Preclinical characterization of the protection and immune response elicited by immunizations with *Schistosoma mansoni* cathepsin B

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Abstract

Schistosomiasis is a neglected tropical disease caused by freshwater flatworms. The parasite eggs become trapped in host tissues and, as a consequence of the stimulated immune response, promote fibrosis leading to severe organ damage. Schistosomiasis is a chronic disease, and it is a major public health concern due to its significant negative impact on quality of life. Mass drug administration with praziquantel is the mainstay of schistosomiasis control. It has long been recognized that the sole use of praziquantel to control schistosomiasis has important limitations. In particular, drug treatment does not prevent re-infection; thus, requiring constant drug delivery to endemic regions which is unsustainable in the long term. Furthermore, overuse of praziquantel raises concerns of drug resistance. There is therefore a strong incentive to develop a vaccine against schistosomiasis. Even a partially effective vaccine, could significantly decrease disease morbidity and transmission. Our group has expressed the candidate vaccine antigen *Schistosoma mansoni* Cathepsin B (Sm-Cathepsin B). This protein is an immunogenic cysteine peptidase found in the parasite gut. The overall goal of this thesis was to characterize Sm-Cathepsin B vaccine formulations. Our first objective was to assess the protective potential of a Sm-Cathepsin B formulation containing CpG. We used a C57BL/6 mouse model of schistosomiasis. Mice immunized with our Sm-Cathepsin B formulation had significantly lower parasite burden compared to control mice (54%-59% protection levels). The Sm-Cathepsin B + CpG immunizations elicited a biased Th1 response characterized by elevated levels of IgG2c, IFNɣ, and TNFα. Next, we sought to examine the effect of a less immune-biasing adjuvant. To this end, we used the mouse model of schistosomiasis to test a Sm-Cathepsin B vaccine formulated with the squalene based adjuvant Montanide ISA 720 VG. Using this formulation, we achieved 56%-62% protection. The formulation elicited a mixed Th1/Th2 response consisting of elevated levels of IFNɣ, TNFα, IL-4, IL-5, and IgG1. Both tested Sm-Cathepsin B formulations generated similar protection levels. However, they induced very different immune responses. To fully understand the vaccine-induced protection, we needed to characterize the underlying immune mechanisms. Using an *in vitro* parasite killing assay, we showed that the anti-parasitic effect in the Sm-Cathepsin B + Montanide immunized animals required the presence of both $CD45^{+}$ cells (leukocytes) and antibodies; thus, suggesting an antibody-dependent cell-mediated mechanism.

Differently, high parasite killing in the Sm-Cathepsin B + CpG group, as well as an unadjuvanted Sm-Cathepsin B group, was independent of the presence of antibodies; thus, suggesting a dominant cellular effect. A more in-depth characterization revealed the cell populations mediating the protection induced by the different vaccine formulations. The anti-parasitic effect observed with the Sm-Cathepsin B + Montanide group was dependent on the presence of $CD4^+$ T cells and Natural Killer (NK) cells. $CD4^+$ T cells are likely activating the NK cells which are then involved in antibody-dependent cell-mediated cytotoxicity. For the Sm-Cathepsin B + CpG group, $CD8^+$ T cells were shown to be the main effectors mediating parasite killing. Lastly, parasite killing in the unadjuvanted Sm-Cathepsin B group was mediated by $CD4^+$ T cells. In summary, we have demonstrated the protective efficacy of two different Sm-Cathepsin B vaccines, and we have begun to characterize the underlying immune mechanisms mediating Sm-Cathepsin B vaccine-induced protection. Overall, the results of this thesis represent significant progress in the development and understanding of a potential vaccine formulation against schistosomiasis.

Résumé

La schistosomiase est une maladie tropicale négligeé causée par des vers plats d'eau douce. Les oeufs du parasite sont piégés dans les tissus de l'hôte et, en conséquence de la réponse immunitaire stimulée, ils favorisent la fibrose entraînant des lesions organiques sévères. La schistosomiase est une maladie chronique qui représente une préoccupation majeure de santé publique en raison de son impact négatif sur la qualité de vie. L'administration de praziquantel est le pilier de la lutte contre la schistosomiase. Depuis longtemps, il a été reconnu que l'utilisation unique de praziquantel pour lutter contre la schistosomiase présente des limites importantes. En particulier, le traitement avec praziquantel n'empêche pas la reinfection; nécessitant ainsi l'administration constante de médicaments dans les régions endémiques, ce qui est insoutenable à long terme. En outre, le recours excessif au praziquantel suscite des inquiétudes sur la pharmacorésistance. Il existe donc une forte incitation pour le développement d'un vaccin contre la schistosomiase. Même un vaccin partiellement efficace pourrait réduire, de manière significative, la morbidité et la transmission de la maladie. Notre groupe a exprimé l'antigène *Schistosoma mansoni* Cathepsine B (Sm-Cathepsine B) comme candidat potentiel. Cette protéine est une peptidase à cystéine très immunogène trouvée dans l'intestin du parasite. L'objectif global de cette thèse était de caractériser les formulations de vaccins contenant Sm-Cathepsine B. Notre premier objectif était d'évaluer le potentiel protecteur d'une formulation de Sm-Cathepsine B contenant du CpG. Nous avons utilisé des souris C57BL/6 pour notre modèle animal de schistosomiase. Le nombre de vers adultes et d'oeufs chez les souris immunisées avec notre formulation de Sm-Cathepsine B étaient nettement inférieure à celle des souris témoins (54%-59% de niveaux de protection). Les immunisations avec Sm-Cathepsine B + CpG ont provoqué une réponse Th1 biaisée caractérisée par des taux élevés d'IgG2c, d'IFNɣ et de TNFα. Ensuite, nous avons examiné l'effet d'un adjuvant moins polarisant l'immunité. À cette fin, nous avons utilisé notre modèle animal de la schistosomiase pour tester un vaccin Sm-Cathepsine B formulé avec l'adjuvant, à base de squalène, Montanide ISA 720 VG. En utilisant cette formulation, nous avons obtenu une protection de 56% à 62%. La formulation a provoqué une réponse Th1/Th2 mixte consistant des taux élevés d'IFNɣ, de TNFα, d'IL-4, d'IL-5 et d'IgG1. Les deux formulations de Sm-Cathepsine B testées ont généré des niveaux de protection similaires. Cependant, elles ont

induit des réponses immunitaires très différentes. Pour bien comprendre la protection induite par le vaccin, il fallait caractériser les mécanismes immunitaires sous-jacents. En utilisant un test *in vitro* de mort parasitaire, nous avons montré que l'effet antiparasitaire chez les animaux immunisés avec Sm-Cathepsine B + Montanide nécessitait la présence à la fois de cellules $CD45^+$ (leucocytes) et d'anticorps; suggèrant ainsi un mécanisme à médiation cellulaire dépendant d'anticorps. De manière différente, la mortalité élevée des parasites dans le groupe Sm-Cathepsine B + CpG, ainsi qu'un groupe Sm-Cathepsine B sans adjuvant, était indépendante de la présence d'anticorps; suggèrant donc un effet cellulaire dominant. Une caractérisation plus approfondie a révélé les différentes populations cellulaires responsable de la protection induite par les diverses formulations vaccinales. L'effet antiparasitaire observé avec le groupe Sm-Cathepsine B + Montanide était dépendant de la présence de cellules T $CD4^+$ et de cellules tueuses naturelles (NK). Les cellules T $CD4^+$ activent probablement les cellules NK qui sont ensuite impliquées dans la cytotoxicité à médiation cellulaire dépendante des anticorps. Pour le groupe Sm-Cathepsine B + CpG, les cellules T $CD8^+$ étaient les principaux effecteurs intervenant dans la destruction des parasites. Enfin, la mort des parasites dans le groupe Sm-Cathepsine B sans adjuvant était médiée par des cellules T $CD4^+$. En résumé, nous avons démontré l'efficacité protectrice de deux différents vaccins contenant Sm-Cathepsine B et nous avons commencé à caractériser les mécanismes immunitaires responsable de la protection induite par nos vaccins Sm-Cathepsine B. Dans l'ensemble, les résultats de cette thèse représentent des progrès significatifs dans le développement et la comprèhension d'une formulation vaccinale potentielle contre la schistosomiase.

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Contributions of authors

The candidate has chosen to present a manuscript-based thesis. This thesis contains three manuscripts and is in accordance with the "Guidelines for Thesis Preparation" provided by the Faculty of Graduate and Postdoctoral Studies of McGill University. The candidate, A Ricciardi, is recognized as the principal author and to have performed the majority of the work of the manuscripts presented. The specific contributions of authors are as follows:

Chapter 1:

Section 1 contains extracts from **Ricciardi A**, Ndao M (2015) Diagnosis of parasitic infections: what's going on? J Biomol Screen 20(1): 6-21.

Extracts from this review are reprinted in this thesis from the Journal of Biomolecular Screening with permission from SAGE Publications. The percentage contributions of authors in the preparation of the final manuscript were as follows: A Ricciardi (70%), M Ndao (30%).

Section 4 contains **Ricciardi A**, Ndao M (2015) Still hope for schistosomiasis vaccine. Hum Vaccin Immunother 11(10): 2504-2508.

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Chapter 2:

Ricciardi A, Dalton JP, Ndao M (2015) Evaluation of the immune response and protective efficacy of Schistosoma mansoni cathepsin B in mice using CpG dinucleotides as adjuvant. Vaccine 33(2): 346-353.

This manuscript is reprinted from the journal Vaccine with permission from Elsevier. The experiments were designed by M Ndao and A Ricciardi. JP Dalton provided the initial tools needed for protein expression and provided advice concerning experimental design. A Ricciardi performed 100% of the experiments. The percentage contributions of authors in the preparation of the final manuscript were as follows: A Ricciardi (75%), JP Dalton (5%), M Ndao (20%).

Chapter 3:

Ricciardi A, Visitsunthorn K, Dalton JP, Ndao M (2016) A vaccine consisting of Schistosoma mansoni cathepsin B formulated in Montanide ISA 720 VG induces high level protection against murine schistosomiasis. BMC Infect Dis 16: 112.

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Chapter 4:

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The experiments were designed by M Ndao and A Ricciardi. JP Dalton provided the initial tools needed for protein expression. The percentage contributions of authors in the experiments performed were as follows: A Ricciardi (85%), N Zelt (10%), K Visitsunthorn (5%). A Ricciardi performed all of the experiments. N Zelt and K Visitsunthorn helped with the animal immunizations and cell isolations/depletions. The percentage contributions of authors in the preparation of the final manuscript were as follows: A Ricciardi (75%), JP Dalton (5%), M Ndao (20%).

Contributions to original knowledge

The work presented in this thesis contributes original knowledge to the fields of anti-schistosomiasis vaccine design and immune mechanisms of vaccine induced protection. The specific contributions are as follows:

1. We showed that immunizations with recombinant Sm-Cathepsin B in combination with CpG dinucleotides significantly reduced parasite burden (worms, hepatic eggs, and intestinal eggs) in a mouse model of schistosomiasis.

2. We demonstrated that Sm-Cathepsin B + CpG immunizations elicited a biased Th1 immune response characterized by the dominant presence of the IgG2c antibody subclass and increased secretion levels of Th1 cytokines.

3. We showed that parasite burden (worms, hepatic eggs, and intestinal eggs) was also significantly reduced by immunizations with recombinant Sm-Cathepsin B + Montanide ISA 720 VG.

4. Protection by immunizations with Sm-Cathepsin B + Montanide ISA 720 VG was associated with a mixed Th1/Th2 response characterized by robust IgG1 titers and increased secretion levels of both Th1 and Th2 cytokines.

5. We provided evidence that protection induced by unadjuvanted Sm-Cathepsin B immunizations is dependent on $CD4^+$ T cells.

6. We demonstrated that $CD8^+$ T cells are the main effectors mediating protection elicited by Sm-Cathepsin B + CpG immunizations. Moreover, we suggested that NK cells play a supportive role, and that the mechanism is antibody independent.

7. We showed that protection induced by Sm-Cathepsin B + Montanide ISA 720 VG immunization is antibody dependent and mediated by NK cells that are potentially activated by $CD4^+$ T cells.

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List of abbreviations

ADCC	Antibody-dependent cell-mediated cytotoxicity
AL	Alabama
Ala	Alanine
APC	Allophycocyanin
Arg	Arginine
Asn	Asparagine
Asp	Aspartic acid
ASP-2	*Ancylostoma* Secreted Protein 2
BC	Before Christ
BMGY	Buffered Glycerol-complex Medium
BUV	Brilliant Ultraviolet
ºC	degree Celsius
CA	California
CAA	Circulating Anodic Antigen
CB	Cathepsin B
CCA	Circulating Cathodic Antigen
CCL5	C-C motif Chemokine Ligand 5
CCR5	C-C Chemokine Receptor type 5
CD	Cluster of Differentiation
cDNA	complementary Deoxyribonucleic Acid
CNS	Central Nervous System
CO_2	Carbon dioxide
CTGF	Connective Tissue Growth Factor
CTL	Cytotoxic T Lymphocyte
CXCR4	C-X-C Chemokine Receptor type 4

Cys	Cysteine
DALY	Disability-Adjusted Life Year
DC	Dendritic Cell
DC-SIGN	Dendritic Cell-Specific Intracellular adhesion molecule-3-Grabbing Non-integrin
DNA	Deoxyribonucleic Acid
EC-2	Extracellular loop 2
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-Linked Immunosorbent Assay
FBS	Fetal Bovine Serum
FcγR	Fc gamma receptor
fg	femtogram
FGS	Female Genital Schistosomiasis
FITC	Fluorescein isothiocyanate
GLA	Glucopyranosyl Lipid Adjuvant
Glu	Glutamic acid
GM-CSF	Granulocyte Macrophage Colony-Stimulating Factor
GMP	Good Manufacturing Practice
HBSS	Hank's Balanced Salt Solution
HBV	Hepatitis B Virus
HCV	Hepatitis C Virus
HHVI	Human Hookworm Vaccine Initiative
His	Histidine
HIV	Human Immunodeficiency Virus
HLA	Human Leukocyte Antigen
HRP	Horseradish Peroxidase
HSP	Heat Shock Protein
IARC	International Agency for Research on Cancer

IFNɣ	Interferon gamma
IgE	Immunoglobulin E
IgG	Immunoglobulin G
IHA	Indirect Heamagglutination
IL	Interleukin
iNOS	inducible Nitric Oxide Synthase
JPD	John Pius Dalton
kDa	kilodalton
KO	Knock-Out
KOH	Potassium Hydroxide
lac	lactose
M	Molar
MA	Massachusetts
MALDI-TOF	Matrix-Assisted Laser Desorption Ionization Time-of-Flight
MD	Maryland
MDA	Mass Drug Administration
MEM	Minimum Essential Medium
ml	milliliter
mM	milliMolar
MO	Missouri
mRNA	messenger Ribonucleic Acid
MS	Mass Spectrometry
N	Normality
NaCl	Sodium chloride
Na/K	Sodium/Potassium
NF-κB	Nuclear Factor kappa-light-chain-enhancer of activated B cells
ng	nanogram

Ni-NTA	Nickel-Nitrilotriacetic Acid
NIAID	National Institute of Allergy and Infectious Diseases
nirB	nitrite reductase B
NJ	New Jersey
NK	Natural Killer
nm	nanometer
ns	not significant
NTD	Neglected Tropical Disease
OD	Optical Density
ON	Ontario
OXA	Oxamniquine
PA	Pennsylvania
pagC	phoP activated gene C
PBS	Phosphate Buffered Saline
PCR	Polymerase Chain Reaction
PE	Phycoerythrin
PerCP	Peridinin-chlorophyll proteins
pH	potential of Hydrogen
Phe	Phenylalanine
Pro	Proline
PZQ	Praziquantel
QC	Quebec
RA	Radiation Attenuated
RAG	Recombination Activating Gene
RNAi	Ribonucleic Acid interference
rpm	revolutions per minute
rRNA	ribosomal Ribonucleic Acid

SE	Stable Emulsion
SE	Standard Error
SEA	Soluble Egg Antigens
SELDI-TOF	Surface-Enhanced Laser Desorption Time-of-Flight
Ser	Serine
Sh28GST	*Schistosoma haematobium* 28 Glutathione-S-Transferase
siRNA	small interfering Ribonucleic Acid
SjCD	S*chistosoma japonicum* Cathepsin D
SGTP	Schistosome Glucose Transporter Protein
SmAE	*Schistosoma mansoni* Asparagyl Endopeptidase
SmCB	*Schistosoma mansoni* Cathepsin B
SmCC	*Schistosoma mansoni* Cathepsin C
SmCD	*Schistosoma mansoni* Cathepsin D
SmCE	*Schistosoma mansoni* Cercarial Elastase
SmCL	*Schistosoma mansoni* Cathepsin L
SmDLC	*Schistosoma mansoni* Dynein Light Chain
SmGPX	*Schistosoma mansoni* Glutathione Peroxidase
SmPrx1	*Schistosoma mansoni* Peroxiredoxin 1
SmSOD	*Schistosoma mansoni* Superoxide Dismutase
Smteg	Schistosomulum tegument
SmTOR	*Schistosoma mansoni* Tetraspanning Orphan Receptor
Sm-TSP-2	*Schistosoma mansoni* Tetraspanin 2
SMYB1	*Schistosoma mansoni* Y-box-binding protein
SNP	Single Nucleotide Polymorphism
SopE2	*Salmonella* outer protein E2
STAT	Signal Transducer and Activator of Transcription
STH	Soil-Transmitted Helminth

SVLPB	*Schistosoma* very low-density lipoprotein-binding protein
TDR	Programme for Research and Training in Tropical Diseases
TDU	Tropical Disease Unit
Th	T-helper
TIP	T cell Immunomodulatory Protein
TLR	Toll-Like Receptor
TPI	Triose Phosphate Isomerase
Treg	Regulatory T cell
UCP-LF	Up-Converting Phosphor-Lateral Flow
µg	microgram
µl	microliter
µm	micrometer
USA	United States of America
USD	United States Dollar
UT	Utah
Val	Valine
WHO	World Health Organization
x g	times gravity
YLD	Years Lived with a Disability
YLL	Years of Life Lost due to premature mortality

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Chapter 1: Literature review and research objectives

1.1 Schistosomiasis general considerations: from ancient civilizations to the golden age of medical research

1.1.1 Brief history

Schistosomiasis is a fresh-water-borne parasitic disease caused by trematode worms of the genus *Schistosoma*. There are three species, *S. mansoni*, *S. haematobium*, and *S. japonicum*, which account for the majority human schistosomiasis cases [1]. Schistosomiasis is a disease that affected the great civilizations of antiquity, and it likely evolved with the evolution of mankind. It is believed that the origin of schistosomes lies in the region of the African great lakes [2]. The disease is suggested to have developed in central Africa where both humans and monkeys acted as definitive hosts for the parasite [2]. At some time in history, the parasite spread to Egypt as there are ancient Egyptian texts describing a disease similar to schistosomiasis as early as the sixteenth century B.C. [3]. The disease was called "â-a-â" and was mentioned numerous times in medical papyri [3, 4]. The parasite likely spread to Egypt sometime between the eighteenth (1543-1292 B.C.) and twentieth (1182-1151 B.C.) dynasties when Egyptians embarked on frequent voyages to the Punt trading emporium on the coast of eastern Africa [2]. Monkeys and slaves, likely infected with schistosomes, were transported back to Egypt. The cargoes arriving from Punt were sent to Thebes, the capital of Upper Egypt, and goods, as well as slaves, were then distributed to other regions in Upper and Lower Egypt. In order to thrive in a geographic region, schistosomes require snail intermediate hosts. It is believed that the snails originated from Ethiopia, and were then naturally carried down along the Nile [2]. Once the snails reached Upper Egypt, they were likely disseminated throughout the Delta region with the help of traveling Egyptians. It was only in 1851 that German physician Theodore Bilharz observed *S. haematobium* worms while working in Cairo. During an autopsy, he discovered the adult worms in the pelvic veins of a recently deceased person [3]. He went on to establish a causal relationship between these parasitic worms and the hematuria that was plaguing Egypt [3]. By the fourth dynasty, the Egyptians had perfected mummification which can preserve tissues long after death. In 1910, Marc Armand Ruffer identified *S. haematobium* eggs as well as urinary bladder calcifications in two mummies from the twentieth

dynasty; thus confirming the presence of schistosomiasis in ancient Egypt [3, 4]. New techniques have been since developed to detect schistosome antigens in mummified tissues [5].

Schistosomiasis has a long history in China as well. There is little information concerning the arrival of schistosomes in the country. However, in 1971, *S. japonicum* eggs were discovered in a female corpse dating back to the Western Han dynasty, approximately 2100 years ago [6-8]. These observations confirm the presence of schistosomiasis in ancient China. Furthermore, Chinese medical texts dating back to 400 B.C., describe an illness resembling acute schistosomiasis (Katayama Fever) [8]. The first reported clinical diagnosis of schistosomiasis in modern China occurred in 1905 in the Hunan Province. The following year, 1906, the Philippines had their first reported case of schistosomiasis [8].

It is believed that schistosomiasis was brought to South America and Europe through the slave trade [9, 10]. There is currently no evidence of schistosomiasis in Brazil prior to the arrival of Columbus [9]. The first record of intestinal schistosomiasis in Europe is linked to the discovery of *S. mansoni* eggs in a latrine dating back to 1450-1500 in Montbéliard, France [10]. The spread of schistosomiasis in Europe is greatly attributed to the slave trade. However, the *S. mansoni* containing latrine in France belonged to an affluent family [10]. Therefore, it is also possible that this family was infected during their travel to Africa for pleasure.

1.1.2 Lifecycle

The schistosome lifecycle was described by British scientist Robert Leiper in 1915 (Figure 1.1) [11, 12]. The lifecycle begins when parasite eggs are excreted into freshwater by an infected host. The eggs will hatch and release the miracidia (140 x 55 μm) which are the free-swimming larval forms of the parasite that infect the intermediate host [13]. The life cycle of the parasite requires a snail intermediate host. Interestingly, the different *Schistosoma* species infect different snail species; thereby, adding an extra level of specificity to the cycle [14]. For instance, *S. mansoni* miracidia specifically infect *Biomphalaria* species of snails whereas *S. japonicum* miracidia infect *Oncomelania* species of snails and *S. haematobium* is associated to *Bulinus* species of snails. Within the snail, there will be two generations of sporocysts and the generation of cercariae. Depending on the ambient temperature and light, the infected snails will shed cercariae (300 μm-500 μm in length), which are the larval forms of

the parasite that infect humans by penetrating the skin [13]. This penetration is facilitated by the secretion into the epidermis of enzymes and immunomodulating factors contained in the cercarial acetabular glands [15, 16]. Once inside the human host, the cercariae shed their forked tail and replace their tegument membranes in order to transform into the migrating larval schistosomulae. The kinetics of migration through the skin are represented by a mean duration of stay in the epidermis of 53 hours and approximately 18 hours in the dermis [17]. The schistosomulae penetrate venules in the skin, and they will travel to the pulmonary vasculature via venous blood flow in a semi-quiescent metabolic state [18]. The parasite arrives in the lungs between two to seven days post infection. The first passage through the pulmonary capillary bed requires three to six days, and subsequent passages are completed within approximately 35 hours [19]. To adapt to the narrow capillaries, the schistosomulae elongate and lose their mid-body spines [19]. Some migrating schistosomulae may become lost as a result of fragile capillary walls that cause shuttling of the larva to the alveolus [19]. Once the parasite exits the lungs, it travels to the systemic organs (transit time of 11-16 hours) and the splanchnic capillary beds (transit time of 7-9 hours) [19]. Once the parasites reach the portal system, they transform into blood-feeding, growing juveniles. The maturing juveniles undergo a three week stay in the portal vessels before females and males mate and migrate together to their final destination [19]. Adult worms reside *in copula*, the slender female (10 mm-20 mm in length) fitted into the male (6 mm-13 mm in length) gynecophoric canal, in mesenteric venules [13]. Different species prefer different locations in the host. *S. mansoni* adult worm pairs are commonly found in the mesenteric veins draining the large intestine whereas *S. japonicum* and *S. haematobium* adults can be found in the superior mesenteric veins draining the small intestine and the venous plexus of the bladder, respectively. Adult worms can live in their host for over ten years. The adult female worms lay hundreds to thousands of eggs (60 μm-140 μm) per day; *S. mansoni* females lay 100-300 eggs/day, *S. japonicum* females lay 500-3500 eggs/day, and *S. haematobium* females lay 20-200 eggs/day [20]. The different schistosome species can be differentiated by the morphology of their eggs (Figure 1.2). These eggs may be excreted in the urine (*S. haematobium*) or feces (*S. mansoni* and *S. japonicum*); which can perpetuate the lifecycle if released in an appropriate water source harbouring the intermediate host. However, more than half of the eggs become trapped in tissues, such as intestinal, hepatic, or urinary tissues, and cause disease.

1.1.3 Epidemiology

More than 200 million people are infected worldwide and over 700 million people are at risk of infection [21]. Unfortunately, these numbers are believed to be underestimates of the reality of schistosomiasis. Current diagnostic tools at times cannot detect low intensity infections, and due to the remote locations of some endemic villages, several cases go unreported. Schistosomiasis is the second most influential and socioeconomically devastating parasitic disease after malaria [22]. The 2010 estimated global burden of schistosomiasis was reported to be 3.3 million disability-adjusted life years (DALYs) (Table 1.1) [23]. This high DALYs rate is influenced by the various schistosomiasis associated conditions seen in patients such as anemia, chronic pain, diarrhea, exercise intolerance, and malnutrition. Furthermore, in affected children, the infection interferes with growth and cognitive development [24-26].

The six *Schistosoma* species known to infect humans are *S. mansoni*, *S. haematobium*, *S. japonicum*, *S. mekongi*, *S. intercalatum*, and *S. guineensis* (the first three being the major species that infect humans) [1]. The infection is most prevalent in tropical and sub-tropical regions where water sanitation and hygiene conditions are poor; thus schistosomiasis is considered an illness of the poor and is one of the neglected tropical diseases (Figure 1.3) [27, 28]. The geographic distribution of schistosomiasis varies according to the species in questions. The *S. mansoni* distribution includes Africa, the West Indies, and South American countries such as Brazil and Venezuela whereas *S. haematobium* is commonly found in Africa, the Middle East, and India [24, 29]. *S. mekongi* is found exclusively in the Mekong river basin of Laos and Cambodia whereas *S. intercalatum* and *S. guineensis* have limited distributions in Central and West Africa [1]. Over 90% of the schistosomiasis cases occur in sub-Saharan Africa [21, 30]. Differently, *S. japonicum* is found China, Indonesia, Japan, and the Philippines [24, 29]. There are also veterinary considerations when dealing with *S. japonicum* infections. There are over forty domestic and wild animals that can act as reservoirs for the parasite. It is estimated that these animal reservoirs are responsible for over 80% of transmission [31].

Over the years, the geography of schistosomiasis has been significantly impacted by water resource development, movement of populations, and climate change. Steinmann *et al.* have reviewed how the development of water resources can intensify schistosome transmission or introduce the parasite in previously non-endemic areas [21]. The development of irrigated

areas and large dam reservoirs result in the expansion of snail habitats. There are examples of *S. haematobium* prevalence increasing in Côte d'Ivoire, Ghana, and Nigeria after the construction of dams [21]. The most significant effect of schistosomiasis expansion as a result of water resource development, in recent years, occurred in northern Senegal. After the construction of the Diama dam in 1985, *S. haematobium* prevalence increased, and the previously absent *S. mansoni* was introduced in the region [21, 32]. The movement of populations also represents an important factor in the changing geography of schistosomiasis. It is believed that the parasite was introduced in Djibouti by refugees [33]. Portugal experienced *S. haematobium* outbreaks between 1920 and 1967 that were believed to be a result of people travelling between Angola and Portugal [21]. However, there have been no cases of schistosomiasis in Portugal since the 1970s. Since 2013, there have been *S. haematobium* outbreaks on the French Mediterranean island of Corsica. The cases were diagnosed in France and Germany in patients that had never travelled to schistosomiasis endemic regions [34-36]. The cases were all traced back to the Cavu River. Molecular analysis of the parasites found in this location showed a close genetic relationship to strains found in northern Senegal [37]. Senegal was a French colony up until 1958, and there are still close ties between the two countries. There is a lot of travel between Senegal and France as the African country is a popular vacation destination for French nationals [37]. *S. haematobium* cases were detected in Corsica during the summer of 2015 indicating the transmission is persisting [38]. Finally, there is some evidence suggesting that climate changes are expanding snail habitats further north in regions where schistosomiasis was previously absent [39, 40].

Schistosomiasis is not a problem restricted solely to the tropics. The increase in international travel and the arrival of new immigrants from endemic areas has made this parasitic disease a reality in Canada as well. Between November 1997 and June 2003, 3528 Canadians who returned from international travel or newly arrived immigrants were examined at the Tropical Disease Unit (TDU) of Toronto General Hospital and were entered into a database. Schistosomiasis accounted for approximately 5% of the parasitic infections observed at the TDU [41]. Immigrants represented approximately 30% of the schistosomiasis cases whereas Canadians who travelled for touristic reasons made up 35% of the cases observed [41]. These findings demonstrate that *Schistosoma* infections do in fact have an impact on Canadians and a better awareness concerning this parasitic disease is needed. Moreover, species known to

infect birds and mammals in temperate climates can potentially cause cercarial dermatitis, also known as swimmer's itch, in humans [14]. Humans are not this parasite's preferred host, but if the cercariae come into contact with a human, they will burrow into the skin, die immediately, and cause an allergic reaction resulting in a rash.

1.1.4 Spectrum of disease

1.1.4.1 Acute schistosomiasis: Katayama syndrome

The acute form of schistosomiasis, also known as Katayama Syndrome, was first described over one hundred years ago in Japan [42]. This form of the disease presents itself approximately 14-84 days post infection and is most commonly seen in young children or individuals with no previous exposure. However, there are some exceptions as cases of Katayama Syndrome are reported in regions of China where *S. japonicum* is endemic and infection intensities are high [42]. Acute schistosomiasis is a systemic hypersensitivity reaction against the migrating schistosomulae and early oviposition. The syndrome presents itself as nonspecific symptoms such as fever, fatigue, swollen lymph nodes, and malaise [43]. These symptoms subside after 2-10 weeks [42]. However, some individuals develop a persistent form of the syndrome which results in weight loss, general rash, abdominal pain, and hepatomegaly [42].

1.1.4.2 Intestinal schistosomiasis

Manifestation of intestinal schistosomiasis is caused by the *S. mansoni* and *S. japonicum* species. Parasite eggs become trapped in the liver and in the intestines. The inflammation caused by the eggs in the intestinal tissues leads to the formation of severe lesions and colonic polyps [44-46]. The liver is the major area of focus for this form of the disease. Schistosome eggs are carried to the liver by the portal vein blood flow and they become trapped in the hepatic tissue. The release of egg antigens elicits a delayed type hypersensitivity response and the subsequent formation of granulomas (Figure 1.4) [47]. The granulomas protect the host tissues from injury by maintaining and neutralizing the hepatotoxic molecules released by the parasite eggs [48]. Early infection is marked by proliferation of endothelial cells in hepatic tissue and formation of large and edematous granulomas [47, 49-51]. Over time, the granulomas become smaller and fibrotic as seen by the accumulation of

extracellular matrix and collagen. Portal fibrosis is the main feature of intestinal schistosomiasis. This clinical manifestation, which looks like white plaques on the surface of the liver, is often referred to as Symmers pipestem fibrosis after the British pathologist who described it in 1904 [52]. The main consequence of portal fibrosis is portal hypertension which can be followed by splenomegaly, esophageal varices, and ascites (Figure 1.5A) [47]. If left unmanaged, chronic intestinal schistosomiasis can lead to peripheral destruction of the portal vein system, gastric hemorrhage from ruptured esophageal varices, and hepatic failure [47].

1.1.4.3 Urogenital schistosomiasis

More individuals are infected with *S. haematobium* than with any of the other major schistosome species combined [53]. *S. haematobium* is the causative agent of urogenital schistosomiasis. Parasite eggs may become trapped in tissues of the urinary bladder, lower ureters, seminal vesicles, vas deferens, prostate, and female genitals [54]. Similarly to intestinal schistosomiasis, the release of egg antigens elicits a delayed type hypersensitivity response which results in the formation of granulomas in the affected tissues. During the early phase of infection, inflammatory lesions can be observed by cystoscopy. These lesions consist of mucosal granulomas which form tubercles or nodules which can ulcerate [54]. Secondary bacterial infections represent a serious problem. In *S. haematobium* endemic areas, *Salmonella* organisms are the major causative agents of resistant secondary bacterial cystitis [55]. In the later phase of infection, the lesions become fibrotic or calcified. The clinical symptoms associated to these lesions in the urinary bladder include hematuria and painful urination (Figure 1.5B) [54]. Fibrotic changes affecting the bladder's sub mucosa and muscle layers can result in the development of urodynamic disorders. Bladder neck obstruction represents a complication that can arise from the deposition of eggs in the urethra [54]. In some cases, the kidneys may become affected, due to back pressure effects, and cause obstructive nephropathy. Kidney involvement has also been reported with some cases of *S. mansoni* infections. Approximately 12%-15% of individuals with hepatosplenic schistosomiasis develop glomerular lesions [47, 56, 57]. *S. haematobium* eggs can also become trapped in the female lower genital tract and cause inflammatory lesion in the uterus, cervix, vagina, or vulva [58]. Approximately 75% of women infected with *S. haematobium* develop female genital schistosomiasis (FGS)

[59]. FGS causes contact bleeding, pain during intercourse, and decreased fertility [59]. Furthermore, FGS has been linked to increased susceptibility to HIV infection [60, 61].

S. haematobium is classified as a group 1 carcinogen by the International Agency for Research on Cancer (IARC) because of its link to bladder cancer [62]. This association is based on numerous case control studies and correlations between bladder cancer incidence and *S. haematobium* prevalence in different locations [62]. Infections are associated with high incidence rates of squamous cell carcinoma of the urinary bladder [62]. The incidence rate of schistosomiasis associated squamous cell carcinoma is approximately 3-4 cases per 100,000 [59]. This is the most common type of bladder cancer in rural African regions where *S. haematobium* is endemic. In more developed countries, transitional cell carcinoma is the most common form of bladder cancer [59]. Between 1969 and 1990, the evaluation of 1026 cases of bladder cancers in Egypt revealed that squamous cell carcinomas represented 60% of the cases, and *S. haematobium* eggs were found in over 85% of these cases [63]. Another investigation into bladder cancer in Egypt evaluated 10,000 patients and found that the link to *S. haematobium* infection dropped to 55.3%. Moreover, transitional cell carcinoma was the most common form of the malignancy (65.8%) [64]. It has been suggested that this difference over the years might be related to better schistosomiasis management and treatment [54]. These observations in Egypt further strengthen the link between *S. haematobium* infection and squamous cell carcinoma of the urinary bladder. The mechanisms linking schistosomiasis and cancer are not well defined. Individuals infected with *S. haematobium* have elevated levels of estradiol [65]. However, this effect is due to an *S. haematobium*-derived estradiol-like molecule. Furthermore, it was demonstrated that *S. haematobium* worms and eggs produce estrogenic molecules [66]. This could be the link to urinary bladder cancer. Estrogen metabolites can have genotoxic effects, and the estrogen-DNA adduct-mediated pathway could potentially lead to host cell DNA damage [59].

1.1.4.4 Neuroschistosomiasis

Neuroschistosomiasis is one of the most severe manifestations of the disease and can occur during infection with any of the three main schistosome species; *S. mansoni*, *S. haematobium*, *S. japonicum* [67]. It has been reported that less than 5% of schistosomiasis patients present central nervous system (CNS) symptoms [20, 67-69]. Neuroschistosomiasis

can be divided into two clinical manifestations: cerebral schistosomiasis and spinal schistosomiasis. To cause neuroschistosomiasis, the parasite eggs must reach the CNS. During acute schistosomiasis, the female worms are not laying eggs yet; therefore, it is believed that any neurological symptoms at this stage are due to eosinophil-mediated toxicity which causes vasculitis and small-vessel thrombosis [70, 71]. If adult worm pairs migrate to sites close to the CNS and begin oviposition, eggs may be shuttled to the brain or spinal cord via retrograde venous flow in the Batson venous plexus. Differently, massive embolization of parasite eggs can occur from the mesenteric-pelvic system [20, 67, 68, 72]. The entrapment of parasite eggs in CNS tissues leads to a cell-mediated CD4+ T cell driven granulomatous response as seen in other affected tissues (hepatic, intestinal, and urinary tissues) [67]. Fibrotic changes cause severe tissue damage, and large egg clusters will calcify within the CNS.

Cerebral schistosomiasis is most commonly associated to infections with *S. japonicum*. Cerebral schistosomiasis was first described by Yamagiwa in 1889 [67]. Today, approximately 4% of schistosomiasis patients in China have CNS involvement [67]. Patients suffering from cerebral schistosomiasis present with symptoms such as fever, headache, speech disturbances, and motor weakness. The main neurological features of the disease are encephalopathy and seizures [67, 68, 72]. *S. mansoni* and *S. haematobium* eggs are retained in the lower spinal cord due to their larger size and presence of lateral and apical spines, respectively [67]. Patients develop myelopathy and present with symptoms such as lumbar pain, lower limb radicular pain, muscle weakness, sensory loss, and bladder dysfunction [67]. The first case of spinal schistosomiasis was diagnosed in a German patient who had visited Brazil in 1930 [72]. Since then, there have been over 500 reported cases [68]. However, this number is believed to be a significant underestimate as there is very little awareness of the link between schistosomiasis and the CNS. In fact, spinal schistosomiasis can be mistaken for a spinal cord neoplasm or tuberculosis due to the manifestation of progressive paraparesis [67, 72]. *S. mansoni* and *S. haematobium* infection may also be linked to cerebral schistosomiasis involving the cerebral and cerebellar cortex as well as the leptomeninges [67, 68, 72]. *S. mansoni* infections and cerebellum involvement has been increasing in Brazil [73].

