.*, 2014). There is little scientific data to inform how candidate HIV vaccines would perform in the presence of ongoing helminthiasis. Important questions that could be asked are: will successful future HIV vaccines be equally effective in areas where helminths are endemic? Could helminth-induced Th2 responses possibly attenuate the induction of HIV vaccine immunity?

1.5.2 Rationale & use of the animal model

It is expected that successful future HIV vaccines will be given to infants during childhood vaccination programmes to protect them from maternal HIV transmission through breastfeeding. It's also likely that a booster HIV vaccination will be required during adolescence to protect them against contracting HIV by sexual transmission (Pathan *et al.*, 2007; van der Sande *et al.*, 2007; Lu *et al.*, 2009). Of the 230 million people infected with a schistosome, school-going children in rural areas generally have the highest prevalence and intensity of infection (Van Der Werf *et al.*, 2003; Verani *et al.*, 2011; Colley *et al.*, 2014). It is therefore important to investigate if infection with helminth parasites would have a negative impact on the capacity of candidate HIV vaccines to boost primary responses that were generated during childhood vaccinations. This is particularly crucial for SSA and other

developing countries where HIV and helminth infections are co-endemic and children are at risk for contracting these parasitic worm infections.

This study will evaluate whether a candidate HIV vaccine regimen shown to induce humoral and cellular immune responses is negatively impacted by helminth-induced immune responses. The candidate SAAVI MVA vaccine, a T cell vaccine, was demonstrated to induce strong cellular responses to Gag, RT and Env following prime-boost immunisations in mice (Shephard *et al.*, 2008). Addition of a HIV-1 Env protein, gp140, to SAAVI MVA vaccination regimen has been shown to induce both cellular and robust anti-Env serum antibody responses in a rhesus macaque model study (Chege *et al.*, 2017) and clinical trials (Churchyard *et al.*, 2016; Gray *et al.*, 2016). However, unlike the studies by Chege *et al.* (2017), Churchyard *et al.* (2016) and Gray *et al.* (2016), the Env protein used here included intact V1/V2 loops. *Schistosoma mansoni* was chosen as a helminth model parasite due to its availability and established protocols in our collaborators facility, as well as the *S. mansoni* infection model being well established in BALB/c mice (Actor *et al.*, 1993; Da'Dara *et al.*, 2006; du Plessis *et al.*, 2013).

Using a candidate HIV vaccine as a model for combined cell mediated and antibody mediated immune responses, generated data will give detailed information on the potential impact of chronic helminth infection on the immunogenicity induced by HIV vaccines. This study will also justify further investigation using nonhuman primate models and possibly clinical trials in humans impacted by both helminth infections and HIV.

1.5.3 Hypothesis

We hypothesize that chronic infection with *Schistosoma mansoni* will attenuate the capacity of a poxvirus/protein HIV vaccine regimen to boost primary HIV vaccine responses.

1.5.4 Aims and objectives

The aim of this study was to investigate whether an ongoing chronic helminthiasis impacts negatively on the capacity of candidate HIV vaccine (SAAVI MVA and a subunit HIV envelope protein) to mount booster responses in a mouse model. The objectives are as following:

1. To vaccinate groups of mice with SAAVI MVA concurrently with gp140 Env (subunit HIV envelope protein) vaccine formulated with an Alum adjuvant in order to induce both cellular and humoral HIV-specific immune responses.
2. To inoculate half of the mice with *Schistosoma mansoni* cercariae to induce chronic helminthiasis while the control groups remain uninfected (appropriated non-vaccinated and infected controls included).
3. To administer booster vaccinations (appropriate test & control groups) with SAAVI MVA-C in combination with gp140 Env after establishment of chronic helminthic infection.
4. Harvest samples/organs from animals and conduct laboratory analyses to elucidate resultant cellular and humoral immunity as well as confirm chronic infection.
5. Compile data and perform statistical analyses

2. Materials and Methods

2.1. General Laboratory Equipment

All animal work and laboratory experiments were conducted on University of Cape Town premises'. Experimental mice were housed in the animal biosafety level 2 (ABSL2) rooms in the Research Animal Facility (RAF) of the Faculty of Health Science. *Schistosoma* worm parasites were produced from S. mansoni-infected snails that were maintained in the snail facility provided by Prof Frank Brombacher of the International Centre for Genomic Engineering and Biotechnology (ICGEB) – Cape Town Component. Laboratory space and equipment including fridges, freezers, a CO_2 incubator and a class II biosafety cabinet were provided by Prof Anna-Lise Williamson of the Institute of Infectious Disease and Molecular Medicine (IDM). The BD LSRII cytometer was provided by the IDM Flow Cytometry Core Facility.

2.2. Materials

2.2.1. Vaccine

The following two HIV-1 vaccines were used in this study:

1) SAAVI MVA-C; a candidate HIV-1 vaccine that was developed by the research team of Prof A-L Williamson at the University of Cape Town, South Africa, as previously described (Burgers *et al.*, 2008). Briefly, the vaccine contains a polygene *gag,* reverse transcriptase *(rt), tat, nef* (*grttnC*) and *env* (gp150CT) genes derived from HIV subtype C vaccine strains Du151 and Du422 in a single recombinant modified vaccinia Ankara (MVA) virus (Burgers *et al.*, 2008). This vaccine was demonstrated to induce strong cellular responses to Gag, RT and Env following prime-boost immunisations in mice (Shephard *et al.*, 2008) and nonhuman primates (Burgers *et al.*, 2009; Chege *et al.*, 2012, 2017). This vaccine, together with its DNA counter-

part were developed for clinical evaluation in a first phase I placebo-controlled clinical trial, titled HVTN 073/SAAVI 102, performed on HIV-uninfected healthy adult participants in South Africa and the United States of America (Williamson *et al.*, 2012) and has been to be immunogenic (Churchyard *et al.*, 2016; Gray *et al.*, 2016). This trial confirmed the safety and immunogenicity of the vaccine in humans as a prime-boost regimen with SAAVI DNA-C priming. A research lot of SAAVI MVA-C, which was manufactured by Therion Biologics Corporation (Cambridge, MA, USA; now defunct) under Good Manufacturing Practices (GMP) was used in the current study.

2) HIV gp140 Env protein vaccine; gp140 (TV-1)(HIV-1/Clade C) protein was obtained from Immune Technology. This protein was derived from a South African HIV subtype C primary isolate (TV-1) (Lian *et al.*, 2005). This was included in the vaccination regimen to mimic the robust anti-Env serum antibody responses as shown previously in a rhesus macaque model and clinical trials (Gray *et al.*, 2016; Chege *et al.*, 2017). However, unlike the study conducted by Chege (2017) and Gray (2016) and their colleagues, the Env protein used in this study included intact V1/V2 loops. This protein was administered in formulation with an alum-based adjuvant (Imject®, Thermo Scientific)

The candidate SAAVI MVA-C vaccine was administered concurrently with HIV envelope protein, gp140, in order to elicit both HIV-specific cellular and humoral responses.

2.2.2. Parasite

A Puerto Rican strain of *Schistosoma mansoni* and used for infection of *Biomphalaria glabrata* snails. These were kept in regulated light/dark cycle conditions with a constant temperature of 25°C in 1x Lepple buffer (see appendix 1), and were fed commercial fish flakes daily. Infectious *S. mansoni* cercariae were obtained by exposing *S. mansoni* infected *Biomphalaria*

glabrata snails to direct light for 30 minutes. Water containing the cercariae was harvested and the parasites were counted.

2.2.3. Peptides and reagents

Culture medium

Splenocytes and peptides were diluted and stored in culture media prepared under sterile conditions in our laboratory. To prepare 100 mL of the media, 1 mL of Penicillin-Streptomycin (Gibco, UK) and 10 mL heat-inactivated fetal bovine serum (FBS) (Thermo Scientific, South America) was added to 88.9 mL RPMI 1640 (1X) with GlutaMAX™ -I (Gibco by Life Technologies, UK). Thereafter, 100 µL of 50 mM 2-Mercapto Ethanol (2ME) (Sigma-Aldrich) was added. This complete culture medium mixture was termed R10 media.

Peptides

Peptides used in stimulation assays, as well as a negative and a positive control, all listed in table 2.1, were diluted in R10 complete media to final concentration of 2 µg/mL.

Table 2. 1 - Stimulant and control peptides used in experimental assays

Stimulant/Control	Description	Peptide sequence	Manufacturer
ConA	Polyclonal stimulant Assay positive control		Sigma-Aldrich, USA
Irrelevant peptide	H-2K^d binding peptide Assay negative control;	TYSTVASSL	Bachem, Switzerland
Gag CD8	Gag H-2^d-restricted class I peptide (CD8 peptide)	AMQMLKDTI	Bachem, Switzerland
Gag CD4 (13 and 17)	Gag MHC class II-restricted peptides (CD4 peptides)	NPPIPVGRIYKRWIILGLNK and FRDYVDRFFKTLRAEQATQE	Bachem, Switzerland
RT CD8	RT H-2^d-restricted class I peptide (CD8 peptide)	VYYDPSKDLIA	Bachem, Switzerland
RT CD4	RT MHC class II-restricted peptide (CD4 peptide)	KQWPLTEVKIKALTAI	Bachem, Switzerland
Env CD4	Env MHC class II-restricted peptide (CD4 peptide)	YGVPVWREAKTILFCA	Bachem, Switzerland
Env CD8	Env H-2d-restricted class I peptide (CD8 peptide)	RGPGRAFVTI	Bachem, Switzerland
SEA	Soluble egg antigen; a crude extract of heterogenous proteins.		Theodor Bilharz Research Institute, Egypt

2.2.4. Mice

Groups of five or six, 6-8 week old, female BALB/c mice were acquired from the South African Vaccine Producers (SAVP, Johannesburg, South Africa) and were housed in individually ventilated cages under BSL2 conditions at all times within the RAF (Research Animal Facility) of the Health Science Faculty. These BALB/c mice were chosen due to the well-known immune systems. Several epitopes present in HIV-1 Gag, RT and Env have been shown to bind the H-2Kd MHC class 1 and class II molecules of BALB/c mice and able to activate corresponding CD8+ and CD4+ T cells respectively (Mata *et al.*, 1998; Mata & Paterson, 1999; Casimiro *et al.*, 2002). This made it possible to use synthetic peptides shown in Table 2.1 in T cell assays to evaluated HIV-specific CD4+ and CD8+ T cell responses in BALB/c mice. The epitope sequence RGPGRAFVTI from HIV-1 gp120 Env, a mouse H-2d-restricted class I peptide, was specifically included in the SAAVI MVA-C vaccine for evaluation of responses

to Env in BALB/c mice (Burgers *et al.*, 2008). The Gag peptide (AMQMLKETI) has been shown to bind to MHC class I molecules at the glutamine of P3 with pocket D of K^d, and thus, the MHC class I molecule H-2 Kd, present in BALB/c mice, is an important motif for the interaction of CTL epitopes with the gag peptide (Mata *et al.*, 1998). A later study identified epitopes present in the p24 portion of the gag protein in BALB/c mice and found that two of these epitopes are common to the H-2b and H-2d MHC class II molecules (Mata & Paterson, 1999). In addition, characterization of stimulatory peptides from pools of short overlapping peptides indicated that optimized RT gene constructs are able to effectively activate both pol-specific CD4+ and CD8+ T cells in BALB/c mice (Casimiro *et al.*, 2002). These studies demonstrate that a BALB/c mouse is a suitable model for evaluating HIV vaccine responses.

2.3 Methods

2.3.1 Animal ethics

All experiments were performed in accordance to guidelines by the Animal Research Ethics Committee of UCT. Experimental protocols were detailed in the project proposal that was reviewed by the Department of Pathology Research Committee. The animal experiments were approved by the Faculty of Health Sciences Animal Ethics Committee (AEC) and the study was assigned protocol number 014/026. In addition, the experimental protocols were detailed in the project proposal that was reviewed by the Department of Pathology Research Committee.

Mice were monitored for changes in appearance and behaviour once a day during the entire course of the experiment. Each mouse was weighed once a week to monitor for weight loss greater than 5% of original weight as an indicator for waning health. The maximum and minimum temperature of the room was monitored each day and the light intensity was measured once a week to keep conditions controlled for optimal welfare of the animals.

2.3.2. Animal procedures

Animal restraint

Mice were anaesthetised with a mixture of ketamine hydrochloride (120 mg/kg body weight) and xylazine (16 mg/kg body weight) before cercarial infections, vaccinations and euthanasia were carried out. These anaesthetics were diluted with saline to a final concentration of 120 mg/kg ketamine and 16 mg/kg xylazine per mouse. Mice weight generally ranged between 18-22 g. The anaesthesia was administered intraperitoneally.

Vaccine inoculum administration

The SAAVI MVA-C vaccine inoculum stocks were mixed diluted with 1 mM Tris buffer (pH 9.0) to a concentration of 10^7 pfu/mL and administered intramuscularly under anaesthesia. Injections of 50 µL of the inoculum were made by hypodermic needle and syringe into the tibialis muscle of each hind leg (total dosage of 10^6 pfu per mouse). The gp140 (TV-1)(HIV-1/Clade C) protein (Immune Technology) was diluted with PBS to a concentration of 100 µg/mL and formulated with an equal volume of Imject® Alum adjuvant (Thermo Scientific, USA) and sterile PBS and administered intraperitoneally (total of 10 µg per mouse).

Parasite infection

For parasitic cercariae infections, the abdomens of the mice were shaved by hand and the mice were then sedated with ketamine/xylazine mixture at a dosage stated above. The shaved areas were exposed to 30 cercariae in water for 20 minutes. This infection model mimicked the natural route of infection.

Experimental endpoint

At experimental endpoints, mice were anaesthetised with ketamine/xylazine mixture (as detailed above) and blood was collected by cardiac puncture. Mice were then euthanized by

cervical dislocation before the spleens and livers were harvested and stored in R10 media for transportation for weighing and processing in the laboratory.

2.4. Experimental design

The SAAVI MVA-C vaccine and Env gp140 protein were administered concurrently as previously described (see 2.2.1) at the start of the experiment to two groups of 5 or 8 mice. Fourteen days later, one group of vaccinated mice and one group of unvaccinated mice were infected with *S. mansoni* cercariae as previously described (see *2.3.2*). Eight weeks thereafter, the vaccinated mice were then boosted with SAAVI MVA-C and Env gp140 protein at the same dose as the initial priming vaccination. The experimental endpoint was twelve days after the boost vaccination, and the mice were euthanised and samples were collected (see *2.3.2*). This experiment was repeated three times at different dates to ensure reproducibility.

Table 2. 2 - Experiment timeline: Infection model

Group	Day 0 Prime Vaccination	Day 14 Cercariae infection	Day 70 Boost vaccination	Day 82 Euthanasia
Vaccine (n=5)	SAAVI MVA-C; i.m. Env gp140 with adjuvant; i.p.		SAAVI MVA-C; i.m. Env gp140 with adjuvant; i.p.	Collect blood by cardiac puncture; harvest spleen and liver
Vaccine+Sm (n=8)	SAAVI MVA-C; i.m. Env gp140 with adjuvant; i.p.	Infect with 30 live cercariae	SAAVI MVA-C; i.m. Env gp140 with adjuvant; i.p.	Collect blood by cardiac puncture; harvest spleen and liver
Naive (n=5)				Collect blood by cardiac puncture; harvest spleen and liver
Sm (n=8)		Infect with 30 live cercariae		Collect blood by cardiac puncture; harvest spleen and liver

2.5. Laboratory protocols

2.5.1. Processing of samples

Isolation of splenocytes

After harvesting the spleens from each mouse, the spleens were pooled per group onto one sterile wire mesh cell strainer (pore size: 70 µm) (Sigma-Aldrich) in a sterile petri dish and mashed through with a 5 mL syringe plunger. After washing the strainer with RPMI (Gibco

by Life Technologies, United Kingdom), the cell suspension was transferred into a sterile 50 mL tube and washed by two centrifugation and resuspension steps. Cells were counted for each group on a standard 0.1 mm deep hemocytometer (Marienfeld, Germany) using a 20 µL sample stained with Turks solution (Gentian violet and 1-2% acetic acid), which stains the nucleus of cells and hemolyses erythrocytes, and thus enables all nucleated cells to be counted. The viability of live cells was then assessed by staining a separate 20 µL sample with Trypan Blue to count dead cells in the sample. Cells for CBA were removed (1.5×10^7 cells/mL, in 3 mL). The remaining cells were lysed with red blood cell (RBC) lysis buffer (Sigma-Aldrich, United Kingdom) and recounted. Finally, the lysed cell solution was separated for ELISPOT assays (5×10^6 cells/mL, in 4 mL) and flow cytometry (1×10^7 cells/mL, in 1 mL) and aliquoted into their respective plates (100 µL/well).

