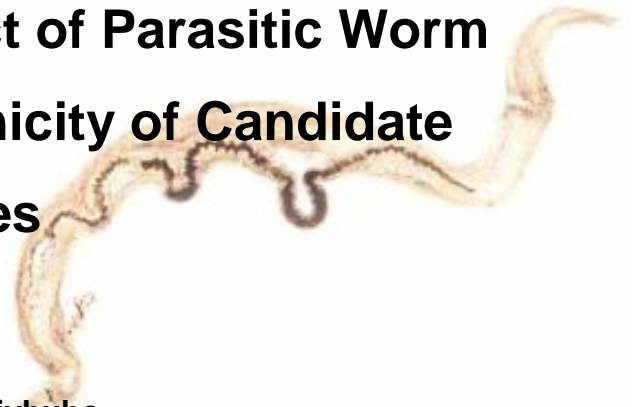




**UNIVERSITY OF CAPE TOWN**  
IYUNIVESITHI YASEKAPA • UNIVERSITEIT VAN KAAPSTAD



# **An Investigation of the Impact of Parasitic Worm Infection on the Immunogenicity of Candidate HIV Vaccines**



By

**Godfrey Azwindini Dzhivhuho**

Thesis submitted to the University of Cape Town in fulfilment of the degree of  
Doctor of Philosophy (PhD) Medical Virology

Division of Medical Virology

Department of Pathology

Faculty of Health Sciences

University of Cape Town

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## Preface and Declaration

All experimental work described in this thesis was carried out in the Divisions of Medical Virology and Immunology, University of Cape Town, from July 2014 to December 2016, under the supervision of Dr Gerald Chege and co-supervision of Prof Anna-Lise Williamson and A/Prof William Horsnell.

I, Godfrey A Dzhivhuho, declare that these studies represent original work by the author and have not otherwise been submitted in any form for any degree or diploma at any University. Where use has been made of the work of others, it is duly acknowledged in the text and reference. Permission of use of Figures and images from other peer-reviewed journals has been granted by the University of Cape Town, provided they are duly acknowledged in the text and reference.

Signed by candidate

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.....

**Student: Mr Godfrey A, Dzhivhuho**

To my loving parents,  
Mr David Dzhivhuho and Mrs Sheila Dzhivhuho

Isaiah 41:10

So do not fear, for I am with you;  
do not be dismayed, for I am your God.  
I will strengthen you and help you;  
I will uphold you with my righteous right hand.

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## LIST OF ABBREVIATIONS AND ACRONYMS

%	Percent	DNA	Deoxyribonucleic acid
°C	Degrees Celsius	ELISA	Enzyme-linked immunosorbent assay
®	Registered	ELISPOT	Enzyme-linked immunospot
µg	Micrograms	Env	Envelope
µl	Microliters	FBS	Foetal bovine serum
µm	Micrometres	g	Grams
Ad5	adenovirus serotype 5	Gag	Group specific antigen
ADCC	antibody-dependent cellular cytotoxicity	GALT	Gut-associated lymphoid tissues
ADCVI	antibody-dependent cell-mediated viral inhibition	GC	germinal centre
AIDS	Acquired Immunodeficiency Syndrome	GI	gastrointestinal
ALB	Albendazole	gp	Glycoprotein
APC	Antigen-presenting cell	HAART	anti-retroviral drugs and highly active antiretroviral therapy
ARV	Anti-retroviral	HIV	human immunodeficiency virus
BALB	Bagg albino	HIV-1C	human immunodeficiency virus subtype C
BCG	Bacille Calmette Guérin	hrs	hours
BFA	Brefeldin A	i.m	intramuscularly
bNAb	Broadly neutralising antibody	i.p	intraperitoneal
CA	Capsid	i.v	Intravenous
CBA	Cytometric bead array	ICS	intracellular cytokine staining
CD4	Cluster of Differentiation antigen number 4	IFN-γ	interferon gamma
CD4+	CD 4 positive effector T lymphocytes	Ig	immunoglobulin
CD8	Cluster of Differentiation antigen number 8	IL	interleukin
CD8+	CD 8 positive effector T lymphocytes	IDUs	Injection drug users
CMV	Cytomegalovirus	LPS	Lipopolysaccharide
CO <sub>2</sub>	Carbon dioxide	LTNPs	Long-term non-progressors
Con A	Concanavalin A	mDCs	Myeloid dendritic cells
CoP	Correlates of protection	MIP-1α	Macrophage inflammatory proteins 1alpha
CTL	Cytotoxic T lymphocyte	ml	Millilitre
DC	Dendritic cell	mM	millimolar

---

MMR	trivalent Mumps/Measles/Rubella	SmE	<i>Schistosoma mansoni</i> eggs
MPER	membrane proximal region	SSA	sub-Saharan Africa
mRNA	Messenger RNA	Tat	trans-activator of transcription
MSM	Men who have sex with men	TB	tuberculosis
MVA	Modified vaccinia Ankara	T <sub>CM</sub>	T cell central memory
Nef	negative infectivity factor	TCR	T cell receptor
ng	Nanograms	T <sub>EM</sub>	T cell effector memory
nAb	neutralizing antibodies	Th1/2	T helper cell type 1/type 2
NHP	non-human primates	TNF- $\alpha$	tumour necrosis factor-alpha
NIAID	National Institute of Allergy and Infectious Disease	U	Unit
nm	nanometre	UCT	University of Cape Town
OD	optical density	Vif	viral infectivity factor
p.i	Post infection	Vpr	viral protein R
PBMC	peripheral blood mononuclear cells	Vpu	viral protein U
PBS	phosphate buffered saline	VZV	Varicella Zoster Virus
pDCs	plasmacytoid dendritic cells	WHO	World Health Organization
pfu	Plaque forming units		
pg	picogram		
Pol	polymerase		
PR	protease		
PZQ	Praziquantel		
RANTES	regulated upon activation, normal T cell expressed and secreted		
Rev	regulator of expression of virion proteins		
RM	rhesus macaques		
RT	Reverse transcriptase		
S.C	subcutaneously		
SAAVI	South African AIDS Vaccine Initiative		
SAVP	South African Vaccine Producers		
SD	standard deviation		
SEA	Soluble egg antigen		
SFU	Spot-forming units		
SIV	simian immunodeficiency virus		
Sm	<i>Schistosoma mansoni</i>		

---

## ABSTRACT

Development of effective and affordable HIV vaccines is one of the best and cost-effective strategies for controlling the HIV epidemic and a top priority in endemic areas. Successful future candidate HIV vaccines are expected to elicit effective antibody and T cell-mediated responses. This is envisaged to be attained through induction of potent T cell-mediated immune responses to control viral replication in the tissues and disease progression as well as a durable antibody immune response which comprises of broadly neutralizing antibodies to block virus entry at the mucosal sites. Both types of immune responses are influenced by a T helper cell type 1 (Th1) immune response. In developing worlds such as Sub-Saharan Africa co-infections of HIV and schistosomiasis are common. Helminth infections such as schistosomiasis induce strong Th2 biased immune responses that have been reported to alter HBV, BCG, Tetanus toxoid and some candidate HIV vaccine-specific immune responses. Because Th1 and Th2 are almost mutually exclusive, it is suggested that, in the presence of chronic helminthic infections, Th1 responses elicited by HIV vaccine may be attenuated, hence, reduce vaccine efficiency. On the other hand, vaccination with an effective HIV vaccine might shift the immune bias towards a Th1 response, resulting in worsening of the helminth-associated pathology, thus, making the vaccine unsafe to the recipients.

This study aimed at investigating if chronic helminth infection (*Schistosoma mansoni*: Sm) has a negative impact on the immunogenicity of HIV vaccine candidates (SAAV DNA-C2, SAAVI MVA-C, and Env gp140 protein) previously shown to induce cellular, mixed and antibody immune responses in mice. The objectives of this study were to (i) infect mice with live *Schistosoma mansoni* infection in order to induce a predominantly Th2 immune response in a mouse model; (ii) evaluate if helminth-induced Th2 immune biasing negatively affects responses to candidate HIV vaccines; (iii) evaluate the ability of antihelminthic chemotherapy in restoring normal responses to HIV vaccines in helminth infected mice; (iv) evaluate if HIV vaccine that predominantly induces strong cellular immune responses result in worsening of the helminth-associated pathology and (v) evaluate if helminth eggs in the absence of live helminth infection drives a Th2-dominant response that can affect HIV vaccine responses.

The BALB/c mouse model has been used extensively at the University of Cape Town for studying the Sm-associated immunology as well as for initial evaluation of candidate HIV vaccines. Female BALB/c mice were either chronically infected with Sm cercariae or inoculated with Sm eggs (SmE) before being subsequently vaccinated twice, 4 weeks apart with three vaccination regimes that elicit cellular (SAAVI DNA-C2 prime + MVA-C boost denoted: DNA+MVA), antibody (gp140 Env protein) and mixed cellular and antibody (SAAVI MVA-C prime + gp140 Env protein boost denoted: MVA+gp140) responses. Some groups of mice infected with live Sm were treated twice with praziquantel (PZQ) prior to vaccination. Spleens, blood and livers were harvested for analysis of vaccine-specific T cell, antibody responses and histological studies using ELISpot, ELISA, CBA, ICS staining, H&E/CAB staining and hydroxyproline assay.

Our findings demonstrated that in a mouse model, chronic Sm-infection induces a predominantly Th2 immune biased response marked with elevated parasite-specific IL-4; IL-6 and IL-10 as well as elevated total IgG1 and IgM, while resulting in decreased Th1 markers. Furthermore, chronic infection significantly inhibited cellular responses to the MVA+gp140 vaccine regimen shown by IFN- $\gamma$  and IL-2 ELISpot; CBA and ICS staining. Similarly, in DNA+MVA vaccinated mice, a significant reduction in vaccine-specific responses was observed in Sm-infected groups compared to uninfected vaccinated groups shown by IFN- $\gamma$  and IL-2 ELISpot; CBA and ICS against HIV immunogens. antihelminthic treatment with PZQ resulted in the partial restoration of Th1-Th2 balance in the Sm-infected hosts, with the levels of vaccine-induced IFN- $\gamma$ ; TNF- $\alpha$  and IL-2 being partially restored despite the presence of elevated Th2 cytokines after treatment with PZQ. A significant overall decrease in Env gp140 specific IgG, IgG1, IgG2a and IgG2b antibody responses was observed in the Sm-infected mice vaccinated with gp140 or MVA+gp140 vaccine regimen compared to uninfected vaccinated controls. Surprisingly, antihelminthic treatment did not restore vaccine-induced antibody responses. Our histology data showed that DNA+MVA vaccinated mice develop increased liver pathology during chronic schistosomiasis compared to unvaccinated Sm-infected groups shown by larger livers; spleen and enlarged granuloma formation. However, no significant difference in collagen content (a marker of fibrosis) measured by hydroxyproline assay in both vaccinated and unvaccinated infected groups was observed. Our findings further demonstrated that in a mouse model, inoculation with SmE also induces a predominantly Th2 immune biased response marked with elevated parasite-specific IL-4; IL-6 and IL-10 while resulting in decreased Th1 markers. No significant impact on cellular responses evaluated by ELISpot and CBA were observed. However, gp140 specific IgG, IgG1 IgG2a and IgG2b antibody responses were significantly reduced in groups challenged with SmE.

Overall, these findings show that chronic helminthiasis in the mouse model induces a strong Th2 biasing which is associated with attenuation of both T cell and antibody response to HIV vaccines. Elimination of helminths by chemotherapy may partially restore T cell responses, but not necessarily antibody responses. These findings further suggest that vaccinating helminth infected individuals with HIV vaccines that induce strong cellular responses may increase the pathology induced by the parasites, rendering the vaccine unsafe in helminth endemic areas. Furthermore, this study suggested that in the absence of an active chronic Sm-infection, SmE left trapped in the tissues following antihelminthic treatment, may continue to induce strong Th2 responses which are capable of downregulating vaccine-specific responses, especially the antibody-mediated responses. This study strongly recommends that development of HIV vaccines should also focus on designing vaccines that can overcome helminth-induced immunity.

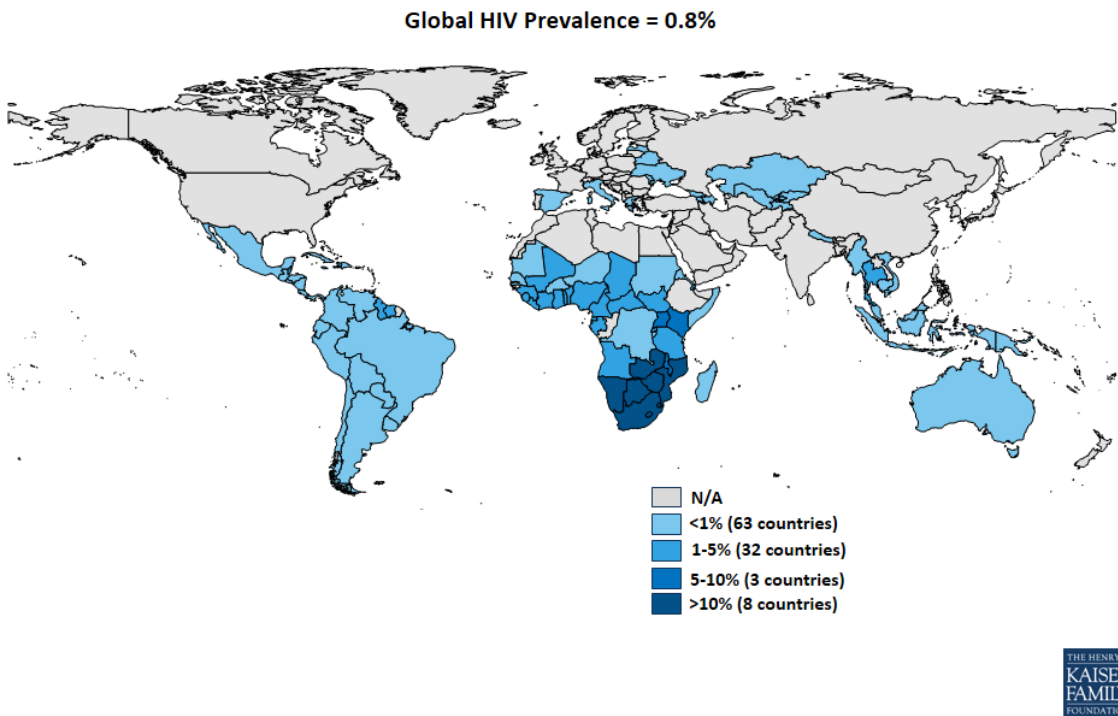


## CHAPTER 1

### 1. BACKGROUND INFORMATION AND LITERATURE REVIEW

#### 1.1. Global burden of HIV/AIDS

The high prevalence of human immunodeficiency virus (HIV) infection continues to pose a global health problem. In 2015, the joint United Nations Program on HIV/AIDS (UNAIDS) and the World Health Organization (WHO) published data showing that there were an estimated 36.7 million people living with HIV/AIDS globally, end of 2015 (WHO, 2015, UNAIDS, 2016). Sub-Saharan Africa (SSA) remains the region most heavily affected by HIV and accounts for more than two-thirds (about 25.8 million) of infections worldwide. In addition, the UNAIDS reported that 70% of the 1.5 million new HIV infections worldwide occurred in SSA (UNAIDS, 2015). Furthermore, South Africa has the highest number of HIV-positive people in the world; with an estimated 7.0 million people living with HIV (UNAIDS, 2016) (Fig 1.1). Only 37% of HIV-infected individuals in sub-Saharan Africa are receiving life-saving antiretroviral therapy (ART) (UNAIDS, 2014). Because of the major disproportionate burden of disease inflicted by HIV in SSA, there is substantial potential for improvement of HIV prevention and diagnosis. Disease management could be further optimized.



**Figure 1.1: Adult HIV prevalence: adapted from Kaiser-Family Foundation, based on UNAIDS, AIDS information. Accessed on 28 July 2016: <http://kff.org/global-health-policy/fact-sheet/the-global-hivaids-epidemic/>**

## 1.2. Background on HIV

### 1.2.1. The origin and history of HIV

Human Immunodeficiency Virus (HIV) is an RNA virus belonging to the *Retroviridae* family, subfamily *Lentiviridae* (Nye and Parkin, 2003). It was first isolated in 1983, (Barre-Sinoussi et al., 1983) and soon discovered to be associated with weakening of the immune system leading to what today is termed the acquired immunodeficiency syndrome (AIDS) (Gallo et al., 1984, Groopman, 1984, Walgate, 1986). The origin of HIV is believed to be the Congo River area in central Africa and was transfected from primates to humans during the late 19<sup>th</sup> or early 20<sup>th</sup> century (Keele et al., 2006, Worobey et al., 2008). The transmission was most likely through hunting and handling uncooked meat infected with simian immunodeficiency viruses (SIV) (Peeters et al., 2002). HIV type 1 (HIV-1) and type 2 (HIV-2) are the two known lineages of HIV with the former being distributed worldwide and the latter found primarily in West Africa (Romieu et al., 1990, Marlink et al., 1994, Popper et al., 1999, Sharp and Hahn, 2011). HIV-1, comprising of groups M, N, O, and P, is by far the most virulent of the two lineages with group M being responsible for the current global HIV pandemic (Robertson et al., 2000, Hemelaar et al., 2011). Group M is further subdivided into ten recognised phylogenetic subtypes (A, B, C, D, F, G, H, J, K, and L) (Robertson et al., 2000, Buonaguro et al., 2007, Hemelaar et al., 2011). HIV-2 is made up of groups A to H and unlike HIV-1; it is closely related to the SIV with cases reported mostly in West Africa (Franchini and Bosch, 1989, Marlink et al., 1994, Popper et al., 1999, de Silva et al., 2008, Sharp and Hahn, 2011, Campbell-Yesufu and Gandhi, 2011). Although the degree of virulence may be less for HIV-2 (Rowland-Jones and Whittle, 2007, Campbell-Yesufu and Gandhi, 2011, Thiébaud et al., 2011), both HIV-1 and HIV-2 infects helper CD4 T cells resulting in their gradual decline and consequent immune-suppression, thus associated with AIDS (Clavel et al., 1987, Campbell-Yesufu and Gandhi, 2011, Reeves and Doms, 2002).

### 1.2.2. HIV-1 structure and genomic organization

The HIV-1 virion is on average ~120nm wide and is roughly spherical in structure. It comprises of the external envelope-glycoprotein complex made up of the external surface membrane (SU, also called gp120) and transmembrane part (TM, also called gp41) of the envelope (Env), the inner membrane (matrix) and the core capsid (CA, also called p24). The core CA contains viral genomic RNA, reverse transcriptase (RT, also called p18), integrase (p32), protease (PR) (p11) and other enzymes needed for the replication cycle (Fig 1.2 B) (Gelderblom, 1997, Rubbert A, 2011, Foley et al., 2013).

The HIV-1 genome consists of three structural genes namely; *gag*, encoding for the matrix enclosing a nucleocapsid (p24); *pol*, encoding enzymes responsible for PR, RT, and integration; and *env*, encoding Env-glycoproteins of the viral membrane (Fig 1.2 A). Six other genes (trans-activator of transcription (*tat*), *rev*, negative infectivity factor (*nef*), viral infectivity factor (*vif*), viral protein R (*vpr*) and viral protein U (*vpu*))

encode proteins responsible for host cell regulation, immune evasion, and viral gene expression. Though *Nef*, *vif*, *vpr* and *vpu* were classified as accessory genes in the past, as they were not absolutely required for replication *in vitro*, their proteins have been studied and characterized in more detail over the past few years (Girard et al., 2011). The accessory genes *nef*, *tat*, and *rev* are all produced early in the viral replication cycle (Shum et al., 2013, Christian, 2015, Altfeld; et al., 2015).

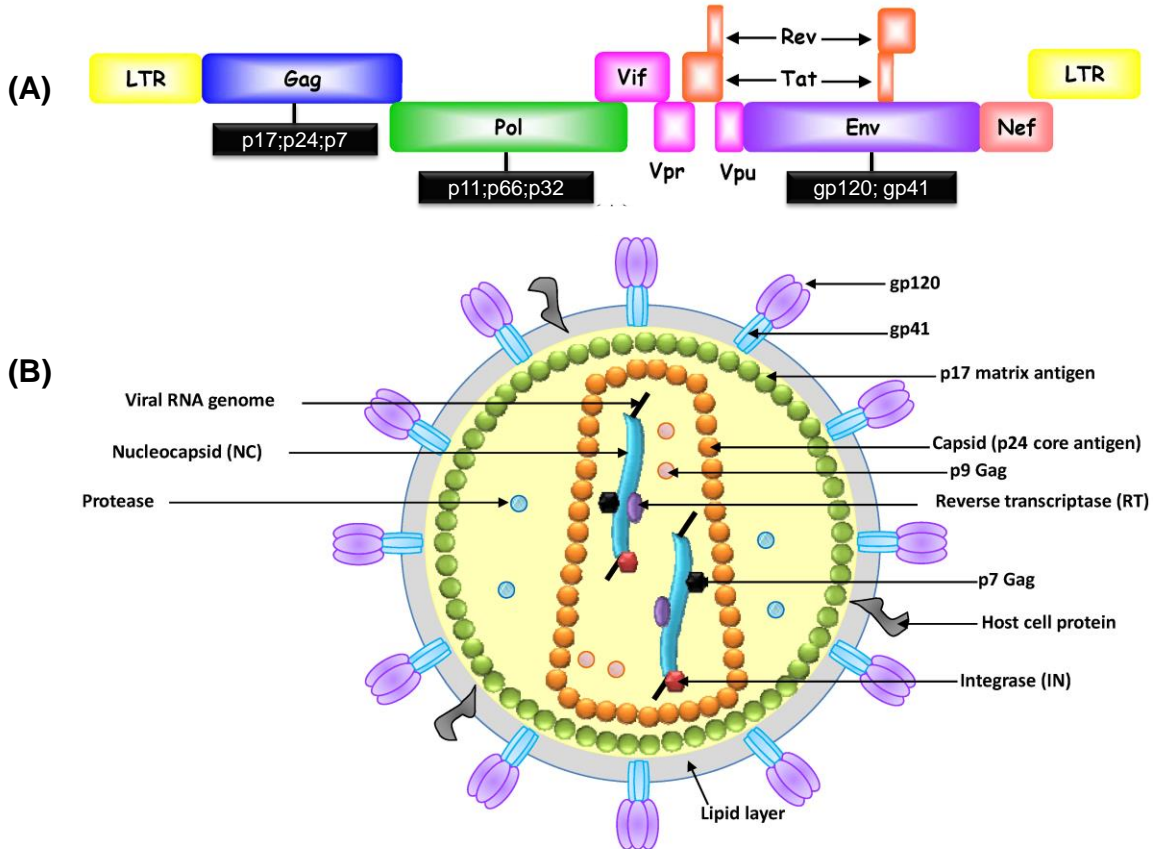
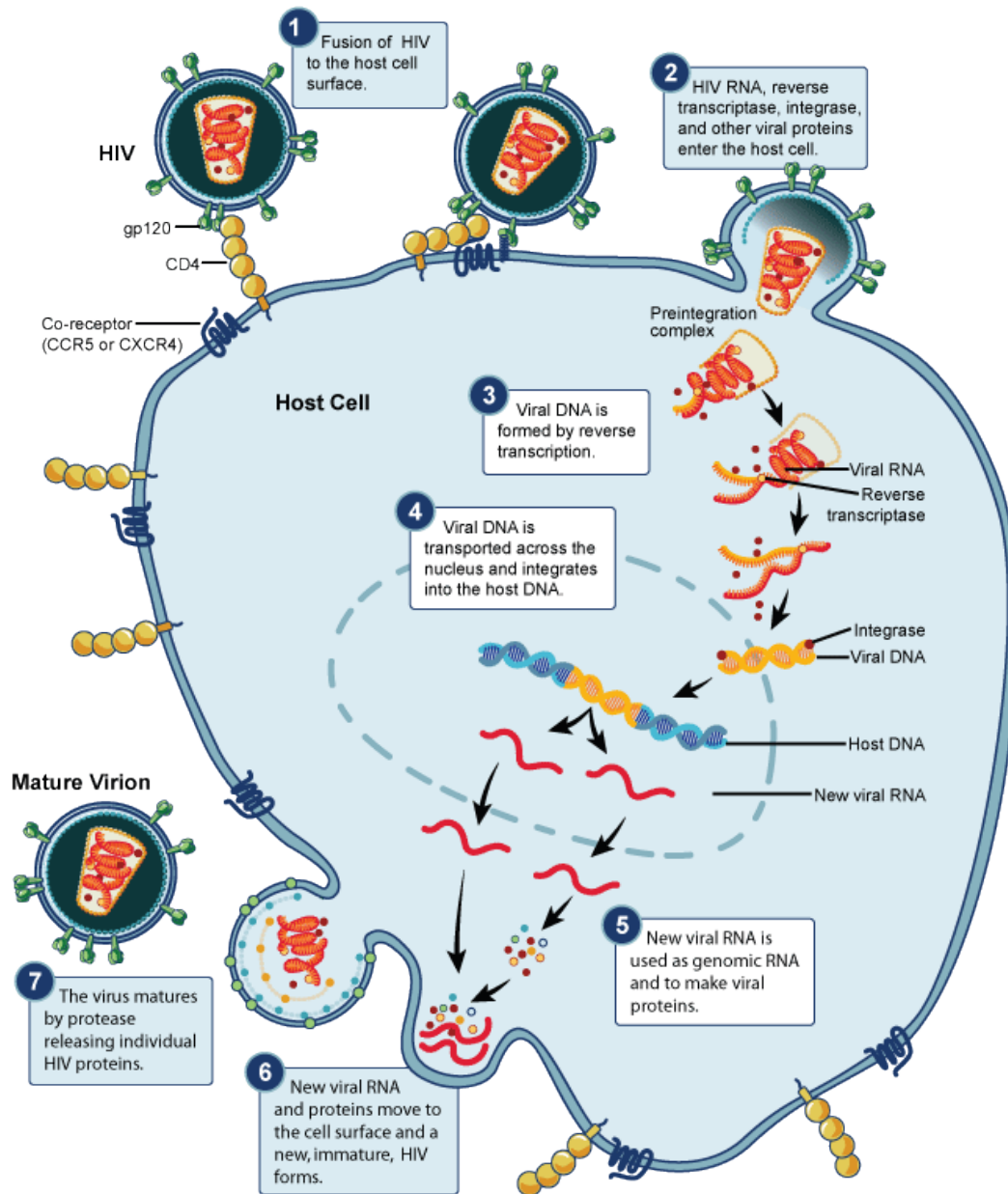


Figure 1.2: (A) HIV-1 genome and (B) HIV-1 virion and potential antiviral targets. Taken from (Shum et al., 2013).

### **1.2.3. HIV-1 replication cycle**

HIV-1 replication cycle occurs in 7 major steps. First, the transmembrane protein gp120 on the surface of the virus interacts with the CD4+ of the T helper cell as well as the monocytes. With a help of co-receptor (CXCR4 and CCR5) fusion of the plasma membrane with the virus capsid to the host cell surface occurs. Then the viral genome (HIV RNA, reverse transcriptase, integrase and other viral proteins) along with the enzyme machinery enter the cell leaving the coat outside. After entering the cell, reverse transcriptase begins to make viral RNA-cDNA hybrid from the information of ssRNA template, which is turned into viral DNA. The viral DNA is then transported to the nucleus and integrates into the host DNA by the integrase enzyme forming a provirus stage. The viral transcription factors now stimulate the host cell transcription and make ssRNA followed by the translation to make necessary proteins to make new viral RNA and proteins. The new viral RNA and proteins assemble with other proteins including gp41 and gp120, which moves to the cell surface and buds out forming viral coat and a new immature HIV. The virus finally matures by protease releasing individual HIV proteins which will seek healthy cells to infect to keep the infection cycle going (Fig 1.3) (Freed, 2001, Korber et al., 2001, Miyauchi et al., 2009, NIAID, 2012, Engelman and Cherepanov, 2012).



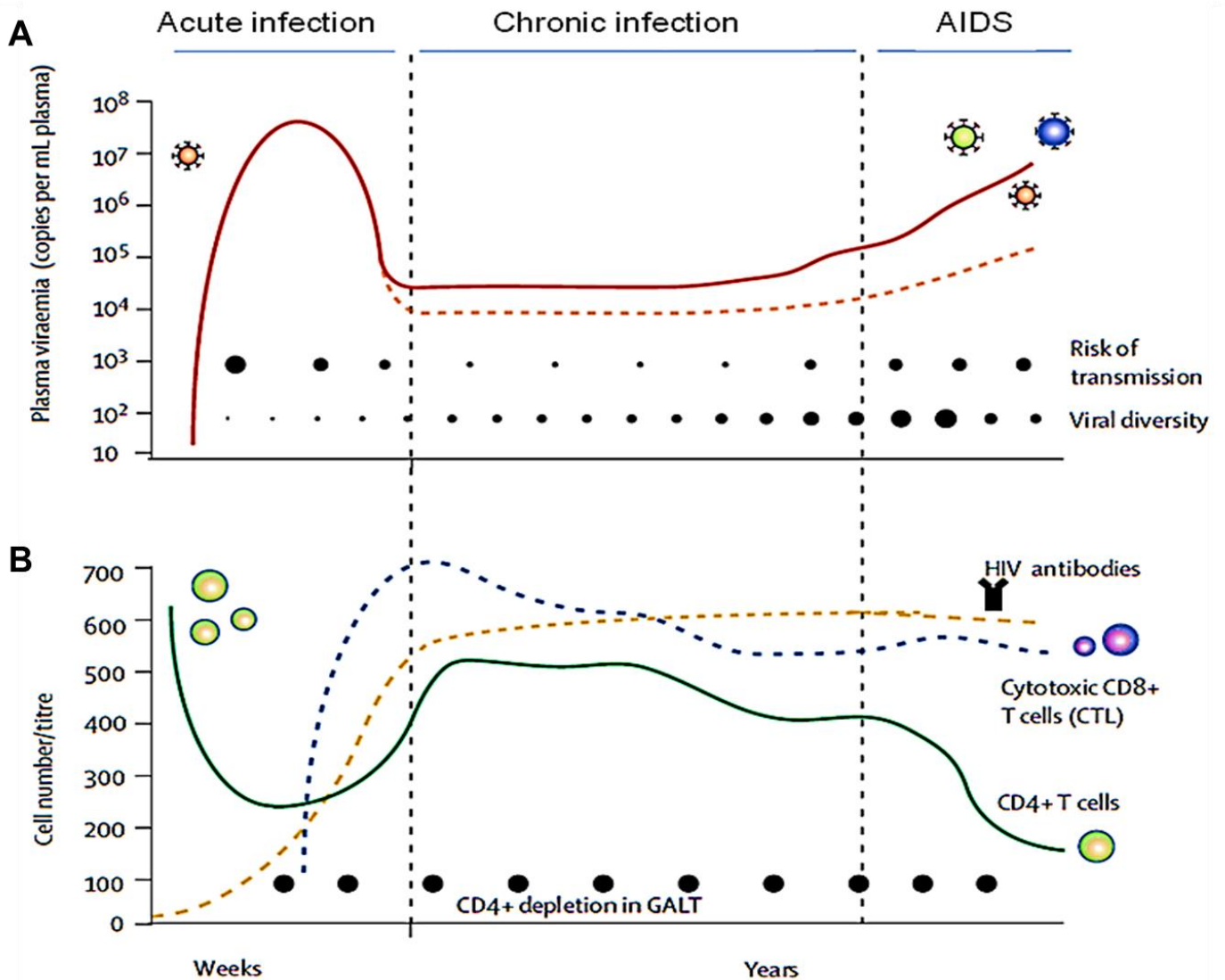
**Figure 1.3: HIV replication cycle.** Taken from (NIAID, 2012).

#### 1.2.4. HIV-1 infection and pathogenesis

HIV-1 is mainly spread by heterosexual people, men who have sex with men (MSM); infection among injection drug users (IDUs) and mother to child transmission occurring in the form of cell-free or cell-associated virus via vaginal secretions, semen, rectal sections, or blood (Royce et al., 1997, Shaw and Hunter, 2012). However, sexual transmission of HIV-1 remains most common route (Royce et al., 1997, Pope and Haase, 2003, McMichael et al., 2010, Cohen et al., 2011b, Girard et al., 2011, Sagar, 2014).

After transmission of the HIV-1 virus into a new host, progressive immunodeficiency is established from a depletion of T lymphocytes that express a cell surface molecule called CD4 (the main target cells for infection by HIV) and from general chronic immune activation brought about by active viral replication, which causes cell death (Ho et al., 1995, Berges et al., 2008, Douek et al., 2009, McMichael et al., 2010, Girard et al., 2011, Xu et al., 2013). A pathophysiological event within days after primary infection determines the course of the disease (Douek et al., 2009). With waning CD4 cell numbers, the adaptive immune system collapses, as a result, a specific immunodeficiency emerges, which permits infection with a range of virulent diseases and opportunistic infections (those capable of disease in immuno-compromised individuals) (Moore and Chaisson, 1996, Okoye and Picker, 2013, Luo et al., 2016).

The first signal of an immune response to HIV-1 is an appearance of an acute phase which is characterized by high plasma viral loads in the first few weeks paired with a decline in CD4+ T cells, followed by an increase in HIV-specific CD8+ CTLs (Cohen et al., 2011b, Maartens et al., 2014). Continuous loss of CD4+ T lymphocytes and systemic immune activation leads to a chronic HIV-1 infection, when untreated, leads to disease progression and sustained viral replication (Grossman et al., 2006, Haas et al., 2011). Stages of HIV infection can be summarized in Fig 1.4 below.



**Figure 1.4: Clinical HIV-1 Infection:** Plasma viraemia (A) and the changes in the CD4 + T-lymphocyte compartments (B). Primary infection is characterised by high plasma viraemia (red line, A), low CD4 cells (green line, B) and absence of HIV-1 specific antibodies (orange line, B). Plasma viraemia drops as cytotoxic CD8+ T-lymphocytes (CTL) develop (blue line, B) and an individual viral-load set point is reached during chronic infection. Viral set points differ greatly among individuals (eg, red dotted line, A) and predict disease progression. Viral diversity increases throughout the disease (closed circles, A). The risk of transmission is highest in the first weeks when viraemia peaks (closed circles, A). GALT=gut-associated lymphoid tissues. Taken from (Simon et al., 2006).

Symptoms of acute HIV-1 infection arise 2-4 weeks post-transmission, often last 3-4 weeks and resemble influenza-like or mononucleosis-like symptoms, which may include fever, headache, sore throat, swollen lymph nodes, joint or muscle pain, malaise, rash, and weight loss (Tindall et al., 1988, Daar et al., 2001, Hecht et al., 2002, Daar et al., 2008, Hoenigl et al., 2016). During chronic HIV infection, immune dysfunction causes some individuals to experience fatigue, weight loss, night sweats, oral lesions (hairy leukoplakia), oral and vaginal candidiasis, and symptoms associated with herpesvirus reactivation (Kuritzkes, 2007). In addition, muscle wasting, dermatological conditions, neurological complications, and multiple organ dysfunctions, including cardiovascular disease, are also observed (Coopman et al., 1993, Nahlen et al., 1993, Simpson and Tagliati, 1994, Wanke et al., 2000, Kuritzkes, 2007).

There is minimal change in viral load and CD4+ T cell counts at the beginning of the chronic phase of infection, however over time, a slow and quasi-steady increase in viral load is in conjunction with a progressive and gradual decrease in the CD4+ T cell population (Fig 1.4) (Simon et al., 2006, Coffin and Swanstrom, 2013).

In the GALT, the depletion of the CD4+ T cell compartment is maintained during chronic HIV-1 infection (Fig 1.4). HIV-1 preferentially infects and causes profound depletion in the Th17 and Th22 CD4+ T cell subsets, which defend against microbes and play a role in maintaining the integrity of the gastrointestinal (GI) tract mucosal barrier, by preventing microbial translocation from the GI tract to the periphery (Brenchley et al., 2008, Raffatellu et al., 2008, Prendergast et al., 2010, Dandekar et al., 2010, Kaul et al., 2011, Kim et al., 2012). When these cells are depleted, the guts permeability becomes enhanced, allowing for microbial translocation, which in turn leads to increased and sustained systemic immune activation, a hallmark of chronic HIV-1 infection (Brenchley et al., 2006a, Raffatellu et al., 2008, Okoye and Picker, 2013).

Multiple factors that promote the widespread production of pro-inflammatory factors and increased viral replication in activated T cells are associated with chronic immune activation (Moir et al., 2011). This cellular hyperactivity leads to persistent high T cell, B cell, and NK cell turnover, which further contributes to increased immune dysfunction marked by cellular exhaustion, senescence, and limited renewal capacity (Mohri et al., 1998, Lempicki et al., 2000, McCune et al., 2000, De Boer et al., 2003, Moir et al., 2011, Wherry, 2011). In this regard, chronic systemic immune activation has a poor prognosis for survival and is associated with a more rapid progression to AIDS (Giorgi et al., 1999, Hazenberg et al., 2003, Brenchley et al., 2006b), the immunosuppression stage that allows infections to surface, which can include oral candidiasis, pneumococcus, tuberculosis, and the reactivation of latent herpesviruses, such as herpes simplex virus, varicella zoster virus, or cytomegalovirus (CMV) (Kuritzkes, 2007).

### 1.2.5. Immune responses to HIV Infection

The early stages of HIV infection are characterized by a short-lived symptomatic illness in most cases and are associated with high levels of virus replication (Perreau et al., 2013). The source of infection is commonly from transmission of a single virus variant (Keele et al., 2008, Perreau et al., 2013). Symptoms at this stage are not specific to HIV but typical of an acute viral syndrome which may be followed by wide range of illnesses (Tindall et al., 1988, Daar et al., 2001, Hecht et al., 2002, Daar et al., 2008, Hoenigl et al., 2016). The symptomatic phase persists for 2–4 weeks at a normal rate of disease progression while prolonging in rapid disease progression (Perreau et al., 2013). Once chronic HIV infection is established, the outcome of the infection will be affected by hosts genetic, immunological and virological factors (O'Brien and Nelson, 2004).

Ever since the discovery of HIV, the host immune responses to HIV have been extensively studied and the immune correlates of protection have been extensively explored (Tomaras and Plotkin, 2017). It is now known that the immune system mounts a robust response to HIV infection; however, the immune response to HIV differs among infected individuals, with the course of the infection determined by several factors including viral fitness or virulence, the host genetic factors such as the association between the different HLA alleles (Liu et al., 1996, Carrington and O'Brien, 2003) and CCR-5 mutation (Schmitz et al., 1999) with resistance, as well as a predominance of type-1 cytokine production.

The immune responses to virus infection are mainly innate, cellular, and/or humoral responses, however, not all contribute to protective immunity (Tomaras and Plotkin, 2017). The first line of immune responses to the virus is mediated by innate immunity (Freed and Gale, 2014) encompassed by (i) natural killer cells (NK cells), which have been shown to control HIV-1 infection of macrophages through soluble factors and cellular contacts in the human decidua in pregnant women, and also playing an important role in viral clearance (Quillay et al., 2016, Lam and Lanier, 2017), (ii) plasmacytoid dendritic cells (pDCs), which are indirectly impaired by HIV through suppression of IFN- $\alpha$  production induced by TLR-7 agonist (Dhamanage et al., 2017). (iii) Cellular restriction factors such as APOBECs and Trim5 $\alpha$  (Rehwinkel, 2014, Simon et al., 2015), SAMHD1 which act by hampering the induction of an efficient immune response directed against HIV-1, (iv) myeloid dendritic cells (mDCs), which function as antiviral restriction factors (Herrmann et al., 2016) as well as inhibitor of HIV-1 reverse transcription in some cell types (Ayinde et al., 2012); and (v) antiviral cytokines such as type I interferons, IL-15 and IL-18 (McMichael et al., 2010, Desimmié et al., 2014, Rustagi and Gale, 2014, Dhamanage et al., 2017). Despite the fact that the innate control of infection is important, it is facilitated by inflammation, which also recruits HIV target cells during acute infection, impairs CD4<sup>+</sup> T cell recovery, and promotes disease progression (Tomalka et al., 2016).

The second line of defence is the adaptive immune response, which is a specialized form of defences occurring as cellular and/or humoral immune responses (Iwasaki and Medzhitov, 2010). This type of an

immune system depends on the generation of a diverse repertoire of antigen receptors on T and B lymphocytes and subsequent activation as well as the clonal expansion of infected or activated cells. The induction of adaptive immunity not only depends on direct antigen recognition by the antigen receptors but also relies on essential signals that are delivered by the innate immune system. This type of immune responses is capable of clearing bacterial and viral infection (Schenken and Medzhitov, 2011). However, despite the induction of both humoral and cellular immune responses, infection with HIV does not get eliminated (Jones and Walker, 2016). Virus-specific CD8<sup>+</sup> T cells play a role in the elimination of viruses, which occurs by recognition of processed viral proteins (Jones and Walker, 2016). A cascade of activation events is initiated by recognition through T cell receptor (TCR) leads to the release of granzymes and perforin and killing of the infected cell, which can occur before infectious progeny virions are produced (Yang et al., 1997, Voskoboinik et al., 2015). Furthermore, TCR activation leads to the release of a variety of effector cytokines including IFN- $\gamma$ , TNF- $\alpha$ , macrophage inflammatory proteins 1 $\alpha$  and 1 $\beta$  (MIP-1 $\alpha$  and MIP-1 $\beta$ ), and RANTES (regulated upon activation, normal T cell expressed and secreted [also known as CCL5]), which have antiviral effects (Jones and Walker, 2016).

A number of studies agree that HIV-specific CD8<sup>+</sup> T cells exert potent antiviral effects and have shown that the magnitude and rapidity of HIV-specific CD8<sup>+</sup> T cell activation inversely correlates with viral load set point in a hyper-acute infection (Ndhlovu et al., 2015), thus mediating an antiviral pressure during peak viremia (Borrow et al., 1994, Koup et al., 1994). The evidence for an antiviral effect of these cells has been demonstrated using *In vitro* models and showed that the cells are potently capable of inhibiting viral replication (Yang et al., 1997, Chen et al., 2009). This further explains why depletion of CD8<sup>+</sup> T cells following acute infection leads to high-level viremia that decreases as CD8<sup>+</sup> T cells reappear (Schmitz et al., 1999). Taking together genetic studies, which indicate that HLA class I alleles are associated with differences in set-point viremia (Fellay et al., 2007); studies of viral fitness, which suggest that CD8<sup>+</sup> T cell-induced mutations can diminish viral fitness, the conclusion is that CD8<sup>+</sup> T cells are capable of potent antiviral function and provide a strong rationale for enlisting these responses in eradication strategies such as vaccines (Jones and Walker, 2016).

Another arm of the immune system that mounts during primary HIV infection is the humoral responses to HIV through the induction of binding and or neutralising antibodies (Moir and Fauci, 2017). The large majority of these antibodies are produced during the first months of natural HIV-1 infection and are generally either non-neutralizing or strain-specific neutralizing antibodies (Wei et al., 2003, Richman et al., 2003, Gray et al., 2007, Tomaras et al., 2011, Mikell et al., 2011). It is only after 2-4 years after primary infection that the emergence of broadly neutralising antibodies (bNabs) occurs, with just about 20% of infected individuals developing these antibodies (Doria-Rose et al., 2010, Walker et al., 2010, Gray et al., 2011). However, a minority of infected individuals (1–2%) develop a more potent form of bNabs capable of neutralizing a large proportion (>70%) of virus isolates (Noto and Pantaleo, 2017). A small percentage of HIV-infected individuals have developed a way of managing a stable disease, with no decline in CD4 T-cell

counts, and able to maintain viral replication below 1000 HIV RNA copies/ml for an extended period of time in the absence of antiretroviral therapy (ART) (Noel et al., 2016). Such individuals are referred to as long-term non-progressors (LTNPs) (Sáez-Cirión and Pancino, 2013). They make up between 2 and 5 % of all HIV-seropositive individuals and maintain low levels of viraemia with elevated CD4+ T-cell levels in contrast to rapid progressor HIV-1 subjects who quickly develop AIDS in the absence of ART (Noel et al., 2016). Furthermore, another group of infected individuals called Elite controllers can suppress HIV infection below 50 HIV RNA copies/ml in the absence of treatment for an unspecified period of time ranging from months to years (Joglekar et al., 2016). However, Elite controllers can undergo immunodeficiency and opportunistic infections as a result of CD4+ T-cell depletion (Hunt, 2009). A better understanding of the mechanisms underlying this viral control is important in the development of therapeutic interventions capable of achieving HIV-1 remission in other patients (Noel et al., 2016).

It is unclear whether these bNabs exert any protective effect during HIV chronic infection since HIV-1 is capable of escaping antibody responses during natural infection, considering the fact that bNabs only appear later on during infection (Noto and Pantaleo, 2017). It is also not clear why bNabs generation is delayed during infection (Noto and Pantaleo, 2017).

Little is known concerning initial responses of B cells during an acute phase. This is due to the fact that it is difficult to access patients with acute HIV infection since most do not come to medical attention until the acute phase has passed (Moir and Fauci, 2017). During the course of HIV infection, hypergammaglobulinemia, which reflects systemic immune-activating effects of HIV on B cell differentiation is common as well as defects in memory B cells, which also reflect defects in CD4+ T cell help (Lane et al., 1983, Buckner et al., 2013). To determine the development and regulation of B cells during early HIV diagnosis, it is clinically important to track seroconversion (Fiebig et al., 2003). During a typical viral infection, short-lived plasmablasts elicited prior to germinal centre (GC) formation and T cell help is the most likely source of this initial antibody response (Fink, 2012). This is also true for HIV, HIV-specific IgM in the first week of infection can be detected (Tomaras et al., 2008). Furthermore, an enrichment of IgG3 antibodies [strongly associated with protection in the RV144 trial (Yates et al., 2014, Chung et al., 2015)] occur during this time (Yates et al., 2011) and are also thought to derive from GC-independent class switching of B cells that have not undergone affinity maturation (Wirths and Lanzavecchia, 2005, Fecteau et al., 2006, Budeus et al., 2015). Although these early responses reflect an inadequate T cell help response, marked by rapid lymphoid tissue damage in environment where cognate B and T cell interact (Levesque et al., 2009), HIV-specific IgG3 antibodies also decline rapidly in early HIV-infected individuals (Yates et al., 2011) including in vaccines of the RV144 trial [reviewed in (Haynes, 2015)].

The transmembrane glycoprotein gp41 is one of the earliest targets of the antibody response following HIV infection (Tomaras et al., 2008). Circulating plasmablasts have been identified as a source of these antibodies (Liao et al., 2011). This is so because the frequencies of plasmablasts in the peripheral blood

are highest in early HIV infection, despite a large portion of these are not specific for HIV (Buckner et al., 2013). Nonetheless, HIV-specific antibodies cloned from these cells were shown to recognize gp41 (Liao et al., 2011). Epitopes within the membrane proximal region (MPER) of gp41 have been shown to elicit bNAbs (Stiegler et al., 2001, Huang et al., 2012). Generally, such antibodies may be very difficult to induce following vaccination in normal, non-immunocompromised individuals due to compromise of immune tolerance caused by self-reactive antibodies (Verkoczy and Diaz, 2014). Nonetheless, a study by Huang et al., showed that potent gp41 MPER-specific neutralizing antibodies can arise in HIV-infected individuals in the absence of autoreactivity (Huang et al., 2012). The chronic phase of HIV, triggered mostly in the majority of HIV-infected individuals not taking antiretroviral therapy (ART) during the early phase of infection has been associated with a broadening of the B-cell response, with a minor percentage of individuals developing a response that is both broad and potent (Burton and Mascola, 2015). The forces involved in driving breadth of B cells of these infected individuals is often referred to as elite neutralizers and have been considered as a pathway to the “holy grail” of immunogen design for an effective vaccine (Moir and Fauci, 2017). However, elite neutralizers are not particularly elite in their HIV disease status (Doria-Rose et al., 2010) as there are few virologic and clinical factors that influence this. This includes increased neutralization breadth with high viral load in early infection (Piantadosi et al., 2009, Gray et al., 2011) or later in infection (Doria-Rose et al., 2010); decline in CD4+ T cells (Gray et al., 2011); HIV subtype and host genotype (Landais et al., 2016) and the quality of CD4+ T cell responses against HIV (Ranasinghe et al., 2016). However, CD4+ T cell studies suggest that a strong cellular response may be beneficial for humoral immunity in HIV-infected individuals.

Apart from bNAbs, non-neutralizing (nonNab) against HIV Env viable domain 1 and 2 (V1V2) have been associated with modest protection from HIV infection in the RV144 trial (Rerks-Ngarm et al., 2009). The outcomes of this trial demonstrated that vaccine-induced IgG antibody responses to the V1V2 region of HIV Env inversely correlated with lower risk of HIV infection in the absence of bNAbs (Montefiori et al., 2012, Tomaras and Plotkin, 2017). Furthermore, V1V2 specific IgG antibodies have been associated with the induction of ADCCs, which have been shown to play 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). ADCC cells have been known to occur when antibody forms a bridge between a target cell bearing foreign antigens on its surface and an effector cell expressing Fc receptors, leading to lysis or apoptosis of the target cell (Forthal et al., 2001). Like CTL activity, ADCC could eliminate infected cells and thereby reduce viral burden. These antibodies have been shown to appear at the same time as CTLs became detectable in acutely infected patients (CONNICK et al., 1996). These findings taken all into account suggested that at least partial protection observed in the RV144 trial was also due to ADCC-mediated antibodies. However, these observations were only in vaccinees of low IgA antibodies to the Env, suggesting that Env specific IgA correlated with high risk of infection (Bonsignori et al., 2012, Kim et al., 2015, Tomaras and Plotkin, 2017).

In a number of nonhuman primate (NHP) studies, protection against SIV acquisition in the absence of bNab has been reported (Letvin et al., 2011, Barouch et al., 2012, Barouch et al., 2013), thus, further supporting the fact that functional vaccine-induced Env-specific antibodies may provide protection against HIV acquisition even in the absence of bNab.

Furthermore, these studies demonstrate that nonNab can result in increased antiviral activity, through recognition of virus-infected cells, infectious virion capture and ADCC (Pollara et al., 2014). Taken together, studies on natural infections demonstrate that the body is principally capable of controlling virus replication through effective CTL and antibody responses. Developing combination therapies that bring together both the cellular and humoral arms of adaptive immunity with innate immune mechanisms is of great importance in order to understand how to trigger immune responses in healthy individuals, therefore, providing a way to control or prevent subsequent HIV infection.

### **1.3. Need for HIV-1 vaccine**

The introduction of anti-retroviral drugs and highly active antiretroviral therapy (HAART) brought a significant decline in morbidity and mortality (WHO, 2013, HIV/AIDS, 2016), and have been shown to prevent HIV transmission (Grant et al., 2010, Cohen et al., 2011a, Baeten et al., 2012). However, the persistence of a latent viral reservoir during HIV infection makes it difficult for treatment to eradicate HIV infection (Finzi et al., 1999, Siliciano et al., 2003). Thus, a continuous need for ART is inevitable, however, the cost is substantial (UNAIDS, 2014, Granich et al., 2015) and may be difficult to sustain (Deeks et al., 2016). Furthermore, adherence to treatment across different populations is critical, as it affects the efficacy and efficiency of biomedical preventive interventions (Doblecki-Lewis et al., 2015). However, the use of ARTs for HIV treatment and prevention can be compromised by HIV-1 drug resistance mutations (DRMs), especially in resource-limited settings where virological monitoring is not routinely performed as part of clinical management (Gupta et al., 2009, Hosseinipour et al., 2009, Sigaloff et al., 2011). High levels of drug resistance have been reported in individuals with prolonged first-line ART failure, including complex nucleoside reverse transcriptase inhibitor (NRTI) resistance profiles (Marconi et al., 2008, Hosseinipour et al., 2009, Wallis et al., 2010, Sigaloff et al., 2011). Also, antiretroviral agents can be responsible for a wide range of toxicities that may present anything from negligible to life-threatening side effects (Organization, 2010). To date, a full spectrum of ARV toxicities observed in adults has also been reported in children as well. Thus, the development of an effective HIV vaccine remains one of the best hopes for controlling the HIV pandemic and is essential as a more sustainable solution (Cohen and Dolin, 2013, Fauci and Marston, 2014). Undoubtedly, the ultimate goal for an HIV vaccine would be to elicit sterilizing immunity, thus completely preventing or aborting HIV infection.

### 1.3.1. HIV vaccines

To date, designing a universal effective HIV vaccine remains an exceptionally difficult biomedical challenge. Ideally, an effective HIV vaccine is expected to be capable of inducing both potent T cell-mediated immune responses as well as a durable humoral immune response through antibody production (Chhatbar et al., 2011, Haynes and McElrath, 2013, Cohen and Dolin, 2013, McMichael and Koff, 2014). However, there are few important factors that make it difficult to develop an effective HIV vaccine. First, there is insufficient knowledge of causal mechanisms of protection against HIV infection (Pantaleo and Koup, 2004, Letvin, 2006, Johnston and Fauci, 2011); second, the extreme diversity between HIV strains and subtypes can differ by up to 35% in their envelope (Env) proteins (Korber et al., 2001, Gaschen et al., 2002). To generate an efficacious global vaccine, immunogens capable of generating protective innate, cellular, and/or humoral responses covering most major strains are required. However, not all responses contribute to protective immunity (Tomaras and Plotkin, 2017). Correlates of protection (CoP) for most pathogens is generally difficult to determine, but research efforts have contributed knowledge of what constitutes protective immunity for many of these infectious pathogens (Tomaras and Plotkin, 2017).

Immunological correlates of protection are immune parameters that measure responses to vaccination that predicts vaccine efficacy for a given clinical outcome and are usually complex (Plotkin, 2010, Plotkin, 2013). To date, the RV144 vaccine efficacy trial remains the only trial in which correlates of HIV-1 risk involving multiple immune responses have been identified. However, these responses are not yet accepted to be CoP until they have been confirmed in another vaccine efficacy trial (Tomaras and Plotkin, 2017). Nonetheless, follow-up studies for determining if RV144 correlates of HIV risk are protective or not are underway.

Currently licenced vaccines induce immune responses that associated with correlates of protection (Plotkin, 2008, Plotkin, 2010, Thakur et al., 2012, Iwasaki, 2016) (Table 1.1). These immune responses include binding antibody responses, functional antibody responses (ie neutralization/pathogen inhibition, opsonophagocytosis) and cellular immunity (CD4+ T cell, lymphoproliferation). Nevertheless, prevention of infection correlates with the induction of antibodies in most vaccines (Plotkin, 2008, Thakur et al., 2012) with exception to BCG, Zoster and Malaria which act through T cell responses or with both reviewed in (Tomaras and Plotkin, 2017).

Table 1.1: Types of immune responses associated/correlated with protection/infection risk: **Taken from** (Tomaras and Plotkin, 2017).

<b>Immune response</b>	<b>Vaccine</b>
Antibody binding	Hepatitis A, Hepatitis B, Human Papilloma Virus, Measles, Pertussis, Rubella, Varicella, Zoster#, HibPolysaccharides/Conjugate, Lyme disease, Tick borne encephalitis, Pneumococcus#, <i>HIV-1#</i> (not licensed)
Antibody function	<b>Neutralization or Pathogen/Toxin inhibition</b> Anthrax, Diphtheria, Influenza, Japanese Encephalitis, Measles, Meningococcal, Mumps, Polio, Rabies, Smallpox, Tetanus, Yellow Fever <b>Opsonophagocytosis</b> Pneumococcus# <b>ADCC</b> <i>HIV-1#</i>
Cellular response	<b>CD4 T cell, Lymphoproliferation</b> Zoster#, BCG <b>CD4+ T-Cell polyfunctionality</b> <i>HIV-1#</i>

Categories of identified immune correlates for licensed vaccines, identified correlates of risk for HIV-1 are also shown.

#Both antibody and cellular correlates, ***Italics***: not licensed vaccines.

This was thought to be the case with initial HIV-1 vaccine approach during the VAX003 and VAX004 clinical trial which focused primarily on the generation of neutralizing antibodies (nAb) (Berman et al., 1990, El-Adam et al., 1995, Berman et al., 1996).

VAX003 and VAX004 were double-blind, randomized trials which used a bivalent vaccine composed of rgp120 from subtype B, conducted in injecting drug users (IDU) in Thailand and among men who have sex with men (MSM) and women at high risk for heterosexual transmission of HIV-1 in North America and The Netherlands respectively (Flynn et al., 2005, Pitisuttithum et al., 2006). Both vaccines did not demonstrate protection; however, they showed an association of higher nAb to HIV-1MN, CD4 blocking Ab and antibody-dependent cell-mediated viral inhibition (ADCVI) and reduced infection rates among vaccine recipients in VAX004 (Gilbert et al., 2005, Forthal et al., 2007). Furthermore, these trials also highlighted supported the importance of cell-mediated immunity in controlling viral replication in rhesus macaques (RM) (Jin et al., 1999, Schmitz et al., 1999, Shiver et al., 2002) and human elite controllers (Klein et al., 1995, Altfeld et al.,

2006). This turned attention to the use of T-cell vaccines to induce HIV-specific cellular immune responses which were implemented in the STEP and the Phambili vaccine trial.