1.1.4.5 Schistosomiasis-associated conditions

The schistosomiasis morbidity burden is greatly worsened by disease-associated conditions such as anemia and cognitive impairment. Severe *S. mansoni* infections, characterized by hepatosplenomegaly, are linked to consistent hematologic changes such as the development of anemia, neutropenia, and thrombocytopenia [74]. Although more thorough investigations are needed, *S. mansoni* associated anemia is suggested to be a result of hypersplenism and hemodilution from the massive splenomegaly [74]. In the case of *S. haematobium* infections, persistent hematuria is a hallmark of the disease, and it is known to result in the development of iron deficiency anemia. Furthermore, children affected by schistosomiasis demonstrate features associated with anemia of chronic disorders [74]. The negative impact of schistosomiasis on children goes beyond physical health. Numerous studies have demonstrated that parasitic helminth infections in children result in decreased cognitive functions and poorer performances in school [75-80]. *S. mansoni*, *S. haematobium*, and *S. japonicum* have shown to have a negative impact on the cognitive development of children. A study conducted in the Kafr El Sheikh Governate of Egypt compared the cognitive capabilities of *S. mansoni* infected and uninfected children (9-12 years old) [81]. The study revealed that infected children obtained poorer scores on comprehension, vocabulary, and picture completion tests when compared to their uninfected counterparts [81]. Similarly, a study performed in Tanzania demonstrated that school children (9-14 years old) with heavy *S. haematobium* infections obtained significantly lower scores on verbal short-term memory tests and reaction time tests compared to control groups [82]. Interestingly, Nokes *et al.* demonstrated that treatment could significantly ameliorate some infection-related cognitive damage in younger children [83]. The study was conducted in Sichuan, China where *S. japonicum* is endemic. Three months after treatment, primary school children (5-7 years old) showed significant improvements in the following cognitive function tests: Fluency, Free Recall, and to a lesser extent Picture Search [83]. These are all components of the central executive part of working memory. Therefore, it is suggested that schistosomiasis treatment of young children could improve the impaired development of working memory [83].

1.1.5 Immunopathology

Schistosomiasis pathology results from the host's immune response to parasite eggs becoming trapped in tissues (Figure 1.6). The parasite egg secretions are a source of constant

antigen stimulus and elicit a granulomatous response that is collagen rich [84]. The granuloma represents a compromise as it has a host-protective function as well. Especially for *S. mansoni*, the egg secretions are hepatotoxic. The granuloma contains these molecules and neutralizes them to prevent excessive tissue damage. Mouse models of schistosomiasis have demonstrated that immunocompromised mice, such as T cell deficient mice, cannot mount a granulomatous response and therefore die sooner compared to wild type mice [85, 86]. The granulomas around the parasite eggs prevent acute death of the host; thus, allowing parasite persistence. During schistosome infection, there is a striking shift from a Type 1 to a Type 2 T-helper response (Th1 to Th2) with the onset of oviposition [87]. The pathology due to development of fibrosis during schistosomiasis is mediated by Th2 cytokines [84, 88, 89]. Mouse models have been crucial in elucidating the importance of Th2 responses in schistosomiasis pathology. Studies using IL-4Rα and signal transducer and activator of transcription (STAT) 6 deficient mice have shown that the IL-4/IL-13 mediated STAT6 pathway is essential for the development of the granulomatous response and subsequent pathology in schistosomiasis [90]. Interestingly, a polarized Th1 response is not beneficial to mice challenged with the parasite. In fact, the unchecked Th1 response that occurs in mice lacking both IL-10 and IL-4 results in 100% mortality by week nine post infection [91]. In mice that are lacking IL-10 and IL-12, and thus have a polarized Th2 response, there is significant mortality by 12-15 weeks post infection [91]. These observations indicate that any polarized immune response during schistosomiasis is detrimental. The role of the Th17 response during schistosomiasis has only begun to be elucidated. However, studies have shown that elevated levels of IL-17 in a mouse model result in severe liver pathology after infection [92].

The immune response elicited by the parasite eggs promotes an increase in alternatively activated macrophages found in granulomas. Th1-skewed mice that have greater inducible nitric oxide synthase (iNOS) responses, and thus more classically activated macrophages, possess smaller granulomas and have elevated mortality rates [93, 94]. L-Arginine is a macrophage substrate. Alternatively activated macrophages hydrolyse arginine to promote the formation of collagen; therefore, linking the Th2 immune response, the increase in alternatively activated macrophages, and the development of fibrosis [84]. The role of alternatively activated macrophages during schistosomiasis pathology is two-fold. First, they are involved in the initial granulomatous response which sequesters the parasite eggs. Second, in the later stages of

infection, the alternatively activated macrophages can take on a more suppressive role. They are capable of rapidly consuming arginine; thus, starving other cells that require arginine such as B cells and T cells [95]. Therefore, during the chronic stage of the disease, the macrophages may have an anti-inflammatory role and suppress other effector cells [84].

IL-13 mediates fibrosis during schistosomiasis. Mice infected with *S. mansoni* have elevated levels of IL-13Rα2 in their serum and liver [96, 97]. IL-13Rα2 is a soluble high affinity binding protein that inhibits IL-13 [98]. Increases in IL-13Rα2 during schistosomiasis are driven by the elicited Th2 response to decrease the levels of IL-13 and control IL-13 mediated pathology by acting as a decoy receptor [84]. *S. mansoni* infected IL-13Rα2 knock-out (KO) mice have exacerbated fibrosis [94, 97]. The excessive pathology in these KO mice can be reversed by administering soluble IL-13Rα2-Fc [97]. During the chronic stage of the disease, the granulomas eventually begin to involute. The granulomas start to decrease in size as of week 12 post infection in mice [84]. In IL-13Rα2 KO mice, granulomas continue increasing in size, even during the chronic stage, which results in increased mortality [97]. All of this evidence indicates that IL-13 is tightly regulated during schistosomiasis.

Regulatory T cells (Tregs) limit collateral damage by managing the magnitude of the immune response to an invading pathogen. At four weeks post schistosome infection, there is a significant expansion of Tregs in the mesenteric lymph nodes of mice followed by Treg accumulation in the liver and spleen [99, 100]. Since the ratio of Tregs to effector T cells does not change after infection, it is still uncertain whether the expansion is directly induced by the parasite or simply occurring in parallel as a response to the expansion of effector cells [84, 99, 101]. Lysophosphatidylserine isolated from the parasite has been shown to stimulate IL-10 secretion by Tregs via Toll-like receptor 2 (TLR-2) signalling on dendritic cells (DCs) [102]. This suggests that the parasite can directly affect Tregs. *S. mansoni* infection of RAG KO mice that received adoptive transfers of CD25 depleted $CD4^+$cells (Tregs are depleted) results in increased weight loss, hepatotoxicity, and mortality [103]. These observations suggest that Tregs are needed to control liver pathology during schistosomiasis.

1.1.6 Co-infections

1.1.6.1 Malaria

Polyparisitism is a common phenomenon in some of the poorest regions of the world, especially in sub-Saharan Africa. Evaluating *Schistosoma* and *Plasmodium* co-infections is of particular importance as both pathogens have overlapping geography. Furthermore, the diseases which they cause, schistosomiasis and malaria, are the two most important human parasitic infections due to the devastatingly high morbidity and mortality which they are responsible for. Epidemiological studies assessing the outcome of disease in individuals co-infected with helminths and *Plasmodium* species have been contradictory. Studies performed in Senegal and Mali suggest that *S. haematobium* infections protect against severe forms of malaria [104-106]. Differently, another study performed in Senegal observed enhanced clinical malaria in individuals who were co-infected with *S. mansoni* [107]. To further complicate any interpretation, another study performed in Senegal demonstrated that the pathological outcomes associated with schistosomiasis and malaria co-infections differ between children and adults [108]. There are numerous factors to consider when dealing with human populations. These include age, parasite exposure, medical history, and duration of infections. Randomized control studies are necessary, but difficult to attain when individuals are involved due to ethical constraints. Animal models are more convenient and have been used to study schistosomiasis co-infections with malaria. Mouse models of co-infection have also generated contradicting results. Some studies suggest that schistosomiasis protects against malaria morbidity while others state that malaria pathology is enhanced during co-infections [109-111]. There are several factors that can influence the outcomes of an animal study. Some factors include the mouse strain, the parasite species, the inoculum size, the route of malaria administration, and the intensity of the helminth infection. Recently, Nyakundi *et al*. have used a baboon model to study schistosomiasis and malaria co-infections [112]. Baboons are excellent models for co-infections as they are natural hosts for *S. mansoni* and they can be experimentally infected with *P. knowlesi* [113]. Moreover, baboons develop immune responses and pathology that mimic what is observed in human schistosomiasis [114, 115]. In their study, Nyakundi *et al*. demonstrated that chronic *S. mansoni* infection protects baboons against severe malaria caused by *P. knowlesi*. The survival rate for co-infected baboons was 81.25% compared to 25% observed in *P. knowlesi* mono-infections [112]. There is a need for better experimental models to study this co-infection as both schistosomiasis and malaria have significant public health impacts.

1.1.6.2 HIV

Sub-Saharan Africa is the unfortunate common geographic setting for some of the worst infectious diseases. As previously mentioned, approximately 90% of schistosomiasis cases occur in this region of the world. In addition, over 68% of people living in sub-Saharan Africa are infected with human immunodeficiency virus (HIV) [116]. This overlap in geography poses a risk for potential co-infections. Numerous epidemiological studies in rural Africa have focused on establishing a link between FGS and HIV infection. Studies conducted in different sub-Saharan African countries concluded that FGS significantly increased a woman's odds of acquiring HIV [61, 117-119]. A recent study revealed that *S. haematobium* exposure increased the probability of an HIV-positive status by three times among females in Mozambique [120]. FGS is considered a risk for HIV infection [121]. The increased risk is believed to be linked to the inflammatory urogenital lesions caused by the parasitic infection. *S. haematobium* increases the density of $CD4^+$ T cells in the female genitals; thus, creating the optimal setting for HIV transmission [122]. Moreover, *in vitro* studies have demonstrated that an active schistosome infection increases the cell surface densities of HIV coreceptors CCR5 and CXCR4 making the cells more susceptible to the viral infection [123]. Future research needs to focus on determining whether the treatment of urogenital lesions caused by schistosomiasis can diminish HIV transmission. Differently, there is currently no significant evidence linking *S. mansoni* infections to elevated HIV transmission [116, 124].

Studies focusing on co-infections have demonstrated that individuals who have schistosomiasis and then acquire HIV experience a decrease in parasite egg excretion [125-127]. Although the number of excreted eggs decreases in co-infected individuals, the number of adult worms is comparable to schistosomiasis mono-infected individuals. Once individuals begin antiretroviral therapy and their $CD4^+$ T cell counts rise, schistosome egg excretion increases as well [128]. It would be logical to assume that HIV and schistosomiasis co-infected individuals develop more severe pathology because they are retaining a greater number of parasite eggs. However, compared to schistosomiasis mono-infected individuals, co-infected people have similar levels of fibrosis and of glutamic oxaloacetic transaminase (marker of parenchymal damage) in their serum [129]. Furthermore, the efficacy of schistosomiasis treatment is not affected by a co-infection with HIV [130]. However, a longitudinal study

performed in Kenya showed that schistosomiasis patients who were co-infected with HIV do not acquire age-dependent resistance to schistosome reinfection [131]. This observation suggests a role for $CD4^+$ T cells in resistance to reinfection.

1.1.6.3 Soil-transmitted helminths

Schistosomiasis endemic regions are often also endemic for soil-transmitted helminths (STH) such as *Ascaris lumbricoides*, *Trichuris trichiura*, and hookworm (*Necator americanus* and *Ancylostoma duodenale*). Most individuals living in these regions are often infected with several helminth species. Integrated control programs implementing the mass administration of praziquantel for schistosomiasis and albendazole for the STH are considered the best approach to the helminthiasis problem in developing countries [132-135]. Animal models of helminth co-infections have elucidated both synergistic and antagonistic relationships between parasites. For instance, in mice, it was shown that a *S. mansoni* infection followed by a *Trichuris muris* infection resulted in the expulsion of the latter parasite from the small intestine [136]. This antagonistic response may be related to the strong Th2 response elicited by the schistosome infection. Similarly, infecting mice with *S. mansoni* followed by *Strongyloides venezuelensis* led to decreased recovery of the latter from the small intestine of the mice [137]. These examples of helminth co-infections in the mouse model suggest that primary infections with *S. mansoni* create a negative environment for incoming intestinal nematodes. Differently, mice carrying an established *T. murius* infection, who were then co-infected with *S. mansoni*, had significantly higher *S. mansoni* worm and hepatic egg burdens [138]. *T. murius* seemed to alter lung cytokine expression as well as the inflammatory foci that surround the schistosomulae [138]. In this model of helminth co-infection, a chronic gastrointestinal nematode infection facilitates the migration and survival of *S. mansoni*. Animal models are indispensable research tools. However, they represent an overly simplified and controlled version of the reality. For instance, in regions endemic for schistosomiasis and STH, it is difficult to determine the sequence of infection. In the first *T. murius* [136] and the *S. venezuelensis* [137] examples, the animals were initially infected with *S. mansoni* and the STH exposure was second. In reality, this is likely not the case. Individuals living in STH endemic regions are often exposed to the parasites within their first year of life [139]. Schistosomiasis exposure depends on contact with contaminated water sources. Therefore, this is likely to occur later when children begin to play

and swim in local water sources. Several epidemiological studies have attempted to define the outcomes of different helminth co-infections. Different studies, conducted in both Africa and South America, described a positive correlation between *S. mansoni* and hookworm co-infections based on parasite egg counts [140-142]. Webster *et al.* demonstrated that individuals infected with *S. mansoni* were more susceptible to hookworm infections than their schistosome-free counterparts [143]. Differently, schistosomiasis is negatively associated to both *A. lumbricoides* and *T. trichiura* infections [142]. Future research should continue to clarify the clinical impact of helminth co-infections as well as their effect on the host immune response.

1.1.6.4 Hepatitis

Globally, Hepatitis B virus (HBV) and Hepatitis C virus (HCV) infect 1 in 12 people [144-146]. The diseases caused by these viruses result in liver damage characterized by fibrosis, cirrhosis, and decompensation. Furthermore, both viruses are associated with primary liver cancer. There is high prevalence of HBV and HCV infections in areas which are also endemic for schistosomiasis. Egypt is notable for having high prevalence of all three infections. [146] HBV/HCV co-infections with *S. mansoni* and S. *japonicum* are of particular interest as infections with these *Schistosoma* species can result in liver damage. The co-infection of *Schistosoma* species with HBV or HCV has been extensively reviewed by Abruzzi *et al*. [146]. The review examines key studies published in English between 1975 and January 2015. It has been shown that having one disease, schistosomiasis or hepatitis, does not necessarily predispose to the other [146]. Any increased risk of HBV and HCV infection in schistosomiasis patients was associated to past mass parenteral anti-schistosome therapy [147-149]. The spread of viral infections was linked to the insufficient sterilization of syringes used to administer treatment. This was especially a problem in Egypt [146]. However, it has been demonstrated that individuals co-infected with *Schistosoma* and HBV/HCV have a more severe clinical course. *Schistosoma* and HBV co-infection results in a prolonged viral carriage state and chronic hepatitis with increased levels of cirrhosis as well as mortality [146]. *Schistosoma* and HCV co-infections promote more advanced liver disease characterized by rapid development of fibrosis and greater mortality rates. Furthermore, co-infection is associated with reduced spontaneous resolution of the viral infection [146]. A prospective cohort study revealed, during

a 72-76 month follow up, that mortality rates for *S. mansoni* and HCV co-infected, HCV mono-infected, and S. *mansoni* mono-infected subjects were 48%, 12%, and 3% respectively [150]. A later prospective cohort study examined rates of liver fibrosis during 96 months of follow up [151]. The rates for *S. mansoni* and HCV co-infected, HCV mono-infected, and S. *mansoni* mono-infected subjects were 0.58 units/year, 0.1 units/year, and less than 0.1 units/year respectively [151]. These observations indicate a multiplicative rather than additive effect; thus, suggesting a synergistic relationship between *Schistosoma* and HCV during a co-infection. It has been shown that the rapid development of fibrosis during co-infection with *S. mansoni* and HCV is associated with the inability to develop early multi-specific HCV-specific $CD4^+$ Th1 responses [151]. Moreover, *S. mansoni* and HCV co-infections in individuals with hepatocellular carcinoma leads to a more aggressive course of disease [152]. Co-infected individuals developed multifocal tumors that were larger in size when compared to tumors present in HCV mono-infected patients [152]. Increased primary prevention for HBV and HCV is required in *Schistosoma* endemic regions. Awareness campaigns and greater use of the HBV vaccine could decrease the number of *Schistosoma*-HBV/HCV co-infections; thus, reducing disease severity in at risk populations.

1.1.7 Diagnostics

Excerpt adapted from *Journal of Biomolecular Screening* with permission from SAGE publications.

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1.1.7.1 Microscopy

The standard methods involved in diagnosing helminth infections rely on direct examinations of a parasite structure either by microscopy or other imaging techniques. In the case of intestinal schistosomiasis (*S. mansoni* and *S. japonicum*), diagnosis is achieved by observing parasite eggs in stool samples using the Kato-Katz technique [153]. This method involves sieving the stool sample before staining it and performing microscopic examination. It continues to be used because it is quantitative, simple, and inexpensive. However, the sensitivity of the Kato-Katz technique suffers when infection intensity is low [154]. The sensitivity of the Kato-Katz technique also suffers in the weeks post-treatment of

schistosomiasis likely due to a lower worm burden and consequently a lower egg output [155]. The FLOTAC technique has also been validated for the diagnosis of *S. mansoni* infections [156]. The protocol involves the homogenization of stool in formalin followed by filtration and the addition of a flotation solution that brings the eggs into flotation. The eggs can then be visualized by microscopy. The FLOTAC technique requires a large-bucket centrifuge; therefore, making it unsuitable for use in the field. However, the modified Mini-FLOTAC does not have this equipment restriction and still has a detection limit of 10 eggs per gram of stool [157]. Comparisons in field testing demonstrated that the Mini-FLOTAC technique detected *S. mansoni* infections with better accuracy than the Kato-Katz method [157]. Differently, urinary schistosomiasis (*S. haematobium*) is diagnosed by microscopically detecting the parasite eggs in the patient's urine. Ten milliliters of mid-day urine sample is passed through a syringe, and eggs are trapped onto a polycarbonate filter with a fine pore size of 8 µm-30 µm. The eggs are then stained with Lugol's iodine and counted by microscopy [158]. Microscopic diagnosis for both intestinal and urinary schistosomiasis is easy to perform. However, this technique lacks sensitivity when dealing with light intensity infections. Furthermore, multiple patient samples over consecutive days need to be tested in order to account for the day to day variation of egg output [158].

1.1.7.2 Serology

Serology-based diagnostic tools could allow for faster diagnosis of helminth infections. Moreover, in situations where microscopic results are confounding, serological exams could provide additional insight. Serology-based assays are important for travellers who have suspected cases of schistosomiasis. However, most reference laboratories use their own tests as the few commercially available antibody detection kits are not standardized among companies. Immunodiagnostic assays have been tested for the detection of imported schistosomiasis [159]. Indirect haemagglutination (IHA) using erythrocytes coated with *S. mansoni* worm antigens has been evaluated using sera from individuals with egg-confirmed schistosomiasis (*S. mansoni* or *S. haematobium*) as well as patients with other parasitic infections as controls for specificity. The IHA had a sensitivity of 88% for *S. mansoni* and 80% for *S. haematobium*. The sensitivity to detect co-infections was 86%. The assay had a specificity of 94.7% [159]. The same group that tested the IHA assay also evaluated the diagnostic potential of using an enzyme-linked

immunosorbent assay (ELISA) coated with *S. mansoni* egg antigens. The ELISA had a sensitivity of 93.3% for *S. mansoni* and 92.0% for *S. haematobium*. The sensitivity to detect co-infections was 93.0%. The specificity of the assay was 98.2% [159]. These two assays were also performed in combination, which increased the overall diagnostic potential. The combination of the two assays resulted in a sensitivity of 98.7% for *S. mansoni,* 96.0% for *S. haematobium*, and 98.0% for co-infections. The specificity of the assay was 97.2% [159]. These results demonstrate the high level of sensitivity and specificity that can be achieved in diagnosing schistosomiasis with a serology-based approach. Dipstick assays have also been developed for the diagnosis of schistosomiasis. One available dipstick which employs a monoclonal antibody against *S. mansoni* adult worm tegumental antigen was compared to an ELISA sandwich assay which uses the same monoclonal antibody [160]. The sensitivity and specificity of the dipstick test using urine samples were 86.7% and 90.0% respectively. Using serum samples, the sensitivity and specificity were 88.3% and 91.7% respectively [160]. The dipstick assay was compared to the sandwich ELISA (diagnostic efficacy was 90.8% and 93.3% in urine and serum samples respectively), and their diagnostic powers were statistically comparable [160]. Therefore, in a field setting, the dipstick method would be a more practical choice due to its rapid nature and simplicity. Recently, a serological assay using cercarial transformation fluid has been developed and was shown to be comparable to assays using adult worm products [161]. The use of cercarial products for an assay is more practical as they are easier to obtain than adult worm products. The cercarial transformation fluid has been used for the development of a rapid diagnostic test which was validated in different settings and demonstrated good sensitivity (75.0% and 66.7% for *S. mansoni* and *S. haematobium* infections respectively) [162]. The urine circulating cathodic antigen (CCA) dipstick represents another rapid diagnostic method for schistosomiasis. This assay yielded sensitivity and specificity ranging from 52.5%-63.2% and 57.7%-75.6% respectively [163]. Sousa-Figueiredo *et al.* demonstrated that this test achieved much higher sensitivity levels than that observed with microscopy (16.7%) [163]. Therefore, the dipstick assay may be a better choice in areas of low transmission. There is a new and promising monoclonal antibody based antigen detection assay, which uses up-converting phosphor-lateral flow (UCP-LF) technology to detect parasite excreted circulating anodic antigen (CAA) or CCA in serum or urine at low levels [164]. The UCP-LF CAA assay was used in China for the screening of *S. japonicum* cases and

demonstrated that, in comparison, the Kato-Katz method underestimated infection prevalence by a factor of ten (11.8% positive results for serum UCP-LF CAA versus 1.4% positive results for Kato-Katz) [165]. The UCP-LF CAA test was also used in Zanzibar, United Republic of Tanzania, to detect low infection intensity cases of *S. haematobium* in areas close to elimination [166]. The assay has also been converted to a dry reagent format which can be stored at ambient temperature; therefore, making it suitable for worldwide shipping and use [167].

1.1.7.3 Molecular techniques

Molecular techniques could render the diagnosis of helminth infections more sensitive and specific. Polymerase chain reaction (PCR) protocols have been developed in order to diagnose schistosomiasis. Abath *et al.* were able to detect *S. mansoni* in fecal specimens by using primers designed to amplify a tandem repeat DNA sequence [168]. No amplification was observed with other helminths; thereby, demonstrating the specificity of the reaction. Furthermore, the detection limit of the PCR was shown to be 2 eggs/gram of sample [168]. It would not be possible to detect such a low concentration of parasite eggs by the Kato-Katz technique. A real-time PCR protocol has also been developed using primers that target the small subunit rRNA of *S. mansoni* [169]. The specificity of the reaction was shown by the lack of amplification of non-schistosome trematode DNA. Moreover, the limit of detection was determined to be 10fg of purified genomic DNA. This amount represents less than one parasite cell [169]. For *S. haematobium*, a real-time PCR procedure targeting the Dra1 sequence of the parasite has been tested [170]. This procedure could amplify parasite DNA from urine, stool, and serum samples. A multiplex real-time PCR that could detect *S. mansoni* and *S. haematobium* DNA from fecal samples collected in Northern Senegal was developed by ten Hove *et al* [171]. The species-specific primers and probes used for this assay were designed to target the cytochrome c oxidase gene. This multiplex real-time PCR was shown to have specificity of 100% and a detection rate of 84.1% [171]. The utilization of this technique can be advantageous due to its high throughput potential. The need for a gel electrophoresis step in order to visualize PCR products represents a disadvantage of this molecular technique for diagnosis. Additional steps are time consuming and are not practical for rapid diagnosis of an infection. Akinwale *et al.* have developed a technique that allows for the visualization of *S.*

mansoni and *S. haematobium* DNA amplicons through the use of an oligochromatographic dipstick [172]. Parasite tandem repeat sequences are amplified and then hybridized with gold conjugated probes. This method is species specific, it has a low detection limit (10fg of genomic DNA), and results become visible within ten minutes [172]. The dipstick also contains a control line which is essential in order to validate the migration occurring in the dipstick. Recently, Lodh *et al.* have shown that *S. mansoni* and *S. haematobium* DNA could be detected in urine sediment obtained by filtration [173]. The DNA was extracted from urine sediment samples collected in Southern Ghana, and the PCR procedure was performed using species-specific primers targeting repeat fragments. The assay was shown to have a sensitivity of 99%-100% and specificity of 100% [173]. Assays that can simultaneously detect *S. mansoni* and *S. haematobium* are greatly coveted because infections with these two schistosome species can overlap geographically. Being able to simultaneously detect different parasite species in one diagnostic assay is a favourable characteristic; results are obtained rapidly, less assays need to be performed, and consequently, the use of reagents is reduced.

1.1.7.4 The future of schistosomiasis diagnostics

Although several serological and molecular diagnostic tools are being tested and used by certain reference laboratories, results are often confirmed by microscopy which remains the gold standard (Table 1.2). Many newer assays have not been standardized yet; thus, forcing diagnosticians to rely on microscopic observations. The evolution of diagnosis in the field of parasitology has been slow to progress. However, in recent years, several groups have focused their research on the improvement of diagnostics. The use of serology-based techniques has revolutionized clinical diagnostics. Many different assays, such as ELISA, are being used in reference laboratories. The introduction of rapid diagnostic tests represents a bridge that can connect the laboratory and the field. These tests could allow for the mass screening of endemic populations and for the monitoring of control programs; thereby, improving quick diagnosis and helping to reduce disease transmission. Furthermore, some assays, such as multiplex real-time PCR, can simultaneously detect several parasites. Molecular methods are promising tools for the future of diagnostics; however, additional work must concentrate on rendering molecular diagnostics more accessible. Although relatively new at the moment, proteomic platforms seem to have an important place in the future of parasitic diagnosis. These new

techniques can identify biomarkers which can categorize susceptible individuals, distinguish between the different stages of an infection, and monitor whether treatments lead to cure. Some techniques that can be used to analyze protein expression include matrix-assisted laser desorption ionization time-of-flight mass-spectrometry (MALDI-TOF MS), surface-enhanced laser desorption ionization time-of-flight mass-spectrometry (SELDI-TOF MS), liquid chromatography combined with mass-spectrometry, isotope-coded affinity tags, and isobaric tags for relative and absolute quantification [174].

There are many different avenues to be investigated in the field of parasitic diagnostics. In order to achieve major advances in this field, knowledge and collaborations are essential. Clinicians should be familiar with the "tropical" diseases in order to report them efficiently. Time and effort should be dedicated to educating and communicating with individuals living in endemic areas in order to ameliorate the impact of control programs. Finally, multicenter studies involving clinical diagnostic laboratories as well as reference laboratories could accelerate the standardization of the assays. Diagnostic research has made much progression; however, there is still a lot of work to be done and improvements to be made. In order to better the diagnosis of parasitic infections, research plus communication is the answer.

1.1.8 Treatment

For over thirty years, the treatment of schistosomiasis has relied on one single drug: praziquantel (PZQ). PZQ is highly effective at treating schistosomiasis caused by all three main schistosome species. PZQ's popularity may be attributed to its efficacy, safety, operational convenience, and price [175]. Although treatment with PZQ seldom achieves a complete cure (100%), its general efficacy is reported to be between 85%-90% [1, 175, 176]. This efficacy range has been argued to be an overestimate due to the low sensitivity of available diagnostic tests when dealing with low intensity infections. Nonetheless, the use of PZQ continues to be the mainstay of schistosomiasis treatment. Furthermore, the use of the drug is especially favourable in areas of polyparasitism as it is also effective against other species of trematodes and certain cestodes [177]. The standard 40mg/kg dose of PZQ is considered the safest of all antihelmintic drugs, and it is also recommended that pregnant as well as lactating women be treated as the benefits outweigh the hypothetical risks [175]. PZQ is easily distributed as tablets that are taken orally. Moreover, its safety and minimal side effects do not require monitoring of

patients. The low cost, at US$ 0.20/treatment, is another advantage of PZQ use. Furthermore, Merck KGaA continues to donate 250 million tablets of PZQ every year [175]. Although PZQ seems like the perfect solution to the schistosomiasis problem, it has numerous limitations that still have not been resolved. For instance, PZQ is only effective at killing the adult worm stage of the parasite and has no effect on the immature stages. This represents a significant issue as treatment has no prophylactic power and does not prevent re-infection. This is especially troublesome in endemic areas with high transmission where infection prevalence returns to baseline as early as 18 months after PZQ distribution has ceased [178]. Furthermore, once drug treatment is interrupted, rebound morbidity can occur upon re-infection resulting in a more aggressive form of the disease [179]. Programs relying on PZQ chemotherapy alone are not sustainable because they depend on the constant distribution of the drug. The overuse of PZQ has also led to the constant fear of eventual drug resistance. To date, there are no concrete field cases of schistosomes resistant to PZQ. However, there are numerous examples of field recovered and experimentally-induced isolates with reduced susceptibility to PZQ [180- 187]. This problem has been further complicated by the lack of certainty concerning the mechanism of action of PZQ. The effect of PZQ treatment on parasite worms has been thoroughly evaluated: calcium influx in the parasite is followed by muscle contraction and then surface modifications [175]. Schistosomes have two regulatory β subunits of voltage-activated calcium channels. Interestingly, one of these is referred to as the variant subunit as it lacks two serine residues that make up putative phosphorylation sites [175]. Co-expression of this variant subunit in *Xenopus* oocytes with the mammalian α_1 subunit channel resulted in novel PZQ sensitivity and increased calcium current in the presence of the drug [175]. It is strongly believed that this variant subunit plays an important role in the mechanism of action of PZQ.

In the hopes of alleviating the stress surrounding potential PZQ resistance, over the years, an extensive list of compounds have been tested against schistosomes. Different PZQ derivatives have been evaluated, but proved to be unsuccessful. Modifications in the aromatic ring and substitutions in the cyclohexyl group both decreased the drug's activity [188, 189]. Prior to PZQ becoming the drug of choice against schistosomiasis, oxamniquine (OXA) was used in South America against intestinal schistosomiasis. Unfortunately, OXA is only effective against *S. mansoni*. Furthermore, OXA resistance was observed in Brazil. This resistance to OXA was a recessive trait controlled by a single autosomal gene [190]. This also led to the

discovery of a parasite sulfotransferase that was required to convert OXA to its active form [191, 192]. Using all of this knowledge, structure-based redesign projects can be undertaken in order to extend the activity of OXA. Antimalarial drugs have also been proposed as PZQ alternatives for the treatment of schistosomiasis. Mefloquine was shown to be effective against the immature stage of the parasite [193]. Artemisinin derivatives were also shown to be effective against immature schistosomes. Clinical trials have demonstrated that the combination of artemisinin derivatives with PZQ was more effective than PZQ alone [194]. This is likely due to the fact that the drugs target different stages of the parasite. However, there is concern that the overuse of antimalarial drugs could lead to drug resistant *Plasmodium* species in co-endemic regions [175]. Furoxan is yet another molecule that is being tested against schistosomiasis. The drug targets that parasite's thioredoxin-glutathione reductase. Furoxan has been shown to be effective against juveniles and adults both *in vitro* and *in vivo* [195]. Continued research in the development of new drugs for the treatment of schistosomiasis is essential in order to ensure that we are never left unarmed.

1.1.9 Schistosomiasis control programs

Preventative chemotherapy through PZQ mass drug administration (MDA) represents the mainstay of schistosomiasis control programs today. Morbidity control is the main goal of MDA. In mid-2001, the World Health Assembly encouraged the deworming of at least 75% of children living in schistosomiasis endemic regions [1]. These control programs normally operate out of local schools. This set up is not optimal because as many as 40% of children in sub-Saharan Africa do not attend school [1]. Furthermore, this is excluding pre-school aged children. In 2012, MDA coverage was assessed and it was estimated that only 14.4% of infected individuals were treated [175]. Although PZQ has significantly helped reduce the morbidity associated to schistosomiasis, chemotherapy alone is not adequate to reduce disease transmission [1].

The complex schistosome lifecycle allows for different points of disease control: the snail releasing the infective stage into the water; humans coming into contact with the infective stage; the parasite living inside the human, the humans releasing the eggs back into the environment. The norm for control programs today seems to be only chemotherapy using PZQ; thus, dealing with the parasite living within the host. Control efforts of the past focused a great

deal on snail control. Niclosamide was the molluscicide of choice, and it was very effective. However, it was toxic to fish and it was costly [1]. Certain groups are currently looking into using plant derivatives. Snail control can also be achieved through environmental changes that alter the snail habitats or by introducing snail predators such as ducks and certain fish [1]. Improving hygiene and water sanitation is crucial to the success of any schistosomiasis control program. Eliminating public defecation will prevent the release of parasite eggs into water sources. This can be achieved by the introduction of latrines. However, health education is essential to obtain behavioural modifications. In order not to fear the latrines, it is essential to explain their use to the people [196]. Finally, successful control programs require constant monitoring and surveillance in order to ensure optimal allocation of resources [197-199].

There is much to learn from the success stories of schistosomiasis control. The Caribbean islands of St. Kitts, Martinique and Guadeloupe were able to eliminate their schistosomiasis problem by combining treatment and intermediate host control [200]. Schistosomiasis was also eliminated in Tunisia using the same strategy as the Caribbean [1]. In 1977, schistosomiasis was declared to be eliminated in Japan. However, they maintained their rigorous surveillance until the early 1990s. They achieved elimination by implementing snail control through the use of molluscicides and environmental changes such as the lining of their canals with cement. They combined snail control with target treatment and increased standard of living [1, 201].

1.2 The worm: know your enemy

1.2.1 Parasite anatomy

1.2.1.1 Cercariae

Cercariae are the free-swimming infective stage of the parasite. The structures of the cercariae must be water-tolerant and satisfy the vigorous contracting and elongating movements of the parasite. The three main regions of the cercariae are the anterior organ, the body segment, and the tail [202]. The anterior organ contains the oral sucker, mouth, and acetabulum. The bifurcated tail is a hallmark characteristic feature of the cercariae. The parasite is covered by a filamentous surface coat known as the glycocalyx which overlays the surface membrane. The surface of the parasite, with the exception of the oral apex, is also covered in

posteriorly-directed spines [202]. The surface membrane of the cercariae is approximately 7 nm thick and trilaminate [202]. Subjacent to the surface membrane is the cercarial tegument. It is a continuous syncytium, 0.2µm-0.5µm thick, which covers the entire length of the parasite. The tegument has a granular, anucleated cytoplasmic region which contains scattered mitochondria and cercarial-type inclusion bodies [202]. The tegument turns inward to form the lining of other structures such as the mouth, oral cavity, esophagus, osmoregulatory pores, and distal areas of multiciliated pits [202]. The cercaria is made up of approximately 1000 cells, the majority being muscle cells [202]. The muscle cells extend throughout the cercaria in myofibers. The cercarial tail is a locomotor organ made up of muscle packets that have two to three layers of longitudinal muscle cells underlying an outer circular layer [202]. The cercarial nervous system comprises a central ganglion and six pairs of long main nerve trunks. The nerve fibers are unsheathed and contain numerous vesicles [202]. The cercaria has approximately 76 sensory papillae; most of which are concentrated on the oral tip. The sensory papillae are terminals of nerve processes, and they interact with the external environment either by cilia or through openings in the tegument [202]. The function of several papillae is unknown; however, they are likely involved in photo-, chemo-, and/or mechano-reception.

The cercarial acetabulum, or ventral sucker, is located ventrally in the body segment. It is a cup-shaped structure made up of longitudinal and circular muscles. The acetabulum is involved in skin exploration and penetration. It attaches to the skin during exploration, and it serves as an anchor during the penetration process [202]. The cercariae possess five pairs of acetabular glands which provide secretions involved in skin penetration. The parasites also have four pre-acetabular glands which are rich in secretory granules containing proteolytic enzymes. The cercariae possess six post-acetabular glands which contain large secretory granules made up mucosubstances involved in skin adhesion [202]. An important cercarial serine protease involved in the degradation of skin macromolecules can be found in acetabular gland secretions. Finally, the cercarial osmoregulatory system is made up of peripherally placed flame cells, collecting tubules, excretory bladder, and excretory pores [202].

1.2.1.2 Schistosomulae

The cercarial/schistosomulum transformation occurs when the parasite penetrates the host skin and sheds its bifurcated tail [203]. Once inside the host, the parasite undergoes several

morphological and biochemical changes in order to develop into the schistosomulum [204]. Upon penetration, the contents of the pre- and post-acetabular glands are released and the heptalaminate surface membrane which contains modified surface antigens begins to form [205, 206]. Once the schistosomulum has reached the lungs, it has completely lost the highly immunogenic cercarial glycocalyx [204]. In the lung, the schistosomulum is an easy target for host immune responses. It has been suggested that the parasite evades these immunological attacks by confining its antigenic molecules to specific lipid-rich sites on the outer surface membrane [204, 207]. The cercarial/schistosomulum transformation also includes a switch from aerobic to anaerobic metabolism and reconstruction of the parasite tegument. The lung schistosomulum tegument is in a dynamic state of remodelling and turnover [208].

1.2.1.3 Adult worms

Male and female adult worms can be easily differentiated by morphology. Males are dorsoventrally flattened and possess a gynaecophoric canal whereas females are more cylindrical, slender, and longer. Adult worms possess anterior and ventral suckers which are involved in blood vessel attachment and intravascular movement [205, 209]. There are large tubercles containing intracellular spines composed of actin bundles on the dorsal surface of the adult male [210]. The rest of the male surface contains pits, some spines, and sensory papillae. The gynaecophoric canal surface is rigid and covered with small spines [209]. The adult female surface resembles the male surface; however, females have fewer spines and lack large tubercles [209]. Furthermore, surface spines are mostly concentrated on the posterior end of females [205]. The worm tegument is one of the major interfaces with the external environment. The tegument cytoplasmic layer contains small mitochondria and two types of secretory inclusions: discoid bodies and multilaminate vesicles [209]. Numerous cell bodies can be found subjacent to the peripheral muscle layers, and they are connected to the tegument cytoplasm via cytoplasmic processes. Proteins and carbohydrates meant for export are synthesized in these cell bodies [209]. The tegument surface consists of an apical plasma membrane and an outer secreted membranocalyx [209]. There are several invaginations and folds in the tegument surface which are believed to contribute to its flexibility and absorptive properties [205, 209]. Numerous parasite surface proteins such as enzymes, receptors, structural proteins, and nutrient transporters have been discovered on adult worms [209, 211-

213]. The adult tegument is also rich in phospholipids and cholesterol [214]. Several studies have also demonstrated the presence of glycoproteins and/or glycolipids containing complex oligosaccharides on the membranocalyx [209, 215-217]. However, it is difficult to determine whether these glycans are of host origin, parasite origin, or a mixture of both [209]. A number of host molecules have also been discovered on the adult worm surface. These molecules include blood group antigens, major histocompatibility complex glycoproteins, immunoglobulin, skin antigens, fibronectin, α2-Macroglobulin, and complement factors [209, 211, 212].