Separation of serum

Blood samples were collected in serum microtainer tubes (BD Bioscience, USA) and stored at room temperature for 4-8 hours to allow clotting before centrifuging (2000 rpm for 10 minutes). Serum was collected and aliquoted into 10 tubes and stored in a -20°C freezer until use in ELISA assays.

Preparation of livers for histology

Harvested livers were collected in R10 media, weighed and subsequently transferred to paraformaldehyde solution (4% paraformaldehyde in PBS) in separate tissue containers. These were then stored at room temperature until they were sent to a service provider (Department of Surgery, UCT) for sectioning and preparation of histological slides.

2.5.2. Enzyme-linked immunospot (ELISPOT) assay

The enzyme-linked immunospot (ELISPOT) assay was developed from traditional enzyme-linked immunosorbent assay (ELISA) techniques in the early 1980s (Sedgwich & Holt, 1983).

It was initially an assay for detecting antibodies secreted from B cells and gradually evolved to a method for detecting cytokines or other soluble mediators secreted from various cell types. This assay allows the direct quantitative measurement of number of cytokine secreting cells at the single cell level. The function of cells can be determined from the types of cytokines secreted by cells in response to different stimuli. The ELISPOT assay is viewed as robust, reliable, and reproducible immunological tool (Tanguay & Killion, 1994).

We used ELISPOT assays to measure T cell-mediated immune responses by measuring the number of CD4+ and CD8+ T cells secreting the cytokine after being stimulated by an array of various antigens. This type of assay utilizes the sandwich (ELISA) technique whereby a monoclonal or polyclonal capture antibody which is specific to the chosen cytokine is coated onto a polyvinylidene difluoride (PVDF) backed microtitre plate. Stimulated cells are incubated in the wells and, during this incubation period, the immobilized antibody coated onto the plate binds the cytokine secreted by the cells. A biotinylated polyclonal antibody, alkaline-phosphatase conjugated to streptavidin is added which binds to the cytokine already bound to the capture antibody. The streptavidin shows up the localization of the bound cytokine as spots after addition of a colour developer (such as NovaRed), whereby one spot generated represents one T cell producing the cytokine. Thus, this assay can directly measure a T cell mediated immune response and thus the presence of the vaccine-induced immunity.

In this study, we used the ELISPOT technique to measure IFN-γ- and IL-2-producing T cells. A BD Mouse IFN-γ ELISPOT kit and a BD Mouse IL-2 ELISPOT kit (both BD Bioscience, USA) were performed according to established laboratory protocols (Shephard *et al.*, 2008). Peptides, as well as a negative and a positive control, all listed in table 2.1, were diluted in R10 complete medium (to final concentration of 2 µg/mL) before incubating with 500 000 splenocytes per well. This was conducted in triplicate wells for each group of mice. The reaction was stopped after 22-24 hours incubation at 37°C with 5% CO_2. Nova Red substrate

(Vector Laboratories, USA) was used to develop the spots, according to manufacturer's instructions, and thereafter the plates were analysed using the CTL ImmunoSpot system (Cellular Technology LTD, USA).

After the plates were analysed using the CTL ImmunoSpot system (Cellular Technology LTD, USA), the spots on the images were visually inspected to determine if debris spots were counted by the CTL reader's software. If such debris spots are identified, these are deleted manually on the software. Thereafter, the mean number of spots per group per peptide as well as the standard deviation were calculated. The background was removed (double the negative control, maximum negative control value of 15 spots or sfu < 30) and the remaining positive results were expressed as spot forming units per million splenocytes (sfu/10^6).

2.5.3. Intracellular cytokine staining (ICS)

Intracellular cytokine staining (ICS) is the process of staining for intracellular markers such as cytokines, and for surface markers that determine the cell phenotype. The staining uses antibodies that are conjugated to a fluorochrome which make it possible for detection by a flow cytometer. Fluorescence-activated cell-sorting (FACS) flow cytometry enables one to measure and analyse the characteristics of a single particle as it moves through a laser. The laser light excites the fluorochrome which generates different light signals that are collected via a system of optics and filters for detection which allows for measurement of the particle's size, granularity and fluorescence.

The flow cytometer utilizes a pressurised fluidic system to allow cells to be taken up through the flow cell. The cells pass the laser individually and the antibody-conjugated fluorochromes that are bound to a cell become excited by the laser and emit light at particular wavelengths, which are then directed by the optics of the flow cytometer to detectors. These wavelengths are

measured by the photomultiplier tubes. These detectors convert the optical signals into digital data, ready to be analysed by the FlowJo® program (Tree Star Inc., USA).

Cytokines, IFN-γ, TNF-α and IL-2, as well as the proportionality of activated CD8+ and CD4+ T cells was measured by ICS to identify the memory and function phenotypes of T cell subsets recognising the vaccine/parasite peptides. The production of these Th1 cytokines due to peptide stimulation of splenocytes was measured by ICS and flow cytometry.

Splenocytes were diluted to 1 million cells/well in R10 media and stimulated with peptides (Table 2.1), with a negative control of unstimulated splenocytes was also included for each group. The plates were incubated at 37°C for 2 hours before brefeldin-A (BFA; Sigma-Aldrich, USA) was added to each well (4 µg/well, 20 µg/mL), and incubated for a further 4 hours. Cells were washed with PBS and stained with ViViD (0.05 µg/well) (Invitrogen, USA) for 20 minutes at room temperature in darkness. The cells were then washed again with PBS and blocked with 50 µL blocking solution (0.12 µL normal mouse serum, 0.12 µL normal rat serum, 0.16 µg CD16/32 (BD Biosciences, USA)) at 4° for 15 minutes. Cells were then incubated at 4°C for 20 minutes in a final volume of 100 µL stain buffer (BD Biosciences, USA) containing 0.05 µg of each of the following fluorochrome-conjugated surface antibodies: CD3 - Alexa 700, CD4 - PE Cy-7, CD8 - APC-Cy7, CD44 – FITC and CD62L - APC. After incubation, unbound antibodies were removed from solution using a wash step before fixation by incubation at 4°C for 30 minutes with fixation/permeabilization buffer (BD Biosciences, USA). Fluorochrome-conjugated cytokine antibodies were diluted in wash buffer (BD Biosciences, USA) and 100 µL (0.2 µg TNF - PE, 0.06 µg IFN-γ - PE and 0.06 µg IL-2 - PE) was added to each tube before incubating at 4°C for 20 minutes. After washing twice, cells were resuspended in CellFix (BD Biosciences, USA. Acquisition was then conducted on a BD LSRII (BD Biosciences, USA), and data was analysed using FlowJo V10. The gating strategy is illustrated

below (Figure 2.1). Cytokine positive (PE+) cells were undetectable and this was therefore excluded from the gating strategy.

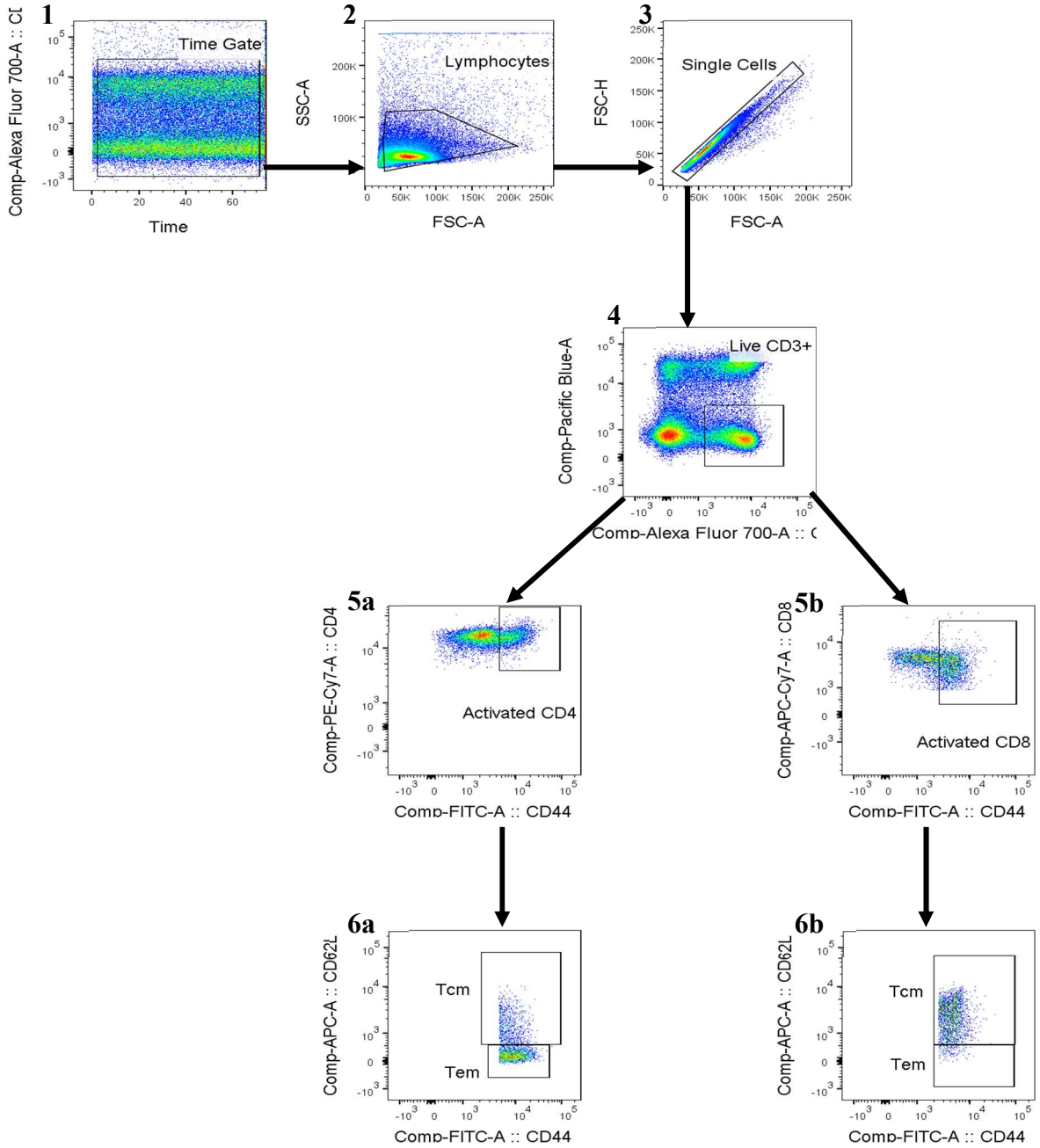

Figure 2.1 - Gating strategy used for flow cytometry analysis of vaccine peptide-specific activated CD4+ and CD8+ cells. Time gate, lymphocytes, singlets, live CD3 cells, which subsequently bifurcates activated CD4 cells and activated CD8 cells and, finally, CD4 T memory cells (Tcm and Tem) gated on activated cells and CD8 T memory cells (Tcm and Tem) gated on activated cells. Splenocytes were stimulated with PMA (positive control), Gag, Pol, Env (CD4), Env (CD8), SEA or R10 media only (unstimulated). Time gates (1) were plotted for each laser. Lymphocytes (2) were gated based on the relative size and granularity of the cells and plotted as forward scatter area (FSC-A) versus side scatter area (SSC-A). Singlet gates (3) based on forward scatter height (FSC-H) and forward scatter area (FSC-A) were used to exclude doublet cells. Live CD3+ cells (4) were gated on ViViD negative populations and CD3 positive populations. The activation of live CD4+/CD8+ lymphocytes (5a/b) was then determined by the CD4+/CD8+ population positive for CD44. The memory phenotype of activated CD4+/CD8+ T cells (6a/b) was then delineated by the CD62L marker as central memory (CD44+CD62L+) and effector memory (CD44+CD62L−) T cell populations.

The number of activated CD44+ T cells was calculated based on the percentage data collected from FlowJo®, worked out per 100 000 cells (the number of events counted). This was then applied to the number of splenocytes counted per spleen. After the spleens were pooled, the concentration of splenocytes per ml were counted and extrapolated to total number of splenocytes. This could then be divided by the original number of spleens pooled per group. Positive responses were those above a cut-off value of 0.05% CD44-expressing T cells.

2.5.4. Cytometric bead array (CBA)

The cytometric bead array (CBA) (BD Bioscience, South Africa) was used to quantify the concentrations of particular extracellular antigen-specific cytokines in a sample using flow cytometry to determine if bias toward a Th1 or Th2 cytokine response was occurring. This assay utilizes flow cytometry as an analytical tool that allows for the discrimination of different particles on the basis of size and fluorescence. The CBA kit uses a variety of beads with discrete fluorescence intensities that have been coated with capture antibodies specific for particular cytokines and their proteins in order to detect multiple cytokines from a single sample. The "capture beads" are mixed with the phycoerythrin (PE)-conjugated detection antibodies and incubated with recombinant protein standards as well as test samples. This will form "sandwich" complexes that can then be analysed by a flow cytometer.

Processed splenocytes (as described in 3.4.1) were pooled per group and stimulated with peptides, as listed in table 2.1, in a 96 well round-bottomed plate. This was done in duplicate for each group of mice. The plate was incubated at 37°C with 5% CO_2 for 48 hours, thereafter the supernatants were harvested without disturbing the cell pelleted at the bottom of the wells. The supernatants were then stored at -20°C until the CBA was commenced, and then the duplicates were pooled together for the assay. The CBA Mouse Th1/Th2/Th17 cytokine kit (BD Bioscience, USA) measures Th1 cytokine (IFN-γ, TNF-α, IL-2), Th2 cytokine (Il-4 and IL-6) and regulatory cytokine (IL-10) levels and was conducted according to the

manufacturer's instructions. Data was acquired using a BD LSRII (BD Bioscience, USA) and analysed by FCAP Array Software (Soft Flow Inc., Hungary).

The FCAP Array software located clusters of fluorescence (to which analytes have been assigned) and then determined the median fluorescence intensity (MFI) of the detector antibody for each analyte. Cytokine standards (provided in the kit and prepared as per manufacturer's instructions (BD Science, South Africa)) were serially diluted and the MFI of these standards enabled the software to calculate a curve from which the concentrations for each of the measured analytes in the samples are calculated. The results were calculated as picogram of cytokine per mL (pg/mL) using the standard curve extrapolated from the set of standards. Finally, the results from the stimulation of the splenocytes with the irrelevant peptide were used as the cut off for positive responses. Any reading above 2x the average of the results for the irrelevant peptide stimulation and were deemed positive. This was further confirmed if the results were higher than the theoretical limits of detection were 0.1 pg/mL for IL-2, 0.03 pg/mL for IL-4, 1.4 pg/mL for IL-6, 0.5 pg/mL for IFN-γ, 0.9 pg/mL for TNF, 0.8 pg/mL for IL-17A, and 16.8 pg/mL for IL-10 (Wei *et al.*, 2014, BD Bioscience CBA Mouse Th1/Th2/Th17 cytokine kit manual).