The STEP and the Phambili trial focused on eliciting HIV-1 gag/pol/nef sub-type B cellular responses aimed to reduce viral load in breakthrough infections (Buchbinder et al., 2008, Gray et al., 2014). These vaccine induced antigen-specific T-cell responses, however, did not prevent HIV-1 infection and had no effect on plasma viral load (Buchbinder et al., 2008). Instead, it was associated with an increased incidence of HIV-1 acquisition in male recipients who were Ad5 seropositive pre-vaccination or were uncircumcised [reviewed in (Gray et al., 2010)]. thus, was stopped after the first interim analysis (Buchbinder et al., 2008).

In 2003, the field was reinvigorated by the optimistic results of the RV144 trial published in 2009, following the failures of the Step and Phambili trial. The RV144 trial was a large Phase III double-blinded randomized study comprised 16,395 HIV negative participants in a multicenter study with enrolment in Thailand (Rerks-Ngarm et al., 2009). The trial was a collaborative effort between trial site (Thailand: Thailand Ministry of Public Health) and the sponsor (United States: US Army, and National Institute of Allergy and Infectious Disease [NIAID]). The trial enrolled Men and women between 18-30 years of age who were at community risk for acquiring HIV. In an effort to induce both a cellular and humoral immune response, the vaccine was given in a prime-boost vaccination (Lu, 2009) with a recombinant canarypox vector expressing HIV Gag, Pol, and Env proteins (ALVAC-HIV) delivered as a prime and the VaxGen recombinant gp120 subtype B and E bivalent protein (AIDSVAX B/E®) was given as a boost (Nitayaphan et al., 2004). The vaccination regimen included four priming inoculations with ALVAC-HIV at weeks 0, 4, 12 and 24, and two booster inoculations with AIDSVAX B/E® at weeks 12 and 24 which took place over 6 months. HIV infections were monitored every semester for 3 years. The primary endpoints for the study included prevention of HIV-1 infection, and upon infection, the impact of the vaccine on HIV-1 viral load (Rerks-Ngarm et al., 2009).

To date, HVTN 505 trial is the last efficacy trial to be conducted. This was a randomized, placebo-controlled trial of a prime-boost, DNA/rAd5 vaccine consisting of a 6-plasmid DNA vaccine (expressing clade B Gag, Pol, and Nef, and Env proteins from clades A, B, and C) with rAd5 vector boost (expressing clade B Gag-Pol fusion protein and Env glycoproteins from clades A, B, and C) (Hammer et al., 2013). The trial was brought prematurely to a stop due to lack of efficacy. Albeit the vaccine-induced both cellular and humoral responses, these were not associated with protection (Hammer et al., 2013). To date, none of the completed vaccine efficacy trials managed to induce strong bnAb responses. However, CD8+ T cell responses in STEP, Phambili and HVTN505 studies was induced, despite not associated with protection. Only one trial, RV144 demonstrated efficacy and protection were associated with functional binding antibodies such as vaccine-induced IgG antibody binding to the V1V2 of Env and thus inversely correlated with associated with of HIV infection but IgA against Env was directly linked to greater risk of infection (Rerks-Ngarm et al., 2009, Tomaras and Plotkin, 2017). Despite the V1V2-specific IgG not being broadly neutralizing, they are associated with mediation of ADCC (Pollara et al., 2011, Montefiori et al., 2012,

Tomaras and Plotkin, 2017). When Env-specific IgA levels are low, these ADCCs are suggested to confer protection, particularly those belonging to IgG1 and IgG3 subclasses (Montefiori et al., 2012, Tomaras and Plotkin, 2017, Pollara et al., 2011). However, efficacy was of a suboptimal magnitude and was not durable.

In summary, the current major goal in HIV vaccine design is to elicit a protective immune response mediated primarily by antibodies that are able to recognize a range of diverse strains. Such type of antibodies includes broadly neutralizing antibodies (bnAb) with activities against major strains that are common in human transmission. Another approach is the induction of protective functional antibodies as well as T cell responses through prime-boost strategies (Chhatbar et al., 2011, Haynes and McElrath, 2013, Cohen and Dolin, 2013, McMichael and Koff, 2014, Zolla-Pazner, 2014, Safrit et al., 2016, Stephenson et al., 2016).

### **1.3.2. Challenges in vaccine research and development**

Although there is continuous progress in vaccine design and development, as well as advanced methodologies for selection of candidate antigens and new technologies for vaccine delivery put in place, fully effective vaccines against widespread infectious diseases including HIV-AIDS, malaria, and tuberculosis remains unachieved. The complexity of the infection/life cycles of these pathogens makes it difficult to determine when during the pathogen's life cycle to intervene, and how to target vaccine delivery such that protective immune responses are induced (Taylor-Robinson, 2002, Good and Doolan, 2010, Douradinha and Doolan, 2011, Stanisic et al., 2013, Schiffner et al., 2013, Chowdhury and Silvestri, 2013, Excler et al., 2014).

Previous clinical studies have shown that humoral immunity plays a role in preventing infection by HIV as well as influencing certain stages of malaria infection, making it an important immune correlate of protection (Mascola et al., 2000, Moorthy and Ballou, 2009, da Silva et al., 2012). However, Th1 cells, CD8+ T cells, or both, also been demonstrated to play a critical role in preventing and controlling these infections (López et al., 2011, Killian et al., 2011, O'Connell et al., 2011, Hansen et al., 2011, Krzych et al., 2012, Mudd et al., 2012, Killian et al., 2013, Zarling et al., 2013, Yan et al., 2013, Chuang et al., 2013).

One of the challenges to vaccines success in target populations is the fact that the human immune system matures during early childhood and then begins to senesce around 60 years of age. Therefore, a question of how to immunize neonates, young children, and the elderly arises. Children younger than 6 months old and preterm infants have immature immune responses (Morein et al., 2002, Morein et al., 2007, Pastoret, 2007, Siegrist, 2007, Pichichero, 2014). This goes for adults over the age of 65 who will have an aging immune system that could result in defects in immune response to vaccinations (Lefebvre and Haynes, 2013, Lowery et al., 2013, Scholz et al., 2013, Shaw et al., 2013, Wong and Goldstein, 2013). Vaccines success is also affected in immune-compromised and/or chronically ill individuals. This is because immune

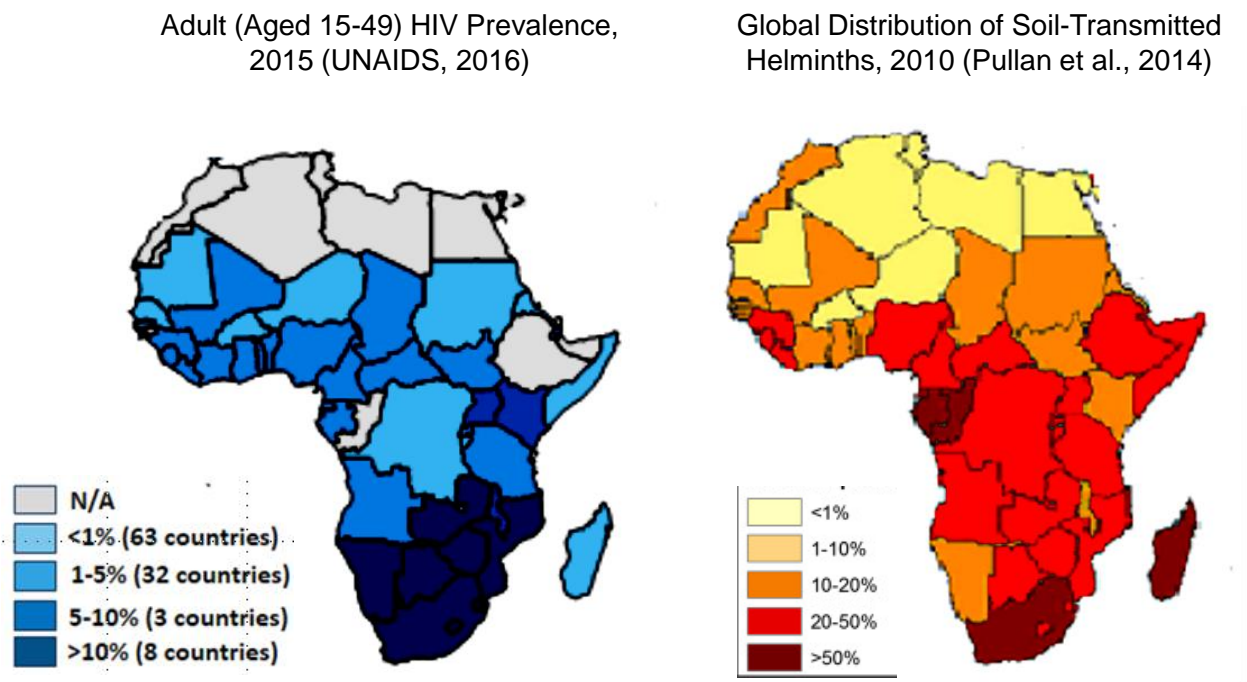
systems can be compromised or suppressed by an infection, as consequence, failure to mount desirable vaccine responses. As most vaccines rely on the bodies infection pathway, their form of infection does not cause illness, but it does cause the immune system to produce T-lymphocytes and antibodies. In some instances, minor symptoms such as a fever can be observed following vaccination. Such minor symptoms are normal and usually expected as the body builds immunity. It is at this stage that cellular or humoral responses are activated. Once the imitation infection is cleared, memory T-lymphocytes, as well as B-lymphocytes that will remember how to fight that disease in the future, are generated (Schenten and Medzhitov, 2011). Immune-compromised individuals usually have challenges in building up these memory responses to vaccination, since most of the resources required will be depleted or exhausted (Schenten and Medzhitov, 2011, Gisbert et al., 2012, Guan et al., 2013). Helminth infections, found in a large percentage of humans, may also suppress immune responses to vaccines as previously suggested (Actor et al., 1993, Sabin et al., 1996). These attenuated responses to vaccines have serious complications for HIV vaccine development in areas where helminth infections are prolific. Challenges posed by helminth infections to vaccine development will be discussed further in section 1.4.1

### **1.3.3. Helminth infection**

Helminths affect one-quarter of the world's population, where the majority of these infections are chronic and occur in the developing world (WHO, 2016). Helminth parasites cause a significant morbidity as they compete with the host for nutrients, causing damage as they migrate through host tissues, but rarely resulting in the death of their host. In particular, schistosomes and the filarial nematodes are associated with severe pathology (McSorley and Maizels, 2012). Protective immunity and clearance of helminths are mediated by Th2 cells, type 2 (M2) macrophages, type 2 innate lymphoid cells (ILC2) and eosinophils (Pearce, 2016). Infected individuals usually develop immunopathology mediated by Th1 or Th17 cells as a result of failure to mount type 2 immune responses against the parasites (Maizels and McSorley, 2016). Over time, helminths have found a way to escape host immune system and prolong their survival in the by a wide variety of approaches for immune suppression, especially the generation of regulatory T cells and anti-inflammatory cytokines IL-10 and TGF- $\beta$ . This ability has the bystander effect of modulating immune responses to unrelated antigens (Maizels and Yazdanbakhsh, 2008). Epidemiology studies in humans have shown that infection with helminth parasites is associated with a low incidence of allergy and autoimmune diseases in developing countries. They do this by suppressing Th2 and Th1/Th17 responses which are mediators of allergy and autoimmunity respectively. Their ability to suppress immune system have been associated with impairment of vaccinations (Piessens et al., 1981, Colley et al., 1986, Grogan et al., 1996, Elias et al., 2001, Abaitua et al., 2006, Ghosh et al., 2006, Da'dara and Harn, 2010, Chen et al., 2012).

#### 1.4. Helminth co-infection with HIV/AIDS

Helminth infections are common in tropical and subtropical areas of the globe and affect the poorest and most deprived communities living in regions with substandard water, sanitation, and hygiene services (Worrell et al., 2016, WHO, 2016). Worldwide, more than 2 billion people are infected with at least one helminth species (Hotez et al., 2008, Walson et al., 2010, WHO, 2016). A significant geographic overlap exists between HIV-1 and helminth infection in sub-Saharan Africa and other developing countries (Hotez et al., 2008, Borkow and Bentwich, 2008, Means et al., 2016). More than 22.5 million people in sub-Saharan Africa alone are estimated to be co-infected with HIV-1 and at least one species of helminth (Fig 1.5) (De Silva et al., 2003, Walson et al., 2009, Pullan et al., 2014, UNAIDS, 2016).



**Figure 1.5: Soil-transmitted helminthiases and HIV epidemics in Africa.**

The interaction between chronic helminth and HIV-1 coinfection as well as their ability to have a profound effect on the host immune system and has sparked research interest in the virology field. Most soil-transmitted helminth infections are asymptomatic, especially in adults. In children, they are a cause of physical and intellectual growth retardation and malnutrition. Helminths induce tissue reactions, such as granuloma, and provokes intestinal obstruction or rectal prolapses, especially in children (Aranzamendi et al., 2013). Some studies have also shown that soil-transmitted helminth infection has profound effects on school performance and attendance and future economic productivity (Brooker et al., 2000, Anderson et al., 2013). Despite all these consequences, helminth infection is an area which remains largely neglected.

A typical helminth infection induces a dominant type 2 T-helper–cell systemic immune system response, accompanied by an induction of regulatory T cells; activation of immunosuppressive cytokines, including interleukin (IL)-4, IL-5, and IL-10; increased production of immunoglobulin IgE; and degranulation of mast cells (Webb et al., 2012, Siegel and Simon, 2012, Lankowski et al., 2014). On the other hand, HIV-1 is controlled by natural killer (NK) cells, CD8 T cells, and antibodies and destroys the host immune system by direct CD4 T cells infection (Siegel and Simon, 2012). Continuous depletion of CD4 T cells leads to immunodeficiency and down-regulation of type 1 helper T cells that are important for modulating the progression to AIDS (Siegel and Simon, 2012, Webb et al., 2012). The intense loss of Th1 immune cells during the AIDS phase is balanced with minimal reduction of Th2-associated cytokine activity (Webb et al., 2012). This immunologic shift from Th1 to Th2 cytokine response in patients with HIV may serve as a marker of HIV progression (Webb et al., 2012). Also, shifting the balance between Th1 and Th2 activation, during coinfection with HIV and different helminth species can influence the likelihood of pathogen establishment, growth, replication, and clearance; disease severity; and transmission (Fenton, 2013) especially since both HIV and chronic helminth infections significantly alter the host immune system. Data on Interactions between chronic helminth infections and HIV infection remains inconclusive.

Coinfection with helminths in patients with HIV infection has been shown to increase cellular susceptibility to HIV-1 infection, which increases the susceptibility to HIV transmission, and downregulating control of HIV-1 replication, which can increase viral replication and accelerate disease progression (Kjetland et al., 2006, Mayer et al., 2007, Walson et al., 2009, Mbabazi et al., 2011, Downs et al., 2012, Fenton, 2013, Lankowski et al., 2014, Colley et al., 2014). Furthermore, a link between helminth infection and increased risk of mother-to-child transmission of HIV has been previously shown (Mayer et al., 2007, Webb et al., 2012). Conversely, the depletion of CD4 T-cells from HIV-1 infection could also increase susceptibility to or reduced clearance of helminth infection (Webb et al., 2012). However, some studies have found no effect of helminth infection such as *S. mansoni* on the risk of HIV transmission (Sanya et al., 2015). A study in an HIV-1-seropositive adult cohort from Uganda, also showed that there was no correlation between helminth infection and exacerbated HIV infection (Elliott et al., 2003).

In addition, helminths can also sabotage host defences against other major pathogens, such as *Mycobacterium tuberculosis* (Salgame et al., 2013, Mishra et al., 2014). Ironically, in the case of malaria, however, the consequences of helminth infection are more different, with evidence of increased susceptibility combined with moderated inflammatory responses and hence attenuated disease severity (Hartgers et al., 2009, Dolo et al., 2012). Also, in a baboon study, chronic *S. mansoni* was shown to attenuate the severity of *Plasmodium knowlesi*, suggesting that infection with *S. mansoni* may provide protection against other diseases (Nyakundi et al., 2016).

T cell up-regulation in the intestinal mucosa has been shown to be capable of inhibiting the ability of CD8 cells to control HIV viral replication, increasing HIV progression in concurrency with schistosomiasis (Bustinduy et al., 2014). In patients with intense HIV infection with low CD4 counts, it has been demonstrated that the excretion efficiency of eggs is suppressed even with heavy schistosomiasis burden is suppression of egg excretion efficiency (Karp and Auwaerter, 2007, Ndeffo Mbah et al., 2013, Bustinduy et al., 2014).

As CD4 counts decline due to a progressive HIV infection, the granulomatous response to *Schistosoma* eggs stops and migration of the eggs through the mucosa becomes inhibited (Karp and Auwaerter, 2007). This, in turn, reduces egg excretion in HIV-1 infected individuals with low CD4 counts, leading to a gross underestimate of schistosomiasis prevalence when conventional stool egg count methods are used for detection (Webb et al., 2012).

Certain anthelmintic treatment regimens have been shown to be less effective in clearing helminth infections in HIV-1 infected persons with low CD4 counts (Kallestrup et al., 2006) compared to HIV-1 uninfected patients. Reinfection has also been shown to be more common after anthelmintic treatment in HIV-infected persons with low CD4 counts (Webb et al., 2012). Highly active antiretroviral therapy (HAART) has been shown to significantly reduce the prevalence of ascariasis, trichuris, hookworm infection, and strongyloidiasis, suggesting that HAART improves immunologic protection and control of helminth infection (Walson et al., 2010).

Despite these associations, it remains unclear whether the overall relationship between helminths and HIV vary with helminth burden and the specific helminth pathogen (Fenton, 2013). It is, therefore, paramount to acquire better understanding of the interactions between helminths and HIV could influence HIV-1 acquisition, disease progression, interaction with helminth vaccination, and eradication of helminth infection (Webb et al., 2012, Downs and Fitzgerald, 2016, Kroidl et al., 2016).

#### **1.4.1. Helminth infection reduces vaccine efficacy**

Vaccine development is essential to the control and prevention of chronic health challenges such as HIV-1 and helminth infections in endemic areas worldwide (Maizels and McSorley, 2016, Tomaras and Plotkin, 2017). Clinical trials and animal experiments indicate that a protective vaccine against HIV-1 will be able to protect a large helminth-coinfected population (Webb et al., 2012). However, the effects of helminths on vaccine-induced immune responses remain an area of uncertainty (Maizels and McSorley, 2016). Elimination of helminths in the host, such as albendazole treatment of ascariasis enhanced the response to oral cholera vaccine in children (Cooper et al., 2000a), and treatment of *S. mansoni*-infected mice with praziquantel had enhanced interferon gamma response to an HIV-1 clade C DNA vaccine (Da'dara and Harn, 2010). These studies suggest that the presence of helminth infection may affect vaccine

immunogenicity. Furthermore, it has been suggested that helminth vaccination may be impeded in people with poorly controlled HIV infection, depending on the type of vaccine. Vaccinations in children with HIV has been shown to be less immunogenic than in children without HIV and protective antibody titres, and they have rapid waning of vaccine-induced immunity (Bustinduy et al., 2014).

A neglected challenge to the development of vaccines for use in developing countries is helminth infection in the human host. Studies show that populations with the highest prevalence of malaria, tuberculosis, and HIV-1 mainly reside in regions of SSA and South America (Haynes et al., 2012). Unfortunately, these same populations are also often endemic for one or more helminth parasites (Hotez et al., 2008, Obuku et al., 2016). Failure to mount efficient immune responses to oral vaccines such as those for cholera, polio and rotavirus in developing countries, initiated the hypothesis that the presence of helminths in the gastrointestinal tract might have affected efficient uptake of these vaccines (Cooper et al., 2000b). In an oral cholera vaccine study, it was found that *A. lumbricoides*, "giant roundworm" of humans, decreased seroconversion, hence impairing vaccine efficiency (Cooper et al., 2000b, Cooper et al., 2001).

In Ethiopia, helminth infections have been shown to negatively impact the response to vaccines such as Bacille Calmette Guérin (BCG) vaccine (Elias et al., 2008). One study showed an improved PPD-specific Interferon gamma (IFN- $\gamma$ ) production and T-cell proliferation from peripheral blood mononuclear cells (PBMCs) in albendazole (ALB) treated healthy subjects with intestinal helminths compared to controls vaccinated with BCG (Elias et al., 2001). It was further shown that BCG-vaccinated mice with prior *S. mansoni* infection had significantly higher tuberculosis (TB) bacillary load as measured by viable count after challenge with TB (Elias et al., 2005a). In addition, lower levels of IFN- $\gamma$  and nitric oxide together with increased levels of IL-4 and IL-5 were observed in mice with prior *S. mansoni* infection (Elias et al., 2005a).

Considerable amount of literature demonstrates that population infected with helminth, have an impaired ability to respond to infections with other pathogens, especially those that require cytotoxic effector cells (Kullberg et al., 1992, Actor et al., 1993, Sabin et al., 1996, Cooper et al., 1999, Gopinath et al., 2000, La Flamme et al., 2002, Chenine et al., 2005, Su et al., 2005). Similarly, immune responses to vaccines designed to drive Th1-type CD4+ and CD8+ T cell responses, have been shown to be diminished in helminth-infected populations (Kullberg et al., 1992, Actor et al., 1993, Sabin et al., 1996, Cooper et al., 1999, Cooper et al., 2001, Chen et al., 2012).

A study by *La Flamme et al.* (La Flamme et al., 2002) showed that mice co-infected with the protozoan parasite *Leishmania* and *S. mansoni* had reduced *Leishmania*-specific IFN- $\gamma$ , TNF- $\alpha$  and nitric oxide production concurrent with increased production of IL-4. In terms of the ability to respond to unrelated antigens, *Kullberg et al.* (Kullberg et al., 1992) demonstrated that schistosome-infected mice had significantly reduced production of sperm whale myoglobin-specific interleukin (IL)-2 and IFN- $\gamma$  per CD4+ T cell coincident with a three-fold increase in CD4+ T cell IL-4 compared to responses from non-infected mice. In a mouse study by Haben and colleagues, which investigated the impact of an infection with

*Litomosoides sigmodontis* at different life stages on the efficacy of an experimental vaccine against *Plasmodium*, it was demonstrated that the helminth infection was able to attenuate accessory T helper cells, resulting in the suppression of antibody-producing B cells, thus suppressing antibody production to the vaccine (Haben et al., 2014).

With regards to the effect of helminth infection on immune responses to HIV-1 antigens, significant downregulation of virus-specific cytotoxic CD8+ T cell responses in schistosome-infected mice have been demonstrated in several studies. In a study by Actor et al., examining immune responses to vaccinia expressing HIV-1 gp160 in schistosome-infected vs non-infected mice, a significant decrease in the frequency of vaccinia and HIV-1 gp160-specific cytotoxic CD8+ T cells in schistosome-infected mice compared to vaccinia infected naïve mice was demonstrated (Actor et al., 1993). Similarly, Da'dara et al. also demonstrated that vaccination of *S. mansoni* infected mice with naked plasmid DNA encoding the TD158 HIV-1 antigens failed to induce significant viral-specific CTL responses (Da'Dara et al., 2006). The lack of gp160-specific cytotoxic CD8+ T cells and response to the TD158 vaccine in helminth-infected vaccine recipients is in agreement with multiple studies suggesting the negative effects of helminth infection on tetanus, BCG and viral vaccines (Sabin et al., 1996, Cooper et al., 1999, Cooper et al., 2001, Elias et al., 2001, Elias et al., 2005a).

## **1.5. Schistosomiasis**

### **1.5.1. History, causative agents, geographical distribution, and life cycle**

Schistosomiasis is a helminth-associated disease caused by digenetic trematodes of the genus *Schistosoma* primarily infecting man. During the mid-1800s, Egyptian pharaohs wrote of urinary disturbances (haematuria) which became common in classically young boys and was once thought to be a sign of puberty. In 1851, came along a young German pathologist, Theodore Bilharz who discovered the causative parasite; *Schistosoma* (El Khoby et al., 1998) following which he described the worm "*Distoma haematobium*" (subsequently *S. haematobium*) and published his findings together with his teacher, Professor Siegbold in Breslau. The connection to urinary disease (haematuria) with the presence of eggs in urine was made later on. There are three main species that infect human being; *S. haematobium*, *S. mansoni*, *S. japonicum*, and two minor species: *S. intercalatum*, and *S. mekongi*. Schistosomes are unique amongst plathyhelminth parasites as they are not hermaphroditic.

Urogenital schistosomiasis such as *S. haematobium* is a water-borne parasitic blood fluke, that has been reported to cause ulcerative lesions and inflammation of the cervix and vagina in females, and leukocytospermia and gross hematospermia in males (Barsoum et al., 2013). It has also been shown that the chronic inflammatory disease caused by granulomatous reactions to *Schistosoma* eggs increases the

risk of HIV transmission by three to four times (Karp and Auwaerter, 2007, Webb et al., 2012, Ndeffo Mbah et al., 2013, Kleppa et al., 2014). The distribution of Urogenital Schistosomiasis species is common in areas of high prevalence of HIV throughout the world. Of the 220 million people in sub-Saharan Africa who are infected with the parasite, 112 million are infected with *S. haematobium* (Ndeffo Mbah et al., 2013). In endemic areas, up to 75% of infected individuals have genital schistosomiasis, acquired primarily during childhood (Ndeffo Mbah et al., 2013).

Hepatic and intestinal schistosomiasis, *S. mansoni*, is most prevalent in certain tropical and subtropical areas of sub-Saharan Africa, the Middle East, South America and the Caribbean (Elbaz and Esmat, 2013). Characterised by liver and gut damaging through inflammatory inducing egg deposition (Elbaz and Esmat, 2013). Its ability to modulate immune responses has been shown to have effects on the immune modulation of HIV (Bustinduy et al., 2014).

However, geographical distribution is dependent on the distribution of the appropriate snail vectors (Gryseels et al., 2006), normally in warm climatic conditions mainly developing worlds. *Schistosoma haematobium* primarily transmitted by aquatic pulmonate snails of the genus *Bulinus* is distributed mainly in Africa and the Middle East. On the other hand, *S. mansoni* (intestinal schistosomiasis) primarily transmitted by aquatic snails of the genus *Biomphalaria* has a wide distribution including Africa, the Caribbean, South America and the middle East (Gryseels et al., 2006). Similar in transmission *S. japonicum* is mainly dominant in China, Indonesia, and the Philippines while the minor *S. intercalatum* and *S. mekongi* are found in limited areas of SSA and Southeast Asia, respectively (Gryseels et al., 2006). Schistosomiasis is typically an infection of the rural dwellers; however, urban schistosomiasis is an increasing problem in many countries especially in Africa (Steinmann et al., 2006). However, man-made ecological changes such as irrigation and the migration of infected populations further contribute to the epidemiology of schistosomiasis (Jordan and Webbe, 1993). Transmission is linked to poor hygiene, human water contact patterns and the presence of the snail vector which can only breed. This involves multiple developmental stages, both in the snail intermediate host and in the human definitive host (Fig 1.6). Schistosome eggs, excreted into fresh water, hatch to release miracidium larvae that seek snail hosts, where they replicate asexually to produce multiple generations of sporocysts (Gryseels et al., 2006, CDC, 2012). Sporocysts mature into cercariae with a characteristic bifurcated tail and are then released from snails into fresh water to seek their definitive host. At this time, cercariae typically live for 24-48 hours (hrs) and can penetrate unbroken skin, shed its tail and transform into a Schistosomulae, which then migrate through tissue into the host bloodstream, the lung, and ultimately the portal vein, resulting in human infection (Lawson and Wilson, 1980, Gryseels et al., 2006). They will mature in the portal vein for 4-6 weeks into adult worms and, migrate preferentially to venules of the urinary and genital tracts (*S. haematobium*) or the gastrointestinal tract (*S. mansoni*, *S. japonicum*). Once they reach the adult stage, they will form reproductive male-female pairs, producing hundreds to thousands of eggs per day and living an average of 3-5 years (Macedonia and Mosimann, 1994, Gryseels et al., 2006). Approximately one-third of eggs produced by worms ultimately

arrive at the lumen of the bladder or intestine and are excreted in urine or stool (Wynn et al., 1994); the remaining eggs are trapped in the tissues or are embolized to distant organs (Gryseels and Nkulikyinka, 1988). To complete the cycle, the eggs must again hatch into miracidium which must penetrate a freshwater snail (intermediate host) and develop into cercariae that are shed into the fresh water. Repeated exposure of the host to schistosome-infected fresh water will maintain the life cycle (CDC, 2012) and progressively increases the number of worms and eggs as well as the severity of the infection (Utzing et al., 2001). Each species has its own specific snail host, which explains why the disease follows the distribution of the respective snails (Sturrock, 1993).

## Schistosomiasis

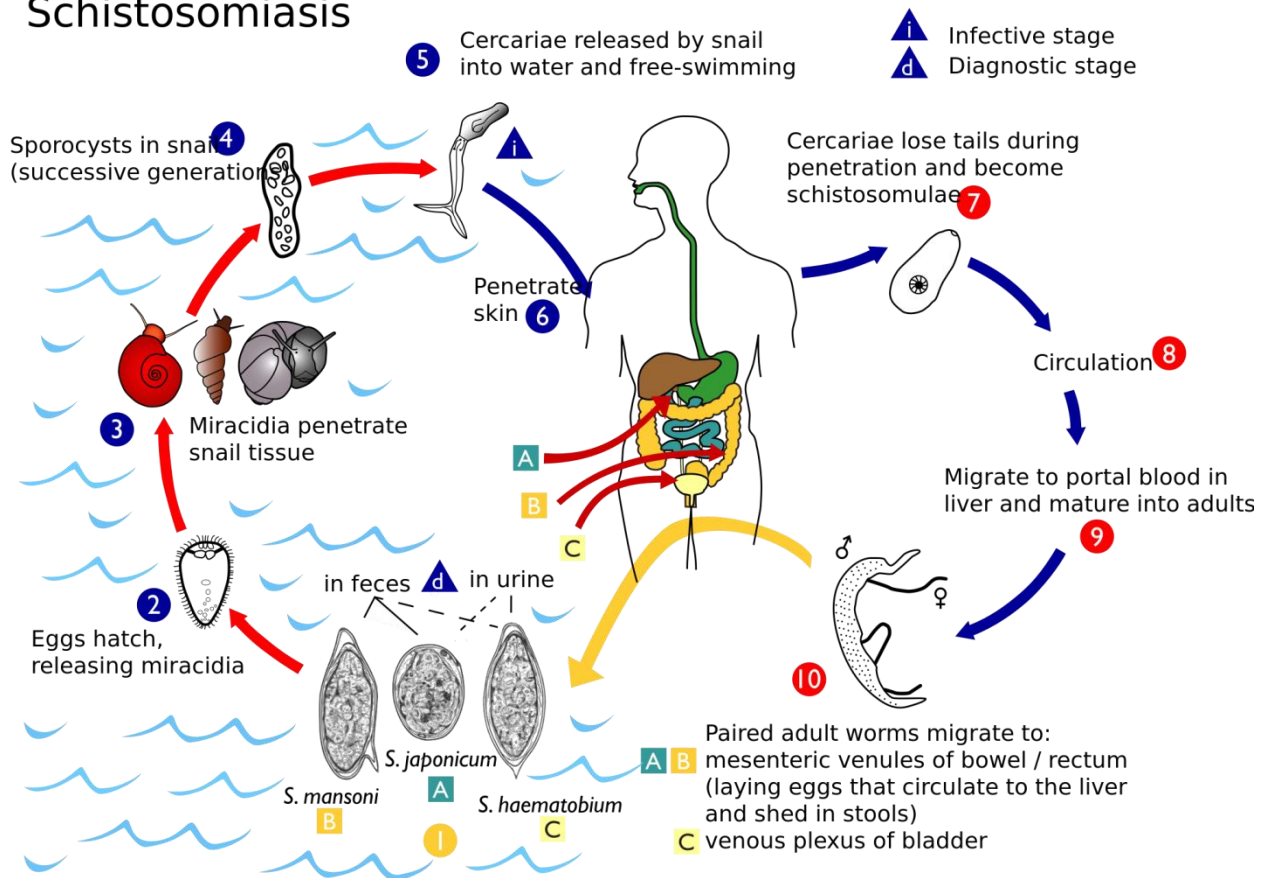


Figure 1.6: The Schistosomiasis life cycle. Taken from

<https://www.cdc.gov/parasites/schistosomiasis/biology.html> (Courtesy of CDC, 2010)

### 1.5.2. Pathogenesis and immune responses

Helminths are extracellular eukaryotic parasites from an evolutionarily ancient and diverse group (Garnier et al., 2013), but show species independent modes of immune modulation in clinical and experimental studies. This gives them the ability to escape the host defence system through suppression of both Th1 and Th2 associated host immunity and establishing chronic infections (Aranzamendi et al., 2013, Maizels and McSorley, 2016). Usually, parasitism leads to complications caused by the blockage of internal organs or the effects of pressure exerted by growing parasites. Parasite eggs migrating through body tissues also cause direct tissue damage, as well as initiate hypersensitivity reactions leading to indirect tissue damage (La Flamme et al., 2002, Borkow and Bentwich, 2004). Immune modulation due to helminth infection results in general immune hyporesponsiveness and anergy of the host (Borkow and Bentwich, 2004). Helminths induce a strong Th2-type immune response characterized by production of cytokines such as IL-4, IL-5, IL-9, and IL-13 and increased levels of circulating IgE antibodies, eosinophils, and mast cells (Kamal and El Sayed Khalifa, 2006, Moreau and Chauvin, 2010, Ortiz et al., 2011, Aranzamendi et al., 2013), creating an ideal environment for chronic infection. The polarized Th2 response, in turn, down-regulates Th1 responses along with associated cell and cytokine subsets (Kamal and El Sayed Khalifa, 2006). CD4<sup>+</sup> Th2-lymphocytes and T-reg cells are the central players in the helminth-induced immune response by the production of cytokines and chemokines such as CCR3.

Previous studies showed that depletion of CD4<sup>+</sup> T-cells discouraged mounting of a protective immune response following vaccination, and lacked the ability to expel the intestinal helminths (Koyasu et al., 2010, Saenz et al., 2010). IL-10 and TGF- $\beta$  mediated suppression of competing Th1 and Th17 cell populations play a critical role in the maintenance of Th2 dominance in many forms of helminth infections (Elias et al., 2008). This has been shown in a mouse filariasis model using *L. sigmodontis* where the chronic phase of infection is marked by T-cell anergy, loss of proliferative responses to parasite antigen challenge, reductions in effector cytokine levels, and elevated expression of inhibitory immune molecules and cells such as IL-10, TGF- $\beta$  and Tregs. In some animal models, the infective stage of the parasite stimulates a Th1-like response, and it is only after the infection is established and adult worms are present that a switch to Th2 occurs (Maizels and Yazdanbakhsh, 2003). However, this observation is not observed in all animals.

Helminths use proteins as an important product to interact with host innate immune responses (Table 1.2). Their interaction can occur through dendritic cells, toll-like receptors, and costimulatory receptors to trigger Th2 and T regulatory responses. Dendritic cells (DC) are key players in the polarization of T cell development towards Th1, Th2, or T-reg through their signaling and antigen presenting. Helminths and helminth products modulate DC function, such as DC maturation, as well as those involved in signaling through TLRs, such as TLR-2,3, and 4. TLRs mostly associated with signaling Th1 responses are activated by helminth products to signal Th2 and regulatory responses (van Riet et al., 2007). Furthermore, helminth products are able to avoid the normal TLR-induced MAP kinase and NF- $\kappa$ B signaling pathways, resulting

in immature antigen presenting cells (APC) and decreased pro-inflammatory cytokines (Harnett and Harnett, 2010). The use of helminth products to stimulate anti-inflammatory immune responses is being investigated to treat auto-immune diseases and severe allergies (Harnett and Harnett, 2010).

**Table 1.2: Immunomodulatory products of helminths: Taken from Harnett and Harnett** (Harnett and Harnett, 2010)

<b>Product</b>	<b>Helminth</b>	<b>Mechanism of action</b>
Lysophosphatidylserine	<i>S. mansoni</i>	TLR2-dependent activation of DCs promotes Treg cell development
Phosphatidylserine	<i>S. mansoni</i> and <i>A. lumbricoides</i>	TLR2-dependent activation of DCs promotes TH 2-type anti-inflammatory responses
LNFPiIII	<i>S. mansoni</i>	Production of IL-10 and prostaglandin E2 by B-1 B cells; TLR4- and C-type lectin-dependent activation of APCs promotes TH 2-type anti-inflammatory responses
ES62	<i>A. viteae</i>	Inhibition of B-2 B cell proliferation and induction of B-1 B cell-dependent IL-10 secretion; TLR4-dependent activation of APCs promotes TH 2-type anti-inflammatory responses; TLR4-dependent inhibition of mast cell degranulation and inflammatory mediator production
dsRNA	<i>S. mansoni</i>	Modulates DC function, through TLR3, to suppress TH2 cell priming of ovalbumin-specific T cells
Cathepsin cysteine proteases	<i>S. mansoni</i> and <i>F. hepatica</i>	Inhibits LPS-induced nitric oxide, IL-6, IL-12 and TNF production by macrophages by inhibition of TRIF signalling through endosomal degradation of TLR3
smCKBP	<i>S. mansoni</i>	Blocks CXCL8-induced migration and infiltration of neutrophils in a mouse air pouch model and in a chemotaxis model
IPSE	<i>S. mansoni</i>	IgE-binding factor that induces IL-4 production from basophils
Omega-1	<i>S. mansoni</i>	T2 ribonuclease that matures DCs to prime TH2 cell responses in an MYD88- and TRIF-independent manner
Peroxiredoxin	<i>F. hepatica</i> and <i>S. mansoni</i>	Promotes TH2 cell responses through induction of alternatively activated macrophages
Cystatin	<i>A. viteae</i> and <i>O. volvulus</i>	Inhibition of T cell responses by macrophage-derived IL-10
Calreticulin	<i>H. polygyrus</i>	Promotes TH2 cell-responses by interacting with scavenger receptor A
DiAg	<i>D. immitis</i>	CD40-dependent polyclonal IgE production

### 1.5.3. Acute and chronic infection

Schistosomiasis morbidity depends on the schistosome species involved, the intensity of infection, the topographic site affected by sequestered eggs and the immune responsiveness of the host (Harms and Feldmeier, 2002, Vennervald and Dunne, 2004). Acute schistosomiasis is a systemic hypersensitivity reaction against migrating schistosomula which occur 3 to 5 weeks after cercarial penetration (Akhiani, 1996, Bottieau et al., 2006). The clinical manifestations reflect developmental stages of the parasite and host responses to toxic or antigenic substances derived from the parasite and eggs. During this early stage of intestinal schistosomiasis (caused by *S. mansoni* and *S. japonicum*), the schistosomula grow into adult worms that lay eggs, which then pass through the intestinal walls. Only about 50% of the eggs are trapped in the walls and mediate an immune reaction that cause mucosal granulomatous inflammation and formation of pseudopolyps and scars around the trapped eggs (Vennervald and Dunne, 2004, Gryseels et al., 2006) leading to an inflammatory reaction that causes a sudden feverish syndrome with severe systemic illness referred to as Katayama fever (Gryseels et al., 2006). This is accompanied by malaise, weight loss, gastrointestinal symptoms, intermittent abdominal pains, high eosinophilia, headache, localised oedema, unproductive irritating cough, loss of appetite, nausea, vomiting, bloody diarrhea and muscular pains (Cheever et al., 2000, van der Werf et al., 2003, Danso-Appiah et al., 2004, Gryseels et al., 2006, Booth et al., 2006). Acute schistosomiasis is short-term conditions that usually resolve without treatment (Lambertucci, 1993).

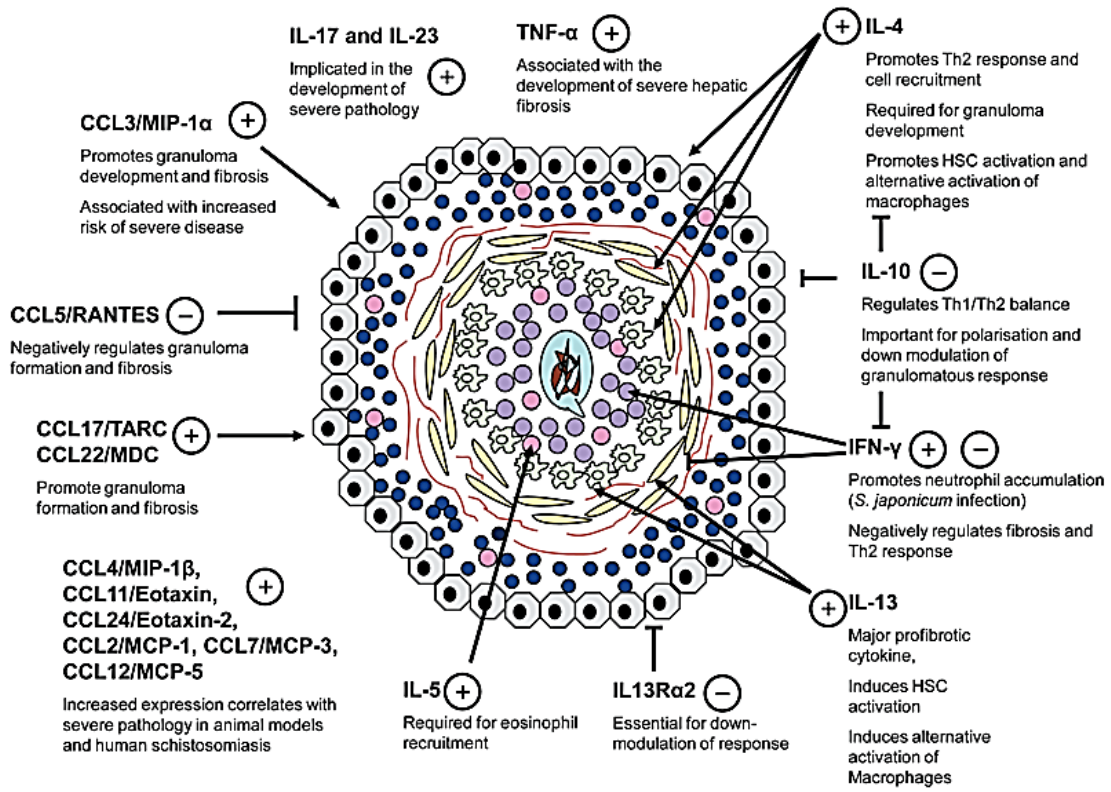
In mice, the acute phase of infection is also associated with the antigen-specific production of Th1 cytokines (IFN- $\gamma$ , IL-2, and TNF- $\alpha$ ) (Grzych et al., 1991), but this switches to a Th2 dominated response, in which there is an underlying Th1 response as soon as eggs are produced (Rutitzky et al., 2001). Failure to develop a Th2 response to regulate the initial pro-inflammatory response associated with acute schistosomiasis has been shown to be lethal. This was first observed when C57BL/6 IL-4 deficient mice were infected with *S. mansoni*. These mice suffered from cachexia and significant mortality on the onset of parasite egg production (Brunet et al., 1997) even though they developed relatively normal hepatic granulomas.

Continuous egg deposition and retaining of eggs that are carried to the liver via the portal circulation where they get trapped in the presinusoidal periportal spaces in the liver trigger a chronic infection (Pearce and MacDonald, 2002, Gryseels et al., 2006). This is characterized by hepatosplenomegaly, although the development of polyps or mucosal proliferation of the intestine is observed in most cases. The eggs contain a developing miracidium, which releases proteolytic enzymes, which give rise to typical eosinophilic inflammatory and granulomatous reactions that result in granuloma formation (Mitchell, 1990, Gryseels et al., 2006). In about 6-8 weeks, the miracidium in the egg dies, decreasing the antigen load, which sometimes leads to shrinking of the granuloma. Egg granulomas are replaced by fibrotic tissues which are prominent in the periportal areas that lead to the development of periportal fibrosis (Rumbley and Phillips, 1999, Oliveira and Andrade, 2001). The granulomatous reactions and the fibrotic lesions may result in hepatosplenic schistosomiasis, which may be accompanied by increased portal pressure. The severity of

these symptoms is associated with the intensity of infection and related to the type of immune responses generated by the infected individual (Pearce and MacDonald, 2002, Vennervald and Dunne, 2004, Gryseels et al., 2006). Hepatosplenic schistosomiasis is more common in children and adolescents than in adults (Gryseels and Polderman, 1991, Vennervald and Dunne, 2004).

#### **1.5.4. The egg granuloma**

Granuloma formation is a result of a delayed type hypersensitivity reaction against schistosome egg antigens. The egg antigens induce CD4+ T cell-dependent response which orchestrates the development of granulomatous lesions, composed of collagen fibers and cells, including macrophages, eosinophils and CD4+ T cells around the individual eggs (Dunne and Pearce, 1999, Layland et al., 2005, Wilson et al., 2007, Layland et al., 2010) (Fig 1.7). The granulomas usually resolve as the eggs die, leaving fibrotic plaques. In *S. mansoni* infection, the schistosomal granuloma is a dynamic process in which suppressor T lymphocytes drive the granulomatous reaction, closely regulated by mononucleated cells (Perrin and Phillips, 1989, Elliott et al., 1990, Lundy and Lukacs, 2013, Pearce, 2016). Both T and B cells have been shown to play a major role in immune responses against *S. mansoni* ova (Chensue and Boros, 1979, Bentley et al., 1982, Pearce, 2016), and plasma cells may produce antibodies targeted at *S. mansoni* antigens (Lichtenberg, 1964). A severe consequence of infection with *S. mansoni* is the result of an increase in portal blood pressure as the liver becomes fibrotic, congested and harder to perfuse (Silva et al., 2000, Wilson et al., 2007).

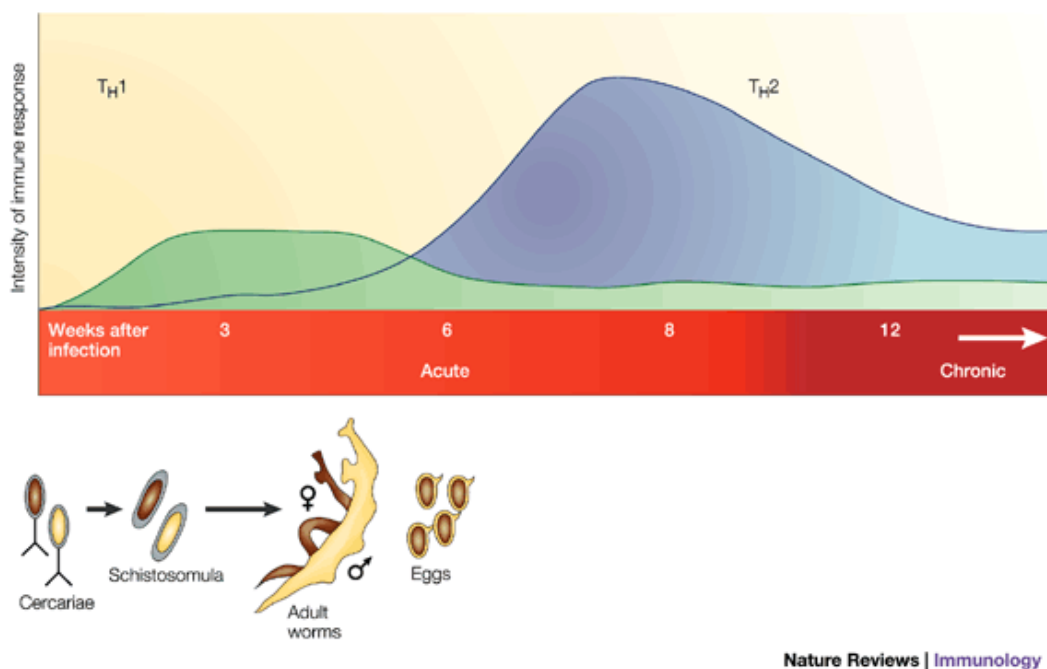


**Figure 1.7:** Major components of the granulomatous response to Schistosome eggs in the host liver and the main cytokines and chemokines that regulate this response. Taken from Burke et al., (Burke et al., 2009).

### 1.5.5. Th1/Th2 responses to Schistosome eggs

Most of the immune responses in schistosomiasis have been deduced from studies of murine *S. mansoni* infection (Burke et al., 2009). A closely regulated immune response against schistosomes acts to suppress the parasites' virulent effects, and may further be essential for host survival (Brunet et al., 1997, Fallon and Dunne, 1999). In murine *S. mansoni* infection model, the first 4-6 weeks of infection are characterised by a moderate T helper 1 cell (Th1) dominated immune response characterized by increased levels of circulating pro-inflammatory cytokines including TNF- $\alpha$ , IL-1, IL-6 and IFN- $\gamma$  (La Flamme et al., 2002, Wynn et al., 2004, Bartley et al., 2006, Wilson et al., 2007). These Th1 cells are recruited in response to schistosomula and immature adult worms and produce cytokines involved in macrophage activation (La Flamme et al., 2002). This is then followed by a shift to a T helper 2 cell (Th2) dominated immune reaction reviewed in (Burke et al., 2009). Th2 cells, are activated in response to ova deposition and produce interleukins such as IL-4, IL-5 and IL-13 involved in establishing eosinophilic and granulomatous reactions and in generating fibrosis (Cheever et al., 1991, Fallon et al., 2000, Pearce and MacDonald, 2002, Colley et al., 2014) as well

as the secretion of immunoglobulin (Ig)E and IgG4 isotypes by plasma cells (Hagan et al., 1991, Mulu et al., 2015). Following a peak at around 8 weeks post-infection (p.i), the Th2 dominant response is then downmodulated towards the chronic stage of infection (Burke et al., 2009). Regulatory T lymphocytes (T regs or CD4+CD25+ T cells) by IL-10 production, regulates the shift from Th1 to Th2 dominant immune responses, preventing severe pathology due to excessively polarised responses (Burke et al., 2009). Previous studies suggest that the immune profile in chronic helminth infections could protect against diseases such as asthma, allergy and autoimmunity (van den Biggelaar et al., 2000, Weinstock et al., 2004, Zaccone et al., 2006). The Th1/Th2-CD4+ T cell biased immune response is well represented in a mouse model by Pearce and MacDonald in Fig 1.8 below.



**Figure 1.8: The development of immune response in schistosomiasis in a mouse model. Taken from (Pearce and MacDonald, 2002).**

### 1.5.6. Control of schistosomiasis by anti-helminth chemotherapy

Despite the abundance and spread of helminth infections, helminth infections are easily eliminated by drug treatment, praziquantel (PZQ) and Oxamniquine for schistosomiasis (Shaheen et al., 1989, Utzinger and Keiser, 2004, Tallima and El Ridi, 2007) and ivermectin or mebendazole for geohelminths (Cañete et al., 2009, Churcher et al., 2009, Geary, 2012). In a mouse model, once treated, the host immunity will usually return to a normal mixed Th1-Th2 system within four to six weeks post-treatment, however, this depends on the length of time post-treatment, and in the absence of subsequent re-infection, (Da'dara and Harn,

2010, Shollenberger et al., 2013b). One of the ways in which treatment (Oxamniquine) works is by causing the worms to shift from the mesenteric veins to the liver, where the male worms are retained. Here the cellular host responses finally eliminate the male worms. On the other hand, PZQ works by increasing the permeability of the membranes of schistosome cells towards calcium ions. This causes contractions of the parasites, leading to paralysis in this contracted state. Therefore, the parasites dislodge from their active site, and are released into the systemic circulation or are destroyed by phagocytosis. There is always caution to be taken since it has been shown that treatment using PZQ was associated with increased viral replication in Ugandan population (Brown et al., 2005). Furthermore, the antifibrotic properties of PZQ have been shown to be inferior compared to Paeoniflorin, a novel experimental drug used for treating schistosomiasis and been shown to reduce and prevent liver fibrosis following liver injury due to *Schistosoma mansoni* (Abd El-Aal et al., 2017).

### **1.6. Problem statement**

Helminthiasis and HIV infections are co-endemic in many regions of sub-Saharan Africa (SSA). Globally, soil-transmitted helminths (STH) and schistosome infections are estimated to infect over 1 billion people (Lustigman et al., 2012) with a third of STH infections and over 90% of schistosomiasis cases occurring in Africa (WHO, 2014). In addition, the geographical distribution of these infections vastly overlaps with regions with high HIV prevalence in SSA (Webb et al., 2012). Therefore, it is very likely that successful future HIV vaccines will be administered to people who already have ongoing helminthiasis or have been previously infected and treated. Helminths are potent modulators of host immune response that drives Th2-dependent pathways to mediate their protection from the host (Anthony et al., 2007, Allen and Maizels, 2011). Chronic helminth infection can result in altered response to non-parasite antigens (Kullberg et al., 1992), and has been previously shown to impair specific immunity induced by a number of vaccines including Tetanus toxoid (Sabin et al., 1996), cholera (Cooper et al., 2000a), *Salmonella typhi* (Muniz-Junqueira et al., 1996), BCG (Buck et al., 1970, Kilian and Nielsen, 1989, Stewart et al., 1999, Elias et al., 2008) and two HIV vaccines, including a DNA-based vaccine construct (Actor et al., 1993, Da'Dara et al., 2006). However, there is lack of consistent scientific data to inform how candidate HIV vaccines would perform in the presence of ongoing helminthiasis given that some studies show that helminthic infections down-regulate vaccine-specific Th1 immune responses to HIV (Da'Dara et al., 2006), while other studies show the opposite is true (Shollenberger et al., 2013a).

Preventive chemotherapy is recommended by WHO and partner organizations for the control of schistosomiasis in many endemic countries. Although effective antihelminthic drugs are available (such as PZQ for schistosomiasis and ALB for gastrointestinal helminths), re-infection after treatment is possible and frequent in endemic areas, thus, vaccinees in developing countries will already have an ongoing chronic helminthiasis or previous infection before receiving the vaccines. This means that helminth infection will

continue to pose a significant problem for the development of HIV-1 vaccines designed to induce viral-specific Th1- type CD4+ and cytotoxic CD8+ T cell responses (Colley et al., 2014).

However, there is a lack of consistency in data to inform the effect of helminth treatment on the recovery of vaccine-specific responses. Some studies show that anthelmintic treatment restores vaccine responses (Da'dara and Harn, 2010, Chen et al., 2012), while others show that there is no difference between treated and untreated individuals (Walson et al., 2012, Lankowski et al., 2014). Furthermore, because both HIV-1 and *S. mansoni* rely on mutually exclusive T helper responses, it is not known whether immunization in infected individuals may contribute to helminth-induced pathogenesis or vice versa. There is therefore, a lack of scientific data to inform how candidate HIV vaccines would perform in the presence of ongoing chronic helminthiasis or whether the integration of helminthic control programmes with future HIV vaccinations would result in beneficial vaccination outcomes.

### **1.7. Rationale of the study**

Schistosomiasis and HIV-1 are endemic to sub-Saharan Africa and co-infection with both is common. Although no effective vaccines are available for both HIV-1 and Schistosomiasis, effective anti-helminthic drugs such as praziquantel are available for schistosomiasis. However, re-infection after successful treatment is very common in endemic areas. Therefore, it is likely that vaccinees will already have an ongoing chronic schistosomiasis or have previous exposure to schistosomiasis during future successful HIV immunization. Typical helminth infections are characterized by an induction of a strong T helper 2 (Th2) immune responses, increased levels of regulatory cells and the induction of specific and generalized immune hyporesponsiveness (anergy) (Borkow et al., 2000, Hoerauf et al., 2005). The performance of HIV vaccines in an ongoing chronic helminthiasis has not been clearly documented and scientific data on whether the integration of helminthic treatment with future HIV vaccinations would result in beneficial vaccination outcomes remains to be elucidated. It is thus important to investigate if whether Schistosomiasis could have an impact on the protective features of HIV vaccines.

At the University of Cape Town (UCT), two candidates HIV vaccines (SAAVI DNA-C2, SAAVI modified vaccinia Ankara [MVA-C]) have been developed (Burgers et al., 2006, Burgers et al., 2008) and have been demonstrated to generate robust T-cell immune responses in mice and NHP when given as a prime-boost vaccination regimen (Shephard et al., 2008, Burgers et al., 2009). It has been further shown that a prime-boost vaccination with SAAVI MVA-C and HIV gp140 protein vaccines induces strong mixed T-cell and antibody immune responses in NHP (Chege et al., 2017). The current study sought out to evaluate whether the immunogenicity of these candidate HIV vaccines are impacted by helminth-induced immune responses in a mouse model. *Schistosoma mansoni* was chosen as a prototype helminthic worm due to its model

being well established in BALB/c mice (Actor et al., 1993, Da'Dara et al., 2006, du Plessis et al., 2013) and its availability and established protocols in our collaborator's facility.