The adult schistosome worms obtain their nutrients by either feeding across the body surface or via the alimentary tract. The tegument is the main site of nutrient uptake in male adults whereas nutrient uptake is balanced between the tegument and the gut in adult females [218]. The tegument contains nutrient transporters such as the schistosome glucose transporter proteins (SGTP) [219-221]. Furthermore, approximately 80%-100% of amino acid absorption occurs via the tegument [222-226]. Female worms take up less glucose than males, but they ingest greater amounts of amino acids [218]. The alimentary tract is made up of the mouth, the esophagus, and the absorptive gut which ends blindly. The gut is lined with an epithelial layer referred to as the gastrodermis [218]. Male and female adult worms ingest approximately 39,000 and 330,000 erythrocytes per hour, respectively [227]. The bolus of ingested blood is delivered from the anterior esophagus to the anterior gut by a wave of peristalsis [218]. The esophageal gland contains several crystalloid vesicles that release products suggested to play a role in red blood cell lysis into the lumen [228]. The aquaporins and the Na/K pump in the tegument are involved in maintaining the worm's water-salt balance [218]. For both males and females, the gut is the main area of lipid uptake [218]. Schistosome worms do not possess an anus; therefore, the waste products of metabolism must be expelled via the mouth. Hemozoin is a major product that is regurgitated by the adult worms. Since free heme is toxic to cells, it is converted to the insoluble crystalline product hemozoin. The glycoproteins circulating anodic antigen and circulating cathodic antigen are two prominent products also found in the worm vomitus [218].

Schistosomes are the only trematodes to have two different sexes. Furthermore, to ensure proper sexual development of the female adult worm, constant pairing with the male is

necessary [229, 230]. The vitellarium is the largest organ in adult female worms [231]. The differentiation of vitelline cells may be reversed when the female is uncoupled with the male [231, 232]. The ovaries of mature females contain oocytes of various sizes [231]. The sperm in male adult worms is stored in the sperm vesicle [231]. Unpaired males appear almost identical to paired males. However, by microscopy, it was revealed that paired adult males have a reduced testes diameter compared to their unpaired counterparts [233].

1.2.1.4 Eggs

Schistosomiasis pathology is associated to the deposition of parasite eggs in host tissues. Immature eggs released by female worms in the host veins consist of an eggshell of cross-linked proteins which contains an ovum and vitelline cells [234]. During egg maturation within the host, the ovum develops into the miracidium, the Von Lichtenberg's envelope forms on the inside of the eggshell, and proteins formed in the sub-shell area are secreted into the environment through pores found in the shell [235, 236]. The egg also contains hatching fluid and the soluble egg antigens; however, these components are only released once the egg dies in the host tissues [234]. The immunological roles associated with schistosome eggs are primarily mediated by protein-linked glycans. These molecules mediate interactions with C-type lectin receptors and antigen uptake by antigen presenting cells [234].

1.2.2 Peptidases

The schistosome life cycle requires switching between different parasite stages and migrating through major parts of the host body; thus exposing the parasites to several potential obstacles. Peptidases, enzymes hydrolyzing peptide bonds, are present throughout all stages of the schistosome life cycle, and they are essential for parasite development as well as for the establishment of infection [237]. Schistosome peptidases have been described to play critical roles in parasite invasion, migration, nutrient acquisition, and immune modulation [237]. Over the years, these enzymes have been the research focus of several laboratories due to their potential to become targets for new diagnostic assays, drugs, and vaccines. Peptidases can be classified according to their catalytic type. Due to the abundance of schistosome peptidases, only the major ones belonging to the different catalytic types will be discussed in this section.

1.2.2.1 Serine peptidases: cercarial elastase

Trematode serine peptidases are not as numerous as those found in vertebrates and invertebrates [237]. *S. mansoni* cercarial elastase (SmCE) is the most studied trematode serine peptidase. SmCE is a chymotrypsin-like serine peptidase with the His68/Asp126/Ser218 catalytic triad in its active site [237, 238]. The preferred substrate of SmCE is Z-Ala-Ala-Ala-Pro-Phe-AMC [238]. SmCE plays a pivotal role during cercarial invasion and penetration of the host skin by cleaving insoluble elastin which is a major component of the skin dermis [239]. SmCE can degrade other skin macromolecules such as keratin, collagen, laminin, and fibronectin [240-242]. Incubating cercariae with inhibitors of cercarial elastase reduces their capability to penetrate human skin by over 75% [239, 243]. SmCE may also play a role in host immune evasion via the degradation of immunoglobulins [237]. Through immunohistochemistry, SmCE was localized to the preacetabular penetration glands of the cercaria [239, 244]. Furthermore, micro-array analysis identified SmCE transcripts in the sporocyst, egg, and adult (female) stages of the parasite [245, 246]. Cercarial elastase has also been described in *S. haematobium* [237]. Interestingly, there has been no cercarial elastase orthologue identified in *S. japonicum* [247]. The absence of this serine peptidase does not seem to affect the penetration ability of *S. japonicum* cercariae as they have been shown to penetrate host skin more rapidly than *S. mansoni* and *S. haematobium* cercariae [248-250]. It has been suggested that a *S. japonicum* cysteine peptidase acts as the major molecule in host skin invasion [237, 247, 249].

1.2.2.2 Aspartic peptidases: Cathepsin D

Cathepsin D is a pepsin-like aspartic peptidase that has been described in both *S. mansoni* (SmCD) and *S. japonicum* (SjCD) adult worms [251-253]. Cathepsin D peptidases possess the Asp33/Asp231 highly conserved active site amino acid residues which are responsible for substrate specificity [252]. SmCD and SjCD have been localized to the gastrodermis of adult worms, and it has been demonstrated that they function optimally at acidic pH [237, 251, 253-255]. SmCD and SjCD are involved in adult worm digestion as they are part of a network of peptidases found in the parasite gut that degrade ingested proteins [256]. SmCD and SjCD participate in hemoglobin degradation as they cleave the hemoglobin alpha-chain between the Phe36-Pro37 amino acids [253, 254, 257-259]. Experiments targeting SmCD by RNAi resulted in decreased hemoglobin digestion by the parasite; thereby,

elucidating an important role for SmCD in the host hemoglobin degradation network [260]. An immune evasion role for Cathepsin D has also been suggested because this peptidase can also cleave immunoglobulin G (IgG) and the C3 factor of complement [261]. Recombinant SjCD was tested as a potential vaccine candidate in a mouse model of schistosomiasis. However, only modest worm burden reductions were achieved (21%-38%) [262]. It has also been proposed to develop specific inhibitors against SmCD and SjCD to treat schistosomiasis [237].

1.2.2.3 Cysteine peptidases: Cathepsins

Cysteine peptidases are the most annotated and best characterized group of trematode peptidases [237]. Schistosome cysteine peptidases possess numerous functions that include tissue invasion, nutrient acquisition, and immune evasion. Most of the cathepsins represent papain-like cysteine peptidases. These peptidases have a cysteine residue as the main catalytic amino acid in their Cys/His/Asn catalytic triad [237]. The His imidazole ring and the Cys side chain sulphydryl group form the reactive thiolate-imidazolium charged couple attacking the peptide bond [263, 264]. Schistosome cathepsins are abundantly expressed; thus, they have been experimentally tested as potential vaccine candidates and serodiagnostic markers.

1.2.2.3.1 Cathepsin B

The first recombinant peptidases for *S. mansoni* were cloned and expressed in the late 1980s [265, 266]. They were the previously described immunodominant, serodiagnostic molecules Sm31 and Sm32 [267]. Sm31 was found to be *S. mansoni* Cathepsin B1 (SmCB1). It is a papain-like peptidase with an optimum pH range of 4.5-6.0 and the preferred substrate Z-Arg-Arg-AMC [237]. Cathepsin B peptidases have the unique characteristic of possessing an occluding loop at one end of their active cleft. The occluding loop contains a catalytic dyad (His110-His111) that is responsible for the exopeptidase activity [237, 268]. SmCB1 is expressed as a pro-enzyme. *In vitro* studies have demonstrated that enzyme processing is needed to obtain the fully active and mature SmCB1. The processing involves an initial trans-activation step by *S. mansoni* asparagyl endopeptidase (SmAE) followed by a second processing step by rat cathepsin C [268]. SmAE was initially identified as the immunodominant molecule Sm32. It is also referred to as Legumain as it was confirmed as a peptidase after the

identification of its orthologue in leguminous plants [269]. SmAE is associated with the parasite gut and is believed to have a role in processing other molecules [237].

SmCB1 is expressed in adult worms and schistosomula. It has been identified in the parasite gut and in vomitus [237, 245, 257]. SmCB1 is the main peptidase in the network involved in the digestion of host blood macromolecules (Figure 1.7) [258]. Mass-spectrometry analysis of the parasite gut contents also demonstrated that SmCB1 is involved in the digestion of host serum albumin and IgG [258]. Silencing of SmCB1 by RNAi results in decreased peptidase activity and parasite growth retardation [270]; thereby, demonstrating its importance in the schistosome development. For *S. japonicum* species, cathepsin B activity was also detected in the penetration gland contents of cercariae [247]. Since, *S. japonicum* cercariae do not seem to express cercarial elastase, it has been suggested that a cysteine peptidase, possibly cathepsin B, acts as the main skin penetrating enzyme.

SmCB1 is a parasite molecule that is highly expressed and released in the excretory/secretory products; therefore, making it a promising serodiagnostic marker. SmCB1 has been studied as a potential tool to improve schistosomiasis diagnostics, and it has been repeatedly shown that this antigen generates a positive reaction with sera from infected humans and mice [267, 271- 277]. SmCB peptidases belonging to different schistosome species as well as different trematodes have also been studied as potential vaccine candidates. A *S. japonicum* CB DNA vaccine in combination with an IL-4 plasmid has been tested in mice, and has been shown to decrease parasite worm and egg burdens by 42% and 77% respectively [237, 278]. A DNA vaccine of the liver fluke, *Fasciola hepatica*, has also been tested in sheep and was able to stimulate both humoral and cellular immune responses [279]. Our group has chosen to focus on SmCB1 (simply referred to as Sm-Cathepsin B in our manuscripts) as a potential vaccine candidate [280-282]. Our formulations as well as our results are discussed in later chapters.

S. mansoni expresses a second distinct cathepsin B cysteine peptidase, SmCB2, which was sequenced and expressed by Caffrey *et al.* [283]. SmCB2 is biochemically similar to SmCB1; however, it is restricted to the adult worm parenchyma and tegumental tubercules [237, 283]. It has been suggested that SmCB2 has a role at the host-parasite interface [283].

1.2.2.3.2 Cathepsin L

The cathepsin L peptidases have been more thoroughly studied in *Fasciola hepatica* as they represent promising vaccine candidates [284-286]. For *S. mansoni*, cathepsin L2 (SmCL2) has been comprehensively studied. SmCL2 is expressed in eggs, sporocysts, cercariae, and adults. It possesses the typical papain-like catalytic triad Cys26/His163/Asn183 [237, 287]. In the cercaria, SmCL2 has been immunolocalized to the vesicles of postacetabular glands; thereby, suggesting that this peptidase may be involved in host skin penetration [288]. SmCL2 is found in both female and male adult worms. In the females, it is expressed in reproductive structures, whereas in the males, it is localized to the sub-tegumental region of the gynaecophoric canal [289]. The location of SmCL2 in the adult worms suggests that it may play a role in parasite reproduction.

More recently, *S. mansoni* cathepsin L3 (SmCL3) was identified and characterized as another gastrodermal cysteine peptidase [290]. The catalytic triad of the peptidase is formed by Cys172/His317/Asn337 [290]. SmCL3 is predominantly expressed in adult worms and schistosomulae. It was localized to the parasite gut and exists mainly as a zymogen [290]. Transcript analysis revealed that SmCL3 mRNA is less abundant relative to other known *S. mansoni* gut peptidases [256, 257, 290]. SmCL3 was shown to efficiently cleave albumin and hemoglobin; thus, suggesting that it may belong to the network of gut peptidases involved in nutrient acquisition [290].

1.2.2.3.3 Cathepsin F

Schistosome cathepsin F is expressed in adult worms and is detected in the intestine as well as intestinal contents which are secreted from the gut epithelium into the lumen [237, 291]. The location of cathepsin F in the adult parasites suggests that the peptidase is involved in blood digestion and nutrient acquisition. Cathepsin F likely belongs to the network of gut-dwelling peptidases that mediate host hemoglobin degradation. Female adult worms have higher cathepsin F expression than males [291]. This may be associated with their greater uptake of red blood cells. It has been suggested that cathepsin F is stored, in its inactive, immature form, in the nascent digestive tract of cercariae, and it is processed to its active form when the schistosomulae begin feeding [291]. Cathepsin F has also been detected in the worm sub-tegument region; therefore, suggesting a role in immune evasion potentially by cleaving host IgG [292].

1.2.2.3.4 Cathepsin C

Schistosome cathepsin C is also known as di-peptidyl-peptidase I exopeptidase [237]. Exopeptidases exert their function by removing N-terminal dipeptides from substrates. Schistosome cathepsin C is expressed in adult worms, and its activity has been detected in the gastrodermis, the male testes, and the female vitelline cells [293]. One report also states that cathepsin C can be found in schistosomulae [294]. *In vitro* studies have demonstrated that the rat orthologue for cathepsin C mediates the final processing step to generate the active form of SmCB1. The rat cathepsin C removes the remaining Val87-Glu88 dipeptide which exposes the N-terminal sequence of mature SmCB1 [268]. Since *S. mansoni* cathepsin C (SmCC) is 43% identical to rat cathepsin C, SmCC is suggested to have processing functions. Specifically, SmCC is believed to be responsible for the final processing step of SmCB1 in the gut [257, 268].

1.3 Immunomodulation: the parasite's shield

The complex schistosome life cycle has been adapted to survive multiple parasite stage maturations, migration through its human host, and constant exposure to immune attacks in sites such as the skin, lung, and blood. The parasite has learned to defend itself in harsh host environments to avoid death and clearance. However, it is important to acknowledge the schistosome well developed and perfected counterattack: immunomodulation.

1.3.1 Parasite stages & their tools to subvert/modulate the immune response

Every schistosome stage within the host has developed its own mechanisms to evade and modulate the immune responses blocking the establishment of infection. There are several secreted or surface molecules that are stage specific, and they possess immunomodulating functions. The cercarial penetration of host skin is expected to cause a strong inflammatory environment; thus, making the cercariae prime targets for host immune cells. However, cercariae are successful in passing through this phase of infection and go on to transform into migrating schistosomulae. There are specific cercarial molecules that have been suggested to play immunomodulating roles; thus helping the parasite to cross the host skin layer unharmed by immune attacks. Sm16 is a secreted cercarial protein that possesses anti-inflammatory properties. This molecule is suggested to suppress the elicited inflammation upon host skin

penetration [295-297]. Other research groups have argued that Sm16 acts as a pro-apoptotic molecule once it is internalized by host cells [298, 299]. It has also been demonstrated that cercariae secrete three sperm-coat domain proteins which are members of a family of wasp venom homologues [296, 300]. Homologues of these proteins in the hookworm *Necator americanus* share structural similarities with host chemokines; thus, suggesting a role for these proteins in dermal immunomodulation [301]. During the transformation from cercaria to schistosomula, the parasite is vulnerable to host immune attacks. SmKK7 is released during this stage. This molecule is believed to have immunomodulating properties as it has homology to potassium-channel blockers in scorpion venom which are known to inhibit T cell activation by interfering with calcium influx [293]. The lung stage schistosomulae have been shown to express a fourth sperm-coat domain protein [302]. Microarray analysis has also demonstrated that the lung schistosomulae express Antigen 5 [302]. This molecule has been shown to have immunomodulatory functions in *Echinococcus* infections [303]. The presence of these molecules indicates that the migrating schistosomulae are capable of immunomodulation.

For the adult worms, most of the immune subversion is believed to take place at the parasite surface. There is quite some evidence suggesting that the adult worms have developed mechanisms to evade the host's complement system. Complement factors have been identified on the parasite surface; however, factors C5-C9, which make up the membrane attack complex, are consistently missing [19, 211, 212]. This observation suggests that the complement cascade is being inhibited. Immunohistochemistry of the adult worm tegument revealed the presence of host decay accelerating factor which functions to block the formation of the membrane attack complex [304]. Furthermore, schistosomes possess six human CD59 homologues. These molecules are inhibitors of the membrane attack complex. Four of these parasite homologues have been detected on the adult worm tegument [19]. In addition to complement related molecules, a schistosome integrin with homology to human T cell immunomodulatory protein (TIP) has been identified on the parasite tegument [19]. TIP has been demonstrated to modulate cytokine secretion by T cells [19]. There are many other molecules that have been identified on the adult worm surface and that share homology with known immune inhibitors [19]. However, there is a need for functional studies in order to properly characterize these molecules.

1.3.2 Eggs & Th2 polarization

Schistosomiasis is a chronic disease whereby the adult worms can live within the host for years. It is in the parasite's best interest to keep the host alive; the schistosomes thrive in the blood and transmission via release of the eggs in the environment continues. The formation of the granuloma around the parasite egg is a Th2 driven event. Granulomas possess a host-protective role as they sequester toxic molecules released by the entrapped eggs. The main Th2 cytokine IL-4 has been shown to have a protective role during schistosomiasis as mice lacking IL-4 develop severe pro-inflammatory responses, and the infection is lethal [305, 306]. Alternatively activated macrophages are associated to wound healing and reduction of inflammation. It is suggested that they possess protective and immunoregulatory roles during schistosomiasis [307-309]. The optimal immune response to avoid host death and promote parasite persistence involves promotion of wound healing, inhibition of pro-inflammatory classically activated macrophages, and prevention of hyper-responsiveness within the Th2 T cell compartment [310].

The host immune response during schistosomiasis becomes Th2 polarized with the onset of oviposition at approximately six weeks post-infection (Figure 1.6). A significant amount of evidence has demonstrated that the parasite eggs are responsible for this shift in immune response. Both the parasite eggs and the soluble egg antigens (SEA) are capable of inducing a strong Th2 response when injected into naïve mice [87, 311]. Dendritic cells (DCs) are believed to be important mediators in the development of Th2 responses [312]. DCs pulsed with SEA generate SEA-specific Th2 responses when injected into mice [310, 313]. Treatment of DCs with SEA inhibits classical TLR-initiated activation and maintains the DCs in an immature state [313]. SEA conditioned DCs do not make IL-12 and do not upregulate the co-stimulatory molecules CD80 and CD86 [314]. These functional changes result in impaired DC-T cell interactions and suboptimal T cell activation. A default hypothesis has been described whereby sub-threshold T cell receptor signalling promotes Th2 polarization [315]. Several research groups have linked the major Th2 immunomodulating effects of parasite eggs to glycoproteins that interact with DCs [316-319]. These interactions are associated to the Lewis-X glycan moiety found on egg glycoproteins. This moiety interacts with the C-type lectin DC-SIGN and inhibits IL-12 secretions by DCs [310, 320]. Omega-1, a T2 ribonuclease, is a major secreted glycoprotein and is believed to be one of the main Th2-polarizing egg antigens to interact with host DCs [321]. Omega-1 induces a Th2 response when injected into naïve mice

[322]. DCs conditioned with Omega-1 can Th2 polarize $CD4^+$ T cells *in vitro* by impairing the DC-T cell interactions [323]. Removal of the Omega-1 ribonuclease activity abolished the Th2 polarizing effects [323]. Omega-1 is believed to be taken up by DCs via a C-type lectin Mannose receptor where it then inhibits protein synthesis by degrading RNA [324]. This is believed to result in weak signal delivery to T cells and therefore Th2 polarization [315]. Other mechanisms leading to Th2 polarization via DC modulation may include degradation of endosomal TLRs by internalized schistosome peptidases, and modulation of NF-kB translocation via C-type lectin interactions [310]. Other parasite egg antigens believed to modulate Th2 polarization include IPSE(alpha-1) and Sm-peroxiredoxin1 (SmPrx1). IPSE(alpha-1) binds IgE and activates naïve basophils resulting in histamine release and secretion of the Th2 cytokines IL-4 and IL-13 [325]. SmPrx1 is an antioxidant molecule that leads to the activation of alternatively activated macrophages independently of IL-4 and IL-13 and promotes Th2 polarization when injected into naïve mice [326].

All evidence suggests that parasite eggs are the main mediators in Th2 polarization during schistosomiasis. However, there is value in examining the potential roles for the other life stages in priming the immune response. Several important antigens are shared among all parasite life stages. Therefore, it is possible that the migrating larva and the maturing worms prime a Th2 response that permits for an effectively biased response upon oviposition [310]. Certain research groups have provided evidence for this hypothesis. For instance, naïve mice are unable to form granulomas around eggs injected directly into their liver [327]. However, in mouse single sex infections, where worms mature but no eggs are produced, normal size granulomas form around eggs injected into livers [327]. Furthermore, naïve mice can be sensitized to form granulomas by vaccinations with either worm or egg antigens [327]. These results were also seen in the pulmonary granuloma model where prior sensitization with parasite eggs was needed in order to obtain normal size granulomas around eggs in the lungs [328]. These observations suggest that the entire parasite life is immunomodulatory.

An unchecked Th2 response will lead to excessive fibrosis and organ failure. After strong Th2 polarization with the onset of egg production, the chronic phase of schistosomiasis should represent hypo-responsiveness in order to minimize adverse consequences of the Th2-biased response (Figure 1.6). *In vitro* conditioning of human DCs with the *S. mansoni* egg and

worm lipid lyso-phosphatidylserine promotes the development of IL-10 secreting Tregs [102]. IL-10 is believed to be necessary for immune balance during chronic schistosomiasis as both IL-4/IL-10 and IL-12/IL-10 double deficient mice rapidly die of the infection [91].

1.4 The Host: immunity & genetics

1.4.1 Protective immunity

1.4.1.1 Acquired immunity

When analyzing the relationship between infection and age for populations living in schistosomiasis endemic regions, it is obvious that children under the age of fifteen are the most susceptible age group. Infection intensities peak at age twelve before starting to fall to lower levels observed in adults [329, 330]. Children are especially susceptible to re-infection after treatment. Decreases in infection susceptibility begin during the mid-teen years and continue to decrease into adulthood until a natural age-dependent partial immunity develops [331-333]. This trend is also observed in high exposure populations such as the fishing communities in schistosomiasis endemic regions [330]. This natural acquired immunity requires decades to develop. Several years of mass drug administration to treat schistosomiasis have revealed that chemotherapy with praziquantel accelerates the development of acquired immunity [334]. Over the years, after multiple rounds of exposure, infection, treatment, and re-infection, there is a resistance to schistosomiasis that develops (Table 1.3) [334-337]. IgE antibodies targeting adult antigens have been linked to acquired immunity and resistance to re-infection [338-341]. There is an association between the elevated presence of parasite specific IgE and low re-infection rates for all three major schistosome species infecting humans [338, 339, 342, 343]. For *S. mansoni*, specific IgE targets have been localized to the adult worm tegument [344, 345]. Parasite specific IgE responses have been shown to increase both with age and after drug treatment; thus, linking this antibody to acquired immunity. Children also experience this increase in parasite specific IgE levels after drug treatment [334, 346]. Individuals who are susceptible to schistosome infection have been shown to have higher levels of parasite specific IgG4 [347]. IgG4 has been partly linked to the slow development of immunity to schistosomiasis as it is considered a blocking antibody [339, 348]. Furthermore, IgG4 can inhibit IgG1-mediated schistosomulae killing by eosinophils [349].Therefore, resistance to re-infection rests on a favourable balance towards IgE production. Acquired

immunity is also associated with enhanced Th2 responses such as the recruitment of eosinophils and the increased secretion levels of IL-4 and IL-5 [350, 351]. Enhanced Th2 responses to the adult worms are also observed after praziquantel treatment [352].

High levels of re-infection after praziquantel treatment have been a major obstacle in all mass drug administration programs aiming to eliminate schistosomiasis. This failure to generate a protective recall response after clearing a primary infection has been demonstrated in humans as well as in mice [353, 354]. Several groups have focused on understanding the immunological determinants of this observed delay in immunity. It is believed that the antigens required for the development of resistance to re-infection are not accessible to the host immune system [355]. Furthermore, schistosomes are not like bacteria or viruses as they do not replicate within the host. Therefore, the immunogenic antigens are less in quantity. During schistosomiasis, the eggs are the major source of antigens. However, the eggs are sequestered in the tissues, and their antigens are mostly carbohydrates and polysaccharides that do not stimulate responses favouring immunity [355]. Parasite proteins represent the best antigens to stimulate protection. However, most of these antigens, with the exception of excretory/secretory products, are only exposed to the host immune system once the adult worms die. Praziquantel targets the adult worms; thus, when individuals are treated, they are exposed to a large number of dead worms which subsequently provides a favourable immune stimulus. Repeated rounds of mass drug administration in endemic regions result in repeated priming and boosting of the immune response; thus, mimicking multiple vaccinations [355]. This would explain why drug treatment accelerates acquired immunity. Resistance to re-infection is the result of exposure and curative treatment cycles.

As previously mentioned, the prolonged susceptibility to re-infection represents a major complication for the control of schistosomiasis. Some research groups have wondered whether there are other mechanisms contributing to this delay. It has been suggested that opposing immunoregulatory responses may be responsible for the slow development of immunity. Wilson *et al.* observed whether the activation of an opposing regulatory response was disrupting the development of resistance to re-infection after drug treatment [353]. Infected mice were treated with praziquantel and re-challenged with the parasite. Although the treatment stimulated immune responses favouring resistance to re-infection, mice were not protected

from the re-challenge [353]. Upon treatment and re-challenge, mice had an increase in a specific cell population expressing IL-10. In a separate set of experiments, infected mice were once again treated, but anti-IL-10R antibodies were delivered along with the praziquantel. When these animals were re-challenged, there was a worm burden reduction of over 50% compared to the animals that received praziquantel alone [353]. Blocking IL-10 signalling resulted in the recruitment of eosinophils as well as increased adult worm specific Th1, Th2, and Th17 cellular responses. Furthermore, there was an increase in anti-worm IgG1, IgG2b, and IgE in these mice [353]. In this system of treatment in combination with IL-10 signaling interference, a mixed immune response is elicited. Increased IL-10 post praziquantel treatment has also been reported in humans [352]. In fact, longitudinal field studies have gathered evidence which suggests that elevated levels of IL-10 represent a major risk factor for re-infection [356]. The results from Wilson *et al.* showing that IL-10 responses impede the development of resistance to re-infection are not entirely unexpected as there is significant immunological evidence suggesting that IL-10 interferes with the development of immunity to infection both naturally or following vaccination [357-361]. In the case of acquired immunity to schistosomiasis, as Wilson *et al.* demonstrated in the mouse model, blocking IL-10 signaling while treating with praziquantel could potentially accelerate the development of resistance to re-infection [353].

1.4.1.2 Natural immunity

There is a small but well-defined cohort living in Siqueira, Brazil which has been defined as endemic normal (Table 1.3). These people live in a region of Brazil which is endemic for schistosomiasis; however, they have been infection free for over five years, as determined by stool examination, despite never being treated with anti-helminth drugs [362]. These individuals possess a natural immunity to schistosomiasis. Individuals who are chronically infected as well as those who have gained drug-induced acquired immunity mount mainly a Th2 response to schistosome antigens [362, 363]. In the case of endemic normal individuals, their response to schistosome antigens involves both Th1 and Th2 components; thus, making them an immunologically distinct group [362, 364-366]. Adult worm specific IgE levels in endemic normal individuals are comparable to levels observed in individuals with acquired immunity after drug treatment. However, endemic normal individuals have

significantly greater levels of IgE specific for schistosomulae surface antigens compared to individuals with acquired immunity [365, 367]. This difference suggests that, for endemic normal individuals, IgE antibodies act at an earlier stage in the infection. The endemic normal individuals also have high levels of paramyosin specific IgG which is associated with lack of eggs in the stool [362, 368]. Peripheral blood mononuclear cells from endemic normal individuals proliferate vigorously and secrete high levels of interferon gamma (IFNɣ) when they are stimulated with schistosome antigens (either adult worm or egg antigens) [364, 366]. $CD4^+$ T cells have been shown to be the main source of IFNɣ [369]. This elevated production of the Th1 cytokine IFNɣ in the endemic normal population is reminiscent of results obtained with the irradiated cercarial vaccine. This vaccine has been used to study favourable immune mechanisms generating protection to schistosomiasis. In the mouse model for the irradiated cercarial vaccine, there is a dominant Th1 profile and lung stage attrition of the parasite [370]. Some immune mechanisms proposed by the irradiated cercarial vaccine include parasite killing by oxidative burst from activated macrophages and killing via cytotoxic T cells [371-373]. These could potentially be the mechanisms mediating the natural immunity observed in the endemic normal cohort. Based on the information we know about these individuals so far, IgE antibodies may act early on by targeting the skin stage larva, and IFNɣ may mediate the immune response later in the lungs. Along with the irradiated cercarial vaccine model, immune studies concerning the endemic normal cohort have provided important insights regarding optimal immunity to schistosomiasis. In addition, the endemic normal individuals have been used to identify two vaccine candidates found on the parasite tegument; Sm-TSP-2 and Sm29 [374, 375]. These two antigens were preferentially recognized by the serum of endemic normal individuals. Understanding the immune responses generated by this particular cohort in response to the parasite can help guide vaccine discovery [376].

1.4.1.3 Concomitant immunity

In schistosomiasis endemic regions, individuals are constantly exposed to the parasite. Infected individuals are still continuously in contact with cercariae present in the water. However, super infections do not occur. Concomitant immunity is a phenomenon whereby the host is able to mount an effective immune response against the invading schistosome larval stages while being incapable of clearing established adult worms [377]. Concomitant immunity

benefits the adult worms as it diminishes competition within the host and prevents crowding; thereby increasing the productivity of the established worms [378]. Smithers and Terry were the first to suggest that the adult worms are the mediators of concomitant immunity [379]. In their experiments, they transferred adult worms to naïve rhesus monkeys, and then exposed these animals to a cercarial challenge. The monkeys were almost completely resistant to the cercarial challenge; however, they were unable to clear the transferred adult worms [379]. Later work by Dumont *et al.* provided further evidence suggesting that the adult worms are responsible for the anti-larval immune response which results in concomitant immunity [380]. They demonstrated that five successive schistosome unisexual infections resulted in significant protection against re-infection [380]. Oviposition does not occur during unisexual infections; therefore, the study by Dumont *et al.* demonstrated that adult worms alone could stimulate immunity against a larval challenge [380]. Adult schistosome worms are long lived within the host. The worms could provide a stimulus for concomitant immunity as several antigens, especially those found in the excretory/secretory products, are shared between life cycle stages. The worms could be vaccinating their host against an incoming larval challenge. It has been shown that sera from infected humans as well as infected laboratory animals can recognize schistosomulae surface antigens mostly raging from 30kDa-40kDa in size [381]. Adult worm products compete with these larval antigens for the same specific antibodies [382]. These observations indicate that the two parasite stages share epitopes; thus, adult worms can induce the production of antibodies targeting schistosomulae [383]. This phenomenon was demonstrated by Dissous and Capron as sera from rats injected with adult worms recognized both a 115kDA worm product and a 38kDa schistosomulae surface antigen [383]. *In vitro* killing assays using schistosomulae as targets have suggested a role for macrophages in concomitant immunity [384]. In a mouse model, macrophages were activated as a consequence of *S. mansoni* adult worms. These activated macrophages were consequently capable of killing schistosomulae *in vitro* [384]. Other work has suggested that tumor necrosis factor alpha (TNFα) may be a necessary molecule in schistosomiasis concomitant immunity [385, 386]. It has been demonstrated that TNFα levels are elevated during schistosomiasis [386, 387]. This increase has been linked to either the release of adult worm antigens into the blood or endotoxin leakage from bacteria in the gut as a result of endothelium damage caused by parasite eggs entering the lumen [386]. Immune serum has the ability to kill schistosomulae *in*

vitro [385]. James *et al.* demonstrated that TNFα is the larvicidal factor in the immune serum [385]. Recombinant TNFα exerted direct toxicity onto the schistosomulae. Furthermore, adding anti- TNFα antisera to a culture of schistosomulae and activated macrophages abrogated the larvicidal effect previously described [384, 385]. Further research is required in order to characterize the mechanisms mediating the important phenomenon of concomitant immunity observed in schistosomiasis.

1.4.2 Genetic background and susceptibility

There is much variability concerning infection intensities, characterized by fecal egg counts, in schistosomiasis endemic regions. Epidemiological studies have discovered high infection levels clustered in certain families [388]. This high variability is also observed when focusing on the severity of disease pathology. For instance, Caucasian Brazilians are five times more likely to develop the hepatosplenic form of the disease than Brazilians of African descent despite comparable egg counts [389]. These different outcomes based on family and ethnic backgrounds suggest that host genetics contribute to the outcome of disease. Initial studies in a Brazilian population linked the variability in schistosomiasis infection intensity to the 5q31-q33 region of the genome [390, 391]. Similar studies in a Senegalese population found the same association between infection intensity and this region of the genome [392]. An association has also been revealed between symptomatic schistosomiasis cases caused by *S. japonicum* in the Poyang Lake region, China, and 5q31-q33 [393]. A specific gene in this chromosomal region, *SM1*, is said to regulate the intensity of infection phenotype for schistosomiasis [394]. Furthermore, loci in this chromosomal region contain a Th2 cytokine gene cluster which includes the genes encoding IL-4, IL-5, and IL-13 [390, 394]. Variations in Th2 related genes have been shown to modulate the outcome of schistosomiasis. Single nucleotide polymorphism (SNP) variants at *IL5* have been associated with symptomatic schistosomiasis in the Poyang Lake region, China [393]. SNPs at *IL13* have been linked to both *S. mansoni* and *S. haematobium* infection intensities and to *S. mansoni* re-infection susceptibility after drug treatment [395- 399]. Moreover, a population study in Mali revealed that *IL13* gene promoter polymorphisms affected *S. haematobium* infection intensity [396]. The *IL13-1055C* allele was associated with high infection intensity and was preferentially transmitted to individuals with elevated infection burdens [396]. Differently, *IL13-1055T* allele was linked to resistance to

infection [396]. Still focusing on Th2 related genes, variations at the *STAT6* locus have been shown to be linked to *S. haematobium* infection intensity [397]. This gene encodes a major Th2 associated transcription factor.

Other genome regions have also been linked to schistosomiasis outcome. Based on a field study in Nigeria, variants in the gene *COLEC11*, found on chromosome 2p25.3, are linked to susceptibility to urinary schistosomiasis [400]. *COLEC11* encodes Collectin Kidney 1 which is a pattern recognition molecule of the lectin complement pathway. This molecule is mainly expressed in the kidneys, and it is believed to bind to the fucosylated schistosome tegument [400]. Chromosome 6 has also been implicated in schistosomiasis pathology. A major locus in the 6q22-q23 genome region has been linked to susceptibility to *S. mansoni* induced severe hepatic fibrotic disease in a Sudanese population [401]. The linkage peak is close to the *IFNGRI* gene which encodes the alpha-chain of the IFNɣ receptor [401]. A separate study in Egypt confirmed the involvement of this gene in severe schistosomiasis pathology [402]. IFNɣ is known to possess antifibrogenic activity and SNPs in *IFNG* are associated with severe liver fibrosis in Sudan [403]. *CTGF*, also found in the 6q22-q23 region, encodes the connective tissue growth factor which has profibrogenic properties. Variants affecting the binding of this growth factor are associated with severe fibrosis in Chinese, Sudanese, and Brazilian populations [404].

Studies focusing on human leukocyte antigen (HLA) genetics and schistosomiasis outcome have been difficult to interpret [405]. In the context of schistosomiasis, some HLA alleles have been described as protective whereas others have been considered risk factors for pathology [406]. For instance, a study in Egypt discovered an association between the class 1 allele B5 and hepatosplenic disease [405]. Additional studies looking at different HLA alleles are needed in order to fully describe their genetic contribution in schistosome infections.

1.5 Still hope for a schistosomiasis vaccine

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Ricciardi A, Ndao M (2015) Still hope for schistosomiasis vaccine. Hum Vaccin Immunother 11: 2504-2508.

Vaccines or at least chemotherapy combined with vaccination, present the best strategy for long-term sustained control of schistosomiasis. Since the parasites do not replicate within the human host, even a partially effective anti-schistosome vaccine could significantly contribute to morbidity reduction and disease elimination [407, 408]. A multi-component, integrated control program is likely the best option, and could include approaches such as vaccination, drug administration, environmental modifications, improved sanitation, and intermediate host control by molluscicides.

New antigen discovery has been aided by the major advances in schistosome molecular biology; the genome, the transcriptome, and the tegument proteome. The genomes of *S. mansoni* and *S. japonicum* were sequenced in 2009 followed by the genome of *S. haematobium* in 2012 [409-411]. The accessibility of this information in combination with the improving postgenomic technologies has the potential to identify plenty of new vaccine candidates. The most relevant targets are the excretory/secretory products as well as molecules on the surface of the worm. These are the molecules that constantly and directly interact with the host immune system.

One of the most important contributions in antischistosomal vaccine studies was the radiation-attenuated (RA) vaccine. Studies demonstrated that a single immunization with radiation attenuated cercariae can attain protection levels of 60-70% in animal models, and these levels can be increased further with repeated immunizations [115, 412-414]. The findings from RA vaccine studies provided crucial knowledge concerning the induction of protective immunity. The RA vaccine is a proof of concept that has paved the way for the development of molecular vaccines against schistosomiasis. These encouraging results along with major advances in molecular technologies prompted the World Health Organization (WHO), approximately 20 years ago, to commence the testing of the most promising vaccine candidates. In order to be considered significant, protection levels needed to reach the 40% threshold established by the WHO [415].

1.5.1 Sh28GST

Recombinant glutathione S-transferase cloned from *S. haematobium* (Sh28GST) was the first schistosome antigen to enter into clinical trials. The vaccine named Bilhvax is

formulated with an alum hydroxide adjuvant. In the pre-clinical studies, the vaccine significantly inhibited female worm fecundity and egg viability [416]. The Institut Pasteur and the French Institut National de la Santé Et de la Recherche Médicale took Bilhvax through both Phase I and II clinical testing. September 1998 marked the start of the Phase I clinical trial in Lille, France. The volunteers did not experience any adverse effects and no cross-reactivity was observed with the human GST(Pi) [416]. Immunogenicity results from the Phase I trial in healthy adults demonstrated that the vaccine elicited a Th2 response. This conclusion was supported by the increased production of Th2 cytokines (Il-5 and IL-13) and the presence of a predominant specific IgG1 response in vaccinated individuals [417]. Furthermore, serum from vaccinated participants was used to successfully inhibit Sh28GST enzymatic activity [417]. This particular observation was promising as the inhibition of schistosome 28GST has been linked to reduced worm fecundity and egg viability, which could potentially reduce pathology [417]. The Phase Ib trials were carried out in St. Louis, Senegal known to be endemic. The trial consisted of two groups of twelve healthy African children, between the ages of 6 and 10 years old. The children received two injections and they tolerated the vaccine with no sign of adverse effects [416]. Bilhvax was being marketed as a pediatric vaccine. The Phase III trial involving infected school children aged 6 to 9 years old began in 2009, in St. Louis. This clinical trial was designed to investigate whether Sh28GST vaccination with praziquantel administration could significantly delay pathologic recurrence. The trial should have come to an end in 2012. Unfortunately, no new information has been made available about the status of the vaccine; thereby, rendering specialists in the field skeptical of its future.