2.5.5. Enzyme-linked immunosorbent assay (ELISA)

Enzyme-linked immunosorbent assays (ELISA), are routinely used to measure the concentration of peptides, antibodies, cytokines and antigens in a solution. An enzyme is covalently bonded to a specific monoclonal/polyclonal antibody that recognises and binds to an antigen target. The antibody-enzyme complex that forms will catalyse a colour-changing reaction. Therefore, the amount of colour change that occurs is directly proportional to the presence of antigen. The indirect ELISA that was employed here allows for both the detection and comparable quantification, whereby antigen protein is coated onto wells of a plate, and

antibodies in the sample are allowed to bind. The enzyme-linked detection antibodies bind to the bound sample antibodies and, when substrate is added, the colour-changing reaction occurs.

Immunoglobulin (Ig) production in response to Env gp140 was quantified by ELISAs conducted on serum collected from each mouse. Maxisorp™ 96-well plates (Nunc, USA) were coated with 0.5 μg/mL (50 ng/well) of TV-1 HIV-1C Env gp140 protein (Immune Technologies) and incubated at 4°C overnight. Serum was diluted to 1:5000 in 2.5% skimmed milk (2.5g dehydrated milk in 100 mL PBS) and added to duplicate wells. Aliquots of pooled sera from gp140 Env-immunized mice (which had high OD values) were used as a standard to generate a standard curve. Thereafter, antibody unit values, starting with a maximum value of 1000, were allocated to the OD values of the serially diluted standard serum and used to enumerate the curve. To determine the antibody values of the test samples, antibody values were extrapolated from the standard curve using the test sample OD values.

The standard sample was serially diluted two-fold, twelve times, starting at a dilution of 1:200 in 2.5% skimmed milk. This was also done in duplicate. The serum aliquots collected from naïve mice were pooled and included as a negative control at a dilution of 1:5000 in 2.5% skimmed milk, this was done in triplicate. Once the test and control sera were aliquoted, the plate was once again incubated at 4°C overnight to increase time for binding. Serum IgG, IgG1, IgG2a and IgG2b were detected using anti-IgG/IgG1/IgG2a/IgG2b conjugated to horse radish peroxidase (HRP) (Southern Biotech, USA) diluted to 1:1000 in 5% skimmed milk (5g dehydrated milk in 100 mL PBS) and developed using tetramethyl-benzidine (TMB; KPL, Gaithersburg, MD). The absorbance was measured on a VersaMax microplate reader (Molecular Devices Corporation, Sunnyvale, CA)) at 450 nm with a reference filter at 540 nm. The OD values from the reference filter were subtracted to give net OD values. The net OD from the standard serum dilutions were assigned antibody units and used to generate a standard curve. To generate the antibody units for the test samples, their net OD values were

extrapolated from the standard curve. The triplicate negative controls were averaged, and the average net OD values plus 2 standard deviations was defined as the cut-off value. Any test sample OD value that was less than the cut-off value was assigned a zero antibody unit. The final results are reported as gp140 Env antibody units.

Another set of ELISA experiments were performed to evaluate the total immunoglobulin levels (non-specific antibody immune responses) in the mouse sera in order to elucidate the Th1/Th2 bias of the different mouse groups. Thus, to quantify total IgG_1, IgG_{2a} and IgG_{2b} levels in mouse sera, Maxisorp™ 96-well plates (Nunc, USA) were coated with 1 µg/mL (0.1 µg/well) goat anti-mouse Ig capture antibody (Southern Biotech, USA) and incubated at 4°C overnight. Sera from test mice were diluted to 1:5000 in 2.5% skimmed milk and added to duplicate wells. The standards provided were diluted to 1 µg/mL and then serially diluted two-fold, twelve times across the plate. This was also done in duplicate on every plate. Once diluted sera were aliquoted, the plate was once again incubated at 4°C overnight to increase time for binding. The levels of IgG, IgG1, IgG2a and IgG2b in the sera were detected using anti-IgG/IgG1/IgG2a/IgG2b conjugated to horse radish peroxidase (HRP) (Southern Biotech, USA) diluted to 1:1000 in 5% skimmed milk and developed using tetramethyl-benzidine (TMB; Abcam, USA). The absorbance was measured using the VersaMax microplate reader as described above. Two blank wells (incubated with 100 µL 2.5% skimmed milk without sera) were included as negative controls. The average OD value of these negative controls plus 2 standard deviations was used as the negative cut off for this assay. Any test sample OD value that was less than the cut-off value was assigned a zero antibody unit

2.5.6. Histology

Chronic schistosomiasis has shown to be a consequence of granuloma formations in tissue. The eggs of *S. mansoni* are carried in the circulatory system into the liver, principally, and the eggs become lodged within the tissue. The CD4+ T lymphocyte response induced by the egg

antigens further promotes the formation of granulomatous lesions around each of the helminth eggs in the tissues. These granulomas are comprised of collagen fibres, macrophages, eosinophils and the aforementioned CD4+ T cells. As the eggs die within the tissues, the surrounding granulomas are replaced by fibrous plaques. As such, confirmation and the degree of chronic infection can be measured by the presence and multitude of granulomas as well as the extent of hepatic fibrosis (Pearce & MacDonald, 2002). In this experiment, observation of granulomas in the liver were used to confirm chronic infection.

Liver tissue samples were stored in 4% formaldehyde solution (diluted in PBS) and sent to the Department of Surgery (UCT) for preparation of histological slides (i.e. embedded the samples in paraffin and cut to 5-7 µm sections and stained with haematoxylin and eosin (H&E)). These slides were examined under a light microscope (Nikon 90i Automated compound microscope with a camera (DCT DS-SMc)) and micrographs of liver granuloma were captured using a Nikon 5.0 mega pixel colour digital camera (DCT DS-SMc).

2.6. Data analysis and statistics

In order to accurately assess the effect of schistosomiasis on the immunogenicity of the candidate HIV vaccine to boost cellular and humoral immune responses, statistical analyses were conducted to compare across the groups (experimental and control groups). Responses to vaccine peptides were compared between the vaccinated groups that were either infected or uninfected. The responses to SEA were compared between infected mouse groups that were vaccinated or unvaccinated. Results were analysed in Prism® (GraphPad Software, USA), using two-tailed Student t-tests or Mann–Whitney–Wilcoxon tests for significance. Statistical p values < 0.05 were considered to be significant.

3. Results

3.1. Introduction

Our aim was to investigate whether an ongoing chronic helminthiasis attenuates the capacity of candidate HIV vaccine (SAAVI MVA and a subunit HIV envelope protein) to mount booster responses in a mouse model. As such, we analysed the cell-mediated and humoral responses for mice infected with *S. mansoni* (Sm), vaccinated with SAAVI MVA-gp140 (Vaccine), naïve or both vaccinated and infected (Vaccine + Sm). The physical differences in the livers and spleens between these groups was also investigated by histology and cell counts.

It was determined previously that peak responses would be elucidated twelve days after MVA-gp140 boost inoculation (Shephard *et al.*, 2008; Chapman *et al.*, 2013; Jongwe *et al.*, 2016). As such, mice were euthanised at this time point and the spleens were pooled within each group.

3.2. Cell-mediated Responses

3.2.1. Magnitude of IFN-γ-producing T cell responses to vaccine peptides by ELISPOT assay

An IFN-γ ELISPOT assay was used to quantify the T cells secreting IFN-γ in response to vaccine peptides. Results from the positive control (ConA) and the negative control (the irrelevant peptide) are not shown (Figure 3.1). The unvaccinated mouse groups did not elicit any responses above the background responses, with regards to vaccine peptides in this assay.

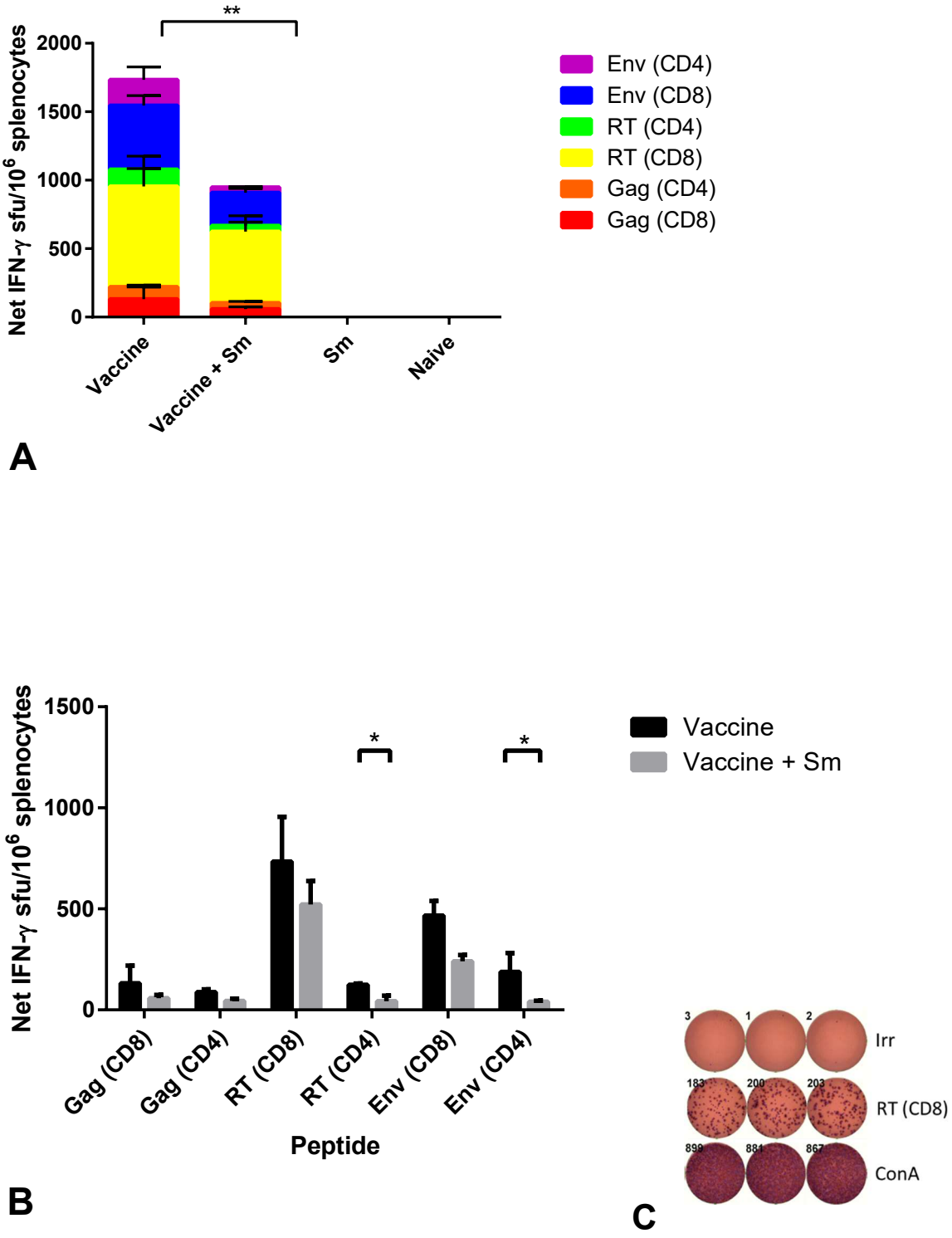

Figure 3.1 - Quantification of T cells secreting IFN-γ in response to vaccine peptides. Cumulative (A) and individual (B) IFN-γ ELISPOT CD4+ and CD8+ responses of Vaccine mice compared to Vaccine+Sm mice. This assay was conducted twelve days after SAAVI MVA-gp140 boost, on pooled splenocytes stimulated with six different vaccine peptides. Bars represent the magnitude of net responses to individual peptides, expressed as spot forming units (sfu) per million splenocytes after removing background responses, with SEM shown. (*$p < 0.05$ **$p=0.0087$). Exemplar wells for the Vaccine+Sm group are also shown (C).

The Vaccine mouse group elicited the higher IFN-γ responses (1733 sfu/10^6) than Vaccine+Sm group (948 sfu/10^6). Infection with helminths was shown to significantly decrease the number of T cells responding with IFN-γ to vaccine peptides ($p<0.01$). And, while all peptides elicited high IFN-γ-production responses in the Vaccine group, the highest responses were produced in response to the RT (CD8) peptide (734 ± 221 sfu/10^6). This RT (CD8) peptide also elicited the highest responses in the Vaccine+Sm mouse group of all the other peptides (521 ± 116 sfu/10^6).

Responses to each peptide were decreased, no peptide was able to maintain the same responses as the Vaccine group. Nevertheless, statistically significant differences in IFN-γ producing T cells were only observed for the RT (CD4) (Vaccine; 124 ± 7 sfu/10^6, Vaccine+Sm; 43 ± 27 sfu/10^6) and Env (CD8) (Vaccine; 467 ± 73 sfu/10^6, Vaccine+Sm; 241 ± 31 sfu/10^6) peptides (*$p < 0.05$).

The Vaccine group elicited an average of 1333 and 400 net IFN-γ sfu/10^6 splenocytes to CD8+ and CD4+ peptides respectively. These results were 1.6 and 3.1 fold higher, respectively, than Vaccine+Sm group, but this was not statistically significant.

3.2.2. Magnitude of IL-2-producing T cell responses to vaccine peptides by ELISPOT assay

An IL-2 ELISPOT assay was used to quantify the T cells secreting IL-2 in response to vaccine peptides. Results from the positive control (ConA) and the negative control (the irrelevant peptide) are not shown (Figure 3.2). The unvaccinated mouse groups did not elicit any responses above the background responses, with regards to vaccine peptides in this assay.