Findings from this study will describe the potential impact of chronic helminth infection on the immunogenicity of candidate HIV vaccines as well as inform whether the integration of helminths control programs will be beneficial to mass vaccination programmes in endemic areas. Also, this study will further demonstrate if HIV vaccinations are safe for helminth-infected people. Such outcomes would have a major impact on health policy regarding the current vaccines in clinical use and future HIV vaccines. In addition, this study is likely to provide justification for further investigations using a nonhuman primate model aimed at providing further information on the impact of schistosomiasis and future HIV vaccinations. The study hypotheses are:

- 1: Chronic schistosomiasis reduces the quantity and quality of HIV vaccine-induced responses to vaccination.
- 2: Effective HIV vaccination worsens the schistosome worm-induced pathology.
- 3: Chemotherapeutic elimination of schistosome worms restores normal responses to HIV vaccination.

### **1.8. Aims of the study**

The aim of the study was to investigate the effects of *S. mansoni* infection (schistosomiasis) on the immunogenicity of candidate HIV vaccines in mice and to determine if chemotherapeutic elimination of schistosome worms using praziquantel reverses these effects. In addition, this study aimed to examine if immunizations with these vaccines worsen the pathological conditions associated with schistosomiasis.

## CHAPTER 2

### 2. An investigation of cellular and humoral HIV vaccine-induced responses in Sm-infected mice: a preliminary study.

#### 2.1. Introduction

The aim of this objective was to conduct a preliminary study to confirm successful reproducibility of the mouse model of chronic schistosomiasis in order to validate the HIV vaccination outcomes and to give an indication of whether candidate HIV vaccine responses elicited by different vaccination regimens are affected by chronic schistosomiasis. As reviewed in Chapter 1, section 1.4, both HIV and parasitic helminthic worm infections are prevalent and geographically overlap each other considerably in Sub Saharan Africa (SSA), with a large number of inhabitants harboring at least one or more species of parasitic helminth infection (De Silva et al., 2003, WHO and UNAIDS, 2009, UNICEF and WHO, 2011) whilst over 50% of the chronically infected individuals are co-infected with HIV (Bustinduy et al., 2014). It is likely that once successful future HIV-1 vaccine(s) are developed, they will target populations in developing country populations, where the HIV epidemic is higher. However, it is most likely that vaccine recipients will be already infected with parasitic helminthics before they receive HIV immunization during such future mass vaccinations.

Several clinical and animal studies have shown that chronic helminthic worm infections play an important role in modulating host immune responses towards a predominantly Th2 type as a parasite survival mechanism (Urban et al., 1996, Fincham et al., 2003, van Riet et al., 2007). On the other hand, the trend in vaccine design is towards targeting the virus at their most vulnerable sites by robust and timely immune responses to various epitopes (Da'Dara et al., 2006, Rappuoli, 2011, Walker and McMichael, 2012). Most often, they target multiple epitopes at the same time to induce robust T cell responses (Da'Dara et al., 2006, Gray et al., 2016, Chege et al., 2017, Wee et al., 2017, Korber et al., 2017). Also, antibody responses to envelope protein are the essential target of current vaccine design (Rerks-Ngarm et al., 2009, Joseph et al., 2017, Chege et al., 2017).

Th1 and Th2 are almost mutually exclusive, meaning that if a strong Th1 response dominates, a Th2 response will be at a minimal level and *vice versa*. Thus, the immunological consequence of host immune response modulation by helminths may adversely impact standard immunizations, by suppressing immune responses to Th1-type vaccine and impairing the expansion of pathogen-specific cytotoxic T lymphocyte (CTL) responses (Elias et al., 2005a, Kamal and El Sayed Khalifa, 2006, Elias et al., 2008, Labeaud et al., 2009, Wammes et al., 2010).

As previously described in Chapter 1, section 1.3.3, several vaccine studies have suggested that helminth infections are capable of impairing vaccine efficiency and efficacy (Actor et al., 1993, Elias et al., 2005a, Da'Dara et al., 2006, Elias et al., 2008, Labeaud et al., 2009, Chen et al., 2012). For example, *S. mansoni*

infection in mice has been shown to down-regulate both HIV-1 gp160 specific CD8+ cytotoxic T lymphocytes and Th1 (IFN- $\gamma$  and IL-2) responses, resulting in delayed clearance of vaccinia virus in the liver, spleen and lungs (Actor et al., 1993). Similarly, *S. mansoni*-infected mice had a poor IFN- $\gamma$  response to an HIV-1 DNA vaccine compared to uninfected group (Da'Dara et al., 2006). However, a recent study showed that HIV-specific responses generated in mice by an attenuated live *L. monocytogenes* expressing HIV-1 Gag were not statistically different between schistosome-free and chronically infected animals (Shollenberger et al., 2013b, Shollenberger et al., 2013a). Furthermore, some studies go as far as showing that the host immune balance is not affected by helminths (Obuku et al., 2016).

Albeit each of the studies has empirical support and opposition, it remains unclear whether or not helminth infection will confound the immunogenicity and efficacy of future HIV vaccination. A number of studies that show no effect of helminth infections have major limitations such as the manner of evaluating vaccine responses and consideration of critical variables that may not render justice to the full evaluation of helminth impact on vaccination if excluded. For example, in mouse study by Shollenberger et al., showed that helminth infections do not affect vaccination with a *Listeria* HIV vaccine, however, immune responses were only observed for one dominant peptide (i.e gag protein responses: Lm-gag) (Mata et al., 2001, Shollenberger et al., 2013a). In another study that evaluated the effect of *Schistosoma mansoni* infection on innate and HIV-specific T cell immune responses in HIV-infected Ugandan fisherfolk, contradicted the theory that *S. mansoni* downregulated HIV-specific Th1 responses in HIV and *S. mansoni*-coinfected human hosts. However, the status of chronic infection was not confirmed as only the egg counts from feces were used to demonstrate Schistosoma infection without the determination of Th1 and Th2 cytokine profiles (Obuku et al., 2016, Storey et al., 2017). An acute and early chronic infection with schistosomes is characterised by elevated Th1 cytokine responses before progressing to late chronic infection which is characterised by the dominant Th2 response (Pearce and MacDonald, 2002, Da'dara and Harn, 2010, Shollenberger et al., 2013a, Shollenberger et al., 2013b). Furthermore, a study by Storey et al., suggested that soil-transmitted helminth infections were not associated with compromised antibody responses to previously administered measles and tetanus vaccines among HIV-1 infected Kenya adults who were not on ARTs. However, the measured responses reported in this study were of unstimulated cells, which may not speak to the impact of these helminths on Th1 cytokine responses (Storey et al., 2017). This is because, for an active secretion of antibodies, memory B cells need to be stimulated (Amanna and Slifka, 2010).

At UCT, two candidate HIV vaccines (SAAVI DNA-C2 and SAAVI MVA-C) have been developed (Burgers et al., 2006, Burgers et al., 2008) and demonstrated to generate robust T-cell immune responses in mice and NHP (Shephard et al., 2008, Burgers et al., 2009) following a prime-boost vaccination regimen. Furthermore, a prime-boost vaccination with SAAVI MVA-C and HIV gp140 protein vaccines was shown to induce strong mixed T-cell and antibody immune responses in guinea pigs and NHP in pre-clinical (Shephard et al., 2008, Chege et al., 2017) and clinical trial (Gray et al., 2016, Churchyard et al., 2016).

The aim of the present study was to determine whether chronic helminthiasis attenuates the immunogenicity of candidate HIV vaccines in the mouse model. Mice are the most widely used animal models in pre-clinical investigation of vaccine and drug development. In this study, BALB/c strain of mice was chosen because of its well-studied immune system. Despite having a less complex immune system than that of human, they make a suitable animal model for evaluating HIV vaccines. For example,

BALB/c mice have both H-2K<sup>d</sup> MHC class I and class II molecules which bind to several epitopes present in HIV-1 Gag, RT and Env and have been shown to activate corresponding CD8<sup>+</sup> and CD4<sup>+</sup> T cells (Mata et al., 1998, Mata and Paterson, 1999, Casimiro et al., 2002). This makes it possible to use synthetic peptides in T cell assays to assess HIV-specific CD4<sup>+</sup> and CD8<sup>+</sup> T cell responses in these mice. Furthermore, both SAAVI DNA and MVA vaccines were designed to contain a peptide sequence, RGPGRFVTI, which is dominant BALB/c Env CD8<sup>+</sup> T cell epitope within HIV-1 gp120 Env (Burgers et al., 2008). Logistically, they are small, have a short gestation period, required small space and are relatively cost-effective. Apart from assessing HIV vaccine responses, BALB/c mice have been used to evaluate immune responses to parasitic infections. Most research in schistosomiasis has focused on *S. mansoni* as a prototype in mice, wherein the entire life cycle can be recapitulated (Fu et al., 2012). Based on the versatility of the BALB/c, this animal model was therefore chosen to assess and compare the results to those previously obtained by our group and others.

Helminth infections have been suggested to negatively affect immune responses to unrelated antigens, particularly Th1 cellular responses. There is little scientific data to inform how candidate HIV vaccines would perform in the presence of ongoing helminthiasis, especially to vaccines that elicit humoral responses to HIV. In this study, three HIV vaccination regimens were selected to elicit either (i) a predominant T cell-mediated response (SAAVI DNA-C2 prime + MVA-C boost denoted DNA+MVA); (ii) a predominant antibody-mediated response (Env-gp140 protein alone) or (iii) mixed cellular and antibody (SAAVI MVA-C prime + Env-gp140 protein boost denoted MVA+gp140) responses. The aims were to investigate whether chronic helminthiasis attenuate either or both types of responses differently or equally. These vaccine regimens were then evaluated in mice chronically infected with *Schistosoma mansoni*. Splenocytes and blood serum were harvested for analysis of vaccine-specific T cell and antibody responses using ELISpot, ELISA, CBA and ICS staining. Data generated from these vaccine regimens was necessary to determine if the different arms of HIV vaccines-generated immune responses are affected differently by ongoing chronic schistosomiasis.

## 2.2. Materials and methods

### 2.2.1. Parasites

*Biomphalaria glabrata* snails infected with the Puerto Rican strain of *S. mansoni* (Sm) were maintained in the laboratory of Prof Frank Brombacher at IDM. To induce shedding of infectious Sm cercariae, 10-15 snails were put in a glass beaker containing Lipper buffered water (water containing CaCl<sub>2</sub>; MgSO<sub>4</sub>; K<sub>2</sub>SO<sub>4</sub>; NaHCO<sub>3</sub> and FeCl<sub>3</sub>.6H<sub>2</sub>O) and placed under a heat lamp (250-Watt) for 30-50 minutes. The harvested cercariae were counted under a dissecting microscope. Three independent counts were taken to get an average dose of 30-35 cercariae per mouse.

### 2.2.2. Vaccines

The following three candidate HIV vaccines were used:

1. SAAVI DNA-C2: composed of two DNA plasmids, pVRCgrttnC, and pVRCgp150CT expressing a human immunodeficiency virus subtype C (HIV-1C) polyprotein comprising *gag*, *reverse transcriptase*, *tat* and *nef* (grttnC) and an HIV-1C truncated *env* (gp150CT) respectively mixed at equimolar concentrations (1mg/ml) (Burgers et al., 2006).
2. SAAVI MVA-C: which expresses the same immunogens as the DNA vaccine with grttnC, is a recombinant MVA virus under the control of the vaccinia virus 40K promoter inserted into the Del III region, and gp150CT, under the control of the vaccinia virus 13 promoter inserted into the 49/50 region (Burgers et al., 2008).

Both the DNA and MVA were developed at UCT and have been shown to generate robust T-cell immune responses in mice (Shephard et al., 2008) and NHP (Burgers et al., 2009, Chege et al., 2017) when given in a prime-boost vaccination regime. These vaccines were manufactured by Therion Biologics Corporation (Cambridge, MA, USA; now defunct) under Good Manufacturing Practices (GMP).

3. HIV-1 gp140 Env protein: The gp140 (TV-1)(HIV-1/Clade C) was purchased from Immune Technology, USA. The Env amino acid consequences were derived from a South African subtype C primary isolate, TV1 (Lian et al., 2005). This vaccine is similar to the Novartis protein subunit vaccine (GSK, Cambridge, MA), an oligomeric V2 loop deleted glycoprotein 140 (gp140ΔV2.TV1) derived from the same HIV isolate, TV1, and shown to induce Env-specific humoral responses in rabbits and mice (Lian et al., 2005).

The above vaccines were manufactured for a clinical trial in South Africa (Williamson et al., 2012). They were demonstrated in a pre-clinical and clinical trial to be safe and to elicit strong CD4<sup>+</sup> responses and modest CD8<sup>+</sup> cellular immunogenicity (Gray et al., 2016, Churchyard et al., 2016, Chege et al., 2017).

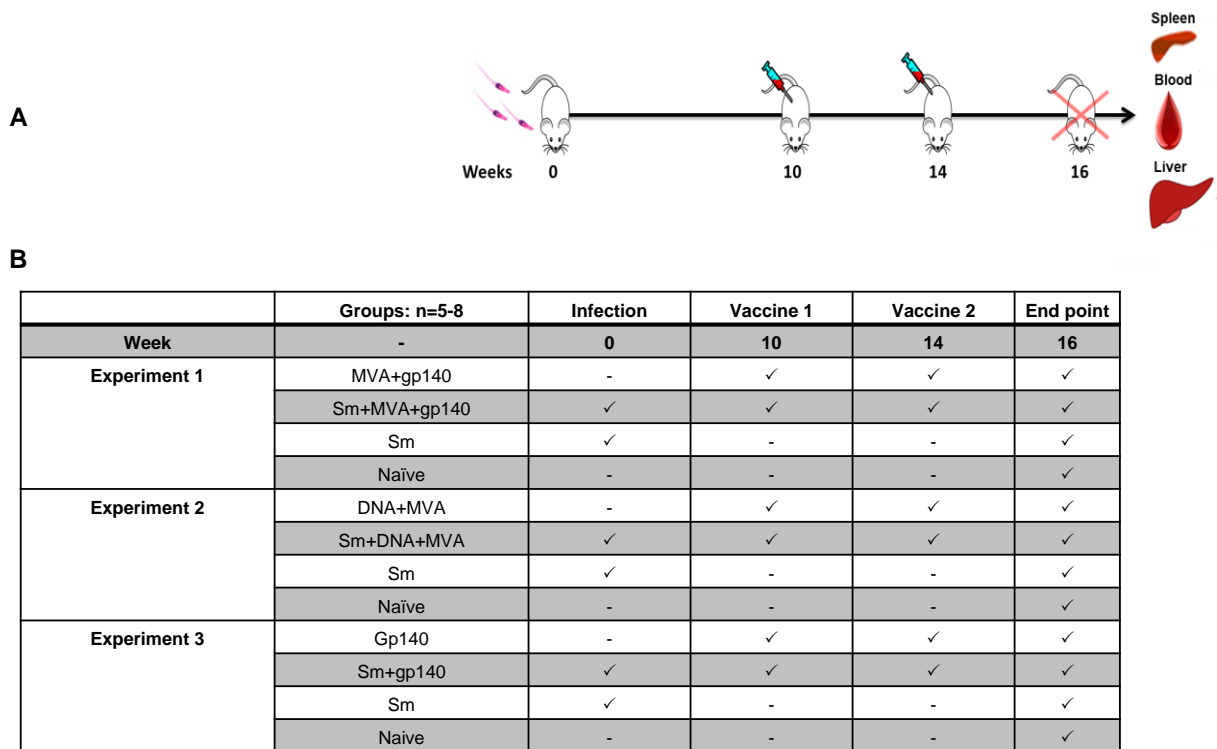
### **2.2.3. Animals**

Female BALB/c mice (6–8 weeks old) were purchased from South African Vaccine Producers (SAVP) (Johannesburg, South Africa), housed in Animal Biosafety Level 2 and maintained in accordance with the South African national guidelines for Use of Animals for Scientific Purposes (SANS Code 10386: 2008). Mice were allowed to acclimate for one week before commencing any experimentation. The experimental protocols performed in this study were reviewed and approved by the Animal Ethics Committee of the University of Cape Town (UCT AEC: protocol number: 014/026).

### **2.2.4. Experimental design**

#### **2.2.4.1. Immunization schedule**

Three pre-clinical experiments were conducted as a preliminary study to indicate if chronic schistosomiasis in mice can be reproduced and whether immune responses elicited by different vaccination regimes are differently affected by schistosomiasis. Briefly, BALB/c mice were divided into two groups of 5 mice (Sm-free) or two groups of 8 mice (Sm-infected). Mice were infected with Sm cercariae at week 0 and subsequently vaccinated twice, four weeks apart with appropriate vaccines at week 10 and 14 respectively. Twelve days after last vaccination (week 16), mice were euthanised, and samples (blood; spleen and liver) were collected for analysis of vaccine-specific T cell; antibody and mixed immune responses (Fig 2.1).



**Figure 2.1: Immunisation schedules:** A) Representation of infection and immunization strategy. B): experiment 1 (mixed cellular and antibody response vaccine regimen); experiment 2 (cellular response vaccine regimen) and experiment 3 (antibody response vaccine regimen).

#### 2.2.4.2. Infection of mice with Sm-cercariae

Mice were anaesthetized by intraperitoneal injection of a mixture of ketamine hydrochloride (120mg/kg body weight) and xylazine (16mg/kg body weight) in saline. Mouse abdomen was shaved and a metal ring (1 cm in diameter) was placed on the wet shaved area. 30-35 live Sm cercariae parasite were placed in water held inside the metal ring for 20 min to allow natural skin penetration.

#### 2.2.4.3. Vaccination of mice with SAAVI DNA-C2; SAAVI MVA and HIV Env-gp140

Vaccinations with SAAVI DNA-C2 and SAAVI MVA-C were given intramuscularly while vaccination with the Env-gp140 protein was given intraperitoneally. The Env-gp140 vaccine protein was administered at a dose of 10µg protein in 100µl PBS containing 1µg Gag VLPs (per mouse). Gag VLPs were included in the protein vaccine to provide adjuvant properties as has been observed (Gerald Chege; personal communication). All intramuscular vaccinations were performed under anaesthesia with ketamine and xylazine mixture. The DNA vaccine (100µg per mouse) was administered intramuscularly in a total volume of 100µl PBS by needle injection, 50µl in the tibialis muscle of each hind leg. The MVA vaccine (10<sup>6</sup> plaque forming units [pfu] per mouse) in a total volume of 100µl 1mM Tris (Sigma Aldrich, USA) per mouse was administered by needle

injection, 50µl in the tibialis muscle of each hind leg. Administration of vaccines was according to the immunisation schedule (Fig 2.1).

### **2.2.5. Preparation of splenocytes and serum**

Spleens harvested from animals of the same group were pooled for the preparation of a single cell suspension of splenocytes as previously described (Shen, 2010). Red blood cells were removed by lysing them with Red Blood Cell Lysing Buffer (Sigma-Aldrich, USA). The resulting splenocytes were used in IFN-γ / IL-2 ELISpot (section 2.2.6), flow cytometry and intracellular cytokine staining (ICS) (section 2.2.7) and cytometric bead array (CBA) assay (section 2.2.8). Serum was separated by spinning down mouse blood at 2000 g for 10 minutes and stored at -22°C until analysed for antibodies using an antibody ELISA assay (section 2.2.9).

### **2.2.6. Interferon (IFN)-γ and interleukin-2 (IL-2) ELISpot assay**

To measure the number of IFN-γ and IL-2 secreting CD4+ and CD8+ T cells, in response to re-stimulation with HIV-1 peptides or *S. mansoni* soluble egg antigen (SEA) and concanavalin A (Con A: Sigma-Aldrich, USA), IFN-γ and IL-2 ELISpot were carried out according to an established and optimised protocol in the lab (Shephard et al., 2008). Briefly, freshly isolated splenocytes were plated in triplicates at half a million cells per well previously coated with either IFN-γ or IL-2 capture antibody. Two micrograms per millilitre (2µg/ml) of an individual peptide (Bachem, Switzerland) was used for *in vitro* stimulation of cells followed by detecting responding cells (Table 2.1). As for the Gag CD4 peptides, MRC13 and MRC17 were pooled together. (The Env CD8 peptide (V3 CTL) was not available for use in the preliminary study but it was used in succeeding assays described in Chapters 3 and 4). BALB/c mice express the H-2K<sup>d</sup> gene in their major histocompatibility complex (MHC) which allows them to bind and respond to specific HIV-1 epitopes. Cells were also stimulated with 2µg/ml of an irrelevant peptide (hemagglutinin [influenza A virus-derived]: negative control); 1µg/ml Con A (positive control) and 20µg/ml *S. mansoni* SEA (parasite control). Membrane-bound cytokines were detected using biotinylated IFN-γ /IL-2 secondary antibodies. Spots were developed using the Nova Red (Vector Laboratories, USA) substrate following manufacturer's instructions. The number of spots was counted and analysed with a CTL ImmunoSpot analysing system (Cellular Technology Limited, USA). The average of triplicate counts of IFN-γ and IL-2 spot forming unit (SFU) was calculated for each stimulant and normalised to 10<sup>6</sup> splenocytes to give SFU/10<sup>6</sup> splenocytes. The results were reported as net IFN-γ or IL-2 SFU/10<sup>6</sup> splenocytes after subtracting the background SFU/10<sup>6</sup> splenocytes obtained from cells stimulated with an irrelevant peptide (negative control). A cut-off value of 2X background spots (responses in irrelevant peptide stimulation) plus 2X standard deviation (SD) was set to determine a positive response. Any response with an SFU/10<sup>6</sup> below the cut-off value was assigned a magnitude of zero (0 SFU/10<sup>6</sup>).

**Table 2.1: Control and peptide stimulants used in the ELISpot, ICS and CBA assays**

<b>Stimulant</b>	<b>Source of stimulant</b>	<b>Description</b>	<b>Peptide sequence</b>
<b>Con A</b>	Sigma-Aldrich, USA	Non-specific polyclonal stimulus positive control	N/A
<b>Env CD4 (Env 6)</b>	Bachem, Switzerland	Env MHC class II-restricted CD4 epitope	-YGVPVWREAKTILFCA-
<b>Env CD8 (V3-CTL) peptide</b>	Bachem, Switzerland	Env H-2D <sup>d</sup> -restricted CD8 peptide	-RGPGRAFVTI-
<b>Gag CD4 (MRC13) peptide</b>	Bachem, Switzerland	Gag MHC class II-restricted peptide (CD4 peptide)	-NPPIPVGDIIYKRWIILGLNK-
<b>Gag CD4 (MRC17) peptide</b>	Bachem, Switzerland	Gag MHC class II-restricted peptide (CD4 peptide)	-FRDYVDRFFKTLRAEQATQE-
<b>Gag CD8 peptide</b>	Bachem, Switzerland	Gag H-2K <sup>d</sup> – restricted class I peptide (CD8 peptide)	-AMQMLKETI-
<b>Irrelevant peptide</b>	Bachem, Switzerland	Negative peptide control	-H-TXSTVASSL-OH-
<b>PMA/Ionomycin</b>	BD Biosciences, USA	Leukocyte Activation cocktail containing PMA, Ionomycin and Brefeldin A: Positive control	N/A
<b>RT CD4 peptide</b>	Bachem, Switzerland	MHC class II-restricted CD4 peptide	-PKVKQWPLTEVKIKALTAI-
<b>RT CD8 peptide</b>	Bachem, Switzerland	H-2K <sup>d</sup> -restricted RT peptide	-VYYDPSKDLIA -
<b>SEA</b>	Theodore Bilharz Research Institute, Egypt	Soluble Egg Antigen: (crude extract of heterogeneous proteins)	N/A

### 2.2.7. Flow cytometry and ICS

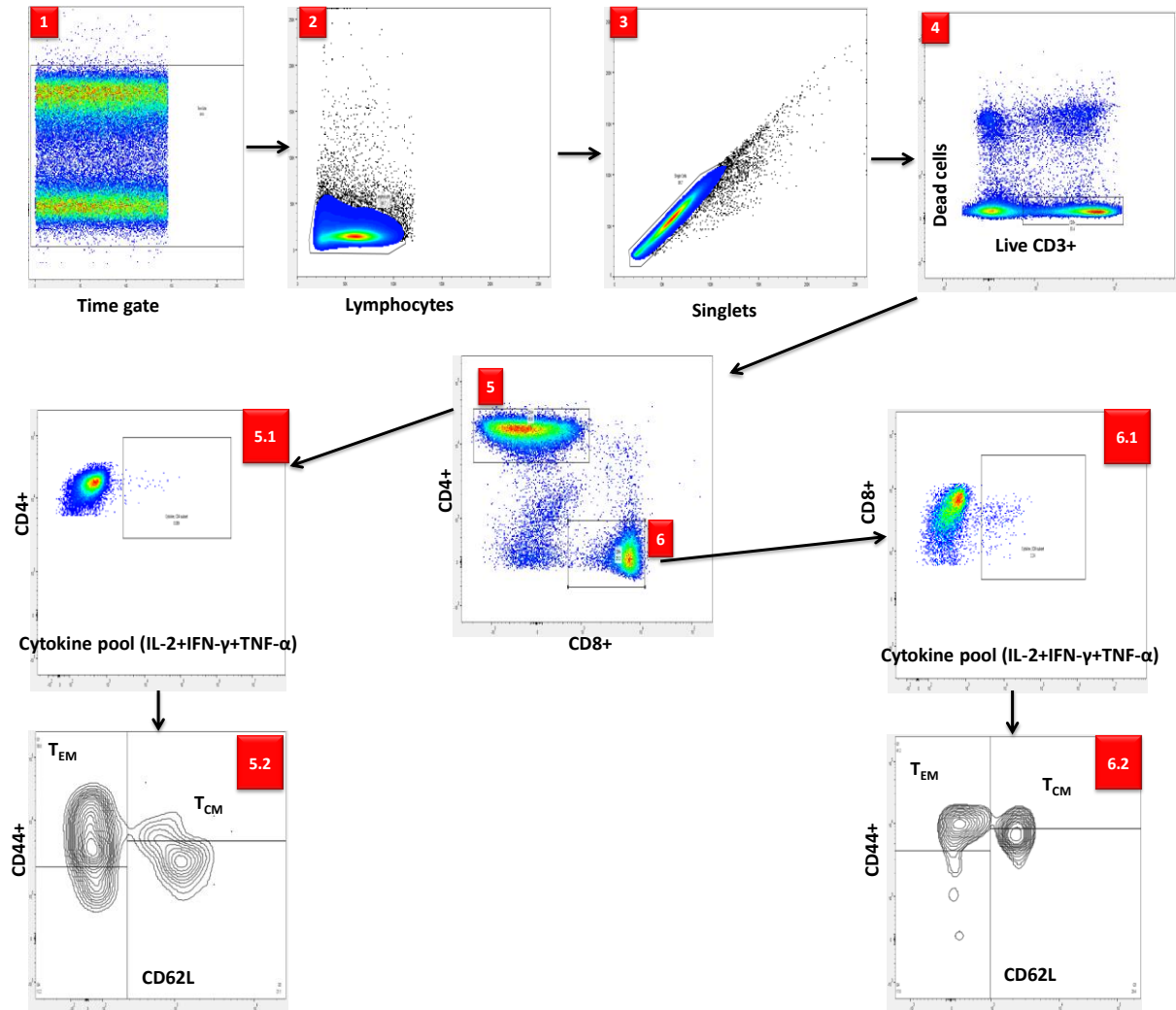
The frequency of IFN- $\gamma$ , IL-2 and TNF- $\alpha$  secreting splenocytes in response to *ex vivo* stimulation with HIV peptides was measured by ICS followed by flow cytometry analysis as modified from previously established

protocol (Jongwe, 2015). Briefly, freshly isolated splenocytes (adjusted to  $2 \times 10^6$  cells/ml) were stimulated with 2 $\mu$ g/ml of HIV Pol (RT CD4+ RT CD8) specific peptides; 20 $\mu$ g/ml of SEA (parasite-specific control) or with 2 $\mu$ l Leukocyte activation cocktail (phorbol 12-myristate 13-acetate + ionomycin [PMA/Ionomycin]) for 2 hrs at 37°C, 5% CO<sub>2</sub>. Each group consisted of a negative control (unstimulated cells). Brefeldin A (BFA) (20 $\mu$ g/ml) was added to the splenocytes and incubated for an additional 4 hrs.

Splenocytes were then dispensed into a V-bottomed 96-well plate and washed twice with 1x PBS (Gibco, Invitrogen) to remove any debris and proteins from the culture medium. This was achieved by spinning the plate in a centrifuge at 840 g for 3 minutes and discarding the supernatant from the wells. Cells were then blocked in 50 $\mu$ l blocking solution (0.12 $\mu$ l normal mouse serum, 0.12 $\mu$ l normal rat serum, 0.16 $\mu$ g CD16/32 [BD Biosciences, USA]) for 15 minutes at 4°C following re-suspending them in 1x PBS (150 $\mu$ l/well) and centrifuging as before.

Cells were re-suspended in ViViD (50 $\mu$ l/well) (Invitrogen) at a pre-determined optimal concentration and the plate was incubated for 20 minutes at room temperature in the dark to stain for the live/ dead cells. Plates were then washed twice with FACS wash buffer and pelleted by centrifugation (840 g for 3 minutes), to remove unbound ViViD before surface staining. Cell surface molecules were stained with the fluorochrome-conjugated antibody (anti-CD3-Alexa 700, anti-CD4-PE-Cy7, anti- $\alpha$ CD8-APC-Cy7, anti-CD62L-APC, and anti-CD44-FITC) diluted to 0.2 $\mu$ g in staining buffer (BD Biosciences, USA) and incubated at 4°C for 20 minutes. Cells were washed and permeabilized with Cytofix/cytoperm and further stained with pooled PE-conjugated anti-TNF (0.2 $\mu$ g) anti-IL-2 (0.06 $\mu$ g) and anti-IFN-g (0.06 $\mu$ g) antibodies diluted in Perm/Wash buffer (BD Biosciences, USA). Cells were washed twice 200 $\mu$ l wash buffer before being re-suspended in 100 $\mu$ l stain buffer and stored at 4°C for until acquisition on a BD LSRII (BD Biosciences, USA). All the antibodies except anti-CD3-Alexa 700; anti-CD62L-APC and anti-CD44-FITC (eBioscience, USA) were obtained from BD Biosciences) (Table 2.2).

Data was acquired using an LSRII flow cytometer running FACS Diva (BD Biosciences, USA) and analysed in FlowJo version 10 software (FlowJo LLC, USA). The gating strategy and representative plots are illustrated below (Fig 2.2). Responses to individual stimulants were considered positive for cytokine production if the frequency of cytokine-producing cells was  $\geq 0.05\%$  (with a minimum of 50 events). Cytokine-positive memory T cells (memory phenotypes of the cytokine positive cells determined by their relative expression of CD44 [effector] and CD62L [central]) were considered positive if >20 events were detected. Thus, any responses not meeting the set cutoff were assigned 0 (negative). One experiment was conducted and the mean frequency of cytokines expressing T cells was plotted.



**Figure 2.2: Gating strategy used for flow cytometry analysis of vaccine-specific CD8+ and CD4+ cytokine-producing cells.** Splenocytes were stimulated with appropriate stimulant or left unstimulated (negative control). Time gates were plotted and used to exclude artefacts or aggregates (if any) (**plot 1**). Lymphocytes were gated based on the relative size and granularity of the cells and plotted as forward scatter (FSC) versus side scatter (SSC) (**plot 2**). Singlet gates based on forward scatter height (FSC-H) and forward scatter area (FSC-A) were used to exclude doublet cells (**plot 3**). Live CD3+ cells were gated on ViViD negative populations (**plot 4**). CD4+ and CD8+ T cells populations were identified (**plot 5** and **plot 6** respectively). Cytokine-producing CD4+ and CD8+ cells were identified (**plot 5.1** and **plot 6.1** respectively) as well as, effector (T<sub>EM</sub>) and central (T<sub>CM</sub>) memory for both cytokine-producing CD4+ and CD8+ T cells (plot 5.2 and 6.2 respectively).

**Table 2.2: Antibodies used in Flow cytometry and ICS of this study modified from previously published thesis (Jongwe, 2015).**

Ab	Working concentration	Manufacturer
anti-CD3-Alexa 700	0.2µg	eBioscience
anti-CD4-PE-Cy7	0.2µg	BD Biosciences
anti-αCD8-APC-Cy7	0.2µg	BD Biosciences
anti-CD62L-APC	0.2µg	eBioscience
anti-CD44-FITC	0.2µg	eBioscience
anti-TNF-PE	0.2µg	BD Biosciences
anti-IL-2-PE	0.06µg	BD Biosciences
anti-IFN-g-PE	0.06µg	BD Biosciences
anti-CD19-Parc Blue	0.2µg	BD Biosciences

### 2.2.8. Quantification of secreted cytokines by CBA

The cytometric bead array (CBA) assay (BD Biosciences, USA) is a powerful tool used for quantifying cytokine production in cells. Unlike ELISA assays, the CBA assay is cost effective and can measure multiple cytokines at a time while requiring less sample volume. The BD CBA mouse Th1/Th2/Th17 kit (BD Biosciences, USA) provides an affordable way to determine whether an immune response elicited is a Th1 or Th2 biased, with IFN-γ, IL-2, and TNF-α being associated with a Th1 response, while IL-4, IL-6, and IL-10 are associated with a Th2 response (Murphy and Weaver, 2016). Freshly isolated splenocytes pooled from 5-8 mice /group were adjusted to  $15 \times 10^6$  cell/ml) with R10 medium. One hundred microlitres (100µl) of the splenocyte suspension ( $1.5 \times 10^6$  splenocytes) was added to each well in a 96 well round-bottom microtiter plate followed by the addition of 100µl of R10 medium (RPMI medium supplemented with 10% heat-inactivated fetal bovine serum (FBS), 15mM 2-mercaptoethanol, 100U penicillin per ml, and 100µg streptomycin), Con A / SEA or HIV peptides (described in Table 2.1). The Gag CD4 peptides comprised of MRC13 and MRC17 were pooled together. (The Env CD8 peptide (V3 CTL) was not used in the preliminary study but used in succeeding assays in Chapters 3 and 4). The plates were incubated at 37°C; 5% CO<sub>2</sub> in a humidified air incubator for 48 hours. The cells were cultured in duplicate for each group of mice. The supernatants were carefully harvested (150µl) without disturbing the cell pellets and stored at -22°C until the day of cytokine detection using a CBA mouse Th1/Th2/Th17 cytokine kit (BD Biosciences, USA) that measures Th1 (IFN-γ, TNF-α, IL-2) Th2 (IL-4, IL-6), Th17 (IL-17A) and non-classical Th2 (IL-10) cytokines. The CBA assay was carried out according to the manufacturer's protocol. Briefly, lyophilized mouse Th1/Th2/Th17 standards were reconstituted in 2ml of assay diluent and allowed to equilibrate at room temperature for 15 minutes. Serial dilutions (1:2, 1:4, 1:8, 1:16, 1:32, 1:64, 1:128 and 1:256) of the reconstituted cytokine standards were prepared in FACS tubes including a tube with assay diluent only

(negative control). Individual capture beads (A1-A7: IL-2, IL-4, IL-6, IFN- $\gamma$ , TNF- $\alpha$ , IL-17A and IL-10 respectively) were vortexed vigorously before being pooled together in a FACS tube (10 $\mu$ l of each type of capture bead per assay). Fifty microliters (50 $\mu$ l) of capture bead mixtures and 50 $\mu$ l of test sample supernatants were mixed in a FACS tube. Also, fifty microliters (50 $\mu$ l) of each standard dilution were added into allocated tubes. Fifty microliters (50 $\mu$ l) of PE detection reagent was then added into each of the tubes containing standards and test samples following which the tubes were incubated at room temperature for two hours in the dark. All the tubes were washed with 1ml wash buffer and each bead pellet was re-suspended with 300 $\mu$ l of wash buffer. The samples were analysed using an LSRII flow cytometer running FACS Diva (BD Biosciences, USA).

The average values of cytokine produced were calculated using FCAP array software (BD Biosciences, USA) and expressed as picogram per millilitre (pg/ml) of supernatant. Cytokine values were considered positive if they were greater than twice the background values (cytokine secreted by splenocytes in the presence of an irrelevant peptide per group). Furthermore, values below the cut-off (detection limit per cytokine described in Table 2.3) were set to zero. Th17 cytokine (IL-17A) was excluded from the final results. The sum of cytokine values obtained with the individual HIV peptide stimuli (cumulative cytokine response) for all vaccinated groups was plotted and expressed as pg/ml. The theoretical limit of detection of the CBA mouse Th1/Th2/Th17 Cytokine Kit (Table 2.3) is comparable to conventional ELISA. By applying the 4-parameter curve fit option, it was possible to extrapolate values for sample intensities not falling within the limits of the standard curve. All responses not meeting the set cut-off were assigned zero (negative).

Table 2.3: Cytometric bead array (CBA) kit (BD Biosciences): Limit of cytokine detection

<b>Cytokine</b>	<b>Limit of detection (pg/ml)</b>
IL-2	0.1
IL-4	0.03
IL-6	1.4
IFN- $\gamma$	0.5
TNF- $\alpha$	0.9
IL-17A	0.8
IL-10	16.8

## **2.2.9. Quantification of total and Env-gp140 specific antibody isotypes by ELISA assay**

### **2.2.9.1. Total IgG antibody ELISA**

Ninety-six-well MaxiSorp ELISA plates (Nunc, USA) were coated with goat anti-mouse IgG capture antibody (Sigma Aldrich, USA) at a concentration of 2.5µg/ml in coating buffer (PBS) and incubated at 4°C overnight. The wells were washed five times with 200µl washing buffer (PBS containing 0.05% Tween-20: Sigma Aldrich, USA), then blocked for 60min at room temperature with 200µl blocking buffer (PBS containing 5% skimmed milk). A standard dilution series for IgM; IgG1; IgG2a and IgG2b were prepared by serially diluting standards in dilution buffer (PBS containing 2.5% skimmed milk) and plated in duplicates. Samples to be tested were diluted (1:5000) and plated in duplicates for 2 hours at room temperature. Plates were washed five times with 200µl wash buffer following which matching secondary antibodies conjugated to horseradish peroxidase (HRP) (1:500) were added. The plates were incubated for 60min at room temperature in the dark. After incubation with appropriate secondary antibodies, plates were washed five times with washing buffer followed by developing with 100µl/well tetramethyl-benzidine substrate (TMB; KPL, USA). The reaction was stopped by addition of 100µl/well stop solution (1N H<sub>2</sub>SO<sub>4</sub>). Immediately after stopping the reaction, the optical density (OD) was measured at 450nm and a reference of 540nm with a microplate reader (Molecular Devices Corporation) running SOFTmax v6 software (Molecular Devices Corporation, USA). Curve fitting was approximated by a 4-parameter hyperbolic curve (SOFTmax PRO ver.6; Molecular Devices Corporation, USA) and data was presented as ng/ml.

### **2.2.9.2. Env-gp140 specific antibody ELISA**

To measure the level of HIV gp140 Env-specific antibodies in mouse sera, a standardised ELISA assay was established according to the ELISA method by (Cheung et al., 2015). The method was based on assigning antibody units (at a fixed dilution) extrapolated from a standard curve of a reference sample. To prepare the reference sample to be used in the current study, sera from mice (previously vaccinated twice with HIV gp140 Env protein) selected for relatively high antibody titer to Env-gp140 were pooled and diluted 1:100 with PBS. Diluted and aliquoted (50µl per aliquot) sera were stored at -22°C until required. To perform the standardized ELISA assay, flat-bottom 96-well ELISA plates (Nunc, USA) were coated with 0.5µg/ml of gp140 protein diluted in phosphate buffered saline (PBS) and incubated overnight at 4°C. Plates were blocked with 200 µl/well of blocking buffer for 2 hours at room temperature. Independent duplicate dilutions (1:1000) of each test mouse serum was prepared in dilution buffer (PBS containing 2.5% skimmed milk). Diluted test sera (1:1000) and 12 two-fold dilutions of reference sample were added to gp140-coated wells (100µl/well) and incubated for 2 hours at room temperature. Plates were washed five times with washing buffer (PBS containing 0.05% Tween-20: Sigma Aldrich, USA) using an automatic plate washer followed by addition of 100µl/well of the appropriate secondary antibody conjugated with horseradish peroxidase (HRP) for 2 hours at room temperature. The secondary antibodies included 3 anti-Mouse IgG isotypes (IgG1; IgG2a and IgG2b: Southern Biotechnology). After incubation with appropriate secondary

antibody, plates were washed five times following developing with 100µl/well tetramethyl-benzidine substrate (TMB; KPL, USA). Plates were incubated at room temperature for 5-10 minutes in the dark. Reactions were terminated by adding 100µl/well of stopping buffer (1N H<sub>2</sub>SO<sub>4</sub>). Immediately after stopping the reaction, the optical density (OD) was measured at 450nm and a reference of 540 nm with a microplate reader (Molecular Devices, USA) running SOFTmax v6 (Molecular Devices Corporation, USA).

To assign antibody units (AUs), first, one of the aliquots was serially diluted in two-fold steps from 1:100 to 1:204,800 (12 dilutions) with dilution buffer. Mouse sera from naïve (unvaccinated) animals were used as a negative control. Next, the ELISA steps were performed as described above. Based on the constants of the standard curve generated from the serially diluted reference sample, the reciprocal dilution giving an OD=1 (against gp140) was assigned 1000 antibody units and the negative control was assigned a reciprocal dilution of 0. Curve fitting was approximated by a 4-parameter hyperbolic curve (SOFTmax PRO ver.6; Molecular Devices Corporation, USA). This method was validated by comparing AUs derived from single dilutions versus multiple serial dilutions of the same test serum and confirming a positive correlation between reciprocal dilutions with OD=1 and calculated AUs. Thereafter, a reference sample was used on each ELISA plate to generate a standard curve from which the assigned antibody units were used to extrapolate for test samples at a fixed dilution of 1:1000. A cut-off value for positive antibody responses was set at 2 x the OD value of the negative control serum (naïve) and those below the cut of value were assigned an antibody unit of zero.

#### **2.2.10. Statistical analysis**

In order to measure the effect of schistosomiasis on the immunogenicity of the candidate HIV vaccine that induced antibody immune responses, statistical analyses were conducted to compare between the vaccinated groups that were either infected or uninfected. Results were analysed in Prism® version 5.0 (GraphPad Software, USA), using two-tailed Student t-tests for significance. Statistical p values < 0.05 were considered to be significant. The false discovery rate (FDR) with Benjamini-Hochberg-adjusted P < 0.05 was performed as described by (McDonald, 2009). No statistical analysis was conducted for the cellular immune response, as only one repeat experiment of pooled mice was conducted for the preliminary study.

## 2.3. Results

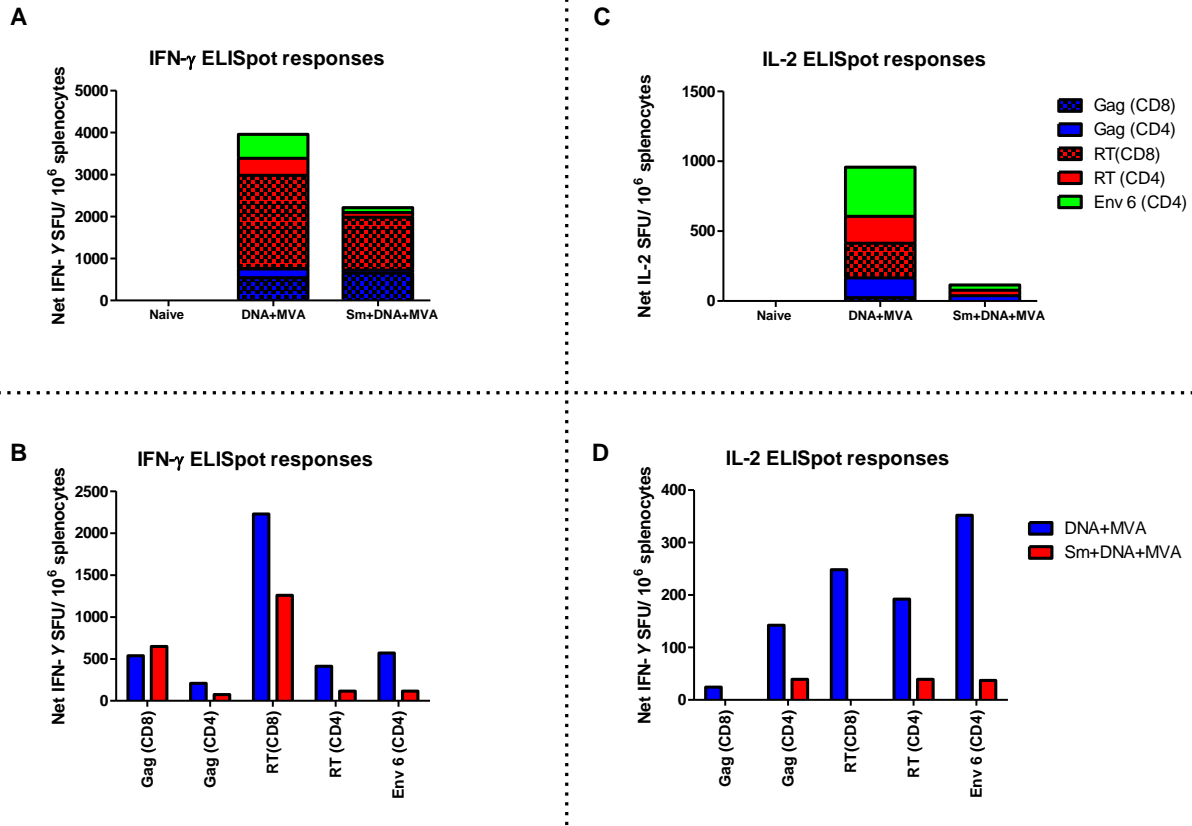
### 2.3.1. Cellular immune responses to the DNA+MVA and MVA+gp140 vaccine regimen in the background of Sm measured by ELISpot

Cellular immune responses were evaluated using IFN- $\gamma$  and IL-2 ELISpot assays to enumerate the magnitude of Gag; RT and Env-specific IFN- $\gamma$  and IL-2 secreting CD8+ and CD4+ T cells in the spleen of mice vaccinated with either the DNA-MVA (DNA+MVA) (Fig 2.3) or MVA-protein (MVA+gp140) (Fig 2.4) vaccines.

#### 2.3.1.1. Vaccine-specific ELISpot responses to DNA+MVA vaccine regimen

Vaccination with DNA+MVA induced high cumulative HIV-1 Gag, RT and Env-specific IFN- $\gamma$  (3957 SFU/10<sup>6</sup> splenocytes) (Fig 2.3 A) and IL-2 ELISpot (958 SFU/10<sup>6</sup> splenocytes) responses in Sm-free vaccinated mice (DNA+MVA). In contrast, vaccination in Sm-infected mice (Sm+DNA+MVA) induced a moderate magnitude of peptide-specific IFN- $\gamma$  (2210 SFU/10<sup>6</sup> splenocytes) and a low magnitude of IL-2 (115 SFU/10<sup>6</sup> splenocytes) ELISpot responses. The same was observed for IFN- $\gamma$  (Fig 2.3 B) and IL-2 (Fig 2.3 D) ELISpot responses to individual Gag (CD4 or CD8); RT (CD4 or CD8) and Env (CD4) specific peptides. IFN- $\gamma$  ELISpot responses to the RT CD8 peptide induced the highest magnitude of IFN- $\gamma$  secreting CD8+ and CD4+ T cells (2228 SFU/10<sup>6</sup> splenocytes) in Sm-free mice. However, a lower magnitude of RT CD8-specific IFN- $\gamma$  (1258 SFU/10<sup>6</sup> splenocytes) was observed in Sm-infected vaccinated mice. The same was observed for all the other peptides except for the Gag CD8 peptide which had slightly higher IFN- $\gamma$  responses in Sm-infected (649 SFU/10<sup>6</sup> splenocytes) compared to Sm-free vaccinated (538 SFU/10<sup>6</sup> splenocytes) group.

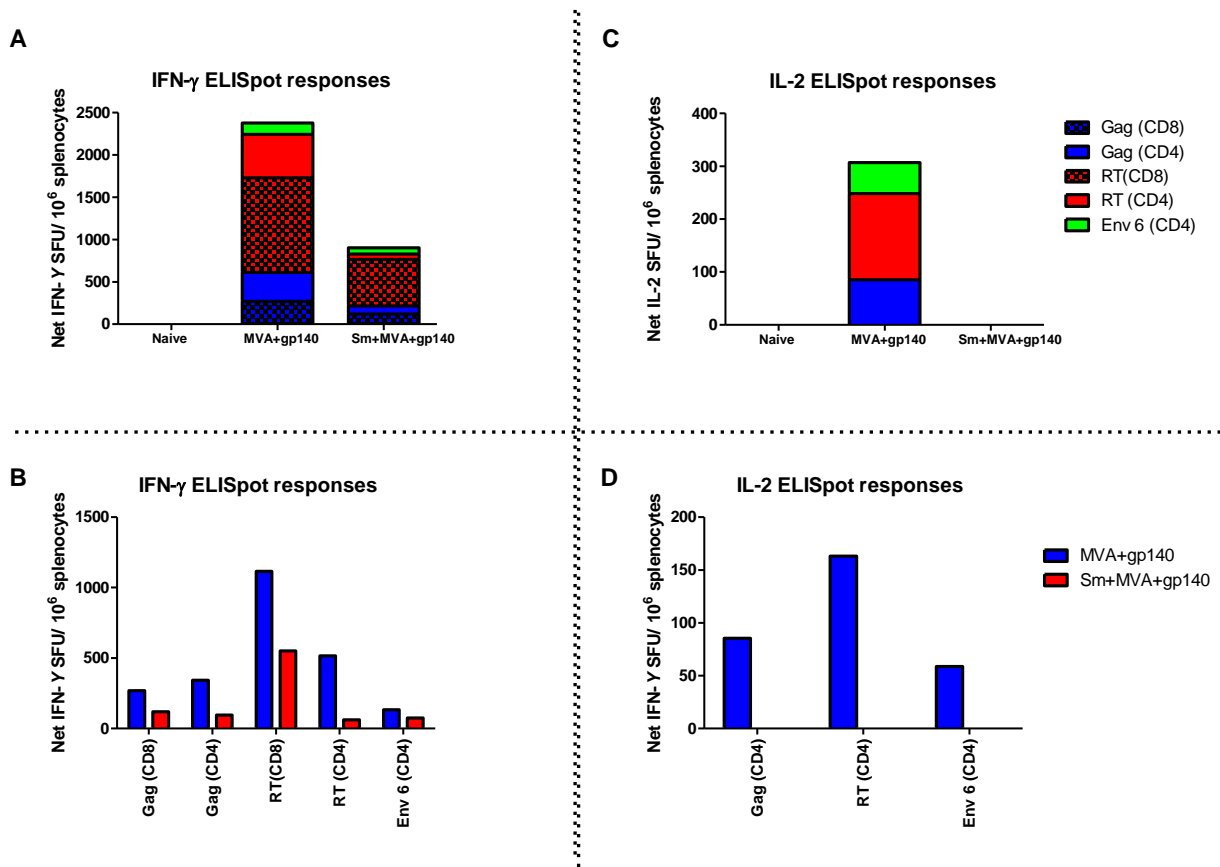
The highest magnitude of IL-2 ELISpot responses was observed in Sm-free vaccinated mice splenocytes (352 SFU/10<sup>6</sup> splenocytes) stimulated with Env CD4 peptide, while in Sm-infected mice, IL-2 responses as low as (37 SFU/10<sup>6</sup> splenocytes) were observed. While all peptides (Gag CD8 and CD4; RT CD8 and RT CD4; and Env CD4) exhibited IL-2 ELISpot response in Sm-free vaccinated mice, they were reduced by almost a half in Sm-infected vaccinated mice. In fact, IL-2 ELISpot responses to the Gag CD8 and RT CD8 peptides were undetectable in Sm-infected mice (Fig 2.3 D).



**Figure 2.3: IFN- $\gamma$  and IL-2 ELISpot analysis of HIV-1 Gag; RT and Env-specific T cell responses induced in the spleen of Sm-infected and Sm-free mice vaccinated with the DNA+MVA vaccine regimen described in Fig 2.1.** At sacrifice, splenocytes were pooled from 5-8 mice per group and stimulated with a control stimulant (irrelevant peptide) or HIV-1 (Gag; RT [CD4 and CD8] and Env CD4) peptides in an IFN- $\gamma$  ELISpot (A and B) and IL-2 ELISpot (C and D) assays. Positive responses to the HIV peptides were considered to be those greater than twice the background response (response to irrelevant peptide stimulation) plus two times the standard deviation (SD) of this response. The magnitude of positive ELISpot responses is expressed as either net cumulative (A and C) or net individual peptides (B and D) responses per  $10^6$  splenocytes. The bars indicate the average net SFU from triplicate wells for  $10^6$  splenocytes.

### **2.3.1.2. Vaccine-specific ELISpot responses to MVA+gp140 vaccine regimen**

Vaccination with MVA+gp140 induced high cumulative HIV-1 Gag, RT and Env-specific IFN- $\gamma$  (2347 SFU/10<sup>6</sup> splenocytes) (Fig 2.4 A) and IL-2 ELISpot (306 SFU/10<sup>6</sup> splenocytes) (Fig 2.4 C) responses in Sm-free vaccinated mice. Vaccinated Sm-infected groups had a low magnitude of HIV peptide-specific IFN- $\gamma$  (900 SFU/10<sup>6</sup> splenocytes) and undetectable IL-2 (0 SFU/10<sup>6</sup> splenocytes) ELISpot responses. The same was observed for IFN- $\gamma$  (Fig 2.3 B) and IL-2 (Fig 2.3 D) ELISpot responses to individual Gag (CD4 or CD8); RT (CD4 or CD8) and Env (CD4) specific peptides. IFN- $\gamma$  ELISpot responses to the RT CD8 peptide induced the highest magnitude of IFN- $\gamma$  secreting CD8+ and CD4+ T cells (1115 SFU/10<sup>6</sup> splenocytes) in Sm-free mice. However, a lower magnitude of RT CD8 specific IFN- $\gamma$  (550 SFU/10<sup>6</sup> splenocytes) was observed in Sm-infected vaccinated mice. The same reduction was observed for all the other peptides. The highest magnitude of IL-2 ELISpot responses was observed in Sm-free vaccinated mice splenocytes (163 SFU/10<sup>6</sup> splenocytes) stimulated with RT CD4 peptide followed by Gag CD4 (85 SFU/10<sup>6</sup> splenocytes) and Env CD4 (58 SFU/10<sup>6</sup> splenocytes). On the other hand, Sm-infected mice had IL-2 responses below detectable levels (0 SFU/10<sup>6</sup> splenocytes).



**Figure 2.4: IFN- $\gamma$  and IL-2 ELISpot analysis of HIV-1 Gag; RT and Env-specific T cell responses induced in the spleen of Sm-infected and Sm-free mice vaccinated with the MVA+gp140 vaccine regimen described in Fig 2.1.** At sacrifice, splenocytes were pooled from 5-8 mice per group and stimulated with a control stimulant (irrelevant peptide) or HIV-1 (Gag; RT and Env [CD4 and CD8]) peptides in an IFN- $\gamma$  ELISpot (A and B) and IL-2 ELISpot (C and D) assays. Positive responses to the HIV peptides were considered to be those greater than the background response (response to irrelevant peptide stimulation) plus two times the standard deviation (SD) of this response. The magnitude of positive ELISpot responses is expressed as either net cumulative (A and C) or net individual peptides (B and D) responses per  $10^6$  splenocytes. The bars indicate the average net SFU from triplicate wells for  $10^6$  splenocytes.