1.5.2 Sm14

The *S. mansoni* fatty acid binding protein Sm14 is the second candidate to enter clinical trials. In mice, the recombinant Sm14 protein generated protection levels of 67% against *S. mansoni* challenge, and complete protection against *Fasciola hepatica* challenge [418]. A vaccine formulated with Sm14 would have the potential to provide protection against two parasitic diseases, but also prevent economical losses caused by veterinary fascioliasis which causes annual losses of over USD 3 billion to the livestock industry [419]. The stability of the Sm14 recombinant protein was further improved in order to avoid dimerization and subsequent aggregation [420]. The protein was successfully expressed in *Pichia pastoris* for scale-up with

good manufacturing practice (GMP) [421]. The Sm14 clinical trials are the result of an initiative between Oswaldo Cruz Foundation, the Brazilian governmental financial agency, and Alvos Biotecnologia [419]. The Phase I clinical trial commenced in 2011, in Rio de Janeiro, Brazil. This initial phase was meant to determine the safety and tolerability of the vaccine composed of recombinant Sm14 with glucopyranosyl lipid adjuvant (GLA) and a squalene emulsifying agent (SE) in healthy adult volunteers [422]. GLA is a synthetic adjuvant known to stimulate Th1 responses, and squalene is used for the formation of oil-in-water emulsions. The data collected from the Phase I clinical trial demonstrated that the vaccine formulation was safe as there were no serious adverse events observed [421]. Furthermore, the formulation was shown to be immunogenic. Vaccinated individuals had significant levels of antigen specific total IgG antibodies present in their serum. There were significant increases in levels of the IgG1 and IgG3 isotypes after the first dose. IgG2 and IgG4 levels were significantly increased only after the third dose of the vaccine [421]. Moreover, immunogenicity data from the clinical trial showed a production of mixed Th1/Th2 cytokines and an expansion of single-cytokine producing $CD4^+$ T cells in the immunized individuals. However, a similar expansion was not observed for multi-functional T cells [421]. The data collected from the Phase I clinical trial demonstrated that the Sm14 formulation is safe and immunogenic; thus, allowing the Brazilian group to confidently move on to Phase II clinical trials which are expected to be performed in Brazil and Egypt.

1.5.3 Sm-TSP-2

The *S. mansoni* tetraspanin extracellular loop 2 (EC-2) of Sm-TSP-2 is another vaccine candidate to recently enter clinical trials. Tetraspanins belong to a family of integral membrane proteins abundantly expressed in the tegument of schistosomes; thereby, making them accessible to the host immune response [376]. Sm-TSP-2 is recognized by IgG1 and IgG3 present in the serum of individuals capable of clearing the infection [374]. Mice immunized with the recombinant EC-2 of Sm TSP-2 plus Freund complete (prime) or Freund incomplete (two boosts) adjuvant have a 57% reduction in worm burden and a 64% reduction in hepatic egg burden compared to control mice [374]. The promising results seen with EC-2 of Sm-TSP-2 prompted the Human Hookworm Vaccine Initiative (HHVI) to select EC-2 of Sm-TSP-2 for development as a human vaccine candidate [415]. HHVI is a group dedicated to the

development of vaccines against helminth infections. Tetraspanins have already been used to generate veterinary vaccines; thereby, providing an encouraging precedent for this type of antigen [423]. The promising pre-clinical data and the successful scale-up expression with GMP of the antigen in *P. pastoris* propelled Sm-TSP-2 into Phase I clinical trials [424, 425] which are ongoing in Houston, Texas [426]. Healthy adult males were recruited for the Phase I trial, and two Sm-TSP-2 formulations are being tested (with Alhydrogel® only and with Alhydrogel® plus an aqueous GLA formulation) [427]. The goal of the clinical trial is to determine the safety, reactogenicity, and immunogenicity of both Sm-TSP-2 formulations in healthy adults.

1.5.4 Promising pre-clinical studies

There are currently several other vaccine candidates which are generating promising results in pre-clinical studies and have the potential to enter into clinical testing in the near future (Table 1.4). The *S. mansoni* calpain large subunit, Sm-p80, is another vaccine candidate generating promising results in pre-clinical studies. Worm burden and hepatic egg burden were reduced by 70% and 75%, respectively, in mice immunized with a formulation of recombinant Sm-p80 and CpG dinucleotides compared to controls [428]. The promising results observed in the mouse model, prompted the group to continue their experiments in a nonhuman primate model; the baboon. Experimentally immunized baboons had a 58% decrease in worm burden compared to the controls [429]. These levels of protection have previously never been seen with a schistosome antigen in large animal models. In addition to prophylactic efficiency, the Sm-p80 immunizations could also induce killing of established adult worms and protect against urinary schistosomiasis caused by *S. haematobium* [430]. Furthermore, follow-up studies have demonstrated that Sm-p80 vaccinations generate long-lasting humoral responses. In the mouse model, antigen specific IgG titers were detected for up to sixty weeks post vaccination. In baboons, Sm-p80 specific IgG antibodies were detected five to eight years following the initial vaccination [431].

Our group has chosen to target *S. mansoni* Cathepsin B (Sm-Cathepsin B) as a vaccine candidate. Immunization of mice with Sm-Cathepsin B adjuvanted with CpG oligodeoxynucleotides, a known Th1 agonist, conferred a 59% decrease in worm burden. Hepatic and intestinal egg burdens were decreased by 56% and 54% respectively compared to

control groups [281]. Sm-Cathepsin B formulated with Montanide ISA 720 VG, a squalene based water-in-oil adjuvant, decreased worm, hepatic egg, and intestinal egg burdens by 60%, 62%, and 56% respectively compared to control groups [282]. Antibody production was significantly augmented in the vaccinated mice; both formulations elicited Sm-Cathepsin B specific total IgG endpoint titers that were greater than 120,000. Furthermore, analysis of cytokine secretion levels revealed that immunization with Sm-Cathepsin B plus CpG resulted in a Th1 biased response whereas immunization with Sm-Cathepsin B plus Montanide ISA 720 VG led to a mixed Th1/Th2 response. Our results highlight the potential of Sm-Cathepsin B as a strong vaccine candidate against schistosomiasis [281, 282]. Details of the Sm-Cathepsin B studies performed by our group will be discussed in later chapters.

Sm29 is another *S. mansoni* tegument protein which has been extensively studied in pre-clinical studies. In a mouse model of schistosomiasis, recombinant Sm29 with Freund's adjuvant induced a Th1 biased response and significantly reduced numbers of worms, intestinal eggs, and liver granulomas (51%, 60%, 50%, respectively) [432]. It was also demonstrated that Sm29 immunizations protected mice that were previously exposed to a schistosome challenge [433]. This study attempted to recreate conditions that would be encountered in the field as individuals living in endemic areas are constantly exposed to the parasite and have likely been treated with praziquantel at least once. Additional pre-clinical studies have tried to increase the protective potential of Sm29 by combining it with other *S. mansoni* antigens such as Sm14 and Sm-TSP-2 [434-437]. Interestingly, DNA vaccinations and protein fusion approaches with Sm-TSP-2 have generated only partial protection against schistosomiasis [436, 437]. A multi-antigen approach with Sm14 and Sm29 resulted in significant protection; however, it did not surpass levels that were originally seen with Sm29 alone [434, 435].

Pre-clinical studies have generated an impressive list of vaccine candidates capable of eliciting either significant or partial protection against schistosomiasis. The protective potential of antioxidant enzymes has been studied. More specifically, *S. mansoni* Cu-Zn superoxide dismutase and glutathione S peroxidase have been tested as DNA vaccine formulations. They have been shown to elicit protection in both a mouse and non-human primate model of schistosomiasis [438, 439]. Proteins exposed to the host immune system are attractive vaccine candidates. A DNA vaccine based on the surface antigen Sm23 elicited protection in

immunized mice [440]. A prime boost approach using the DNA vaccine formulation and recombinant Sm23 did not improve protection levels [441]. Partial protection was obtained by immunizing mice with a formulation of the newly transformed schistosomulum tegument (Smteg) with Freund's adjuvant [442]. Interestingly, this protection was dependent on the presence of the adjuvant [443]. Smteg was also tested in the presence of alum-CpG; however, only partial protection was achieved [444]. Other *S. mansoni* antigens being tested in pre-clinical vaccine studies include members of the dynein light chain family (SmDLC), tetraspanin orphan receptor (SmTOR), a recombinant component of the adult protein fraction PIII (rP22) [445-447].

1.5.5 *S. japonicum*: a possible veterinary vaccine

There are also several *S. japonicum* antigens which are undergoing pre-clinical studies for vaccine development. There is an added level to consider when researching vaccine development for *S. japonicum*; zoonotic transmission from reservoir hosts. For this schistosome species, there is the possibility of developing a transmission-blocking veterinary vaccine. Most of the *S. japonicum* antigens being tested are membrane proteins, muscle components, or enzymes [14]. Examples of some vaccine candidates include Sj26GST [448-453], Sj23 [454, 455], Paramyosin (Sj97) [456-459], *S. japonicum* very low-density lipoprotein binding protein (SVLBP) [460], Serpins [461], *S. japonicum* triose-phosphate isomerase (TPI) [462, 463], *S. japonicum* heat shock protein (SjHSP70) [464], *S. japonicum* UDP-glucose-4-epimerase [465], and *S. japonicum* insulin receptor (SjLD2) [466]. *S. japonicum* pre-clinical studies have tested both DNA and recombinant protein vaccines in mouse models as well as in water buffalo and sheep in field studies.

1.5.6 *S. haematobium*: the neglected schistosome

The list of *S. mansoni* and *S. japonicum* vaccine candidates is quite extensive. However, with the exception of Sh28GST, there are almost no *S. haematobium* antigens being targeted in pre-clinical studies. Any research addressing the development of a vaccine against *S. haematobium* infection focuses on *S. mansoni* candidates that have cross-protection potential. *S. haematobium*, the causative agent of urogenital schistosomiasis, accounts for approximately two thirds of all human schistosome infections [53]. In fact, the actual number of urogenital

schistosomiasis cases is believed to be underestimated; thereby, potentially making it the most common infection and health condition in sub-Saharan Africa [467]. *S. haematobium* infections have also been linked to Africa's AIDS and emerging cancer epidemics [468-471]. There is abundant evidence indicating that urogenital schistosomiasis is a top public health concern. However, *S. haematobium* has long been considered the neglected schistosome. A PubMed search of schistosome publications between 2008 and 2012 revealed that there were 644 papers published for every million cases of *S. japonicum*, 25 papers for every million cases of S. *mansoni*, and only 3 papers for every million cases of *S. haematobium* (Table 1.5) [472]. If *S. haematobium* is responsible for the majority of schistosomiasis cases, and it is a co-factor for other diseases, then why is it so understudied? Some explanations for this stagnation include the lack of an adequate mouse model for urogenital schistosomiasis, the absence of *in vitro* systems to study the parasite, and the delay in completing the *S. haematobium* genome [472]. Recent advances in *S. haematobium* research have begun to correct these weaknesses. For instance, microinjecting *S. haematobium* eggs into the bladders of mice can reproduce the histopathology found in human urogenital schistosomiasis; thus, providing a reliable mouse model [473]. Furthermore, the development of techniques using short interfering ribonucleic acids (siRNAs) to genetically manipulate the parasite has improved *S. haematobium in vitro* studies [474, 475]. Finally, the completion of the parasite genome in 2012 revealed a number of *S. haematobium* unique genes not present in the other two main schistosome species affecting humans [411, 476, 477]. With these new tools in the *S. haematobium* research arsenal, there is still hope for the development of a vaccine to combat urogenital schistosomiasis.

1.5.7 Hurdles to Overcome

The introduction of praziquantel had a significant impact on schistosomiasis control. Mass administration of praziquantel resulted in never before seen reductions in morbidity, and thereby creating an illusion that schistosomiasis was a disease of the past. In consequence, research for a schistosomiasis vaccine suffered from a lack of funding due to the misconception that this disease was not an urgent public health issue. This lack of interest from the scientific community presented a major hurdle in the development for a vaccine against schistosomiasis as such research is costly. The funding situation has slightly improved over the last years greatly due to the reassessment of its effect on the global burden of disease and the inability of

praziquantel to disrupt disease transmission. Furthermore, the schistosomiasis vaccine field has recently garnered attention as a result of a Gates Foundation grant of USD 2.85 million. The grant was awarded to Dr. Afzal A. Siddiqui of Texas Tech University Health Sciences Center to support a proof of concept trial of Sm-p80/GLA-SE schistosomiasis vaccine [478].

Human correlate studies have represented a major step forward in the development of an anti-schistosome vaccine. These studies revealed that a balance between positive and negative responses rather than biased responses correlate with resistance to infection. This lack of a straight-forward response has rendered the testing of potential vaccine antigens difficult to standardize. Independent testing has revealed that certain schistosome antigens require Th2 responses for protection whereas others require Th1 responses [479-482]. Once the antigen has been selected, one must consider formulation. The adjuvant used is important for the stimulation of the desired immune response. Several adjuvants have been tested for schistosomiasis vaccine formulation: CpG dinucleotides, alum, Freund's complete/incomplete adjuvant are just a few examples. The adjuvant selection is one of many factors that influence the results of pre-clinical studies. Other factors include the animal model, the vaccination schedule, the vaccine delivery route, and the dose for parasite challenge. A standardized method to test upcoming antigens is necessary in order to obtain consistently reproducible data. Next, we must ask whether our method of testing one antigen at a time is correct. Developing an anti-parasitic vaccine is not a simple task. These pathogens are complex and, they have developed an arsenal of mechanisms to evade our defenses and establish an infection. Studies developing vaccines against other parasitic infections, such as malaria and leishmaniasis, suggest that an optimal anti-parasitic formulation might be composed of more than a single antigen and a defined adjuvant [483, 484]. Perhaps testing multi-antigen vaccine formulations earlier on in pre-clinical trials may accelerate the discovery of the most effective composition.

Once the vaccine candidate has been selected and shown to elicit high levels of protection in animal models, it must be produced with GMP and in large scale; if it cannot, it is of little value. Some very promising antigens, such as paramyosin and MAP4, have been abandoned because they could not pass this rate limiting step [407]. This implies that we might have to compromise antigen efficacy for the ability to be scaled-up with GMP. It is important not to overlook the importance of the production process as it can be as challenging to navigate

through as the initial antigen discovery and pre-clinical stages. Furthermore, a schistosomiasis vaccine would be delivered to some of the poorest populations; therefore, it would need to be produced cheaply. A schistosomiasis vaccine would require costing below USD 1-2 per dose, which restricts the use of certain techniques and equipment [415]. Therefore, the focus should be on expressing the proteins of interest in low cost yeast and bacterial expression vectors such as *Pichia pastoris* and *Escherichia coli*.

Schistosomiasis vaccine development is tedious and costly. Maintaining the life cycle of the parasite requires the appropriate housing of the intermediate snail host. The pre-clinical phase usually involves immunological and protection studies carried out in the mouse model. There are worries that the mouse model is too dissimilar from human schistosomiasis, and that results from vaccine pre-clinical studies cannot translate to real life applicability. One of the major concerns stems from the fact that laboratory animals living in a controlled environment are being used to test a vaccine that will be administered to populations living in schistosomiasis endemic regions. These models do not take into account co-infections. The majority of individuals infected with a schistosome species also suffer from malaria, HIV, or soil-transmitted helminth infections [139, 485, 486]. The laboratory mice are pathogen free; therefore, these studies do not take into account an underlying infection's effect on vaccine efficacy. Furthermore, individuals living in schistosomiasis endemic regions have likely already been infected and treated with praziquantel at least once in their lives. Usually, pre-clinical studies do not analyze vaccine success in previously infected mice that were cured with praziquantel. These different real life scenarios highlight the complexity of introducing a new vaccine in endemic settings. Furthermore, it is important to learn lessons from the ASP-2 based hookworm vaccine trial. The vaccine was safe and well tolerated in U.S. volunteers. However, once introduced in a hookworm endemic region of Brazil, the vaccine elicited IgE mediated allergic responses in the volunteers; thereby, halting the clinical trial [179, 487]. These individuals had parasite specific IgE antibodies which had developed from past infections. The unfortunate outcome of this study needs to be kept in mind when developing a schistosomiasis vaccine. It is essential to perform human correlate studies in order to test a candidate antigen's reactivity with IgE in serum samples from high-risk endemic populations. In terms of better models for vaccine efficacy, baboons would be a good choice because they develop a disease which is similar to that seen in humans [488, 489]. However, they are significantly more

expensive than mice and they require more extensive maintenance. Furthermore, there are more ethical hurdles from institutional review boards and regulatory agencies to overcome when conducting studies on baboons.

1.5.8 Still hope

Schistosomiasis affects a large portion of the world's population and many more are at risk of infection. Due to its morbidity and impact on public health, schistosomiasis is the most important helminth infection; yet, it is a neglected tropical disease. The development of a vaccine against schistosomiasis has the potential to contribute a long-lasting decrease in disease spectrum and transmission. A vaccine would lessen the morbidity of the disease by decreasing worm burden and egg production. Furthermore, used in combination with praziquantel, it could prolong the intervals between required drug treatments. The feasibility of developing a vaccine against schistosomiasis is supported by convincing arguments. For instance, immunization of mice with a single dose of cercarial RA vaccine induces high levels of protection. Further supporting evidence includes the effective development and application of veterinary recombinant vaccines against parasitic worms. Bilhvax, Sm14, and Sm-TSP-2 are the only antischistosome vaccines to have entered clinical trials. There are currently several antigens that are in the research pipeline and are advancing in pre-clinical trials. This growth in new antigen discovery is the result of advances in schistosome molecular biology. Based on their pre-clinical data, Sm-p80 and Sm-Cathepsin B appear to be promising vaccine candidates. The vaccine formulations based on these antigens are being refined, and hopefully, they will enter into clinical trials in the near future. Once the technical and economical hurdles of vaccine development are surpassed, the next step would be to determine the best way to make the vaccine globally accessible.

1.6 Rationale and research objectives

The World Health Organization considers schistosomiasis the most important human helminth infection. Treatment of schistosomiasis relies exclusively on the single drug praziquantel which raises concerns of potential drug resistance. Moreover, praziquantel does not provide prophylactic protection and does not prevent re-infection. The lack of therapeutic drugs and preventative measures, as well as the high disease burden caused by the infection are justifications for developing a vaccine against schistosomiasis. The following chapters describe

the candidate's contribution to novel research focusing on a Sm-Cathepsin B based anti-schistosomiasis vaccine.

Sm-Cathepsin B possesses characteristics that make it a favourable vaccine candidate. It is expressed by the migrating larva as well as the adult, it is highly immunogenic, and it is secreted. Therefore, the first objective of this thesis was to investigate the protective efficacy of Sm-Cathepsin B. Different vaccine formulations were tested and we evaluated their ability to reduce parasite burden in a mouse model of schistosomiasis. Given the importance of the adjuvant in a vaccine formulation, the immune responses elicited by the different formulations were also analyzed. The second objective of this thesis was to describe the type of immune responses generated by the protective Sm-Cathepsin B vaccine formulations. We focused on both antigen-specific antibody production and cytokine secretion levels.

Finally, we wanted to expand our studies beyond simple measures of efficacy and immunogenicity. Given the different vaccine formulations tested and their diverse respective immunological effects, mechanistic studies are both scientifically and commercially valuable. Moreover, regulatory authorities are typically very interested in mechanistic data in support of Phase II and Phase III clinical trials. Therefore, the final objective of this thesis was to identify the underlying mechanisms of Sm-Cathepsin B mediated protection for the different vaccine formulations.

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1.8 Tables

Table 1.1 Breakdown of the estimated DALYs of the neglected tropical diseases (NTDs)

Disease	DALYs (in millions)	DALY rank	YLLs (in millions)	YLL rank	YLDs (in millions)	YLD rank
NTDs	26.06		7.90		18.22	
Soil-transmitted helminths	5.19	1	0.20	7	4.98	1
Hookworm disease	3.23	4	0	-	3.23	2
Ascariasis	1.32	8	0.20	7	1.11	6
Trichuriasis	0.64	10	0	-	0.64	7
Leishmaniasis	3.32	2	3.19	1	0.12	12
Schistosomiasis	3.31	3	0.32	5	2.99	3
Lymphatic Filariasis	2.78	5	0	-	2.77	4
Food-borne trematodiases	1.88	6	0	-	1.87	5
Rabies	1.46	7	1.46	2	<0.01	16
Dengue	0.83	9	0.81	3	0.01	15
African Trypanosomiasis	0.56	11	0.55	4	0.08	14
Chagas Disease	0.55	12	0.24	6	0.30	11
Cysticercosis	0.50	13	0.05	8	0.46	9
Onchocerciasis	0.49	14	0	-	0.49	8
Trachoma	0.33	15	0	-	0.33	10
Echinococcosis	0.14	16	0.03	9	0.11	13
Yellow Fever	<0.001	17	<0.01	10	<0.01	16

Disability-adjusted life years (DALYs) represent a time based measure that combines years of life lost due to premature mortality (YLLs) and years lived with a disability (YLDs). The numbers represented in this table are from the Global Burden of Disease Study 2010. The table is adapted from [23].

Table 1.2 Diagnostic tools for the detection of schistosomiasis

	Method			
Pathogen	**Microscopy**	**Serology**	**Molecular**	**Proteomic**
***Schistosoma* species**	Kato-Katz on stool for intestinal schistosomiasis[153], Detection of eggs in urine for urinary schistosomiasis	IHA, ELISA[159], Dipstick[160,163]	PCR[168,173], real-time PCR[169,170], multiplex real-time PCR[171], Dipstick[172]	-

The information from this table is adapted from **Ricciardi A**, Ndao M (2015) Diagnosis of parasitic infections: what's going on? J Biomol Screen 20: 6-21.

IHA: indirect hemagglutination; ELISA: enzyme-linked immunosorbent assay; PCR: polymerase chain reaction.

Table 1.3 Immunity to schistosomiasis [14]

Acquired immunity after drug treatment	Naturally immunity: endemic normal individuals
• Acquired with age • Accelerated by chemotherapy with praziquantel • Mediated by Th2 response • IgE stimulates eosinophils to release cytotoxins	• Parasite negative for over 5 years • Never treated with antischistosome drugs • Continually exposed to the parasite • Mount vigorous cellular & humoral responses to crude antigen • Peripheral blood mononuclear cells secrete both Th1 & Th2 cytokine • Th1 response believed to be key

Table 1.4 ***Schistosoma mansoni*** **vaccine candidates tested in preclinical studies**

Antigen	Identity	Outcome
Sm14	Fatty acid binding protein	Mice: 67% protection against *S. mansoni* challenge [418] Phase I clinical trial: demonstrated that the Sm14 formulation is safe and immunogenic [421]
Sm-TSP-2	Tetraspanin integral membrane protein	Mice: 57% worm and 64% hepatic egg reductions [374] Phase I clinical trial: ongoing in healthy adult males in Houston, Texas [426,427]
Sm-p80	Calpain; neutral cysteine peptidase	Mice: 70% worm and 70% hepatic egg reductions [428] Baboons: 58% worm reduction [429] Ongoing: preparation for human clinical trials [490]
Sm-Cathepsin B	Gut cysteine peptidase	Mice: 54-62% protection against *S. mansoni* challenge [281,282]
Sm29	Glycoprotein on parasite tegument	Mice: 51% worm, 50% liver granuloma, and 60% intestinal egg reductions [432]
Sm-SOD	Superoxide dismutase	Mice: 54% worm reduction [438] Baboons: 0-20% worm, 2-34% hepatic egg, and 22-45% intestinal egg reductions [439]
Sm23	Tetraspanin integral membrane protein	Mice: 21-44% worm reduction [440]
Smteg	Newly transformed schistosomula tegument	Mice: 43-48% worm and 65% hepatic egg reductions [442]
SmDLC	Member of the dynein light chain family	Mice: 43% & 51% worm and 70% liver granuloma size reductions [445]
SmTOR	Tetraspanin orphan receptor	Mice: 45% & 64% worm and 50% hepatic egg reductions [446]
rP22	Component of the adult protein fraction PIII	Mice: 51% worm, 23% hepatic egg, 60% liver granuloma size, and 71% fibrosis reductions [447]

Sm-TSP-1	Tetraspanin integral membrane protein	Mice: 34% worm and 52% hepatic egg reductions [374]
Sm28-GST	Glutathione S-transferase	Mice: 30-60% protection against *S. mansoni* challenge [491]
Sm28-TPI	Triose phosphate isomerase	Mice: 30-60% protection against *S. mansoni* challenge [491]
Sm97 paramyosin	Paramyosin	Mice: 30% protection against *S. mansoni* challenge [491]
SmGPX	Glutathione S-peroxidase	Mice: 43% worm reduction [438] Baboons: 17% worm, 24% hepatic egg, and 31% intestinal egg reductions [439]
SMYB1	Y-box-binding protein	Mice: 26% worm and 28% hepatic egg reductions [492]

Table 1.5 ***Schistosoma*** **species and number of PubMed citations between 2008 and 2012**

Schistosoma **species**	**Estimated number of human infections**	**Number of PubMed citations between 2008 and 2012**[a]	**PubMed citations per number of human infections**
S. japonicum	1 million	644	644
S. mansoni	54 million[b]	1,371	25
S. haematobium	112 million[b]	342	3

[a]The PubMed search was conducted on July 14, 2012.

[b]Numbers of human infections in Sub-Saharan Africa only.

The information presented in this table is adapted from [472].

1.9 Figures and legends

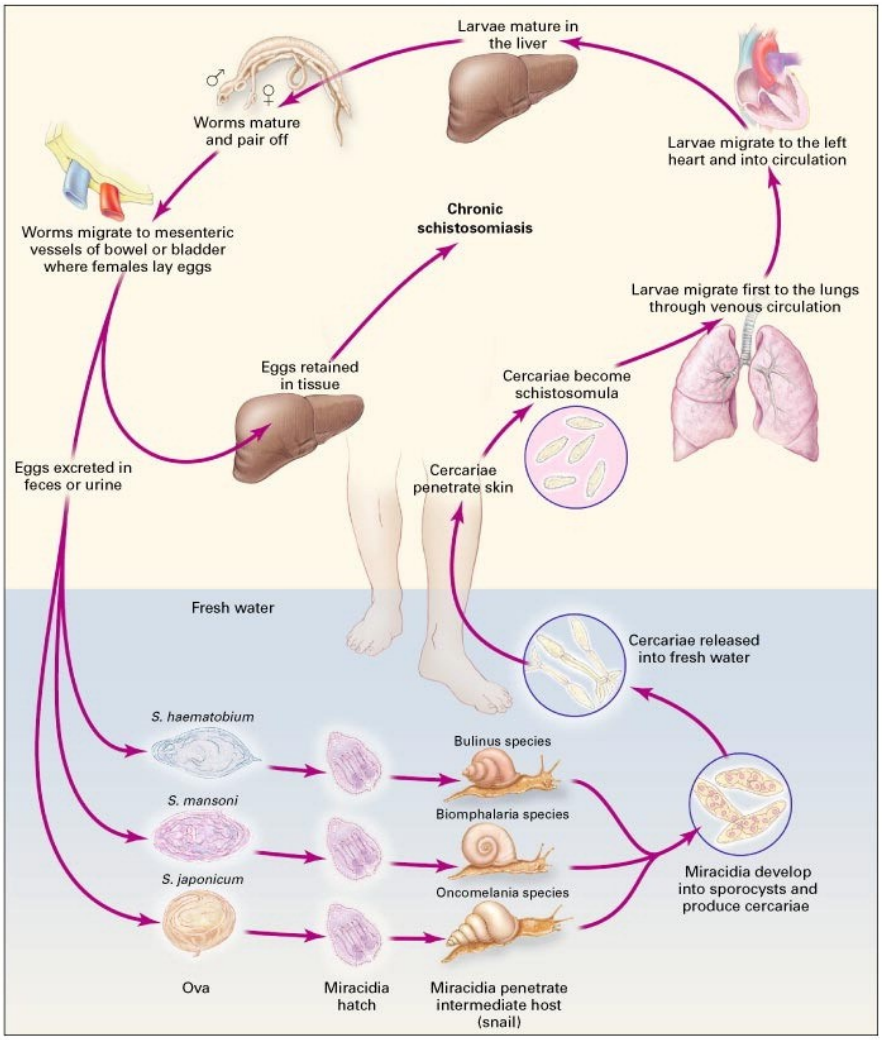

Figure 1.1 *Schistosoma* lifecycle

Fresh water dwelling *Schistosoma* cercaria infect the definitive host through skin penetration. Inside the host, the cercaria transform into the migrating schistosomula which travel through the circulatory system as they mature. Female and male adult worms pair off and the female begins to produce hundreds to thousands of eggs per day. The eggs are released into the environment via the feces (*S. mansoni* and *S. japonicum*) or the urine (*S. haematobium*). The eggs will hatch in fresh water; thereby, releasing the miracidia which can infect the intermediate snail host. Reproduced with permission from Ross AG, Bartley PB, Sleigh AC, Olds GR, Li Y, et al. (2002) Schistosomiasis. N Engl J Med. 346: 1212-1220. doi: 10.1056/NEJMra012396, Copyright Massachusetts Medical Society.

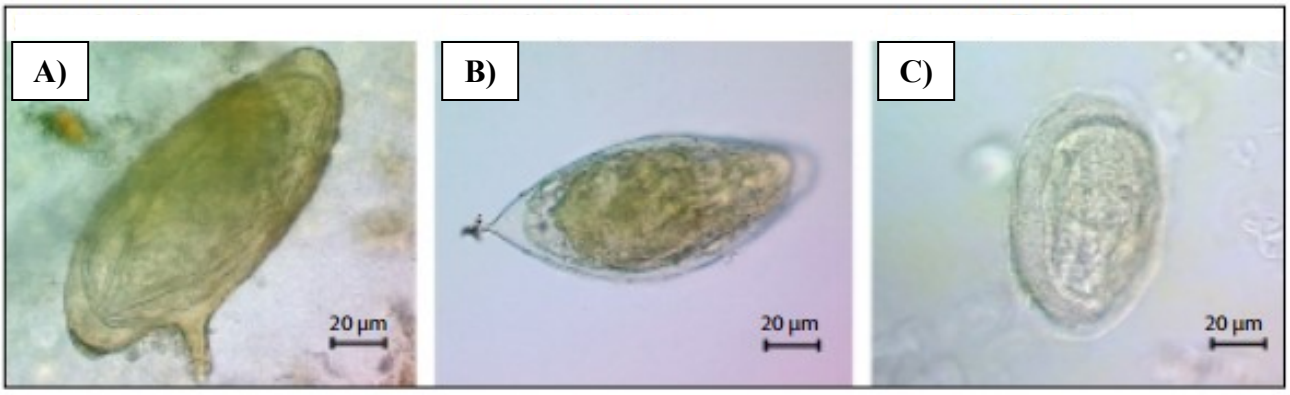

Figure 1.2 *Schistosoma* eggs

The three main *Schistosoma* species affecting humans can be differentiated through the morphology of their eggs. **(A)** *S. mansoni* eggs have a characteristic lateral spine, **(B)** *S. haematobium* eggs possess an apical spine, and **(C)** *S. japonicum* eggs are distinguished by their lack of a spine.

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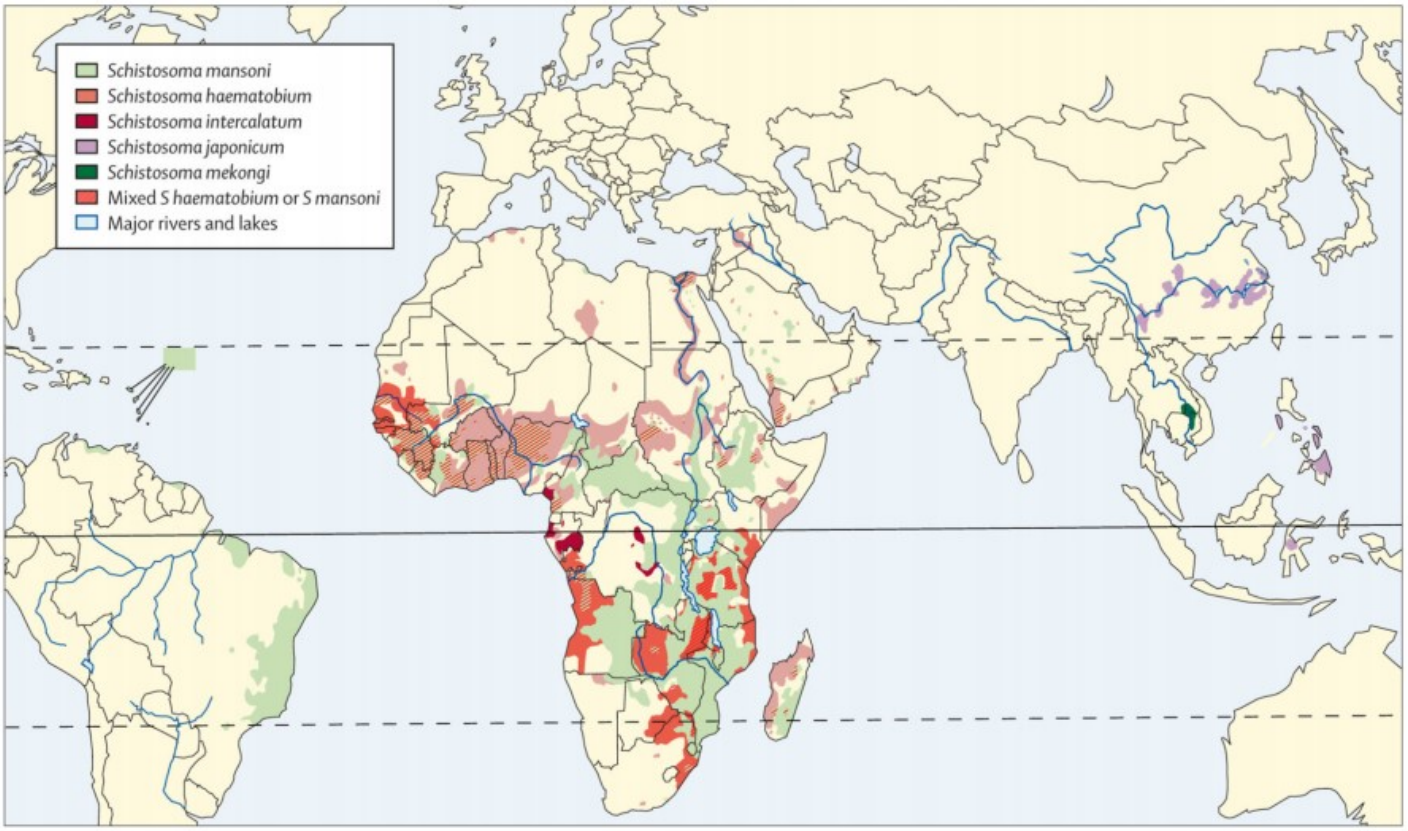

Figure 1.3 Geographic distribution of schistosomiasis

Schistosomiasis is found mainly in the tropical and subtropical regions of the world. *S. mansoni* is the only species present in the Americas. Both *S. mansoni* and *S. haematobium* cause disease in Africa and the Middle East. In recent years, there have been confirmed cases of *S. haematobium* infections in Corsica, France (not shown on map). *S. japonicum* occurs in Asia, primarily China and the Philippines. *S. mekongi* also causes human disease; however, it is restricted to the Mekong River basin.

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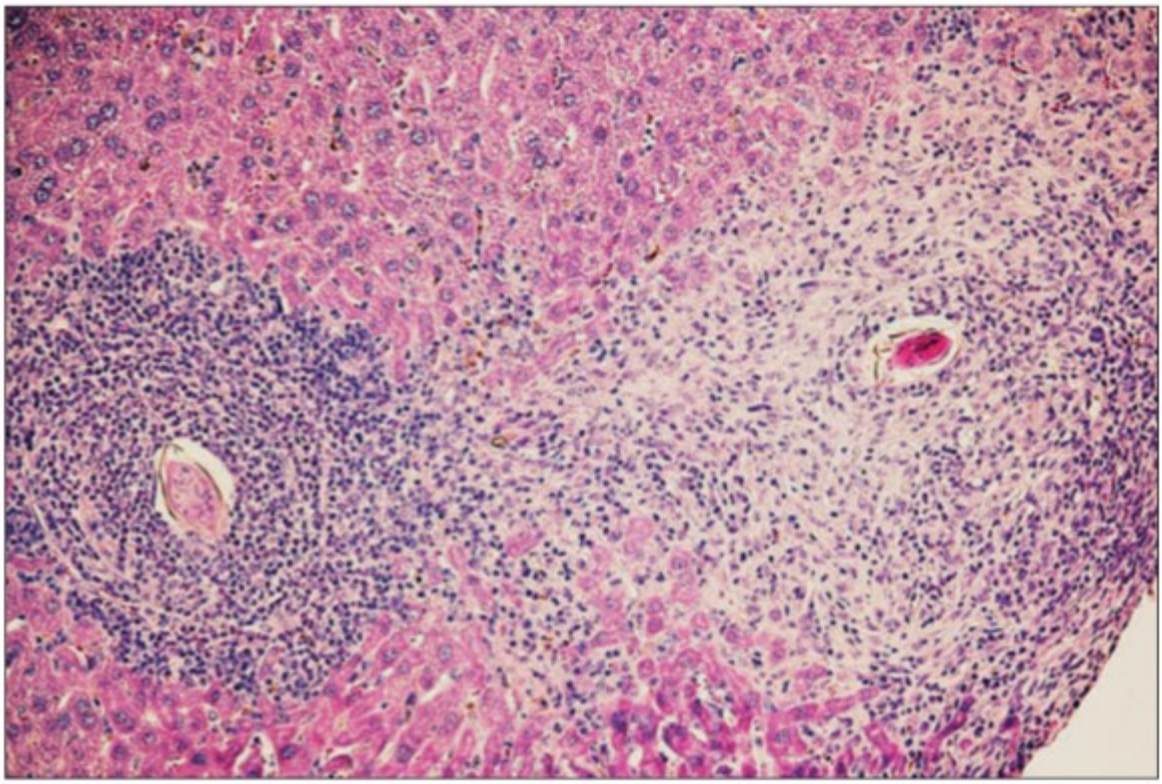

Figure 1.4 *Schistosoma mansoni* egg induced granulomas

Approximately half of the eggs produced by the female adult worm become trapped in the host tissues. The antigens released by these trapped eggs results in a delayed type hypersensitivity response and subsequent formation of granulomas. The image represents *S. mansoni* egg induced granulomas in the liver of an infected mouse.