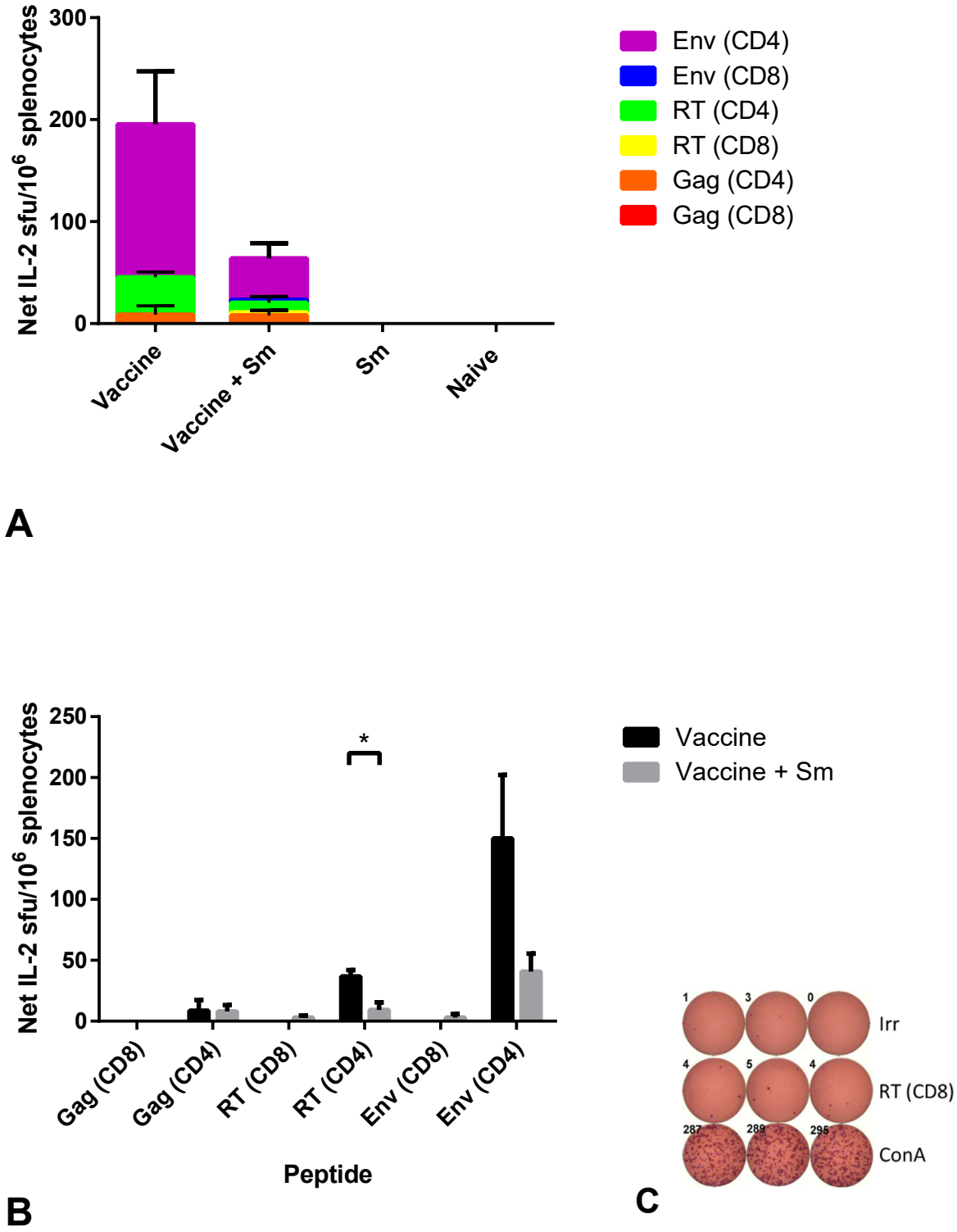

Figure 3.2 - Quantification of T cells secreting IL-2 in response to vaccine peptides. Cumulative (A) and individual (B) IL-2 ELISPOT CD4+ and CD8+ responses of Vaccine mice compared to Vaccine+Sm mice. This assay was conducted twelve days after SAAVI MVA-gp140 boost, on pooled splenocytes stimulated with six different vaccine peptides. Bars represent the magnitude of net responses to individual peptides, expressed as spot forming units (sfu) per million splenocytes after removing background responses, with SEM shown. (*$p < 0.05$). Exemplar wells for the Vaccine+Sm group are also shown (C).

The Env (CD4) peptide elicited the greatest IL-2-producing T cell responses in both vaccinated mouse groups (Vaccine; 150 ± 52 sfu/10^6, Vaccine+Sm; 41 ± 15 sfu/10^6) however, the levels were not statistically significant between the groups. Responses by way of IL-2-producing cells were highest in the Vaccine group (195 sfu/10^6), with the Vaccine+Sm group having slightly supressed responses (64 sfu/10^6), but not significantly. Two peptides failed to elicit IL-2-producing T cell responses above the background: Gag (CD8) and Env (CD8). Again, responses to each vaccine peptide were decreased in the Vaccine+Sm group and no peptide was able to produce the same responses as the Vaccine group. But, only one peptide, RT (CD4), elicited significantly lower IL-2 producing T cells in the Vaccine+Sm group (9 ± 6 sfu/10^6) when compared to the Vaccine group (37 ± 5 sfu/10^6) (*$p < 0.05$). Overall, the Vaccine group produced 3.4 fold greater net CD4+ IL-2 sfu/10^6 splenocytes than Vaccine+Sm group ($p=0.045$).

3.2.3. Magnitude of IFN-γ and IL-2 T cell responses to helminth egg antigens

To determine the effect of vaccination on the magnitude of T cell responses to helminth antigen, SEA was used to stimulate splenocytes from all mouse groups in the IFN-γ ELISPOT as well as the IL-2 ELISPOT assays, in exactly the same concentration as the other peptides used in the assay. As expected, uninfected groups did not elicit T cell responses to the SEA peptide.

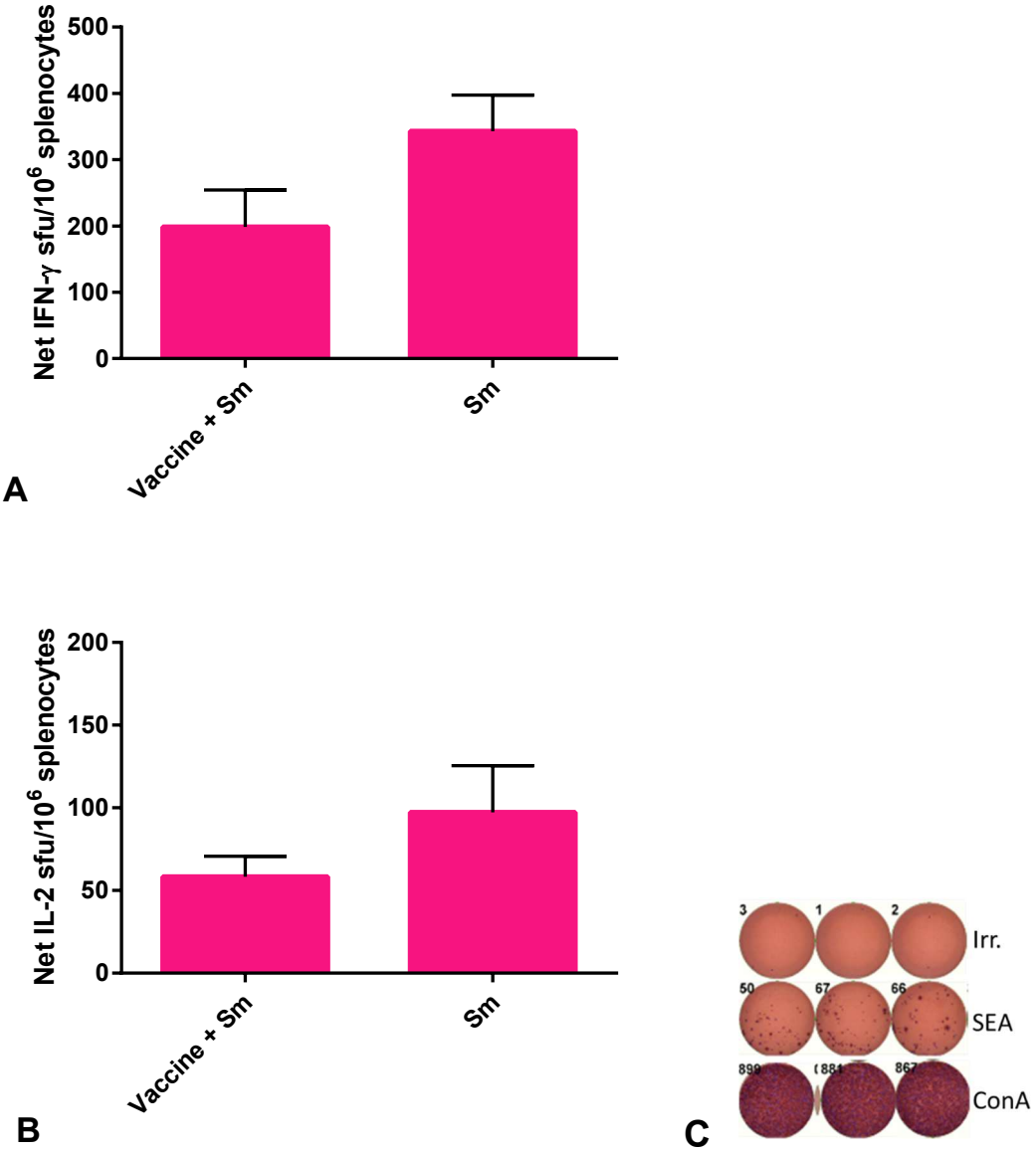

Figure 3.3 - Quantification of T cells secreting cytokine in response to SEA. IFN-γ ELISPOT (A) and IL-2 ELISPOT (B) T cell responses of Sm mice compared to Vaccine+Sm mice. This assay was conducted twelve days after MVA-gp140 boost, on pooled splenocytes stimulated with the SEA peptide. Bars represent the magnitude of net responses expressed as spot forming units (sfu) per million splenocytes after removing background responses. Exemplar wells for the Vaccine+Sm group are also shown (C).

The Sm group showed higher IFN-γ T cell responses (343 ± 54 sfu/10^6) than the Vaccine+Sm group (199 ± 55 sfu/10^6). This was also the case for IL-2-producing T cells, between the Sm group (97 ± 28 sfu/10^6) and the Vaccine+Sm group (58 ± 12 sfu/10^6). However, no significant differences exist between these mouse groups. Thus, both infected groups had similar recall immune responses to the helminth egg antigen.

3.2.4. Quantification of secreted cytokines in response to vaccine peptides

Ex vivo secretion of Th1 cytokines (IFN-γ, TNF-α and IL-2) as well as Th2 cytokines (IL-4 and IL-6) and regulatory cytokine (IL-10) by the mouse splenocytes were quantified by a cytometric bead array using a mouse CBA Th1/Th2/Th17 kit as described in the Methods section (2.5.4.). The production of these cytokines was measured following 48 hour stimulation in culture with one of the vaccine peptides (table 2.1) or SEA.

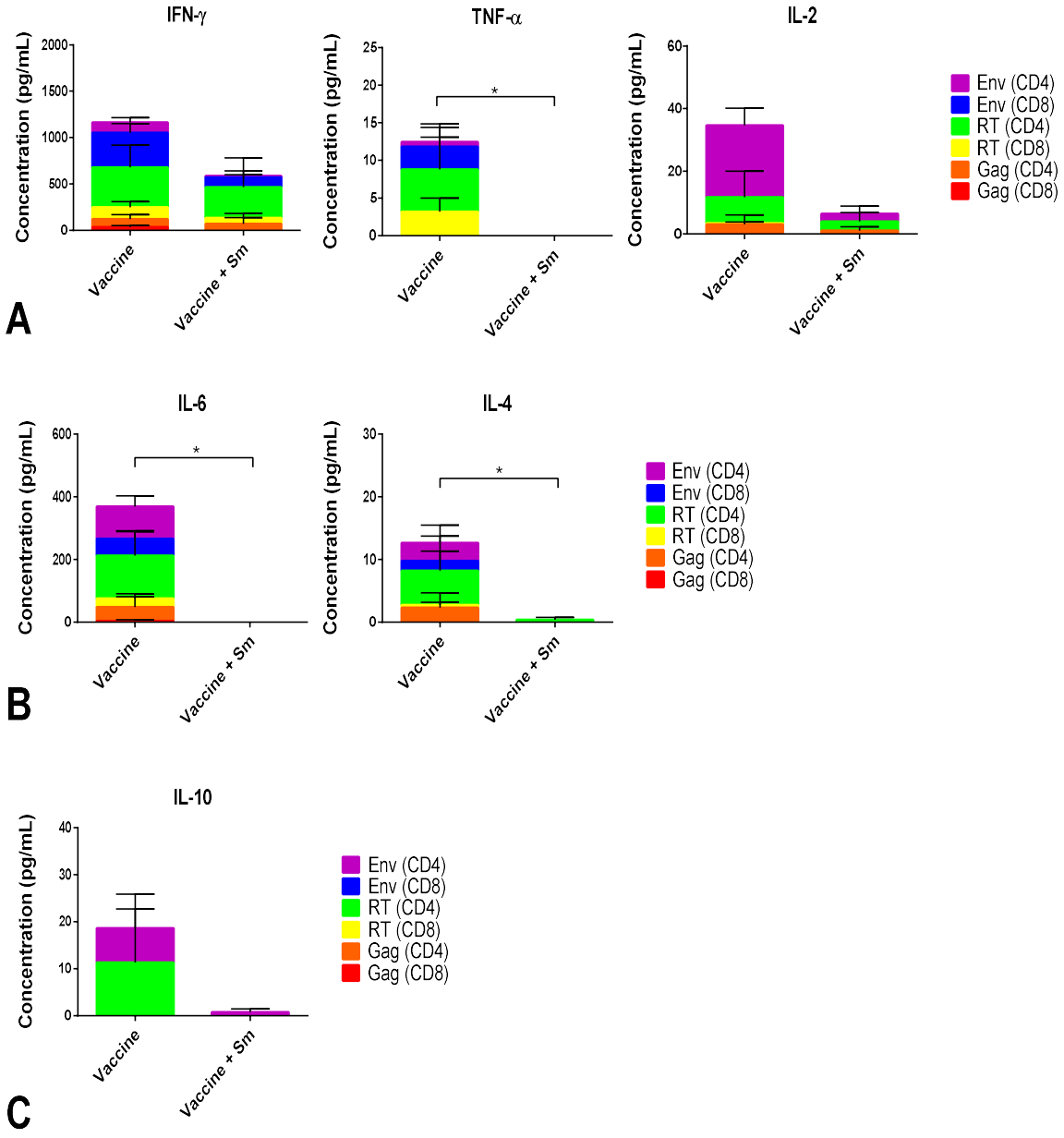

Figure 3.4 - Cytokine secretion from mouse splenocytes to CD8+ and CD4+ vaccine peptides. A cytometric bead array was used to quantify the secretion of Th1 (A: IFN-γ, TNF-α and IL-2), Th2 (B: IL-6 and IL-4) and regulatory (C: IL-10) cytokines 12 days after SAAVI MVA-gp140 boost. Bars represent concentration of cytokines secreted in response to vaccine peptides, expressed as pictograms per millilitre (pg/mL) after removing background responses ($p < 0.05$).

The cumulative concentrations of various cytokines vary widely, with IFN-γ and IL-6 having the highest concentrations. However, TNF-α, IL-4, IL-10 and IL-2 had comparatively low concentrations in all samples. Cytokine responses were consistently present specific to

RT (CD4) peptide, with TNF-α responses in the Vaccine+Sm group being the only exception. In fact, this group elicited no TNF-α above detection levels.

As shown in Figure 3.4A, production of the Th1 and Th2 cytokines as well as IL-10 was suppressed in the Vaccine+Sm group, when compared to the Vaccine group. All the Th1 cytokines, IFN-γ, TNF-α, and IL-2, by splenocytes from Vaccine+Sm mice were generally decreased (IFN-γ; 585.587 pg/mL, TNF-α; 0 pg/mL, IL-2; 6.457 pg/mL) when compared to Vaccine mice (IFN-γ; 1165.56 pg/mL, TNF-α; 12.46 pg/mL IL-2; 34.663 pg/mL). Secretion of TNF-α was below detection levels for the Vaccine+Sm mouse group, and this was the only Th1 cytokine with statistical significance, whereby this Vaccine+Sm group secreted significantly lower levels of TNF-α than the Vaccine mouse group ($p = 0.048$). For individual peptides, the only statistical significance that appeared between the two mouse groups was the secretion of IL-2 in response to Env (CD4), whereby IL-2 was significantly lower in the Vaccine+Sm group (2.477 ± 2.477 pg/mL) when compared to the Vaccine group (22.927 ± 5.451 pg/mL) ($p = 0.0269$).

With regards to the Th2 cytokines, IL-6 was secreted significantly more in response to vaccine peptides by the splenocytes of the Vaccine mice (369.757 pg/mL) when compared to the Vaccine+Sm mice (0 pg/mL) (*$p=0.012$). The background for IL-6 secretion in Sm mice, as determined from the IL-6 responses to the irrelevant peptide, was notably high and many peptides could not exceed this. The other Th2 cytokine detected, IL-4, was also secreted to significantly higher levels to vaccine peptides by the splenocytes of the Vaccine mice (12.653 pg/mL) when compared to the Vaccine+Sm mice (0.37 pg/mL), however both of these concentrations are generally low (*$p=0.031$).