## **2.3.2. Th1 Th2 responses to non-specific stimulation (Con A) and parasite-specific (SEA) in mice vaccinated with MVA+gp140 and DNA+MVA vaccine regimens**

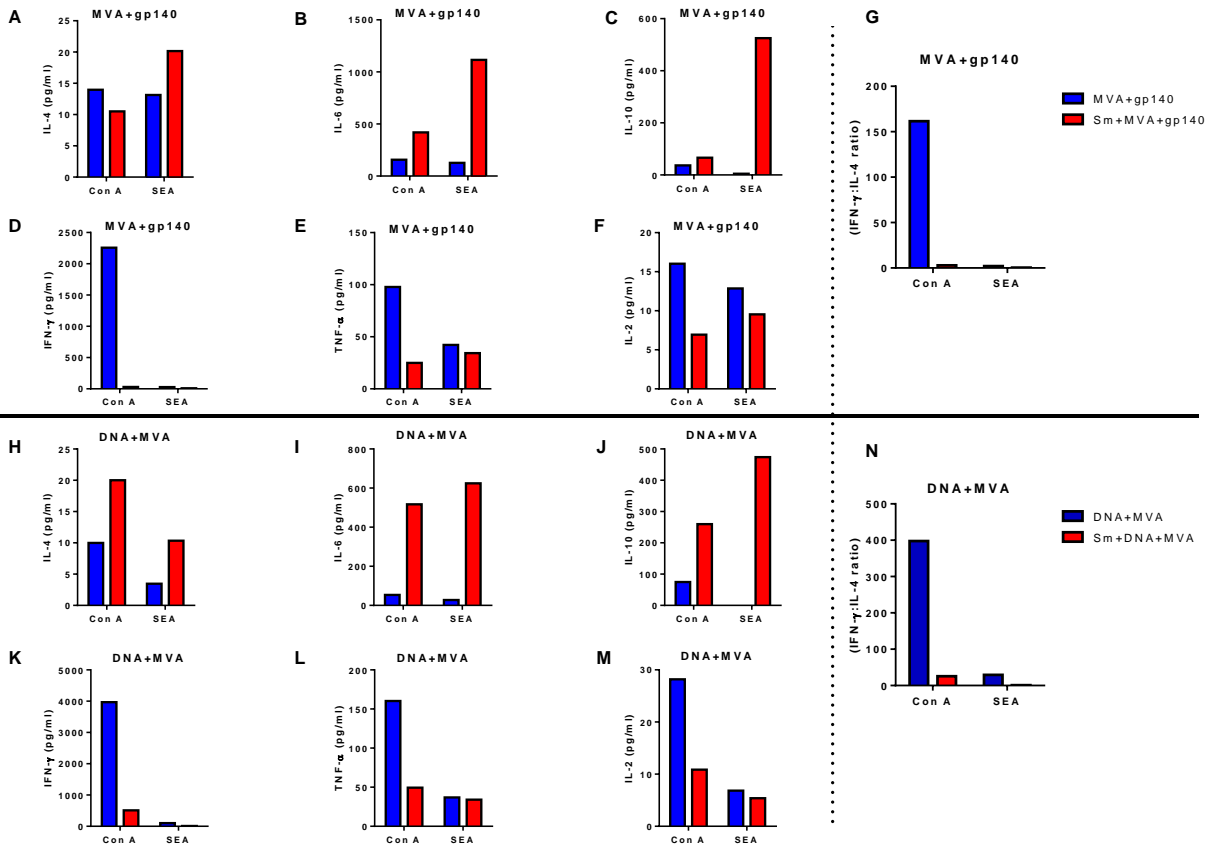
### **2.3.2.1. Cytokine responses to Con A and SEA**

The amount of Th1 and Th2 cytokines secreted by T cells after stimulation with Con A (non-specific stimulation) and SEA (parasite-specific stimulation) (Fig 2.5) were quantified for MVA+gp140 (A-G) and DNA+MVA (H-N) vaccinated mice using CBA. As shown in Fig 2.5 A-C, an increased skewing towards Th2 of Sm-infected compared to Sm-free mice vaccinated with MVA+gp140 marked with IL-6 (418.77 pg/ml) and IL-10 (66.02 pg/ml) respectively in Sm-infected compared to Sm-free (IL-6=157.01 pg/ml) and IL-10 = 36.56 pg/ml) but not IL-4 (10.5 vs 13.97 pg/ml) responses to Con A was observed. Furthermore, an increase in IL-4 (20.15 pg/ml); IL-6 (1115.56 pg/ml) and IL-10 (525.24 pg/ml) respectively in Sm-infected compared to Sm-free (IL-4 = 13.14 pg/ml) IL-6 (127.42 pg/ml) and IL-10 (4.66 pg/ml) in response to the parasite-specific SEA was observed (Fig 2.5 A-C).

However, drastic reduction of Th1 cytokines in Sm-infected compared to Sm-free vaccinated mice stimulated with Con A, marked by IFN- $\gamma$  (29.93 vs 2257.03 pg/ml) IL-2 (6.94 vs 16.03 pg/ml) and TNF- $\alpha$  (24.94 vs 97.86 pg/ml) was observed. Sm-infected and Sm-free vaccinated mice produced close to similar levels of Th1 cytokines (IL-2 [9.54 vs 12.86 pg/ml] and TNF- $\alpha$  [34.34 vs 42.23 pg/ml]) except for IFN- $\gamma$  [9.59 vs 28.0 pg/ml] which was higher in Sm-free after stimulation with SEA (Fig 2.5 D-F). Skewing towards Th2 marked by a decrease in the IFN- $\gamma$ :IL-4 ratio following stimulation with Con A was observed in Sm-infected (2.85) compared to Sm-free (161.56) vaccinated mice (Fig. 2.5 G).

In mice vaccinated with DNA+MVA, an increased Th2 cytokine production marked with IL-4 (20.0 vs 9.98 pg/ml); IL-6 (516.93 vs 53.89 pg/ml) and IL-10 (259.79 vs 74.82 pg/ml) responses to Con A was observed in Sm-infected compared to Sm-free vaccinated mice (Fig 2.5 H-N). Also, an increase in IL-4 (10.3 vs 3.47 pg/ml) IL-6 (624.35 vs 27.48 pg/ml) and IL-10 (473.80 vs 0 pg/ml) in response to the parasite-specific SEA was observed in Sm-infected mice compared to Sm-free vaccinated mice (Fig 2.5 H-J).

Th1 cytokines were lower in Sm-infected compared to Sm-free mice vaccinated with DNA+MVA and stimulated with Con A marked by IFN- $\gamma$  (508.69 vs 3967.68 pg/ml) IL-2 (10.85 vs 28.18 pg/ml) and TNF- $\alpha$  (49.34 vs 160.18 pg/ml). Similar to the mice vaccinated with MVA+gp140, Sm-infected and Sm-free vaccinated mice produced close to similar levels of Th1 cytokines (IL-2 [5.39 vs 6.85 pg/ml] and TNF- $\alpha$  [34.05 vs 36.87 pg/ml]) except for IFN- $\gamma$  [11.37 vs 101.99 pg/ml] which was higher in Sm-free after stimulation with SEA (Fig 2.5 K-M). Th2 biasing marked by a decrease in the IFN- $\gamma$ :IL-4 ratio upon stimulation with Con A was observed in Sm-infected (25.43) compared to Sm-free (397.56) vaccinated mice (Fig. 2.5 N).



**Figure 2.5: Cytokine production by Con A and SEA stimulation in Sm-free and Sm-infected mice vaccinated with MVA+gp140 or DNA+MVA regimen.** Splenocytes were harvested from mice vaccinated with the indicated regimen described in Fig 2.1. They were then stimulated with an irrelevant peptide (negative control) or Con A or with SEA for 48 h. Culture supernatants were collected and the level of Th1 and Th2 cytokines released into the medium for MVA+gp140 (A-C and D-F respectively) and DNA+MVA (H-J and K-M respectively) vaccinated mice was measured using a CBA and flow cytometric analysis. The individual bars represent the magnitude of the net positive cytokine levels in the medium of splenocytes of Sm-free and Sm-infected vaccinated mice. The level of cytokines in the culture medium was considered positive when the level is higher than the detection limit of the respective cytokine described in Table 2.3.

### **2.3.2.2. HIV-Specific cytokine production**

The amount of Th1 and Th2 cytokines secreted by HIV-specific T cells stimulated with HIV Gag (CD4; CD8); RT (CD8; CD4) and Env (CD4) was also quantified (Fig 2.6-2.9). Splenocytes harvested from mice vaccinated according to the regimens indicated in Fig 2.1 were incubated for 48 h with the HIV Gag (CD4; CD8); RT (CD8; CD4) and Env (CD4) T cell peptides or with Con A or SEA and the level of Th1 (IFN- $\gamma$ ; IL-2; TNF- $\alpha$ ) and Th2 (IL-6; IL-4; IL-10) cytokines released into the culture medium was quantified using a Cytometric Bead Array assay (BD Biosciences, USA) and flow cytometric analysis.

### **2.3.2.3. Cytokine responses to DNA+MVA**

The cumulative positive cytokine levels produced in response to HIV peptides for the DNA+MVA vaccination regimen is shown in Fig 2.6. High levels of net Th1 cytokines: IFN- $\gamma$  (4157.38 pg/ml); IL-2 (102.59 pg/ml) and TNF- $\alpha$  (303.55 pg/ml) was released from T cells of Sm-free mice in response to a DNA+MVA vaccination regimen. However, splenocytes of mice infected with Sm produced lower Th1 cytokine levels of IFN- $\gamma$  (469.99 pg/ml); IL-2 (27.06 pg/ml) and TNF- $\alpha$  (71.71 pg/ml) (Fig 2.6 A; B and C respectively). Conversely, net Th2 cytokine levels in Sm-free vaccinated mice were lower compared to Sm-infected vaccinated: IL-6 (301.67 vs 1007.16 pg/ml) and IL-10 (100.5 vs 169.13 pg/ml) with exception to IL-4 which was similar between Sm-free (17.03 pg/ml) and Sm-infected (17.91 pg/ml) vaccinated mice (Fig 2.6 D, E and F).

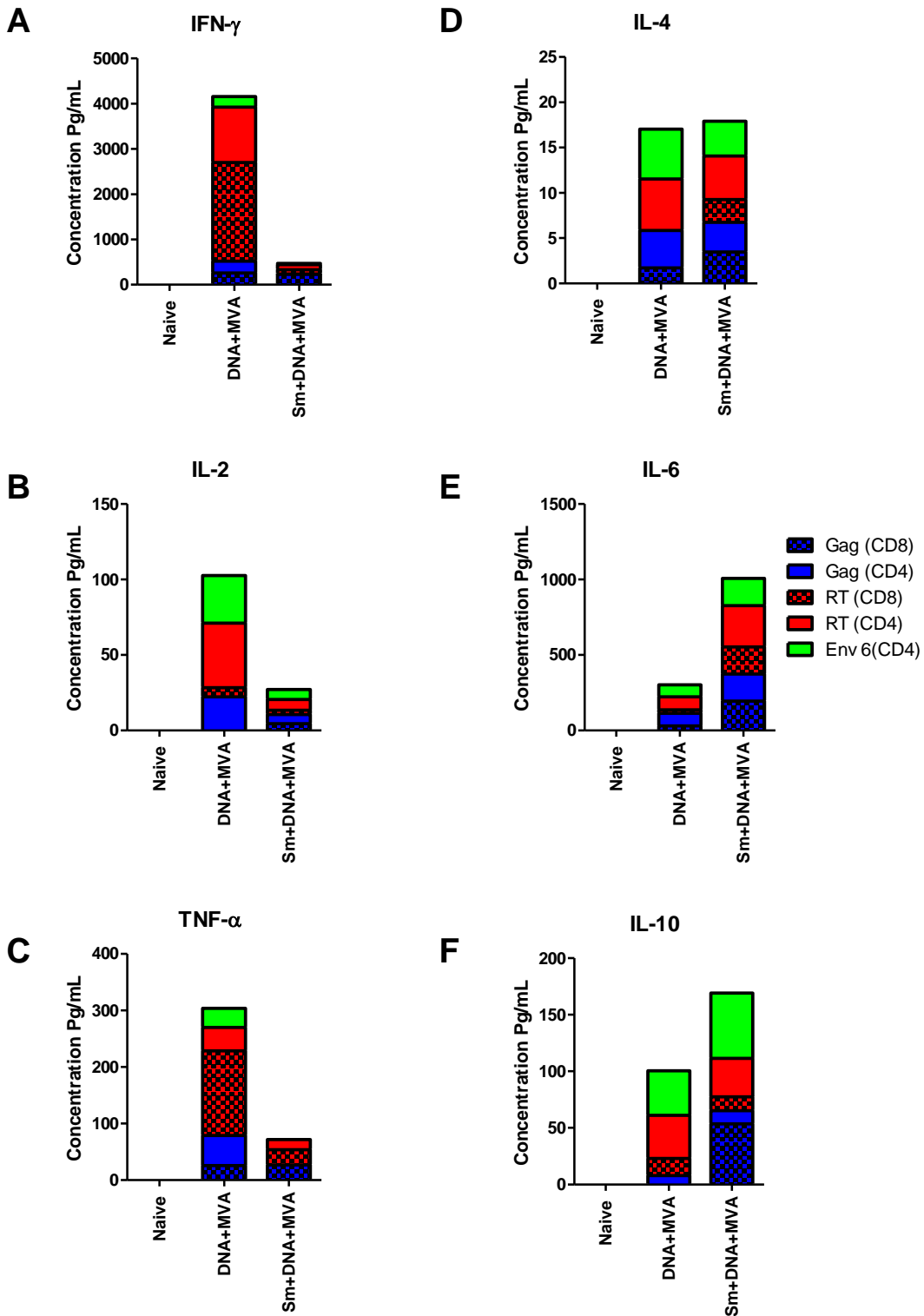


Figure 2.6 Cumulative cytokines produced by HIV peptide-specific T cells of Sm-free and Sm-infected mice vaccinated with DNA+MVA. Splenocytes were harvested from mice vaccinated with the

indicated regimen described in Fig 2.1. They were then stimulated with an irrelevant peptide (negative control) or with the individual HIV specific T cell peptides for 48 h. Culture supernatants were collected and the level of Th1 (A, B and C) and Th2 (D, E and F) cytokines released into the medium was measured using CBA. The individual bars represent the magnitude of the cumulative positive cytokine levels in the medium of splenocytes of Sm-free and Sm-infected vaccinated mice, stimulated with individual HIV peptides, after subtracting the background (splenocytes stimulated with irrelevant peptide). The level of cytokines in the culture medium was considered positive when the level is higher than the detection limit of the respective cytokine described in Table 2.3.

Despite high levels of net IFN- $\gamma$  released from HIV peptide-specific T cells of Sm-free mice vaccinated with a DNA+MVA vaccine, 53% of the responses were attributed to the RT CD8 peptide-specific responses (Fig 2.7 A). However, Sm-infected vaccinated groups had a drastic reduction of the net IFN- $\gamma$  cytokine (95.37 pg/ml) in response to RT CD8 peptide. The same was true for all peptides except for the Gag CD8, which was minimally reduced in Sm-infected (226.73 pg/ml) compared to Sm-free vaccinated mice (256.88 pg/ml). Levels of TNF- $\alpha$  were also drastically reduced in Sm-infected mice in response to all HIV peptides with exception to the Gag CD8 which produced almost similar TNF- $\alpha$  between the Sm-infected (26.8 pg/ml) and Sm-free vaccinated group (25.39 pg/ml) (Fig 2.7 C). The level of IL-2, on the other hand, was not detected in Sm-free mice (0 pg/ml) while they only reached 4.39 pg/ml in Sm-infected mice (Fig 2.7 B).

On the contrary, Th2 cytokines were elevated in Sm-infected compared to Sm-free vaccinated mice. High levels of IL-6 were observed in all cultures of vaccinated Sm-infected cells stimulated with HIV peptides (Fig 2.7 E). Responses to the RT CD4 peptide were the highest (273.65 pg/ml) responses. The other peptides also induced higher levels of IL-6 in Sm-infected compared to Sm-free vaccinated mice.

Higher levels of IL-4 were observed in Sm-free compared to Sm-infected vaccinated mice when stimulated with CD4-specific HIV peptides. On the other hand, stimulation with CD8-specific HIV peptides induced more Gag CD8 (3.47 pg/ml) and RT CD8 (2.53 pg/ml) in Sm-infected compared to Sm-free vaccinated mice, which produced less Gag CD8 (1.71 pg/ml) and no RT CD8 (0 pg/ml) (Fig 2.7 D).

Sm-infected cells stimulated with HIV peptides produced high IL-10 levels compared to Sm-free vaccinated cells (Fig 2.7 F). This was only true for Gag CD8 (53.51 pg/ml vs 0 pg/ml); Gag CD4 (11.69 pg/ml vs 7.94 pg/ml) and Env CD4 (57.76 pg/ml vs 39.5 pg/ml) responses in Sm-infected versus Sm-free vaccinated mice. Cytokine responses to the RT CD4 and CD8 peptide stimulation produced more IL-10 for Sm-free (37.91 and 15.5 pg/ml respectively) versus Sm-infected (33.92 and 12.25 pg/ml respectively) vaccinated mice (Fig 2.7 F).

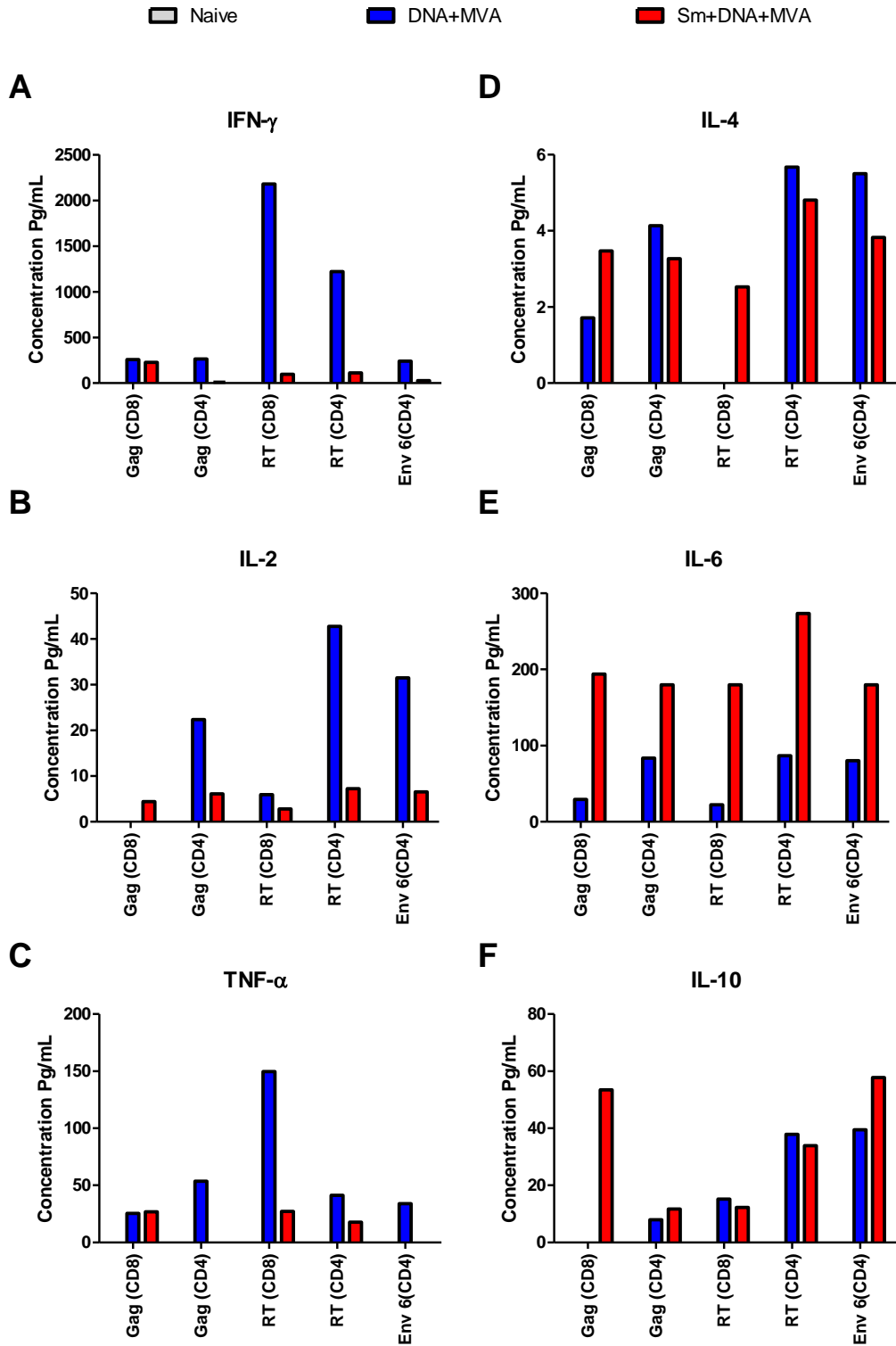


Figure 2.7: Cytokine produced by Gag (CD8 and CD4) RT (CD8 and CD4) and Env (CD4) specific peptides in Sm-free and Sm-infected mice vaccinated with DNA+MVA. Splenocytes were harvested from mice vaccinated with the indicated regimen described in Fig 2.1. They were then stimulated with an

irrelevant peptide (negative control) or with the individual HIV-specific T cell peptides for 48 h. Culture supernatants were collected, and the level of Th1 (A, B and C) and Th2 (D, E and F) cytokines released into the medium was measured using a Cytokine Bead Array assay and flow cytometric analysis. The individual bars represent the magnitude of the net positive cytokine levels in the medium of splenocytes of Sm-free and Sm-infected vaccinated mice, stimulated with individual HIV peptides, after subtracting the background (splenocytes stimulated with irrelevant peptide). The level of cytokines in the culture medium was considered positive when the level is higher than the detection limit of the respective cytokine described in Table 2.3.

#### **2.3.2.4. Cytokine responses to MVA+gp140**

The cumulative positive cytokine responses to the HIV peptides for the MVA+gp140 vaccination regimen are shown in Fig 2.8. High levels of net Th1 cytokines: IFN- $\gamma$  (2165.44 pg/ml); IL-2 (138.88 pg/ml) and TNF- $\alpha$  (217.29 pg/ml) was released from HIV peptide-specific T cells of Sm-free mice vaccinated with the MVA+gp140 vaccination regimen compared to Sm-infected vaccinated mice which produced lower Th1 cytokine levels of IFN- $\gamma$  (142.63 pg/ml); IL-2 (34.34 pg/ml) and TNF- $\alpha$  (78.2 pg/ml) (Fig 2.8 A; B and C respectively). Conversely, net Th2 cytokine levels in Sm-free vaccinated mice were lower compared to Sm-infected vaccinated: IL-4 (35.82 vs 108.56 pg/ml) and IL-10 (58.63 vs 115.88 pg/ml) with exception to IL-6 which was similar between Sm-free (928.4 pg/ml) and Sm-infected (891.15 pg/ml) vaccinated mice (Fig 2.8 D, E and F).

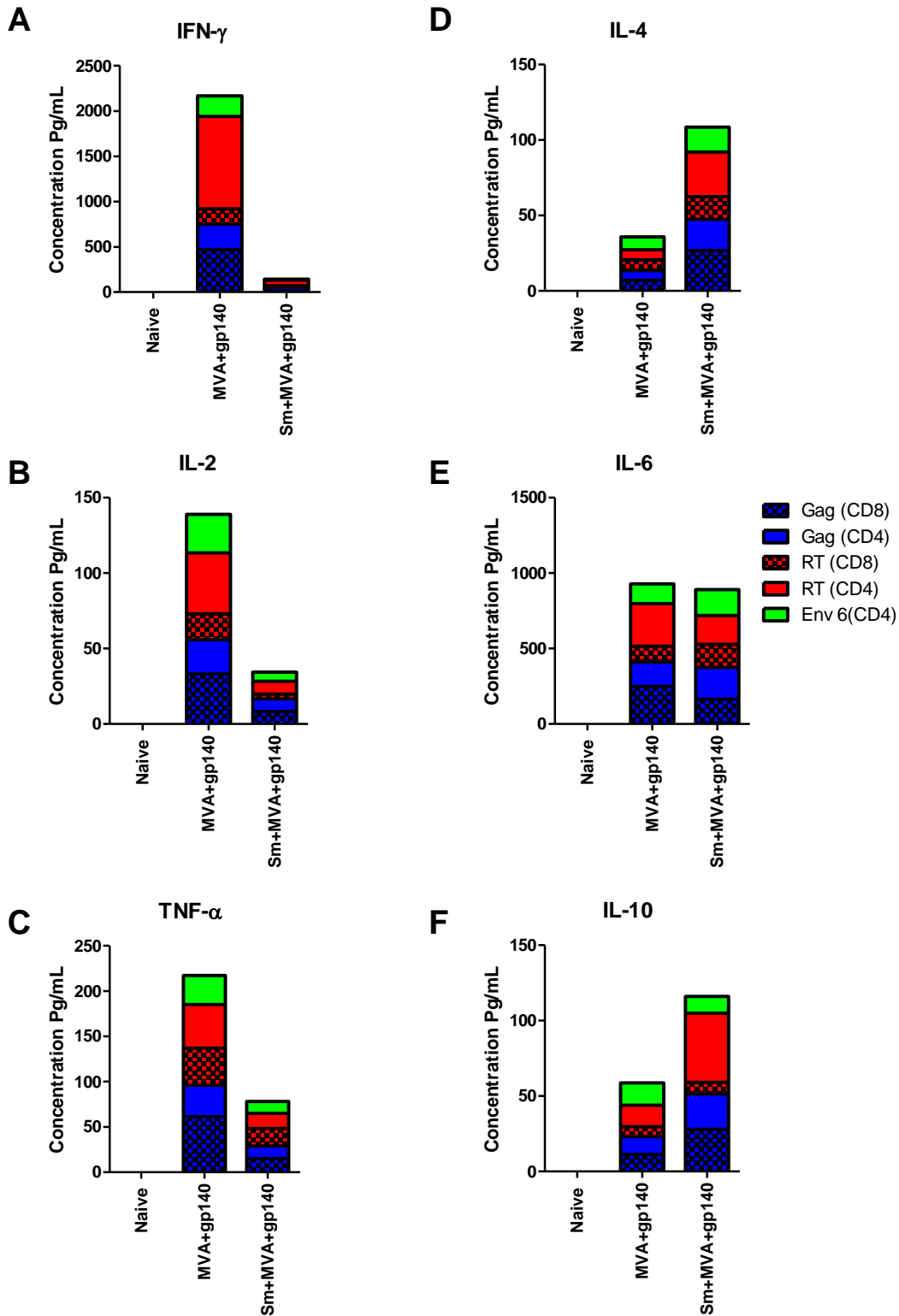


Figure 2.8: Cumulative cytokines produced by HIV peptide-specific T cells of Sm-free and Sm-infected mice vaccinated with MVA+gp140. Splenocytes were harvested from mice vaccinated with the

indicated regimen described in Fig 2.1. They were then stimulated with an irrelevant peptide (negative control) or with the individual HIV specific T cell peptides for 48 h. Culture supernatants were collected and the level of Th1 (A, B and C) and Th2 (D, E and F) cytokines released into the medium was measured using a Cytokine Bead Array assay and flow cytometric analysis. The individual bars represent the magnitude of the cumulative positive cytokine levels in the medium of splenocytes of Sm-free and Sm-infected vaccinated mice, stimulated with individual HIV peptides, after subtracting the background (splenocytes stimulated with irrelevant peptide). The level of cytokines in the culture medium was considered positive when the level is higher than the detection limit of the respective cytokine described in Table 2.3.

Although Sm-free mice vaccinated with an MVA+gp140 vaccine produced high levels of net IFN- $\gamma$  in response to the HIV peptides, 47% (1018.30 pg/ml) of the responses were attributed to the RT CD4 peptide-specific responses (Fig 2.9 A). However, Sm-infected vaccinated groups had a drastic reduction of the net IFN- $\gamma$  cytokine (61.77 pg/ml) in response to RT CD4 peptide. The drastic drop in IFN- $\gamma$  cytokine was also observed for all peptides (Fig 2.9 A). Levels of TNF- $\alpha$  were also reduced by more than half in Sm-infected mice (15.04 vs 61.31 pg/ml) in response to the Gag CD8 peptide. This was true for all the other HIV peptides (Fig 2.9 C). Levels of IL-2 in Sm-infected mice were also drastically reduced by more than half compared to the Sm-free vaccinated mice (Fig 2.9 B).

Th2 cytokines were higher in Sm-infected compared to Sm-free vaccinated mice particularly IL-4 and IL-10 (Fig 2.9 D and F respectively). High levels of IL-4 were observed in all cultures of vaccinated Sm-infected cells compared to those of Sm-free stimulated with HIV peptides (Fig 2.9 E). Responses to the RT CD4 peptide were the highest in Sm-infected (29.54 pg/ml) and reduced in Sm-free mice (6.67 pg/ml). The other peptides relatively stimulated more or less similar levels of IL-4 responses to other peptides (Gag CD8 [26.86 pg/ml]; Gag CD4 [20.6 pg/ml]; RT CD8 [15 pg/ml] and Env CD4 [179.90 pg/ml] in Sm-infected mice was reduced in Sm-free vaccinated mice (Gag CD8 [7.01 pg/ml]; Gag CD4 [6.53 pg/ml]; RT CD8 [7.08 pg/ml] and Env CD4 [8.53 pg/ml]).

Increased IL-10 levels were observed in Sm-infected cells while being decreased in cells of Sm-free vaccinated mice (Fig 2.9 F). This was the case for responses to all peptide with exception to the Env CD4 which was higher in Sm-free (14.69 pg/ml) versus Sm-infected (11.05 pg/ml) cultures. Cytokine responses to the RT CD4 peptide stimulation produced more IL-10 for Sm-infected (45.72 pg/ml) versus Sm-free (14.23 pg/ml) vaccinated mice. A minimal decrease in IL-10 levels was observed in cultures of Sm-free (6.69 pg/ml) versus Sm-infected (7.8 pg/ml) vaccinated.

High levels of IL-6 were detected in both Sm-infected and Sm-free vaccinated group for all peptides (Fig 2.9 E). The RT CD4 stimulated cells produced the highest amount of IL-6 cytokine in Sm-free (284.15 pg/ml)

mice compared to Sm-infected (192.41 pg/ml) vaccinated mice. The Gag CD8 peptide also stimulated higher levels of IL-6 in Sm-free mice (249.04 pg/ml) than in Sm-infected (164.74 pg/ml) vaccinated mice. Only in Gag CD4 (210.21 pg/ml); RT CD8 (152.57 pg/ml) and Env CD4 (171.22 pg/ml) stimulated cells did Sm-infected have higher cytokine production compared to Sm-free vaccinated: Gag CD4 (161.6 pg/ml); RT CD8 (103.77 pg/ml) and Env CD4 (129.84 pg/ml).

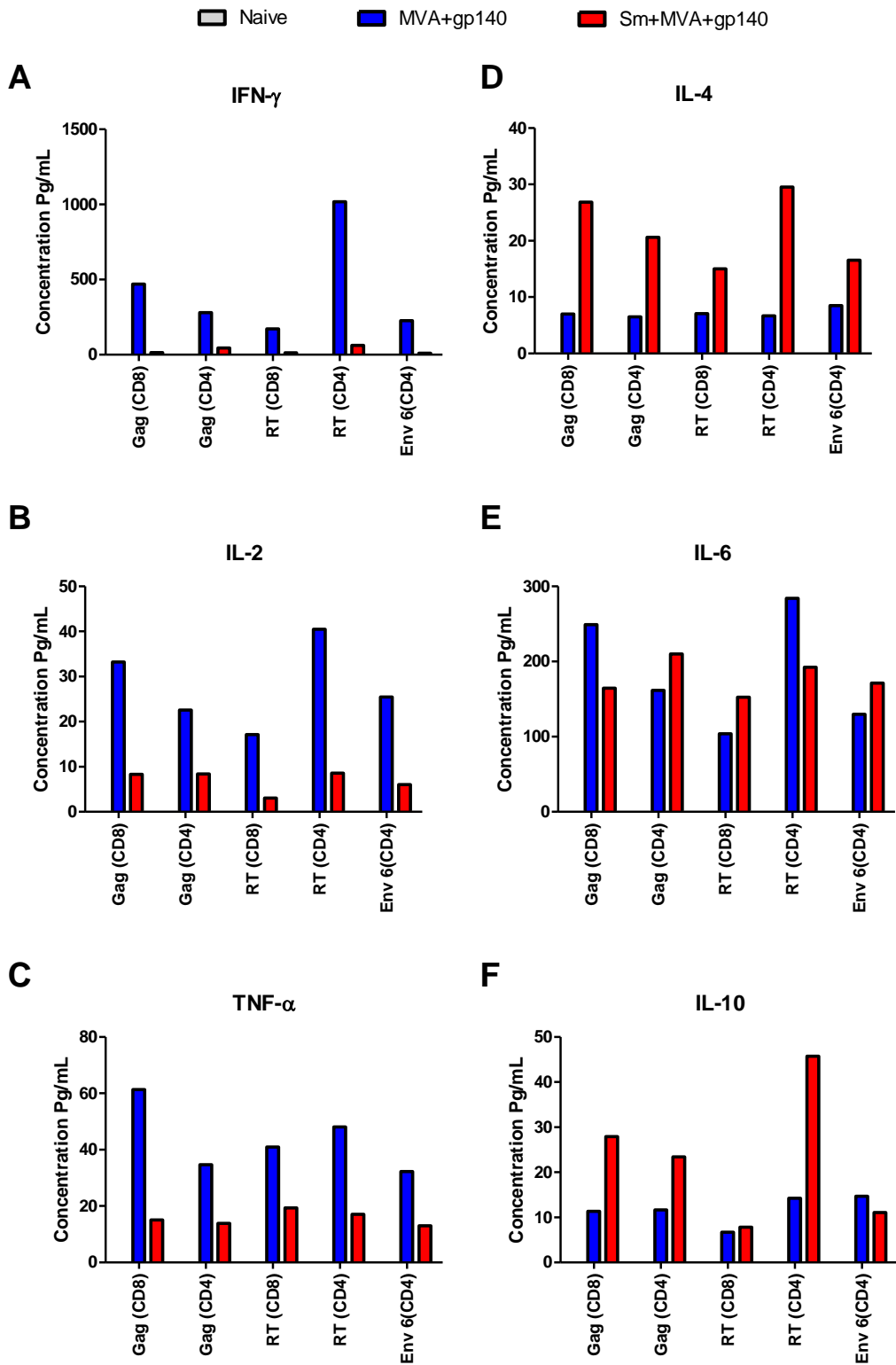


Figure 2.9: Cytokine produced by Gag (CD8 and CD4) RT (CD8 and CD4) and Env (CD4) specific peptides in Sm-free and Sm-infected mice vaccinated with MVA+gp140. Splenocytes were harvested

from mice vaccinated with the indicated regimen described in Fig 2.1. They were then stimulated with an irrelevant peptide (negative control) or with the individual HIV-specific T cell peptides for 48 h. Culture supernatants were collected and the level of Th1 (A, B and C) and Th2 (D, E and F) cytokines released into the medium was measured using a Cytokine Bead Array assay and flow cytometric analysis. The individual bars represent the magnitude of the net positive cytokine levels in the medium of splenocytes of Sm-free and Sm-infected vaccinated mice, stimulated with individual HIV peptides, after subtracting the background (splenocytes stimulated with irrelevant peptide). The level of cytokines in the culture medium was considered positive when the level is higher than the detection limit of the respective cytokine described in Table 2.3.

### **2.3.3. Cellular immune responses to the SAAVI DNA-C2 and SAAVI MVA-C vaccine regimen in the background of Schistosomiasis measured by ICS and FACS**

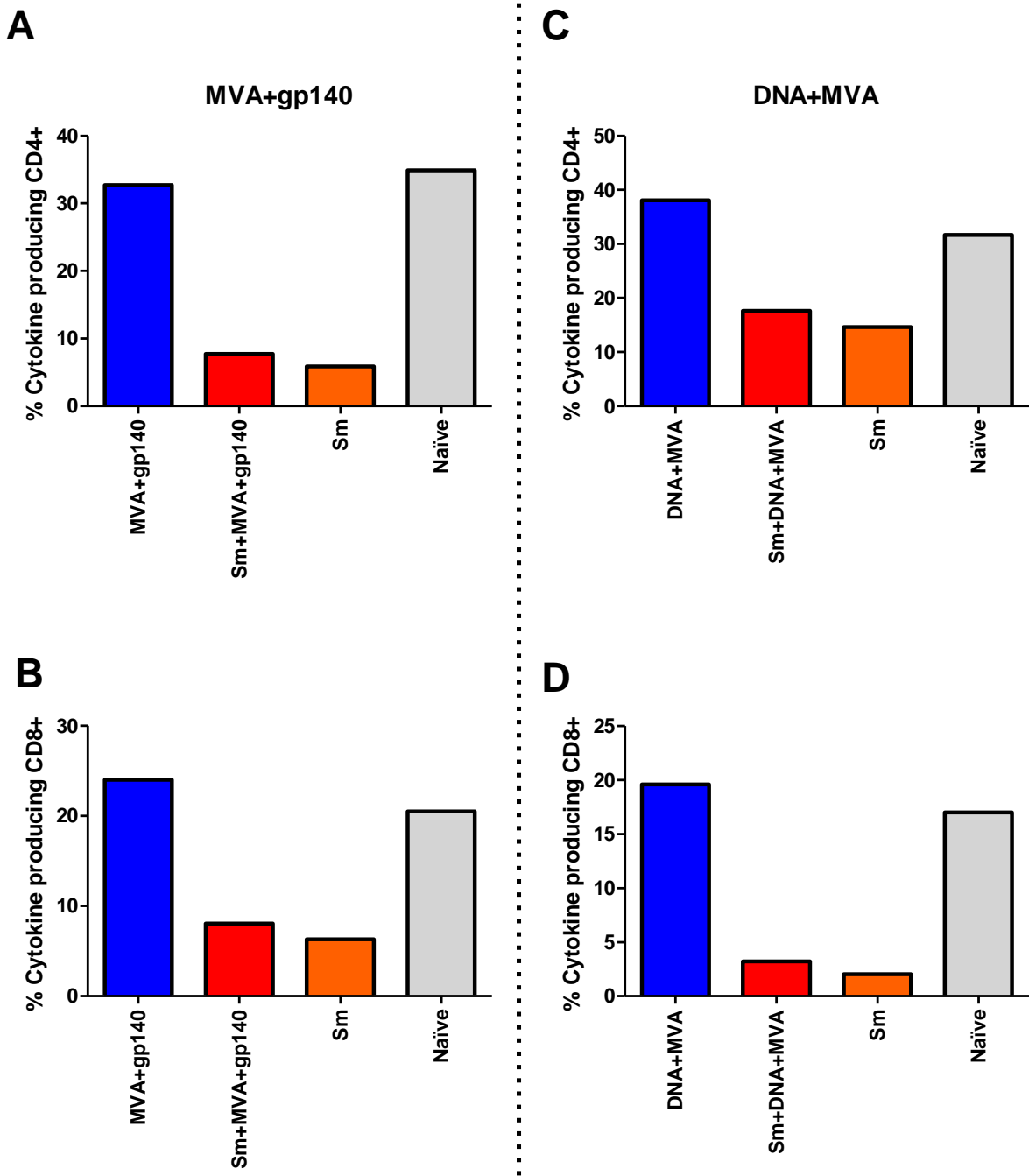
The proportions of cytokine (pool of IFN- $\gamma$ , IL-2 and TNF- $\alpha$ ) producing T cells in response to a non-specific stimulant (PMA/Ionomycin) were determined by ICS. In both MVA+gp140 (Fig 2.10 A and B) and DNA+MVA (Fig 2.10 C and D) vaccination regimen, a high number of cytokine-producing CD4+ and CD8+ T cells was observed in Sm-free mice whilst a decrease in a number of cytokine-producing CD4+ and CD8+ T cells was observed in Sm-infected mice (Fig 2.10). However, the proportion of CD4+ T cells producing cytokines was greater than that of CD8+ T cells in DNA+MVA and MVA+gp140.

#### **2.3.3.1. Cellular immune responses to PMA in MVA+gp140 vaccine regimen**

The frequency of cytokine-producing CD4+ T cells was higher in Sm-free mice (32.7%) compared to Sm-infected (7.71%) vaccinated mice (Fig 2.10 A). The same was observed for Sm-free (34.9%) mice compared to Sm-infected (5.87%) mice without vaccination. A similar pattern was observed for cytokine-producing CD8+ T cell (Fig 2.10 B). Cytokine-producing CD8+ T cells were high in Sm-free mice (24%) compared to Sm-infected vaccinated mice (8.05%). Unvaccinated Sm-free mice also had higher cytokine-producing CD8+ T cells compared to Sm-infected mice (20.5% vs 6.3%).

#### **2.3.3.2. Cellular immune responses to PMA in DNA+MVA vaccine regimen**

The frequency of cytokine-producing CD4+ T cells was also higher in Sm-free mice (38.1%) compared to Sm-infected (17.6%) vaccinated mice (Fig 2.10 C). The same was observed for Sm-free (31.7%) mice compared to Sm-infected (14.6%) mice without vaccination. This was also true for cytokine-producing CD8+ T cell (Fig 2.10 D). Cytokine-producing CD8+ T cells were high in Sm-free mice (19.6%) compared to Sm-infected vaccinated mice (3.21%). Unvaccinated Sm-free mice also had higher cytokine-producing CD8+ T cells compared to Sm-infected mice (17% vs 2.03%).

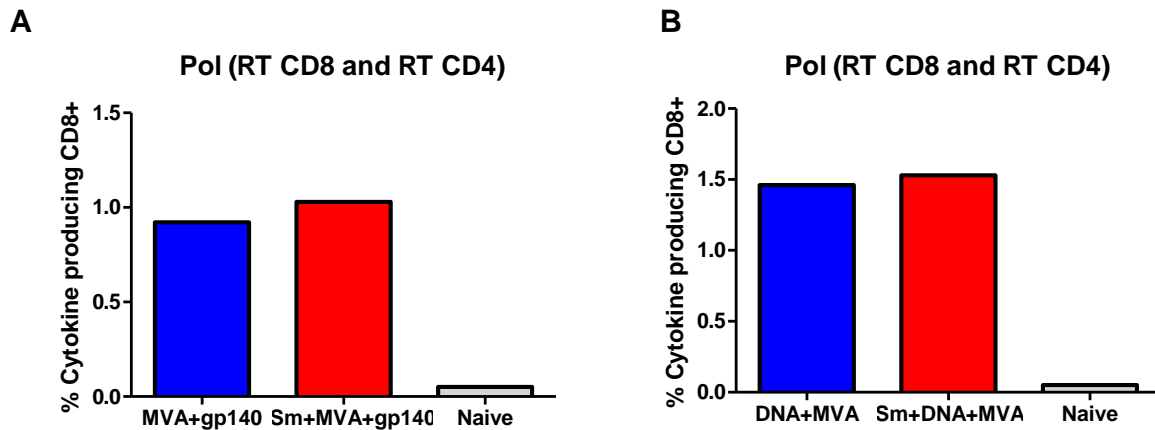


**Figure 2.10: Assessment of cytokine-producing T cells (CD4+ and CD8+) following stimulation with a Leukocyte Activation Cocktail (PMA/Ionomycin).** (A and B) Total frequency of T cells (CD4+ and CD8+) producing cytokines (IFN- $\gamma$ , IL-2, and TNF- $\alpha$ ), in response to PMA/Ionomycin stimulation following vaccination with MVA+gp140 regimen. (C and D) Total frequency of T cells (CD4+ and CD8+) producing

cytokines (IFN- $\gamma$ , IL-2, and TNF- $\alpha$ ), in response to PMA/Ionomycin stimulation following vaccination with DNA+MVA regimen. Bars show positive responses above a cut-off value of 2x background, 50 events or 0.05% cytokine-producing T cells.

### 2.3.3.3. Vaccine-specific cellular responses to MVA+gp140 and DNA+MVA measured by ICS

The proportions of cytokine (pool of IFN- $\gamma$ , IL-2 and TNF- $\alpha$ ) producing T cells in response to pooled HIV peptides (Pol: [RT CD4 + RT CD8]) were determined by ICS. In both MVA+gp140 (Fig 2.11 A) and DNA+MVA (Fig 2.11 B) vaccination regimen, a moderate number of cytokine-producing CD8+ T cells was observed in Sm-free mice and Sm-infected vaccinated mice (Fig 2.11). Cytokine-producing CD4+ T cells above the cutoff value were not detected in this experiment. In the MVA+gp140 vaccination regimen, the frequency of cytokine-producing CD8+ T cells slightly higher in Sm-infected mice (1.03%) compared to Sm-free (0.92%) vaccinated mice (Fig 2.11 A). In the DNA+MVA vaccination regimen, the frequency of cytokine-producing CD8+ T cells was almost similar in Sm-infected mice (1.53%) and Sm-free (1.46%) vaccinated mice (Fig 2.11 B).

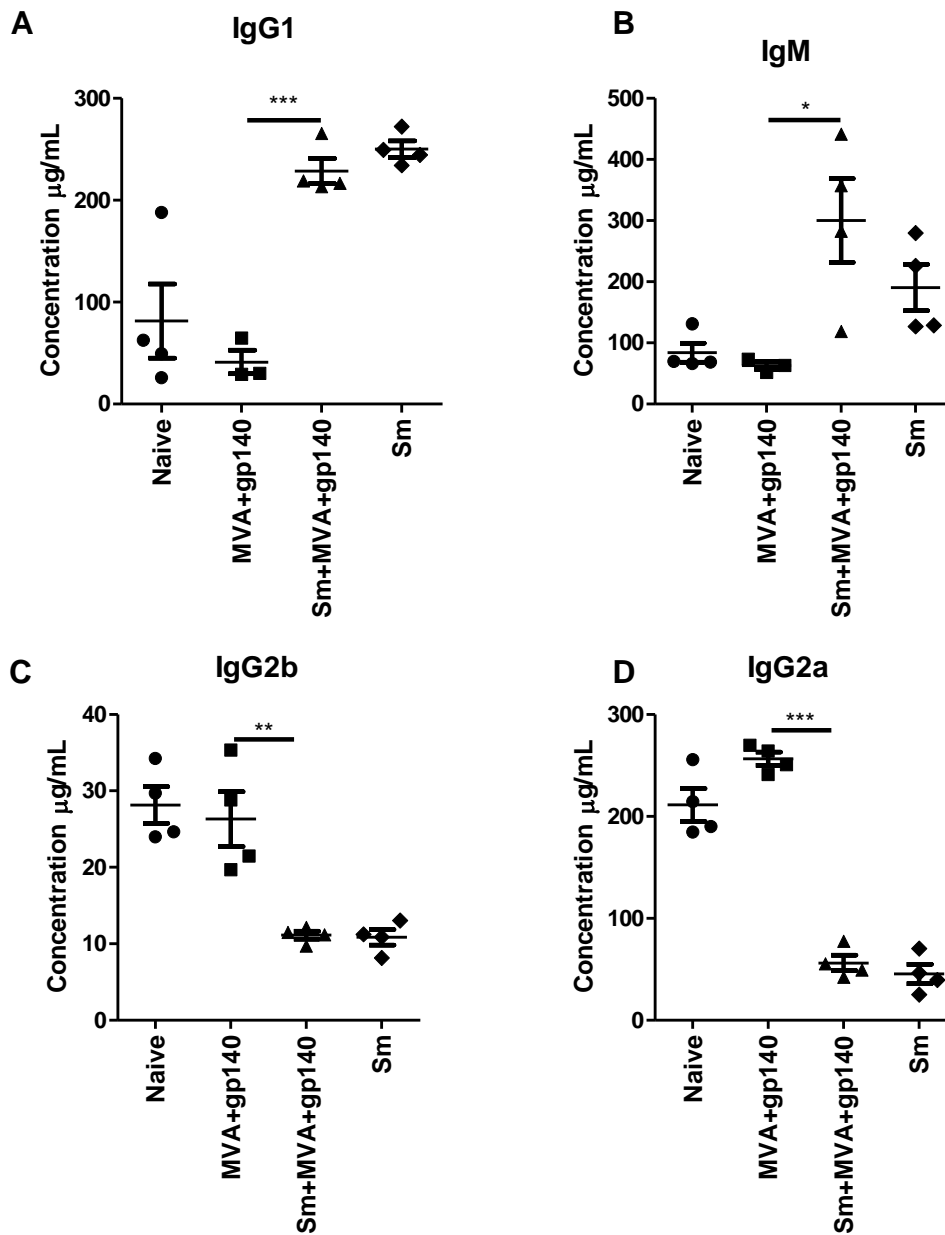


**Figure 2.11: Assessment of cytokine-producing T cells (CD8+) following stimulation with pooled HIV peptides (Pol: [RT CD4 + RT CD8]).** (A) Total frequency of T cells CD8+ producing cytokines (IFN- $\gamma$ , IL-2, and TNF- $\alpha$ ), in response to Pol stimulation following vaccination with MVA+gp140 regimen. (B) Total frequency of T cells CD8+ producing cytokines (IFN- $\gamma$ , IL-2, and TNF- $\alpha$ ), in response to Pol stimulation following vaccination with DNA+MVA regimen. Bars show positive responses above a cut-off value of 2x background, 50 events or 0.05% cytokine-producing T cells.

## **2.3.4. Assessing antibody responses in mice vaccinated with MVA+gp140 and with gp140 alone**

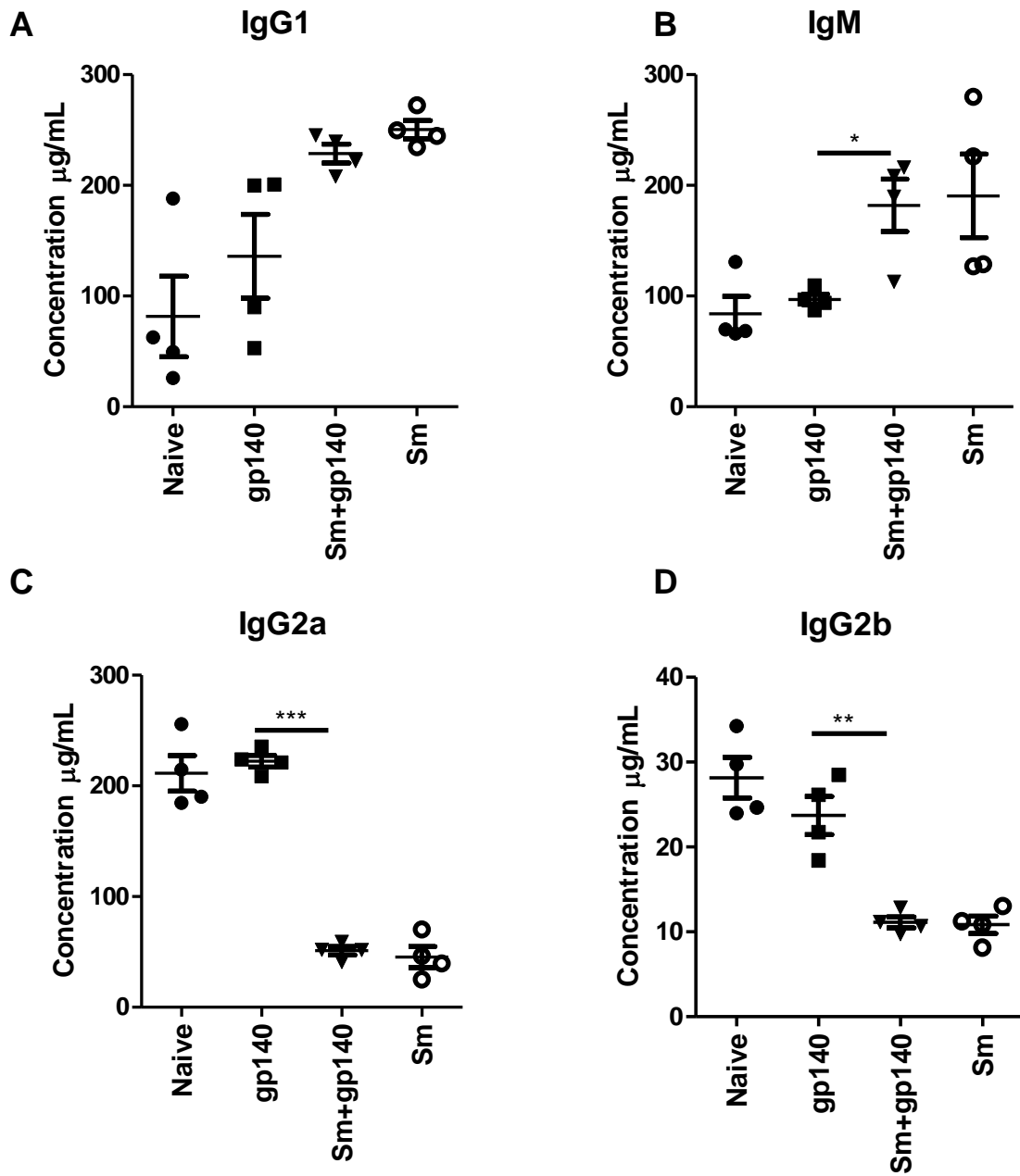
### **2.3.4.1. Total antibody responses**

Total IgG (IgG1; IgG2a; IgG2b and IgM) antibody isotypes in mice serum were measured using ELISA. For the MVA+gp140 regimen, Sm-free mice vaccinated with the MVA+gp140 vaccines induced significantly lower total IgG1 ( $41.25 \pm 11.65 \mu\text{g/ml}$ ) responses compared to Sm-infected vaccinated mice, which induced significantly higher total IgG1 ( $228.7 \pm 12.39 \mu\text{g/ml}$ ) responses (Fig 2.12 A). This was true in unvaccinated Sm-free controls. Total IgM in Sm-infected mice vaccinated with the MVA+gp140 was also significantly higher ( $300.1 \pm 68.63 \mu\text{g/ml}$ ) than in Sm-free ( $62.55 \pm 6.220 \mu\text{g/ml}$ ) vaccinated mice (Fig 2.12 B). This was also the case in unvaccinated controls. On the contrary, IgG2a was significantly elevated in Sm-free mice ( $256.6 \pm 6.463 \mu\text{g/ml}$ ) compared to Sm-infected mice ( $56.15 \pm 7.613 \mu\text{g/ml}$ ) vaccinated with MVA+gp140 (Fig 2.12 C). This was true for unvaccinated controls. A significantly higher total IgG2b was observed in Sm-free ( $26.33 \pm 3.596 \mu\text{g/ml}$ ) vaccinated mice compared to Sm-infected ( $11.14 \pm 0.5159 \mu\text{g/ml}$ ) vaccinated mice (Fig 2.12 D). A similar trend was observed in unvaccinated controls.



**Figure 2.12: Antibody responses in Sm-infected and Sm-free mice vaccinates with MVA+gp140.** Serum samples were collected two weeks post last vaccination. Total serum IgG isotypes (IgG1 [A], IgM [B], IgG 2b [C] IgG2a [D],) were analysed by ELISA. Values are expressed as mean  $\pm$  SEM. Statistical differences between the groups were calculated by unpaired t-test (two-tailed) followed by FDR for multiple comparisons, \* $P < 0.05$ ; \*\* $P < 0.01$ ; \*\*\* $P < 0.0001$ .

For the gp140 regimen, Sm-free mice vaccinated with the gp140 vaccines induced lower total IgG1 ( $135.8 \pm 37.94 \mu\text{g/ml}$ ) responses compared to Sm-infected vaccinated mice, which induced higher total IgG1 ( $228.7 \pm 8.487 \mu\text{g/ml}$ ) responses (Fig 2.13 A). However, the difference was not statistically significant. The same observation was true for unvaccinated controls. Total IgM in Sm-infected mice vaccinated with the gp140 was also significantly higher ( $181.9 \pm 23.66 \mu\text{g/ml}$ ) than in Sm-free ( $96.89 \pm 4.742 \mu\text{g/ml}$ ) vaccinated mice (Fig 2.13 B). This was also the case in unvaccinated controls. On the contrary, IgG2a was significantly higher in Sm-free mice ( $222.2 \pm 5.450 \mu\text{g/ml}$ ) compared to Sm-infected mice ( $51.01 \pm 3.801 \mu\text{g/ml}$ ) vaccinated with gp140 (Fig 2.13 C). This was true for unvaccinated controls. A significantly higher total IgG2b was observed in Sm-free ( $23.71 \pm 2.248 \mu\text{g/ml}$ ) vaccinated mice compared to Sm-infected ( $11.11 \pm 0.6483 \mu\text{g/ml}$ ) vaccinated mice (Fig 2.13 D). A similar trend was observed in unvaccinated controls.

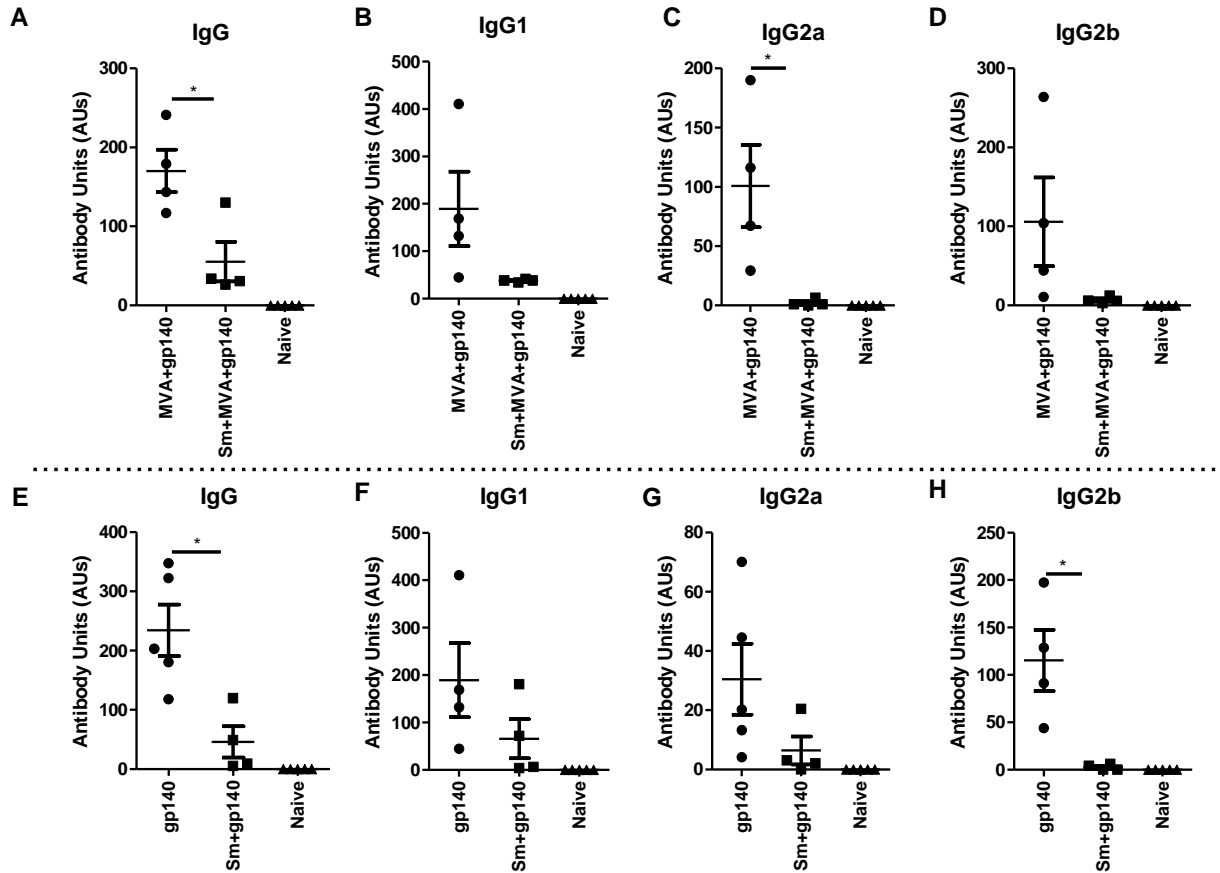


**Figure 2.13: Antibody responses in Sm-infected and Sm-free mice vaccinated with gp140.** Serum samples were collected two weeks post last vaccination. Total serum IgG isotypes (IgG1 [A], IgM [B], IgG 2b [C] IgG2a [D],) were analysed by ELISA. Values are expressed as mean  $\pm$  SEM. Statistical differences between the groups were calculated by unpaired t-test (two-tailed) followed by FDR for multiple comparisons, \* $P < 0.05$ ; \*\* $P < 0.01$ ; \*\*\* $P < 0.001$ .