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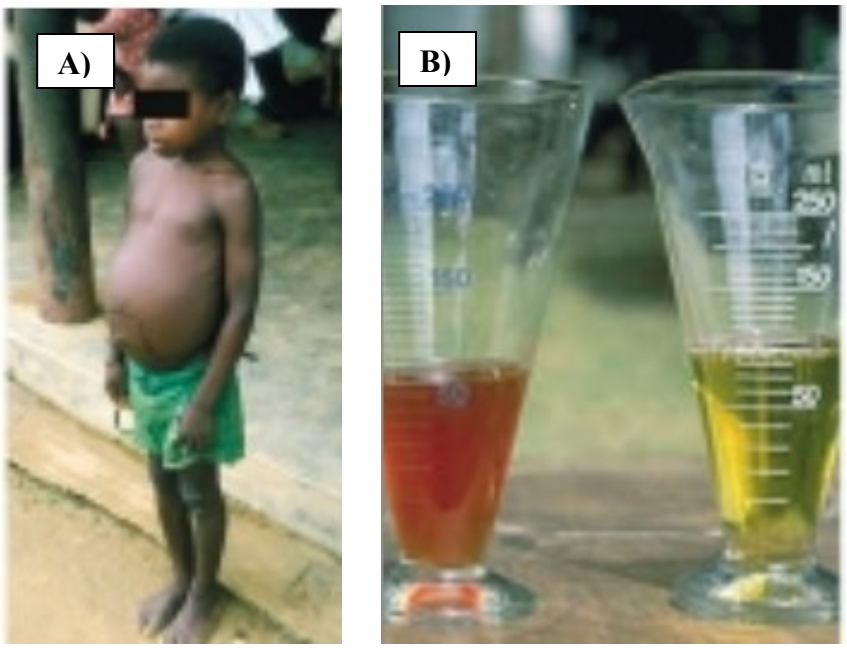

Figure 1.5 *Schistosoma* disease manifestation

(A) Enlargement of the liver and spleen is commonly observed in advanced cases of intestinal schistosomiasis. **(B)** Blood in urine, also known as hematuria, is the classic symptom of urogenital schistosomiasis.

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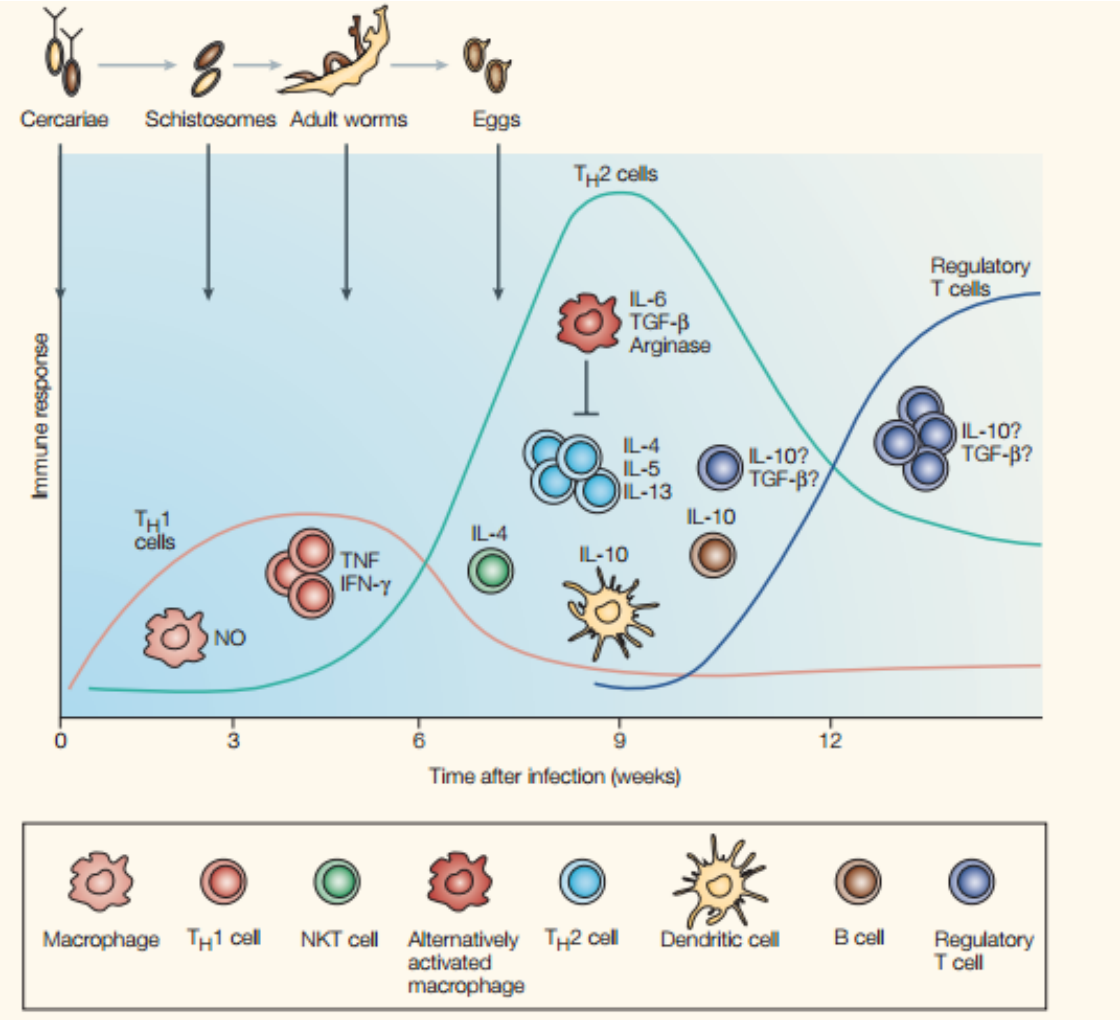

Figure 1.6 Immune response to *Schistosoma*

The initial immune response after infection is Th1 dominant. Approximately six weeks post infection (the start of oviposition), the immune response is skewed towards a Th2 cell response. This shift is mediated by antigens released from tissue entrapped parasite eggs. The chronic phase of schistosomiasis is a hypo-responsive state characterized by the development of IL-10 secreting regulatory T cells and alternatively activated macrophages.

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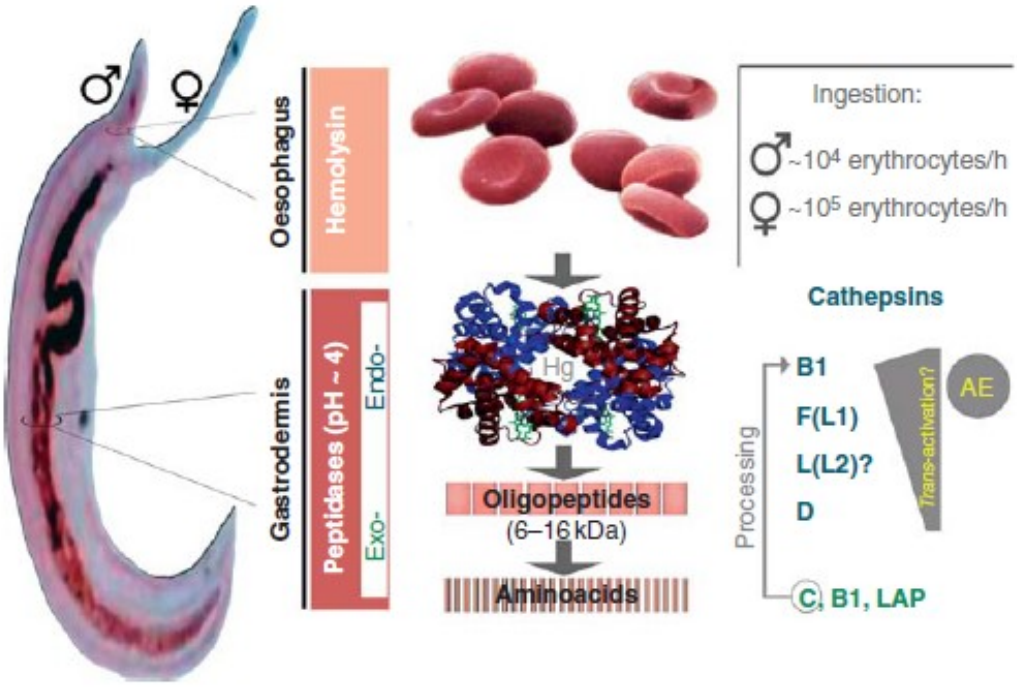

Figure 1.7 *Schistosoma mansoni* peptidases involved in blood digestion

The adult worm possesses a network of peptidases that mediates the degradation of host blood proteins, such as hemoglobin, into absorbable peptides and amino acids.

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Chapter 2: Evaluation of the immune response and protective efficacy of *Schistosoma mansoni* Cathepsin B in mice using CpG dinucleotides as adjuvant

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2.1 Preface

Schistosoma mansoni Cathepsin B (Sm-Cathepsin B) is the most abundant cysteine peptidase in the parasite gut. It is the main component of the network of peptidases involved in hemoglobin digestion and it is essential for proper parasite development. Sm-Cathepsin B is also detected in the parasite's excretory/secretory products; thus, making it a promising vaccine candidate as it is in constant interplay with the host's immune system. Our collaborator, Dr. John P. Dalton, previously demonstrated that Sm-Cathepsin B possesses inbuilt adjuvant properties, and that immunizations with the recombinant protein alone results in a decrease in parasite burden. However, the formulation's immunogenicity and protection efficacy could be improved. Immunizations with unadjuvanted Sm-Cathepsin B elicited low levels of parasite-specific antibodies, and did not decrease intestinal egg burden. We sought to optimize the

vaccine formulation by adding a Th1 promoting adjuvant. To this end, we immunized mice with a formulation composed of recombinant Sm-Cathepsin B and CpG dinucleotides. The animals were then exposed to a cercarial challenge. The following chapter describes the protection efficacy analyzed using a mouse model of schistosomiasis. The chapter also describes our assessment of the antigen-specific immune response by looking at antibody production and cytokine secretion levels.

2.2 Abstract

Schistosomiasis is the most important human helminth infection due to its impact on public health. Worldwide, schistosomiasis is estimated to infect at least 200 million individuals while 700 million are at risk. The clinical manifestations are chronic and significantly decrease an individual's quality of life. Infected individuals suffer from long-term organ pathologies including fibrosis which eventually leads to organ failure. The development of a vaccine against this parasitic disease would contribute to a long-lasting decrease in disease spectrum and transmission. Our group has chosen to target *Schistosoma mansoni* Cathepsin B as a prospective vaccine candidate. The recombinant protein was tested in the presence of synthetic oligodeoxynucleotides containing unmethylated CpG dinucleotides, which are Toll-like receptor 9 agonists known to stimulate a Th1 response. This formulation conferred a 59% decrease in worm burden as well as a reduction in egg burden. Hepatic egg burden and intestinal egg burden were decreased by 56% and 54% respectively. Immunizations with the formulation elicited robust production of Sm-Cathepsin B specific antibodies, both IgG1 and IgG2c but with the latter predominating. Furthermore, splenocytes isolated from the immunized animals, compared to control animals, had increased secretion levels of key Th1 cytokines, IFN-γ and TNF-α, as well as the chemokine CCL5 when stimulated with recombinant Sm-Cathepsin B. These results highlight the potential of Sm-Cathepsin B/CpG as a vaccine candidate against schistosomiasis.

2.3 Introduction

Schistosomiasis is a fresh-water borne parasitic disease caused by trematode worms belonging to the *Schistosoma* genus. There are currently approximately 200 million people infected, and over 700 million individuals who are at risk of infection. Infections caused by *Schistosoma mansoni* are geographically focused in Sub-Saharan Africa, Brazil, Venezuela,

Egypt, and the Arabic peninsula [1, 2]. The pathology associated with schistosomiasis is due to the deposition of parasite eggs in tissues that elicit a delayed type hypersensitivity response leading to granuloma formation and ultimately culminating in organ damage and failure. This neglected disease is of great public health importance due to the severe morbidity it causes [3]. Current schistosomiasis control strategies rely on mass treatment with praziquantel which has greatly helped decrease disease related morbidity. However, control programs solely relying on this drug struggle with effectiveness and sustainability [1, 4, 5]. Treatment with praziquantel does not prevent re-infections, and infection intensities often return to baseline approximately six months after chemotherapy treatments are stopped [6]. Furthermore, in areas of high transmission, rebound morbidity following drug treatment poses a great threat, especially in children [7, 8]. These observations point to a constant need for drug administration, questioning the long-term sustainability of praziquantel-based control programs. This continuous drug pressure also raises concerns for potential emergence of resistance. These fears are strengthened by the presence of parasites with decreased sensitivity to praziquantel in Egypt, Kenya, and Senegal [9, 10], and the ability to generate resistant strains in the laboratory [11, 12]. The development of a vaccine against this parasitic disease has the potential to contribute a long-lasting decrease in disease spectrum and transmission. During the mid-1990s, the WHO Special Programme for Research and Training in Tropical Diseases (TDR/WHO) planned for the independent testing of various *Schistosoma mansoni* antigens with the hopes of uncovering an optimal vaccine candidate. The committee's goal was to find an antigen that could consistently induce 40% protection or better [6]. Current available annotated parasite genomes represent an invaluable tool for new antigen discovery and testing for vaccine candidates as well as drug targets [13].

S. mansoni Cathepsin B, previously known as Sm31, is the most abundant papain-like cysteine protease found in the parasite gut and is involved in digestion of host hemoglobin, serum albumin, and immunoglobulin G (IgG) [14-16]. Knock-down of Cathepsin B transcription by RNAi results in decreased protease activity and parasite growth retardation [17], thereby demonstrating its importance in schistosome development. Serum belonging to mice immunized with radiation attenuated cercariae can identify the Sm-Cathepsin B specific band by western blot analysis [18]. Sm-Cathepsin B has been studied as a potential tool to better schistosomiasis diagnostics [19-21]. Cathepsin B proteases belonging to different

schistosome species as well as different trematodes have been studied as vaccine candidates. A *Schistosoma japonicum* Cathepsin B DNA vaccine in combination with an IL-4 plasmid has been tested in mice, and has been shown to decrease parasite worm and egg burdens [22]. Furthermore, a DNA vaccine of the liver fluke, *Fasciola hepatica*, has been tested in sheep and was able to stimulate both humoral and cellular immune responses [23]. Due to its essential role in parasite development, its immunogenicity, and its continuous interplay with the host immune system, our group has chosen to focus on Sm-Cathepsin B as a potential vaccine candidate. Moreover, we have chosen synthetic oligodeoxynucleotides containing unmethylated CpG dinucleotides as the adjuvant. The use of CpG dinucleotides as adjuvants has shown promise in experimental vaccines against other parasitic infections such as malaria and leishmaniasis [24-28]. Furthermore, these Toll-like receptor 9 agonists are known to stimulate Th1 responses. Th1 responses have long been believed to play key roles in protection against schistosomiasis. This belief stems from the research carried out using the radiation-attenuated cercarial vaccine [29, 30] as well as the investigations involving the cohort of putative resistant individuals living in schistosomiasis endemic regions of Brazil [31]. To our knowledge, we are the first to use Sm-Cathepsin B in the presence of CpG, and, in this present communication, we seek to determine whether this formulation has any protective effect in a mouse model of schistosomiasis.

2.4 Materials and Methods

2.4.1 Sm-Cathepsin B recombinant protein preparation

Schistosoma mansoni Cathepsin B (Genbank accession number M21309.1) cDNA was synthesized by GenScript in pUC57 with modifications in order to be suitable for expression in the yeast *Pichia pastoris*. The glycosylation site at position 183 was altered by changing the asparagine to a glycine. The Sm-Cathepsin B secretion signal was removed because the vector used possessed its own. Furthermore, Mly I and Kpn I restriction sites were added to the sequence as well as a 6x His tag and a stop codon at the 3' end. The expression vector was constructed using Sm-Cathepsin B cDNA and the pPinkα-HC vector. The expression vector was transformed into yeast cells belonging to the PichiaPink^{TM} strain 1. The recombinant Sm-Cathepsin B expression was carried out following the manufacturer's recommendations for the PichiaPink expression system (Invitrogen, Burlington, ON). Briefly, yeast cells were cultured

in a flask containing buffered complex glycerol medium (BMGY) (1% yeast extract [Fisher Scientific, Ottawa, ON], 2% peptone [BD Biosciences, Mississauga, ON], 100mM potassium phosphate pH 6.0 [Bioshop Canada Inc., Burlington, ON], 1.34% yeast nitrogen base without amino acids with ammonium sulphate [Bioshop Canada Inc.], 0.00004% biotin [Supelco, Bellefonte, PA], 1% glycerol [Bioshop Canada Inc.]) at 28°C with shaking (250rpm) until it reached log phase growth (OD_{600} = 6). The cells were harvested by centrifugation at 3,000 x g for five minutes at room temperature. The pellet was resuspended in 200ml induction media; buffered complex methanol medium (same recipe as BMGY except 1% methanol [Fisher Scientific, Ottawa, ON] was used instead of glycerol). The culture was grown for three days at 28°C with shaking (250rpm). Every 24 hours, methanol was added to a final concentration of 0.5%. After the three days, the cells were harvested by centrifugation at 3,000 x g for five minutes at room temperature. The supernatant was collected and filtered before proceeding to purification via Ni-NTA affinity chromatography. The elute was analyzed by Coomassie blue staining of polyacrylamide gel and Western Blot using antibodies directed against the His-tag.

2.4.2 Immunization protocol

Six week old female C57BL/6 mice were purchased from Charles River Laboratories (Senneville, QC). Three groups of mice, containing ten mice each, were immunized intramuscularly in the thigh with 50µl of the different formulations. Group 1, saline control: mice were immunized with phosphate-buffered saline (Wisent Bioproducts, St-Bruno, Qc). Group 2, adjuvant control: mice were immunized with 40µg of adjuvant, synthetic oligodeoxynucleotides containing unmethylated CpG dinucleotides of class B (5'-tcgtcgttttgtcgttttgtcgtt-3') (Catalog# HC4039, Cedarlane, Burlington, ON). Group 3, experimental: mice were immunized with 20µg recombinant Sm-Cathepsin B and 40ug of adjuvant CpG. All mice in each group were immunized at week 0 and boosted with the same formulation at weeks 3 and 6. Prior to selecting this final immunization regiment, several optimization studies were conducted in order to determine the optimal antigen dose, antigen-adjuvant ratio, immunization schedule, and route of delivery. All animal procedures were performed in accordance with Institutional Animal Care and Use Guidelines and were approved by the Animal Care and Use Committee at McGill University.

2.4.3 *Schistosoma mansoni* challenge and worm/egg burden recovery

Biomphalaria glabrata snails infected with the *Schistosoma mansoni* Puerto Rican strain were obtained from the Schistosomiasis Resource Center of the Biomedical Research Institute (Rockville, MD). Three weeks after the final immunization (week 9), all of the mice were challenged with 150 cercariae via tail exposure. The mice were sacrificed seven weeks post challenge. The adult worms were perfused from the hepatic portal system and counted manually as described previously [32]. The number of worms collected from the experimental group was compared to the control group in order to calculate the percent reduction in worm burden. The livers and intestines of each mouse were also collected. These organs were weighed, and then digested overnight in 4% potassium hydroxide as previously described [32]. The following day, the eggs present in these tissues were enumerated. The number of eggs per gram of tissue was recorded, and the experimental group was once again compared to the control group in order to calculate percent reduction in egg burden. Reductions in worm and egg burden in immunized mice were calculated using the following formula:

$$\%\ \text{worms or eggs reduction} = \left(1 - \frac{\text{Mean number of worms or eggs recovered in immunized mice}}{\text{Mean number of worms or eggs recovered in control mice}}\right) x100\%$$

2.4.4 Humoral response measured by enzyme-linked immunosorbent assays (ELISA)

Throughout the immunization timeline, blood was collected by saphenous bleed from each mouse at week 0, 3, 6 and 9. Sera were obtained by centrifugation. The humoral responses elicited by the vaccinations were analyzed by enzyme-linked immunosorbent assay (ELISA). Briefly, 96-well plates were coated with the recombinant Sm-Cathepsin B (10ng of recombinant protein/well). Plates were incubated for 90 minutes with 100µl/well of blocking buffer (PBS, 5% bovine serum albumin [Sigma-Aldrich, St. Louis, MO], 0.1% Tween 20 [Sigma-Aldrich]]) at 37°C. The plates were washed three times with PBS (pH 7.4; 0.01 M phosphate buffer, 0.14 M NaCl) containing 0.05% Tween 20 (Sigma-Aldrich). Serial dilutions of the sera were added in duplicate to wells (50µl/well) and incubated for two hours at 37°C. The secondary antibodies used were conjugated with horseradish peroxidase. Secondary antibodies used were: goat anti-mouse IgG- horseradish peroxidase (HRP) (Jackson Immunoresearch Laboratories, West Grove, PA), goat anti-mouse IgG1-HRP (Southern Biotechnologies Associates, Birmingham, AL), and goat anti-mouse IgG2c-HRP (Southern Biotechnologies Associates). Sm-Cathepsin B specific total IgG was analyzed as well as the

IgG1 and IgG2c classes. After washing the plates three times with PBS/0.05% Tween 20, 50µl/well of the optimally diluted secondary antibody was added and incubated for one hour at 37°C. After a final washing step, 50µl/well of the substrate, 3,3',5,5'-Tetramethylbenzidine (Millipore, Billerica, MA), was added. The plates were incubated ten minutes and the reaction was stopped by adding 25µl/well of 1N sulphuric acid (Sigma-Aldrich). The plates were read at wavelength 450nm. The samples were analysed by ELISA in duplicates and data confirmed by a single repeat. The results are expressed as endpoint titers ±standard error [33].

2.4.5 Cytokine production

The spleens of the sacrificed animals were collected and splenocytes were isolated using a modified protocol of Plante et al. [34]. Briefly, the organs were mashed using a syringe plunger and cell strainer which was washed with 10ml Hank's balanced salt solution (HBSS) (Wisent Bioproducts, St. Bruno, QC). The suspension was centrifuged at 4°C, 400 x g for five minutes. The red blood cells were lysed using 3ml ammonium-chloride potassium lysing buffer and then the reaction was stopped by adding 11ml HBSS. The cells were harvested by centrifuging at 4°C, 400 x g for five minutes. The supernatant was decanted and the cells were resuspended in 5ml HBSS and centrifuged at 4°C, 400 x g for five minutes. Washing and centrifugation steps were repeated once. Finally, the cells were resuspended in 1ml complete media (RPMI-1640, 10% fetal bovine serum [Wisent Bioproducts], 50µg/ml Gentamycin, 0.05mM 2-Mercaptoethanol) and passed through a strainer. 10^6 cells/well determined by Trypan Blue exclusion were seeded into 96-well plates and incubated at 37°C in the presence of 2µg/ml of recombinant Sm-Cathepsin. After 72 hours, 100µl of supernatant from each well was collected. The splenocyte supernatant was used to assess cytokine production by QUANSYS multiplex ELISA (Quansys Biosciences, Logan, UT).

2.4.6 Statistical analysis

Data was analyzed by the two-tailed unpaired *t* test using the software GraphPad Prism 5 (La Jolla, CA). *P* values less than 0.05 were considered significant.

2.5 Results

2.5.1 Expression of recombinant Sm-Cathepsin B

The expression of recombinant Sm-Cathepsin B was carried out using the *Pichia Pastoris* expression system PichiaPink™ (Invitrogen). Expression of the recombinant protein is shown in Figure 2.1 by Coomassie blue stained polyacrylamide gel (Figure 2.1A) and by western blot analysis using antibodies against the His tag (Figure 2.1B). A discrete band representing recombinant Sm-Cathepsin B at 39kDa can be visualized in both cases.

2.5.2 Protective potential of Sm-Cathepsin B

Immunized mice received a primary dose of Sm-Cathepsin B (20µg) in the presence of CpG dinucleotides and two boosts of the same formulation at week 3 and week 6. The control mice, kept on the same immunization schedule, received CpG dinucleotides alone or saline alone. All of the mice were sacrificed seven weeks after the cercarial challenge.

There was a 59% reduction in worm burden in the mice immunized with the recombinant protein in the presence of CpG dinucleotides when compared to the adjuvant control animals ($p<0.0001$) (Figure 2.2A). When compared to the saline group, the experimental group had a 63% decrease in worm burden ($p<0.0001$). There were significant decreases in egg burdens as well. Compared to the adjuvant control group, in the Sm-Cathepsin B plus CpG dinucleotides group, there was a 56% decrease in hepatic egg burden ($p= 0.0010$), and a 54% decrease in intestinal egg burden ($p= 0.0008$) (Figure 2.2B and 2.2C respectively). Hepatic and intestinal egg burdens were decreased by 63% and 64% respectively in the Sm-Cathepsin B plus CpG dinucleotides group compared to the saline group ($p<0.0001$). Overall, there were no significant changes in parasitological burdens between the adjuvant and saline control groups (Figure 2.2).

2.5.3 Antibody response

Sera collected throughout the immunization timeline were analyzed by ELISA in order to determine antigen-specific antibody production levels. There were no detectable levels of Sm-Cathepsin B specific total IgG in the control animals (administered CpG dinucleotides or saline alone). In the experimental group having received the recombinant protein in the presence of adjuvant, antigen-specific total IgG titers underwent a marked increase after week 3 (after the first boost). Robust antibody production was observed in these mice, reaching mean endpoint titers of 128,000 at week 9 (Figure 2.3A).

The titers of IgG subtypes IgG1 and IgG2c were also analyzed. The adjuvant and saline control mice did not have any detectable levels of either IgG1 or IgG2c specific for Sm-Cathepsin B. In the mice immunized with the recombinant protein and the adjuvant, antigen specific IgG1 titers started to rise at week 3 and the mean endpoint titers achieved before cercarial challenge at week 9 were 9,840 (Figure 2.3B). Antibody levels of Sm-Cathepsin B specific IgG2c were also detected in the mice immunized with the protein in the presence of adjuvant. Titers for this IgG subtype were more robust than those observed for IgG1. IgG2c titers began to rise at week 3 and reached the highest level at week 9 before the cercarial challenge (Figure 2.3C). The mean IgG2c endpoint titers at week 9 were 61,440.

2.5.4 Cytokine Production

Murine splenocytes from the vaccinated groups were stimulated *ex vivo* with recombinant Sm-Cathepsin B in order to analyze the differences in cytokine secretion levels. There were no significant changes in cytokine secretion levels between the saline and adjuvant control groups for all of the cytokines analyzed. Compared to the two groups of control mice, the mice that had been immunized with the formulation containing the recombinant protein had significantly higher Th1 cytokine secretion levels (Figure 2.4). IFN-γ (Figure 2.4A) and TNF-α (Figure 2.4B) were both significantly higher in these vaccinated animals compared to the adjuvant controls (p=0.0015 and p<0.0001, respectively) as well as the saline controls (p=0.0013 and p<0.0001, respectively). By contrast, there were no significant differences in the levels of the Th2 cytokines when comparing the group immunized with the recombinant protein plus adjuvant to either control group (Figure 2.5). IL-4 (Figure 2.5A) and IL-5 (Figure 2.5B) levels did not vary significantly between the experimental group and the adjuvant control group (p=0.7571 and p=0.1666, respectively) nor between the experimental group and the saline control group (p=0.1192 and p=0.3412, respectively). The group immunized with Sm-Cathepsin B in the presence of adjuvant also had significant increases in IL-10 (Figure 2.6A) and CCL5 (Figure 2.6B) secretion levels compared to the adjuvant control group (p=0.0037 and p=0.0381, respectively) and the saline group (p=0.0021 and p=0.0265, respectively).

2.6 Discussion

This study sought to determine whether the formulation of recombinant Sm-Cathepsin B in the presence of synthetic oligodeoxynucleotides containing unmethylated CpG could elicit a protective effect in mice against a challenge infection with *S. mansoni* cercariae. A group immunized with the adjuvant alone served as a control group when comparing parasite burdens and immunological observations. Immunizations with the experimental formulation resulted in a 59% reduction in worm burden, a 56% decrease in hepatic egg burden, and a 54% decrease in intestinal egg burden when compared to the adjuvant control group. These induced protection levels exceed the 40% threshold established by the TDR/WHO committee [6].

Immunizations with the recombinant protein in the presence of CpG dinucleotides resulted in the robust production of Sm-Cathepsin B specific IgG antibodies. Prior to cercarial challenge, at week 9, the mean antigen-specific IgG endpoint titers were 128,000. The protective role of antibodies in schistosomiasis has been previousy demonstrated. It has been shown that antibody titers at the time of cercarial challenge inversely correlate with worm burden [35]. The importance of antibodies was further supported by the demonstration that protection in baboons immunized with radiation attenuated cercariae is proportional to antibody titers [35]. Moreover, several passive transfer studies have shown that both wild type and immunologically deficient animals have decreased parasite burden and pathology when they received antibodies from chronically infected or immunized wild type animals [36-39]. Therefore, the elevated antigen-specific IgG titers elicited by the formulation of recombinant Sm-Cathepsin B in the presence of CpG dinucleotides may be a crucial factor contributing to the decreased parasite burden observed at the time of perfusion and organ collection. The antibodies specific for Sm-Cathepsin B may be performing a neutralizing task; thus, blocking nutrient uptake and leading to parasite starvation. Immunoproteomic studies concerning schistosomiasis self-cure in rhesus macaques revealed that gut digestive enzymes were common IgG targets in high responder animals [40]. This observation strengthens the potential role of Sm-Cathepsin B specific antibodies in protection.

The titers of IgG subtypes IgG1 and IgG2c were also analyzed from the serum of immunized mice. For both subtypes, titers began to rise after the first boost. Significantly, at week nine, IgG1 endpoint titers were 9,840 whereas IgG2c endpoint titers were 61,440. This substantial difference between the two subtypes may be a marker of the type of immune

environment dominating at the time of cercarial challenge. IFN-γ is known to stimulate the synthesis of IgG2c while IL-4 stimulates the production of IgG1. These observations suggest that the invading parasite will enter an immunization-induced Th1 primed environment upon infection.

The mice immunized with Sm-Cathepsin B in the presence of CpG dinucleotides had significantly higher IFN-γ and TNF-α secretion levels compared to the control animals. Both cytokines are capable of activating macrophages, and TNF-α may act to trigger nitric oxide production by macrophages. Immunological studies using the radiation-attenuated cercariae vaccine indicate that cell-mediated immunity involving $CD4^+$ effector cells with a Th1 phenotype is essential for protection against *Schistosoma* infections [29, 30, 41]. Th1 responses are especially important for targeting of the migratory schistosomulae by forming an inflammatory focus in the lungs around the parasite. The ability of the Sm-Cathepsin B formulation to elicit strong Th1 cytokine secretion is therefore a promising characteristic. We found no significant differences in Th2 cytokine secretion levels (IL-4 and IL-5) when comparing the experimental and control groups. During the course of infection, there is a switch to a dominant Th2 response around week six [42]. This shift is directly mediated by egg antigens [43-45]. The formulation used to immunize the animals in this study was able to elicit an elevated Th1 response, but left the Th2 cytokines unchanged; indicating, that in the immunized animals, the altered immune response was not in favour of parasite establishment within the host. The levels of IL-10 were significantly increased in the immunized animals. IL-10 acts as a regulatory cytokine and can block the development of severe pathology caused by a polarized immune response [46, 47]. The observed increase in IL-10 secretion may be in response to the strong Th1 environment elicited by the immunizations. IL-10 deficient mice infected with *S. mansoni* have been shown to develop unchecked Th1 responses which lead to detrimental hepatotoxic effects [47]. Perhaps, the higher levels of IL-10 in the experimental group allow for the beneficial effects of a dominant Th1 response to take place while preventing the unwanted liver damage that may follow an unregulated polarized response. Levels of CCL5 were also significantly increased in the experimental group. This chemokine recruits T cells as well as certain granulocytes. It can also act on Natural Killer (NK) cells to induce their proliferation and activation. NK cells are innate immune cells that can play an important role against schistosomiasis. They can be a source of IFN-γ and mediate antibody

dependent cell mediated cytotoxicity. Moreover, they have been shown to be key mediators of protection against schistosomiasis in the elderly [48].

In summary, immunizations with recombinant Sm-Cathepsin B in the presence of CpG dinucleotides significantly reduced both worm and egg burdens. The formulation was also able to elicit robust antibody titers and increase secretion levels of key cytokines. These observations support the exploitation of Sm-Cathepsin B as a promising vaccine candidate and encourage further work.

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2.9 Figures and legends

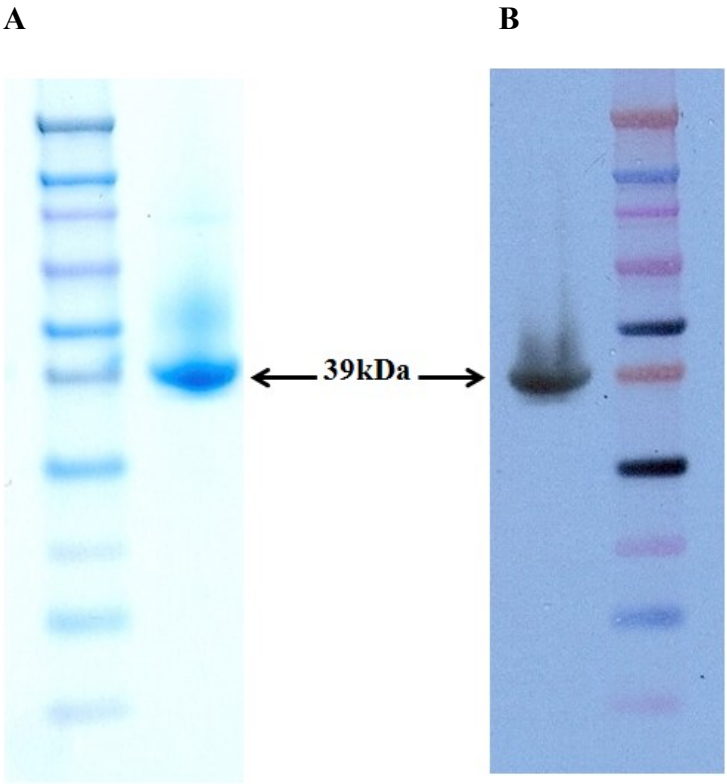

Figure 2.1 Recombinant Sm-Cathepsin B expression.

Recombinant Sm-Cathepsin B was expressed using the *Pichia pastoris* system. The Coomassie stained polyacrylamide gel shows the representative band at 39kDa **(A)**. This band was detected using anti-His tag antibodies in western blot analysis **(B)**.

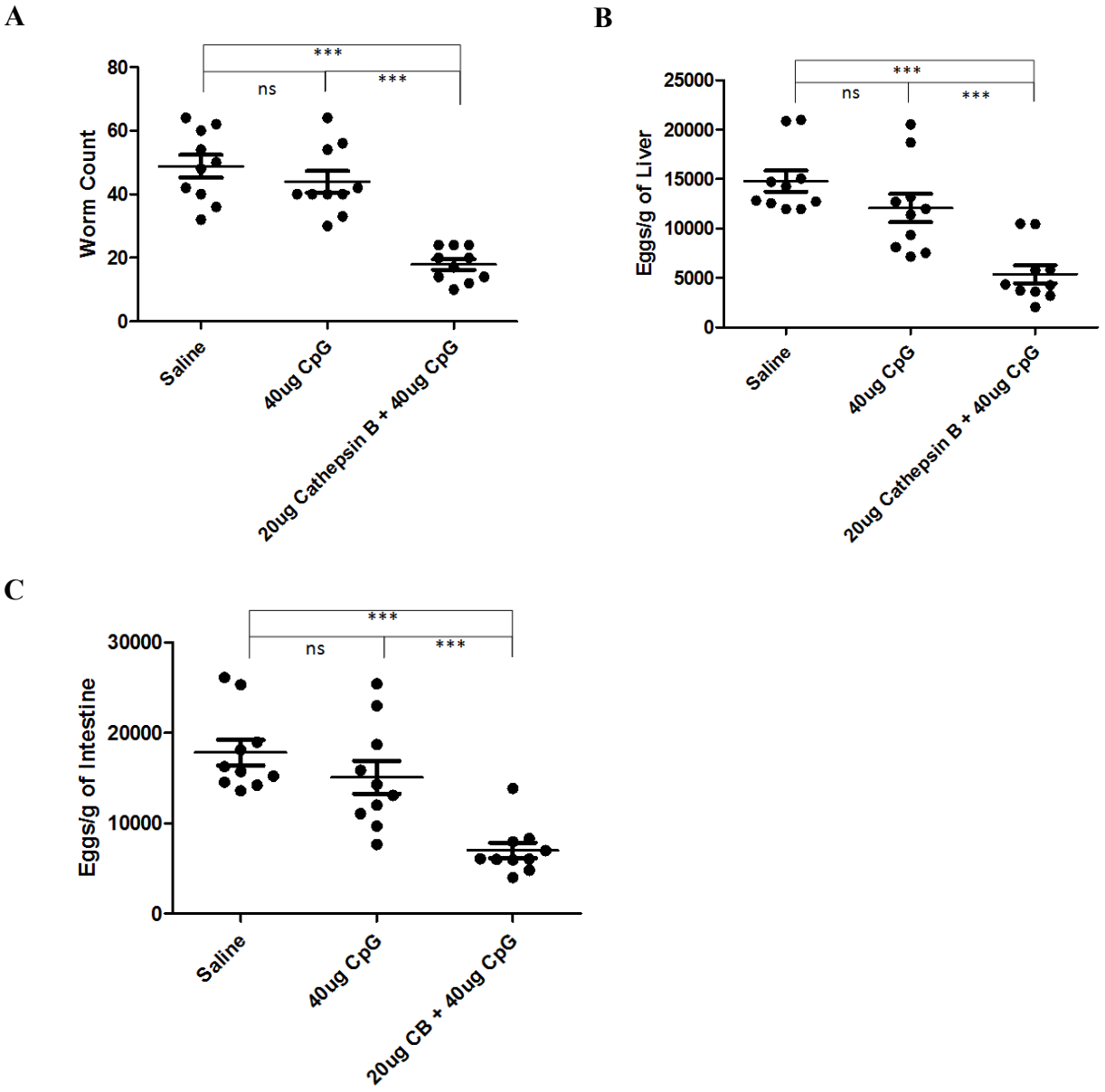

Figure 2.2 Assessment of parasitological burden.