The regulatory cytokine, IL-10, did not show any definitive difference between the two mouse groups. Only two of the vaccine peptides elicited secretion of IL-10; RT (CD4) and Env (CD4).

The IL-10 production in response to RT (CD4) was generally reduced in Vaccine+Sm mice (vaccine; 11.367 ± 11.367 pg/mL, vaccine + Sm; 0 pg/mL). This trend was also seen with IL-10 secretion in response to Env (CD4) (vaccine; 7.257 ± 7.257 pg/mL, vaccine + Sm; 0.737 ± 0.737 pg/mL).

3.2.5. Quantification of cytokines produced in response to SEA

The analysis of the production of cytokines in response to the parasitic infection was also conducted using the cytometric bead array. This assay was conducted using the supernatants of splenocytes from each group stimulated with SEA in the same conditions and concentrations as those used to analyse the vaccine peptides. The groups that were not infected did not respond to the SEA stimulation and thus did not yield any results above the background in this regard.

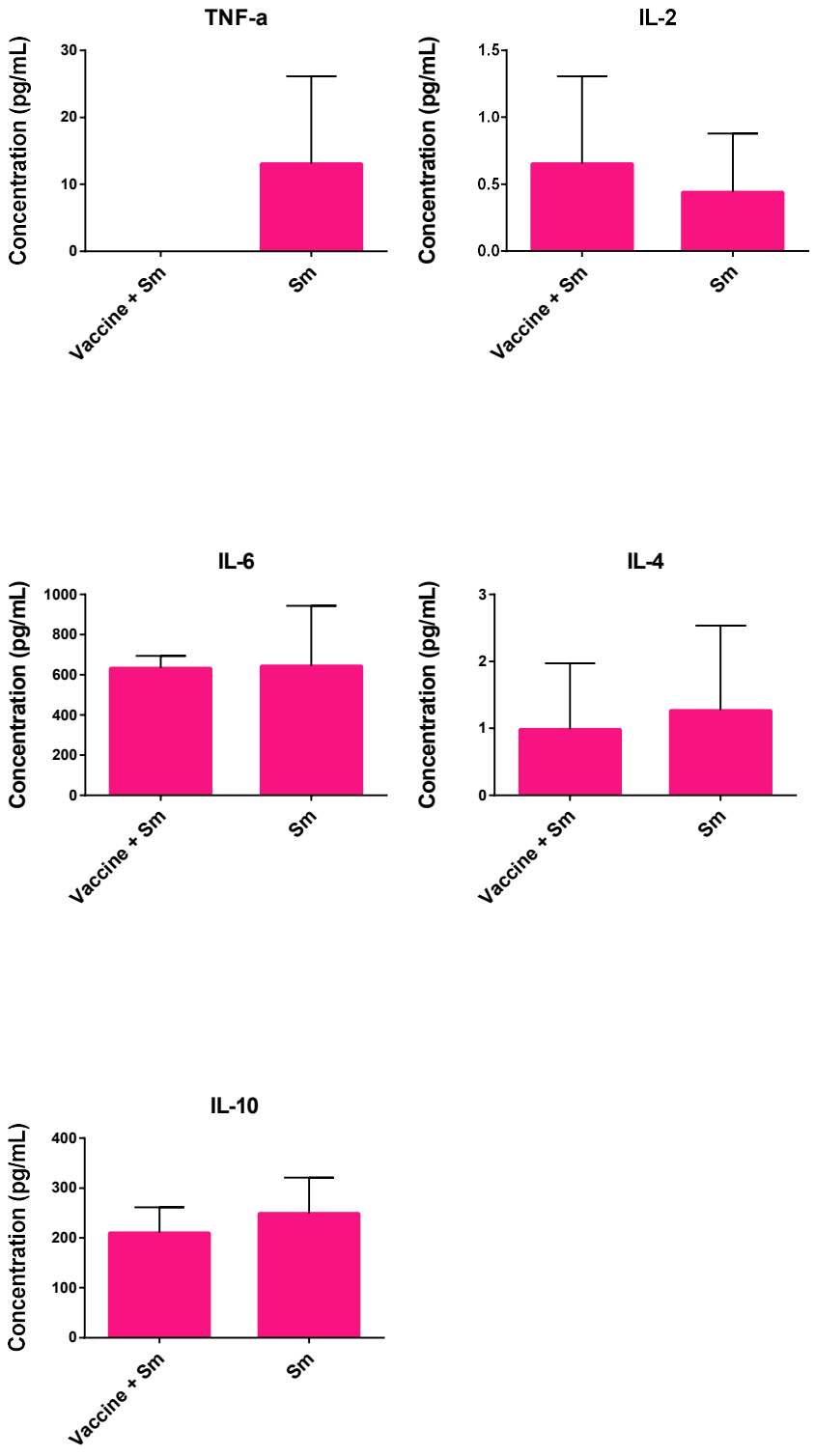

Figure 3.5 - Cytokine secretion from mouse splenocytes to helminth egg antigen. A cytometric bead array was used to quantify the secretion of Th1 (A: TNF-α and IL-2), Th2 (B: IL-6 and IL-4) and regulatory (C: IL-10) cytokines 12 days after SAAVI MVA-gp140 boost. Bars represent concentration of cytokines secreted in response to SEA peptide, expressed as pictograms per millilitre (pg/mL) after removing background responses.

The Vaccine+Sm mouse group showed generally increased concentrations of both Th1 cytokine, IL-2 in response to SEA (0.653 ± 0.653 pg/mL) when compared to the Sm group (0.44 ± 0.44 pg/mL). However, this is incredibly low and not statistically significant. The secretion of TNF-α in response to SEA was generally, but not significantly, higher in the Sm mouse group (13.07 ± 13.07 pg/mL) as opposed to the levels observed in Vaccine+Sm mice (0 pg/mL). The levels of IFN-γ secreted in response to SEA was not within detectable limits.

It was observed that the Sm group had slightly more elevated IL-6 levels (642.6 ± 300 pg/mL) than the Vaccine+Sm mouse group (632.6 ± 61.57 pg/mL). Similarly, for IL-4, the Sm mouse group had marginally higher levels in response to SEA (1.267 ± 1.267 pg/mL) when compared to the Vaccine+Sm group (0.986 ± 0.986 pg/mL). However, both of these Th2 did not show significance differences between the two infected mouse groups.

Levels of IL-10 were some of the highest to SEA. The Sm mouse group had the higher IL-10 levels (248.8 ± 51.14 pg/mL) than the Vaccine+Sm group (210.2 ± 51.14 pg/mL). But again, there was no statistical difference in IL-10 concentrations between these infected mouse groups.

3.2.6. Th1/Th2 bias in cytokine secretion

The bias of the immunity to Th1/Th2-type was evaluated by the ratio of IFN-γ to IL-6 in response to stimulation with the ConA peptide. As this was the positive control, all mouse groups elicited cytokine responses and the T helper type bias could be observed for each mouse group.

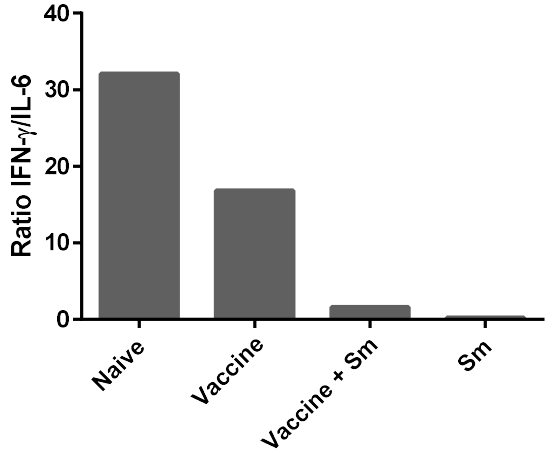

Figure 3.6 - IFN-γ/IL-6 ratio in response to ConA positive control peptide. A cytometric bead array was used to quantify the secretion of Th1 cytokine, IFN-γ and Th2 cytokine, IL-6, 12 days after SAAVI MVA-gp140 boost, after removing background responses.

The highest IFN-γ/IL6 ratio was observed in the naïve mouse group (32.07), showing the greatest Th1 bias. The Vaccine group had the second highest apparent Th1 bias (16.837), almost half the naïve group. The Sm mouse group had a ratio less than one (0.2), showing a greater secretion of IL-6 to ConA as opposed to IFN-γ secretion, and thus a strong Th2 bias. While slightly higher, the Vaccine+Sm mouse group showed a low ratio (1.607) when compared to the uninfected mouse groups.

3.2.7. Distribution of activated T cell memory responses

3.2.7.1 Activated T cells

Flow cytometry analysis was used to quantify the vaccine specific cytokine responses induced by the MVA gp140 vaccine. Unstimulated cells were included as a negative control, and this was controlled at 0.1% CD4+/CD8+ cells producing IFN-γ, TNF-α and IL-2 cytokines to confirm correct gating. The unvaccinated mouse groups elicited no cytokine responses to the vaccine peptides tested, as expected. However, no cytokine positive cells were detected for the other groups above this cut-off value using this gating strategy.

We then modified the gating strategy to determine the number of activated CD8+ and CD4+ T lymphocytes and the distribution of memory phenotypes for each group.

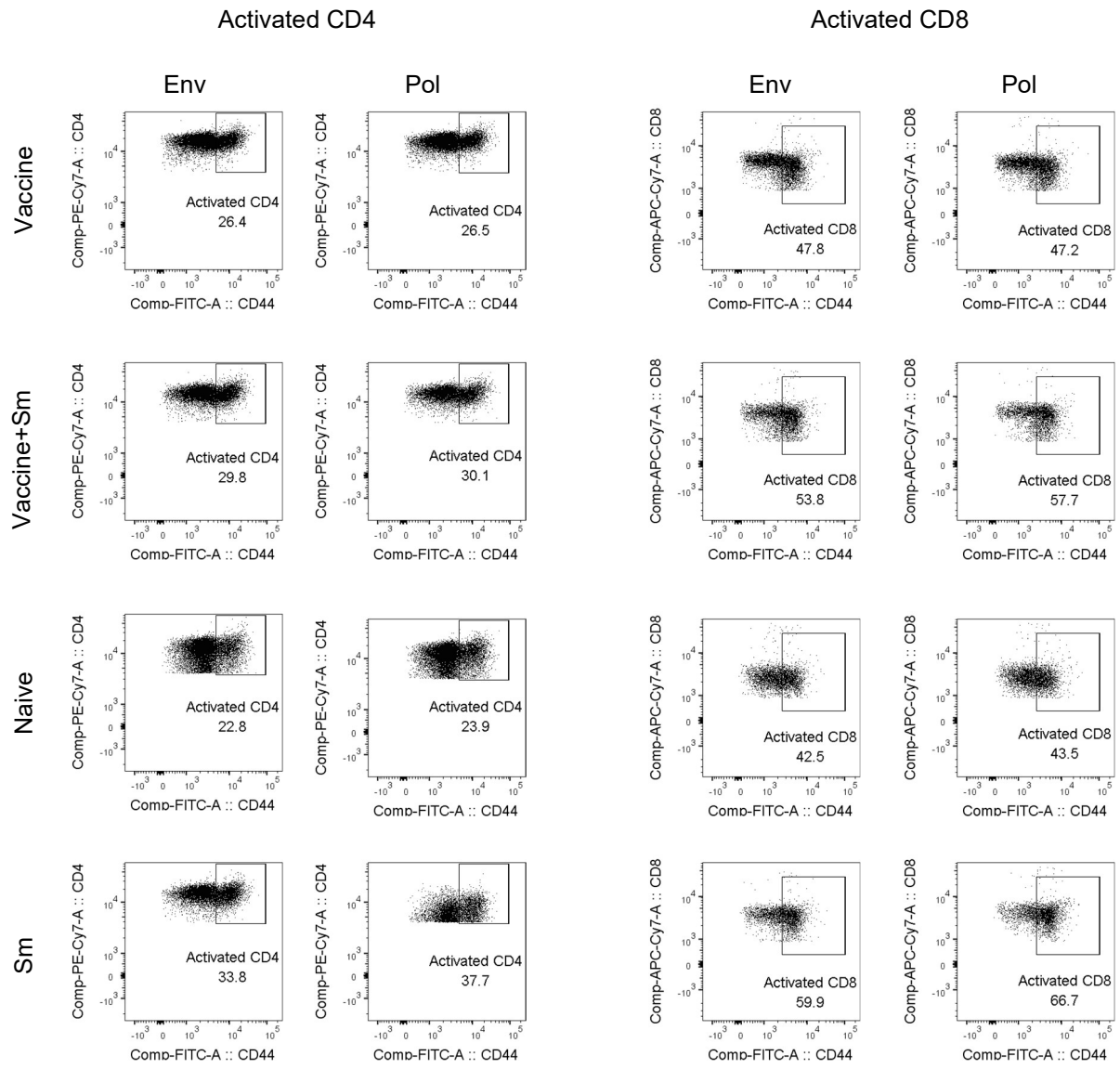

Figure 3.7 - Representative dot plot graphs showing HIV-1 peptide (Env, Pol) activated CD4+ and CD8+ T cells in Vaccine, Vaccine+Sm, Sm and Naïve groups. Splenocytes were stimulated with HIV peptides (Pol and Env) for 6hrs at 37°C, 5% CO_2. Staining of surface markers and ICS were performed using established protocol. The expression of CD44 (activation marker) were determined for CD4+ (left panel) and CD8+ (right panel) T cells gated on live CD3.

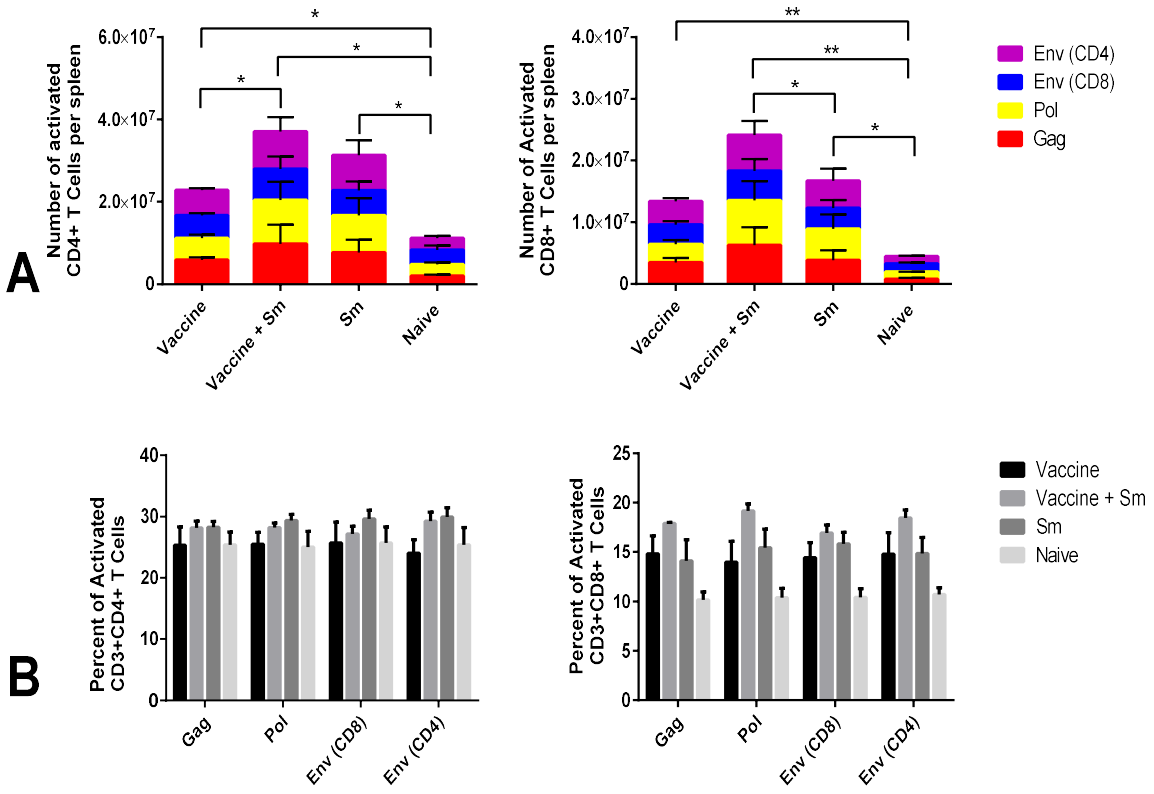

Figure 3.8 - Activated T cell responses to vaccine peptides. Vaccinated mouse T cell responses to vaccine peptides with or without infection. Magnitude of activated CD8+ and CD4+ T cells 12 days after SAAVI MVA-gp140 boost. Bars represent A) number of activated T cells per mouse spleen, and B) percent of activated T cells of live CD3+ cells. Representative averages and standard deviation of three independent experiments. (**p <0.01; *p < 0.05)

The accumulated activated CD8+ T cell populations elicited by the Vaccine mice were significantly greater than the naïve group ($p < 0.01$). The Vaccine+Sm group was significantly greater than the Sm ($p < 0.05$) and the naïve ($p < 0.01$) groups. However, the Vaccine group and the Vaccine+Sm groups did not differ with any statistical significance. Again, with the percentage of activated CD8+ T cells within the live CD3+ population, the Vaccine+Sm group roused a higher proportion of activated CD8+ T lymphocytes than the other three groups, but not with any significance.