#### **2.3.4.2. Env-gp140 specific antibody responses to MVA+gp140 and gp140 vaccine regimen**

ELISA determined antibody immunity in response to immunisation with either MVA+gp140 or gp140 is shown in Fig 2.4. In mice vaccinated the MVA+gp140, a significant amount gp140 specific-IgG antibody units were observed in Sm-free ( $170.2 \pm 26.85$ ) vs Sm-infected ( $55.33 \pm 25.02$ ) mice (Fig 2.14 A). Sm-free vaccinated mice ( $100.7 \pm 34.70$ ) also elicited a significant amount of IgG2a compared to Sm-infected mice ( $2.156 \pm 1.508$ ) (Fig 2.14 C). Levels of gp140 specific IgG1 and IgG2b were also higher in Sm-free vaccinated mice ( $189.2 \pm 78.32$  and  $105.8 \pm 56.12$ ) compared to Sm-infected mice ( $38.27 \pm 1.552$  and  $6.878 \pm 1.963$ ). However, the difference was not significant (Fig 2.14 B and D respectively).

Similarly, in mice vaccinated with gp140, higher levels of gp140 specific IgG was observed in Sm-free ( $234.4 \pm 43.59$ ) vs Sm-infected ( $45.99 \pm 26.47$ ) vaccinated mice (Fig 2.14 E). Sm-free vaccinated mice ( $115.3 \pm 32.37$ ) also elicited a significant amount of IgG2b compared to Sm-infected ( $2.614 \pm 1.550$ ) mice (Fig 2.14 H). Levels of gp140 specific IgG1 and IgG2a were also higher in Sm-free vaccinated mice ( $189.2 \pm 78.32$  and  $30.47 \pm 11.97$ ) compared to Sm-infected mice ( $65.73 \pm 41.51$  and  $6.426 \pm 4.742$ ). However, the difference was not significant (Fig 2.14 F and G respectively).



**Figure 2.14: Humoral immune responses in mice immunised with MVA+gp140 and gp140.** HIV-1 gp140-specific IgG (A and E), IgG1 (B and F), IgG2a (C and G), and IgG2b (D and H) serum antibody responses two weeks after the last vaccination with either MVA+gp140 (A-D) or gp140 (E-H) analysed using ELISA. Values are plotted and expressed as mean antibody units (AUs)  $\pm$  SEM for the 3-5 animals in each group. Statistical differences between the groups were calculated by unpaired t-test (two-tailed) followed by FDR for multiple comparisons, \*P<0.05.

## 2.4. Discussion

Helminthiasis and HIV infections are co-endemic in many regions of sub-Saharan Africa (SSA). Globally, soil-transmitted helminths (STH) and schistosome infections are estimated to infect over 1 billion people (Lustigman et al., 2012) with a third of STH infections and over 90% of schistosomiasis cases occurring in Africa (WHO, 2014). In addition, the geographical distribution of these infections vastly overlaps with regions with high HIV prevalence in SSA (Webb et al., 2012). Therefore, it is very likely that successful future HIV vaccines will be administered to people who already have ongoing helminthiasis or have been previously infected and treated. In animal models, schistosomiasis impairs responses to immunisation against hepatitis B (Chen et al., 2012), malaria (Su et al., 2006), mycobacterial infection and BCG immunisation (Elias et al., 2005b, Elias et al., 2005a). There is a lack of consistent scientific data to inform how candidate HIV vaccines would perform in the presence of ongoing helminthiasis given that helminthic infections have been shown to down-regulate Th1 immune responses which are expected to be an important component of successful HIV vaccines (Da'Dara et al., 2006). However, some studies have shown that this is not always true. Shollenberger et al., have demonstrated that the *Listeria monocytogenes* vaccine vector elicited robust and functional HIV-specific cellular immune responses in mice, despite the presence of a chronic helminth infection (Shollenberger et al., 2013a). Furthermore, recent studies have shown that *S. mansoni* egg antigen (responsible for biasing immune responses towards Th2) enhances vaccine-specific T cell responses to *Listeria monocytogenes* vector HIV-1 Vaccine (Beck et al., 2015).

The present study presents immunological data from three experiments, which were conducted to test the impact of helminth infection on the immunogenicity of three candidate HIV vaccine regimens that elicit (1) predominantly cellular (SAAVI DNA-C2 + SAAVI MVA-C: DNA+MVA), (2) Predominantly antibody (Env-gp140 protein) and, (3) Mixed cellular and antibody responses (SAAVI MVA-C + Env-gp140: MVA+gp140). The primary aim of this study was to generate preliminary data that address the possible effect of helminth infection on unrelated antigen stimulation such as vaccine immunogens. The first objective of these experiments was to demonstrate if infection with live helminth infection, induces a predominantly Th2 immune response. This was necessary to confirm if the type of helminth model (*S. mansoni*) was capable of inducing a type 2 response in BALB/c mice as well as to get an understanding of the time of Th2 onset in these mice. The second objective was to evaluate if this helminth-induced Th2 immune biasing negatively affects responses to candidate HIV vaccines. Therefore, three experiments comprising of 4 groups of mice were used. Two groups of mice were chronically infected with Sm, of which one group was subsequently vaccinated twice, 4 weeks apart with appropriate vaccination regimen. The control groups were those with either appropriate vaccine alone without Sm-infection or naïve. These experiments were conducted in BALB/c mice using *S. mansoni* (Sm) as a helminth prototype. The mouse model has been used extensively at the University of Cape Town for studying the Sm-associated disease (Ndlovu, 2013) as well as for initial evaluation of candidate HIV vaccines (Shephard et al., 2008).

#### 2.4.1. Skewing of Th1/Th2 immune balance in chronic Sm infection

Schistosomiasis in mice drives immune responses towards a predominant Th2-type characterized by the production of cytokines including IL-4, IL-5, IL-13, and IL-6. In addition to driving polarized Th2 responses, an increased production of the immunoregulatory cytokine IL-10 is common (MacDonald et al., 2002, Pearce and MacDonald, 2002). To test this in our mouse model, mice were chronically infected with Sm and systemic immune responses to Con A and SEA in Sm-infected and Sm-free vaccinated mice were analysed 16 weeks post-infection (wpi) to assess the induction of Th2 biasing using CBA assay (Fig 2.5). Parasite-specific Th2 cytokines (IL-4; IL-6 and IL-10) went on the rise in Sm-infected mice vaccinated with either an MVA+gp140 (Fig 2.5 A-C) or DNA+MVA (Fig 2.5 H-J) vaccine regimen in comparison with Sm-free vaccinated mice. This was accompanied by a decrease in Th1 cytokines IFN- $\gamma$ ; IL-2 and TNF- $\alpha$  by almost 50% in Sm-infected mice vaccinated with MVA+gp140 (Fig 2.5 D-F) or DNA+MVA (Fig 2.5 K-M) compared to Sm-free vaccinated mice. Furthermore, a significant decrease of the IFN- $\gamma$ :IL-4 ratio was observed in Sm-infected vaccinated mice when compared to Sm-free vaccinated mice (Fig 2.5 G and N), which is considered representative of a trend toward Th2 biasing and immunosuppression advocated by an increase in IL-10; IL-6 and antigen-specific IL-4 in helminth-infected mice (Shollenberger et al., 2013a).

Cytokine data was further complimented with findings from ICS assay, which showed an overall reduction in the frequency of Th1 cytokine-producing CD4+ and CD8+ T cells in Sm-infected vaccinated mice stimulated with PMA/Ionomycin compared to Sm-free vaccinated (Fig 2.10). In Sm-free unvaccinated controls, the frequency of Th1 cytokine-producing CD4+ and CD8+ T cells was also higher compared to Sm-infected and Sm-infected vaccinated mice after stimulation with PMA/Ionomycin. Furthermore, total Ig antibodies were measured to further confirm skewing of host immune responses to Th2 biased. Infection with Sm resulted in increased Th2 Ig antibody markers (IgG1 and IgM) and a decrease in Th1 Ig antibody markers (IgG2a and IgG2b). The findings in the present study are consistent with the literature which reports that Sm infection increases total Th2 antibodies in Sm-infected compared to Sm-free individuals (Maizels and McSorley, 2016). The findings in this study also suggest that there is a correlation between the decrease of cytokine-producing CD8+ cells (Fig 2.10) and IgG2a and IgG2b (Fig 2.13) observed in Sm infected and Sm-infected and vaccinated mice. Previous studies have shown that cytokines produced by CD4+ and CD8+ T cells play an important role in the regulation of the humoral immune response and isotype switching (Kemeny et al., 1994, Burger et al., 1996, Snapper et al., 1997). Moreover, CD8+ depletion study conducted by Cano et al., showed that CD8+ T cells are necessary for optimal clearance of the fungus from tissues of mice infected with *P. brasiliensis* (Cano et al., 2000) which is dependent on IgG2a and IgG2b antibodies (Bueno et al., 2016). It is worth noting that vaccines that induce antibody responses, may result in a slight reduction of non-specific total antibody responses (Fig 2.10) since more antibodies will become specific to epitopes presented by the vaccines. The immunological consequence of a predominant Th2 biasing demonstrated in Sm-infected mice agrees with the concept of Th2 and Th1 mutual exclusiveness as

previously suggested (Finkelman and Urban, 2001, Fincham et al., 2003, Thomas and Harn, 2004). Analysing both cellular responses and antibody responses eliminate ambiguity regarding Th2 dominance status during a chronic infection in the mouse model in the present study.

#### **2.4.2. Vaccine-specific T cell responses induced by the MVA+gp140 and DNA+MVA vaccine regimen in Sm infected mice**

The findings of the present study demonstrated that chronic helminth infection impairs Th1 cellular responses to HIV antigens, which are important for the induction of vaccine-specific cellular responses. Vaccination with the SAAVI-MVA + gp140 or SAAVI-DNA+MVA vaccine regimens induced strong T cell responses in Sm-free vaccinated mice measured by IFN- $\gamma$  and IL-2 ELISpot (Fig 2.3 and 2.4) and CBA (Fig 2.6 -2.9) as previously demonstrated (Burgers et al., 2006, Burgers et al., 2008, Shephard et al., 2008, Burgers et al., 2009). However, the mice with chronic Sm-infection failed to generate high vaccine-specific Th1 cellular responses measured by ELISpot (Fig 2.3 and 2.4) and CBA (Fig 2.6 -2.9) after vaccination with MVA+gp140 and DNA+MVA vaccine regimen. These results suggest that a chronic Sm-infection inhibited the immune responses to the vaccines against CD4+ and CD8+ specific HIV epitopes. Previous reports have linked the downregulation of vaccine-specific Th1 responses with a rise in helminth-induced Th2 responses (Fallon et al., 1996, Urban et al., 1996, Urban et al., 2000, Remoué et al., 2000, Finkelman and Urban, 2001, Fincham et al., 2003, De Silva et al., 2003, Thomas and Harn, 2004). Thus, the results of the present study echo the findings by Da'Dara et al., which demonstrated that Sm infection suppressed T cell responses to a multi-epitope HIV-1 TD158 DNA vaccine (Da'Dara et al., 2006, Da'dara and Harn, 2010).

However, Shollenberger et al, reported no negative impact on the *Listeria* induced CD8+ responses in Sm-infected mice upon Gag stimulation (Shollenberger et al., 2013a). Although not substantial, a similar outcome was observed in the present study when splenocytes of Sm-infected mice vaccinated with DNA+MVA regimen induced higher ELISpot responses to Gag CD8 peptide (Fig 2.3B) compared to Sm-free vaccinated mice. It can be hypothesised that interplay between parasite-induced cytokines may contribute to this upregulation of vaccines specific cellular responses. However, evaluating T cell responses to multiple vaccine immunogens as done in the current study, could give a much clearer picture compared to evaluating responses to only 2 peptides as previously done by Shollenberg et al., (Shollenberger et al., 2013a). Nonetheless, our data along with other published observations shows that, helminth infection may attenuate vaccine-specific responses (Actor et al., 1993, Sabin et al., 1996, Cooper et al., 1999, Cooper et al., 2001, Elias et al., 2005a, Elias et al., 2008, Chen et al., 2012). The reduction of activated CD4+ and CD8+ cells due to infection reiterates the potential negative impact of helminth infection on the hosts T cells, and potentially the immunogenicity of future candidate HIV vaccines. Furthermore, cellular anergy or hyporesponsiveness that result from immunoregulatory cytokines (Prendergast et al., 2015) may be a hurdle that may prevent mounting of vaccine-specific cellular responses in helminth-endemic areas. However, these findings do not address if the early phase of helminth infection could similarly impair cellular responses to vaccines as observed in chronic infection. Thus, future studies may observe how cellular

responses to in acutely infected groups compare to chronically infected groups since there is a paradigm shift of immune responses during these two phases. Surprisingly, slightly higher frequency of cytokine-producing CD8+ T cells in response to stimulation with pol peptide pool (RT CD4 and RT CD8) in mice vaccinated with MVA+gp140 and DNA+MVA regimen were observed (Fig 2.11). However, it is not clear if an increased TNF- $\alpha$  production may attribute to this. A previous study by Magalhães et al., have shown that infection with schistosomiasis may be accompanied by high proportions of TNF- $\alpha$  secreting T cells and a low proportion of T cells secreting IFN- $\gamma$  which is often associated with liver fibrosis (Magalhães et al., 2004). In this study, it was not possible to confirm this because the ICS assay was designed to have IFN- $\gamma$ , IL-2 and TNF- $\alpha$  monoclonal antibodies multiplexed on the same fluorochrome to analyse a pool of all the three cytokines rather than individual cytokines. It is, therefore, tempting to speculate that the slight increase in the frequency of cytokine-producing T cells may just be to other factors other than the vaccines themselves.

#### **2.4.3. Vaccine-specific antibody responses induced by the MVA+gp140 and gp140 vaccine regimen in Sm infected mice**

The implication of inhibited functionality of CD4+ T cells, translates to failure in B cell activation, hence failure in antibody production (Swain et al., 2012). We demonstrated this in chronically Sm-infected mice vaccinated with MVA+gp140 (Fig 2.14 A-D) and gp140 (Fig 2.14 E-F) vaccine regimens. A dramatic decrease in production of serum anti-gp140 antibodies levels of IgG and all subclasses of IgG (IgG1; IgG2a and IgG2b) was observed in Sm-infected compared to Sm-free vaccinated mice (Fig 2.14). A study by Chen et al., demonstrated similar results in mice infected with *S. japonicum* and vaccinated with an HBV vaccine (Chen et al., 2012). These results were attributed to a preexisting Th2-dominated immune profile in the infected host, which is associated with downregulation of anti-HBs antibodies and Th1 cytokines to vaccination (Chen et al., 2012). To our knowledge, there is no reported study evaluating HIV vaccine-specific antibody responses to Env-gp140 in helminth setting. However, it may be hypothesised that because Th1 cellular responses can stimulate B cells into secreting IgG2a while inhibiting IgG1 (Deenick et al., 2005), their reduction may directly affect antibody responses.

#### **2.4.4. Conclusion and recommendations**

The results of this preliminary study were useful in validating successful reproducibility of the schistosomiasis mouse model. The findings of this study demonstrated following:

- (i) Infection with Sm induces a predominantly Th2 biased response marked by increased Th2 cytokines and decreased Th1 cytokines in mice. Also, infection with Sm increases type 2 antibodies (IgG1 and IgM) with a decrease in type 2 total antibodies (IgG2a and IgG2b) in mice.
- (ii) Infection with Sm resulted in a decrease in cellular responses to SAAVI DNA and MVA vaccines in mice measured by ELISpot, CBA and ICS.
- (iii) Infection with Sm significantly reduced the production of env-gp140-specific antibodies in vaccinated mice.

Although these results are preliminary, they suggest that the leading of host immune responses towards biased Th2 responses caused by helminth infections could potentially dampen the efficacy of the vaccine-induced immunity. These results justify further studies to investigate the impact of helminth infection on the immunogenicity of HIV vaccines and to investigate if these adverse effects can be overcome by the elimination of parasites through chemotherapy as previously suggested (Da'dara and Harn, 2010, Chen et al., 2012).

## CHAPTER 3

### 3. Treatment moderately restores T cell responses but not antibody responses

#### 3.1. Introduction

Schistosomiasis is one of the major neglected tropical diseases affecting more than 207 million people in tropical areas. It is well documented that infection with the parasite skews the immune response to a predominantly Th2 immune setting which is capable of dampening responses to unrelated antigens, particularly that of vaccine immunogens. In the preceding chapter, three experiments were conducted in order to identify whether T cell-mediated, antibody-mediated or both vaccine responses are affected by helminthiasis. Furthermore, the preceding chapter also aimed at confirming if the helminth mouse model using *Schistosoma mansoni* as a prototype helminth can be reproduced as previously shown (Ndlovu, 2013).

The outcomes of the preliminary study showed that chronic schistosomiasis could be successfully established in mice and it skewed the host immune system towards a predominantly Th2 profile. Interestingly, this skewing towards Th2, correlated with reduced vaccine-specific cellular responses, including upregulation of IL-10 (suppressive cytokine) which is not considered desirable for an effective HIV vaccine. Similarly, vaccine-specific antibody responses including IgG and all its isotypes (IgG1; IgG2a; IgG2b) were reduced in Sm-infected mice compared with Sm-free mice. This chapter serves as a follow up to the preliminary study further investigate the effect of parasite elimination on the immunogenicity of HIV vaccines.

As reviewed in Chapter 1, section 1.5.3 parasitic helminth infections can be treated with chemotherapeutic drugs such as praziquantel (PZQ) for schistosomiasis (Shaheen et al., 1989, Utzinger and Keiser, 2004, Tallima and El Ridi, 2007) and ivermectin or mebendazole for geohelminths (Cañete et al., 2009, Churcher et al., 2009, Geary, 2012). However, reinfection is common and frequent in endemic populations (N'Goran et al., 2001). We hypothesise that the elimination of these parasites should result in the recovery of immune responsiveness as previously suggested (Bentwich et al., 1999, Cooper et al., 2000a, Borkow and Bentwich, 2006).

Previous vaccine studies have shown in mice that certain candidate HIV vaccines failed in schistosome-infected mice but were functional if the helminth infection was eliminated prior to vaccination (Shollenberger et al., 2013b). Another study by Cooper et al. has demonstrated that *Ascaris lumbricoides* infected patients had diminished Th1 response to cholera toxin B subunit (CT-B) vaccine, however, once treated with ALB, they showed partial recovery of vaccine-induced Th1 responses (Cooper et al., 2001). Similarly, a study by Elias et al. demonstrated that peripheral blood mononuclear cells from de-wormed (ALB treatment) individuals had higher frequencies of BCG vaccine-specific IFN- $\gamma$  and IL-12 producing cells than helminth

infected individuals (Elias et al., 2008). These studies suggest that anthelmintic treatment may correct the overall immune balance in the host immune response, hence restore vaccine responses. However, it remains unclear whether complete restoration and immune balance are entirely achieved (Da'dara and Harn, 2010). The present chapter sought to investigate if vaccine-specific responses in chronically infected mice as shown in the previous chapter can be restored by anthelmintic chemotherapy. In addition, this chapter served as a validity and reproducibility experiment of the preliminary study.

The immune responses of the hosts to chronic helminth infection are generally characterized by a skewed Th2-like response. 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). Parasite-induced Th2 responses are protective to the host against excessive pathology, however, helminths have developed several means of escaping these immune responses, enabling them to persist in the host (McSorley and Maizels, 2012). Conversely, an effective HIV vaccine would be expected to induce high expression of Th1 cytokines such as IFN- $\gamma$ , IL-2 and TNF- $\alpha$  which in turn may inhibit the parasite-induced Th2 responses. This could result in exacerbation of the helminth-associated pathology, thus, making vaccination unsafe in helminth-infected recipients. This chapter further investigates if predominantly Th1 T cell responses induced by HIV vaccination will exacerbate helminth-induced pathology in chronically infected individuals.

The primary markers of immunopathology of the helminth *S. mansoni* are characterized by enlarged granulomas and fibrosing inflammation around parasite eggs in the liver tissue. Thus, the present study was also designed to examine liver tissue of Sm-infected mice vaccinated with a predominantly Th1 inducing vaccine DNA+MVA.

## **3.2. Materials and methods**

### **3.2.1. Parasites**

Infectious *Schistosoma mansoni* cercariae were prepared as described in chapter 2 (section 2.2.1).

### **3.2.2. Vaccines**

Vaccines described in chapter 2 (section 2.2.2) (SAAVI DNA-C2; SAAVI MVA-C and HIV-1 gp140 Env protein) were used for immunisation of mice.

### **3.2.3. Alum adjuvant**

Imject Alum (Thermo Fisher Scientific, USA) was used as an adjuvant for the formulation of Env protein gp140 protein.

### **3.2.4. Animals**

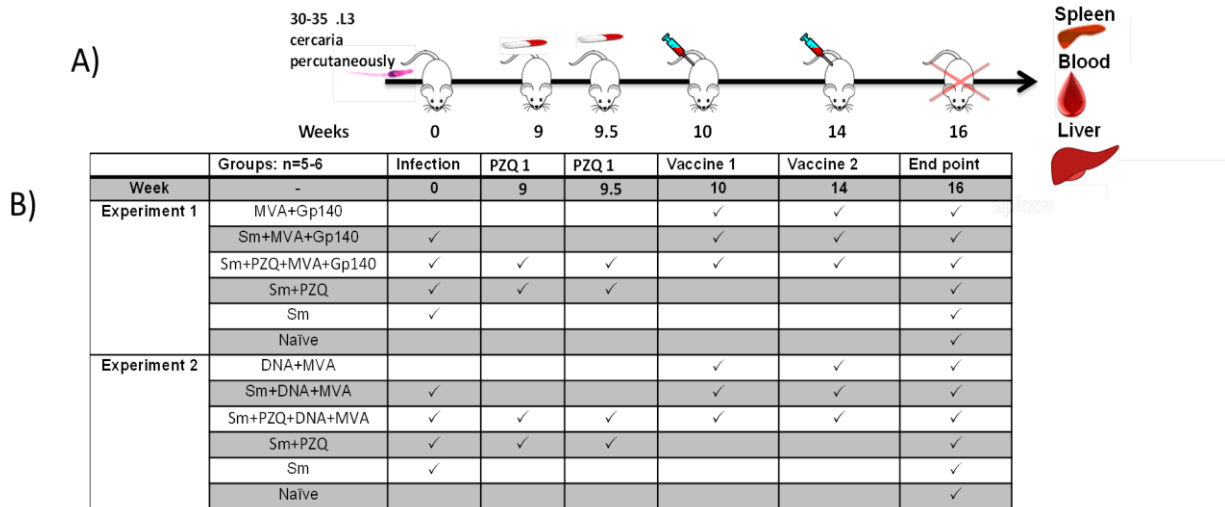
Female BALB/c mice (6–8 weeks old) were purchased from South African Vaccine Producers (SAVP) (Johannesburg, South Africa) and housed as described in chapter 2 (section 2.2.3).

### **3.2.5. Experimental design**

#### **3.2.5.1. Immunization schedule**

Two pre-clinical experiments comprising of 6 groups of mice were used. The first experiment was designed to induce a predominantly HIV-specific T cell response (DNA+MVA) whilst the second experiment was designed to induce a mixed T cell and humoral response (MVA+gp140). The immunogenicity of these vaccines was evaluated in a chronic schistosomiasis mouse model.

Briefly, BALB/c mice were chronically infected with *S. mansoni* (Sm). Nine and a half weeks post infection; half of the infected mice were treated twice with PZQ (Sigma Aldrich, USA) and the other half remained untreated. The two doses of PZQ were administered by oral gavage two days apart. At week 10, half of the PZQ-treated, untreated Sm-infected and Sm-free mice were vaccinated with either MVA+gp140 vaccine regimen, two vaccinations 4 weeks apart (Experiment 1) or vaccinated once with DNA followed by MVA, 4 weeks later (Experiment 2). Groups of Sm-infected but untreated, Sm-infected but PZQ-treated and Sm-free (naïve) mice were left unvaccinated to serve as controls. Twelve days following last vaccination (week16), mice were euthanised and spleens, blood, and livers were collected and evaluated for HIV immune responses and helminth-induced pathology (Fig 3.1).



**Figure 3.1: Infection, treatment and Immunisation schedules:** Groups of BALB/c mice (n=5-8) were chronically infected with *S. mansoni* (Sm) before vaccination with SAAVI MVA-C ( $10^7$  pfu/mouse) concurrently with gp140 Env protein (10 $\mu$ g/mouse, formulated in Imject Alum [Thermo Fisher Scientific, USA]), 4 weeks apart (Experiment 1) or vaccinated once with DNA followed by MVA, 4 weeks apart (Experiment 2). These vaccinations were given with or without (control) prior anti-helminthic treatment with PZQ. Twelve days following last vaccination (week16), mice were euthanised and spleens, blood, and livers were collected and evaluated for HIV immune responses and helminth-induced pathology. A) Representation of infection and immunisation strategy. B) Schedule for infection, treatment, vaccination and experimental endpoint.

### 3.2.5.2. Infection of mice with Sm-cercariae

Infection of mice was performed as described in Chapter 2, section 2.2.4.2.

### 3.2.5.3. Vaccination of mice with SAAVI DNA-C2; SAAVI MVA and HIV Env-gp140

Vaccination of mice was performed as described in Chapter 2, section 2.2.4.3 with modifications to the gp140 formulation. Briefly, the Env-gp140 vaccine protein was administered at a dose of 10 $\mu$ g Env-gp140 formulated in an equal volume of Imject® Alum adjuvant (Thermo Scientific, USA) and given at 100 $\mu$ l mixture per mouse.

### 3.2.6. Preparation of splenocytes and serum

Splenocytes and serum were processed as previously described in chapter 2 (section 2.2.5).

### **3.2.7. Interferon (IFN)- $\gamma$ and interleukin-2 (IL-2) ELISpot assay**

Splenocytes were stimulated with Gag (CD4 and CD8); RT (CD4 and CD8) and Env (CD4 and CD8) specific peptides. Interferon (IFN)- $\gamma$  and IL-2 ELISpot was performed as previously described in chapter 2 (section 2.2.6).

### **3.2.8. Flow cytometry and ICS**

Splenocytes were stimulated with Gag (CD4 and CD8); RT (CD4 and CD8) and Env (CD4 and CD8) specific peptides. Flow cytometry and ICS analysis were performed as described in chapter 2 (section 2.2.7).

### **3.2.9. Quantification of secreted cytokines by CBA**

Splenocytes were stimulated with Gag (CD4 and CD8); RT (CD4 and CD8) and Env (CD4 and CD8) specific peptides. CBA was used to measure cytokine levels and was performed as described in previous chapter 2 (section 2.2.8).

### **3.2.10. Quantification of total and Env-gp140 specific antibody isotypes by ELISA assay**

Total and gp140 specific antibodies in mice serum were detected and quantified by an ELISA assay as previously described in chapter 2 (section 2.2.9).

### **3.2.11. Liver and spleen weighing**

Spleens and livers were weighed prior to processing for immunological evaluation in the laboratory to determine if HIV vaccination worsens helminth-associated pathology.

### **3.2.12. Histopathological examination**

After sacrifice of different groups of animals, part of the liver tissue was immediately fixed in 4% (v/v) buffered formalin solution. These were further processed in Groote Schuur Hospital for histopathological analysis. Briefly, samples were fixed in formaldehyde in phosphate buffered saline, embedded in wax and processed. Sections (5-7 $\mu$ m) were stained with hematoxylin and eosin (H&E) (Sigma Aldrich, USA) and Chromotrop-aniline blue solution (CAB) (Sigma Aldrich, USA) and counterstained with Weigert's hematoxylin (Sigma Aldrich, USA) for collagen staining. Micrographs of liver granuloma were captured using a Nikon 90i widefield microscope using a 5.0-megapixel colour digital camera running Nikon's NIS-Elements v. 4.30 software (Nikon Instruments Inc., USA). The area of each granuloma containing a single egg was measured with the ImageJ 1.34 software (National Institutes of Health, USA). A total of 25-30

granulomas per slide per mouse were included in the analyses of all groups with liver granulomas. Data was presented as a mean area of each granuloma containing a single egg.

### **3.2.13. Hydroxyproline content assay**

Hydroxyproline content, which is a direct measure of collagen content was determined using a modified protocol (Bergman and Loxley, 1963). Briefly, liver samples were weighed, hydrolyzed and added to a 40mg Dowex/Norit mixture (20g Dowex [200 to 400 mesh; Sigma Aldrich, USA] with 10g Norit-A [Thermo Fisher Scientific, USA]). The supernatants were neutralised with 1% phenolphthalein and titrated against 10 M NaOH. An aliquot was mixed with isopropanol and added to chloramine-T/citrate buffer solution (pH 6.5). Erlich's reagent (95% ethanol containing dimethylaminobenzaldehyde (DMAB) and concentrated hydrochloric acid) was added and absorbance was read at 570 nm. Hydroxyproline levels were calculated using 4-hydroxy-L-proline (Sigma Aldrich, USA) as a standard, and results were expressed as  $\mu$ moles hydroxyproline per weight of tissue that contained  $10^4$  eggs. The number of eggs per gram of liver was determined by counting individual eggs from hydrolysed liver under a microscope.

### **3.2.14. Statistical analysis**

In order to determine the effect of schistosomiasis on the immunogenicity of the candidate HIV vaccine to induce cellular and humoral immune responses, statistical analyses were conducted to compare across experimental and control groups (vaccinated Sm-free groups with vaccinated Sm-infected groups). To determine if treatment restores vaccine-specific responses, vaccinated Sm-infected-PZQ treated groups were compared with untreated-Sm-infected vaccinated groups. However, to determine if HIV vaccination worsened helminth-associated pathology, the pathology of vaccinated Sm-infected groups was compared with unvaccinated Sm-infected groups. Furthermore, Con A and SEA responses between Sm-free and Sm-infected groups were compared to determine the skewing of Th1/Th2 balance. Statistical analysis was performed using Prism version 5.0 (GraphPad Software, USA). The t-test for independent unpaired non-parametric comparisons was applied to assess the level of significance between means  $\pm$ SEM of groups. Three independent experiments were conducted and all tests were two-tailed. P values  $<0.05$  were considered significant.

### **3.3. Results**

#### **3.3.1. Cellular immune responses to Con A in Sm-infected mice vaccinated with the DNA+MVA and MVA+gp140 vaccine regimen measured by ELISpot**

To evaluate the Th1-Th2 balance cellular immune responses were evaluated using IFN- $\gamma$  and IL-2 ELISpot assays to enumerate the magnitude of Con A-stimulated IFN- $\gamma$  or IL-2 secreting CD8+ and CD4+ T cells in the spleen of mice vaccinated with either the DNA vaccine (DNA+MVA) or MVA-protein (MVA+gp140) (Fig 3.2).

##### **3.3.1.1. ELISpot responses to Con A in DNA+MVA vaccine regimen**

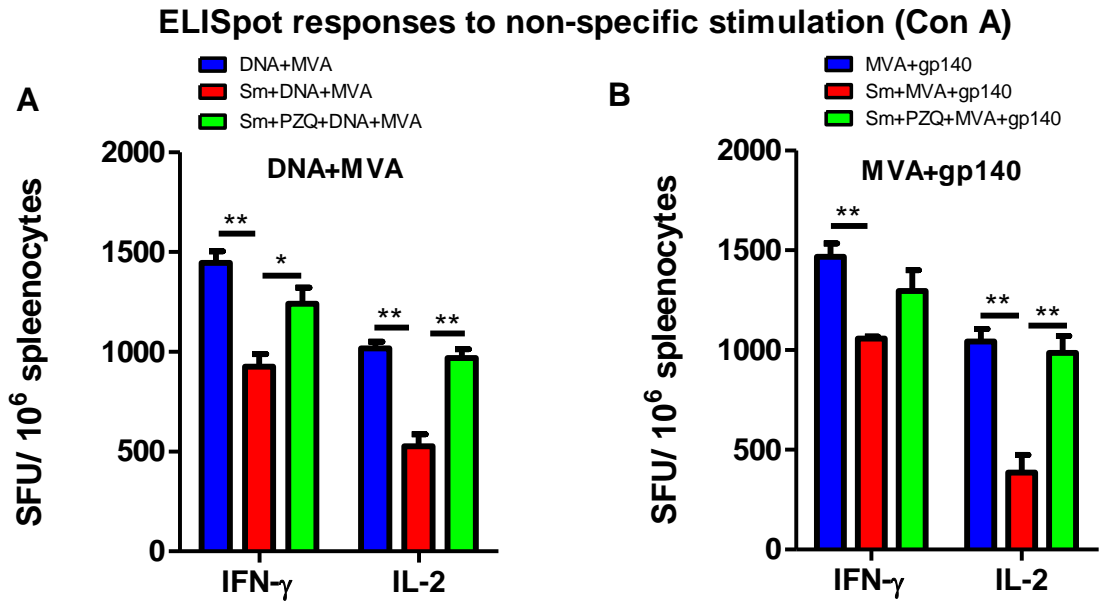
A significant number of IFN- $\gamma$  secreting T cells was observed in Sm-free ( $1446 \pm 59.07$  SFU/ $10^6$  splenocytes) compared to Sm-infected vaccinated ( $925.3 \pm 64.17$  SFU/ $10^6$  splenocytes) mice ( $p < 0.01$ ). Vaccinated Sm-infected-PZQ treated ( $925.3 \pm 64.17$  SFU/ $10^6$  splenocytes) mice also had a significant number of IFN- $\gamma$  secreting T cells compared to Sm-infected vaccinated mice ( $P < 0.05$ ). However, Sm-free vaccinated mice had a slightly higher number of IFN- $\gamma$  secreting T cells compared to vaccinated Sm-infected-PZQ treated mice, albeit insignificant (Fig 3.2 A).

The same pattern was observed for IL-2 ELISpot responses. Vaccinated Sm-free ( $1017 \pm 33.98$  SFU/ $10^6$  splenocytes) and Sm-infected-PZQ treated ( $969.2 \pm 44.31$  SFU/ $10^6$  splenocytes) vaccinated mice had a significantly higher number of IL-2 secreting T cells compared to Sm-infected ( $526.7 \pm 60.77$  SFU/ $10^6$  splenocytes) vaccinated mice ( $P < 0.01$ ). The difference between vaccinated Sm-free and vaccinated Sm-infected-PZQ treated mice was insignificant (Fig 3.2 B).

##### **3.3.1.2. ELISpot responses to Con A in MVA+gp140 vaccine regimen**

A significant number of IFN- $\gamma$  secreting T cells was observed in vaccinated Sm-free ( $1468 \pm 66.94$  SFU/ $10^6$  splenocytes) compared to Sm-infected vaccinated ( $1058 \pm 9.292$  SFU/ $10^6$  splenocytes) mice ( $P < 0.01$ ). Vaccinated Sm-infected-PZQ treated ( $1241 \pm 80.83$  SFU/ $10^6$  splenocytes) mice also had higher but not significant number of IFN- $\gamma$  secreting T cells compared to Sm-infected vaccinated mice, Sm-free vaccinated mice also had a higher number of IFN- $\gamma$  secreting T cells compared to vaccinated Sm-infected-PZQ treated mice, but the difference was not significant (Fig 3.2 B).

The same pattern was observed for IL-2 ELISpot responses. Sm-free ( $1042 \pm 64.30$  SFU/ $10^6$  splenocytes) and Sm-infected-PZQ treated ( $985.9 \pm 85.26$  SFU/ $10^6$  splenocytes) vaccinated mice had a significantly higher number of IL-2 secreting T cells compared to Sm-infected ( $386.7 \pm 88.04$  SFU/ $10^6$  splenocytes) vaccinated mice ( $P < 0.01$ ). The difference between vaccinated Sm-free and vaccinated Sm-infected-PZQ treated mice was insignificant (Fig 3.2 B).



**Figure 3.2: IFN- $\gamma$  and IL-2 ELISpot analysis of Con A stimulated T cell responses in the spleen of Sm-free vaccinated; Sm-infected vaccinated and Sm-infected-PZQ treated mice vaccinated with DNA+MVA (A) or MVA+gp140 (B) vaccine regimen described in Fig 3.1.** At sacrifice, splenocytes were pooled from 5-8 mice per group then stimulated with Con A and analysed in an IFN- $\gamma$  and IL-2 ELISpot assays. Bars are the mean number of spots of triplicate reactions in the irrelevant peptide (background) or to an individual peptide for  $10^6$  splenocytes with indicated standard deviation (SD) of the mean. Positive responses to the Con A stimulation were considered to be those greater than the background response (response to irrelevant peptide stimulation) plus two times the standard deviation (SD) of this response. Results represent 3 independent experiments. Statistical analysis was performed using unpaired, two-tailed t-test analysis followed by FDR for multiple comparisons, \* $p < 0.05$ , \*\* $p < 0.01$ .

### 3.3.2. HIV-specific cellular immune responses to the DNA+MVA and MVA+gp140 vaccine regimen in the background of Sm measured by ELISpot

Cellular immune responses were evaluated using IFN- $\gamma$  and IL-2 ELISpot assays to enumerate the number of Gag; RT and Env-specific IFN- $\gamma$  or IL-2 secreting CD8+ and CD4+ T cells in the spleen of mice vaccinated with either the DNA vaccine (DNA+MVA) or MVA-protein (MVA+gp140) (Fig 3.3).

#### 3.3.2.1. Vaccine-specific ELISpot responses to DNA+MVA vaccine regimen

Vaccination with DNA+MVA induced high cumulative HIV-1 Gag, RT and Env-specific IFN- $\gamma$  ( $2014 \pm 177.4$  SFU/ $10^6$  splenocytes) (Fig 3.3 A) and IL-2 ELISpot ( $174.1 \pm 71.13$  SFU/ $10^6$  splenocytes) (Fig 3.3 D) responses in Sm-free compared to Sm-infected vaccinated mice which had significantly reduced IFN- $\gamma$  ( $1420 \pm 61.54$  SFU/ $10^6$  splenocytes) (Fig 3.3 A) and no IL-2 ELISpot (0 SFU/ $10^6$  splenocytes) (Fig 3.3 D)

responses. Also, cumulative HIV-specific IFN- $\gamma$  ELISpot responses in Sm-free were significantly higher ( $P < 0.05$ ) compared to Sm-infected-PZQ treated ( $1290 \pm 146.4$  SFU/ $10^6$  splenocytes) vaccinated mice. However, cumulative HIV specific IL-2 ELISpot responses in vaccinated Sm-infected-PZQ treated ( $185.0 \pm 25.86$  SFU/ $10^6$  splenocytes) mice were slightly lower but not significant when compared with Sm-free vaccinated mice (Fig 3.3 D).

The same was also true for IFN- $\gamma$  (Fig 3.3 B) and IL-2 (Fig 3.3 E) ELISpot responses to individual Gag (CD4); RT (CD4 or CD8) and Env (CD4) specific peptides. IFN- $\gamma$  ELISpot responses to the RT (CD8) peptide induced the highest number of IFN- $\gamma$  secreting CD8+ and CD4+ T cells ( $1019 \pm 217.3$  SFU/ $10^6$  splenocytes) in Sm-free mice. However, reduced number of RT (CD8) specific IFN- $\gamma$  was observed in vaccinated Sm-infected ( $686$  SFU/ $10^6$  splenocytes) and Sm-infected-PZQ treated vaccinated mice. The same was observed for all the other peptides except for the Gag CD8 peptide which had higher IFN- $\gamma$  responses in Sm-infected ( $228$  SFU/ $10^6$  splenocytes) compared to Sm-free vaccinated ( $141$  SFU/ $10^6$  splenocytes) and Sm-infected-PZQ treated vaccinated mice. Vaccinated Sm-infected-PZQ treated mice only had slightly higher response compared Sm-infected mice when stimulated with Gag CD4 ( $109$  vs  $53$  SFU/ $10^6$  splenocytes); RT CD8 ( $731$  vs  $686$  SFU/ $10^6$  splenocytes) and Env CD4 ( $255$  vs  $203$  SFU/ $10^6$  splenocytes) (Fig 3.3 B).

The highest number of IL-2 ELISpot responses was observed in Sm-free ( $67$  SFU/ $10^6$  splenocytes) and Sm-infected-PZQ treated ( $69$  SFU/ $10^6$  splenocytes) vaccinated mice splenocytes stimulated with Env CD4 peptide, while in Sm-infected mice, no IL-2 responses above cutoff were observed for all the peptides ( $0$  SFU/ $10^6$  splenocytes). While Gag CD4; RT CD4; and Env CD8 exhibited IL-2 ELISpot response in Sm-free vaccinated mice, vaccinated Sm-infected-PZQ treated mice also exhibited IL-2 responses to these peptides except for the Env CD8 peptide (Fig 3.3 E).

### **3.3.2.2. Vaccine-specific ELISpot responses to MVA+gp140 vaccine regimen**

Vaccination with MVA+gp140 also induced significantly high cumulative HIV-1 Gag, RT and Env-specific IFN- $\gamma$  ( $1838 \pm 173.3$  SFU/ $10^6$  splenocytes) (Fig 3.3 A) and IL-2 ( $197.7 \pm 20.12$  SFU/ $10^6$  splenocytes) ELISpot responses in Sm-free compared to Sm-infected vaccinated mice which had significantly reduced IFN- $\gamma$  ( $1166 \pm 132.2$  SFU/ $10^6$  splenocytes) (Fig 3.3 A) and IL-2 ELISpot ( $11.89 \pm 5.951$  SFU/ $10^6$  splenocytes) (Fig 3.3 D) responses. Also, cumulative HIV-specific IFN- $\gamma$  ELISpot responses in vaccinated Sm-infected-PZQ treated ( $1685 \pm 251.3$  SFU/ $10^6$  splenocytes) mice were higher compared to Sm-infected vaccinated mice albeit insignificant (Fig 3.3 A). However, cumulative HIV-specific IL-2 ELISpot responses in vaccinated Sm-infected-PZQ treated ( $185.0 \pm 25.86$  SFU/ $10^6$  splenocytes) mice were significantly higher when compared with Sm-infected vaccinated mice but slightly lower than Sm-free vaccinated mice.

The same was also true for IFN- $\gamma$  (Fig 3.3 C) and IL-2 (Fig 3.3 D) ELISpot responses to individual Gag (CD4 and CD8); RT (CD4 and CD8) and Env (CD4 and CD8) specific peptides. IFN- $\gamma$  ELISpot responses to the Env (CD8) peptide induced the highest number of IFN- $\gamma$  secreting CD8+ and CD4+ T cells ( $553.3 \pm 55.86$  SFU/ $10^6$  splenocytes) in Sm-free mice. However, significantly reduced the number of Env (CD8) specific IFN- $\gamma$  was observed in Sm-infected ( $450.0 \pm 25.53$  SFU/ $10^6$  splenocytes) vaccinated mice (Fig 3.3 C). Vaccinated Sm-infected-PZQ treated ( $703$  SFU/ $10^6$  splenocytes) mice had higher IFN- $\gamma$  ELISpot responses when compared to Sm-infected vaccinated mice but no significant difference was observed (Fig 3.3 C). The number of IFN- $\gamma$  secreting CD8+ and CD4+ T cells in response to all the peptides was reduced in Sm-infected mice, whilst been recovered back after treatment with PZQ (Sm-infected-PZQ treated mice). However, responses in vaccinated Sm-infected-PZQ treated were not higher than responses in Sm-free vaccinated mice except a slight increase when stimulated with Gag CD8.

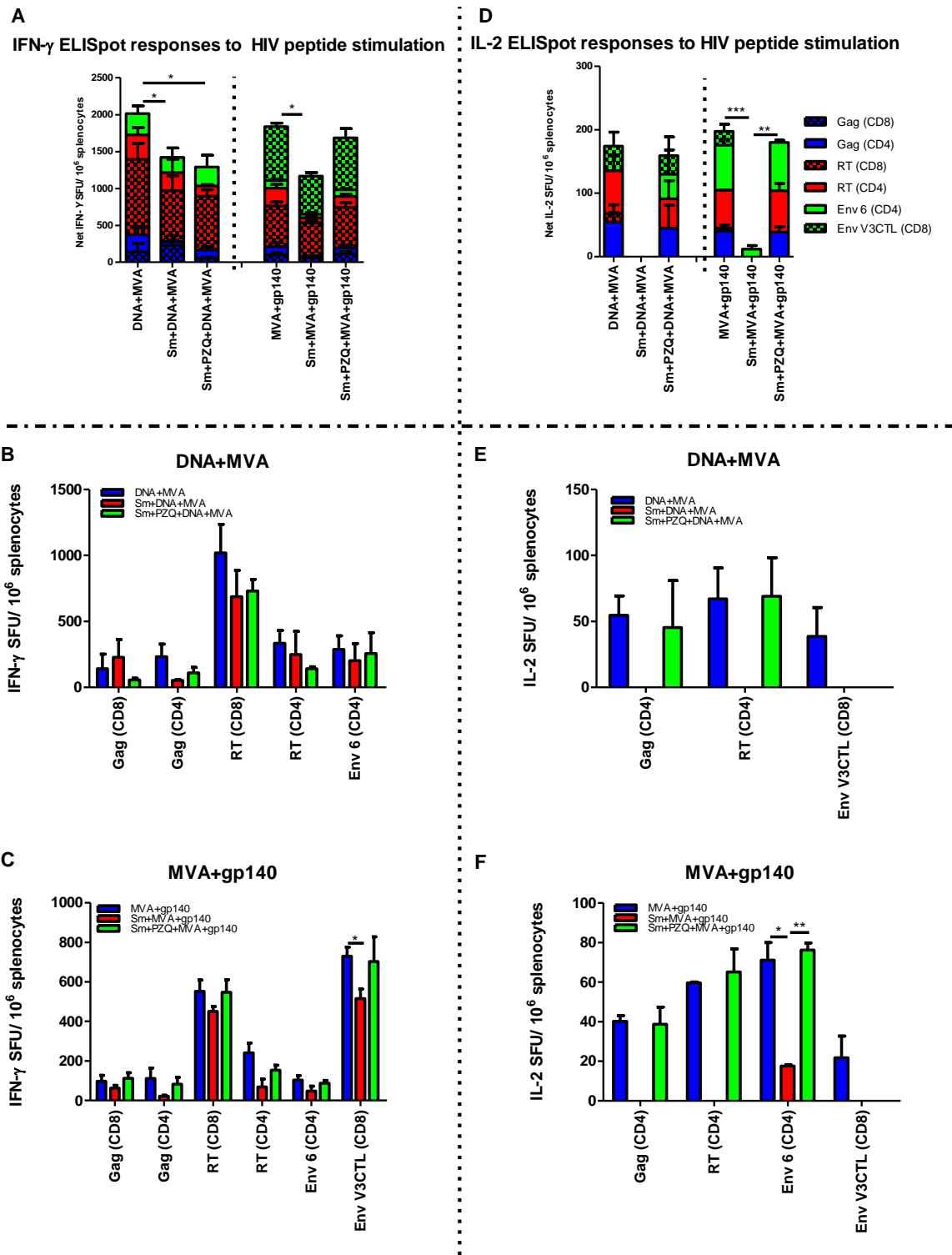


Figure 3.3: IFN- $\gamma$  and IL-2 ELISpot analysis of HIV-1 Gag; RT and Env-specific CD8+ and CD4+ T cell responses induced in the spleen of Sm-infected; Sm-infected-PZQ treated and Sm-free mice vaccinated with either the DNA+MVA or the MVA+gp140 vaccine regimen. At endpoint (2 weeks after

last vaccination) splenocytes were pooled from 5-8 mice per group then stimulated with an irrelevant peptide or stimulated with HIV-1 Gag; RT; Env CD8 and CD4 T cell peptides in IFN- $\gamma$  and IL-2 ELISpot assays. Cumulative proportions of positive IFN- $\gamma$  (A) and IL-2 (D) as well as individual positive IFN- $\gamma$  and IL-2 ELISpot responses induced by the DNA+MVA (B and E respectively) and MVA+gp140 (C and F respectively) vaccination regimens as described in Fig 3.1. The individual sections of each bar indicate the average net SFU  $\pm$ SD from triplicate wells for  $10^6$  splenocytes to an individual CD8 or CD4 peptide. Results represent 3 independent experiments after subtraction of background (irrelevant peptide responses) and at a minimum of 2X background SFU/ $10^6$  splenocytes considered positive. Statistical analysis was performed using unpaired, two-tailed t-test analysis followed by FDR for multiple comparisons, \* $p < 0.05$ , \*\* $p < 0.01$ , \*\*\* $p < 0.001$ .

### **3.3.3. Th1 and Th2 cytokine responses in MVA+gp140 and DNA+MVA vaccine regimen in Sm-infected-PZQ treated mice measured by Cytometric Bead Array (CBA)**

To assess the skewing towards Th2 profile, the amount of Th1 and Th2 cytokines secreted by T cells after being stimulated with Con A (non-specific stimulation) and SEA (parasite-specific stimulation) (Fig 3.4) were quantified for MVA+gp140 (A-G) and DNA+MVA (H-N) vaccinated mice by CBA.

#### **3.3.3.1. Th1 and Th2 cytokines in MVA+gp140 vaccinated mice**

As shown in Fig 3.4 A-C, there was more skewing towards Th2 in Sm-infected compared to Sm-free mice vaccinated with MVA+gp140 marked with increased IL-4 (4.28 vs 2.15 pg/ml); IL-10 (54.11 vs 33.97 pg/ml) and IL-6 (350.57 vs 113.3 pg/ml) responses to Con A. Furthermore, an increase in IL-4 (5.1 vs 1.3 pg/ml); IL-6 (559.27 vs 49.76 pg/ml) and IL-10 (236.91 vs 1.3 pg/ml) was observed in Sm-infected mice compared to Sm-free vaccinated mice when stimulated with the parasite-specific SEA (Fig 3.4 A-C).

Interestingly, vaccinated Sm-infected-PZQ treated mice also showed skewing towards a Th2 profile marked with a significant increase in IL-6 (308.48 pg/ml); IL-10 (56.62 pg/ml) and high IL-4 (9.15 pg/ml) compared to Sm-free mice in response to the Con A stimulation. Furthermore, vaccinated Sm-infected-PZQ treated mice also had a significant increase in IL-6 (619.43 pg/ml); IL-10 (402.5 pg/ml) and high IL-4 (5.86 pg/ml) responses to the parasite-specific SEA compared to Sm-free and Sm-infected mice (Fig 3.4 A-C).

Expectedly, Th1 cytokines were drastically reduced in Sm-infected compared to Sm-free vaccinated mice stimulated with Con A marked by decreased IFN- $\gamma$  (70.19 vs 2732 pg/ml); TNF- $\alpha$  (25.47 vs 75.58 pg/ml) and a significant decreased IL-2 (4.29 vs 13.79 pg/ml) cytokine production. The same pattern was observed when splenocytes were stimulated with SEA. Sm-infected vaccinated mice produced lower IFN- $\gamma$  (10.17 vs 53.8 pg/ml); IL-2 (4.98 vs 9.86 pg/ml) but higher TNF- $\alpha$  (18.44 vs 10.33 pg/ml) production, when compared to Sm-free, vaccinated mice (Fig 3.4 D-F).

Interestingly, in vaccinated Sm-infected-PZQ treated mice, Th1 cytokines levels were higher compared to untreated Sm-infected vaccinated groups despite elevated Th2 cytokines. Significantly higher levels of IFN- $\gamma$  ( $290.2 \pm 47.74$  pg/ml) and higher TNF- $\alpha$  ( $35.5$  pg/ml) and IL-2 ( $9.1$  pg/ml) when compared to Sm-infected vaccinated mice in responses to Con A. However, when stimulated with SEA, Th1 responses in Sm-infected-PZQ treated mice were similar to responses in Sm-infected vaccinated mice (Fig 3.4 D-F).

Th2 biasing marked by a significant decrease in the IFN- $\gamma$ :IL-4 ratio upon Con A stimulation was observed in vaccinated Sm-infected ( $20.38 \pm 8.11$ ) and vaccinated Sm-infected-PZQ treated ( $44.41 \pm 3.52$ ) relative to Sm-free ( $648.1 \pm 96.38$ ) vaccinated mice. However, vaccinated Sm-infected-PZQ treated mice had higher IFN- $\gamma$ :IL-4 ratio compared to Sm-infected vaccinated mice. No difference was observed in the IFN- $\gamma$ :IL-4 ratio when stimulated with SEA except for higher levels observed (Fig. 3.4 G).

### 3.3.3.2. Th1 and Th2 cytokines in DNA+MVA vaccinated mice

As shown in Fig 3.4 H-J, an increased Th2 biasing in Sm-infected compared to Sm-free mice vaccinated with DNA+MVA was marked with increased IL-4 ( $15.4$  vs  $7.9$  pg/ml); IL-10 ( $178.05$  vs  $76.86$  pg/ml) and significant increased IL-6 ( $450.54$  vs  $100.44$  pg/ml) responses to Con A. Furthermore, a significant increase in IL-4 ( $6.803 \pm 0.46$  vs  $2.09 \pm 1.06$  pg/ml); IL-6 ( $522.6 \pm 52.36$  vs  $47.88 \pm 19.07$  pg/ml) and IL-10 ( $413.7 \pm 30.07$  vs below detectable [ $0.3433 \pm 0.3433$ ] pg/ml) was observed in Sm-infected mice compared to Sm-free vaccinated mice when stimulated with the parasite-specific SEA (Fig 3.4 H-J).

Interestingly, vaccinated Sm-infected-PZQ treated mice had significantly increased Th2 biasing marked with a significant increase in IL-4 ( $18.95 \pm 3.795$  pg/ml); IL-10 ( $222.2 \pm 38.13$  pg/ml) and higher IL-6 ( $256.41$  pg/ml) when compared to Sm-free mice in response to the Con A stimulation. Furthermore, vaccinated Sm-infected-PZQ treated mice also had a significant increase in IL-6 ( $278.3 \pm 40.90$  pg/ml); IL-10 ( $472.7 \pm 76.94$  pg/ml) and IL-4 ( $6.803 \pm 0.46$  pg/ml) responses to the parasite-specific SEA compared to Sm-free vaccinated mice (Fig 3.4 H-J).