The worm counts per individual mouse **(A)** as well as the egg load per gram of liver **(B)** and per gram of intestine **(C)** are represented for the saline control mice, the adjuvant control mice that received three doses of 40µg CpG dinucleotides alone, and for the experimental group that received three doses of 20µg Sm-Cathepsin B plus 40µg CpG dinucleotides. The mice were sacrificed seven weeks post cercarial challenge. The figure illustrates the mean ±standard error for each group. ***$p \leq 0.001$

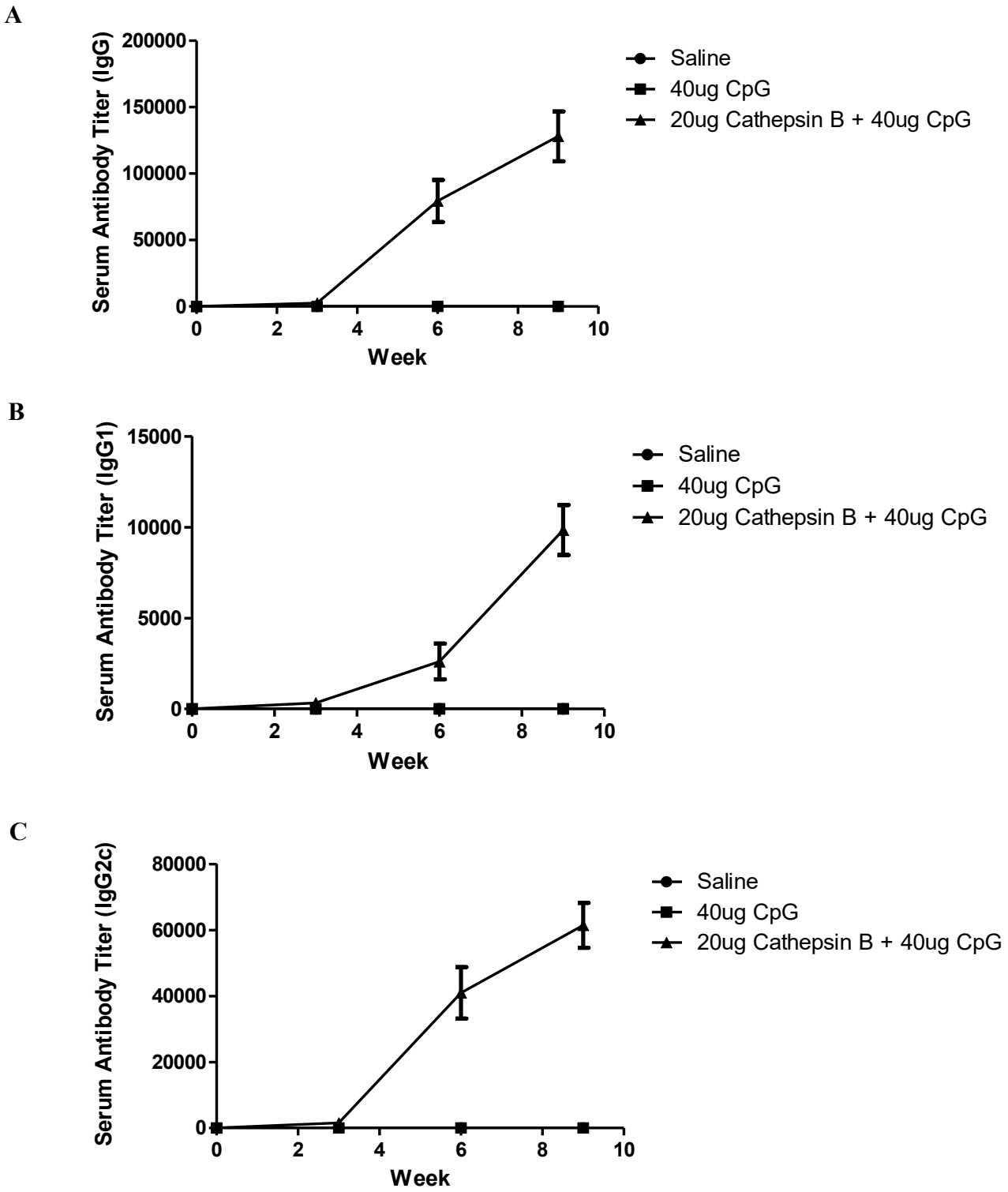

Figure 2.3 Sm-Cathepsin B specific antibody production.

Sm-Cathepsin B specific total IgG **(A)**, IgG1 **(B)**, and IgG2c **(C)** in immunized mice: saline control animals received three doses of saline, adjuvant control animals received three doses of 40µg CpG dinucleotides, and the experimental animals received three doses of 20µg Sm-

Cathepsin B plus 40µg CpG dinucleotides. Serum from individual mice was analyzed by ELISA. The results are expressed as the mean endpoint titers within a group ± standard error. Endpoint titers were determined for week 0, week 3, week 6, and week 9.

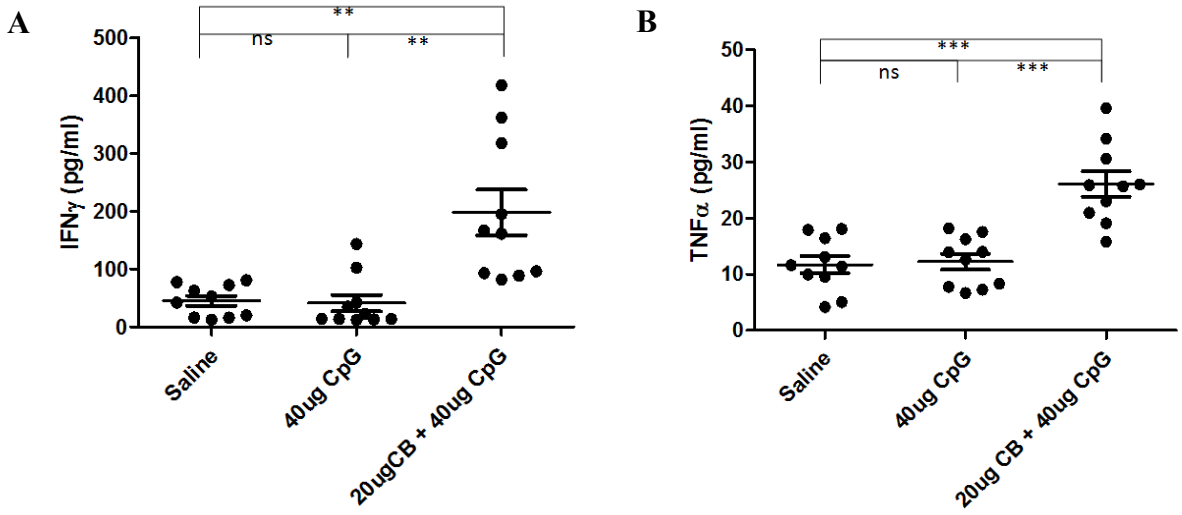

Figure 2.4 Sm-Cathepsin B specific cytokine response: Th1 Cytokine levels.

IFNγ (p=0.0015) **(A)** and TNFα (p<0.0001) **(B)**, produced 72 hours after stimulating splenocytes *ex vivo* with recombinant Sm-Cathepsin B. Splenocytes were isolated from all C57BL/6 mice subjected to cercarial challenge belonging to each group: saline control, CpG dinucleotides control, and Sm-Cathepsin B with CpG dinucleotides. Cytokine production was analyzed by QUANSYS multiplex ELISA. The figure illustrates the mean ±standard error for each group. **p≤0.01; ***p≤0.001

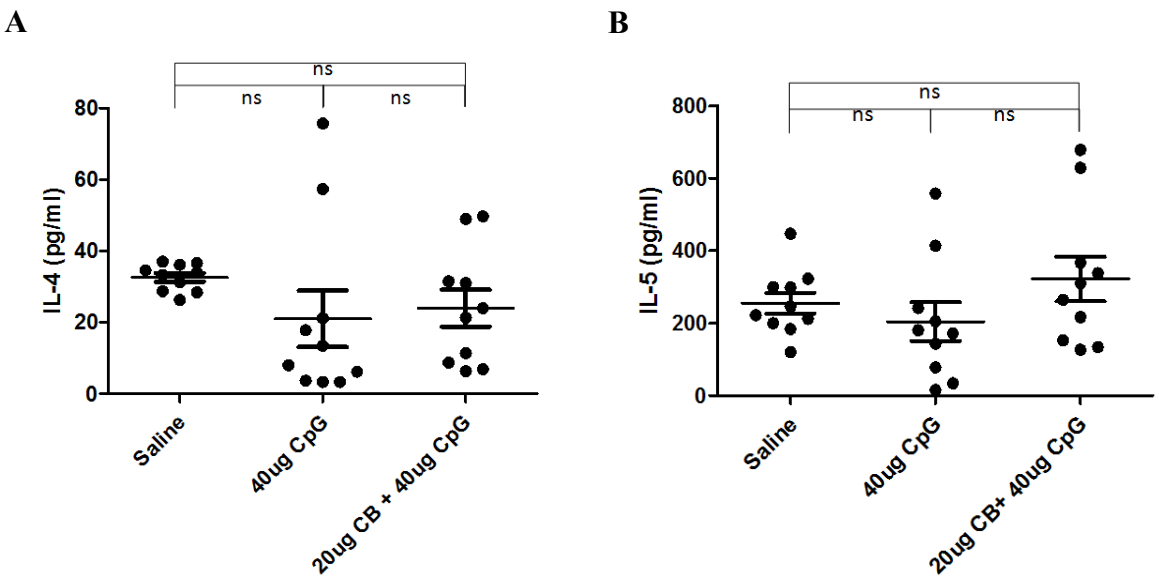

Figure 2.5 Sm-Cathepsin B specific cytokine response: Th2 Cytokine levels.

IL-4 (p=0.7571) **(A)** and IL-5 (p=0.1666) **(B)**, produced 72 hours after stimulating Splenocytes *ex vivo* with recombinant Sm-Cathepsin B. Splenocytes were isolated from all C57BL/6 mice subjected to cercarial challenge belonging to each group: saline control, CpG dinucleotides control, and Sm-Cathepsin B with CpG dinucleotides. Cytokine production was analyzed by QUANSYS multiplex ELISA. The figure illustrates the mean ±standard error for each group. ns: not significant (p> 0.05)

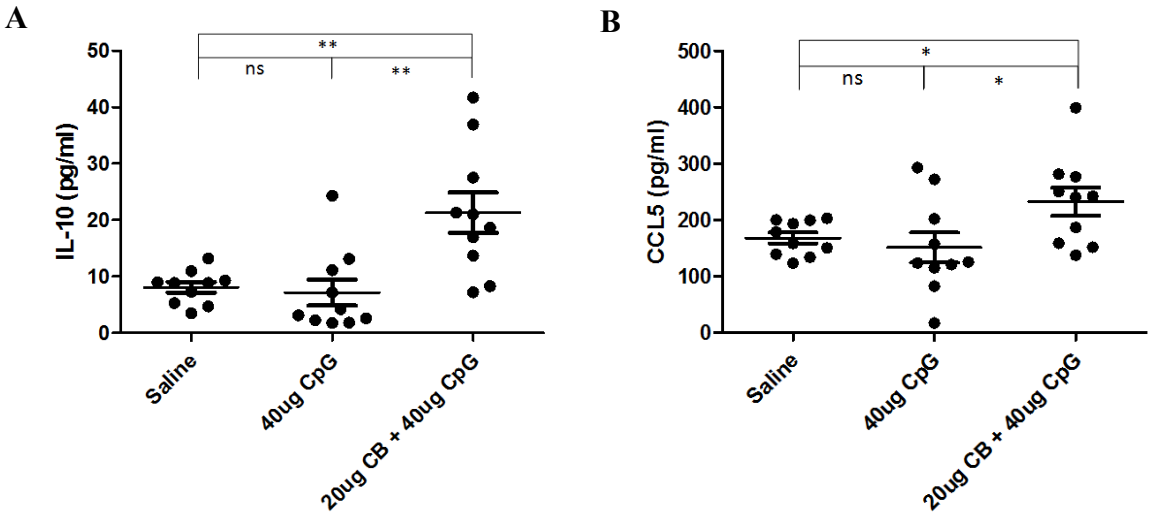

Figure 2.6 Sm-Cathepsin B specific cytokine response: Regulatory and Chemotactic Cytokine levels.

IL-10 (p=0.0037) **(A)** and CCL5 (p=0.0381) **(B)**, produced 72 hours after stimulating splenocytes *ex vivo* with recombinant Sm-Cathepsin B. Splenocytes were isolated from all C57BL/6 mice subjected to cercarial challenge belonging to each group: saline control, CpG dinucleotides control, and Sm-Cathepsin B with CpG dinucleotides. Cytokine production was analyzed by QUANSYS multiplex ELISA. The figure illustrates the mean ±standard error for each group. *$p \leq 0.05$; **$p \leq 0.01$

Chapter 3: A vaccine consisting of *Schistosoma mansoni* cathepsin B formulated in Montanide ISA 720 VG induces high level protection against murine schistosomiasis

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3.1 Preface

In Chapter 2 of this thesis, we described the effect of Sm-Cathepsin B + CpG immunizations using a mouse model of schistosomiasis. The formulation significantly reduced all forms of parasite burden which include worms, hepatic eggs, and intestinal eggs. The formulation elicited a biased Th1 immune response. We next sought to analyze whether a broader acting adjuvant, such as Montanide ISA 720 VG, could increase protection levels. Montanide ISA 720 VG is a squalene based adjuvant that forms water-in-oil droplets. In the following chapter, we assess the protection potential of the formulation containing recombinant Sm-Cathepsin B and Montanide ISA 720 VG using a mouse model of schistosomiasis. In addition to determining the formulation's effect on parasite burden, we also describe the immune response elicited analyzing antigen-specific antibody and cytokine production.

3.2 Abstract

Schistosomiasis is the most important human helminth infection due to its impact on public health. The clinical manifestations are chronic and significantly decrease an individual's quality of life. Infected individuals suffer from long-term organ pathologies including fibrosis which eventually leads to organ failure. The development of a vaccine against this parasitic disease would contribute to a long-lasting decrease in disease spectrum and transmission. Our group has chosen *Schistosoma mansoni* (Sm) cathepsin B, a peptidase involved in parasite feeding, as a prospective vaccine candidate. Our experimental formulation consisted of recombinant Sm-cathepsin B formulated in Montanide ISA 720 VG, a squalene based adjuvant containing a mannide mono-oleate emulsifier. Parasitological burden was assessed by determining adult worm, hepatic egg, and intestinal egg numbers in each mouse. Serum was used in ELISAs to evaluate production of antigen-specific antibodies, and isolated splenocytes were stimulated with the antigen for the analysis of cytokine secretion levels. The Sm-cathepsin B and Montanide formulation conferred protection against a challenge infection by significantly reducing all forms of parasitological burdens. Worm burden, hepatic egg burden and intestinal egg burden were decreased by 60%, 62%, and 56%, respectively in immunized animals compared to controls ($P = 0.0002$, $P < 0.0001$, $P = 0.0009$, respectively). Immunizations with the vaccine elicited robust production of Sm-cathepsin B specific antibodies (endpoint titers = 122,880). Both antigen-specific IgG1 and IgG2c titers were observed, with the former having more elevated titers. Furthermore, splenocytes isolated from the immunized animals, compared to control animals, secreted higher levels of key Th1 cytokines, IFN-γ, IL-12, and TNF-α, as well as the Th2 cytokines IL-4 and IL-5 when stimulated with recombinant Sm-cathepsin B. The Th17 cytokine IL-17, the chemokine CCL5, and the growth factor GM-CSF were also significantly increased in the immunized animals compared to the controls. The formulation tested in this study was able to significantly reduce all forms of parasite burden, stimulate robust production of antigen-specific antibodies, and induce a mixed Th1/Th2 response. These results highlight the potential of Sm-cathepsin B/Montanide ISA 720 VG as a vaccine candidate against schistosomiasis.

3.3 Introduction

Schistosomiasis, caused by trematodes belonging to the *Schistosoma* genus, is an important neglected tropical disease with a substantial impact on public health. Over 200 million people are infected with approximately half of these being school aged children [1-3]. It has long been argued that the schistosomiasis disease burden is greatly underestimated, and it has been suggested that the number of infected individuals surpasses 400 million [4]. This underestimation is likely due to the inability of current diagnostic methods to detect light infections. Schistosomiasis is a chronic illness that significantly diminishes quality of life, and, in affected children, interferes with growth and cognitive development [2, 5, 6]. Treatment of schistosomiasis relies solely on the drug praziquantel which is distributed in mass drug administration control programs. However, program outcomes have been disappointing [3]. In 2011, of the 112 million children in need of praziquantel, only 16 million received treatment [7]. School-based mass drug administration programs do not take into account that as many as 40% of children in sub-Saharan Africa are not enrolled in school [8]. It has been shown that less than 5% of the schistosomiasis affected population is treated with praziquantel, indicating that control programs are not reaching sufficient coverage [9]. Furthermore, these mass drug administration programs are not sustainable because praziquantel does not prevent re-infection. Therefore continuous drug distribution at an optimal timing is necessary in order to maintain disease control and break the transmission cycle [8-10]. The current amount of praziquantel donated by the various sources, such as the Schistosomiasis Control Initiative, MedPharm, and Merck KgaA, is not sufficient to meet the global need [9]. Vaccines, and/or chemotherapy combined with vaccination, present the best strategy for long-term sustained control of schistosomiasis. An anti-schistosome vaccine could contribute to decreased disease spectrum and transmission by reducing worm and egg burdens. It has been stated that even a partially effective anti-schistosome vaccine could significantly help accelerate the elimination of schistosomiasis if incorporated with other control efforts such as mass drug administration, intermediate host control, and improved sanitation [11, 12].

Our group has chosen to focus on *Schistosoma mansoni* (Sm) cathepsin B as a vaccine candidate. This cysteine peptidase, originally cloned and identified by Klinkert *et al*., [13, 14] is predominantly found in the adult worm and migratory larva. Sm-cathepsin B is involved in

the digestion of blood macromolecules. It plays a key role in hemoglobin degradation and is involved in digestion of host serum albumin or immunoglobulin G [15-19]. The physiological importance of Sm-cathepsin B has been highlighted by RNAi technology which demonstrated that suppression of cathepsin B results in impeded parasite growth [20]. The present study was designed to investigate the protective potential of a Sm-cathepsin B formulation with the adjuvant Montanide ISA 720 VG (SEPPIC Inc., Fairfield, NJ, USA) in a mouse model of schistosomiasis. Montanide is a squalene based adjuvant containing a mannide mono-oleate emulsifier. The adjuvant forms water-in-oil droplets that allow for slow antigen release at the injection site. Montanide was chosen as an adjuvant because it is acceptable for use in humans; it is both safe and well-tolerated. The different Montanide adjuvants have been used in over fifty clinical trials including malaria, cancer, and HIV vaccine trials [21-23]. These adjuvants have also continuously shown to elicit robust antibody responses. In this communication, we report that an immunization regimen involving a formulation of recombinant Sm-cathepsin B and Montanide ISA 720 VG can significantly reduce worm and egg burdens.

3.4 Materials and Methods

3.4.1 Cloning, expression, and purification of Sm-cathepsin B

The recombinant *Schistosoma mansoni* cathepsin B protein was generated as described elsewhere [24]. Briefly, *Schistosoma mansoni* cathepsin B (Genbank accession number M21309.1) cDNA was synthesized by GenScript in pUC57 with modifications in order to be suitable for expression in the yeast *Pichia pastoris* [25]. The modification process used the following primers: primer a, 5'-CAACTTGTGTGTGATTCTTTCGAAC-3' and primer b, 5'-GTTCGAAAGAATCACACACAGGTTG-3' [25]. Sm-cathepsin B cDNA was inserted in the pPinkα-HC vector and then transformed into yeast cells belonging to the PichiaPink^{TM} strain 1. The recombinant Sm-cathepsin B expression was carried out following the manufacturer's recommendations for the PichiaPink expression system (Invitrogen, Burlington, ON). Briefly, yeast cells were cultured in a flask containing buffered complex glycerol medium (BMGY) (1% yeast extract [Fisher Scientific, Ottawa, ON], 2% peptone [BD Biosciences, Mississauga, ON], 100mM potassium phosphate pH 6.0 [Bioshop Canada Inc., Burlington, ON], 1.34% yeast nitrogen base without amino acids with ammonium sulphate [Bioshop Canada Inc.], 0.00004% biotin [Supelco, Bellefonte, PA], 1% glycerol [Bioshop Canada Inc.]) at 28°C with

shaking (250rpm) until it reached log phase growth (OD_{600} = 6). The cells were harvested by centrifugation at 3,000 x g for five minutes at room temperature. The pellet was resuspended in 200ml induction media: buffered complex methanol medium (same recipe as BMGY except 1% methanol [Fisher Scientific, Ottawa, ON] was used instead of glycerol). The culture was grown for three days at 28°C with shaking (250rpm). Every 24 hours, methanol was added to a final concentration of 0.5%. After the three days, the cells were harvested by centrifugation at 3,000 x g for five minutes at room temperature. The resulting expressed recombinant protein was purified using Ni-NTA affinity chromatography (Ni-NTA Superflow by QIAGEN, Venlo, Limburg, Netherlands). Expression quality of recombinant Sm-cathepsin B was analyzed by Coomassie staining of polyacrylamide gel and western blot using antibodies directed against the His-tag (Sigma-Aldrich, St. Louis, Mo) followed by a HRP-conjugated secondary antibody (anti-mouse IgG-HRP from Jackson Immunoresearch Laboratories, West Grove, PA)

3.4.2 Immunization protocol

Female 6-8 week old C57BL/6 mice were purchased from Charles River Laboratories (Senneville, Qc). There were three immunization groups containing 10 mice each. The immunization groups were as follows: saline control mice were injected with 50 µl of phosphate buffered saline (PBS) (Wisent Bio Products, Saint-Jean-Baptiste). Adjuvant control mice received 50 µl of a 70% volume formulation of Montanide ISA 720 VG (SEPPIC Inc., Fairfield, NJ). The experimental mice were immunized with a 50 µl solution of 20 µg recombinant Sm-cathepsin B in a 70% volume formulation of Montanide ISA 720 VG. Each mouse was immunized intramuscularly at weeks 0, 3 and 6. The immunization timeline and the vaccine formulation (antigen: adjuvant ratio) were determined based on previous small scale immunization/challenge pilot studies. All animal procedures were performed in accordance with Institutional Animal Care and Use Guidelines and were approved by the Animal Care and Use Committee at McGill University (Animal Use Protocol 7625).

3.4.3 *Schistosoma mansoni* challenge

Biomphalaria glabrata snails infected with the *Schistosoma mansoni* Puerto Rican strain were provided by the NIAID Schistosomiasis Resource Center of the Biomedical Research Institute (Rockville, MD). Cercariae, the infectious stage of the parasite, were

collected from the shedding of the infected *B. glabrata* snails. Three weeks after the final immunization, all of the mice were challenged with 150 cercariae via tail exposure lasting one hour. The mice were sacrificed seven weeks post cercarial challenge. The adult worms were perfused from the hepatic portal system and counted manually [26]. The number of worms retrieved from the experimental group was compared to the control groups in order to calculate the percent reduction in worm burden. The livers and intestines of the sacrificed mice were also collected. The intestines were cut open to expose the lumen, and the contents were cleaned out by repeated rinsing with saline. The livers and intestines were then weighed and digested overnight in 4% potassium hydroxide (KOH) [26]. The following day, the eggs present in these tissues were counted by microscopy. The number of eggs recorded from the experimental group was once again compared to the control groups in order to calculate percent reduction in egg burden. Burden reductions were calculated as follows:

$$\text{Percent worms or eggs reduction} = \left(1 - \frac{\text{Mean number of worms or eggs recovered in immunized mice}}{\text{Mean number of worms or eggs recovered in control mice}}\right) \textit{multiplied by } 100 \textit{ percent}$$

3.4.4 Humoral response: enzyme-linked immunosorbent assays

Blood collection via saphenous bleed was performed on each mouse prior to each immunization as well as on the day of the cercarial challenge. Sera were obtained by allowing the collected blood to clot and then removing it by centrifugation. The humoral responses elicited by the immunizations were analyzed by enzyme-linked immunosorbent assay (ELISA) as described elsewhere [24]. Briefly, 96-well plates were coated with the recombinant Sm-Cathepsin B (10ng/well). Serial dilutions of the sera were prepared and incubated in the antigen coated plates. Sm-cathepsin B specific total IgG was analyzed as well as the isotypes IgG1 and IgG2c using secondary antibodies conjugated with horseradish peroxidase (HRP) (total IgG-HRP from Jackson Immunoresearch Laboratories, West Grove, PA; both IgG1-HRP and IgG2c-HRP from Southern Biotechnologies, Birmingham, AL). Following the addition of the substrate, 3,3',5,5'-tetramethylbenzidine (Millipore, Billerica, MA), the plates were read at wavelength 450nm. The samples were analysed by ELISA in duplicates and data confirmed by a single repeat. The results are expressed as mean endpoint titers ±standard error. Endpoint

titers refer to the reciprocal of the highest dilution that gives a reading above the cut-off [27]. Based on the number of negative controls used in the ELISAs, the cut-off was roughly equal to two standard deviations above the mean negative values [27].

3.4.5 Cytokine production: multiplex ELISA

The animals were sacrificed seven weeks post cercarial challenge. The spleens of each animal were collected and splenocytes were isolated as described elsewhere [24, 28]. Briefly, the spleens were crushed and the red blood cells were lysed using ammonium-chloride potassium lysing buffer. Following repeated wash and centrifugation steps, the cells were resuspended in complete media (RPMI-1640, 10% fetal bovine serum [Wisent Bio Products], 50 µg/ml Gentamycin, 0.05 mM 2-Mercaptoethanol). Cell counts were determined by trypan blue exclusion, and 10^6 cells/well were seeded into 96-well plates. The cells were incubated at 37°C in the presence of 2 µg/ml of recombinant Sm-cathepsin B. After 72 hours, 100 µl of the supernatant was collected. The splenocyte supernatant was used to asses cytokine production by QUANSYS multiplex ELISA (16-plex) (Quansys Biosciences, Logan, UT) following the manufacturer's recommendations.

3.4.6 Statistical analysis

Statistical analysis was done using the Mann-Whitney test (nonparametric test) to compare different groups of data. Antibody and cytokine data are presented on semi-log plots showing geometric mean and 95% confidence intervals. The software GraphPad Prism 5 (La Jolla, CA) was used. P values less than 0.05 were considered significant.

3.5 Results

3.5.1 Expression of recombinant Sm-cathepsin B

The expression of the recombinant Sm-cathepsin B protein used for the immunizations was shown by both Coomassie staining of polyacrylamide gel (Supplementary Figure 3.1A) and Western Blot analysis (Supplementary Figure 3.1B). By both methods, a single and clear band representing the protein can be observed at the expected 39 kDa region. Lipopolysaccharide testing revealed no detectable levels of the endotoxin.

3.5.2 Protective potential of Sm-cathepsin B formulation

In order to determine the protective potential of the Sm-cathepsin B and Montanide ISA 720 VG formulation, an immunization regimen was tested using C57BL/6 mice as described above. The average worm number from the group that received the Sm-cathepsin B and Montanide ISA 720 VG formulation (16.4 ± 2.5 worms) was compared to average from the adjuvant control group (41.1 ± 0.8 worms). Therefore, worm burden was reduced by 60.1% in the mice that received the Sm-cathepsin B/adjuvant formulation compared to the adjuvant control mice ($P = 0.0002$) (Figure 3.1A). Compared to the saline control group (48.8 ± 3.5 worms), the mice given the Sm-cathepsin B/adjuvant formulation had a 66.4% reduction in worm burden ($P < 0.0001$). There were no statistically significant differences in worm burdens between the two control groups ($P = 0.1285$).

Schistosomiasis pathology is linked to the parasite eggs becoming trapped in tissues. Hepatic and intestinal egg burdens were determined for both the experimental and control mouse groups. Comparisons between the adjuvant control group (mean egg count = 11,876.0 ± 1,197.9 eggs/gram of liver) and the Sm-cathepsin B/adjuvant formulation group (mean egg count = 4,578.5 ± 492.9 eggs/gram of liver) revealed a 61.5% reduction in hepatic egg burden for the mice given the vaccine ($P < 0.0001$) (Figure 3.1B). When comparing the experimental group and the saline control (mean egg count = 14,791.6 ± 1,080.1 eggs/gram of liver) group, hepatic egg burden is decreased by 69.1% in the mice that received Sm-cathepsin B/adjuvant formulation ($P < 0.001$). There also seems to be a significant decrease in hepatic egg burden in the adjuvant control group compared to the saline group ($P = 0.0185$).

Intestinal eggs were also enumerated from the digested organ using microscopy. The group containing the animals immunized with the Sm-cathepsin B/adjuvant formulation (mean egg count = 7,292.8 ± 1,173.1 eggs/gram of intestine) had a 56.1% reduction in intestinal egg burden compared to the group that received adjuvant only (mean egg count = 16,606.6 ± 1,590.0 eggs/gram of intestine) ($P = 0.0009$) (Figure 3.1C). Compared to the saline group (mean egg count = 17,800.2 ± 1,421.8 eggs/gram of intestine), the experimental group had a 59.0% decrease in intestinal egg burden ($P < 0.0001$). There were no statistically significant differences in intestinal egg burdens between the two control groups ($P = 0.4813$). All of the results elucidating the protective potential of the Sm-cathepsin B formulation are summarized in Supplementary Table 3.1 and Supplementary Table 3.2.

3.5.3 Antibody response

The serum collected throughout the immunization regimen was used in order to analyze and compare the antibody production pattern between the three groups of mice used in this study. ELISAs were performed to determine antibody titers. In both the adjuvant control and saline control mice, there were no detectable levels of Sm-cathepsin B specific antibodies (total IgG or IgG subtypes IgG1 and IgG2c). In the Sm-cathepsin B/adjuvant formulation group, Sm-cathepsin B specific total IgG titers rose sharply after the first boost at week 3, reaching mean endpoint titers of 40,160 at week 6, and continued to rise until the challenge at week 9 (mean endpoint titers = 122,880) (Figure 3.2A). The sera collected from the experimental group revealed robust production of Sm-cathepsin B specific IgG1 that rose sharply after the first boost at week 3. At week 6, the IgG1 mean endpoint titer was 24,960, and it continued to rise until the challenge at week 9 (mean endpoint titer = 48,640) (Figure 3.2B). By contrast, Sm-cathepsin B specific IgG2c production was much lower. As seen with the total IgG and IgG1 antibody production patterns, IgG2c titers had the most substantial increase after the first boost at week 3. The mean endpoint titer at week 9 was found to be 1,840 (Figure 3.2C).

3.5.4 Cytokine Production

Analysis of the cytokine panel using QUANSYS multiplex ELISA array revealed no significant differences in cytokine secretion levels between the two control groups ($P > 0.05$). There was an increase in Th1 cytokine production in the group that was immunized with Sm-cathepsin B with Montanide ISA 720 VG compared to the control groups. There was a significant increase in IFNγ secretion levels in the experimental group compared to the Montanide adjuvant control group ($P = 0.0021$) and the saline group ($P < 0.0001$) (Figure 3.3A). There were also significant increases in TNFα ($P < 0.0001$) and IL-12 ($P = 0.0185$) levels in the experimental group compared to the adjuvant control group (Figures 3.3B and 3.3C, respectively). These increases in TNFα and IL-12 levels were also seen when comparing the experimental group to the saline group ($P < 0.0001$ and $P = 0.0185$, respectively). Th2 cytokine production was analyzed as well. There were statistically significant increases in IL-4 levels between the experimental and adjuvant groups ($P = 0.0288$) as well as between the experimental and the saline groups ($P = 0.0147$) (Figure 3.4A). IL-5 secretion levels were also significantly increased ($P = 0.0015$) in the group immunized with the Sm-cathepsin B and

Montanide ISA 720 VG formulation compared to the adjuvant alone control group (Figure 3.4B). This significant increase was also observed when comparing the experimental group to the saline-only group ($P < 0.0001$). IL-17 levels were observed to gain insight into a possible Th17 involvement. Secretion levels of IL-17 were significantly increased in the experimental group compared to the adjuvant control group ($P = 0.0433$). However, this statistically significant increase was not seen when comparing the experimental group to the saline control group ($P = 0.0892$) (Figure 3.5A). Other cytokine secretion levels that were significantly increased in the experimental group compared to the adjuvant control group include CCL5 levels ($P = 0.0002$) and GM-CSF levels ($P = 0.0147$) (Figures 3.5B and 3.5C, respectively). Secretion levels of CCL5 and GM-CSF in the experimental group were significantly higher when compared to the saline group as well ($P = 0002$ and $P = 0.0089$, respectively).

3.6 Discussion

Due to its essential role in parasite growth and development and its continuous interplay with the host immune system, our group has focused on Sm-cathepsin B as a potential vaccine candidate. During the mid-1990s, the WHO Special Programme for Research and Training in Tropical Diseases (TDR/WHO) planned for the independent testing of various *Schistosoma mansoni* antigens with the hopes of uncovering an optimal vaccine candidate. The committee's goal was to find an antigen that could consistently induce 40% protection or better [29]. Recently, our collaborators have demonstrated the inbuilt adjuvant properties of Sm-cathepsin B. Immunizations with unadjuvanted Sm-cathepsin B could decrease both worm and hepatic egg burdens in a mouse model of schistosomiasis by 66% and 51%, respectively, when compared to saline control mice [30]. However, the intestinal egg burden was not significantly reduced (24.7%). The pathological importance of egg burden in the intestines cannot be neglected. The inflammation surrounding eggs trapped in intestinal tissues results in the formation of severe lesions and colonic polyps [31-33]. Furthermore, a decrease in intestinal egg burden could reflect a consequent decrease in transmission by reducing the passage of parasite eggs from the mesenteric veins to the intestinal lumen. The development of an anti-schistosome vaccine needs to target all forms of parasitological burden: worm, hepatic egg, and intestinal egg burdens. The data presented in this manuscript for the formulation of Sm-

cathepsin B plus Montanide ISA 720 VG shows comparable protective results for worm burden; decreased by 60% compared to adjuvant controls and 66% compared to saline controls. The reduction in hepatic egg burden was greater in the animals immunized with antigen plus adjuvant (reduction of 62% compared to adjuvant controls and 69% compared to saline controls). In this study, we showed that animals immunized with Sm-cathepsin B plus Montanide ISA 720 VG had a significant decrease in intestinal egg burden (56% compared to the adjuvant control animals and 59% compared to saline controls). The Montanide-adjuvanted recombinant protein formulation was capable of achieving significant reductions of all parasitological burdens (worms, hepatic eggs, and intestinal eggs). Therefore, using Montanide ISA 720 VG as an adjuvant with the recombinant Sm-cathepsin B improves the protective potential of Sm-cathepsin B.

The *S. mansoni* radiation attenuated (RA) cercariae vaccine has been essential to study immune effector mechanisms associated with protection as well as discovering potential parasite points of vulnerability. The RA vaccine is considered the gold standard for reproducible induced anti-worm immunity. Exposure to the attenuated cercariae can elicit significant protection levels against challenges with normal cercariae. Protection levels have been shown to range from 56% all the way to 80% when multiple vaccinations are performed in the mouse model [34-37]. The protection levels attained in this study with Sm-cathepsin B plus Montanide ISA 720 VG, 56-69%, are beyond the 40% threshold established by the TDR/WHO, and are comparable to those generated by the RA vaccine.

Several studies have demonstrated that IgG antibodies play an important role in protection against schistosomiasis. The passive transfer of sera from chronically infected or protected animals results in significantly reduced parasite burdens in the recipient animals [38-42]. Studies utilizing the olive baboon and rhesus macaque models have shown that high IgG titers at the time of cercarial challenge correlated with low worm burdens [43, 44]. Furthermore, the worms collected from the macaques, upon perfusion, were physiologically crippled. The compromised structure of these worms is suggested to be a result of antibody-dependant mechanisms where IgG has a blocking or stimulatory effect leading to parasite starvation or exhaustion, respectively [44, 45]. *In vitro* work using immune sera has also highlighted antibody dependant cell mediated cytotoxicity and antibody dependant complement

mediated cytotoxicity as potential effector mechanisms involved in protection against schistosomiasis [41, 46]. Immunizations with the recombinant Sm-cathepsin B in the presence of Montanide ISA 720 VG resulted in the production of elevated antigen-specific IgG titers in the experimental mice (mean endpoint titers at week 9 = 122,880). Immunization with Sm-cathepsin B alone yielded endpoint titers of approximately 3,500 [30] (data not shown) thereby, demonstrating that the addition of the adjuvant Montanide ISA 720 VG increases the production of Sm-cathepsin B specific antibodies by a factor of 35. These values demonstrate this vaccine formulation's substantial impact on humoral immunity. There were prominent differences when comparing endpoint titers of different IgG subclasses. The adjuvanted recombinant protein formulation elicited a significantly stronger production of the IgG1 subclass (mean endpoint titers at week 9 = 48,640) over IgG2c (mean endpoint titers at week 9 = 1,840). The IgG1 subclass is known to be representative of a Th2 phenotype as its production is stimulated by IL-4 whereas the IgG2c subclass is a marker for a Th1 phenotype [47].

It has long been a consensus that Th1 responses play a key role in protection against schistosomiasis. For instance, peripheral blood mononuclear cells isolated from individuals, who are considered naturally resistant to schistosomiasis, produce high levels of IFN-ɣ when stimulated with schistosome antigen [29, 48]. Moreover, immunological studies involving the RA vaccine have shown that, in protected animals, the migration of the schistosomulae is terminated in the lungs due to the formation of inflammatory foci consisting of monocytes and $CD4^+$ T-cells with Th1 characteristics [37, 45, 49]. In the RA model, IL-12 was shown to prime the Th1 response by inducing IFN-ɣ, which then acts in an autocrine manner to amplify the response [37, 45, 49]. The RA vaccine model has also elucidated the important role of TNF-α in protection against schistosomiasis [50]. Both Th1 cytokines IFN-ɣ and TNF-α are secreted by cells in the inflammatory foci, and they are both capable of activating macrophages. Other studies with promising *S. mansoni* vaccine candidates have shown an association between protection against parasite challenge and an increased IFN-ɣ production [51-53]. The formulation of Sm-cathepsin B plus Montanide ISA 720 VG was able to significantly increase the secretion levels of the three Th1 cytokines IFN-ɣ, TNF-α, and IL-12 compared to both the saline and adjuvant controls. However, our experimental formulation was also able to increase the secretion levels of Th2 cytokines; the secretion levels of IL-4 and IL-5 were significantly increased in the experimental animals compared to the controls. Recent studies have discussed

the importance of inducing a Th2 response as well to achieve protection against schistosomiasis. Inducing such a response aims to involve eosinophils and basophils in the response to the parasite [30, 54]. The formulation of Sm-cathepsin B in the presence of Montanide ISA 720 VG was able to stimulate both a Th1 (IFN-ɣ, TNF-α, and IL-12) and a Th2 response (IL-4, IL-5, and IgG1). This resulting mixed Th1/Th2 response may explain the higher levels of protection obtained in this study compared to our previous study using a CpG adjuvanted Sm-cathepsin B formulation [24]. The CpG formulation generated a more Th1-biased response. Furthermore, a previous study showed that immunization with Sm-cathepsin B alone leads to increased secretion of Th2-associated cytokines such as IL-4, IL-5, and IL-13, but not Th1-associated cytokines such as IFN-γ thus, creating a Th2-biased response [30]. The Sm-cathepsin B + Montanide ISA 720 VG also significantly increased the secretion levels of IL-17 in the experimental animals compared to the adjuvant control animals. This observation highlights the potential role of Th17 responses in protection against schistosomiasis. Increases in IL-17 levels have been correlated to lower worm burdens [55]. IL-17 may contribute to protection by mediating the recruitment and activation of neutrophils which can impede larval migration via their extracellular traps [54]. Furthermore, this implication of IL-17 was not observed in our previously tested formulation of Sm-cathepsin B + CpG [24] thus, demonstrating the multi-faceted immune involvement mediated by this Montanide adjuvanted formulation.