However, the CD4+ T cell activation profiles did show a significant difference between the Vaccine and the Vaccine+Sm mouse groups ($p < 0.05$). The CD4+ T cells were more activated

in Vaccine+Sm mice when compared to those of the Vaccine mice. The naïve mouse group elicited significantly lower responses than the other three groups ($p < 0.05$). The percentage of activated CD4+ T cells within the live CD3+ population showed no significant differences amongst the groups, however, unlike that of the activated CD8+ T cells, the Sm group had the higher percentage of activated T cells per peptide.

3.2.7.2. Memory phenotype of activated T cells

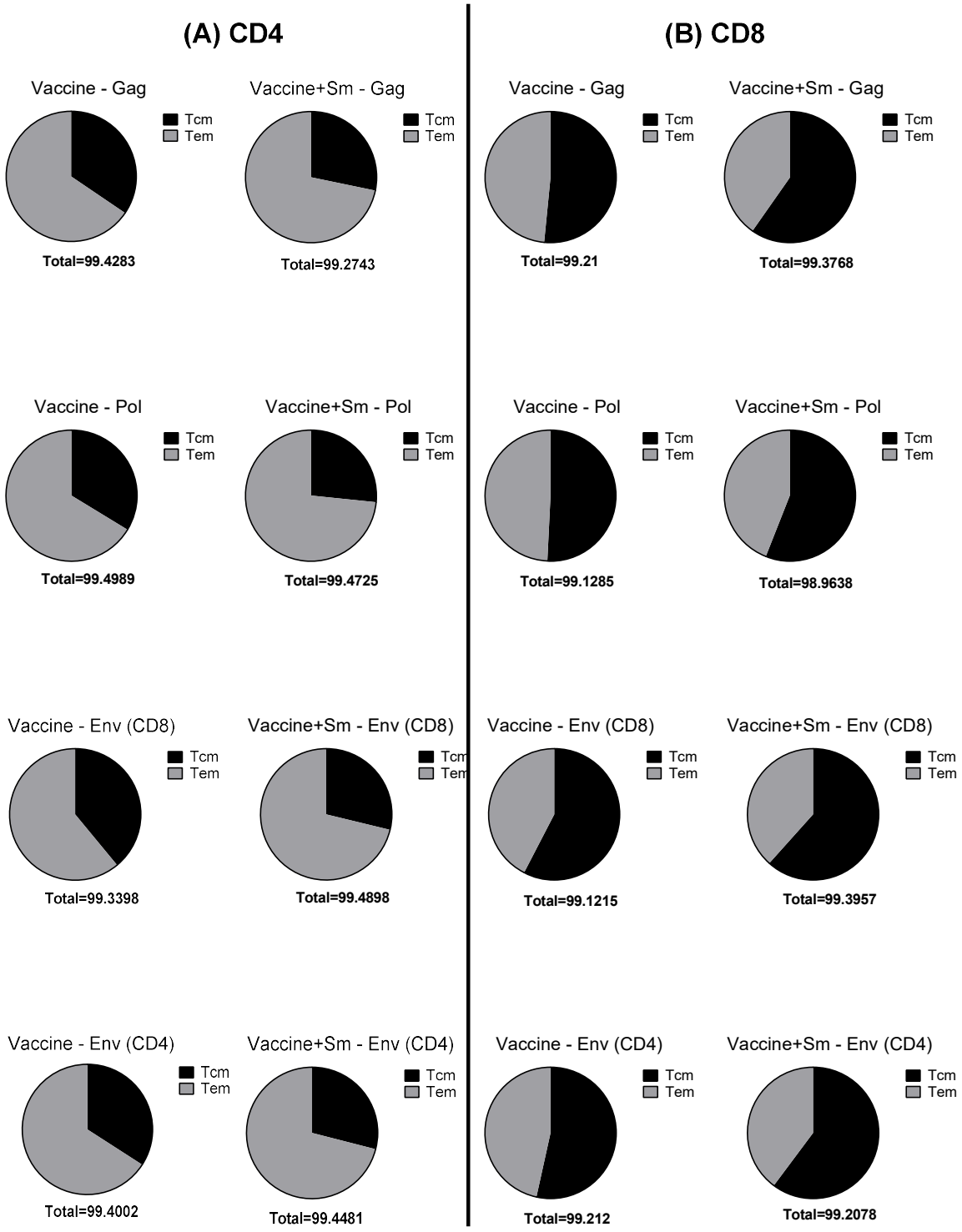

Figure 3.9 – Comparison of memory distribution profiles of activated T cells between Vaccine+Sm and Vaccine groups. The frequencies of effector (Tem) and central (Tcm) memory of A) activated CD4+ and B) activated CD8+ populations after stimulation of splenocytes 12 days after SAAVI MVA-gp140 boost.

The CD8+ T cell memory distribution did not differ greatly between the two mouse groups. The greatest difference between the groups was seen for the Gag peptide, whereby a greater central memory profile was seen for the Vaccine+Sm group (59.37 ± 14.10%) when compared to the Vaccine group (51.156 ± 14.53%). The effector memory profiled at averages between 38% and 43.5% for all the peptides in the Vaccine+Sm, and between 42% and 48.8% for the Vaccine group. The effector memory profile was much larger for globally activated CD4+ T cells, ranging from 65.6% (Env (CD8)) to 66% (Pol) for the Vaccine group, and 70.7% (Env (CD4)) to 73% (Pol) for the Vaccine+Sm group. The central memory phenotype for activated CD4 T cells was decreased in Vaccine+Sm mice (Gag; 28.09 ± 3.95%, Pol; 26.45 ± 4.66%, Env (CD4); 28.79 ± 6.95%, Env (CD8); 28.65 ± 3.29%) as opposed to the Vaccine group (Gag; 34.28 ± 8.35%, Pol; 33.53 ± 6.34%, Env(CD4); 33.92 ± 3.87%, Env (CD8); 38.76 ± 10.52%). Thus, a greater proportion of effector memory CD4+ T cells were observed in the Vaccine+Sm mouse group.

3.3. Humoral Responses

3.3.1. HIV Env protein-specific antibody responses

Env gp140-specific immunoglobulin responses were determined by IgG, IgG1, IgG2a and IgG2b ELISAs. A sample with the highest was OD reading was assigned as the standard and was included on all plates that were run. A plate blank was also included as well as a negative control serum from one of the mice in the naïve group and one of the mice from the Sm group.

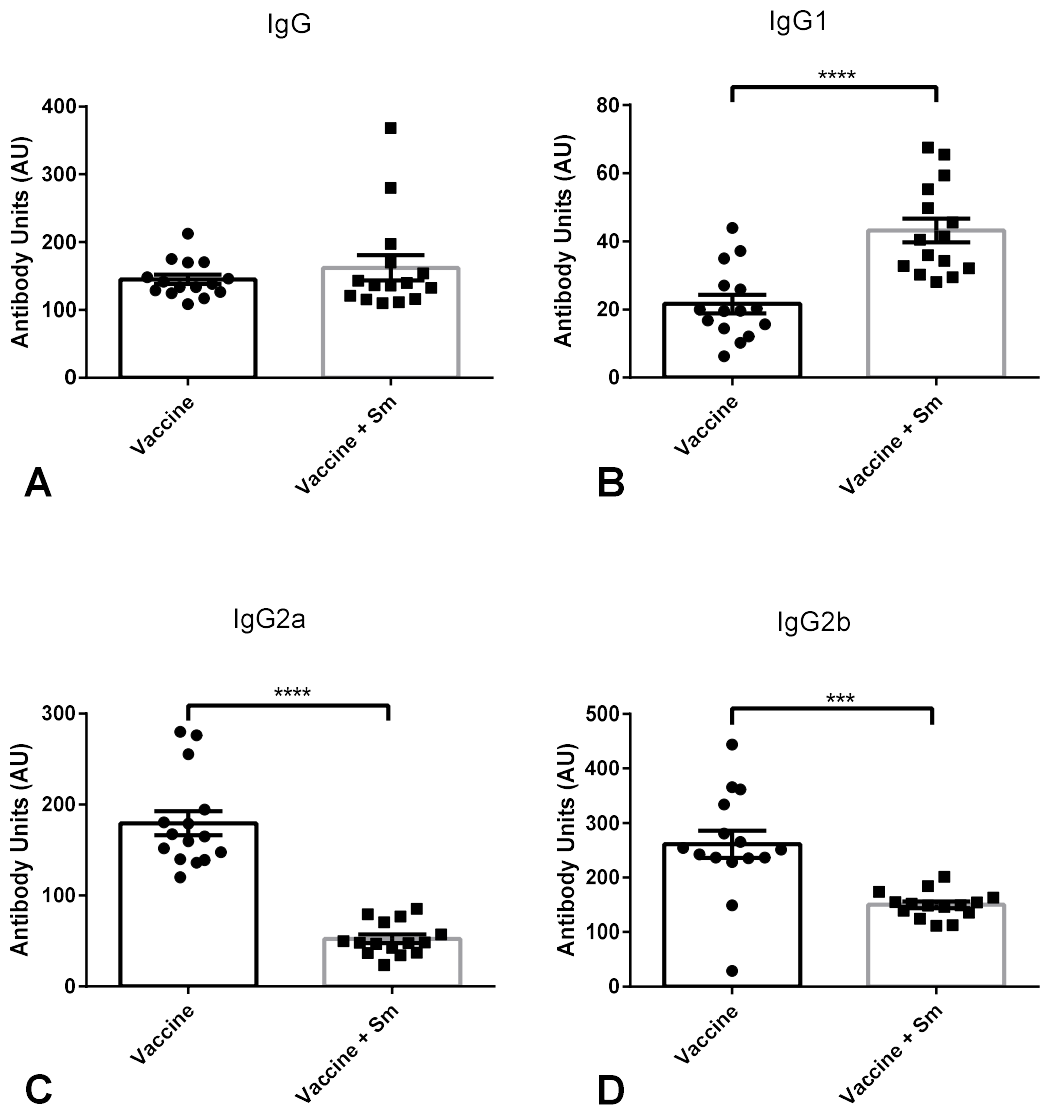

Figure 3.10 - Antibody responses to gp140. Serum of individual mice was run to demonstrate IgG (A), IgG1 (B), IgG2a (C) and IgG2b (D) responses to HIV Env gp140 protein subunit from the vaccination. Serum from each mouse was collected by cardiac puncture 12 days after SAAVI MVA-gp140 boost. Bars represent the average comparable concentrations of Ig for each group in gp140 Env antibody units (AU). (**** $p < 0.0001$; *** $p < 0.001$)

The Vaccine mice presented with lower levels of IgG (Figure 3.10A) in the sera (145.1 ± 6.97 AU) than in the sera of the mice that were Vaccine+Sm (162.2 ± 18.53 AU) but this was not a significant difference. However, IgG1 levels to gp140 were significantly different between the Vaccine mice (21.6 ± 2.71 AU) and the Vaccine+Sm mice (43.17 ± 3.45 AU) ($p < 0.0001$).

The differences in gp140-specific IgG2a levels were also statistically significant between the groups ($p < 0.0001$). The Vaccine+Sm mice group had a much lower IgG2a antibody response to gp140 (52.34 ± 4.67 AU) than the Vaccine mice (179.6 ± 13.21 AU). Similarly, for gp140-specific IgG2b antibody responses, the Vaccine mice had significantly higher levels of IgG2b in response to gp140 (261 ± 24.87 AU) than the Vaccine+Sm mice (150.2 ± 6.35 AU).

3.3.2. Non-specific immunoglobulin responses

Total levels of IgG1, IgG2a and IgG2b were elucidated for all four mouse groups from individual mouse sera. These responses were not differentiated *in vitro* between those prompted by the vaccination nor those brought about by the parasitic infection. All mouse groups demonstrated IgG1, IgG2a and IgG2b responses.

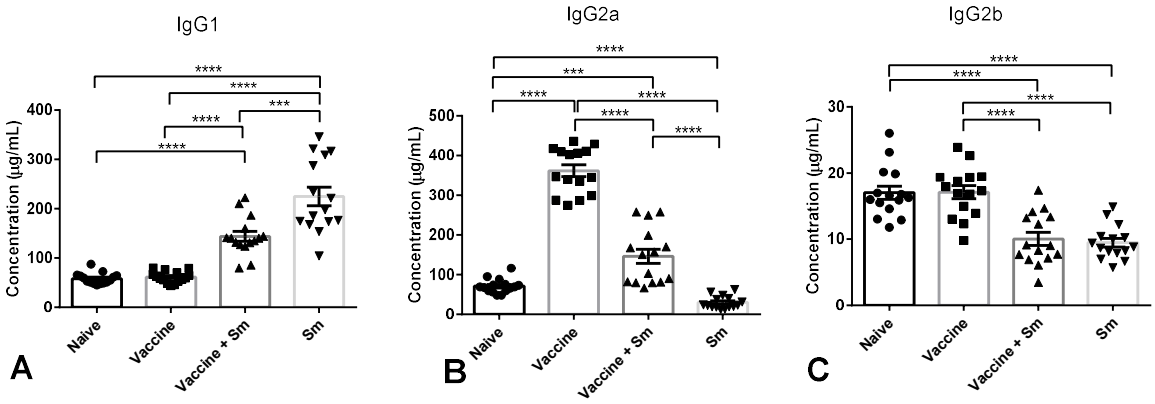

Figure 3.11 - Non-specific antibody responses. The total IgG1 (A), IgG2a (B) and IgG2b (C) levels were captured for each mouse sera which was collected by cardiac puncture 12 days after SAAVI MVA-gp140 boost. Bars represent the average comparable concentrations of Ig for each group (µg/mL). (**** $p < 0.0001$; *** $p < 0.001$)

IgG1 responses were significantly greater in the groups that were infected with the parasite than the uninfected groups. The Sm group elicited the highest IgG1 responses (224.8 ± 19.04 µg/mL) which was significantly higher than all three other mouse groups. The Vaccine+Sm group also demonstrated a significantly higher IgG1 response (143.8 ± 10.07 µg/mL) than the uninfected groups but significantly lower than the Sm group ($p < 0.001$). The naïve mice elicited the lowest average IgG1 responses (58.29 ± 2.91 µg/mL) but this was not significantly lower than the levels produced by the Vaccine mouse group (61.54 ± 2.98 µg/mL).