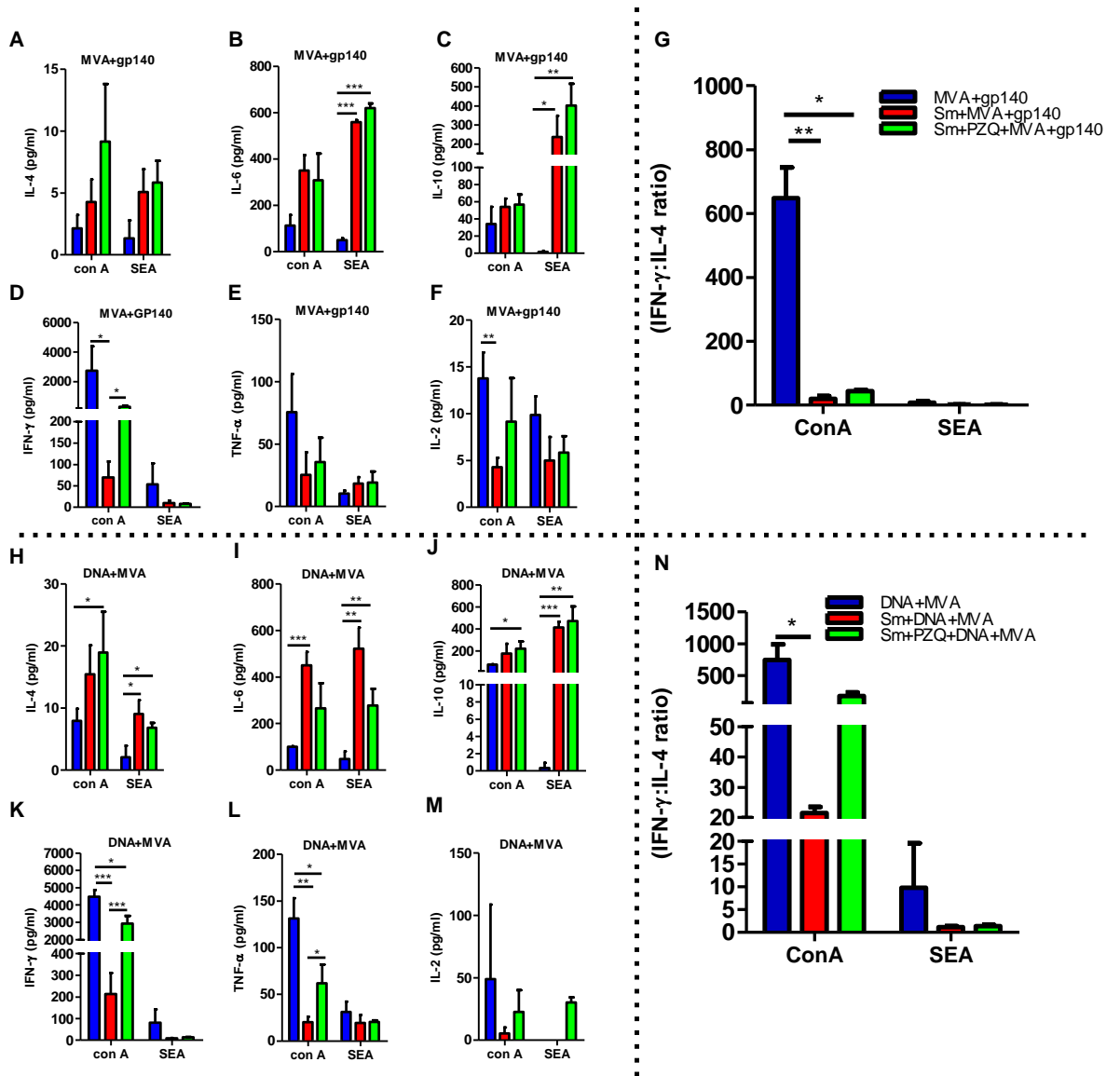
Expectedly, Th1 cytokines were drastically reduced in Sm-infected compared to Sm-free vaccinated mice stimulated with Con A marked by significant decreased IFN- $\gamma$  ( $214.5 \pm 55.71$  vs  $4486 \pm 221.0$  pg/ml); TNF- $\alpha$  ( $20.17 \pm 3.343$  vs  $131.3 \pm 12.50$  pg/ml) and a decreased IL-2 ( $5.3$  vs  $48.9$  pg/ml) cytokine production (Fig 3.4 K-M). When splenocytes were stimulated with SEA, Sm-infected vaccinated mice produced lower IFN- $\gamma$  ( $8.66$  vs  $81.86$  pg/ml); TNF- $\alpha$  ( $19.64$  vs  $31.3$  pg/ml), when compared to Sm-free, vaccinated mice, but no IL-2 ( $0$  pg/ml) was observed in both Sm-infected and Sm-free vaccinated mice (Fig 3.4 D-F).

Interestingly, vaccinated Sm-infected-PZQ treated mice showed recovery of Th1 cytokines despite increased Th2 cytokines. Significantly higher levels of IFN- $\gamma$  ( $2933 \pm 261.8$  pg/ml); TNF- $\alpha$  ( $62.14 \pm 11.42$  pg/ml) and IL-2 ( $22.69$  pg/ml), when compared to Sm-infected, vaccinated mice in responses to Con A were

observed. However, when stimulated with SEA, Th1 responses in vaccinated Sm-infected-PZQ treated mice were similar to responses in Sm-infected vaccinated mice except for IL-2 responses which were only detectable in vaccinated Sm-infected-PZQ treated (Fig 3.4 K-M).

Th2 biasing marked by a significant decrease in the IFN- $\gamma$ :IL-4 ratio upon Con A stimulation was observed in Sm-infected ( $21.53 \pm 2.00$ ) relative to Sm-free ( $745.6 \pm 244.9$ ) vaccinated mice (Fig. 3.4 N). Vaccinated Sm-infected-PZQ treated mice showed a recovery of IFN- $\gamma$ :IL-4 ratio (177.2) when compared to Sm-infected ( $21.53 \pm 2.00$ ). No difference was observed in the IFN- $\gamma$ :IL-4 ratio, when stimulated with SEA except for higher levels observed in Sm-free mice compared to both Sm-infected and Sm-infected PZQ, treated vaccinated mice (Fig 3.4 N).

Th1/Th2 cytokines in response to non-specific and parasite specific stimulation (Con A and SEA)



**Figure 3.4: Cytokine production by Con A and SEA stimulation in Sm-free and Sm-infected mice vaccinated with MVA+gp140 or DNA+MVA regimen.** Splenocytes were harvested from mice vaccinated with the indicated regimen described in Fig 3.1. They were then stimulated with an irrelevant peptide (negative control) or Con A or with SEA for 48 h. Culture supernatants were collected and the level of Th1 and Th2 cytokines released into the medium for MVA+gp140 (A-C and D-F respectively) and DNA+MVA (H-J and K-M respectively) vaccinated mice was measured using a Cytokine Bead Array assay. The individual bars represent the magnitude of the net positive cytokine levels in the medium of splenocyte of vaccinated Sm-free (blue); vaccinated Sm-infected (red) and Sm-infected-PZQ treated (green) vaccinated

mice. The level of cytokines in the culture medium was considered positive when the level is higher than the detection limit of the respective cytokine described in Table 2.3. Results represent 3 independent experiments and plotted as the mean + SEM, and cytokine levels were expressed as pg/ml. Statistical analysis was performed using unpaired, two-tailed t-test analysis followed by FDR for multiple comparisons, \* $p < 0.05$ , \*\* $p < 0.01$ , \*\*\* $p < 0.001$ .

### **3.3.4. HIV-Specific cytokine production**

The amount of Th1 and Th2 cytokine secreted by the Gag; RT and Env-specific CD4+ and CD8+ T cells during culture with the HIV peptides was also quantified for DNA+MVA and MVA+gp140 vaccine regimen (Fig 3.5). Splenocytes harvested from mice vaccinated according to the regimens indicated in Fig 3.1 were incubated for 48h with the Gag; RT and Env- specific CD8+ and CD4+ T cell peptides and the cytokine level released into the culture medium was quantified using a Cytometric Bead Array assay (BD Pharmingen, USA).

#### **3.3.4.1. HIV-Specific cytokine production in DNA+MVA vaccine regimen**

The cumulative positive cytokine levels produced against the HIV peptides for the DNA+MVA vaccination regimen is shown in Fig 3.5. Significantly higher levels of net cumulative Th1 cytokines: IFN- $\gamma$  ( $6523 \pm 282.0$  pg/ml); IL-2 ( $84.86 \pm 0.3147$  pg/ml) and TNF- $\alpha$  ( $251.2 \pm 30.33$  pg/ml) was released from splenocytes of Sm-free mice in response to a DNA+MVA vaccination regimen compared to lower Th1 cytokine levels of IFN- $\gamma$  ( $1899 \pm 244.6$  pg/ml); IL-2 ( $23.55 \pm 4.094$  pg/ml) and TNF- $\alpha$  ( $122.9 \pm 17.45$  pg/ml) released in Sm-infected vaccinated mice (Fig 3.5 A-C).

Conversely, net cumulative Th2 cytokine levels in Sm-free vaccinated mice were drastically lower compared to Sm-infected vaccinated mice: IL-6 ( $339.3 \pm 37.56$  vs  $949.8 \pm 156.2$  pg/ml) and IL-10 ( $68.38$  vs  $121.1$  pg/ml) with exception to IL-4 which was surprisingly higher (though insignificant) in Sm-free ( $25.38$  pg/ml) compared to Sm-infected ( $20.1$  pg/ml) vaccinated mice (Fig 3.5 D-F).

Expectedly, Th1 cytokine recovery was observed in Sm-infected-PZQ treated vaccinated mice marked with a significant increase in IFN- $\gamma$  ( $4729 \pm 673.5$  pg/ml); IL-2 ( $51.49 \pm 7.189$  pg/ml) and higher TNF- $\alpha$  ( $158.4$  pg/ml) when compared to Sm-infected mice. However, Th1 cytokine levels were still lower than those of Sm-free vaccinated mice (Fig 3.5 A-C). Furthermore, Th2 cytokines were lower in vaccinated Sm-infected-PZQ treated compared to Sm-infected vaccinated mice (Fig 3.5 D-F).

#### **3.3.4.2. HIV-Specific cytokine production in MVA+gp140 vaccine regimen**

The cumulative positive cytokine levels produced in response to the HIV peptides for the MVA+gp140 vaccination regimen is shown in Fig 3.5. Significantly high levels of net cumulative Th1 cytokines: IFN- $\gamma$

(2416 pg/ml); IL-2 (63.79 pg/ml) and lower TNF- $\alpha$  (112.48 pg/ml) was released from HIV peptide-specific T cells of Sm-free mice in response to a MVA+gp140 vaccination regimen compared to lower Th1 cytokine levels of IFN- $\gamma$  (789 pg/ml); IL-2 (4.0 pg/ml) and higher TNF- $\alpha$  (123.87 pg/ml) released in Sm-infected vaccinated mice (Fig 3.5 A-C).

Conversely, net cumulative Th2 cytokine levels in Sm-free vaccinated mice were drastically lower compared to Sm-infected vaccinated mice: IL-6 (497.92 vs 1681.88 pg/ml); IL-10 ( $14.95 \pm 2.250$  vs  $68.42 \pm 4.710$  pg/ml) and IL-4 (22.87 vs 27.6 pg/ml) (Fig 3.5 D-F). Expectedly, Th1 cytokine recovery was observed in Sm-infected-PZQ treated vaccinated mice marked with a significant increase in IFN- $\gamma$  (2513.61 pg/ml); IL-2 (38.04 pg/ml) but lower TNF- $\alpha$  (96.14 pg/ml) when compared to Sm-infected mice. Of all the Th1 cytokine levels, only IL-2 was lower compared to Sm-infected vaccinated mice (Fig 3.5 A-C). Furthermore, Th2 cytokines were lower in vaccinated Sm-infected-PZQ treated compared to Sm-infected vaccinated mice (Fig 3.5 D-F).

# Th1/Th2 responses to HIV peptides stimulation

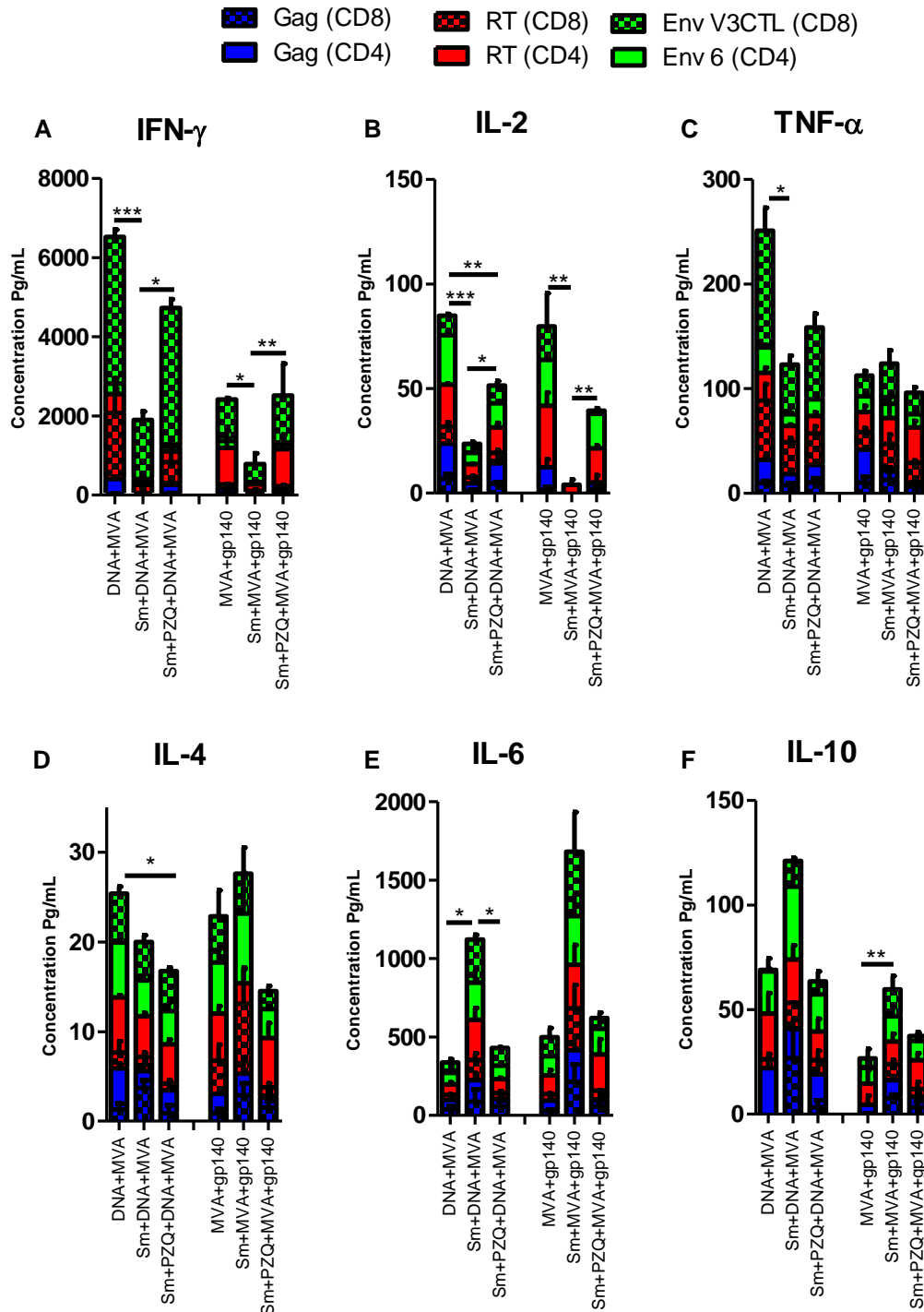


Figure 3.5: Cumulative cytokines produced by HIV peptide-specific T cells of Sm-free and Sm-infected mice vaccinated with DNA+MVA and MVA+gp140. Splenocytes were harvested from mice

vaccinated with the indicated regimen described in Fig 3.1. They were then stimulated with an irrelevant peptide (negative control) or with the individual HIV-specific T cell peptides for 48 h. Culture supernatants were collected, and the level of Th1 (A, B and C) and Th2 (D, E and F) cytokines released into the medium was measured using a Cytokine Bead Array assay and flow cytometric analysis. The individual bars represent the magnitude of the cumulative positive cytokine levels in the medium of splenocytes of vaccinated Sm-free; vaccinated Sm-infected and Sm-infected-PZQ treated vaccinated mice, stimulated with individual HIV peptides, after subtracting the background (splenocytes stimulated with irrelevant peptide). The level of cytokines in the culture medium was considered positive when the level is higher than the detection limit of the respective cytokine described in Table 2.3. Results represent 3 independent experiments and plotted as the mean + SEM, and cytokine levels were expressed as pg/ml. Statistical analysis was performed using unpaired, two-tailed t-test analysis followed by FDR for multiple comparisons, \* $p < 0.05$ , \*\* $p < 0.01$ , \*\*\* $p < 0.001$ .

### **3.3.5. Cytokine responses to individual HIV-1 peptides for the DNA+MVA regimen**

Sm-infected mice (1489.38 pg/ml) vaccinated with a DNA+MVA vaccine had lower levels of IFN- $\gamma$  compared to Sm-free vaccinated mice (3704 pg/ml) in response to Env CD8 peptide stimulations (Fig 3.6 A). Furthermore, net IFN- $\gamma$  cytokine responses to Gag CD4 peptides was significantly lower in Sm-infected ( $31.06 \pm 7.58$  pg/ml) compared to Sm-free ( $385.5 \pm 52.60$  pg/ml) vaccinated mice. Although lower IFN- $\gamma$  responses to the other HIV peptides was observed in Sm-infected compared to Sm-free vaccinated mice, no significant difference was observed (Fig 3.6 A).

Net IFN- $\gamma$  cytokine responses in vaccinated Sm-infected-PZQ treated mice was significantly higher for the Env CD8 ( $3502 \pm 216.1$  pg/ml) compared to Sm-infected ( $1489 \pm 213.8$  pg/ml) but not Sm-free vaccinated mice. IFN- $\gamma$  cytokine responses to the other HIV peptides were higher in vaccinated Sm-infected-PZQ treated mice compared to Sm-free, but not higher than IFN- $\gamma$  cytokine in Sm-free vaccinated mice.

Of all the HIV peptides, Env CD4 induced higher levels of IL-2 in Sm-free ( $23.57 \pm 2.508$  pg/ml) compared to Sm-infected ( $5.390 \pm 0.5727$  pg/ml). Furthermore, Significant levels of Gag CD4 ( $16.74 \pm 1.212$  vs  $3.627 \pm 1.476$  pg/ml); RT CD4 ( $20.16 \pm 0.3842$  vs  $6.243 \pm 0.1683$  pg/ml) and Env CD8 ( $9.287 \pm 0.8467$  vs  $4.250 \pm 1.027$  pg/ml) specific responses were observed in Sm-free vs Sm-infected mice. The same trend for other peptides (Gag CD8 and RT CD8) was observed but the difference was not significant (Fig 3.6 B). Expectedly; vaccinated Sm-infected-PZQ treated mice had significantly higher IL-2 responses to the Gag CD8 ( $4.520 \pm 0.7757$  vs  $1.140 \pm 0.5828$  pg/ml) and Env CD4 ( $11.65 \pm 1.059$  vs  $5.390 \pm 0.5727$  pg/ml) when compared to Sm-infected vaccinated mice. Also, IL-2 responses to other peptides were higher in vaccinated Sm-infected-PZQ treated mice vs Sm-infected vaccinated mice; however, the difference was not significant.

Furthermore, IL-2 responses in vaccinated Sm-infected-PZQ mice were not higher than those of Sm-free vaccinated mice (Fig 3.6 B).

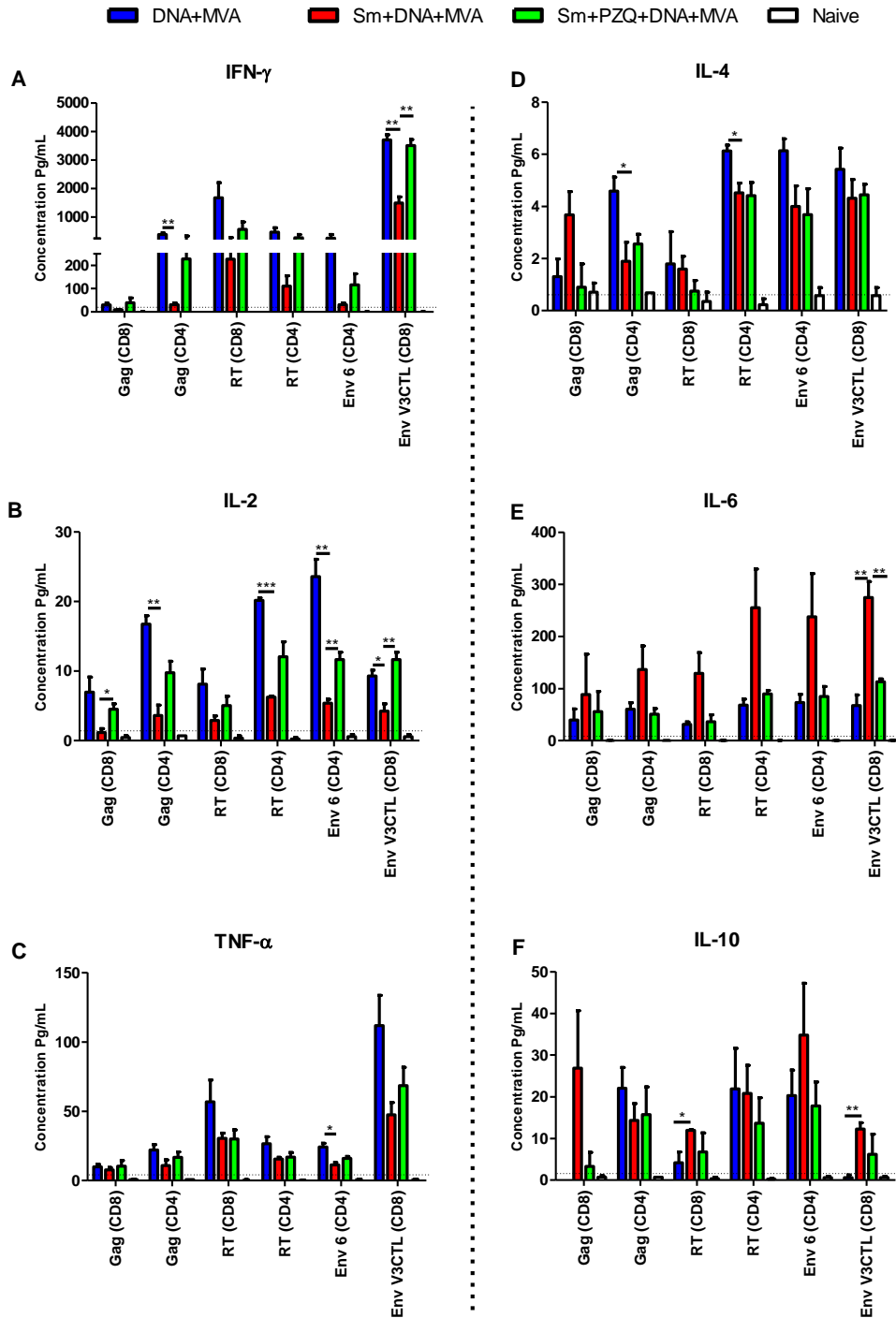
Levels of TNF- $\alpha$  were also drastically reduced in Sm-infected mice in response to all HIV peptides. Responses to the Env CD8 peptide induced the highest responses in Sm-free (111.76 pg/ml) compared to Sm-infected (47.48 pg/ml) vaccinated mice. However, only responses to the Env CD4 were significant when compared with Sm-free vaccinated mice ( $24.25 \pm 2.732$  vs  $11.15 \pm 1.789$  pg/ml). Responses to the HIV peptides in vaccinated Sm-infected-PZQ treated mice were similar to those of Sm-infected with exception to the Env CD8 which had higher TNF- $\alpha$  responses when compared to Sm-infected vaccinated mice. However, none of the responses in vaccinated Sm-infected-PZQ treated mice were higher than responses in Sm-free vaccinated mice (Fig 3.6 C).

Higher levels of IL-6 cytokines in all cultures of vaccinated Sm-infected cells stimulated with HIV peptides compared to Sm-free vaccinated mice was observed. However, a significant difference was only observed in response to the Env CD8 peptide ( $274.5 \pm 30.67$  vs  $67.37 \pm 20.32$  pg/ml) (Fig 3.6 E). Responses in vaccinated Sm-infected-PZQ treated mice had similar responses to all peptides when compared to the response in Sm-free vaccinated mice.

Surprisingly, IL-4 levels in response to Gag CD4 ( $4.590 \pm 0.5372$  vs  $1.890 \pm 0.7314$  pg/ml) and RT CD4 ( $6.127 \pm 0.2270$  pg/ml vs  $4.523 \pm 0.3696$  pg/ml) was significantly higher in Sm-free compared to Sm-infected vaccinated mice. Responses to the RT CD8 (1.79 vs 1.6 pg/ml); Env CD4 (6.1 vs 4.0 pg/ml) and Env CD8 (5.4 vs 4.3 pg/ml) peptides were similar in vaccinated Sm-free mice compared to the Sm-infected vaccinated mice (Fig 3.6 D). Levels of IL-4 in vaccinated Sm-infected-PZQ treated mice was relatively similar to those of Sm-infected with exception to the Gag CD8 which was slightly lower in vaccinated Sm-infected-PZQ treated (0.9 pg/ml) mice compared to Sm-infected (3.68 pg/ml) vaccinated mice.

Levels of IL-10 in response to the Gag CD8 (26.89 vs 0 pg/ml) and Env CD4 (34.81 vs 20.26 pg/ml) were higher in Sm-infected compared to Sm-free vaccinated mice, although not significant. IL-10 response to the RT CD4 (20.81 vs 21.87 pg/ml) and Gag CD4 (14.34 vs 22.06 pg/ml) was relatively similar in Sm-infected compared to Sm-free vaccinated mice (Fig 3.6 F). Levels IL-10 in response to the RT CD8, and Env CD8 was below detectable range. Vaccinated Sm-infected-PZQ treated mice only had detectable levels of IL-10 to the Gag CD8 (15.73 pg/ml) and Env CD4 (17.8 pg/ml) which was closely similar to that of Sm-free vaccinated mice (Fig 3.6 F).

## DNA+MVA



**Figure 3.6: Cytokine produced by Gag (CD8 and CD4) RT (CD8 and CD4) and Env (CD 4) specific peptides in Sm-free; Sm-infected and Sm-infected-PZQ treated mice vaccinated with DNA+MVA. Splenocytes were harvested from mice vaccinated with the indicated regimen described in Fig 3.1. They**

were then stimulated with an irrelevant peptide (negative control) or with the individual HIV-specific T cell peptides for 48 h. Culture supernatants were collected and the level of Th1 (A, B and C) and Th2 (D, E and F) cytokines released into the medium was measured using a Cytokine Bead Array assay and flow cytometric analysis. The individual bars represent the magnitude of the net positive cytokine levels in the medium of splenocyte of Sm-free and Sm-infected vaccinated mice, stimulated with individual HIV peptides, after subtracting the background (splenocytes stimulated with irrelevant peptide). The level of cytokines in the culture medium was considered positive when the level is higher than the detection limit of the respective cytokine described in Table 2.3. Results represent 3 independent experiments and plotted as the mean + SEM, and cytokine levels were expressed as pg/ml. Statistical analysis was performed using unpaired, two-tailed t-test analysis followed by FDR for multiple comparisons, \* $p < 0.05$ , \*\* $p < 0.01$ , \*\*\* $p < 0.001$ .

### **3.3.6. Cytokine responses to individual HIV-1 peptides for the MVA+gp140 regimen**

Despite high levels of net IFN- $\gamma$  released from HIV peptide-specific T cells of Sm-free mice vaccinated with a DNA+MVA vaccine, 43% (1047.59 pg/ml) of the responses are attributed to the Env CD8 peptide-specific responses while 39.8% (926.76 pg/ml) of the responses are attributed to the RTCD4 (Fig 3.7 A). However, Sm-infected vaccinated mice had a drastic reduction of the net IFN- $\gamma$  cytokine (1489.38 pg/ml) in response to Env CD8 (461) and RT CD4 (169.79 pg/ml) peptide, albeit not significant. Furthermore, net IFN- $\gamma$  cytokine responses to all peptides were lower in Sm-infected compared to Sm-free vaccinated mice (Fig 3.7 A). Net IFN- $\gamma$  cytokine responses in vaccinated Sm-infected-PZQ treated mice were relatively similar to those of Sm-free vaccinated mice for all HIV peptides. However, no significant difference was observed (Fig 3.7 A).

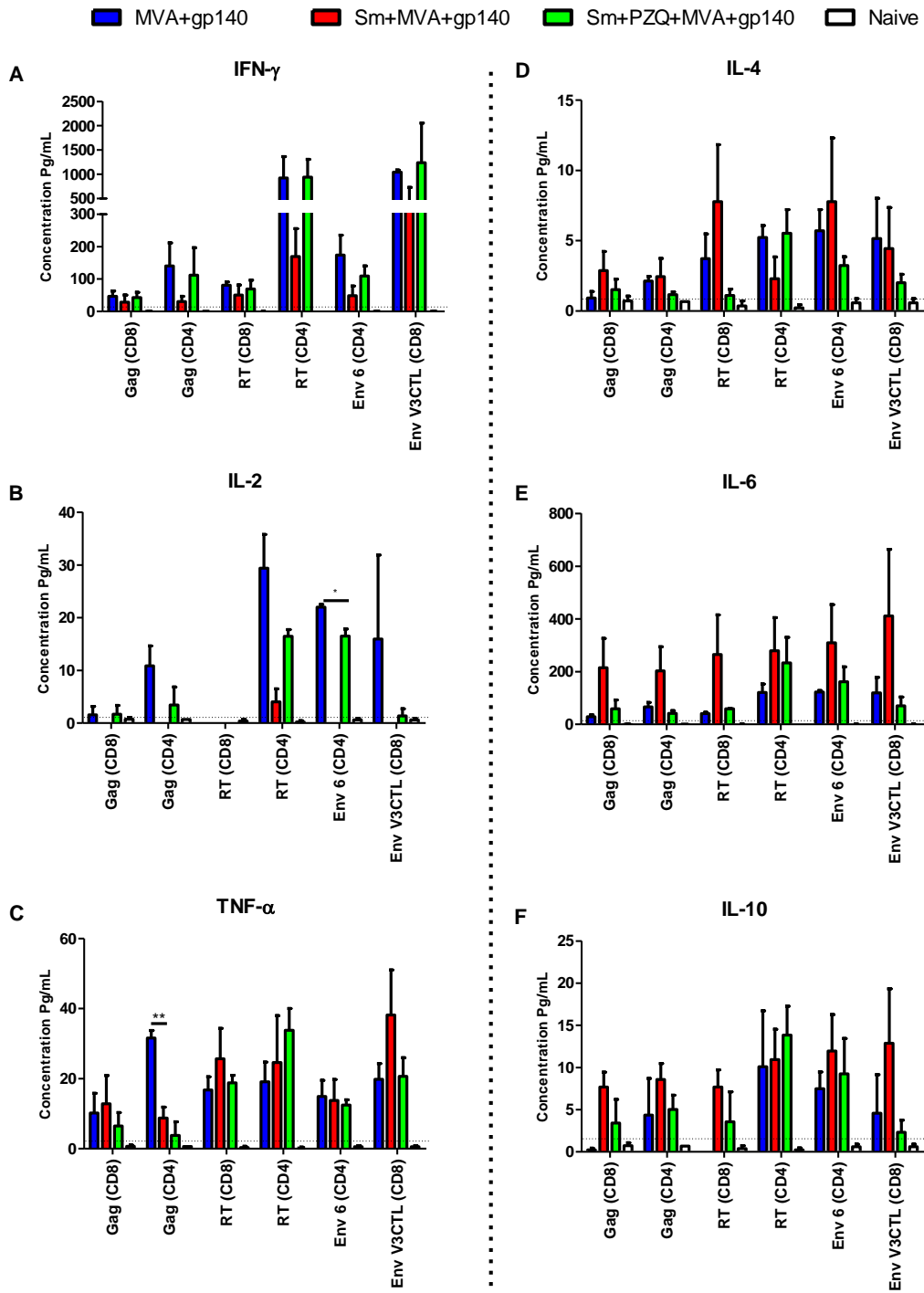
The highest levels of IL-2 were observed in responses to the RT CD4 peptides in Sm-free (29.38 pg/ml) compared to both the Sm-infected (4.0 pg/ml) and Sm-infected-PZQ treated (16.46 pg/ml) vaccinated mice. Of all the HIV peptides, only responses to the RT CD4 were detected in the Sm-infected vaccinee mice while other peptides did not induce a detectable IL-2 cytokine. Interestingly, vaccinated Sm-infected-PZQ treated mice had detectable IL-2 responses to the Gag CD8 (1.6 pg/ml); Gag CD8 (3.4 pg/ml); Env CD4 (16.48) and Env CD8 (1.3 pg/ml), although they were lower than IL-2 responses induced in Sm-free vaccinated mice (Fig 3.7 B).

Levels of TNF- $\alpha$  were significantly lower in Sm-infected ( $8.807 \pm 3.092$  pg/ml) mice in response to Gag CD4 peptides compared to the Sm-free ( $31.60 \pm 2.119$  pg/ml) vaccinated mice (Fig 3.7 C). However, responses to the Gag CD8 (12.8 vs 10.22 pg/ml) RT CD8 (25.64 vs 16.75 pg/ml) RT CD4 (24.61 vs 19.13 pg/ml) and Env CD8 (38.17 vs 19.84 pg/ml) was lower in Sm-free compared to Sm-infected vaccinated mice although not significant. In vaccinated Sm-infected-PZQ treated mice, responses to the RT CD4 had higher TNF- $\alpha$

(33.83 pg/ml) compared to Sm-free (19.14 pg/ml) and Sm-infected (24.61 pg/ml) vaccinated mice. However, for all the peptides, the amount of cytokine produced was similar to that of the Sm-free mice (Fig 3.7 C).

Higher levels of IL-6 cytokines in all cultures of vaccinated Sm-infected cells stimulated with all HIV peptides (Gag CD4 [202.79 vs 66.37 pg/ml]; Gag CD8 [214.84 vs 28.92 pg/ml]; RT CD4 [279.09 vs 120.8 pg/ml]; RT CD8 [264.81 vs 40.54 pg/ml]; Env CD4 [309.31 vs 122.19 pg/ml] and Env CD8 [411.02 vs 119.1 pg/ml]) when compared to Sm-free vaccinated mice was observed. In vaccinated Sm-infected-PZQ treated mice, the levels of IL-6 produced was similar to that of the Sm-free mice except when stimulated with CD4 (233.09 pg/ml) which was quite higher compared to Sm-free mice although lower compared to Sm-infected vaccinated mice. Although cumulative levels of IL-10 in response to HIV peptides were quantified, no levels of IL-10 in response to individual peptides was above the limit of detection, hence considered not positive according to the detection limit as shown in Table 2.3.

## MVA+gp140



**Figure 3.7: Cytokine produced by Gag (CD8 and CD4) RT (CD8 and CD4) and Env (CD 4) specific peptides in Sm-free; Sm-infected and Sm-infected-PZQ treated mice vaccinated with MVA+gp140. Splenocytes were harvested from mice vaccinated with the indicated regimen described in Fig 3.1. They**

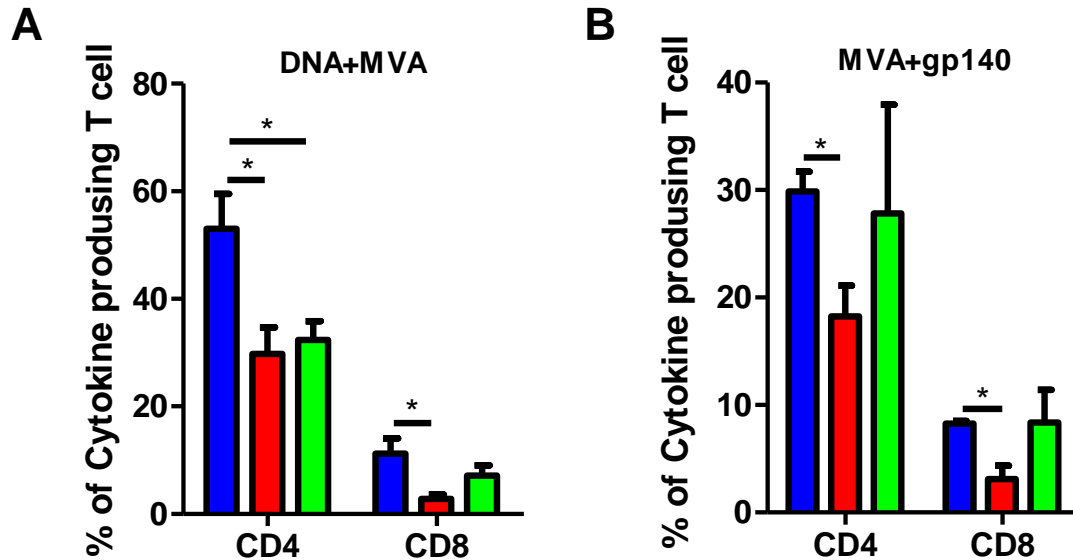
were then stimulated with an irrelevant peptide (negative control) or with the individual HIV specific T cell peptides for 48 h. Culture supernatants were collected and the level of Th1 (A, B and C) and Th2 (D, E and F) cytokines released into the medium was measured using a Cytokine Bead Array assay and flow cytometric analysis. The individual bars represent the magnitude of the net positive cytokine levels in the medium of splenocyte of Sm-free and Sm-infected vaccinated mice, stimulated with individual HIV peptides, after subtracting the background (splenocytes stimulated with irrelevant peptide). The level of cytokines in the culture medium was considered positive when the level is higher than the detection limit of the respective cytokine described in Table 2.3. Results represent 3 independent experiments and plotted as the mean + SEM, and cytokine levels were expressed as pg/ml. Statistical analysis was performed using unpaired, two-tailed t-test analysis, \* $p < 0.05$ , \*\* $p < 0.01$ .

### **3.3.7. Cellular immune responses to PMA in DNA+MVA and MVA+gp140 vaccine regimens in the background of Schistosomiasis measured by ICS and Facs**

To evaluate the Th1/Th2 balance in Sm-infected and Sm-free mice, the frequencies of cytokine (IFN- $\gamma$ , IL-2 and TNF- $\alpha$ ) producing T cells in response to a non-specific stimulant (PMA/Ionomycin) were determined by ICS in the DNA+MVA (Fig 3.8 A) and MVA+gp140 (Fig 3.8 B) vaccination regimen. A significantly higher frequency of cytokine-producing CD4+ ( $53.03 \pm 6.459$  vs  $29.77 \pm 4.924\%$ ) and CD8+ T ( $11.26 \pm 2.823$  vs  $2.82 \pm 0.7903\%$ ) cells were observed in Sm-free mice compared to Sm-infected mice vaccinated with the DNA+MVA vaccine regimen (Fig 3.8 A). In vaccinated Sm-infected-PZQ treated mice, cytokine-producing CD4 cells were similar to that on Sm-infected mice and significantly lower ( $53.03 \pm 6.459$  vs  $32.37 \pm 3.474\%$ ) when compared to that of Sm-free mice. Cytokine-producing CD8 cells in vaccinated Sm-infected-PZQ treated mice were higher (7.19%) than those of Sm-infected (2.82%) mice, however, were lower than that of Sm-free mice (11.26%) (Fig 3.8 A).

In mice vaccinated with the MVA+gp140 vaccine regimen, a significantly higher number of cytokine-producing CD4+ ( $29.90 \pm 1.823$  vs  $18.23 \pm 2.895\%$ ) and CD8+ T ( $8.293 \pm 0.2149$  vs  $3.103 \pm 1.256\%$ ) cells were observed in Sm-free mice compared to Sm-infected mice vaccinated with the MVA+gp140 vaccine regimen (Fig 3.8 B). In vaccinated Sm-infected-PZQ treated mice, cytokine-producing CD4 cells were higher (27.8%) compared to vaccinated Sm-infected mice but not higher than that of vaccinated Sm-free mice. Cytokine-producing CD8 cells in vaccinated Sm-infected-PZQ treated mice were higher (8.39%) than those of Sm-infected (3.1%) mice as well as higher than that of Sm-free mice (8.29%). However, the difference was not significant (Fig 3.8 B).

## Cytokine producing T cells in response to non-specific stimulation PMA/ionomycin



**Figure 3.8: Assessment of cytokine-producing T cells (CD8+ and CD4+) following stimulation with a Leukocyte Activation cocktail (PMA/Ionomycin) in mice vaccinated with DNA+MVA and MVA+gp140 vaccine regimen.** (A) Total frequency of T cells (CD4+ and CD8+) producing cytokines (IFN- $\gamma$ , IL-2, and TNF- $\alpha$ ), in response to PMA/Ionomycin stimulation following vaccination with DNA+MVA regimen. (B) Total frequency of T cells (CD4+ and CD8+) producing cytokines (IFN- $\gamma$ , IL-2, and TNF- $\alpha$ ), in response to PMA/Ionomycin stimulation following vaccination with MVA+gp140 regimen. Bars show positive responses above a cut-off value of 2x background, 50 events or 0.05% cytokine-producing T cells. Results represent 3 independent experiments and plotted as the mean + SEM. Statistical analysis was performed using unpaired followed by FDR for multiple comparisons, two-tailed t-test analysis, \* $p < 0.05$ .

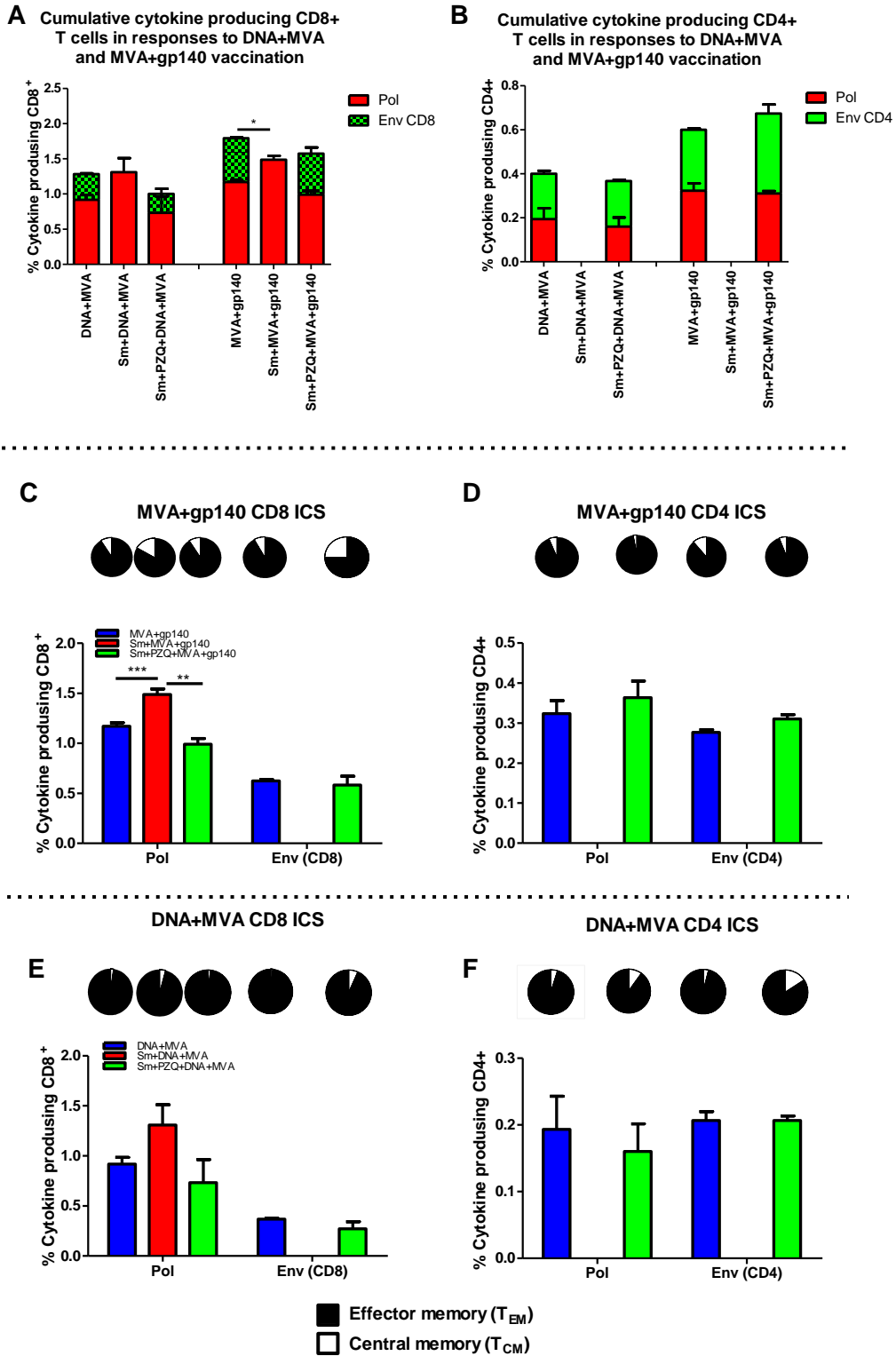
### 3.3.7.1. Vaccine specific-cellular responses to MVA+gp140 measured by ICS

The proportions of cytokine (IFN- $\gamma$ , IL-2 and TNF- $\alpha$ ) producing T cells in response to HIV peptides Pol: (combined RT CD4 and RT CD8 peptides); Env CD4 and Env CD8 were determined by ICS. In the MVA+gp140 vaccination regimen, a significantly higher percentage of cumulative cytokine producing CD8 cells in response to the Pol and Env CD8 peptide stimulation was observed in Sm-free vaccinated ( $1.79 \pm 0.04\%$ ) compared to Sm-infected vaccinated ( $1.49 \pm 0.06\%$ ) mice (Fig 3.9A). Sm-infected-PZQ treated ( $1.57\%$ ) vaccinated mice had similar cumulative cytokine producing CD8 as Sm-infected vaccinated mice but was lower compared to Sm-free vaccinated mice (Fig 3.9 A). However, Sm-infected vaccinated mice

responded only to Pol (RT CD4 + CD8 peptides) in which the frequency was significantly higher than those of Sm-free and Sm-infected-PZQ treated mice (Fig 3.9C) In general, the majority of the CD8+ T cytokine positive cells belonged to the effector memory phenotype (>80%) but the Sm-infected-PZQ treated group had a lower frequency (75%) of effector memory phenotype (Fig 3.9 C: charts). On the other hand, Cytokine producing CD4 cells in response to Pol and Env CD4 peptide was similar in Sm-free vaccinated (0.6%) and vaccinated Sm-infected-PZQ treated (0.67%) mice while not detected in Sm-infected mice vaccinated mice (Fig 3.9 B). In general, CD4+ T cytokine positive cells belonged to the effector memory phenotype (>90%) for both Sm-free and Sm-infected-PZQ-treated mice (Fig 3.9 D charts).

### **3.3.7.2. Vaccine-specific cellular responses to DNA+MVA measured by ICS**

In the DNA+MVA vaccination regimen, In the DNA+MVA vaccination regimen, no significant difference between cumulative cytokine producing CD8 cells in response to the Pol and Env CD8 peptide stimulation was observed in Sm-free vaccinated (1.2%); vaccine Sm-infected-PZQ treated (1.0%) and Sm-infected vaccinated (1.3%) mice. However, Sm-infected vaccinated mice responded only to Pol (RT CD4 + CD8 peptides) in which the frequency was higher than those of Sm-free and Sm-infected-PZQ treated mice albeit insignificant (Fig 3.9 E). The majority of the CD8+ T cytokine positive cells belonged to the effector memory phenotype (>90%) (Fig 3.9 E: charts). On the other hand, Cytokine producing CD4 cells in response to Pol and Env CD4 peptide was similar in Sm-free vaccinated and vaccinated Sm-infected-PZQ treated mice while not detected in Sm-infected mice vaccinated mice (Fig 3.9 B). In general, CD4+ T cytokine positive cells belonged to the effector memory phenotype (>90%) for Sm-free but the Sm-infected-PZQ treated group had a noticeably lower frequency (84%) of effector memory phenotype and Sm-infected-PZQ-treated mice (Fig 3.9 F charts).



**Figure 3.9: Assessment of cytokine-producing T cells (CD8+ and CD4+) following stimulation with HIV peptides (Pol: [RT CD4 + RT CD8]; Env CD4 and Env CD8). Cumulative frequency of CD8+ (A) and**

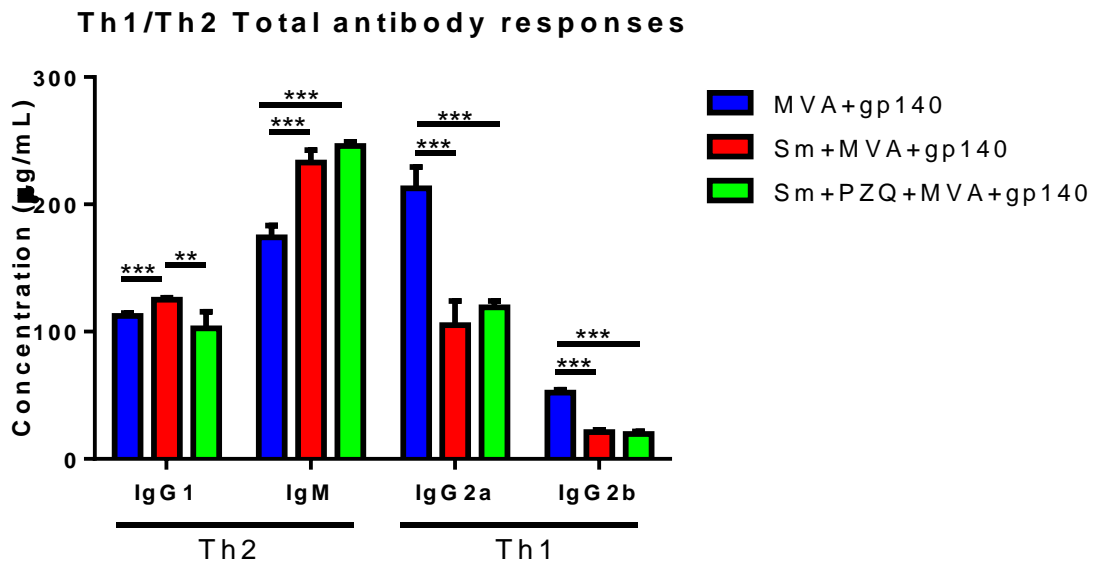
CD4+ (B) producing cytokines (IFN- $\gamma$ , IL-2, and TNF- $\alpha$ ) in response to Pol and Env CD8 stimulation following vaccination with MVA+gp140 and DNA+MVA regimen. (C) Total frequency of T cells (CD8+) producing cytokines (IFN- $\gamma$ , IL-2, and TNF- $\alpha$ ), in response to Pol and Env CD8 stimulation following vaccination with MVA+gp140 regimen. (D) Total frequency of T cells (CD4+) producing cytokines (IFN- $\gamma$ , IL-2, and TNF- $\alpha$ ), in response to Pol and Env CD4 stimulation following vaccination with MVA+gp140 regimen. (E) Total frequency of T cells (CD8+) producing cytokines (IFN- $\gamma$ , IL-2, and TNF- $\alpha$ ), in response to Pol and Env CD8 stimulation following vaccination with DNA+MVA regimen. (F) Total frequency of T cells (CD4+) producing cytokines (IFN- $\gamma$ , IL-2, and TNF- $\alpha$ ), in response to Pol and Env CD4 stimulation following vaccination with DNA+MVA regimen. Bars show positive responses above a cut-off value of 2x background, 50 events or 0.05% cytokine-producing T cells. Pie charts above each corresponding bar per group represent cell phenotype (Central; T<sub>CM</sub> [clear] and effector; T<sub>EM</sub> [solid] memory) of responding cells to HIV peptides. Results represent 3 independent experiments and plotted as the mean + SEM. Statistical analysis was performed using unpaired, two-tailed t-test analysis followed by FDR for multiple comparisons, \*\*p<0.01, \*\*\*p<0.001.

### **3.3.8. Assessing antibody responses in mice vaccinated with MVA+gp140 and with gp140 alone**

#### **3.3.8.1. Total antibody responses**

To assess the antibody Th1/Th2 profile in Sm-infected mice, total IgG (Th1: IgG2a and IgG2b; Th2: IgG1 and IgM) antibody isotypes in serum of Sm-infected; Sm-free and Sm-infected-PZQ treated mice vaccinated with the MVA+gp140 regimen were measured using ELISA.

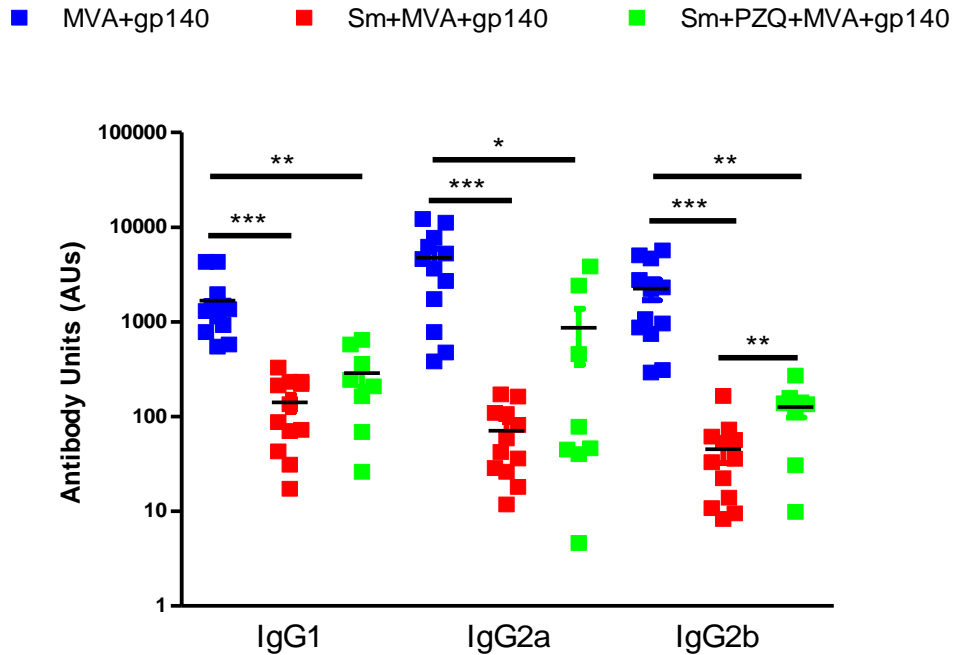
Sm-free mice vaccinated with the MVA+gp140 vaccines had significantly lower total IgG1 ( $11.25 \pm 0.22 \mu\text{g/ml}$ ) antibodies compared to Sm-infected vaccinated mice ( $12.50 \pm 0.16 \mu\text{g/ml}$ ) antibodies. Similarly, vaccinated Sm-infected-PZQ treated mice had significantly lower total IgG1 ( $11.55 \pm 0.19 \mu\text{g/ml}$ ) antibodies compared to Sm-infected vaccinated mice. Also vaccinated Sm-free vaccinated mice had significantly lower ( $17.41 \pm 0.92 \mu\text{g/ml}$ ) total IgM antibodies compared to vaccinated Sm-infected ( $23.30 \pm 0.96 \mu\text{g/ml}$ ) and Sm-infected-PZQ treated ( $24.58 \pm 0.34 \mu\text{g/ml}$ ) vaccinated mice. However, there was no difference between vaccinated Sm-infected and vaccinated Sm-infected-PZQ treated mice. Total IgG2a was significantly lower in both vaccinated Sm-infected ( $11.26 \pm 1.52 \mu\text{g/ml}$ ) and Sm-infected-PZQ treated ( $11.92 \pm 0.4878 \mu\text{g/ml}$ ) compared to Sm-free ( $21.26 \pm 1.67 \mu\text{g/ml}$ ) vaccinated mice. However, there was no difference between vaccinated Sm-infected and vaccinated Sm-infected-PZQ treated mice. Similarly, Total IgG2b was significantly lower in both vaccinated Sm-infected ( $2.11 \pm 0.19 \mu\text{g/ml}$ ) and vaccinated Sm-infected-PZQ treated ( $1.95 \pm 0.21 \mu\text{g/ml}$ ) compared to Sm-free ( $5.20 \pm 0.22 \mu\text{g/ml}$ ) vaccinated mice. However, there was no difference between vaccinated Sm-infected and vaccinated Sm-infected-PZQ treated mice (Fig 3.10).



**Figure 3.10: Antibody responses in Sm-free; Sm-infected and Sm-infected-PZQ treated mice vaccinates with MVA+gp140.** Serum samples were collected two weeks post last vaccination. Total serum IgG isotypes (IgG1, IgM, IgG2a and IgG2b) were analysed by ELISA. Values are expressed as mean  $\pm$  SEM. Statistical differences between the groups were calculated by unpaired t-test (two-tailed) followed by FDR for multiple comparisons, \*\* $P < 0.01$ ; \*\*\* $P < 0.0001$ .

### 3.3.9. Env-gp140 specific antibody responses to MVA+gp140 vaccine regimen

Env-specific antibody immunity in response to immunisation with MVA+gp140 was determined by ELISA. The mean concentration of gp140 Env-specific serum IgG antibody isotypes (IgG1, IgG2a, IgG2b) was determined and compared across the groups (Sm-free vaccinated; Sm-infected vaccinated and Sm-infected-PZQ treated vaccinated). Significantly higher amounts of gp140-specific IgG antibodies were observed in Sm-free vaccinated compared to Sm-infected vaccinated mice across all IgG isotypes (IgG1[1681  $\pm$  373.9 vs 140.8  $\pm$  29.42 AUs]; IgG2a[4746  $\pm$  1154 vs 71.14  $\pm$  15.98 AUs]; IgG2b[2247  $\pm$  553.9 vs 45.23  $\pm$  12.65 AUs]). Also, significantly higher amounts of gp140-specific IgG antibodies were observed in Sm-free vaccinated compared to Sm-infected-PZQ treated vaccinated mice across all IgG isotypes (IgG1[287.0  $\pm$  79.96 AUs], IgG2a[866.1  $\pm$  514.3 AUs], IgG2b[126.3  $\pm$  28.41 AUs]). Furthermore, Sm-infected-PZQ treated vaccinated mice had a higher amount of antibodies compared to Sm-infected vaccinated mice for the IgGb isotype only (Fig 3.11).