Levels of both GM-CSF and CCL5 were also significantly increased in the experimental animals compared to the control animals. GM-CSF is involved in macrophage recruitment whereas CCL5 is involved in the recruitment of T cells and granulocytes. Furthermore, CCL5 mediates proliferation and activation of NK cells, which play a role in schistosomiasis protection in elderly populations [56]. Increased secretion levels of CCL5 were also observed with our previous formulation of Sm-cathepsin B + CpG [24]. However, elevated levels of GM-CSF were not observed in our previous formulation thus, representing another immunological difference generated by the formulation containing the Montanide ISA 720 VG adjuvant.

The results obtained from this study are promising and promote further testing of the vaccine candidate Sm-cathepsin B. In the future, the formulation can potentially be tested in a

non-human primate model of schistosomiasis. These animals manifest a disease that is similar to that observed in humans, and the data from such a study may be more clinically applicable than data from a mouse study.

3.7 Acknowledgements

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3.9 Figures and legends

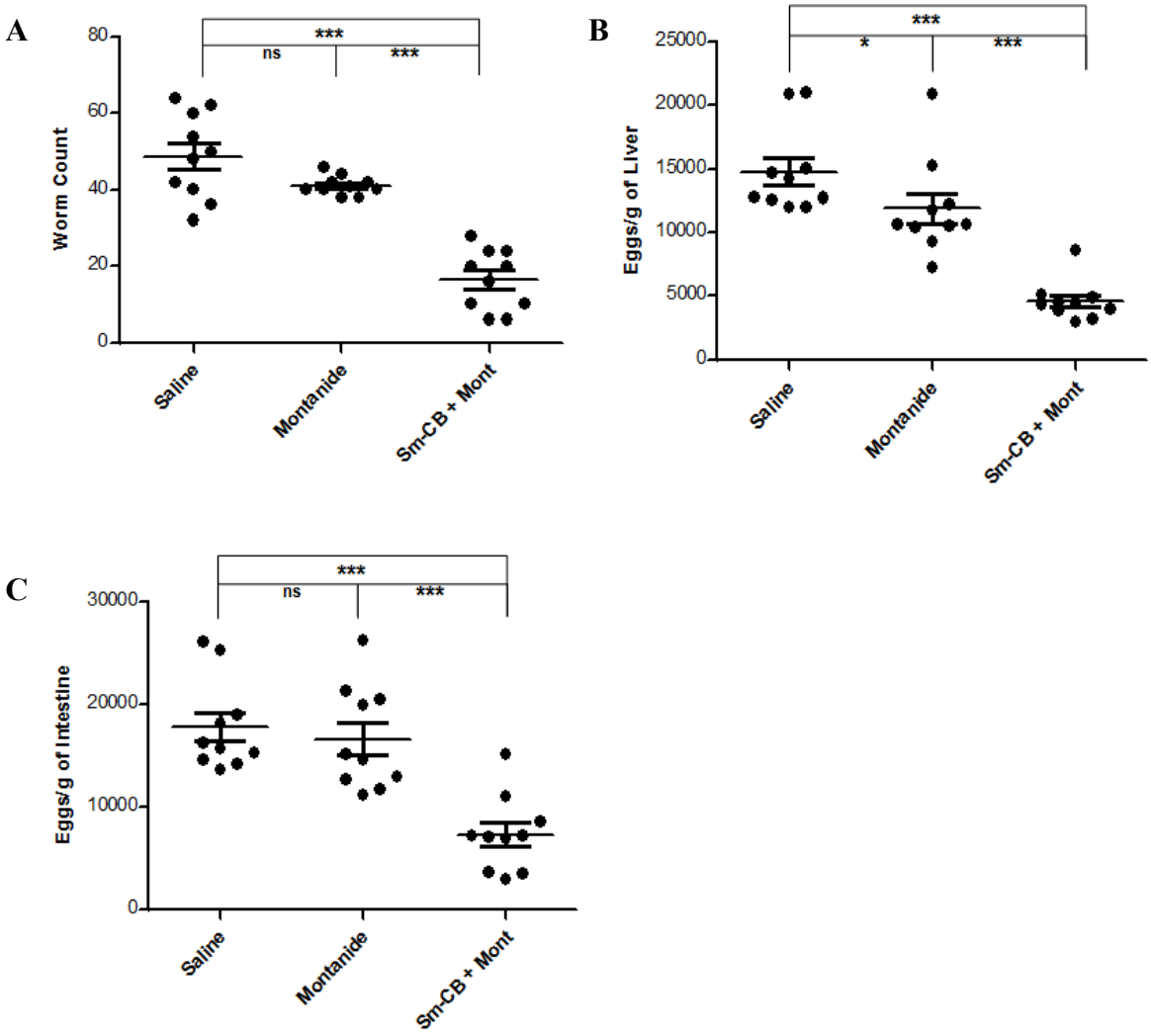
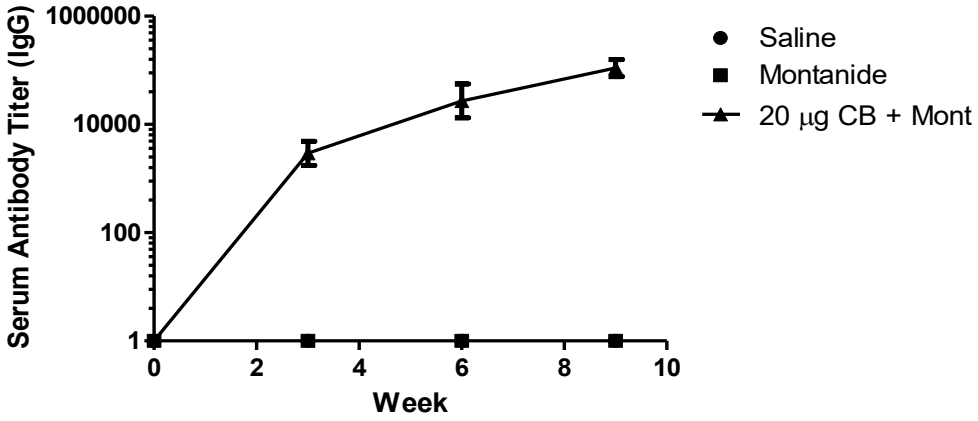


Figure 3.1 Assessment of parasitological burden.

The worm counts per individual mouse ($P = 0.0002$) **(A)** as well as the egg load per gram of liver ($P < 0.0001$) **(B)** and per gram of intestine ($P = 0.0009$) **(C)** are represented for mice in the saline control group, the adjuvant alone control group (Montanide ISA 720VG), and the experimental group (20µg Sm-cathepsin B with Montanide ISA 720 VG). Worm and egg burdens were determined 7 weeks post cercarial challenge. (ns = not significant, * $P < 0.05$, *** $P \leq 0.001$)

A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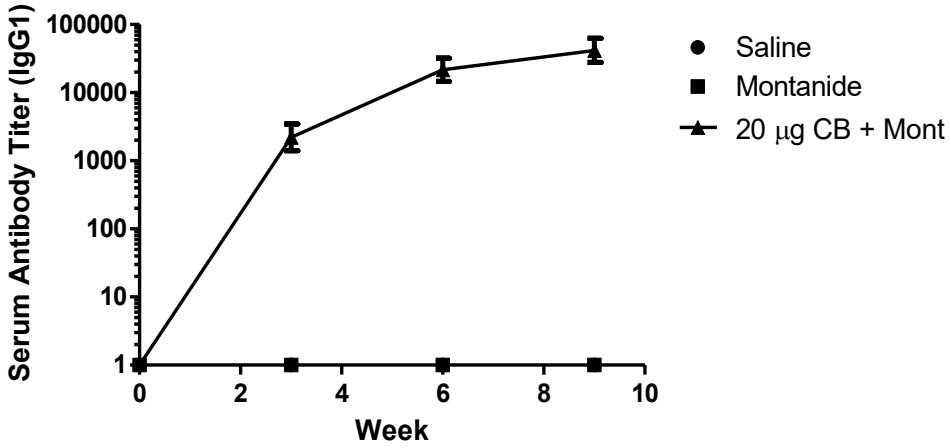
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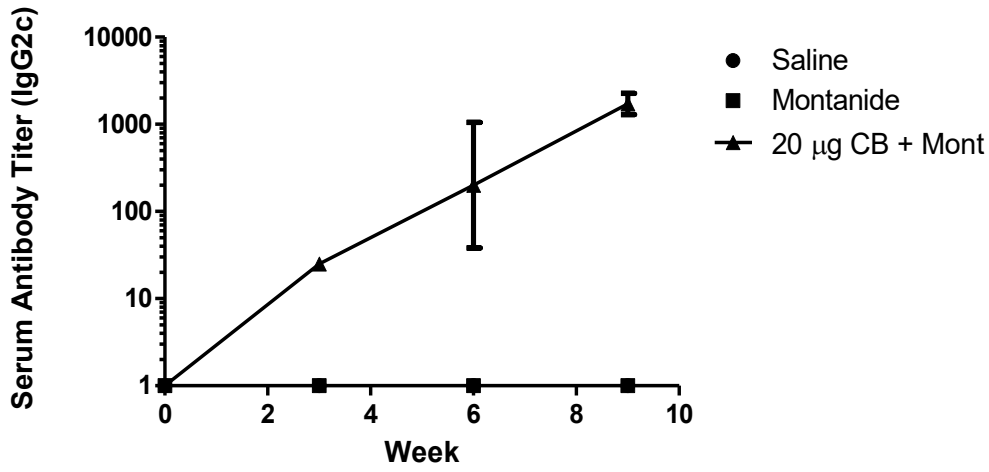
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Figure 3.2 Production of Sm-Cathepsin B specific antibodies.

Sm-cathepsin B specific total IgG **(A)**, IgG1 **(B)** and IgG2c **(C)** in immunized mice: Saline control, Montanide ISA 720 VG adjuvant control, and Sm-cathepsin B with Montanide ISA 720 VG. Serum from individual mice was analyzed by ELISA. Geometric means and 95% confidence intervals are shown. Endpoint titers were determined for week 0, week 3, week 6, and week 9.

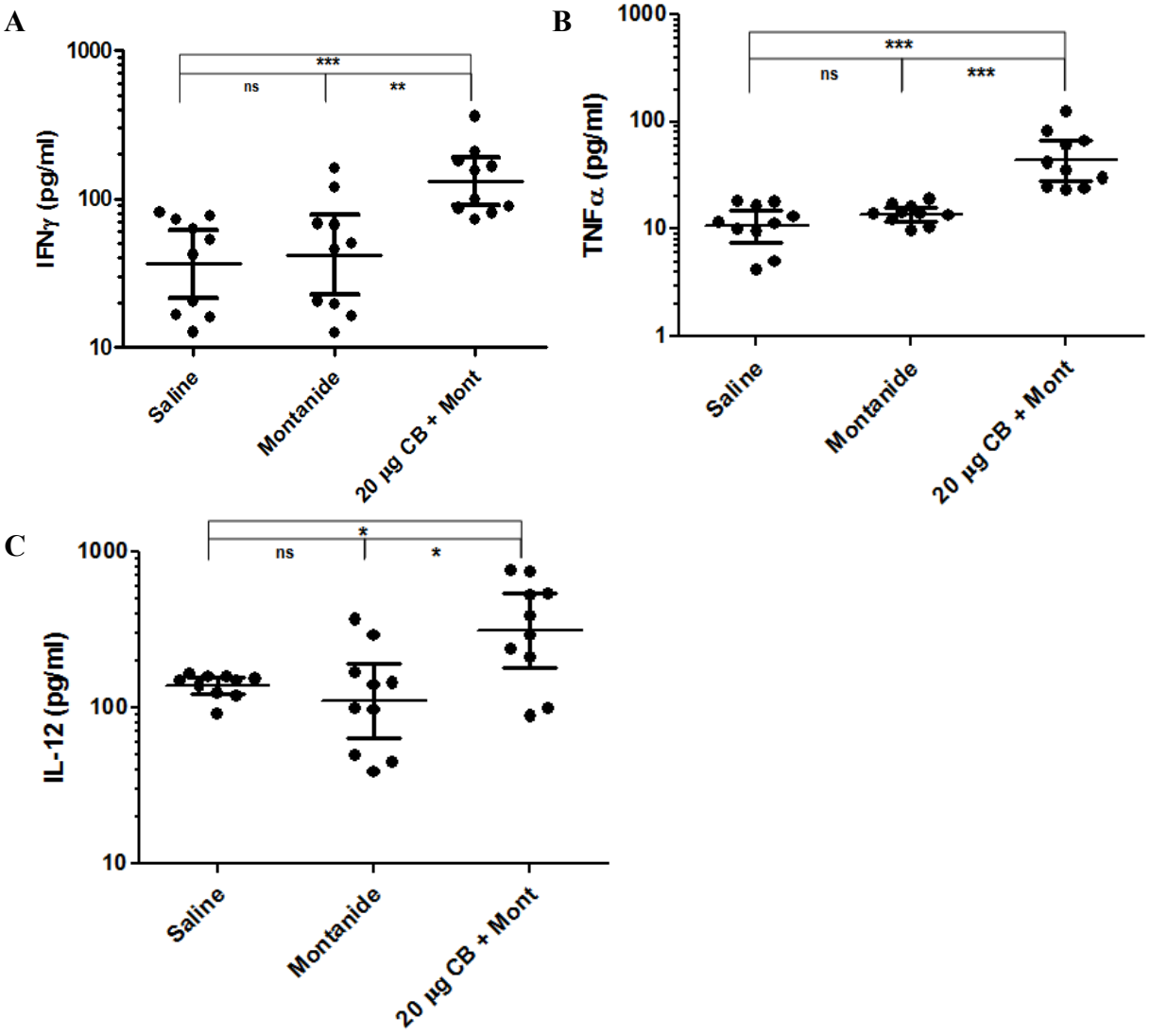


Figure 3.3 Th1 cytokine secretion levels.

IFNγ ($P = 0.0021$) **(A)**, TNFα ($P < 0.0001$) **(B)**, and IL-12 ($P = 0.0185$) **(C)** produced 72 hours after stimulating splenocytes *ex vivo* with recombinant Sm-cathepsin B. Splenocytes were

isolated from every C57BL/6 mice belonging to each group: Saline control (saline), Montanide ISA 70VG control (Montanide), and Sm-cathepsin B in the presence of Montanide ISA 720 VG (SmCB + Montanide). Cytokine production was analyzed by QUANSYS multiplex ELISA. Geometric means and 95% confidence intervals are shown. (ns = not significant, * $P <$ 0.05, ** $P \leq 0.01$, *** $P \leq 0.001$).

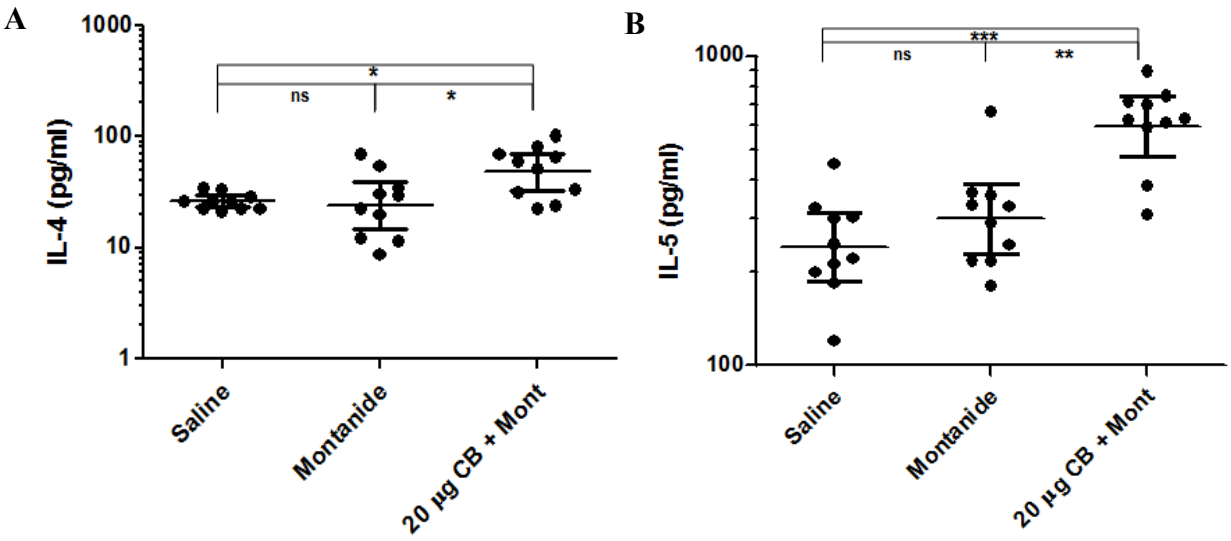


Figure 3.4 Th2 cytokine secretion levels.

IL-4 ($P = 0.0288$) **(A)** and IL-5 ($P = 0.0015$) **(B)** produced 72 hours after stimulating splenocytes *ex vivo* with recombinant Sm-cathepsin B. Splenocytes were isolated from every C57BL/6 mice belonging to each group: Saline control (saline), Montanide ISA 70VG control (Montanide), and Sm-cathepsin B in the presence of Montanide ISA 720 VG (SmCB + Montanide). Cytokine production was analyzed by QUANSYS multiplex ELISA. Geometric means and 95% confidence intervals are shown. (ns = not significant, * $P <0.05$, ** $P \leq 0.01$, *** $P \leq 0.001$).

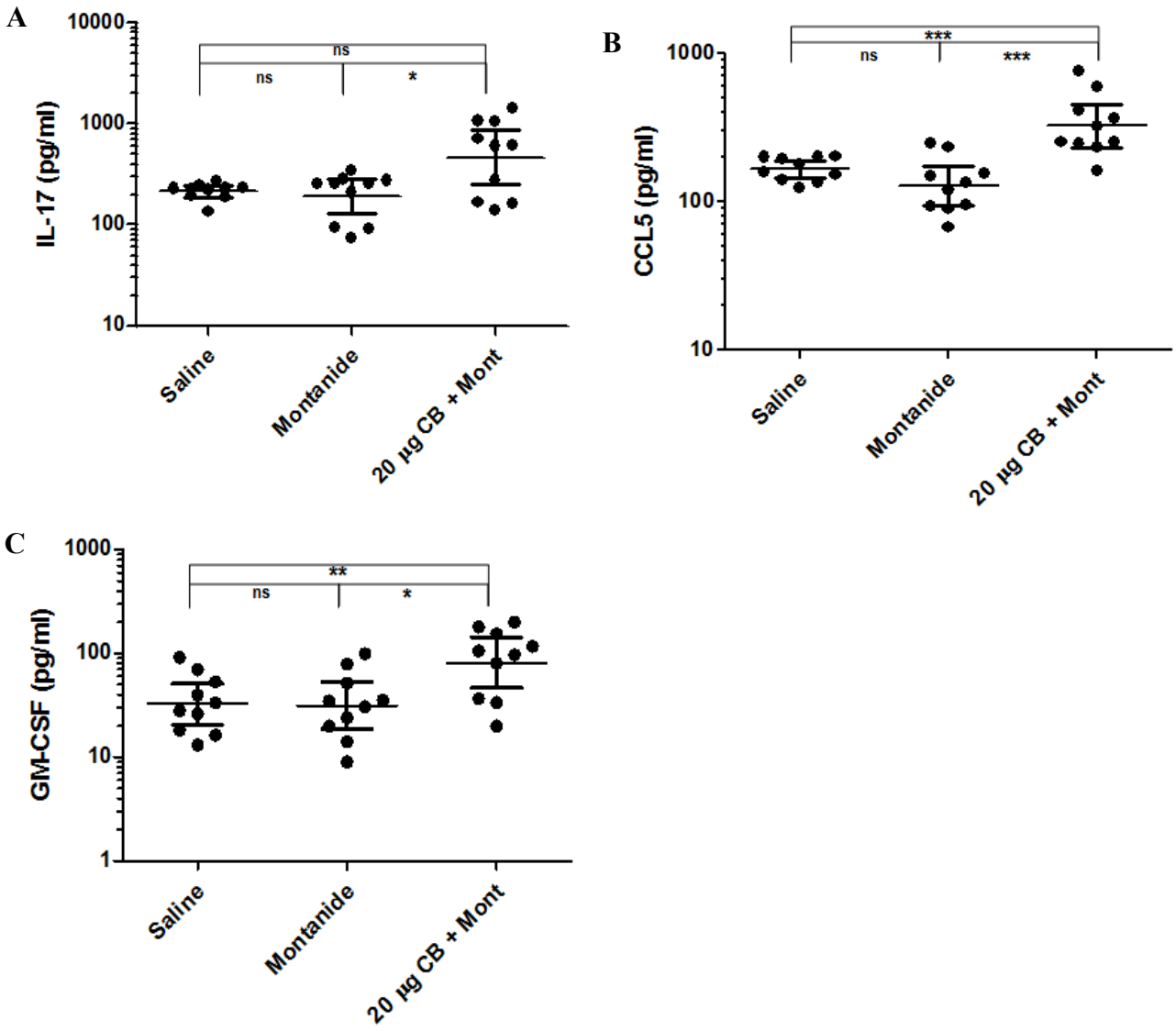
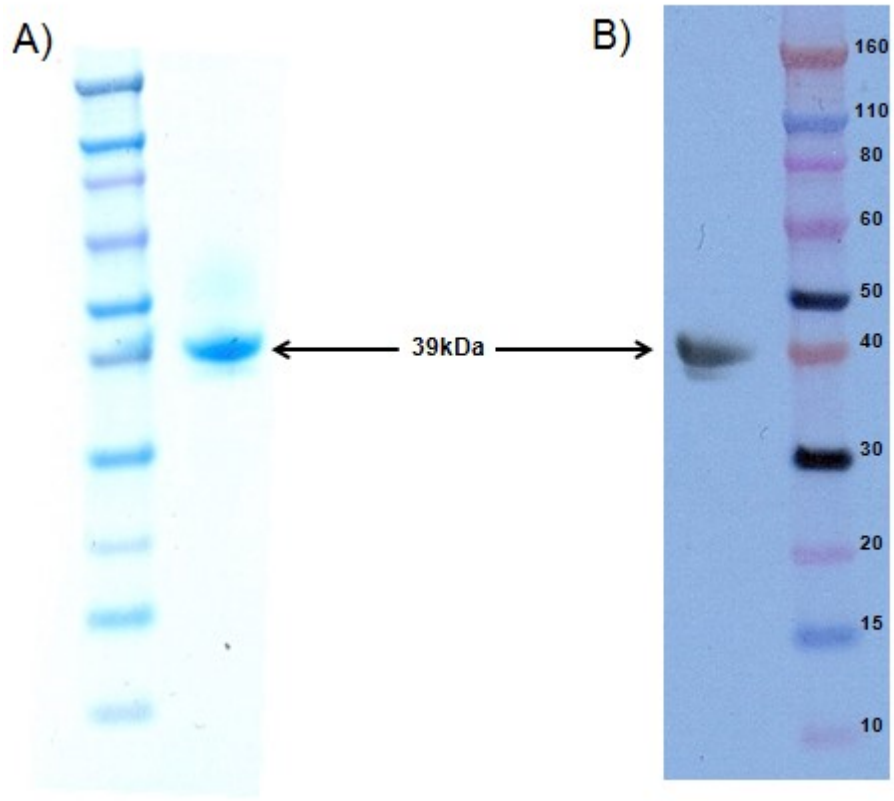


Figure 3.5 Th17, Chemotactic, and Growth Stimulating Cytokine levels.

IL-17 ($P = 0.0433$) **(A)**, CCL5 ($P = 0.0002$) **(B)**, and GM-CSF ($P = 0.0147$) **(C)** produced 72 hours after stimulating splenocytes *ex vivo* with recombinant Sm-cathepsin B. Splenocytes were isolated from every C57BL/6 mice belonging to each group: Saline control (saline), Montanide ISA 70VG control (Montanide), and Sm-cathepsin B in the presence of Montanide ISA 720 VG (SmCB + Montanide). Cytokine production was analyzed by QUANSYS multiplex ELISA. Geometric means and 95% confidence intervals are shown. (ns = not significant, * $P \leq 0.05$, ** $P \leq 0.01$, *** $P \leq 0.001$).

3.10 Supplemental figures and tables



Supplemental Figure 3.1 Recombinant Sm-cathepsin B expression

In panel **A**, the Coomassie stained polyacrylamide gel demonstrated the Sm-cathepsin B representative band at 39 kDa. The same band is also seen upon western blot analysis using anti-His tag antibodies as demonstrated in panel **B**.

Supplemental Table 3.1 Burden reductions observed in mice vaccinated with Sm-Cathepsin B and Montanide ISA 720 VG compared to Saline control mice

Group	Worm Burden (mean ± SE)	Percent Reduction ($p \leq 0.001$)	Eggs/g of Liver (mean ± SE)	Percent Reduction in experimental group ($p \leq 0.001$)	Eggs/g of Intestine (mean ± SE)	Percent Reduction in experimental group ($p \leq 0.001$)
Saline (control)	48.8 ± 3.5	-	14791.6 ± 1080.1	-	17800.2 ± 1421.8	-
Sm-Cathepsin B + Montanide ISA 20VG (experimental)	16.4 ± 2.5	66.4%	4578.5 ± 492.9	69.1%	7292.8 ± 1173.1	59.0%

The percent reduction values stated in Table 1 represent the decrease in parasite burden (worms and eggs) observed in the experimental animals when compared to the saline control animals. SE: standard error

Supplemental Table 3.2 Burden reductions observed in mice vaccinated with Sm-Cathepsin B and Montanide ISA 720 VG compared to Adjuvant control mice

Group	Worm Burden (mean ± SE)	Percent Reduction ($p \leq 0.001$)	Eggs/g of Liver (mean ± SE)	Percent Reduction in experimental group ($p \leq 0.001$)	Eggs/g of Intestine (mean ± SE)	Percent Reduction in experimental group ($p \leq 0.001$)
Montanide ISA 720 VG (adjuvant control)	41.1 ± 0.8	-	11876.0 ± 1197.9	-	16606.6 ± 1590.0	-
Sm-Cathepsin B + Montanide ISA 20VG (experimental)	16.4 ± 2.5	60.1%	4578.5 ± 492.9	61.5%	7292.8 ± 1173.1	56.1%

The percent reduction values stated in Table 2 represent the decrease in parasite burden (worms and eggs) observed in the experimental animals when compared to the adjuvant control animals. SE: standard error

Chapter 4: Immune mechanisms involved in *Schistosoma mansoni*-cathepsin B vaccine induced protection in mice

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4.1 Preface

The experiments performed in previous chapters have demonstrated that the two tested Sm-Cathepsin B formulations, with CpG and with Montanide ISA 720 VG, generated similar protection levels. However, they elicited different immune responses; a biased Th1 response with the CpG adjuvant and a mixed Th1/Th2 response with Montanide. Furthermore, unadjuvanted Sm-Cathepsin B was also able to reduce parasite burden in a mouse model of schistosomiasis. The immunizations elicited a biased Th2 response, which differed from either formulation containing an adjuvant. Given these observations, we wanted to gain further insights on the underlying mechanisms of vaccine-induced protection. Studying these immune mechanisms could enable us to identify an optimal vaccine formulation against schistosomiasis. We therefore performed *in vitro* parasite killing assays using lung cells as well as immune serum from mice immunized with the different Sm-Cathepsin B formulations (with CpG, with Montanide, or unadjuvanted). For these mechanistic studies, schistosomulae were used as targets. In addition, cell population depletions were performed in order to begin deciphering

which effectors mediate vaccine-induced protection. The observations presented in the following chapter provide the first insight into the different immune mechanisms mediating Sm-Cathepsin B vaccine-induced protection for the different formulations tested.

4.2 Abstract

A vaccine against schistosomiasis would contribute to a long-lasting decrease in disease spectrum and transmission. Our previous protection studies in mice using *Schistosoma mansoni* Cathepsin B (Sm-Cathepsin B) resulted in 59% and 60% worm burden reduction with CpG oligodeoxynucleotides and Montanide ISA720 VG as adjuvants, respectively. While both formulations resulted in significant protection in a mouse model of schistosomiasis, the elicited immune responses differed. Therefore, in this study, we aimed to decipher the mechanisms involved in Sm-Cathepsin B vaccine-mediated protection. We performed *in vitro* killing assays using schistosomulae stage parasites as targets for lung-derived leukocytes and serum obtained from mice immunized with Sm-Cathepsin B adjuvanted with either Montanide ISA 720 VG or CpG and from non-vaccinated controls. Lung cells and immune sera from the Sm-Cathepsin B + Montanide group induced the highest killing (63%) suggesting the importance of antibodies in cell-mediated parasite killing. By contrast, incubation with lung cells from Sm-Cathepsin B + CpG immunized animals induced significant parasite killing (53%) independent of the addition of immune serum. Significant parasite killing was also observed in the animals immunized with Sm-Cathepsin B alone (41%). For the Sm-Cathepsin B + Montanide group, the high level killing effect was lost after the depletion of $CD4^+$ T cells or Natural Killer (NK) cells from the lung cell preparation. For the Sm-Cathepsin B + CpG group, high parasite killing was lost after $CD8^+$ T cell depletion, and a reduction to 39% was observed upon depletion of NK cells. Finally, the parasite killing in the Sm-Cathepsin B alone group was lost after the depletion of $CD4^+$ T cells. Our results demonstrate how the different Sm-Cathepsin B formulations influence the immune mechanisms involved in parasite killing and protection against schistosomiasis.

4.3 Introduction

Schistosomiasis is one of the most important human parasitic infections. The number of infected individuals surpasses 200 million, and this number may be an underestimate of the true infection burden [1]. The majority of infected individuals suffer from long-term

schistosomiasis-related conditions such as developmental complications, anemia, and chronic pain while severe morbidity is represented by the hepatosplenic form of the disease [1-3]. Praziquantel is currently the only drug used to treat schistosomiasis, and it is the mainstay of mass drug administration (MDA) programs which benefitted from substantial pharmaceutical donations [1]. However, the MDA programs' impact on disease transmission has often been questioned, and field studies in Kenya have shown no evidence of an overall reduction in schistosome transmission after several rounds of praziquantel treatment [4]. Maintained disease transmission is particularly a problem for phenotypically susceptible individuals [5] who do not acquire resistance to reinfection after multiple rounds of treatment. Moreover, the use of praziquantel is a double edged sword as increasing the proportion of schistosomes exposed to the drug increases selection pressure on drug resistance. The possibility of praziquantel resistant parasite emergence has been discussed for years [6-10] and represents a valid concern as schistosomiasis treatment relies on this one drug. A multifaceted approach to schistosomiasis control that would include drug treatment, snail control, water sanitation, hygiene education, and better disease mapping is suggested [1, 11]. However, the development of an anti-schistosome vaccine would significantly improve schistosomiasis control efforts, especially if it would be incorporated within this multifaceted approach.

Our group has focused on *Schistosoma mansoni* Cathepsin B (Sm-Cathepsin B), the most abundant cysteine peptidase found in the parasite gut, as a potential vaccine candidate. It is needed for schistosome development [12] and it functions in parasite nutrition through the digestion of blood macromolecules [13-17]. It has been demonstrated that immunizations with Sm-Cathepsin B alone can significantly decrease parasite burden in a mouse model of schistosomiasis [18]. With the addition of an adjuvant, either CpG dinucleotides [19] or Montanide ISA 720 VG (SEPPIC Inc., Fairfield, NJ, USA) [20], protection levels were increased when examining all forms of parasitological burden including worm, hepatic egg, and intestinal egg numbers. CpG dinucleotides are Toll-like receptor 9 agonists and they promote a T-helper cell type 1 (Th1) response and have shown promise in vaccine formulations against various parasitic infections [21-25]. Differently, Montanide ISA 720 VG is a squalene based adjuvant which forms water-in-oil droplets that allow for slow antigen release at the site of injection. Montanide adjuvants are acceptable for use in humans, and they have been used in over fifty clinical trials [26-28]. Although both formulations, Sm-Cathepsin B in combination

with CpG and with Montanide ISA 720 VG, elicited significant protection in a mouse model of schistosomiasis, the immune responses which they generated differed. Sm-Cathepsin B with CpG stimulated a Th1-biased response [19] whereas the Montanide ISA 720 VG adjuvanted formulation yielded a mixed Th1/Th2 response [20]. This suggests that the soluble and/or cellular effector mechanisms involved in the vaccine-mediated protection differ between formulations.

In the present study, we sought to determine the antibody-dependant and cellular effectors involved in mediating the protection elicited by our different Sm-Cathepsin B vaccine formulations. We focused on the lung stage parasites, schistosomulae, since studies on radiation-attenuated vaccines have shown that this stage is susceptible to immune-mediated protection mechanisms [29, 30]. Therefore, lung cells obtained from mice vaccinated with Sm-Cathepsin B formulations were studied *in vitro* for their ability to kill schistosomulae in the presence or absence of antibodies. We are the first to report mechanistic data behind the protection observed with the different formulations of Sm-Cathepsin B.

4.4 Materials and Methods

4.4.1 Expression and purification of Sm-Cathepsin B

Expression and purification of the Sm-Cathepsin B recombinant protein were carried out as previously reported [19]. Briefly, the PichiaPink^{TM} expression system (Thermo Fisher Scientific, Waltham, MA, USA) was used and yeast cells were cultured in buffered complex glycerol medium followed by induction in buffered complex methanol medium. Purification of the recombinant protein was performed via Ni-NTA chromatography (Ni-NTA Superflow by QIAGEN, Venlo, Limburg, Netherlands) and the elution was analyzed by Coomassie blue staining of polyacrylamide gel and western blot (Supplemental Figure 4.1).

4.4.2 Immunization protocol

Female, 6-8 week old C57BL/6 mice were purchased from Charles River Laboratories (Senneville, Qc, Canada). Six groups containing twelve to sixteen mice each were immunized intramuscularly with different formulations. Group 1 (saline control): mice received 50 µl of phosphate buffered saline (PBS) (Wisent Bio Products, Saint-Jean-Baptiste, Qc, Canada). Group 2 (antigen control): mice were immunized with 20 µg of recombinant Sm-Cathepsin B.

Group 3 (CpG adjuvant control): mice received 40 µg of synthetic oligodeoxynucleotides containing unmethylated CpG dinucleotides (Catalog# HC4039, Cedarlane, Burlington, ON, Canada). Group 4 (antigen and CpG experimental): mice were immunized with 20 µg recombinant Sm-Cathepsin B and 40 µg CpG adjuvant. Group 5 (Montanide adjuvant control): mice received a 70% volume formulation of Montanide ISA 720 VG (SEPPIC Inc., Fairfield, NJ, USA). Group 6 (antigen and Montanide experimental): mice were immunized with 20µg recombinant Sm-Cathepsin B in a 70% volume formulation of Montanide ISA 720 VG. All mice received an initial immunization of their respective formulation at week 0 and were boosted with the same formulation at weeks 3 and 6. The mice were sacrificed three weeks after the final boost (week 9). Whole lungs and blood samples were collected from all animals. The experiment was repeated four additional times. All animal procedures were performed in accordance with Institutional Animal Care and Use Guidelines, and the Animal Use Protocol 7625 was approved by the Animal Care and Use Committee at McGill University.

4.4.3 Lung cell suspension preparation

Whole lungs were collected from each mouse at week 9. The lungs were cut into very small pieces and transferred into tubes containing 10 ml of collagenase digestion solution: 10 ml PBS (Wisent Bio Products), 300 U/ml collagenase type II (Worthington Biochemical Corporation, Lakewood, NJ, USA), 150 µ1 of 10 mg/ml stock solution of DNase I (Sigma Aldrich, St. Louis, MS, USA). The lungs were digested at 37 ºC for 45 minutes under constant shaking. The lung digests were transferred onto strainers into new tubes and sterile syringe plungers were used to push any remaining lung pieces through the strainers. The strainers were washed twice with 5 ml of Hank's balanced salt solution (Wisent Bio Products). All tubes containing the lung digests were centrifuged for 5 minutes at 300xg and 4 ºC. The supernatants were decanted. At this point, lungs belonging to mice from the same group were pooled (three to five lungs from the same group were pooled). The pooled cell pellets were resuspended using PBS to a final volume of 10 ml. The tubes were centrifuged for 5 minutes at 300xg and 4 ºC. The supernatants were decanted, the pellets resuspended in 10 ml PBS, and centrifuged once again for 5 minutes at 300xg and 4 ºC. The supernatants were decanted and the pellets were resuspended in 10 ml MACS buffer: PBS (Wisent Bio Products), 2 mM EDTA (Sigma

Aldrich), 0.5% Fetal bovine serum (FBS) (Wisent Bio Products). Cell numbers were determined by trypan blue exclusion before proceeding to the cell isolation step.

Mouse CD45 MicroBeads (Miltenyi Biotec, Germany) were used, following the manufacturer's instructions, for the positive selection of leukocytes ($CD45^+$ cells) from the lung cell suspensions. Briefly, pelleted cells were resuspended in 90 µl MACS buffer followed by the addition of 10 µl CD45 MicroBeads and a 15 minute incubation at 4 ºC in the dark. The cells were then washed with 1.5 ml MACS buffer and centrifuged for 10 minutes at 300xg and 4 ºC. Pellets were resuspended in 500 µl MACS buffer and applied to MS columns placed in an OctoMACS^{TM} separator (Miltenyi Biotec). The columns were washed three times with 500 µl MACS buffer. Afterwards, the columns were removed from the separator and placed on clean collection tubes. Then, 1 mL of MACS buffer was added to each column and the magnetically labelled cell fraction ($CD45^+$ population) was flushed out by firmly applying the plunger supplied with the column.

4.4.4 Cell population depletions

Additional Miltenyi Biotec mouse MicroBead kits were used to deplete different cell populations (Miltenyi Biotec): CD4 (L3T4) MicroBeads, CD8a (Ly-2) MicroBeads, CD49b (DX5) MicroBeads, and biotin labelled F4/80 antibody in combination with anti-biotin MicroBeads. For the CD4, CD8a, and CD49b MicroBeads, the procedures were similar. Briefly, the cell suspension was centrifuged at 300xg for 10 minutes and then the supernatant was removed completely. The pelleted cells were resuspended in 90 µl MACS buffer followed by the addition of 10 µl of the respective MicroBeads and 15 minute incubation at 4 ºC in the dark. The cells were then washed with 1.5 ml MACS buffer and centrifuged for 10 minutes at 300xg and 4 ºC. Pellets were resuspended in 500 µl MACS buffer and applied to MS columns placed in an OctoMACS^{TM} separator.