However, the total IgG2a responses were statistically highest in the sera of the Vaccine mice (361.5 ± 14.77 µg/mL). The Vaccine+Sm mice (145.7 ± 18.04 AU) had significantly lower levels of IgG2a ($p < 0.0001$) but statistically higher than the mice in the two groups that were unvaccinated (naïve group $p < 0.001$; Sm group $p < 0.0001$). The Sm mice had the lowest levels of IgG2a in the sera (29.58 ± 4.07 µg/mL), even significantly lower than the levels in the sera of the naïve mice (70.65 ± 4.66 µg/mL) ($p < 0.0001$).

Conversely, the sera collected from the naïve mice appeared to have high IgG2b levels (17.03 ± 0.99 µg/mL) which was significantly higher than the Sm mice ($p < 0.0001$) and the Vaccine+Sm mice ($p < 0.0001$). This was only slightly lower than the average IgG_{2b} levels in

the sera of the Vaccine mice (17.10 ± 0.98 µg/mL). The Sm mouse group had the lowest levels of IgG2b (9.48 ± 0.66 µg/mL), but not significantly lower than the levels in the sera of the Vaccine+Sm mice (10.03 ± 1 µg/mL).

3.4. Histology

3.4.1. Liver and spleen pathology

Livers and spleens were harvested from the mice and, thereafter, weighed before the spleens were homogenised and the livers were stored in formaldehyde. This indicates the probable degree of organ pathology (hepatomegaly and splenomegaly). This would be further confirmed by the identification of granulomas in the liver and total splenocyte count.

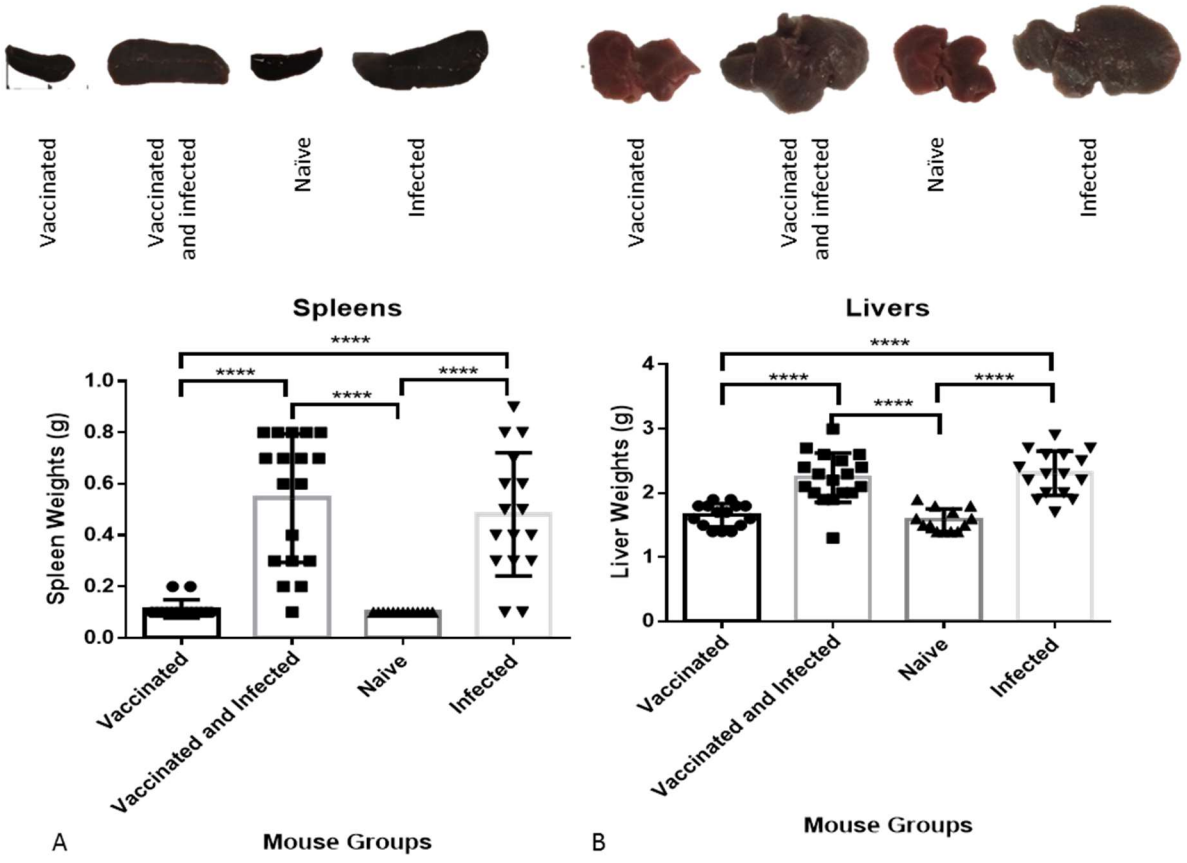

Figure 3.12 - The weights of organs harvested from mouse groups. Spleens (A) and livers (B) were harvested from each mouse 12 days after SAAVI MVA-gp140 boost. Bars represent the average weights for each group in grams. (**** $p < 0.0001$)

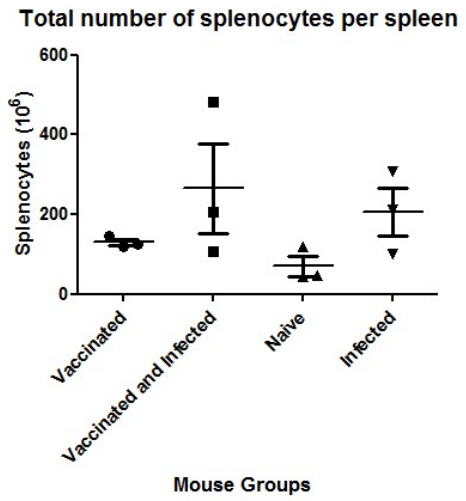

Figure 3.13 - The number of splenocytes per spleen harvested from mouse groups. Spleens were harvested from each mouse 12 days after SAAVI MVA-gp140 boost and pooled into each group. After the spleens were pooled, the concentration of splenocytes per ml were counted and extrapolated to total number of splenocytes. This could then be divided by the original number of spleens pooled per group. Data points represent each experiment repeat.

Spleen weights (Figure 3.12 A) suggested that the parasitic burden in the infected groups caused splenomegaly in these mice. The spleens of Vaccine+Sm group (0.544 ± 0.25 g) and those of the Sm group (0.481 ± 0.24 g) were statistically larger than the spleens of the uninfected groups; the Vaccine group (0.113 ± 0.035 g) and the naïve group (0.1 g) ($p < 0.0001$). Similarly, the average number of splenocytes collected per spleen for each group (Figure 3.13) was greater for infected mouse groups than those of the uninfected mouse groups, although this difference was not significant.

Liver weights (Figure 3.12B) also indicated that infected mice, regardless of whether or not the mice received the vaccination, had some degree of hepatomegaly. The livers of Vaccine+Sm group (2.239 ± 0.384 g) and the livers of the Sm group (2.306 ± 0.345 g) were significantly heavier than the livers of the naïve group (1.577 ± 0.174 g) and the Vaccine group (1.653 ± 0.181 g). This extra weight of infected mouse livers can be accounted for by the presence of granulomas, which was not observed in the livers of mice that were not exposed to *S. mansoni* cercariae (as shown in Figure 3.14).

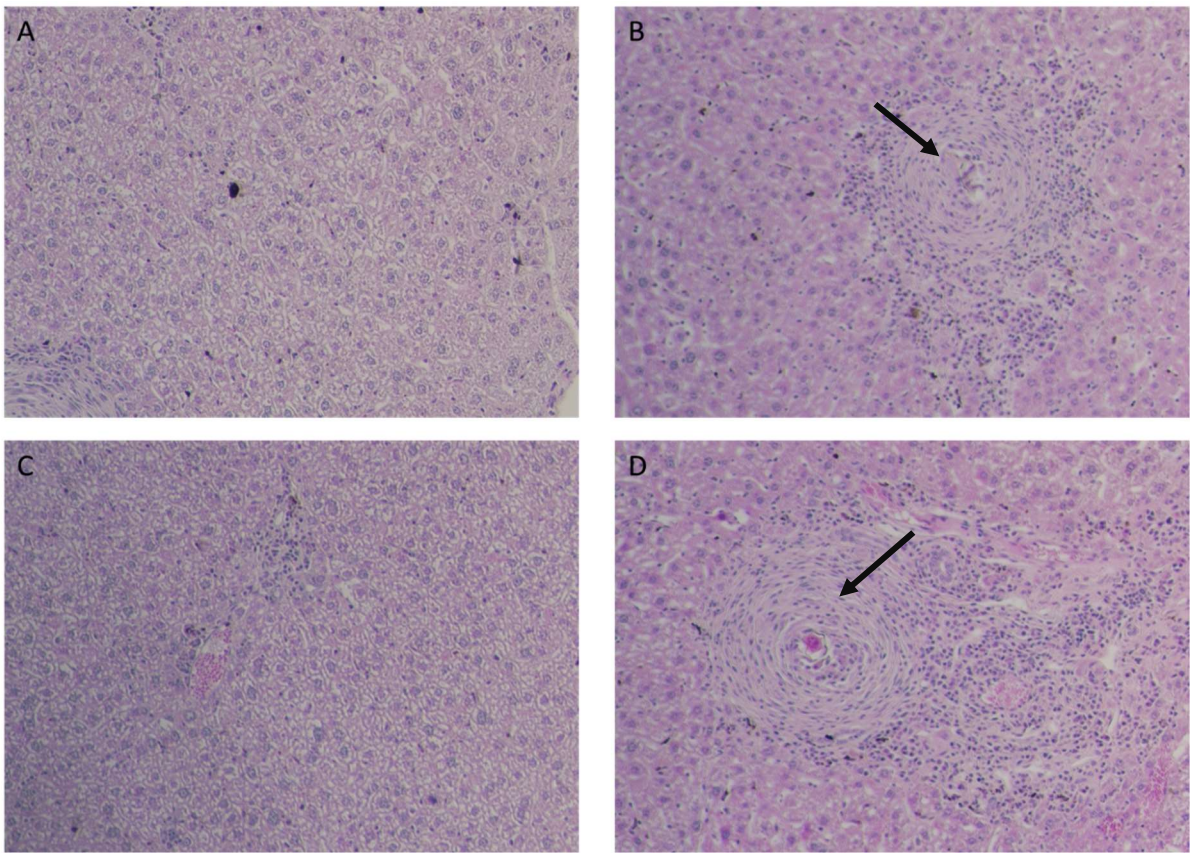

Figure 3.14 - Photographs of liver samples. H&E stained liver samples of A) Vaccine mice, B) Vaccine+Sm mice, C) Naïve mice, and D) Sm mice. Granulomas were observed in the mice that were infected with the parasite, regardless of administration of the vaccine. These granulomas were seen 68 days after infection with *S. mansoni* cercariae. The arrows (B and D) show granuloma formation.

4. Discussion

4.1 Overview

Parasitic worm infections and HIV are major health concerns in sub-Saharan Africa and the prevalence of these infections overlap substantially in many of these countries. It is expected that successful future HIV vaccines will be targeted to people living in these regions. Therefore, it is likely that a large proportion of vaccine recipients, including children, will also be chronically infected with parasitic helminths. It is likely that HIV vaccines will be administered to infants during childhood vaccination programmes to protect them from maternal HIV transmission through breastfeeding, and a booster vaccination will be required during adolescence to protect them against HIV via sexual transmission. Currently, it is not known how an otherwise effective HIV vaccine would perform in the background of ongoing chronic helminthiasis given that helminthic infections have been shown to down-regulate Th1 immune responses which are crucial for controlling viral infections (Reiner & Locksley, 1995; Hirahara & Nakayama, 2016). This is particularly important for SSA and other developing countries where HIV and helminth infections are co-endemic and large portion of children are already infected or at an extremely high risk of contracting these parasitic worm infections (Davis *et al.*, 2014; Shumbej *et al.*, 2015).

This study evaluated the immunogenicity of a candidate HIV vaccine regimen in the background of schistosomiasis to investigate whether helminth-induced immune responses have the potential to attenuate the boosting capacity of an HIV vaccine. We used two candidate HIV vaccines to induce both cell-mediated and antibody-mediated immune responses in a mouse model of schistosomiasis to generate data that would inform on the potential impact of chronic helminth infection on the immunogenicity induced by HIV vaccines.

4.2 Reproducing a mouse model for schistosomiasis infection

Schistosoma mansoni was chosen as a helminth model parasite due to its availability and established protocols in our collaborators facility, as well as the *S. mansoni* infection model being well-established in BALB/c mice (Actor *et al.*, 1993; Da'Dara *et al.*, 2006; du Plessis *et al.*, 2013). However, confirmation of the success of chronic *S. mansoni* infection was necessary before assuming cercariae-exposed mice were in fact chronically infected, and before conclusions could be drawn from the immunological data gathered. In a mouse model, literature describes schistosomiasis pathology in mice as being chiefly associated with a granulomatous responses to parasite eggs that become lodged in the liver and intestinal tissue (Hams, Aviello & Fallon, 2013; Lundy & Lukacs, 2013). Thus, validation of chronic *S. mansoni* infection in the mice challenged with the helminth cercariae could be achieved at the experimental endpoint by observation of granulomatous inflammation in the liver and splenomegaly.

The histology of the livers confirmed chronic infection at a physiological level by the presence of granulomas in the tissue (Figure 3.14). Granulomas were observed only in the mice that were exposed to *S. mansoni* cercariae. Similarly, the livers of helminth-infected mice were observed to be inflamed and of a greater average weight than the livers of unexposed mice (Figure 3.12 B). Additionally, splenomegaly was confirmed by the weights of the spleens and number of splenocytes of the infected mice when compared to the unchallenged mice. As stated before, these physiological deviances are expected of chronic helminth infection and play a role in driving Th2 responses (Pearce *et al.*, 2004; Hotez *et al.*, 2008). Thus, one can conclude that the mice challenged with the *S. mansoni* cercariae in the current study were indeed chronically infected, *vis a vis* the mice that went unchallenged were uninfected.

4.3 Helminth-induced down-regulation of Th1 responses

To demonstrate Th1/Th2 biasing and thus down-regulation of Th1 responses due to chronic helminth infection, the ratio of secreted IFN-γ to IL-6 (the cytokines secreted in the highest levels) in response to ConA was calculated. The results of the current study showed that the naïve mice had a greater Th1 biasing (Figure 3.6). The Vaccine mice had a ratio of almost half the levels in the naïve mice, indicating a slight shift to Th2 due to the vaccine. This may be due to the Th2 cytokines being secreted in response to the vaccine peptides as well as Th1 cytokines. The Sm mice had a ratio below 1. This shows a remarkable Th2 immune biasing in response due to the helminth, a finding supported by previous studies conducted on the immune system of humans, as well as animals (Nutman, Kumaraswami & Ottesen, 1987; Grzych *et al.*, 1991; Pearce *et al.*, 1991, 2004, Araujo *et al.*, 1994, 1996; Sartono *et al.*, 1995; McKee & Pearce, 2004; Yin *et al.*, 2012). Thus, the baseline of mouse immunity to helminth being Th2 biased and mouse immunity to the vaccine being Th1 biased was established. The Vaccine+Sm mice had a ratio of less than 2. This is a vast decrease from the Vaccine mice, indicative of a further shift to Th2 biasing. This finding is supported by an earlier study conducted on human subjects by Sabin and colleagues (1996), where subjects infected with *S. mansoni* were assessed for their ability to mount Th1 IFN-γ responses to a tetanus toxoid (TT) vaccine as compared to an uninfected control group (Sabin *et al.*, 1996). The resulting data reported that the IFN-γ produced in response to TT was significantly diminished in the helminth-infected group (Sabin *et al.*, 1996). A more recent study by Yin *et al.* (2012) demonstrated that if the HIV DNA vaccination was administered before helminth infection, the vaccine is able to significantly reduce IL-13 expression and Th2 biasing induced by the chronic parasitic infection, and enhances expression of Th1 cytokine TNF-α. Interestingly, these authors also reported that the vaccine had no significant influence on IL-4 or IFN-γ expression, when looking at the cytokines in the CD4+ cells (Yin *et al.*, 2012).