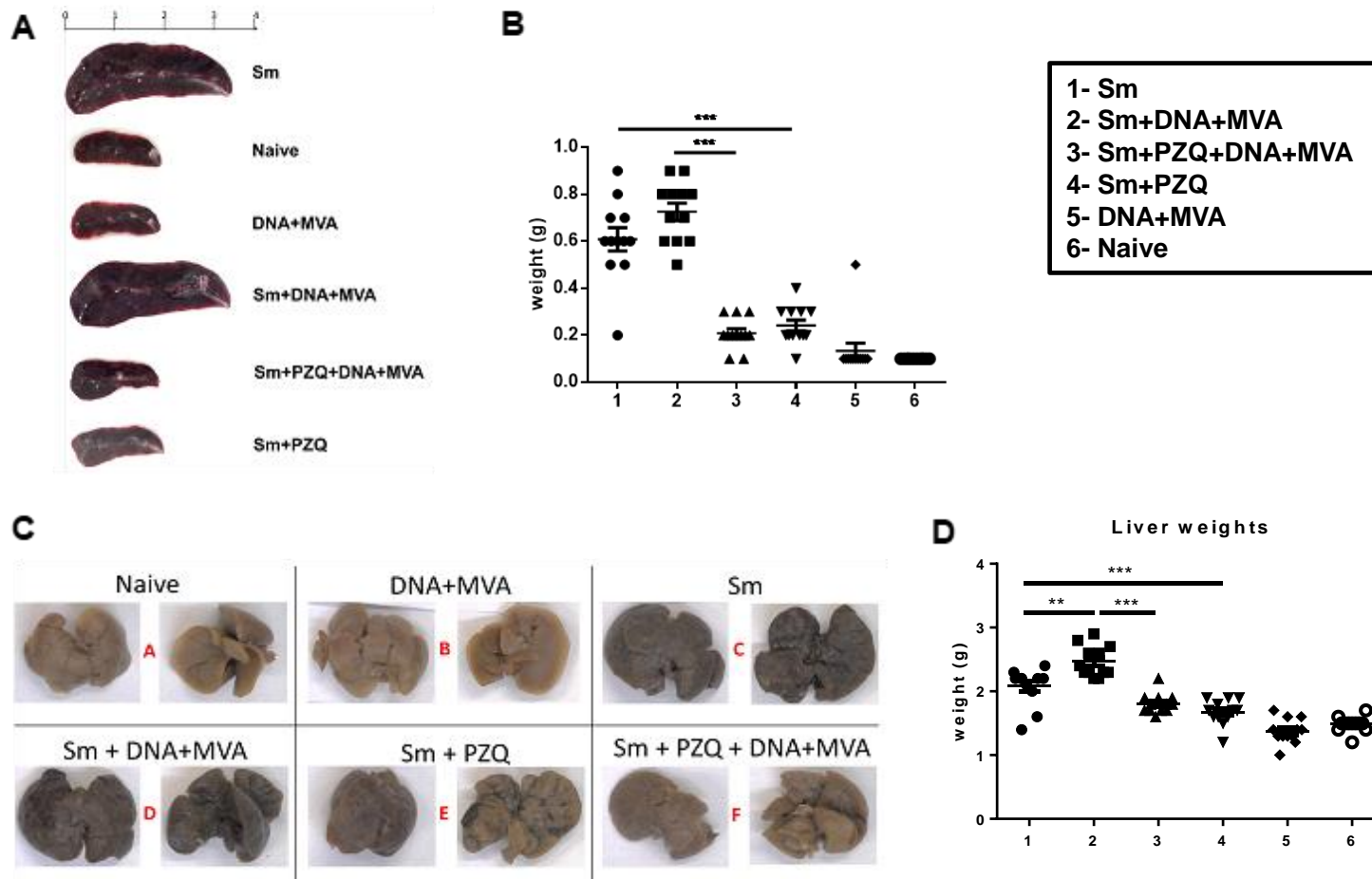
**Figure 3.11: Humoral immune responses in mice immunised with MVA+gp140.** Serum samples were collected two weeks post last vaccination. HIV-1 gp140-specific IgG1, IgG2a and IgG2b serum antibodies were analysed by ELISA. Values are plotted and expressed as mean antibody units (AUs)  $\pm$  SEM for the 8-12 animals in each group. Statistical differences between the groups were calculated by unpaired t-test (two-tailed) followed by FDR for multiple comparisons, \* $P < 0.05$ , \*\* $P < 0.01$ ; \*\*\* $P < 0.0001$ .

### 3.3.10. Liver and spleen weights of Sm-infected mice vaccinated with DNA+MVA

This study sought to investigate if DNA+MVA exacerbate helminth associated pathology in chronically infected individuals. The study also sought to establish if PZQ treatment prior to vaccination can result in less pathology.

Sm-associated pathology was evaluated by measuring the weights and size of the spleen, livers, and granuloma size in unvaccinated Sm-infected; Sm-infected vaccinated, and Sm-infected-PZQ treated vaccinated mice. Naïve and Sm-free vaccinated groups were considered as negative controls as they were uninfected. As shown in Fig 3.12 A, vaccinated Sm-infected and unvaccinated Sm-infected mice had larger spleens compared to vaccinated Sm-free; naïve and vaccinated Sm-infected-PZQ treated mice. The weights of Sm-infected ( $0.6083 \pm 0.04994\text{g}$ ) and Sm-infected vaccinated ( $0.7250 \pm 0.03718\text{g}$ ) mice spleens were significantly higher compared to Sm-free ( $0.2083 \pm 0.01930\text{g}$ ), naïve ( $0.1\text{g}$ ) and Sm-infected-PZQ ( $0.2417 \pm 0.02289\text{g}$ ) treated mice. Spleen weights of Sm-infected vaccinated mice were higher compared to Sm-infected alone ( $0.7250 \pm 0.03718$  vs  $0.6083 \pm 0.04994\text{g}$ ), albeit not significant (Fig 3.12 B).

Similarly, liver sizes and weights in Sm-infected vaccinated and unvaccinated mice were larger compared to Sm-free; naïve and Sm-infected-PZQ treated mice (Fig 3.12 C). The weights of Sm-infected ( $2.083 \pm 0.08424\text{g}$ ) and Sm-infected vaccinated ( $2.467 \pm 0.06999\text{g}$ ) mice livers were significantly higher compared to Sm-free ( $1.375 \pm 0.05522\text{g}$ ), naïve ( $1.483 \pm 0.03445\text{g}$ ) and Sm-infected-PZQ ( $1.808 \pm 0.04516\text{g}$ ) treated mice (Fig 3.12 D). Liver weights of Sm-infected vaccinated mice were significantly higher compared to Sm-infected alone ( $2.467 \pm 0.06999\text{g}$  vs  $2.083 \pm 0.08424\text{g}$ ).

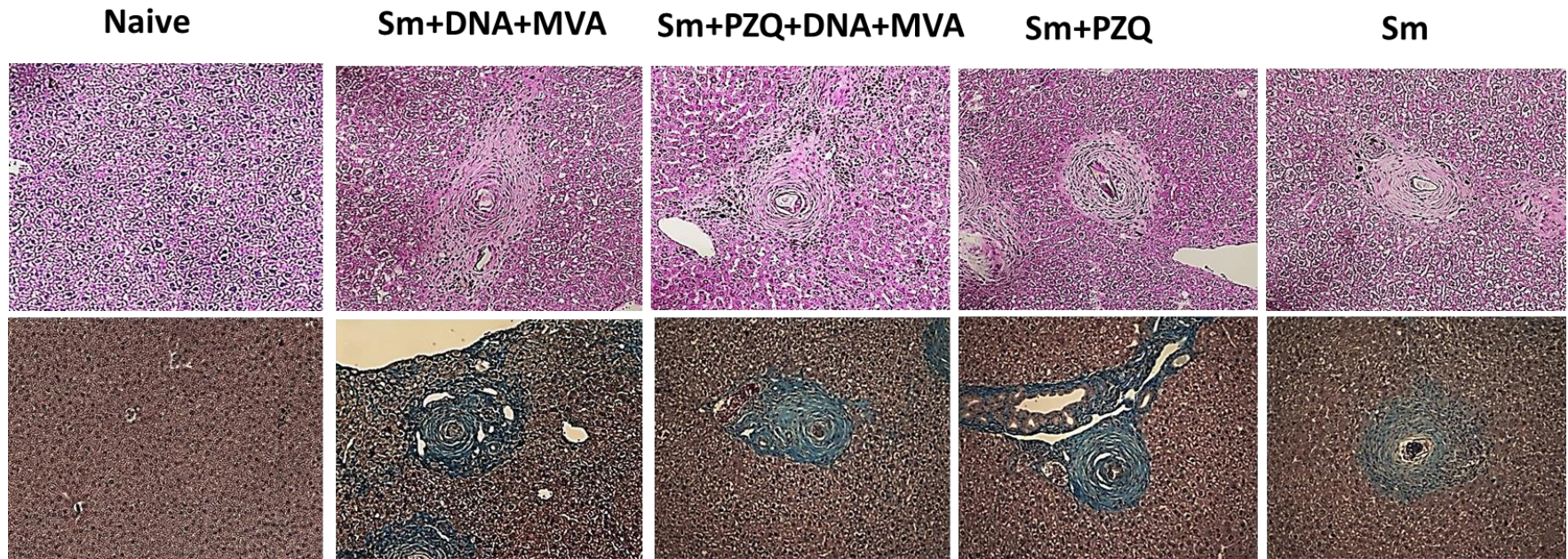


**Figure 3.12: Analysis of liver and spleen weights of Naïve; Sm-infected; Sm-infected-PZQ treated mice vaccinated or unvaccinated with the DNA+MVA vaccine regimen.** Groups of mice were infected with 30-35 Sm cercariae. Nine and a half weeks post infection; half of the infected mice were treated twice with PZQ and the other half remained untreated prior vaccination. Mice were euthanised 12 days after last vaccination and samples (liver and spleen) were collected and weighed for analysis. (A) Representations of freshly harvested spleen. (B) Spleen weights measured during autopsy. (C) Representation of harvested livers of different groups; (D) Liver weights measured during autopsy. Results represent 3

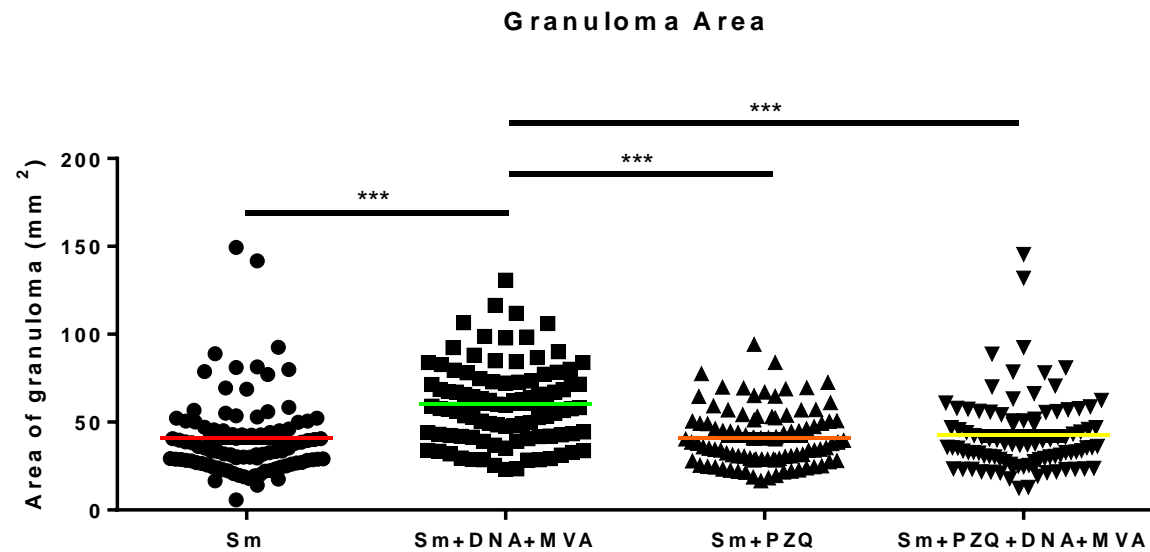
independent experiments as the mean + SEM of weights in grams (g). Statistical analysis was performed using unpaired, two-tailed t-test analysis followed by FDR for multiple comparisons, \*\*p<0.01, \*\*\*p<0.0001.

### **3.3.11. Sm-induced granuloma size in mice vaccinated with DNA+MVA vaccine regimen**

Analysis of the H&E staining revealed that granuloma sizes in Sm-infected vaccinated mice were significantly larger compared to other groups of mice (Fig 3.13 upper panel). Also, Sm-infected vaccinated mice had more collagen staining as indicated by more CAB staining compared to other groups (Fig 3.13 lower panel). Furthermore, Sm-infected vaccinated had significantly larger ( $60.27 \pm 2.368 \text{ mm}^2$ ) granuloma area when compared to vaccinated Sm-infected-PZQ treated ( $41.29 \pm 1.660 \text{ mm}^2$ ); Sm-infected alone ( $40.79 \pm 2.382 \text{ mm}^2$ ) and Sm-infected-PZQ treated vaccinated ( $42.36 \pm 2.259 \text{ mm}^2$ ) mice (Fig 3.14).



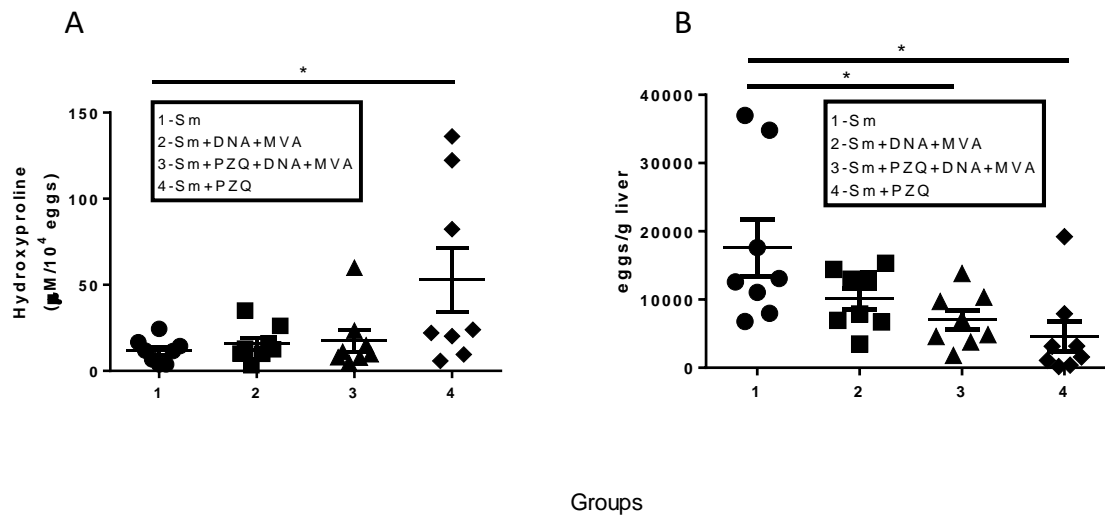
**Figure 3.13: Representative micrographs showing of liver granulomas viewed under the microscope.** Groups of mice were infected with 30 Sm for 10 weeks and a half were treated with PZQ 3 days prior vaccinating with DNA+MVA or left unvaccinated. Mice were euthanised 12 days after last vaccination and samples (Liver) were collected for histology. Upper panel: H&E stained, magnification: X100; Lower panel: CAB stained, magnification: X100. Micrographs of liver granuloma were captured using a Nikon 5.0 megapixel colour digital camera (DCT DS-SMc).



**Figure 3.14: DNA vaccine in Sm-infected groups result in large granuloma sizes:** Groups of mice were infected with 30 Sm for 10 weeks and a half were treated with PZQ 3 days prior vaccinating with DNA+MVA or left unvaccinated. Mice were euthanised 12 days after last vaccination and samples (Liver) were collected for histology. Granuloma area surrounding eggs was quantified by microscopic analysis on H&E stained sections. The area of each granuloma containing a single egg was measured with the ImageJ 1.34 software. An average of 20 granulomas per mouse was included in the analyses. Data is representative of 3 independent experiments, student t-test followed by FDR for multiple comparisons. \*P<0.05; \*\*P<0.01; \*\*\*p< 0.0001.

### 3.3.12. Analysis of hydroxyproline content and egg burden in the liver of mice vaccinated with DNA+MVA

Hepatic fibrosis is a key pathological change of schistosomiasis, and the elevated hydroxyproline content in the liver is an informative biomarker for hepatic fibrosis. Therefore, we assessed hepatic hydroxyproline content to examine liver pathology. We further evaluated the levels of collagen using hydroxyproline assay between groups: Sm-infected vaccinated; Sm-infected-PZQ treated vaccinated; Sm-infected-PZQ treated and Sm-infected mice. Surprisingly, high levels of hydroxyproline content were observed in unvaccinated Sm-infected-PZQ treated mice ( $52.79 \pm 18.70$  hydroxyproline  $\mu\text{M}/10^4$  eggs) groups compared to Sm-infected ( $11.64 \pm 2.485$  hydroxyproline  $\mu\text{M}/10^4$  eggs); Sm-infected vaccinated ( $15.76 \pm 3.581$  hydroxyproline  $\mu\text{M}/10^4$  eggs) and Sm-infected-PZQ treated vaccinated ( $17.42 \pm 6.405$  hydroxyproline  $\mu\text{M}/10^4$  eggs) (Fig 3.15 A). The number of eggs per gram of liver in Sm-infected mice ( $17610 \pm 4161$  eggs/g liver) was significantly higher compared to unvaccinated Sm-infected-PZQ treated ( $4585 \pm 2265$  eggs/g liver) and vaccinated Sm-infected-PZQ treated ( $7003 \pm 1420$  eggs/g liver) mice. Sm-infected vaccinated mice had a higher number of eggs ( $10100 \pm 1540$  eggs/g liver) which was not a significantly higher than the number in Sm-infected-PZQ treated and Sm-infected-PZQ treated vaccinated.



**Figure 3.15: Liver fibrosis measured by hydroxyproline assay.** BALB/c mice were chronically infected with *Schistosoma mansoni*. At 9.5 weeks post-infection, some groups were treated twice with PZQ. At 10 weeks post-infection, groups were primed and boosted with DNA and MVA vaccines 4 weeks apart. Mice were euthanised 12 days after last vaccination and livers were collected for hydroxyproline analysis. Hydroxyproline content (A) and a number of eggs per gram of liver (B) were analysed in 4 mice per group.

Data is representative of 2 independent experiments, student t-test followed by FDR for multiple comparisons. \*P<0.05.

### 3.4. Discussion

#### 3.4.1. Introduction

Helminths are potent modulators of host immune responses that drive Th2-dependent pathways to mediate their protection from the host (Borkow and Bentwich, 2004, Anthony et al., 2007, Allen and Maizels, 2011). In chronically infected individuals, the Th2 biased profile is accompanied by an increase in immunoregulatory responses which have been associated with suppression of non-parasite antigens (Kullberg et al., 1992). Therefore, it is likely that helminth-infected vaccine recipients will have a problem in mounting optimal vaccine-specific immune responses (Finkelman and Urban, 2001, Fincham et al., 2003, Thomas and Harn, 2004). So far, several studies have reported that helminth infections impair the outcome of a variety of vaccines, including BCG (Buck et al., 1970, Kilian and Nielsen, 1989, Stewart et al., 1999, Elias et al., 2008), yellow fever (Buck et al., 1970), tetanus (Prost et al., 1983, Kilian and Nielsen, 1989, Sabin et al., 1996, Cooper et al., 1998), diphtheria toxoid (Haseeb and Craig, 1997), live attenuated oral cholera (Cooper et al., 2000a), and HIV-1 vaccine candidates (Da'Dara et al., 2006). On the other hand, another study showed that HIV-specific responses generated in mice by an attenuated live *L. monocytogenes* expressing HIV-1 Gag were not statistically different between schistosome-free and chronically infected animals (Shollenberger et al., 2013b, Shollenberger et al., 2013a). Albeit each of the studies has empirical support and opposition, it remains unclear whether or not helminth infection will confound the immunogenicity and efficacy of future HIV vaccination. Moreover, the HIV vaccines tested in the above studies expressed only the HIV-1 *gag* gene whilst future successful HIV vaccines are most likely to comprise of multiple HIV-1 genes (de Souza Apostólico et al., 2017).

Granting that there is no vaccine for helminth infections, chemotherapeutic drugs such as praziquantel (PZQ) for schistosomiasis (Shaheen et al., 1989, Utzinger and Keiser, 2004, Tallima and El Ridi, 2007) and ivermectin or mebendazole for geohelminths (Cañete et al., 2009, Churcher et al., 2009, Geary, 2012) are available alternatives that have been associated with recovery of immune responses to unrelated antigens (Piessens et al., 1981, Colley et al., 1986, Grogan et al., 1996, Elias et al., 2001, Abaitua et al., 2006, Ghosh et al., 2006, Da'dara and Harn, 2010, Chen et al., 2012).

The findings from the preliminary study (Chapter 2) demonstrated the capacity to reproduce the BALB/c model of chronic schistosomiasis by using Sm infection to induce a dominant Th2 immune profile. The study also generated preliminary data that strongly suggested that the immune skewing towards Th2 was associated with HIV vaccine-specific Th1 cellular and antibody responses. However, no firm conclusions could be made as the experiments were repeated only once. Furthermore, it was found reasonable to further refine the experiments by introducing a treatment arm to investigate the effect of eliminating the parasite on the immunogenicity of the vaccines and vaccine safety. Therefore, the present study aimed to investigate the impact of chronic helminthic infections on the immunogenicity of HIV vaccines regimens that elicit predominantly cellular (DNA+MVA) and mixed cellular and antibody responses (MVA+gp140) using *S. mansoni* as a prototype helminth.

### 3.4.2. Skewing of Th1/Th2 profile

To confirm that the vaccinated mice with chronic helminth infection also exhibited Th2 biased profile, the skewing of the host immune responses towards a Th2 (an archetypical feature of helminth infection) was determined using cytometric bead array. As seen in the preliminary study (Chapter, Section 2.4.1), vaccinated mice infected with Sm induced high levels of Th2 cytokines compared to Sm-free mice vaccinated with DNA+MVA or MVA+gp140 (Fig 3.4). Conversely, Sm-infected vaccinated mice had lower Th1 cytokine levels compared to Sm-free vaccinated mice. In addition, the reduction of the IFN- $\gamma$ :IL-4 ratio accompanied with increase IL-10 levels in Sm-infected vaccinated mice further indicated that immune profile of Sm-infected vaccinated mice was skewed towards Th2 and most likely immune suppressed (Shollenberger et al., 2013a). These findings are in line with previous studies conducted on the immune system of humans, as well as animals (Nutman et al., 1987, Grzych et al., 1991, Araujo et al., 1994, Sartono et al., 1995, McKee and Pearce, 2004, Yin et al., 2012). However, compared to the preliminary study, the IFN- $\gamma$ :IL-4 ratio of Sm-free vaccinated groups in this study was generally higher for both vaccination regimens (Fig 2.5 vs Fig 3.4).

Furthermore, total immunoglobulin levels (non-specific antibody immune responses) in the mouse sera were evaluated in order to elucidate the Th1/Th2 bias of the different mouse groups. Antibody isotype of serum is useful in determining T-helper lymphocyte bias by virtue of the effects that certain cytokines have on immunoglobulin isotype selection in B cells (McSorley and Maizels, 2012). For example, a Th2 cytokine such as IL-4 is capable of inducing B cells to secrete IgG1, at the same time inhibiting IgG2a production (Deenick et al., 2005). On the other hand, a Th1 cytokine, IFN- $\gamma$  can stimulate B cells into secreting IgG2a while inhibiting IgG1 (Deenick et al., 2005). Thus, the type of cytokine being produced can be extrapolated from the IgG isotype secreted. Therefore, total immunoglobulin isotypes were evaluated between Sm-infected vaccinated and Sm-free vaccinated mice. It was found that IgG2a and IgG2b (Th1 antibodies markers) were significantly decreased in Sm-infected vaccinated groups compared to Sm-free vaccinated groups. On the contrary, IgG1 and IgM (Th2 antibodies markers) were significantly elevated in vaccinated Sm-infected compared to Sm-free vaccinated groups (Fig 3.10), indicative of a very strong chronic helminth-induced Th2 immunity (Deenick et al., 2005, McSorley and Maizels, 2012). These findings confirm the results of the preliminary study and further demonstrated that Sm infected mice had immune responses skewed towards predominantly Th2, which can be attributed to chronic infection (Maizels and McSorley, 2016). However, when compared to the preliminary study, the overall total antibody was lower (~3 fold) in study, particularly IgG1 and IgM (Fig 2.12 vs 3.10).

To evaluate if whether PZQ treatment reduced the Th2 skewing or not, the quantity of Th1 cytokines (IFN- $\gamma$ ; IL-2 and TNF- $\alpha$ ) upon Con A stimulation were evaluated and found to be partially restored when mice were treated with PZQ compared to Sm-infected untreated vaccinated mice (Fig 3.4 D-F, and K-M). However, the Th2 (IL-4; IL-6 and IL-10) particularly parasite-specific IL-4 and IL-10 remain considerably elevated (Fig 3.2 A), thus the IFN- $\gamma$ :IL-4 ratio remained lower even in PZQ treated group vaccinated with

MVA+gp140, albeit partially elevated in DNA+MVA groups (Fig 3.2 C). Because chemotherapy with PZQ only has effects on adult worm, the reduction of the adult worm will reduce the egg burden hence lowering the TH2 responses but only restoring TH1 responses partially since it is not a guaranteed that all adult worms were eliminated. This implies that even after treatment with PZQ, the Th2-biased profile may remain increased. The increase of IL-10 (immune suppressing cytokine) and IL-4 have been reported after treatment with PZQ in mice (Da'dara and Harn, 2010). These findings may further support and explain why IL-10 messenger RNA (mRNA) expression in a study by Helmy et al., was significantly increased in PZQ-treated mice compared to infected mice (Helmy et al., 2009).

### **3.4.3. Vaccine-induced Cellular responses in Sm-infected mice**

Because helminth infections are associated with immunological features that have the potential to impair non-parasite antigens, this raised a question of whether Sm infection could suppress cellular immune responses to the SAAVI candidate HIV vaccine regimens as demonstrated in other previous vaccine studies (Buck et al., 1970, Kilian and Nielsen, 1989, Actor et al., 1993, Sabin et al., 1996, Muniz-Junqueira et al., 1996, Stewart et al., 1999, Cooper et al., 2000a, Da'Dara et al., 2006, Elias et al., 2008). Normally, vaccination with DNA+MVA or MVA+gp140 induces high magnitude Th1 cellular responses. These responses are associated with high cumulative Th1 cytokine production (Fig 3.5 A-C), a high number of responding T cells (Fig 3.3 A and D), and a high number of cytokine-producing CD8+ and CD4+ in response vaccine peptides (Fig 3.9) (Burgers et al., 2006, Burgers et al., 2008, Shephard et al., 2008, Burgers et al., 2009). Cumulative ELISpot, cytokine and cytokine-producing T cell responses to HIV peptides were shown to be impaired in Sm infected mice.

#### **Vaccine-specific Th1 cytokines in Sm-infected mice**

As shown in Fig 3.5 A-C, vaccine-specific Th1 cumulative cytokines levels (IFN- $\gamma$ , IL-2 and TNF- $\alpha$ ) in response to HIV peptides was significantly reduced in vaccinated Sm-infected compared to Sm-free mice vaccinated with DNA+MVA, suggesting a suppression. Similarly, mice vaccinated with the MVA+gp140 induced significantly lower cumulative IFN- $\gamma$  and IL-2 cytokine responses in Sm-infected groups, with exception to TNF- $\alpha$  which was similar in both vaccinated Sm-infected and Sm-free, vaccinated mice (Fig 3.5 A-C). These findings reflect observations in the preliminary study, except for IFN- $\gamma$  which was much higher in this study compared to the preliminary study in DNA+MVA vaccinated groups. However, the vaccine-induced IL-2 and TNF- $\alpha$  in this study were slightly lower than in the preliminary study (Fig 2.6 vs Fig 3.5).

Classical cytokines such as IFN- $\gamma$ , IL-2 and TNF- $\alpha$  are important immune correlates of protection in HIV immunity (Tomaras and Plotkin, 2017) and good indications of polyfunctional CD4 T-cell responses (Lin et al., 2015). Therefore, findings of this study suggest that Sm infection is capable of negatively affecting

protective HIV-1 immunity. This is often attributed to its characteristics of inducing downmodulation of inflammatory responses (Pearce and MacDonald, 2002). As such Th1 cytokines such as IFN- $\gamma$ , IL-2 and TNF- $\alpha$  can be inhibited in Sm-infected vaccine recipients whilst an increase in the levels of IL-4, IL-6 and IL-13 are observed (Yin et al., 2012).

#### **3.4.3.1. Vaccines-specific ELISpot responses in Sm infected mice**

Also, vaccine-specific cumulative IFN- $\gamma$  and IL-2-producing T cell responses measured by ELISpot were significantly lower in Sm-infected vaccinated mice compared to Sm-free mice vaccinated with DNA+MVA and/or MVA+gp140 (Fig 3.3 A and B). These observations agree with a previous study by Actor et al., which suggested that the immune inability to mount specific responses in helminth-infected mice might prevent the mice from clearing viral infections (Actor et al., 1993). Another Sm-infection mouse study by Da'Dara et al., 2006, agreed with these findings reporting a significant decline in the number of IFN- $\gamma$  secreting CD8+ T cells in Sm-infected mice compared to uninfected controls following immunization with DNA vaccine (Da'Dara et al., 2006). Apiwattanakul et al., also reported that the effectiveness and immunogenicity of their pneumococcal vaccine were compromised in helminth-infected mice (Apiwattanakul et al., 2014). Recently, a human study conducted by Riner et al., also agree with the pattern of these findings and showed that *Schistosoma mansoni* infection can jeopardize the duration of protective levels of antibody responses to immunizations against Hepatitis B and Tetanus Toxoid (Riner et al., 2016). These studies alongside with others, conclude that the attenuation of Th1 responses, such as IFN- $\gamma$  production, together with increased production of anti-inflammatory and Th2 cytokines in helminth-infected vaccine recipients correlate with impaired vaccine efficiency (Elias et al., 2001, Elias et al., 2005b, Elias et al., 2008, Da'dara and Harn, 2010, Apiwattanakul et al., 2014). Interestingly, the DNA+MVA vaccination in the preliminary study induced double the ELISpot responses in Sm-free vaccinated groups compared to this study (Fig 2.3 vs Fig 3.3), while MVA+gp140 vaccination was consistent in both studies (Fig 2.4 vs Fig 3.3).

#### **3.4.3.2. Cytokine-producing CD4+/CD8+ T cells in Sm-infected mice**

Furthermore, the ability of the SAAVI HIV vaccines to induce cytokine-producing T cells as well as memory phenotype was determined by flow cytometry in Sm-infected vaccinated mice. As shown in Fig 3.9 A, a significantly higher cumulative cytokine-producing CD8+ cells in response was observed in Sm-free vaccinated compared to Sm-infected mice vaccinated with MVA+gp140 mice. However, no statistical difference between the percentages of cumulative cytokine-producing CD8+ cells was observed between Sm-free vaccinated and Sm-infected mice vaccinated with DNA+MVA. These findings were consistent with the finding in the preliminary study, except that in a preliminary study, no statistical difference could be measured between groups. Polyfunctional CD8+ T cell responses significantly correlate with decreased

HIV-1 risk and control of viral replication as observed in HIV-1 controllers (Appay et al., 2000, Hersperger et al., 2010, Walker and McMichael, 2012, Sáez-Cirión and Pancino, 2013, Tomaras and Plotkin, 2017, Arcia et al., 2017). As such, future preventative HIV vaccines will aim to induce these immune responses (Walker and McMichael, 2012, Hanke, 2014). Therefore, our data alongside with others report the potential drawback helminth infection could cause (Actor et al., 1993, Elias et al., 2007, Elias et al., 2008, Da'dara and Harn, 2010, Chen et al., 2012). A study by Stelekati et al., demonstrated that bystander chronic infection negatively impacted the development of CD8+ T cells memory (Stelekati et al., 2014). Cumulative cytokine-producing CD4+ cells were completely attenuated by Sm-infection in DNA+MVA and MVA+gp140 vaccinated groups (Fig 3.9 B). Polyfunctional CD4+ T cells have been correlated with decreased HIV transmission (Lin et al., 2015, Tomaras and Plotkin, 2017). Furthermore, CD4+ T cells play a major role during HIV infection in that they stimulate the function of CD8 T cells and B cells (NIAID, 2013). Inhibition functionality of CD4+ T cells, translates to failure in B cell activation, thus, failure in antibody production may be expected (Swain et al., 2012). Taking into account the findings of this study, Sm infection may not only affect cellular responses but antibody responses to unrelated antigens.

Furthermore, cytokine-producing T cells were further characterized into effector or central memory T cell populations. The two are similar in expression of many costimulatory molecules with differences mostly noted in their adhesion, and chemokine receptors function. These populations have different functions (Schenkel and Masopust, 2014). Effector memory cells act as the first line of defence at the sites of HIV-1 infection. When effector memory T cells experience viral activation, they mature rapidly into effector cells that secrete large amounts of effector cytokines such as IFN- $\gamma$  (Lefrançois, 2002, Murphy, 2011). Central memory T cells, on the other hand, take longer to differentiate into effector T cells and secrete lower amounts of cytokines compared to effector memory T cells upon viral activation; however, they serve as a backup for effector memory T cells replenishment (Lanzavecchia and Sallusto, 2005, McKinstry et al., 2010, Murphy, 2011). T cell memory phenotype of activated CD4+ T cells was not detected in Sm-infected vaccinated mice. Nevertheless, Sm-infected vaccinated mice had a slight but insignificant increase in central memory CD8+ T cells compared to Sm-free groups vaccinated with the MVA+gp140 regimen (Fig 3.9 A charts). Mice vaccinated with the DNA+MVA exhibited comparable memory profiles of activated CD8+ T cells, which were similarly observed in a study by Shollenberger et al., showing that there were no difference between Sm-infected and Sm-free vaccinated with a *Listeria* vaccine (Shollenberger et al., 2013b). It is not clear whether central memory would be favoured in Sm-infected vaccinated groups, however, further investigation in future may be necessary.

#### **3.4.4. Antibody responses**

The impairment of vaccine-specific cellular immune responses during a chronic helminth infection has been previously reported, however, few studies to date have evaluated how antibody responses to vaccination

perform in the presence of helminth infections (Chen et al., 2012), thus, poorly understood. To our knowledge, no study has evaluated the impact of helminth infections on the antibody responses to HIV. Although the induction of cellular responses is central in vaccine design, correlation with protection for licensed vaccines include binding antibody responses, functional antibody responses and cellular immunity (Plotkin, 2010, Tomaras and Plotkin, 2017). In addition, the RV144 study demonstrated the importance of vaccine-generated Env antibodies in vaccine protection (Haynes et al., 2012). In this study, antibody responses to the Env gp140 protein in Sm-infected vaccinated and Sm-free vaccinated were evaluated using ELISA. The findings revealed that helminth infection significantly downregulated the production of Env gp140 specific binding antibodies in sera. The mean concentration of anti-gp140 antibodies in Sm-infected mice vaccinated with MVA+gp140 was significantly lower than that in Sm-free vaccinated control for all IgG isotypes (IgG1; IgG2 and IgG2b) (Fig 3.11). An HBV vaccines study reported a reduction of anti-HBV antibodies in mice infected with *S. japonicum* and vaccinated with an HBV vaccine (Chen et al., 2012). Another related study showed that helminth-infected cholera patients display decreased mucosal humoral immune responses to *V. cholera* protein antigen (Harris et al., 2009). Recently, other studies have associated duo parasite infections with an impaired ability to develop IgG antibody responses to key protective antigens of Hib and diphtheria in infants of mothers infected with malaria and/or helminths during pregnancy (Malhotra et al., 2015). Antibody responses to specific HIV antigens have been proposed to correlate with protection (Haynes et al., 2012, Tomaras and Plotkin, 2017). In this study, *S. mansoni* infection in mice was associated with decreased antibody immune responses to Env gp140, therefore, demonstrating a potential negative impact of helminthiasis on future antibody-based HIV vaccines. Unlike in the preliminary study which evaluated responses to gp140 protein, only MVA+gp140 vaccine was used evaluated in this study. However, the trend in the preliminary study reflected the observations made in this study.

#### **3.4.5. PZQ treatment and cellular responses**

Elimination of helminth parasites prior to immunization appears to restore normal vaccine T cell responsiveness (Elias et al., 2001, Abaitua et al., 2006, Ghosh et al., 2006, Da'dara and Harn, 2010). Thus, this study also aimed to investigate if the immunogenicity of these candidate vaccines (DNA+MVA and MVA+gp140) can be restored by the chemotherapeutic elimination of worms. For this, two experiments comprising of 6 groups of mice were used. Three groups of mice were chronically infected with Sm, of which two of these groups were further treated twice with PZQ nine and a half weeks post infection. One of the treated groups and the untreated group were subsequently vaccinated twice, 4 weeks apart with appropriate vaccination regimen. The control groups were those with either Sm-free vaccinated; unvaccinated Sm-infected; unvaccinated Sm-infected-PZQ treated or naïve.

This study hypothesised that treatment with PZQ would result in a restoration of cellular immune responses to normal as previously shown in other studies (Da'dara and Harn, 2010, Shollenberger et al., 2013b) by reducing predominating Th2 cytokines (IL-4, IL-6, and IL-10) while enhancing Th1 cytokines (IFN- $\gamma$ ; TNF- $\alpha$  and IL-2). Surprisingly, vaccine-specific cumulative IFN- $\gamma$  ELISpot responses in DNA+MVA vaccinated groups infected with PZQ were not improved by PZQ treatment as anticipated, nevertheless, cumulative IFN- $\gamma$  ELISpot responses in MVA+gp140 Sm-infected vaccinated groups were partially restored after PZQ treatment (Fig 3.3 A). Furthermore, a significant improvement in cumulative IL-2 ELISpot responses induced by DNA+MVA and MVA+gp140 vaccine regimen was observed in vaccinated Sm-infected-PZQ treated mice compared to Sm-infected vaccinated mice (Fig 3.3 D). These results agree with a study by Da'dara and Harn, which showed restored cellular immune responses to the HIV-1C T cell-based DNA vaccine in Sm-infected-PZQ treated vaccinated mice (Da'dara and Harn, 2010).

A significant restoration in cumulative IFN- $\gamma$  and IL-2 cytokines in response to HIV peptides was observed in PZQ treated groups vaccinated with DNA+MVA (Fig 3.5 A and B). However, cumulative TNF- $\alpha$  response to vaccine peptides in PZQ treated group was not different from the Sm-infected group (Fig 3.5 C). Similarly, in MVA+gp140 a vaccinated group, a significantly high cumulative IFN- $\gamma$  and IL-2 cytokines in response to HIV peptides was observed after treatment with PZQ (Fig 3.5 A-B). This was with exception to cumulative TNF- $\alpha$  levels which were not different from the Sm-infected group (Fig 3.5 C). Cumulative vaccine specific Th2 cytokines (IL-4 IL-6 and IL-10) were also seen to decrease after treatment with PZQ in DNA+MVA and MVA+gp140 vaccinated group (Fig 3.5 D-F). However, these are not considered desirable for an effective HIV vaccine.

The effect of treatment on the frequency of cytokine-producing CD4 and CD8 T cells was also evaluated using ICS. As shown in Fig 3.9 B, the cumulative cytokine-producing CD4+ T cells were completely restored to normal after PZQ treatment in MVA+gp140 and DNA+MVA vaccinated groups. On the other hand, there was no difference in cumulative cytokine-producing CD8+ T cells of Sm-infected compared to Sm-infected-PZQ treated mice vaccinated with DNA+MVA or MVA+gp140. However, PZQ treated vaccinated groups had responses to all peptides compared to Sm-infected untreated vaccinated mice which did not have responses to Env CD8 peptide (Fig 3.9 A). Cytotoxic T lymphocytes (CD8+ T cells) play a vital role in clearing HIV infection while CD4+ cells serve as helpers (Sant and McMichael, 2012, Arcia et al., 2017, Tomaras and Plotkin, 2017). Thus, the inability to restore this form of vaccine responses raises potential challenges to future HIV vaccines. This suggests that vaccine responses to different peptides may not be equally restored by treatment. Nevertheless, this could be due to the length of time needed to clear schistosome antigens, and/or for immune cells to cease producing Th2-type cytokines (Da'dara and Harn, 2010).

Although no major difference in memory phenotype was observed between PZQ treated vaccinated mice and untreated Sm-infected vaccinated mice, the treatment seemed to result in increased central memory

CD8+ cells in MVA+gp140 vaccine regimen and central memory CD4+ in DNA+MVA vaccine regimen. The implications of these findings are not yet clear and justify a further future investigation.

The potential of deworming to enhancing immune responses to vaccination has been demonstrated (Bentwich et al., 1999, Cooper et al., 2000a, Borkow and Bentwich, 2006). In this study, our results show that restoration of cellular immune responses is partial, rather than complete as observed in other studies (Da'dara and Harn, 2010). Results from this study are in line with those of Cooper et al., which demonstrated that *Ascaris lumbricoides* infected patients had diminished-Th1 response to cholera vaccine and only partial recovery following albendazole treatment was observed (Cooper et al., 2001). Taken together these findings, it can be concluded that treatment with PZQ may not be a guarantee of a complete restoration of vaccine-specific immune responses unless a longer recovery period after treatment is allowed.

#### **3.4.6. PZQ treatment and antibody responses**

The current study further demonstrated that in chronically Sm-infected mice vaccinated with MVA+gp140 vaccine, production of serum anti-gp140 antibodies levels of subclass IgG1; IgG2a and IgG2b were significantly decrease compared to Sm-free vaccinated mice (Fig 3.11). To our knowledge, no study has evaluated the ability of anthelmintic treatment in the restoration of antibody responses to HIV. A mouse study by Chen and colleagues reported recovery of vaccine HBV- specific antibody responses in mice infected with *S. japonicum* following treatment with PZQ (Chen et al., 2012). Contrary to the proposition that treatment with anthelmintic chemotherapy is expected to restore host immune responses, treatment with PZQ did not recover levels of anti-gp140 antibodies in mice that were Sm-infected. The quantity of serum anti-gp140 antibodies levels of the subclasses IgG1 and IgG2a and IgG2b were significantly suppressed even after treatment with PZQ compared to uninfected vaccinated controls (Fig 3.11).

Responses to vaccination following treatment of helminth infection have been shown to depend on the time given for clearance of helminth-induced responses in this mouse model. A study by Chen et al., showed a recovery of immune balance 16 weeks post-treatment (Chen et al., 2012). Also, findings from the studies conducted by Da'dara's group and Shollenberger's groups, demonstrated that normal immune responses can be achieved 2-10 weeks post-treatment (Da'dara and Harn, 2010, Shollenberger et al., 2013b). However, in this study, normal immune responses were not achieved even after four weeks post-treatment with PZQ. Nevertheless, the phenotype of the mice was restored, just as observed in previous mouse helminth studies (Da'dara and Harn, 2010, Shollenberger et al., 2013b). This study suggests that prior PZQ treatment may restore the phenotype but may not restore immune balance to normal, as such, not confer restoration to HIV vaccination responses. Perhaps, multiple doses and different treatment time points may be explored in future studies. Thus, the findings of this study validate the need to conduct more detailed investigations on whether or not the recovery of immune responses after PZQ treatment is time or even dose-dependent.

### 3.4.7. Helminth-induced pathology

In a mouse model, the literature describes schistosomiasis pathology in mice as being largely associated with granulomatous responses to parasite eggs that become lodged in the liver and intestinal tissue (Hams et al., 2013, Lundy and Lukacs, 2013). Therefore, the sizes and weights of the liver and spleen can be described as markers of exacerbation of schistosomiasis pathology, suggesting that groups with larger mean liver or spleen weights will have more severe pathology than those with lower mean weights. In this study, results suggest that immunization of Sm-infected mice with DNA+MVA vaccination resulted in marked exacerbation of the immunopathology in these mice. All Sm-infected vaccinated mice had larger and heavier spleens and livers compared to unvaccinated Sm-infected groups, thus increased pathology (Fig 3.12 A-D).

To further compare pathology between vaccinated and unvaccinated Sm-infected mice, areas of granulomatous responses to parasite eggs trapped in the liver are considered as primary markers for disease pathology in murine models (Pearce and MacDonald, 2002) humans (Gryseels et al., 2006, Colley et al., 2014). Therefore, granuloma areas of Sm-infected mice vaccinated with DNA+MVA were compared with granuloma areas of unvaccinated Sm-infected mice.

Analysis of the H & E staining revealed that granuloma sizes in Sm-infected vaccinated group were significantly larger than in an unvaccinated Sm-infected group, implicating that vaccination with DNA+MVA worsens the pathology in Sm-infected groups (Fig 3.14). Furthermore, hydroxyproline and egg burden per gram of liver was analysed to further describe pathology between these groups. Hydroxyproline is an amino acid characteristic of collagen metabolism and used as an informative marker to express the extent of liver fibrosis (Souza et al., 2005). No significant difference in hydroxyproline content between unvaccinated Sm-infected and vaccinated Sm-infected mice were observed. However, the number of eggs per gram of liver was higher in unvaccinated Sm-infected compared to Sm-infected vaccinated groups. This result suggests that egg deposition was severe in the unvaccinated Sm-infected group, but the pathology marked by granuloma size was severe in the presence of DNA+MVA vaccination.

Expectedly, PZQ treatment restored liver and splenic sizes back to normal as shown in Fig 3.12, as well as a reduction in the area of liver granulomas (Fig 3.14). This finding entails that PZQ treatment restores and elevates tissue damage from schistosomiasis. Da'dara's et al., have obtained similar results in their study where they showed that PZQ treatment significantly reduced splenomegaly in Sm-infected mice (Da'dara and Harn, 2010).

Surprisingly, the amount of hydroxyproline content in PZQ treated Sm-free mice was significantly higher compared with unvaccinated Sm-infected groups, suggesting that PZQ treatment may contribute to increased fibrosis of the liver (Fig 3.15 A). A study by Brown et al., showed that treatment of *S. mansoni* infection with PZQ in adults coinfecting with HIV-1 in Uganda resulted in a transient increase in viral replication, therefore, increasing pathology (Brown et al., 2005). Another recent study showed that a novel

experimental drug (Paeoniflorin) used for treating Schistosomiasis managed to control sclerosis better than PZQ (Abd El-Aal et al., 2017). This brings into question the ability of PZQ in controlling fibrosis in the livers of infected mice, hence, further studies could evaluate this research avenue. Nevertheless, PZQ treatment resulted in reduced egg per gram of liver when compared with Sm-infected untreated groups (Fig 3.15 B).

### **3.4.8. Limitations of the study**

#### **3.4.8.1. Mouse model limitations**

Although the BALB/c mice are a good model for first line of preliminary investigation, it does not provide full information. For example, The MHC of these animals is only limited to presenting few specific HIV peptides, rather than cumulative magnitude responses. Furthermore, *Schistosoma mansoni* kills mice just after 20 weeks post-infection, while in human schistosomiasis, individuals can live longer with the infection, and thus the mouse model may be exaggerating the pathology.

#### **3.4.8.2. Vaccine immunogenicity**

Cytokine-producing T cells in vaccinated mice were detected using antibodies conjugated to the same fluorophore (PE) and thus making it difficult to determine the polyfunctionality of these candidate HIV vaccines in Sm-infection mouse model. Furthermore, cytotoxicity and T cell avidity were also not evaluated as part of this study in determining desirable immune readouts for a T cell-based vaccine in the Sm-infection mouse model. Cytokines and antibodies such as IL-13 and IL-12 and IgE could give valuable information concerning pathology and host immune switching.

This study was unable to evaluate neutralizing and non-neutralizing antibodies such as ADCC-mediating antibodies elicited by the candidate HIV-1 vaccines standard TZM-bl assay (Barouch et al., 2013, Buchbinder et al., 2014, Goepfert et al., 2014) (Aasa-Chapman et al., 2005, Holl et al., 2006) (Pollara et al., 2011, Sarzotti-Kelsoe et al., 2014). Such antibodies have been associated with protection against HIV infection. These assays have not been established for the mouse model and thus, were unavailable. Nonetheless, this study provides concise empirical data on the impact of helminth infections in the immunogenicity of HIV vaccines as well the role of anthelmintic in restoring immune responses to vaccines.

### **3.4.9. Conclusion and recommendations**

The findings of this study suggest that chronic helminth infections are capable of downregulating the immunogenicity of candidate HIV vaccines. Surprisingly, not only cellular responses are downregulated as previously reported (Da'Dara et al., 2006, Da'dara and Harn, 2010), but antibody responses to HIV Env gp140 were significantly downregulated in Sm-infected individuals. The downregulation of HIV antibody

responses to HIV vaccines, specifically to Env gp140, has not been previously reported. Praziquantel is a useful form of treatment available that has been reported to restore immune responses to other vaccinations. In this study, we demonstrated that partial restoration of cellular responses to HIV vaccination can be achieved; however, responses to antibody responses may not be achieved. Furthermore, this study suggests that vaccination with DNA+MVA candidate vaccines that elicit strong Th1 cellular responses may be detrimental to Sm-infected individuals in that they exacerbate helminth-induced pathology. This study contributes to the body of knowledge concerning the potential challenges helminth infection may pose to future successful candidate HIV vaccines.

The overall study gives an informed report on the impact of helminth infection in HIV vaccines; however, not all immunological aspects could be elucidated. Thus, this study justifies further studies, probably using a nonhuman primate model such as baboons (immune system sequence is highly similar to humans) to obtain a comprehensive understanding of these immune responses.

## CHAPTER 4

### 4. Helminth eggs alone can drive Th2-dominant responses that can affect vaccine-induced responses

#### 4.1. Introduction

In the preceding chapter, we demonstrated that the elimination of helminth worms by chemotherapy only partially restores cellular immune responses but not antibody responses. Although these results showed some improvement in vaccine immunogenicity after treatment, it was not clear if the remaining eggs that are trapped in tissue after treatment contributed to lack of full recovery by continuing to drive a Th2 immune response, capable of downregulating Th1 responses to vaccine antigens. Thus, the present chapter evaluated the effect of *Schistosoma mansoni* eggs (SmE) in the absence of adult Sm worms.

As previously described in chapter 1, section 1.5.5, clinical and animal studies showed that chronic helminthic worm infections exert a predominant Th2 immune response as a parasite survival mechanism (Fincham et al, 2003; Urban et al, 1996). This is as a result of female schistosomes producing large numbers of eggs, many of which become lodged in the liver and intestine where they induce a granulomatous response, a major pathologic manifestation of the disease (Boros, 1989, La Flamme et al., 2002, Caldas et al., 2008).

No vaccines are available for schistosomiasis or other helminthic infections and although effective anti-helminthic drugs are available (such as PZQ for schistosomiasis and ALB for STH), re-infections after successful treatments are also very common in endemic areas (N'Goran et al., 2001). Also, in the case of *Schistosoma*, antihelminth treatment using PZQ only eliminates live worms but not the eggs lodged and trapped in tissue such as liver (Shaheen et al., 1989, Utzinger and Keiser, 2004, Tallima and El Ridi, 2007). Mouse experiments have shown that *Schistosoma* eggs induce strong Th2 immune responses; however, this is only maintained for a short while before the immune response reverts to balanced Th1/Th2 (Wynn et al., 1993, Joyce et al., 2012). In fact, a study in mice showed that a challenge with *Schistosoma mansoni* eggs (SmE) induced a peak Th2 response at 2-3 weeks post challenge (Joyce et al., 2012). Murine studies have shown that following intraperitoneal (i.p) sensitization and intravenous (i.v) challenge, SmEs are transported to the lung via the pulmonary arteries where they are trapped within the lung parenchyma by granulomas composed of lymphocytes, eosinophils, and alternatively activated macrophages, thus, triggering a Th2 response (Sandler et al., 2003, Sandor et al., 2003, Perrigoue et al., 2007, Nair et al., 2009). In the previous Chapter 3, livers from PZQ-treated mice had many Sm eggs (Fig 3.15 B) and the sizes of granulomas were almost the same as those in the livers from untreated Sm-infected mice (Fig 3.14) at least 6 weeks after treatment. In this Chapter, we attempt to inform if Sm eggs left lodged in the tissues after PZQ treatment contributed to the incomplete restoration of the immune responses that were observed in the vaccinated Sm-infected PZQ-treated mice. Concurrently with this experiment, an additional control group was utilized to determine the level of Th1/Th2 immune balance at each vaccination time point. Data

from the additional group would inform on the level of SmE induced Th2 responses at the time when the HIV vaccinations were given.

## **4.2. Materials and methods**

### **4.2.1. Parasites eggs**

*S. mansoni* eggs (SmE) were purchased from the Theodor Bilharz Research Institute (Schistosome Biological Supply Center, Egypt). Upon arrival, the eggs were aliquoted in vials of 50 000 eggs/ml and stored at -80°C until use for inoculation. The integrity and viability of the eggs were determined using a light microscope prior to use.

### **4.2.2. Vaccines**

Vaccines described in Chapter 3 (section 3.2.2 and 3.2.3) (SAAVI MVA-C and HIV-1 gp140 Env protein) were used for immunisation of mice.

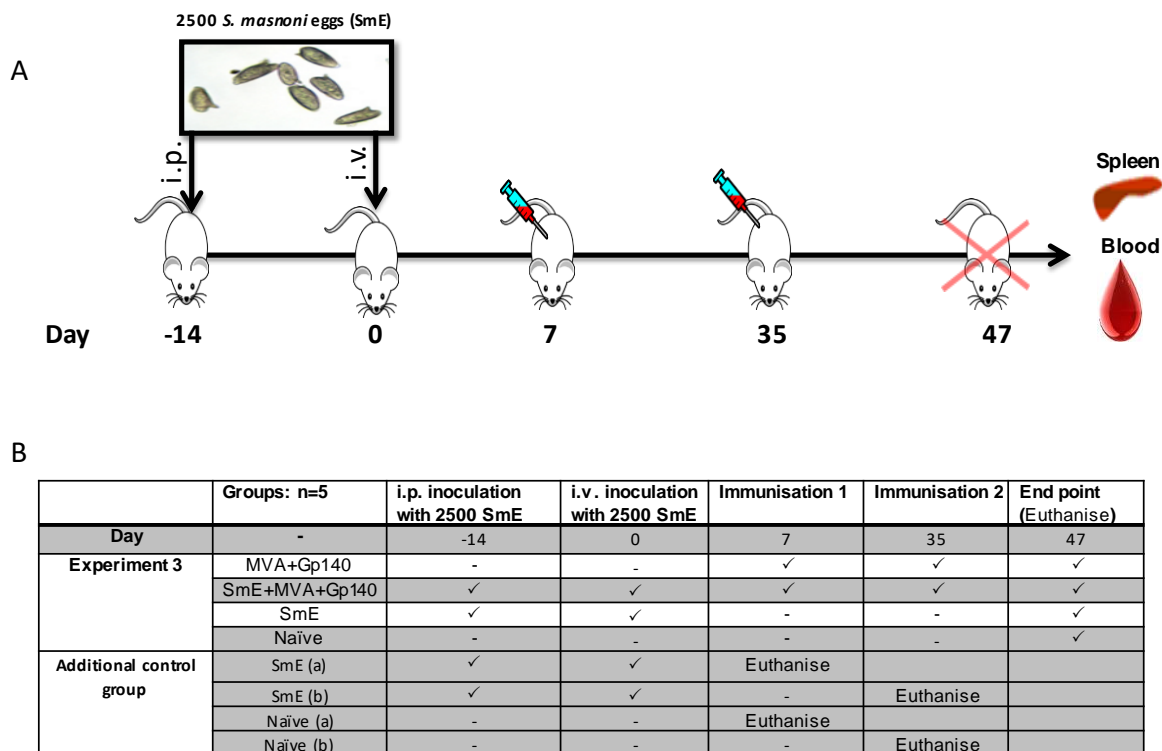
### **4.2.3. Animals**

Female BALB/c mice (6–8 weeks old) were purchased from South African Vaccine Producers (SAVP) (Johannesburg, South Africa) and housed as described in Chapter 2 (section 2.2.3).

### **4.2.4. Immunisation schedule and inoculation of mice with SmE**

Briefly, BALB/c mice were divided into 2 groups of 5 mice/group (SmE-free) and 2 groups of 5 mice/group (SmE-inoculated). Mice were inoculated with 2500 SmE given i.p to sensitise the immune system and challenged with 2500 SmE given i.v 14 days apart. After 7 days, one group of SmE-inoculated and one group of SmE-free was vaccinated with MVA+gp140 vaccine regimen. Mice were boosted 4 weeks later with the same vaccines to induce a mixed vaccine-specific T cell and antibody responses. Twelve days after the last vaccination, blood was collected via cardiac puncture under deep anaesthesia and euthanised by cervical dislocation followed by harvesting of spleen. Cellular and humoral responses were determined by using ELISpot, CBA; flow cytometry and antibody ELISA (Fig 4.1). In a concurrent experiment, a group of 20 mice (additional control group) were divided into 4 groups (5 mice/group). Two groups were sensitized and challenged with SmE in an identical manner (SmE-inoculated) as above while the other 2 groups were left as SmE-free. After 7 and 35 days (coinciding with the HIV vaccination times points), mice in one SmE-inoculated and one of the SmE-free groups were euthanized followed by harvesting of spleens for cellular immune assays.