To further support these data, total IgG1, IgG2a and IgG2b levels in the sera were quantified. It has long been established that the isotype of serum antibodies can be used to determine Th lymphocyte bias by virtue of the effects that certain cytokines have on immunoglobulin isotype selection in B cells (Finkelman *et al.*, 1990; Mcsorley & Maizels, 2012). As such, IL-4, a Th2 cytokine, can induce B cells to produce IgG1 while simultaneously inhibiting IgG2a production (Snapper & Paul, 1987a,b; Deenick, Hasbold & Hodgkin, 2005). Conversely, IFN-γ, a Th1 cytokine, promotes IgG2a secretion and inhibit IgG1 secretion (Stevens *et al.*, 1988; Deenick, Hasbold & Hodgkin, 2005). Thus, the subtype of IgG secreted can indicate the cytokines being produced by T cells and the Th biasing. We therefore also determined the total levels of the three subtypes in all the mouse groups and found that the Vaccine mice had the highest levels of IgG2a and IgG2b and the lowest levels of IgG1 indicating a very strong Th1 immunity, expected from a strong vaccine induced immunity. Alternatively, the Sm mice had the highest levels of IgG1 and the lowest levels of IgG2a and IgG2b, again, indicative of a very strong chronic helminth-induced Th2 immunity. The Vaccine+Sm mice had responses between these groups, showing that the vaccine does induce a Th1 response but that the helminth infection attenuates this response and shifts the immunity. Vaccine+Sm mice had similar IgG2b levels to the Sm mice.

These data indicate that there was a biasing towards a predominant Th2 immune response in mice infected with the helminth. This can be reasoned to be caused by the immune responses caused by the chronic stage of infection (Maizels & McSorley, 2016).

4.4 T cell responses

The ability of the vaccine to induce Gag-, Pol- and Env-specific CD4+ and CD8+ T cell responses was elucidated by IFN-γ and IL-2 ELISPOT, CBA and ICS assays 2 weeks post boost. The IFN-γ T cell responses as well as cytokine secretions to vaccine peptides were

shown to be attenuated in schistosome-infected mice. Also noted was an observed shift from central to effector memory CD4+ T cell phenotype in these infected mice.

4.4.1 IFN-γ- and IL-2-producing T cell responses

As shown in Figure 3.1, vaccine-specific IFN-γ-producing T cell responses were significantly diminished in Vaccine+Sm mice. In contrast, the accumulative IL-2-producing T cells (Figure 3.2) were not significantly diminished in these Vaccine+Sm mice. However, there was a significant decrease in IL-2-producing T cells in response to RT (CD4). These diminished IFN-γ and IL-2 responses to viral antigens are in line with a previous study conducted by Actor and colleagues (1993) where they suggested this immune disability in helminth-infected mice might prevent the mice from clearing viral infections. A study by Da'Dara *et al.* (2006) concurred with this finding with a *S. mansoni*-infection in a mouse model whereby a significant decrease in the frequencies of IFN-γ-producing CD8+ T cells was reported in vaccinated and infected mice compared to vaccinated and uninfected mice. These authors went on to state that the chronic helminth infection nearly rendered the candidate vaccines unable to elicit Gag-specific CD8+ T cell responses. Apiwattanakul and colleagues (2014) reported that they too found that the effectiveness and immunogenicity of their pneumococcal vaccine was compromised in helminth-infected mice. These authors, as with others, conclude that the attenuation of Th1 responses, such as IFN-γ production, together with increased production of anti-inflammatory and Th2 cytokines in helminth-infected vaccine recipients correlate with impaired vaccine efficacy (Elias *et al.*, 2001, 2005, 2008; Resende *et al.*, 2007; Da'dara & Harn, 2010; Apiwattanakul *et al.*, 2014).

Nonetheless, the responses to SEA amongst mice that were vaccinated or not remained statistically unaltered. This indicates that the vaccine does not inhibit the immunity mounted against the parasitic egg deposition in the tissues. As responses to SEA are an indication of egg burden and degree of immunopathology in the host, this data suggests that the candidate

HIV vaccine used in this study does not worsen the schistosomiasis pathology, validating its safety for administration helminth-infected recipients (Abdelbagi & Eltayeb Omer, 2015).

4.4.2 Th1/T2 cytokine secretion

In this study, we also investigated the *ex vivo* secretion of key Th1 and Th2 cytokines in response to vaccine peptides between the Vaccine mouse group and the Vaccine+Sm mouse group. Th2-type responses, commonly associated with chronic schistosomiasis, are typically characterized by increases in the levels of IL-4, IL-6 and IL- 13 (Yin *et al.*, 2012). Conversely, the vaccine would be expected to induce high expression of Th1 cytokines such as IFN-γ, IL-2 and TNF-α which can inhibit the Th2 responses and vice versa. We demonstrated that Th1 cytokine secretion is generally decreased in response to vaccine peptides in the Vaccine+Sm mouse group. The finding of a significant decrease of TNF-α secretion levels in Vaccine+Sm mice is supported by an earlier study by Yin *et al.* (2012) that illustrated a significant decrease in the intracellular levels of this Th1 cytokine in infected and vaccinated mice.

Surprisingly, both Th2 cytokines quantified in this study showed significantly decreased secretion levels to vaccine peptides in Vaccine+Sm mice when compared to the Vaccine mouse group. This suggests that the ability of the vaccine to stimulate IL-6 and IL-4 in response to the peptides was also attenuated by the presence of the helminth. This finding is in contradiction with the study conducted by Yin and colleagues (2012) where by vaccination with their HIV vaccine candidate to mice infected with *S. mansoni* showed no significant differences in the levels of the Th2 cytokine that they assayed; IL-4. This may be due to the fact that these authors assayed intracellular cytokine of only IL-4, and by ICS and FACS analysis, rather than a cytometric bead array, which measures the amount of cytokines excreted out into the medium, as used in our study. Yin *et al.* (2012) measured total cytokine within T cells, whereas this study measured cytokine secreted specifically to vaccine peptides.

Therefore, this study demonstrates that helminth infection may suppress cytokine secretion to vaccine peptides, significantly for Th1 cytokine TNF-α, and Th2 cytokines IL-6 and IL-4.

Da'dara and colleagues (2010) found that when stimulated with SEA splenocytes from infected mice had greater IL-4, IL-10 and IFN-γ secretion than naïve mice. But when the same splenocytes were stimulated with ConA, infected mice only showed higher secretion of the Th2 cytokine (IL-4) and the regulatory cytokine (IL-10), but lower levels of IFN-γ secretion, when compared to the levels of naïve mice. In this study, the cytokines secreted in response to SEA showed no significant differences amongst infected mouse groups. However, IL-4 and IL-10 were slightly elevated in the Sm mouse group.

4.4.3 Activated CD4+/CD8+ T cells

We were unable to detect vaccine peptide-specific cytokine-positive CD4+ and CD8+ populations, most probably because the cytometry panel used was not optimal with regard to compensation for cytokines and their assigned PE fluorochrome. As this is a tiny population of cells, incorrect compensation could have resulted in non-detection by a similar fluorochrome and thereby masking the cytokine fluorescence in the PE channel. Therefore, this study went on to analyse the globally activated T cells (which were non-specific to HIV peptides) and their memory phenotypes of responding for comparisons across the various groups.

The frequencies of globally-activated (CD3+CD4+CD44+) T cells in the spleens of Vaccine+Sm mice were significantly higher than Vaccine mice. This was similar to trends seen for activated CD3+CD8+CD44+ T cells. We delineated these cells further into effector or central memory T cell populations. Although these two broad subsets are similar in expression of many costimulatory molecules, adhesion, and chemokine receptors, these populations have different functions (Schenkel & Masopust, 2014). Central memory T cells (Tcm) cells express CD44+ and CD62L+ and they secrete high levels of IL-2. After

stimulation and proliferation, they differentiate to effector cells able to produce effector cytokines (MacLeod *et al.*, 2009; McKinstry, Strutt & Swain, 2010). Effector T cells (Tem) cells also express CD44+ but not CD62L+ and can produce effector cytokines such as IFN-γ and IL-4 (Lefrancois, 2002).

While there was a slight increase in percentage of central memory CD8+ T cells for the Vaccine+Sm group, the memory profiles of activated CD8+ T cells were comparable between infected and uninfected mice that were vaccinated, a finding supported by the results from the study by Shollenberger and colleagues (2013). However, there was more variation in the memory phenotypes of activated CD4+ T cells whereby Vaccine+Sm mice had a greater proportion of Tem and a smaller population of Tcm memory T cells. It could be proposed that the increased IFN-γ and IL-4 cytokine levels and the lower levels of IL-2 observed in these Vaccine+Sm mice may be due to this shift in memory profile from Tcm to Tem. This shift in memory, and thus cytokine production is also supportive of the Th2 shift expected and observed in helminth-infected mice. However, the memory phenotypes of the activated cells may be different from ELISPOT responding cells, as the activated CD3 cells detected by the ICS may not be secreting cytokine. As such, the observable coincidences would need to be further investigated.

The data is supported by previous studies on T cell memory profiles for helminth infection in mice (Shollenberger *et al.*, 2013; Thawer *et al.*, 2014). In the study by Shollenberger and colleagues (2013), they stated that the biological relevance of this shift in activated CD4+ memory profile was unclear due to their lack of significant difference observed in Gag-specific IFN-γ ELISPOT results between infected and uninfected mice. However, in this study, while we also saw no significant differences in T cell responses to Gag (CD4 and CD8) peptide, we did observe significant differences in the IFN-γ ELISPOT responses to the RT (CD4) and Env (CD4) peptide. This may clear up the uncertainty behind the biological relevance.

4.5 Antibody responses

Certain types of antibody responses to specific HIV antigens have been proposed to correlate with protection (Tomaras & Plotkin, 2017). For example, the RV144 trail highlighted that IgG1 and IgG3 responses to the V1V2 regions of gp120 may be involved in protection against HIV-1 infection (Haynes *et al.*, 2012; Tomaras & Plotkin, 2017).

When stimulated by Th1 cytokines, B cells can produce IgG2a, whereas Th2 cytokines can induce secretion of Th2-type antibodies, such as IgG1. IgG2b antibody is indicative of Treg T cells. As such, the levels of these three isotypes of IgG, as well as complete IgG, specific to gp140 subunit of Env injected as part of the vaccine regimen, were analysed between the mouse groups. These data show that while IgG levels as a whole to gp140 did not show a significant difference, the subclasses showed that the Vaccine+Sm mice had a significant increase in gp140-specific IgG1, and significantly lower levels of gp140-specific IgG2a and IgG2b when compared to the Vaccine mice. Thus, the Vaccine+Sm mice have less Th1-type gp140-specific antibody levels in the sera than Vaccine mice. However, the levels of Th2-type antibodies specific to gp140 were significantly higher in the Vaccine+Sm mice. As these immune interactions are complex, these data can only suggest Th2 biasing caused by the helminth. The observed increase in Th2-type gp140-specific IgG1 levels in schistosome-infected vaccinated mice may not necessarily be detrimental to the efficacy of the vaccine.

4.6 Limitations

Several studies have demonstrated the importance of HIV Env-specific neutralizing (Baba *et al.*, 2000; Mascola *et al.*, 2000; Burton *et al.*, 2004; Barouch *et al.*, 2013; Buchbinder *et al.*, 2014; Goepfert *et al.*, 2014) and non-neutralizing antibodies (Aasa-Chapman *et al.*, 2005; Holl *et al.,* 2006) in protection against HIV. Therefore, generation of high titres of both types of antibodies is considered as an important attribute of HIV vaccine-generated immune

protection. To generate an effective antibody response by vaccination, antigen-stimulated B cells must undergo class switch recombination and affinity maturation following contact with T cells in the germinal centre. Repeated exposure to related immunogens by means of boosting inoculations has been thought to be a possible method to drive the B cells to produce broadly neutralizing antibodies (Dosenovic *et al.,* 2015). Neutralizing and non-neutralizing antibodies such as ADCC-mediating antibodies elicited by the vaccines against HIV-1 could also have been investigated in this mouse model by means of a standard TZM-bl assay (Pollara *et al.*, 2011 and Sarzotti-Kelsoe *et al.*, 2014 respectively). However, this was not possible because these assays have not been established in our laboratory and our resources were limited. Other functional B-cell assays such as evaluation of avidity of gp140-specific antibodies by a sodium thiocyanate-displacement ELISA (Emmer *et al.,* 2016) or by means of a B cell ELISPOT (Chege *et al.,* 2017) were also not available. As such, future studies could assess the vaccine-specific B cell immunity to determine the helminth-associated Th2 biasing as suggested by the current study, attenuates the capacity of candidate HIV vaccines to elicit such responses.

Vaccine-generated cellular responses that are mediated by IFN-γ, IL-2 and TNF-α are also thought to be important for a HIV vaccine (Morel & Turner, 2010; Belisle *et al.,* 2011; Yin *et al.,* 2012). It is therefore important to characterise the phenotypes of T cells producing these cytokines. The current study sought to determine the memory phenotypes and the proportions of cells that produced these cytokines by flow cytometry. This was not successful, possibly because the flow cytometry panel was not optimally functional. In future, an optimized cytokine panel for the detection of intracellular cytokines by ICS and FACS would prove useful to investigate whether helminthiasis changes the magnitude and frequencies of vaccine-specific memory T phenotypes. More accurate indications of IFN-γ, TNF-α and IL-2 producing splenocytes of the mice could clarify more of the immunological responses of the combination of the vaccine and the helminth within the mouse model.

4.7 Conclusion

In conclusion, the findings of this study suggest that contracting a chronic helminth infection in between primary and boost vaccinations may have some disastrous effects on the vaccine-generated Th1 responses such as IFN-γ T cell responses. The secretion of Th1-type cytokines, and indeed Th2 cytokines, may also be inhibited by helminth infection. The memory phenotype of activated CD4+ T cells is shifted by the helminth to a greater effector memory profile, and a greater central memory profile of activated CD8+ T cells. Importantly, and even surprisingly, the Env-specific humoral responses may be altered by helminths, leading to a bias to Th2 responses which could potentially dampen the efficacy of the vaccine-induced immunity. This study justifies further studies, possibly using a nonhuman primate model, to obtain an in-depth understanding of these immune responses.

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Appendix 1

10x Lepple Buffer

- 5.6 g $CaCl_2$
- 12.28 g $MgSO_4.7H_2O$
- 0.43 g K_2SO_4
- 4.2 g $NaHCO_3$
- 0.48 mL $FeCl_3.6H_2O$ solution (2.5 g dissolve in 50 mL H_20)

The chemicals were dissolved in 1 L distilled water, except the $NaHCO_3$, and allowed to stand for 1 hour. The $NaHCO_3$ was added and the solution was made up to 10 L with distilled water.

To make a working stock of 1x Lepple buffer, 1 part 10x Lepple buffer was added to 9 parts distilled water.