Briefly, BALB/c mice were divided into 2 groups of 5 mice/group (SmE-free) and 2 groups of 5 mice/group (SmE-inoculated). Mice were inoculated with 2500 SmE given i.p to sensitise the immune system and challenged with 2500 SmE given i.v 14 days apart. After 7 days, one group of SmE-inoculated and one group of SmE-free was vaccinated with MVA+gp140 vaccine regimen. Mice were boosted 4 weeks later with the same vaccines to induce a mixed vaccine-specific T cell and antibody responses. Twelve days after the last vaccination, blood was collected via cardiac puncture under deep anaesthesia and euthanised by cervical dislocation followed by harvesting of the spleen. Cellular and humoral responses were determined by using ELISpot, CBA; flow cytometry and antibody ELISA (Fig 4.1). In a concurrent experiment, a group of 20 mice (additional control group) were divided into 4 groups (5 mice/group). Two groups were sensitized and challenged with SmE in an identical manner (SmE-inoculated) as above while the other 2 groups were left as SmE-free. After 7 and 35 days (coinciding with the HIV vaccination times points), mice in one SmE-inoculated and one SmE-free groups were euthanized followed by harvesting of spleens for cellular immune assays.



**Figure 4.1: Immunisation schedules:** Mice were inoculated with 2500 SmE given i.p to sensitise the immune system and challenged with 2500 SmE given i.v 14 days apart. After 7 days, one group of SmE-inoculated and one group of SmE-free were vaccinated twice with MVA+gp140 vaccine regimen 4 weeks apart to induce a mixed vaccine-specific T cell and antibody responses. Twelve days after the last vaccination, mice were euthanised and blood and spleen were collected for analysis of cellular and humoral responses. Cellular and humoral responses were determined by using ELISpot, CBA; and antibody ELISA.

A) Representation of SmE inoculation and immunisation strategy. B): experiment 3 (mixed cellular and antibody response vaccine regimen) and time point experiment without the vaccine. Three replicates of Experiment 3 and two replicates of control group experiments were conducted.

#### **4.2.5. Preparation of splenocytes and serum**

Splenocytes and serum were processed as previously described in Chapter 2 (section 2.2.5).

#### **4.2.6. Interferon (IFN)- $\gamma$ and interleukin-2 (IL-2) ELISpot assay**

Interferon (IFN)- $\gamma$  and IL-2 ELISpot was performed as previously described in Chapter 3 (section 3.2.7).

#### **4.2.7. Quantification of secreted cytokines by CBA**

CBA was used to measure cytokine levels and was performed as described in previous Chapter 3 (section 3.2.9).

#### **4.2.8. Quantification of Env-gp140 specific antibody isotypes by ELISA assay**

Antibodies to gp140 in mice serum were detected and quantified using HIV-1 subtype C (strain TV1) gp140 protein (Immune Technology, USA) in an ELISA assay as previously described in chapter 2 (section 2.2.9).

#### **4.2.9. Statistical analysis**

Statistical analysis was performed using Prism version 5.0 (GraphPad Software, San Diego, CA). The t-test for independent unpaired non-parametric comparisons was applied to assess the level of significance between means  $\pm$ SEM of groups. Three independent experiments were conducted and all tests were two-tailed. P values  $<0.05$  were considered significant.

### 4.3. Results

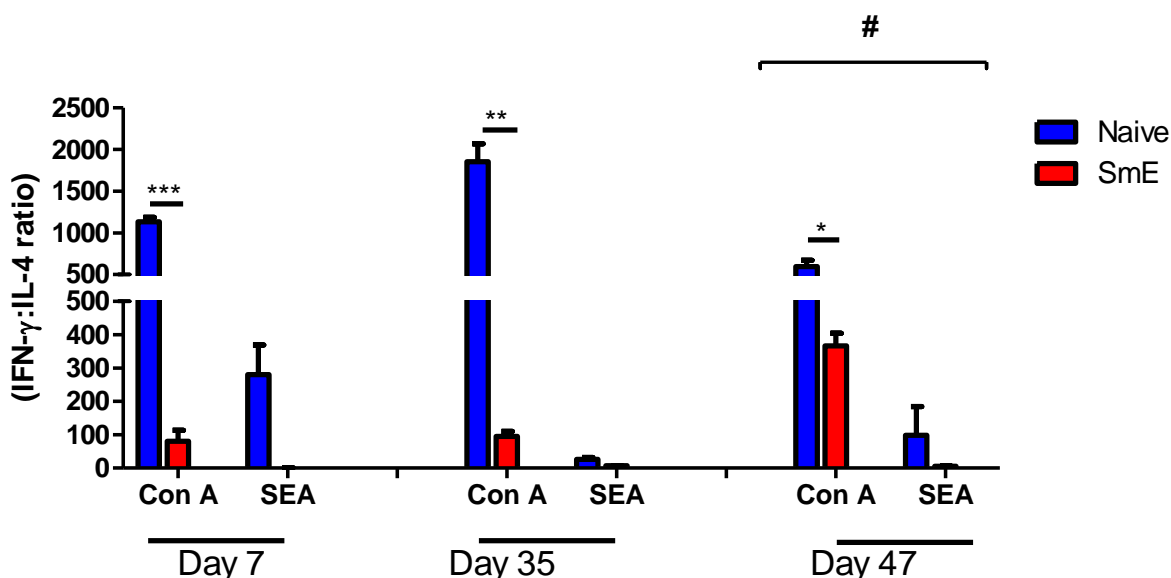
#### 4.3.1. Analysis of Th1/Th2 responses to non-specific stimulation (Con A) and Parasite-specific (SEA) in SmE-inoculated mice vaccinated/unvaccinated with MVA+gp140 vaccine regimen

To determine if SmE alone drive Th2 responses strong enough to attenuate the immunogenicity of HIV vaccines, groups of mice were inoculated with 2500 SmE twice and euthanised at day 7 and day 35 post initial inoculation coinciding with vaccination time points. Data was useful for determining the Th1/Th2 profile in SmE challenged mice. Furthermore, the study was aimed at determining the host immune response at the time of vaccination.

The bias of the immunity to Th1/Th2-type was also evaluated by calculating the ratio of IFN- $\gamma$  to IL-4 in response to stimulation with the Con A and SEA peptide. The full data on Th1 and Th2 cytokine profiles are shown in Appendix 1A-1F (Appendices section), which, in general, show secretion of more Th2 and less Th1 cytokines by splenocytes from SmE-inoculated compared to those of SmE-free mice.

At day 7 post-challenge, the IFN- $\gamma$ :IL-4 ratio was significantly lower in SmE-inoculated ( $80.44 \pm 33.65$  pg/ml) compared to SmE-free ( $1133 \pm 52.68$  pg/ml) mice after stimulation with Con A. Similarly, when stimulated with SEA, the IFN- $\gamma$ :IL-4 ratio was lower in SmE-inoculated mice compared to SmE-free mice was lower. At 35 days post challenge, the IFN- $\gamma$ :IL-4 ratio was also significantly lower in SmE-inoculated ( $95.27 \pm 15.26$  pg/ml) compared to SmE-free ( $1853 \pm 217.0$  pg/ml) mice after stimulation with Con A. Similarly, when stimulated with SEA, the IFN- $\gamma$ :IL-4 ratio was lower in SmE-inoculated mice compared to SmE-free mice. At day 47 post-challenge, the IFN- $\gamma$ :IL-4 ratio was significantly lower in vaccinated SmE-inoculated ( $367.0 \pm 38.34$  pg/ml) compared to SmE-free ( $597.2 \pm 72.93$  pg/ml) vaccinated mice after stimulation with Con A. Similarly, when stimulated with SEA, the IFN- $\gamma$ :IL-4 ratio was lower in SmE-inoculated mice compared to SmE-free vaccinated mice (Fig 4.2).

### Th1/Th2 cytokine in response to non-specific (Con A) and Parasite-specific (SEA) stimulation



**Figure 4.2: Cytokine production by Con A and SEA stimulation in SmE-inoculated and SmE-free mice.** Splenocytes were harvested from mice vaccinated with the indicated regimen described in Fig 4.1. They were then stimulated with an irrelevant peptide (negative control) or Con A or with SEA for 48 h. Culture supernatants were collected and the level of Th1 and Th2 cytokines released into the medium for naïve and SmE challenged mice was measured using a Cytokine Bead Array assay. The individual bars represent the magnitude of the net positive cytokine levels in the medium of splenocytes of SmE-free (blue) and SmE-inoculated (red) mice. #: mice were vaccinated with the MVA+gp140 vaccine. The level of cytokines in the culture medium was considered positive when the level is higher than the detection limit of the respective cytokine described in Table 2.3. Results represent 2-3 independent experiments and plotted as the mean + SEM, and cytokine levels were expressed as pg/ml. Statistical analysis was performed using unpaired, two-tailed t-test analysis followed by FDR for multiple comparisons, \* $p < 0.05$ , \*\* $p < 0.01$ , \*\*\* $p < 0.001$ .

#### 3.3.3. Cytokine responses to HIV-specific peptide stimulation in the background of SmE-inoculation

The cumulative positive cytokine responses to the HIV peptides for the MVA+gp140 vaccination regimen are shown in Fig 4.3. Higher but not significant levels of net Th1 cytokines: IFN- $\gamma$  (3332.96 pg/ml); IL-2 (103.69 pg/ml) and TNF- $\alpha$  (135.55 pg/ml) was released from HIV peptide-specific T cells of SmE-free mice vaccinated with the MVA+gp140 vaccination regimen compared to SmE-inoculated vaccinated mice which

produced lower Th1 cytokine levels of IFN- $\gamma$  (2544.24 pg/ml); IL-2 (62.81 pg/ml) and TNF- $\alpha$  (86.97 pg/ml) (Fig 4.3 A; B and C respectively). No noticeable difference was observed in levels of IL-10 (86.47 vs 87.88 pg/ml) and IL-4 (15.43 vs 15.38 pg/ml) and slightly higher IL-6 in SmE-free (818.86 pg/ml) compared to SmE-inoculation (711.16 pg/ml) vaccinated mice.

# Th1/Th2 responses to HIV peptides stimulation

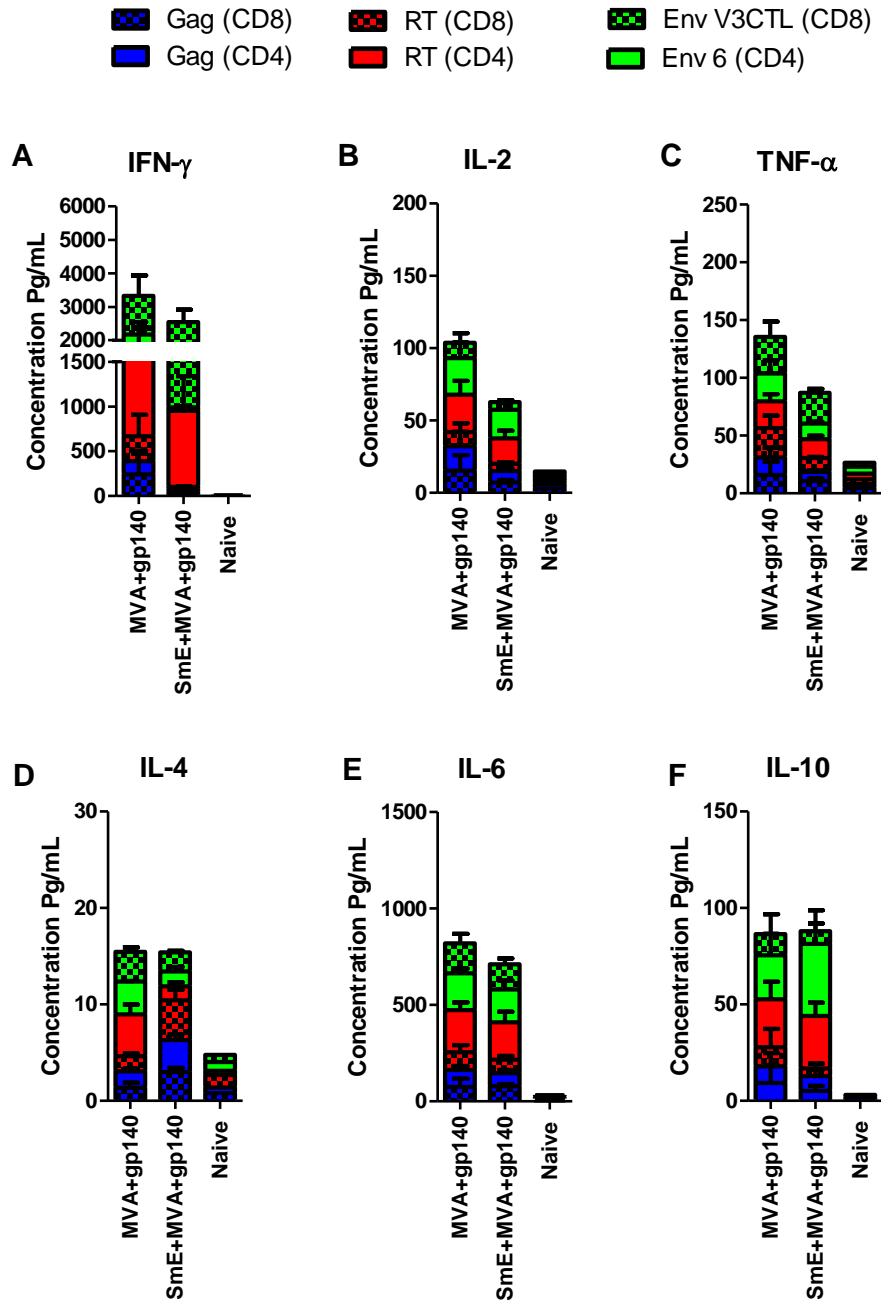
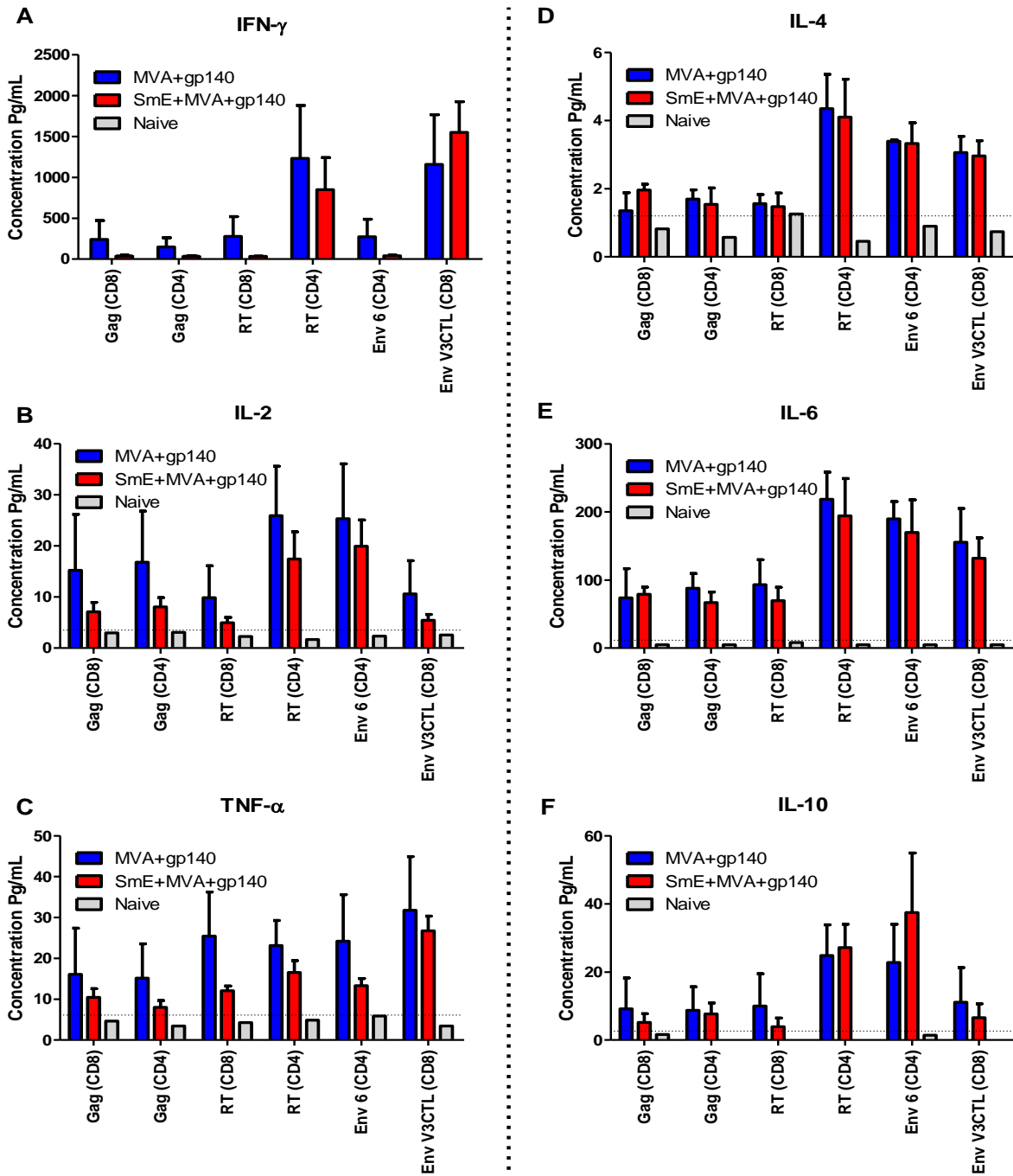


Figure 4.3: Cumulative cytokines produced by HIV peptide-specific T cells of Sm-free and Sm-infected mice vaccinated with MVA+gp140. Splenocytes were harvested from mice vaccinated with the indicated regimen described in Fig 4.1. They were then stimulated with an irrelevant peptide (negative

control) or with the individual HIV-specific T cell peptides for 48 h. Culture supernatants were collected and the level of Th1 (A, B and C) and Th2 (D, E and F) cytokines released into the medium was measured using a Cytokine Bead Array assay and flow cytometric analysis. The individual bars represent the magnitude of the cumulative positive cytokine levels in the medium of splenocytes of SmE-free and SmE-inoculated vaccinated mice, stimulated with individual HIV peptides, after subtracting the background (splenocytes stimulated with irrelevant peptide). The level of cytokines in the culture medium was considered positive when the level is higher than the detection limit of the respective cytokine described in Table 2.3. Results represent 3 independent experiments and plotted as the mean + SEM, and cytokine levels were expressed as pg/ml. Student t-test followed by FDR for multiple comparisons.

#### **4.3.2. Cytokine responses to individual peptides**

Despite high levels of net IFN- $\gamma$  released from HIV peptide-specific T cells of SmE-free mice vaccinated with an MVA+gp140 vaccine, only 37% (1233.51 pg/ml) of the responses are attributed to the RT CD4 peptide-specific responses (Fig 4.4 A). However, SmE-inoculated vaccinated mice had a drastic reduction of the net IFN- $\gamma$  cytokine (850.9 pg/ml) in response to RT CD4 peptide. IFN- $\gamma$  responses to Gag CD8 (242.2 vs 36.47 pg/ml); Gag CD4 (147.78 vs 32.93 pg/ml); RT CD8 (278.01 vs 41.42 pg/ml) in SmE-free vaccinated mice were higher compared to responses in SmE-inoculated vaccinated mice with exception to responses to Env CD8 which were higher in SmE-free (1158.23 vs 1550.36 pg/ml) compared to SmE-inoculated vaccinated mice (Fig 4.4 A). IL-2 (Fig 4.4 B) and TNF- $\alpha$  (Fig 4.4 C) levels were higher in SmE-free mice compared to SmE-inoculated mice; however, the difference was not significant in response to all HIV peptides. No noticeable difference was observed in all Th2 (Fig 4.4 D-F) cytokine levels between SmE-inoculated and SmE-free vaccinated mice in response to all HIV peptides.



**Figure 4.4: Cytokine-produced by Gag (CD8 and CD4) RT (CD8 and CD4) and Env (CD 4 and CD8) specific peptides in SmE-free and SmE-inoculated mice vaccinated with MVA+gp140.** Splenocytes were harvested from mice vaccinated with the indicated regimen described in Fig 4.1. They were then stimulated with an irrelevant peptide (negative control) or with the individual HIV-specific T cell peptides for 48 h. Culture supernatants were collected and the level of Th1 (A, B and C) and Th2 (D, E and F) cytokines released into the medium was measured using a Cytokine Bead Array assay and flow cytometric analysis.

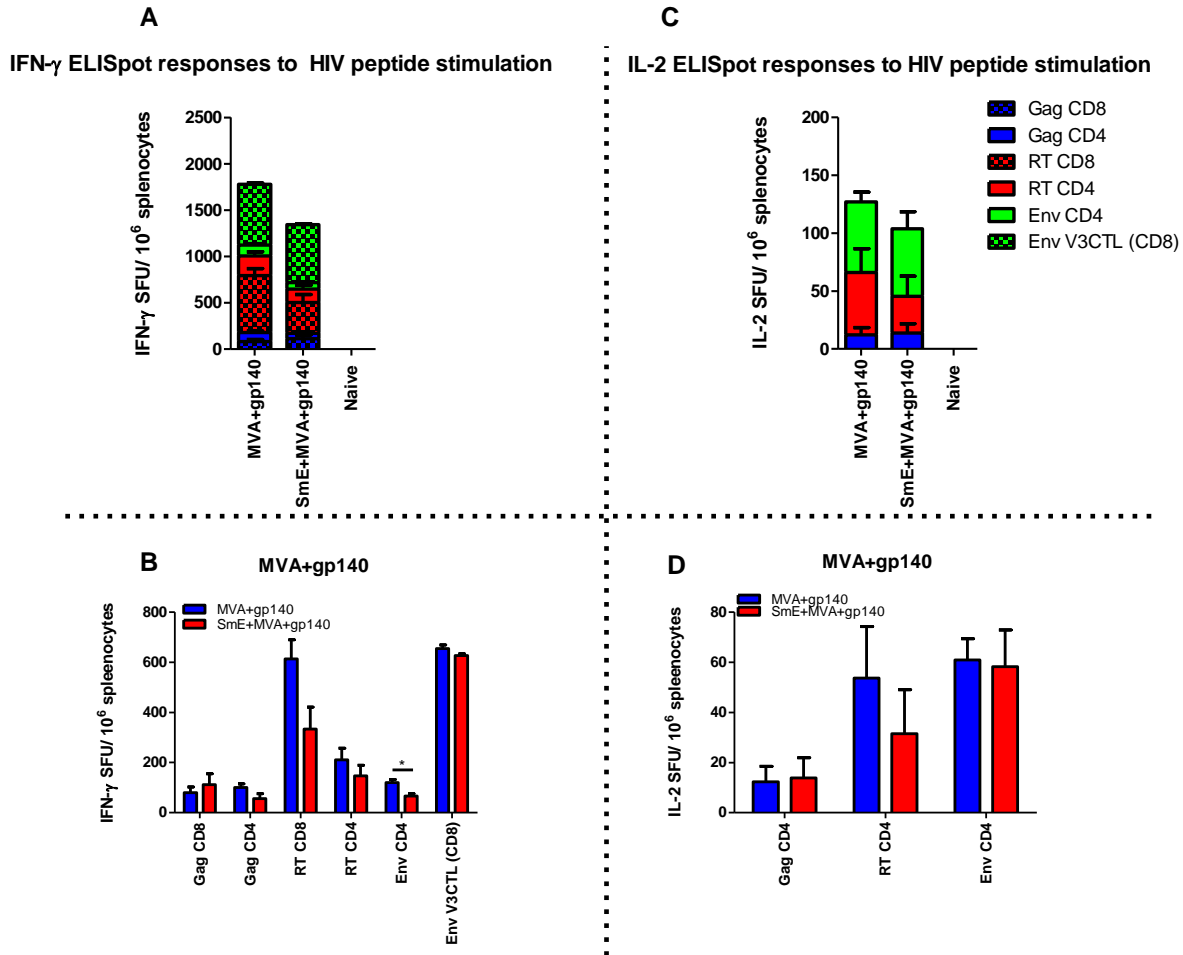
The individual bars represent the magnitude of the net positive cytokine levels in the medium of splenocytes of Sm-free and Sm-infected vaccinated mice, stimulated with individual HIV peptides, after subtracting the background (splenocytes stimulated with irrelevant peptide). The level of cytokines in the culture medium was considered positive when the level is higher than the detection limit of the respective cytokine described in Table 2.3. Results represent 3 independent experiments and plotted as the mean + SEM, and cytokine levels were expressed as pg/ml. Student t-test followed by FDR for multiple comparisons

#### **4.3.3. Cellular immune responses to the MVA+gp140 regimen in the background of SmE inoculation measured by ELISpot**

Cellular immune responses were evaluated using IFN- $\gamma$  and IL-2 ELISpot assays to enumerate the number of Gag; RT and Env-specific IFN- $\gamma$  or IL-2 secreting CD8+ and CD4+ T cells in the spleen of mice vaccinated with MVA-protein (MVA+gp140) (Fig 4.5).

Vaccination with MVA+gp140 induced higher but not significant cumulative HIV-1 Gag, RT and Env-specific IFN- $\gamma$  (1778.78 SFU/10<sup>6</sup> splenocytes) (Fig 4.5 A) and IL-2 ELISpot (126.89 SFU/10<sup>6</sup> splenocytes) (Fig 4.5 C) responses in SmE-free vaccinated mice. In contrast, vaccination in SmE-inoculated mice induced a lower number of peptide-specific IFN- $\gamma$  (1343.56 SFU/10<sup>6</sup> splenocytes) and IL-2 (103.78 SFU/10<sup>6</sup> splenocytes) ELISpot responses.

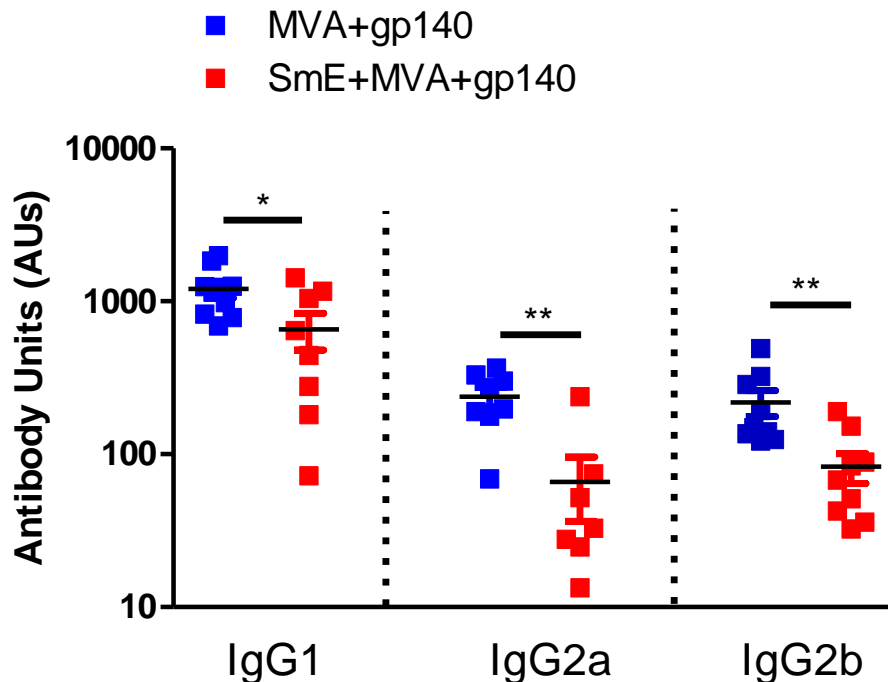
Levels of IFN- $\gamma$  ELISpot responses to individual peptides were also higher in SmE-free vaccinated compared to SmE-inoculated mice except for the ELISpot responses to the Gag CD8 peptide which was slightly higher in SmE-inoculated compared to SmE-free vaccinated mice (Fig 4.5 B). On the other hand, IL-2 ELISpot responses to individual peptides were only positive for Gag CD4; RT CD4 and Env CD4. There was no difference between SmE-free and SmE-inoculated vaccinated mice except for IL-2 ELISpot responses to the RT CD4 which were slightly more in SmE-free (53.67 SFU/10<sup>6</sup> splenocytes) compared to SmE-inoculated (31.56 SFU/10<sup>6</sup> splenocytes) vaccinated mice (Fig 4.5 D).



**Figure 4.5: IFN- $\gamma$  and IL-2 ELISpot analysis of HIV-1 Gag; RT and Env-specific T cell responses induced in the spleen of Sm-infected and Sm-free mice vaccinated with the MVA+gp140 vaccine regimen described in Fig 4.1.** At sacrifice, splenocytes were pooled from 5 mice per group then stimulated with an irrelevant peptide (negative control) or stimulated with HIV-1 Gag (CD4 and CD8); RT (CD8 and CD4) and Env (CD4 and CD8) T cell peptides in an IFN- $\gamma$  ELISpot (A and B) and IL-2 ELISpot (C and D) assays. Bars are the mean number of spots of triplicate reactions in the irrelevant peptide (background) or to an individual peptide for 10<sup>6</sup> splenocytes. Positive responses to the HIV peptides were considered to be those greater than the background response (response to irrelevant peptide stimulation) plus two times the standard deviation (SD) of this response. The magnitude of positive IFN- $\gamma$  ELISpot responses is expressed as either net cumulative (A) or net individual (B) responses per 10<sup>6</sup> splenocytes. The magnitude of positive IL-2 ELISpot responses is expressed as either net cumulative (C) or net individual (D) responses per 10<sup>6</sup> splenocytes. The bars indicate the average net SFU from triplicate wells for 10<sup>6</sup> splenocytes. Results represent 3 independent experiments and plotted as the mean + SEM, and cytokine levels were expressed as pg/ml. Statistical analysis was performed using unpaired, two-tailed t-test analysis followed by FDR for multiple comparisons, \*p<0.05.

#### 4.3.4. HIV gp140 specific ELISA antibody responses in MVA+gp140 regimen

To investigate the impact of SmE on vaccine antibody responses, antibody immunity in response to immunisation with MVA+gp140 was determined by ELISA. Significantly higher amount gp140-specific IgG1 antibody units were observed in SmE-free ( $1203 \pm 152.0$  AUs) compared to SmE-inoculated ( $656.8 \pm 177.1$  AUs). Significantly higher amount gp140-specific IgG2a antibody units were observed in SmE-free ( $238.1 \pm 34.33$  AUs) compared to SmE-inoculated ( $71.14 \pm 15.98$  AUs) vaccinated mice. Similarly, significantly higher amount gp140-specific IgG2b antibody units were observed in SmE-free ( $218.3 \pm 41.86$  AUs) compared to Sm-infected ( $82.73 \pm 18.20$  AUs) vaccinated mice (Fig 4.6).



**Figure 4.6: *Schistosoma mansoni* eggs (SmE) significantly reduce gp140 specific antibody units.**

Mice were sensitised and challenged with 2500 SmE prior vaccination with an MVA-protein (MVA+gp140) HIV vaccine regimen. Blood was collected 12 days after last vaccination from 5 mice per group and analysed for gp140 Env-specific IgG isotypes (IgG1, IgG2a, IgG2b). Previously-generated mouse anti-sera (Gp140 Env-specific with high OD values) were pooled and used to generate a standard curve. Individual mouse sera were tested at a dilution of 1:5,000. A test sample with OD values less than a cut-off value of 2X the OD value of the negative control serum (pooled from unvaccinated, uninfected normal mice) was assigned a zero antibody unit. Each square represents an individual mouse of naïve (blue) or SmE challenged vaccinated (red) group). Groups are compared using unpaired, two-tailed t-test analysis. Data

is representative of 3 independent experiments, student t-test followed by FDR for multiple comparisons. \*P<0.05; \*\*P<0.01; \*\*\*P<0.0001.

#### **4.4. Discussion**

Chronic helminth infections are characterized by skewing host immune response towards a dominant Th2 type, marked by induction of Th2 related cytokines such as IL-4, IL-5 and IL-13 that induce B lymphocytes to switch to IgE antibody production accompanied by expanded T regulatory cell populations and some level of immune suppression (Pearce et al., 1991, Bundy et al., 2000, Voehringer et al., 2004, van Riet et al., 2007, Colley et al., 2014, Lundie et al., 2015, Mulu et al., 2015). Previous studies reported that this immune modulation from Th1 to Th2 induced by chronic helminth infection impaired the specific immunity induced by vaccination (Actor et al., 1993, Sabin et al., 1996, Muniz-Junqueira et al., 1996, Da'Dara et al., 2006, Elias et al., 2008, Da'dara and Harn, 2010, Chen et al., 2012). In schistosomiasis, eggs drive the Th2 immune response (Pearce et al., 1991, Grzych et al., 1991). However, elimination of the worms by treatment has been associated with recovery of immune balance and response to vaccination (Da'dara and Harn, 2010, Chen et al., 2012, Shollenberger et al., 2013b). It is not known if eggs (trapped in tissue) in the absence of an active infection can induce a Th2 immune biased capable of affecting vaccine responses.

This study asked if helminth eggs alone can drive a Th2-dominant response that can affect vaccine-induced responses to a vaccine regimen that induces mixed cellular and antibody responses (SAAVI MVA-C + Env-gp140: MVA+gp140). For this, two experiments comprising of 4 groups of mice were used. The objective of the first experiment was to conduct a time point study to determine the immune profile at different vaccination time points in BALB/c mice model inoculated with Sm eggs (SmE). The second experiment was to evaluate if SmE induced Th2 immune biasing negatively affects responses to candidate HIV vaccines.

##### **4.4.1. Skewing towards Th2 in SmE-inoculated mice**

Our findings demonstrated that SmE induced strong Th2 cytokine responses and downregulation of host Th1 cytokines. The decrease of IFN- $\gamma$ :IL-4 ratio observed in SmE inoculation further demonstrated biasing of host immune system towards predominant Th2 type. These findings coincide with other studies that have shown that biasing immune responses towards Th2 concurs with SmE deposition during live infection (Pearce et al., 1991, Grzych et al., 1991). In the absence of a live Sm-infection, this model shows that the eggs on their own induce strong Th2 immune responses comparable to those induced by live infection (Cheever et al., 2002, Fu et al., 2012, Joyce et al., 2012). It appears that SmE-induced Th2 immune responses wane over time. As shown in Fig 4.4, Day 47 post first inoculation with SmE, Th2 immune responses begin to decrease, marked by an overall decrease of IFN- $\gamma$ :IL-4 ratio. However, the kinetics of this parasite eggs induced immune responses are not well understood past 47 days post first inoculation, thus, few reasons are suggested. Surprisingly, when compared to the treatment experiment in chapter 3,

the IFN- $\gamma$ :IL-4 ratio in the SmE experiment was much higher in SmE-free vaccinated group at early time points (day 7 and 35 post inoculation with SmE) and became similar at 47 days post-inoculation (Fig 3.4 vs Fig 4.2). This observation may suggest that younger mice, elicit higher responses to antigens due to their stronger immune system. Generally, too young mice or too old mice don't mount best immune responses compared to middle-aged groups of mice. Perhaps, this strain of mice responds best at 35 days post inoculation. It would be interesting to evaluate how different vaccination timepoints in younger mice behave.

First, this may be likely due to the number of eggs inducing these Th2 responses in the host. In a live infection, egg deposition is continuous, thus, maintaining strong helminth-induced Th2 (Hams et al., 2013, Colley et al., 2014). As seen in this chapter, SmE-inoculated mouse only received a fixed amount of eggs (+5000 eggs per mouse) compared to (+1000 SmE daily for 4weeks plus) found in livers of mice infected with live infection (Pearce, 2005). It can be concluded that the number of eggs correlates with the strength or intensity of Th2 biasing.

In previous chapter 3, parasitic worms were eliminated using PZQ treatment which does not eliminate the eggs trapped in tissue, which are drivers of Th2 responses, thus only partial restoration of Th1 responses was observed while Th2 responses remained high. Da'dara and Harn reported increased IL-10 (suppressor cytokine following Th2 responses) levels in PZQ treated groups despite the restoration of Th1 responses to their DNA vaccines (Da'dara and Harn, 2010). However, little is known about the consequences of these residual Th2 cytokines.

#### **4.4.2. Vaccine-specific cellular response**

Cumulative vaccine-specific T cell responses in SmE inoculated mice were evaluated using ELISpot. SmE-inoculated vaccinated mice induced slightly lower net IFN- $\gamma$  and IL-2 ELISpot responses compare to SmE-free vaccinated mice, albeit insignificant (Fig 4.7). These results suggest that SmE is downregulating vaccine responses though it may be to a low degree (Fig 4.7). These findings were comparable to the treatment experiment in chapter 3 when comparing SmE-free vaccinated and Sm-free vaccinated groups, except that SmE did not significantly reduce vaccine responses as observed with the live infection with Sm. These findings may explain why PZQ treatment only partially restored responses to vaccines. Because eggs were still trapped in tissue, they may be inducing responses that suppress vaccine-specific cellular responses as observed in this study. These findings are further supported by previous studies that point SmE as the culprit that drives vaccine-specific response suppression, likely due to hyporesponsiveness (Hams et al., 2013, Colley et al., 2014).

The same was true for vaccine-specific cytokine responses measured by CBA. SmE inoculated vaccinated mice had lower cumulative vaccine specific Th1 cytokines IFN- $\gamma$ ; IL-2 and TNF- $\alpha$  compared to SmE-free vaccinated mice, suggesting that SmE is capable of affecting vaccine specific cytokine levels, albeit it

insignificant (Fig 4.5 A-C). In previous Chapter 3, vaccinated Sm-infected-PZQ treated mice showed only partially restored cytokine levels, suggesting that because eggs were still in liver tissue, they induced Th2 cytokines which are known to participate in the reduction of Th1 vaccine-specific responses. These findings further suggest that SmE may only partially reduce vaccine-specific responses rather than completely reducing them as observed in a live infection model.

Conversely, Bui and colleagues showed that *S. mansoni* soluble egg antigens enhance the *Listeria monocytogenes* vector HIV-1 vaccine induction of cytotoxic T cells (Bui et al., 2014). As a follow-up study, Bui and colleagues recently demonstrated that SEA enhances T cell responses to a newly identified HIV-1 Gag H-2<sup>b</sup> epitope (Bui et al., 2015). These findings suggest that there may be two sides of the coin concerning the impact of helminth products on HIV vaccination.

It is worth noting that a considerable level of Th2 cytokines (IL-10; IL-6 and IL-4) were expressed in high levels even in SmE free groups. Even though Th1 are the classical markers for these vaccines. These findings may suggest that these vaccines may have the ability to induce Th2 cytokine responses particularly in younger mice (Fig 4.3). However, they still induce more Th1 cytokines when compared to Th2 despite the presence of SmE.

#### **4.4.3. Antibody responses in SmE-inoculated mice**

To evaluate if egg-induced Th2 responses affect vaccine-specific antibody responses, serum of SmE vaccinated mice was evaluated by ELISA. Surprisingly, antibody responses in SmE vaccinated groups were significantly reduced for all Env gp140 specific isotypes compared to SmE-free vaccinated mice (Fig 4.8). This was not anticipated since only partial reduction of cellular immune responses was observed. Thus, these findings may suggest that the SmE affect critical immune components responsible for antibody production, most likely, the major players in antibody activation and production (T helper B cells) (Swain et al., 2012). These findings may explain why PZQ treated mice remained with defected vaccine-specific antibody responses, while T helper cells were reduced to undetectable levels (Fig 3.9 B). Furthermore, even if there were 10-fold more eggs in PZQ treatment vaccinated compared to SmE mice, PZQ has been shown to increase Th2 responses, which may contribute greatly to the reduction of vaccine antibody responses. The overall findings of this study suggest that even in the absence of a live Sm-infection, SmE can still induce Th2 responses that can potentially affect vaccine-specific responses, particularly antibody responses. To our knowledge, this study is the first to demonstrate the ability of Sm eggs in negatively affecting vaccine specific HIV antibody responses. There was a big difference in vaccine-specific IgG2a and IgG2b between SmE-free experiments and Sm-free experiments. It is not clear why this could be, however, the difference in age may play a role in modifying how antibody responses behave in these mice. One mouse study demonstrated that aged mice were capable of eliciting antibody responses better

compared to younger mice, attributing this to increased autoantibody forming cells found in older mice (Zhao et al., 1995).

Conversely, a vaccine study by Shollenberger and colleagues demonstrated that the *Listeria* vaccine vector induced enhanced antibody responses to HIV Gag in vaccinated mice infected with Sm (Shollenberger et al., 2013b). They attributed these enhancements in the vaccine responses to the soluble egg antigen (SEA) which is produced by Sm eggs.

#### **4.4.4. Limitations of the study**

The egg model does not reproduce *S. mansoni* infection model (characterised by continuous egg deposition) which is performed by infecting mice with infectious cercariae. However, the *S. mansoni* egg model provides a platform for evaluating a series of immunological concepts. In this study, lungs were collected with an aim of determining successful inoculation with the eggs which get trapped in the lungs. However, this was not achievable due to lack of time and resources. Furthermore, comparison of the two models (liver for live infection vs lungs for SmE model) would give information on the types of granulomas formed and estimating egg burden of different tissues. It would have been interesting to evaluate pathological characteristics such as mouse footpads of vaccinated mice compared to unvaccinated mice.

#### **4.4.5. Conclusion and recommendations**

The overall study demonstrates that *S. mansoni* egg (SmE) alone can drive Th2 responses capable of attenuating the immunogenicity of HIV vaccines, particularly humoral responses. These findings propose that Sm eggs left lodged in the tissues after PZQ treatment may be contributors to the incomplete restoration of the immune responses observed in the vaccinated Sm-infected PZQ-treated mice (Chapter 3). These findings further suggest that future mass antihelminthic treatment may not be the immediate solution to counteract the impact of helminth infections on the immunogenicity of HIV vaccines as previously suggested, thus, further investigations may be required to give clarity on method of restoring immune responses. Therefore, this study recommends the implementation of thorough evaluation of immune responses even after antihelminthic therapy to achieve optimal and effective vaccine responses to established and future Th1 based vaccines. This study also calls for HIV vaccine development programs to consider designing vaccines that can overcome helminth-induced immunity as shown by the likes of Bui and Shollenberger (Shollenberger et al., 2013b, Bui et al., 2014, Bui et al., 2015). This study is, however, the first to demonstrate that antibody responses in SmE challenged mice get significantly downregulated.

## CHAPTER 5

### 5. General Discussion

The development of successful HIV vaccines remains a high priority, particularly in the developing Sub-Saharan Africa (SSA) (UNAIDS, 2015). These candidate vaccines are expected to elicit effective humoral and T cell-mediated responses against HIV (Haynes and McElrath, 2013, McMichael and Koff, 2014). T cell responses associated with a predominantly T-cell helper type 1 (Th1) immune response will control viral replication in the tissues and disease progression (Koup and Douek, 2011), while humoral responses associated with durable antibody production neutralise and block virus entry at the mucosal sites (Mascola and Montefiori, 2009). However, the success of these future vaccines may be threatened by the widespread infection with water and soil-transmitted parasitic helminths in SSA (Means et al., 2016, WHO, 2016). This is mainly because helminth infection has been shown to cause CD4+ Th2 immune biased response (Maizels and Yazdanbakhsh, 2003, Maizels et al., 2004, Moreau and Chauvin, 2010, Dauby et al., 2012, Yin et al., 2012), which in turn downregulates the CD4+ Th1 responses that are normally required for vaccine effectiveness (Maizels and Yazdanbakhsh, 2003, van Riet et al., 2007, Bourke et al., 2011). Whether vaccines currently in development for HIV and other diseases will function in populations that have a high prevalence of helminth infection is uncertain, and is something to worry about in the field of vaccine development. Especially with a growing literature showing that infection with helminth parasites causes immune suppression and a CD4+ Th2 skewing of the immune system, thereby impairing Th1-type vaccine efficacy (Buck et al., 1970, Prost et al., 1983, Kilian and Nielsen, 1989, Sabin et al., 1996, Haseeb and Craig, 1997, Cooper et al., 1998, Stewart et al., 1999, Cooper et al., 2000a, Elias et al., 2008, Chen et al., 2012, Apiwattanakul et al., 2014, Bobat et al., 2014, Wajja et al., 2017).

In this study, 3 major experiments were conducted to explore some of the unanswered questions regarding this idea.

The first experiment sought to investigate the effect of helminth infection on the humoral and cellular immunogenicity of two candidate HIV vaccines previously shown to induce specific immune responses in a mouse model system. A preliminary study for the establishment and the validation of a suitable helminth-HIV vaccines experiment was conducted in mice using *Schistosoma mansoni* (Sm) as a helminth model. In this experiment, Sm-infected mice elicited predominantly Th2 responses marked by increased levels of Th2 cytokine and a decreased Th1 cytokine profile. Furthermore, it was demonstrated in Figure 2.10 that cytokine producing CD8 and CD4 T cells are impaired during Sm-infection. This was accompanied by an increase in type 2 total antibodies in serum of Sm-infected mice compared to uninfected groups (Fig 2.12 and 2.13). The phenotype of infected mice was characterized by enlarged spleens and liver. With the model established, infected mice were immunised with SAAVI candidate HIV vaccines, following which immune responses against HIV peptides and proteins were evaluated between Sm-infected vaccinated and Sm-free vaccinated groups. The findings revealed that in the presence of infection, mice failed to induce

adequate vaccine-specific T cell responses. Furthermore, humoral responses to the gp140-env protein were significantly impaired in Sm-infected vaccinated mice (Fig 2.14). This was an interesting finding with far-reaching implications for vaccine development. This led to asking if the removal of the worms by antihelminth chemotherapy could perhaps restore vaccine-specific responses.

The second experiment sought to evaluate how previously infected mice will respond to vaccination after being treated with an antihelminth drug. The findings showed that treating mice prior to vaccination could partially restore T cell responses, particularly IFN- $\gamma$  and IL-2. Surprisingly, the partial recovery of these responses did not translate to the reduction of Th2 responses. Anti-inflammatory cytokines such as IL-10 remained elevated despite treatment with antihelminth. Similar findings were reported in previous schistosome-mouse studies, where PZQ was a drug of choice (Da'dara and Harn, 2010, Shollenberger et al., 2013b).

Even more surprising, findings revealed that antibody responses to the gp140-env protein in Sm-infected mice remained significantly low despite prior treatment with PZQ (Fig 3.11). The reason for such findings could be that there is a disruption of T cell-B cell interaction caused by Sm-associated elements. Liver and spleen evaluation revealed that after treatment, the normal phenotype can be achieved. However, histology showed that eggs remained trapped in tissue even after treatment. Treatment with PZQ has been shown to act upon adult worms, thus lowering the egg burden in treated subjects (Shaheen et al., 1989, Utzinger and Keiser, 2004, Tallima and El Ridi, 2007). Therefore, it can be anticipated that after treatment, the immune responses would return to normal. However, in this study, vaccine-specific cellular responses in treated mice were not fully recovered. Instead, they still had eggs trapped in their livers. Because SmE drives the Th2 biasing induced by Sm, it can be concluded that the recovery of T cell responses can only be partial as a result of the helminth eggs trapped in tissue. This impairment of T cell responses, albeit minimal, may be the reason for significantly impaired vaccine-specific antibody responses observed in this study despite treatment.

This study went further to demonstrate that Th1 cellular responses elicited by DNA+MVA vaccines exacerbated helminth-induced pathology. Sm-infected mice which were vaccinated with DNA+MVA vaccines had significantly larger granuloma sizes (a marker for pathology in Schistosomiasis (Hams et al., 2013, Lundy and Lukacs, 2013)) as well as heavier spleen and livers compared to Sm-infected unvaccinated groups. Treatment significantly reduced the pathology, however, eggs were still present in the tissue. These findings highlighted the scientific challenges in the development of HIV vaccines for Africa, where parasitic helminthiasis is endemic. Furthermore, this raised a question if Sm eggs in the absence of a live infection could result in some downregulation of HIV vaccine-specific responses.

The third experiment sought to further investigate the impact of the presence of helminth eggs alone on vaccine-induced immune responses. Following an established Sm-egg model (Joyce et al., 2012), mice were inoculated with eggs and then vaccinated to evaluate how these eggs affect vaccination. The findings

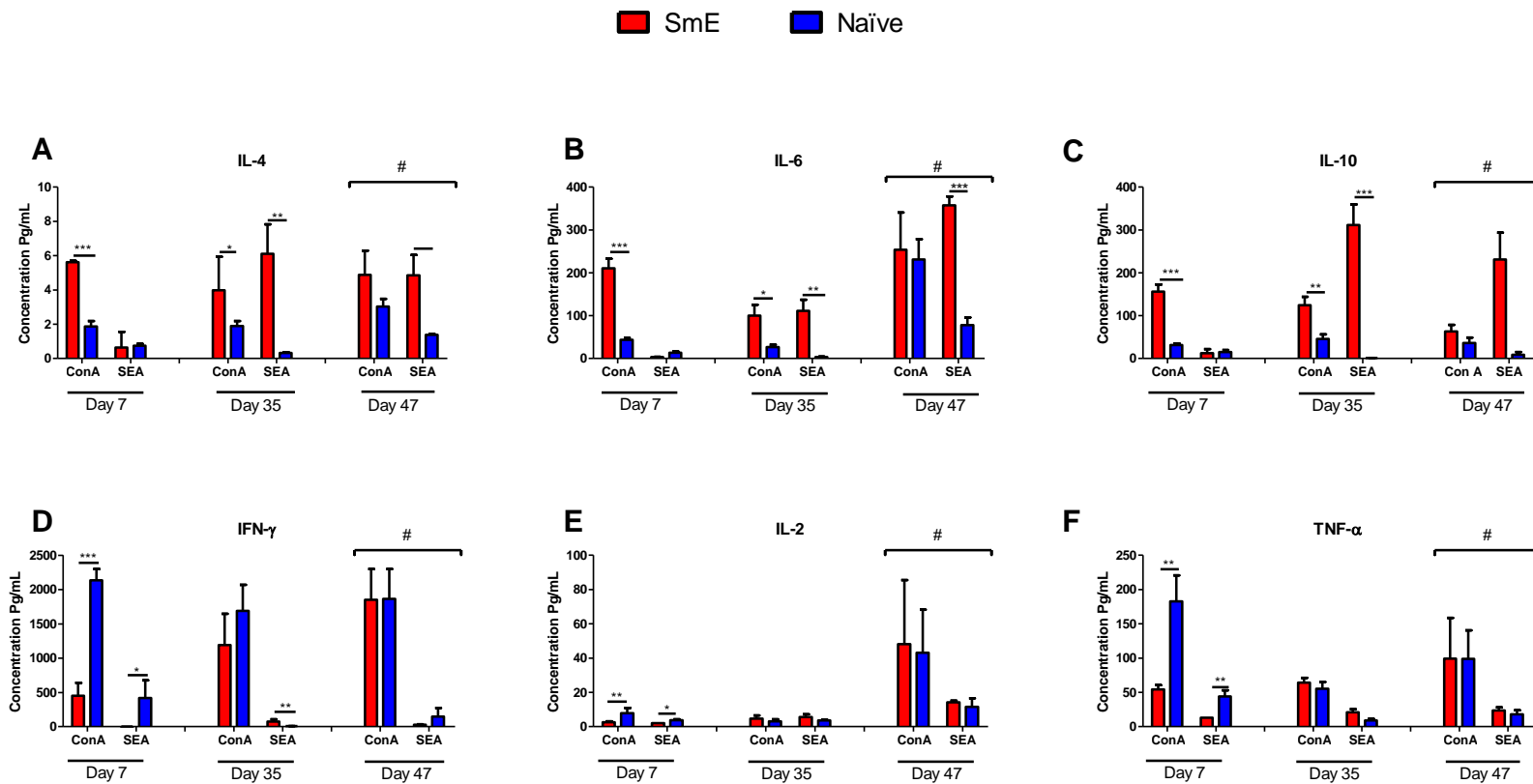
showed that even in the absence of live worms, eggs could drive a considerable Th2 immune response shown by elevated Th2 cytokines. Surprisingly, this immune response only partially affects the T cell compartment. Although reduced, the decrease in vaccine-specific cellular responses observed in SmE inoculated mice was not significant. This may be because the number of eggs used for inoculation were low, hence the minimal downregulation observed. This can also explain why treated-vaccinated groups also induced partially reduced vaccine-specific responses, attributing the effect to be orchestrated by the schistosome eggs. Unexpectedly, antibody responses to HIV env-gp140 were significantly reduced in the presence of SmE. This again may explain why antibody responses in treated groups did not get restored. This finding further highlights another challenge, that even treatment of helminths would not be a definite solution as helminth eggs alone could still attenuate HIV vaccine-induced immune responses.

In light of the findings, this study suggests that elimination of worms can offer an affordable and a simple means to partially restore immune responsiveness to T cell-based vaccines for HIV-1 and other infectious diseases in helminth endemic settings. However, it would be important to consider bacterial vector such as the *Listeria* delivering HIV vaccine, as these have been shown to elicit immune responses without being affected by the presence of helminth infections. Perhaps current and future therapeutic vaccines can benefit from the cooperative use of antihelminthic treatment together with HAART, as this will give the immune system a better chance to mount appropriate responses. The question of how acutely infected patients would respond to HIV vaccination is an avenue to explore, since few such cases may occur. Perhaps such patients could benefit from these drugs as does chronically infected patients.

Furthermore, this study gives an informed report on the impact of helminth infection in HIV vaccines; however, not all immunological aspects could be elucidated. Thus, this study justifies further investigations with use of a nonhuman primate model such as baboons (immune system sequence is highly similar to humans) to obtain a comprehensive understanding of these immune responses. The findings of this study contribute to the body of knowledge concerning the potential challenges helminth infection may pose to future successful candidate HIV vaccines. To our knowledge, there is no reported study evaluating HIV vaccine-specific antibody responses to Env-gp140 in a helminth setting. Furthermore, this study is the first to demonstrate that antibody responses in SmE challenged mice are significantly downregulated.

## Appendices

### Appendix A: Supplementary data



**Appendix A: Th1 and Th2 profile induced by SmE in mice.** Splenocytes were harvested and stimulated with either con A or SEA for 72 hrs at 5% CO<sub>2</sub>, 37°C. Supernatants were harvested and analysed for Th2 cytokines IL-4; IL-6; IL-10 (A-C respectively) and Th1 cytokines: IFN- $\gamma$ ; TNF- $\alpha$

and IL-2 (D-F respectively) measured by CBA. Red bars represent mice challenged with SmE and blue bars represent Naïve groups. Results represent 2-3 independent experiments and plotted as the mean + SEM, and cytokine levels were expressed as pg/ml. #:Groups that received MVA+gp140 vaccination. Statistical analysis was performed using unpaired, two-tailed t-test analysis, \* $p < 0.05$ , \*\* $p < 0.001$ , \*\*\* $p < 0.0001$

## Appendix B Reagents and solutions

### Chloramine T

Dissolve 7 g of chloramine T in 100 ml deionized water. Store for 6 months in the dark at 4°C.

### Citrate-acetate buffer

To 385 ml of isopropanol (2-propanol), add:

57 g sodium acetate.3H<sub>2</sub>O

37.5 g sodium citrate.2H<sub>2</sub>O

5.5 g H<sub>3</sub>citrate. H<sub>2</sub>O

Bring final volume to 1 liter with deionized water

Store indefinitely at room temperature

### Dowex/Norit A mixture

Mix 20 g Dowex (200 to 400 mesh; Sigma, USA) with 10 g Norit-A (Fisher Scientific, USA) in a large beaker. Add 200 ml of 6N HCl and mix well. Transfer mixture to a large Büchner funnel and filter. Wash two times with 6 N HCl. Wash with 95% ethanol and then with 100% ethanol two times. Let dry and store 12 months at room temperature.

### Phenolphthalein, 1%

Add 1 g of phenolphthalein to 100 ml of absolute ethanol. Stable for up to 2 years at room temperature.

### Ehrlich's solution

Dissolve 1g of Para-dimethylamino benzaldehyde in 95ml of 95% ethanol

Add 20 ml of concentrated HCl to the solution from step 1

Mix well

### 10x Lepple Buffer

5.6 g CaCl<sub>2</sub>

12.28 g MgSO<sub>4</sub>.7H<sub>2</sub>O

0.43 g K<sub>2</sub>SO<sub>4</sub>

4.2 g NaHCO<sub>3</sub>

0.48 mL FeCl<sub>3</sub>.6H<sub>2</sub>O solution (2.5 g dissolve in 50 mL H<sub>2</sub>O)

Aqua clean solution

Mix all reagents in 1 L distilled water, except the  $\text{NaHCO}_3$ , and allowed to stand for 1 hour.

Add  $\text{NaHCO}_3$  and make up a solution to 10 L with distilled water.

To make a working stock of 1x Lepple buffer, add 1 part 10x Lepple buffer to 9 parts distilled water and add 2 drops of aqua clean solution.

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