Since F4/80 MicroBeads were not available, the $F4/80^+$ cell population was depleted using an indirect method. Briefly, cells were pelleted and the supernatant was removed. The cells were resuspended in MACS buffer and anti-F4/80-biotin (the manufacturer's recommended antibody dilution was 1:10 for up to 10^6 cells/50 µl of buffer). The resuspended cells were incubated in the dark at 4 ºC for 10 minutes. The cells were washed with 1.5 mL

MACS buffer and centrifuged for 10 minutes at 300xg and 4 ºC. Pellets were resuspended in 500 µl MACS buffer and applied to MS columns placed in an OctoMACS^{TM} separator. For all of the performed depletions, the columns were washed three times with 500 µl MACS buffer. Unlabelled cells passed through the column whereas the magnetically labelled $CD4^+$, $CD8a^+$, $CD49b^+$, or $F4/80^+$ cells were retained within the columns. Therefore, the total column effluent represented the depleted population. All of the cell depletions were followed by CD45 positive selection as previously explained.

4.4.5 Flow cytometry

Flow cytometry analysis was performed in order to confirm the specific cell population depletions. The cell suspension was centrifuged for 5 minutes at 400xg and 4 ºC. The cells were washed with 200 µl PBS and centrifuged for 5 minutes at 400xg and 4 ºC. The cells were stained with viability dye (eBioscience, San Diego, CA, USA) (0.1 µl/10^6 cells in 100 µl PBS) and incubated for thirty minutes at 4 ºC in the dark. After the incubation, the cells were washed three times with 200 µl PBS + 1% FBS. The supernatant was removed, 50 µl of BD Mouse Fc block (2.4G2 Ab) (BD Biosciences, Mississauga, ON, Canada) (1 µl/10^6 cells in 50 µl PBS) was added, and the cells were incubated in the dark for 10 minutes at 4 ºC. Next, 50 µl of the surface stain containing CD45-Buv395 Clone 30-F11 (BD Biosciences), CD4-FITC Clone RM4-5 (eBioscience), CD8a-PerCP-Cy5.5 Clone 53-6.7 (eBioscience), CD49b-PE Clone DX5 (eBioscience), F4/80-APC Clone BM8 (eBioscience) antibody mixture was added, and the cells were incubated in the dark at 4 ºC for 20 minutes. The manufacturers' recommended antibody dilutions were used. The cells were centrifuged for 5 minutes at 400xg and 4 ºC, and then washed with 200 µl PBS + 1% FBS. The supernatant was removed; the cells were resuspended in 100 µl IC Fix buffer (eBioscience) and incubated overnight at 4 ºC. The stained cells were acquired with a BD LSRFortesa (BD Biosciences). Flow cytometry analysis was performed using FlowJo (Tree Star, Inc. USA).

4.4.6 Schistosomulae preparation

Biomphalaria glabrata snails infected with the *Schistosoma mansoni* Puerto Rican strain were obtained from the Schistosomiasis Resource Center of the Biomedical Research Institute (Rockville, MD, USA). Cercariae were collected by placing infected snails in a beaker

containing water and shining a strong light above the beaker for two hours. The collected cercariae were incubated on ice, in the dark for one hour. The settled cercariae were resuspended in 8 ml of alpha-MEM (Thermo Fisher Scientific). The cercariae were vortexed for 1 minute, rested on ice for 3 minutes, and finally vortexed again for 1 minute. The detached parasite tails float at the surface of the media whereas the heads settle on the bottom of a Petri dish. The parasites were then incubated on ice for ten minutes in order to allow the parasite to pellet. The pellet was washed with 10X OPTI media (Opti-MEM Reduced Serum Media (Thermo Fisher Scientific), 0.25ug/ml fungizone (Thermo Fisher Scientific), 100ug/ml streptomycin (Thermo Fisher Scientific), 100U/ml penicillin (Thermo Fisher Scientific)), and then incubated on ice for 8 minutes. The pellet was washed a second time with 10X OPTI media and incubate once again on ice for 8 minutes. A final wash using 1X OPTI media (Opti-MEM Reduced Serum Media (Thermo Fisher Scientific), 0.25ug/ml fungizone (Thermo Fisher Scientific), 10ug/ml streptomycin (Thermo Fisher Scientific), 10U/ml penicillin (Thermo Fisher Scientific), 6% FBS (Wisent Bio Products)) was performed followed by an 8 minute incubation on ice. The parasite pellet was resuspended in 1X OPTI media and schistosomulae were plated in 96-well plates.

4.4.7 *In vitro* killing assay

For the *in vitro* killing assay, the isolated lung cells were the effector cells and the transformed schistosomulae were the targets. In 96-well plates, approximately sixty schistosomula were added to each well. The different cell populations ($CD45^+$, $CD45^+CD4^-$, $CD45^+CD8^-$, $CD45^+CD49b^-$, $CD45^+F4/80^-$) were seeded in 96-well plates as 1 x 10^5 cells/well in a 100 µl cell suspension. The different incubation conditions included media, cells, serum, cells + pre-immune serum, and cells + immune serum. Pre-immune serum was collected by saphenous bleed prior to the first immunization at week 0, and immune serum was collected by cardiac puncture at week 9. Endpoint titers of Sm-Cathepsin B specific total IgG for the experimental groups, antigen + CpG and antigen +Montanide, were above 120,000 [19, 20]. Fifty microliters of serum was added to designated wells to obtain a 1:4 dilution. Incubation at 56ºC for thirty minutes was performed in order to heat inactivate the serum. Every test was plated in duplicate. The plates were incubated for 24 hours at 37 ºC and 5% CO_2, after which,

the percentage of dead schistosomulae was determined by microscopic examination of motility, granularity, shape integrity, and uptake of methylene blue dye by the schistosomulae.

4.4.8 Statistical analysis

Data generated from this study was analyzed by one-way analysis of variance followed by Tukey multiple comparison post-test. Two-way analysis, followed by Bonferroni post-test, was used when comparing original and depleted samples. P values less than 0.05 were considered significant.

4.5 Results

4.5.1 *In vitro* parasite killing in the presence of lung cells and serum

In order to investigate the effector mechanisms involved in vaccine mediated protection, an *in vitro* assay was employed which involved culturing schistosomulae in the presence of immune effector cells with/without serum from vaccinated mice. Schistosomulae were incubated in media alone in order to establish background parasite death. We first showed that heat inactivated serum alone from the different animal groups had no significant effect on parasite viability in culture; thus, antibodies generated by vaccination alone are not cytotoxic to the schistosomulae (Figures 4.1 and 4.2). The parasites were also incubated with lung cells alone, lung cells + pre-immune serum, and lung cells + immune serum obtained from the different control and experimental animal groups. In a separate set of experiments, lung lavage cells were used instead of cells isolated from whole lungs; however, this did not lead to novel observations worth pursuing (Supplemental Figure 4.2).

The experimental vaccine formulation of Sm-Cathepsin B and Montanide ISA 720 VG was compared to the saline control and the Montanide adjuvant control. As shown in Figure 4.1, the highest percentage of parasite killing (63%) was observed when schistosomulae were incubated in the presence of both lung cells and immune serum from the Sm-Cathepsin B and Montanide ISA 720 VG group. The percentage of parasite killing obtained with this condition and group was significantly higher ($p < 0.001$) when compared to the control groups. Supplemental Figure 4.3 depicts the reduced schistosomulae viability in the wells containing lung cells and immune serum from the Sm-Cathepsin B + Montanide ISA 720 VG animals compared to the control wells. These data demonstrate that the addition of immune serum to

cells from the experimental group is necessary to achieve significant levels of parasite killing. Therefore, an antibody dependent cell-mediated cytotoxicity effect may explain the mechanism of protection in mice vaccinated with Sm-Cathepsin B and Montanide ISA 720 VG.

Next, immune serum and cells taken from mice vaccinated with Sm-Cathepsin B alone and the Sm-Cathepsin B + CpG formulation were compared to those taken from mice given saline or CpG adjuvant only. Both groups containing Sm-Cathepsin B (with CpG adjuvant and unadjuvanted) had increased parasite killing compared to the saline and adjuvant controls (Figure 4.2). This increased parasite toxicity was independent of the addition of immune serum to the lung cells (Supplemental Figure 4.2), indicating that the high larval killing observed with the Sm-Cathepsin B and Sm-Cathepsin B + CpG groups is dependent on cellular effectors. The percent parasite killing observed with cells from the Sm-Cathepsin B group, 41%, was significantly higher than all conditions with control groups ($p < 0.001$) (Figure 4.2). The schistosomulae killing recorded with the cells from the Sm-Cathepsin B + CpG group, 53%, was significantly higher compared to the killing obtained with the control groups as well as that obtained with the Sm-Cathepsin B alone group ($p < 0.001$) (Figure 4.2). Examples of parasites with reduced viability in the presence of cells taken from the Sm-Cathepsin B + CpG group can be seen in Supplemental Figure 4.4. These observations suggest that the parasite killing effects elicited by immunizations with Sm-Cathepsin B and Sm-Cathepsin B + CpG are mediated by cellular effectors.

4.5.2 Parasite killing post cell population depletions

In order to begin dissecting the mechanisms involved in Sm-Cathepsin B vaccine mediated protection, *in vitro* parasite killing assays were performed using lung cell preparations that had undergone depletions of specific immune cell populations. Once again the schistosomulae were incubated in conditions that included lung cells alone, lung cells + pre-immune serum, and lung cells + immune serum. All of the cell population depletions were confirmed by flow cytometry (Figure 4.3) (Table 4.1).

When $CD4^+$ T cells were depleted, the parasite killing observed in the presence of serum and lung cells obtained from mice in the Sm-Cathepsin B + Montanide ISA 720 VG group was reduced from 63% to 36% which was similar to the levels of killing observed in the

control groups. Moreover, the presence of immune serum had no effect on the percentage of schistosomulae killing (Figure 4.4A). These observations indicate that $CD4^+$ T cells are important effectors mediating the antibody-dependent parasite killing in Sm-Cathepsin B + Montanide ISA 720 VG immunized mice.

Depletion of $CD8^+$ cells also significantly decreased the parasite killing in the experimental group, from 63% to 50% ($p < 0.05$) (Figure 4.4B). However, in this case, the presence of immune serum in the $CD8^+$ depleted lung cells resulted in significantly higher killing compared to the absence of immune serum and to the saline control group ($p < 0.05$) (Figure 4.4B). This observation suggests that $CD8^+$ T cells participate in parasite killing, although, they are not the primary immune cell effectors.

Depletion of $F4/80^+$ cells (macrophages) resulted in 50% parasite viability ($p < 0.01$) (Figure 4.4C). The highest level of killing was observed when F4/80-depleted lung cells were incubated with immune serum (Figure 4.4C). The antibody dependent effect suggested that macrophages are not the main mediators for the parasite killing mechanism elicited by Sm-Cathepsin B + Montanide ISA 720 VG immunizations.

Upon the depletion of NK cells ($CD49b^+$), the percentage of parasite killing in the experimental group was reduced to levels comparable to the controls (34%) (Figure 4.4D). In this situation, the addition of immune serum to the NK depleted lung cells did not increase parasite killing (Figure 4.4D). These observations indicate a role for NK cells in mediating parasite killing elicited by Sm-Cathepsin B + Montanide ISA 720 VG immunizations.

Levels of parasite killing observed with cells obtained from the Sm-Cathepsin B + CpG mice were slightly reduced from 53% to 46% after $CD4^+$ T cell depletion (Figure 4.5A). Larval killing in the Sm-Cathepsin B + CpG group was still significantly higher compared to the control groups (Figure 4.5A). The conservation of schistosomulae killing upon $CD4^+$ T cell depletion suggests that other cellular effectors are involved in the protection mediated by Sm-Cathepsin B + CpG immunizations. By contrast, the percentage of parasite death with lung cells from the unadjuvanted Sm-Cathepsin B immunized animals decreased to levels comparable to controls (30%) upon depletion of $CD4^+$ T cells ($p < 0.001$) (Figure 4.5A). This

data indicates that $CD4^+$ T cells are necessary effectors in the protection mediated by unadjuvanted Sm-Cathepsin B immunizations.

Parasite killing was reduced to 34% in the Sm-Cathepsin B + CpG after $CD8^+$ T cell depletion ($p < 0.001$) (Figure 4.5B). This level was comparable to that observed in the saline and CpG adjuvant control groups (Figure 4.5B) and indicates an important role for $CD8^+$ T cells in mediating parasite killing in Sm-Cathepsin B + CpG immunized mice. By contrast, larval killing was maintained (40%) in the Sm-Cathepsin B alone group following $CD8^+$ Tcell depletion suggesting that, in this group, $CD8^+$ T cells have a negligible effect on the protection (Figure 4.5B).

When $F4/80^+$ cell populations were depleted from lung cells taken from the Sm-Cathepsin B and Sm-Cathepsin B + CpG groups, significantly higher parasite killing compared to the saline and adjuvant control groups was maintained ($p < 0.001$) (Figure 4.5C). Incubation of schistosomulae with depleted $F4/80^+$ cells from the CpG adjuvanted experimental group resulted in parasite killing levels that were slightly reduced compared original observations; 53% to 45% ($p < 0.01$) (Figure 4.5C). This suggests that although macrophages are not required to maintain high levels of larval death, they may contribute to the parasite killing mechanism elicited by Sm-Cathepsin B + CpG immunizations. By comparison, $F4/80^+$ cell depletion from lung cells taken from the unadjuvanted Sm-Cathepsin B group increased parasite killing from 41% to 55% ($p < 0.001$) (Figure 4.5C). This depletion resulted in significantly higher levels of parasite death in the unadjuvanted group compared to the CpG adjuvanted group ($p < 0.001$) (Figure 4.5C).

Depletion of NK cells resulted in schistosomulae killing observed in the Sm-Cathepsin B + CpG group decreasing from 53% to 39% ($p < 0.001$) (Figure 4.5D). Although this level of killing was still significantly higher than that recorded for the saline and adjuvant control groups, the substantial decrease suggests that NK cells may belong to a network of cellular effectors mediating vaccine induced protective effects (Figure 4.5D). Furthermore, upon the depletion of NK cells, the parasite killing elicited by the Sm-Cathepsin B + CpG group was no longer significantly higher than the killing observed with the Sm-Cathepsin B alone group (Figure 4.5D). The percentage of parasite killing with the cells from the Sm-Cathepsin B alone animals was unaffected by the depletion of NK cells (41%) (Figure 4.5D). The maintenance of

schistosomulae killing upon NK cell depletion suggests that other cellular effectors are involved in protection mediated by unadjuvanted Sm-Cathepsin B immunizations.

4.6 Discussion

Our past vaccine studies using the candidate Sm-Cathepsin B induced promising protection levels against schistosomiasis in mice whether formulated with Montanide ISA 720 VG or CpG dinucleotides adjuvants [19, 20]. Although parasite burden reductions were similar between the different formulations tested, we found that the immune responses elicited were entirely different. Immunizations with recombinant Sm-Cathepsin B in combination with Montanide ISA 720 VG resulted in a mixed Th1/Th2 antigen specific response [20] while immunizations with the antigen formulated in CpG yielded a biased Th1 response [19]. However, both formulations elicited robust Sm-Cathepsin B specific total IgG antibody production pre-challenge. The protective role of antibodies in schistosomiasis has been demonstrated and antibody titers at the time of cercarial challenge are inversely correlated with worm burden [31]. The importance of antibodies is further supported by the demonstration that protection in baboons immunized with radiation attenuated cercariae is proportional to antibody titers [31]. Moreover, several passive transfer studies have shown that both wild type and immunologically deficient animals have decreased parasite burden and pathology when they receive antibodies from chronically infected or immunized wild type animals [32-35]. Therefore, the elevated antigen-specific IgG titers elicited by the previously tested formulations of recombinant Sm-Cathepsin B in the presence of CpG dinucleotides or Montanide [19, 20] (endpoint titers >120,000) may be a crucial factor contributing to the decreased parasite burden observed at the time of perfusion and organ collection. In order to determine whether protection is associated to antibody-dependent effectors, antibody dependent cell mediated cytotoxicity (ADCC) was analyzed in this study.

The schistosomulae are the most vulnerable to an immune attack as they migrate through the lungs, and the level of resistance has an important effect on the establishment of infection [36, 37]. Therefore, we used schistosomulae as targets in order to determine *in vitro* cytotoxicity effects of cells in the presence of antibodies. We found that the highest level of parasite killing was observed when $CD45^+$ lung cells from the Sm-Cathepsin B + Montanide immunized animals were incubated in the presence of immune serum. The requirement of both

lung cells and immune serum from the immunized mice for high parasite killing indicates that the mechanism involved is antibody dependent and cell mediated. Although high parasite killing was also observed with $CD45^+$ lung cells from the Sm-Cathepsin B + CpG mice, this effect was not dependent on the presence of immune serum. All incubation conditions containing lung cells from the Sm-Cathepsin B + CpG vaccinated mice (cells alone, cells + pre-immune serum, and cells + immune serum) led to parasite killing levels that were significantly higher than those observed with cells from the control groups. These observations suggest that the mechanism involved in protection of these mice is cell dependent but antibody independent. This significant difference between the two Sm-Cathepsin B formulations is likely linked to the IgG subclasses involved. Although both formulations elicited robust antigen-specific IgG titers, IgG1 was the dominant subclass present in animals immunized with Sm-Cathepsin B + Montanide, whereas IgG2c was the dominant subclass in animals that received the CpG adjuvanted formulation [19, 20].

ADCC is a known effector function of IgG1 antibodies as they are efficiently bound by Fc gamma receptors (FcɣRs) on effector cells [38]. On the other hand, IgG2c antibodies are not associated with ADCC properties. The antibody independent cellular effect observed in the Sm-Cathepsin B + CpG immunized animals was not unexpected. CpG dinucleotides have been shown to enhance both innate and adaptive cellular responses [39]. Activation of TLR9 by CpG dinucleotides stimulates the migration of plasmacytoid dendritic cells to T cell zones of lymphoid organs where they upregulate the expression of co-stimulatory molecules and promote strong Th1 CD4 T cell and cytotoxic T lymphocyte (CTL) responses [39].

Different cell populations were depleted in order to decipher the main effectors mediating protection in vaccinated animals. The high larvicidal effect observed with the incubation of lung cells and immune serum from the Sm-Cathepsin B + Montanide group was lost upon the depletion of NK cells. As previously mentioned, the necessity of immune serum for the development of high larvicidal activity suggested an ADCC mechanism. Unlike other hematopoietic cells, NK cells do not express inhibitory FcɣRs; therefore, possessing only their activating FcɣRIIIa, they are free to act as key mediators of ADCC [40]. NK cells meditate ADCC by the exocytosis of cytotoxic granules containing perforin and granzyme, or by the release of pro-inflammatory cytokines. The cytotoxic molecules cause direct damage to the

target whereas pro-inflammatory cytokines activate nearby immune cells and promote dendritic cell maturation as well as antigen presentation [41]. Based on the observations from the *in vitro* schistosomulae killing assays, ADCC mediated by NK cells may be the mechanism of protection elicited by Sm-Cathepsin B + Montanide immunizations.

The *in vitro* parasite killing observed with cells taken from the Sm-Cathepsin B + Montanide group was also lost upon the depletion of $CD4^+$ T cells. Activated $CD4^+$ T cells are an important source of IL-2 which potentiates robust activation of NK cells [42]. Activation by IL-2 results in NK cell proliferation, secretion of effector molecules, and enhancement of cytotoxic function [42]. NK cell activation via $CD4^+$ T cell-derived IL-2 may be necessary for schistosomulae killing. The importance of $CD4^+$ T cell presence for NK cell mediated mechanisms has been demonstrated for other parasitic infections. NK cell responses targeting *Leishmania major* require IL-2 from primed antigen-specific $CD4^+$ T cells [43]. Similarly, NK cell targeting of *Plasmodium falciparum* infected red blood cells is dependent on IL-2 from antigen-specific $CD4^+$ T cells [44]. The data from this present study suggests that protection induced by Sm-Cathepsin B + Montanide immunizations involves the killing of schistosomulae via ADCC mediated by NK cells that are activated by $CD4^+$ T cells.

For the Sm-Cathepsin B + CpG group, the high larvicidal effect was entirely abolished upon the depletion of $CD8^+$ T cells, suggesting a main protective effector role for this cell population. As previously mentioned, CpG dinucleotides are known to promote cellular responses such as those including CTLs. CTLs mediate killing of extracellular pathogens by direct recognition, and this function is most often associated with $CD8^+$ cells [45]. Granulysin has been described as the major effector molecule mediating CTL activity as it inserts into the target membrane and disrupts its permeability [46]. CTLs can directly kill *S. mansoni* schistosomulae in a contact dependent manner [47] and can target extracellular *Toxoplasma gondii* in an antigen-specific manner [48]. The data from this study suggests that $CD8^+$ T cells are the main effectors mediating protection elicited by Sm-Cathepsin B + CpG immunizations. The increased larvicidal activity observed with the Sm-Cathepsin B + CpG group was also significantly decreased upon the depletion of NK cells. However, the parasite killing observed with this experimental group was still higher than the control groups. Therefore, although $CD8^+$ T cells could be the main effectors mediating protection, the data suggests that NK cells play an

important supportive role in the network of coordinated immune cells. NK cells are important in schistosomiasis progression and protection, and animal models have shown that NK cells activated during schistosome infection negatively regulate granuloma size as well as liver fibrosis [49-51]. Furthermore, field studies in schistosomiasis endemic regions have shown that NK cells are linked to schistosomiasis protection in elderly individuals in Brazil [52], while in Sudan, impaired NK cell activity showed a direct relationship with patients' parasite loads [53].

The importance of NK cells has also been investigated in the context of immunizations against schistosomiasis. It has been demonstrated that protective immune responses elicited by the immunization of mice with lung stage larval antigens + IL-12 depends upon the presence of NK cells [54]. In the presented study, larvicidal activity with cells from the Sm-Cathepsin B + CpG group was significantly impaired by the depletion of NK cells. In both cases, the vaccine formulations elicited strong Th1 cellular responses [19, 54]. NK cells likely represent the major source of early IFN-ɣ production. NK cell derived IFN-ɣ is believed to regulate IL-12 expression in the context of schistosomiasis as the induction of this cytokine is completely blocked in animals depleted of either IFN-ɣ or NK cells [49]. Since IL-12 is a potent stimulator of $CD8^+$ cells, we suggest that early NK derived IFN-ɣ triggers IL-12 expression, and together these cytokines prompt $CD8^+$ CTL activity targeting schistosomulae.

It has been shown that Sm-Cathepsin B has inbuilt adjuvant properties, and that immunizations with antigen alone are capable of significantly reducing parasite burden [18]. However, these immunizations elicit low levels of antigen-specific total IgG (< 3,500) [18]. In the mouse model, the formulation of Sm-Cathepsin B alone stimulates a Th2 biased immune response characterized by increased secretion levels of IL-4, IL-5, and IL-13, but no detectable levels of antigen-specific IgE [18]. In the present study, it was demonstrated that incubation of schistosomulae with $CD45^+$ lung cells from Sm-Cathepsin B immunized animals resulted in parasite killing levels that were significantly higher than those observed for the saline and CpG controls, but still lower than the Sm-Cathepsin B + CpG group. The larvicidal effect was independent of the presence of immune serum. These results suggest that immunizations with unadjuvanted Sm-Cathepsin B stimulate a cellular response capable of targeting the parasite larva. Depletion of $CD4^+$ T cells abolished the larvicidal effect observed with the unadjuvanted Sm-Cathepsin B group. This observation suggests that $CD4^+$ T cells are important effectors

mediating protection elicited by unadjuvanted Sm-Cathepsin B immunizations. In their study, El Ridi and colleagues suggested that immunizations with unadjuvanted Sm-Cathepsin B boost early adaptive immune responses with $CD4^+$ T cell help [18]. $CD4^+$ T cells could potentially be the major source contributing to the observed increase in Th2 cytokines [18]. The same research group also suggested that schistosomulae will succumb if met by a type 2 cytokine environment [55]. One mechanism which they proposed involved the recruitment of eosinophils as well as basophils to the lungs, and their activation by type 2 cytokines [55]. The presented study yielded an unexpected result upon the depletion of macrophages ($F4/80^+$ cells). Depleting macrophages from the unadjuvanted Sm-Cathepsin B group led to a significant increase in schistosomulae killing. This observation may indicate a function for macrophages in limiting effector cell mediated parasite killing, or it may simply be a result of a significant change in cell population proportions in our *in vitro* assay. Interestingly, the inteplay between alternatively activated macrophages and Th2 $CD4^+$ T cells has been evaluated in the context of schistosomiasis. The Th2 cytokines IL-4 and IL-13 are known to initiate the alternative activation of macrophages. Arginase 1, a key marker of alternatively activated macrophages, converts L-arginine into L-ornithine and urea. Macrophages expressing this enzyme can successfully compete with Th2 $CD4^+$ T cells for arginine; thus, limiting T cell proliferation [56, 57].

In the presented study, it was shown that three different formulations containing the same antigen elicit different protection mechanisms in a mouse model of schistosomiasis. Although the formulations generated similar protection levels, the immune responses they stimulated were different, and this is reflected in the diverse effectors that mediate protection in the immunized animals. Understanding the underlying mechanisms of vaccine induced protection will allow for the selection of a formulation that can stimulate the most optimal immune response.

4.7 Acknowledgements

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4.9 Table

Table 4.1 Depletion of cell populations in Sm-Cathepsin B + Montanide, Sm-Cathepsin B +CpG, and Sm-Cathepsin B groups

	Sm-Cathepsin B + Montanide		Sm-Cathepsin B +CpG		Sm-Cathepsin B	
	Original Sample	**Depleted Sample**	**Original Sample**	**Depleted Sample**	**Original Sample**	**Depleted Sample**
$CD4^+$	12.70%	1.48%	19.50%	0.89%	16.80%	0.94%
$CD8^+$	9.83%	1.38%	21.10%	2.96%	13.50%	1.37%
$F4/80^+$	74.60%	1.97%	60.90%	3.95%	59.50%	2.00%
$CD49^+$	4.65%	0.58%	5.72%	0.31%	6.18%	0.37%

4.10 Figures and legends

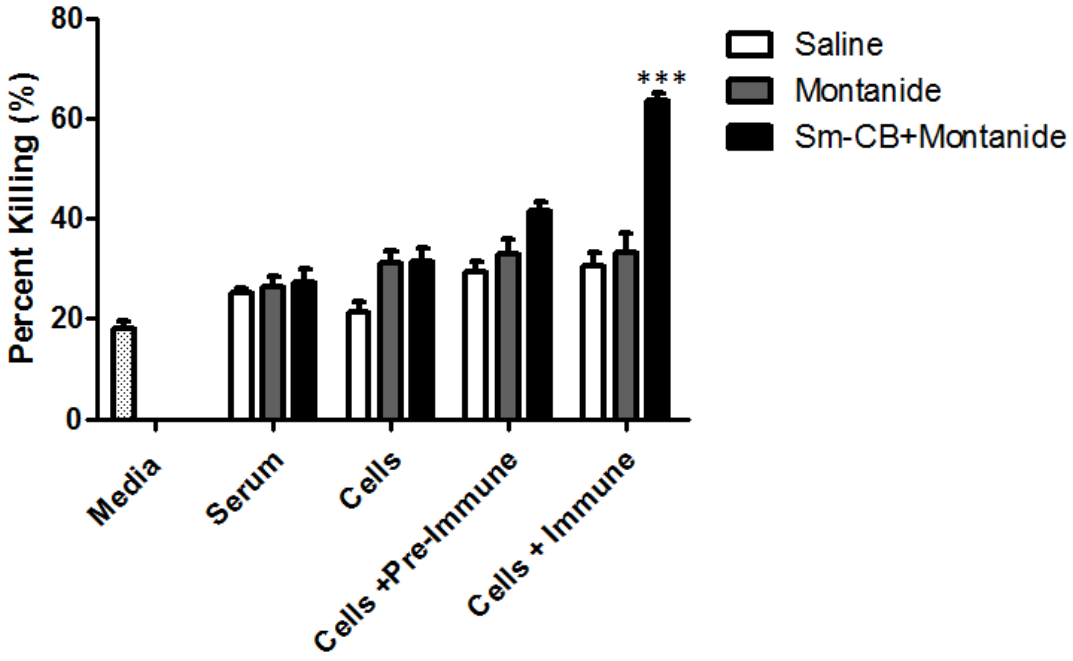


Figure 4.1 Schistosomulae death with Sm-Cathepsin B + Montanide.

Sixty mechanically transformed schistosomulae were incubated for 24 hours at 37 ºC, 5% CO_2 in the presence of media, serum, cells, cells + pre-immune serum, or cells + immune serum. $CD45^+$ lung cells and serum were collected from the different immunized mouse groups: saline, Montanide ISA 720 VG, and Sm-Cathepsin B + Montanide ISA 720 VG. Percent parasite killing was determined for all groups and conditions. n=14 for all groups. ***: $p \leq 0.001$

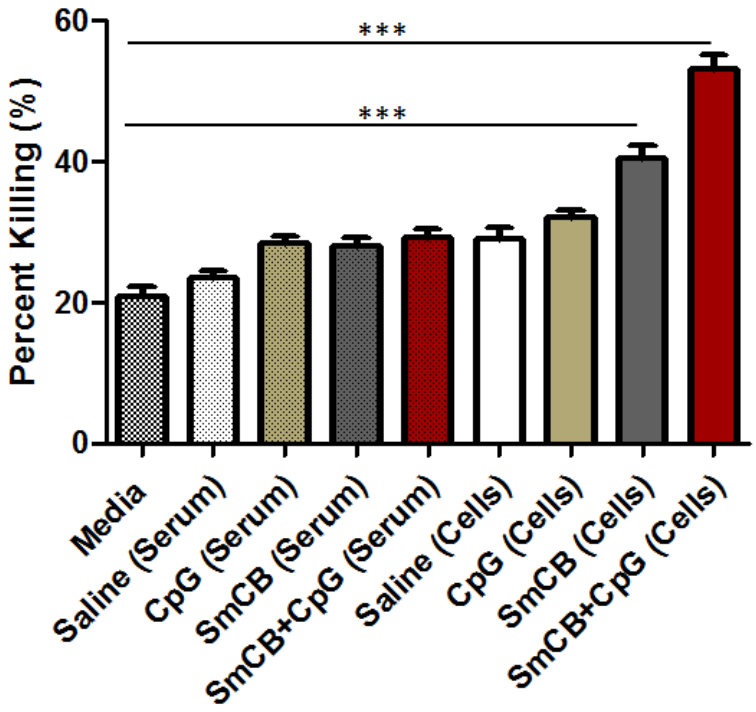
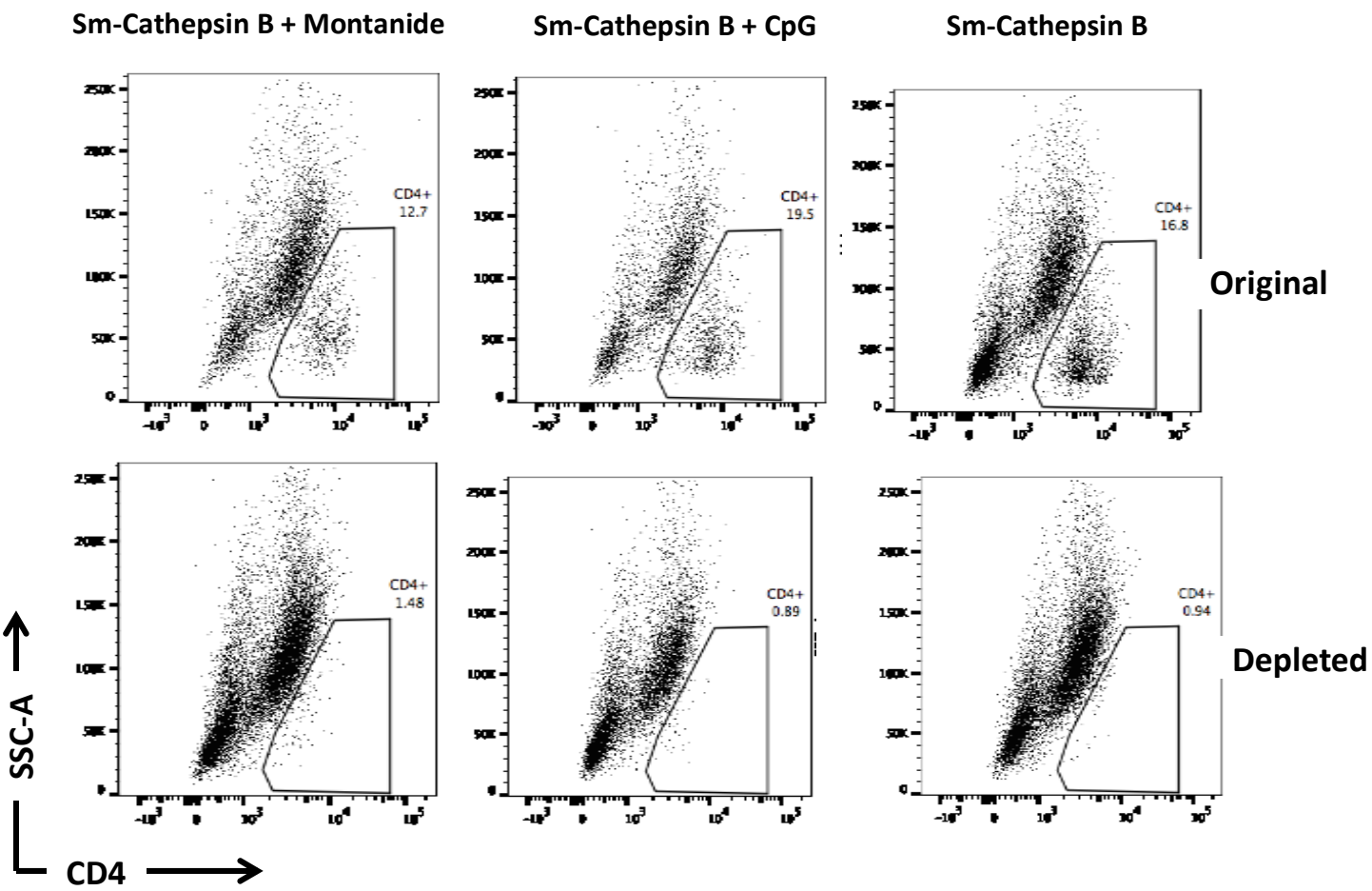


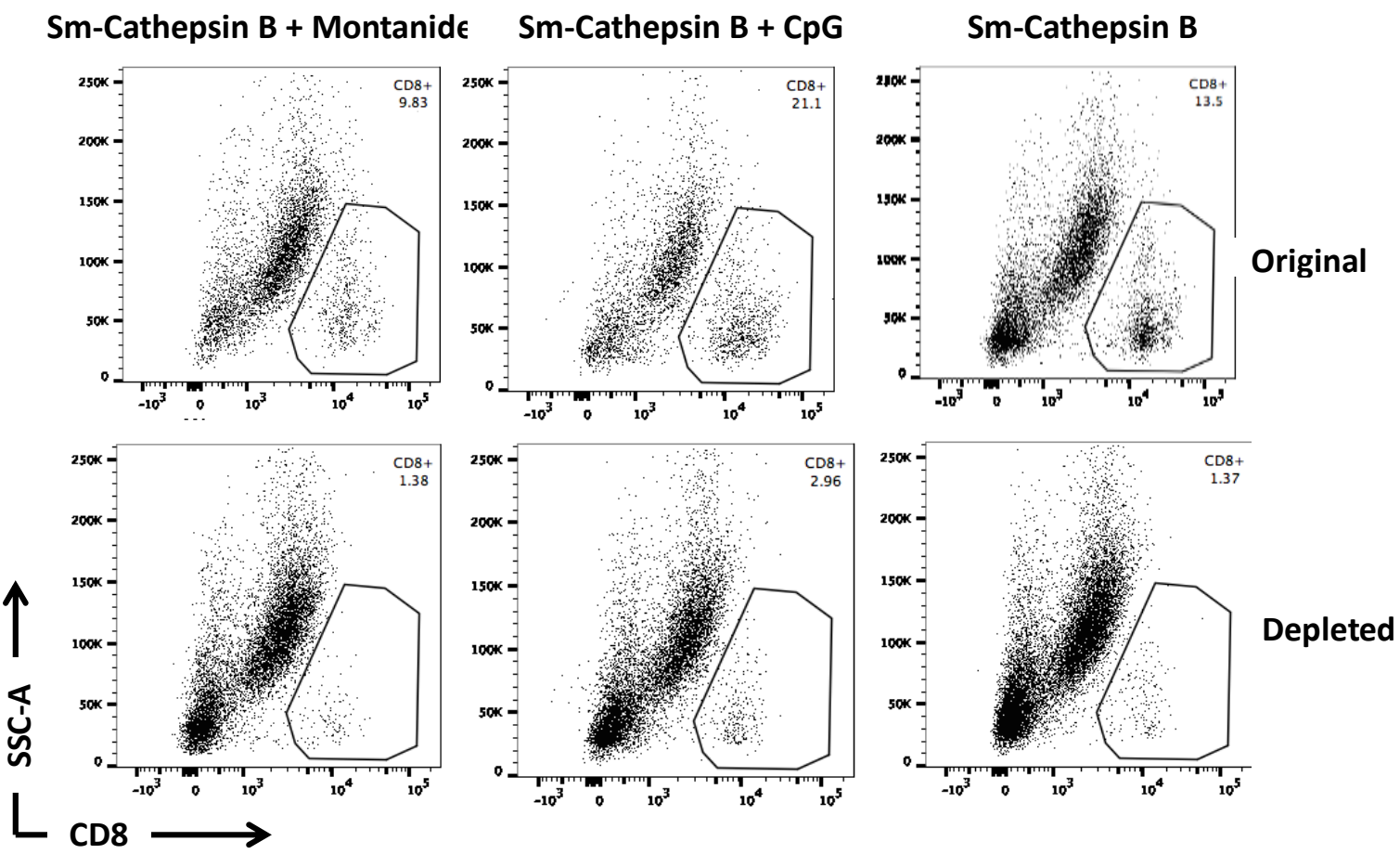
Figure 4.2 Schistosomulae death with Sm-Cathepsin B alone & Sm-Cathepsin B + CpG.

Sixty mechanically transformed schistosomulae were incubated for 24 hours at 37 ºC, 5% CO_2 under different conditions which included: media, serum, and cells. $CD45^+$ lung cells and serum were collected from the different immunized mouse groups: saline, CpG dinucleotides, Sm-Cathepsin B alone, and Sm-Cathepsin B + CpG. Percent parasite killing was determined for all groups and conditions. n=14 for all groups. ***: $p \leq 0.001$

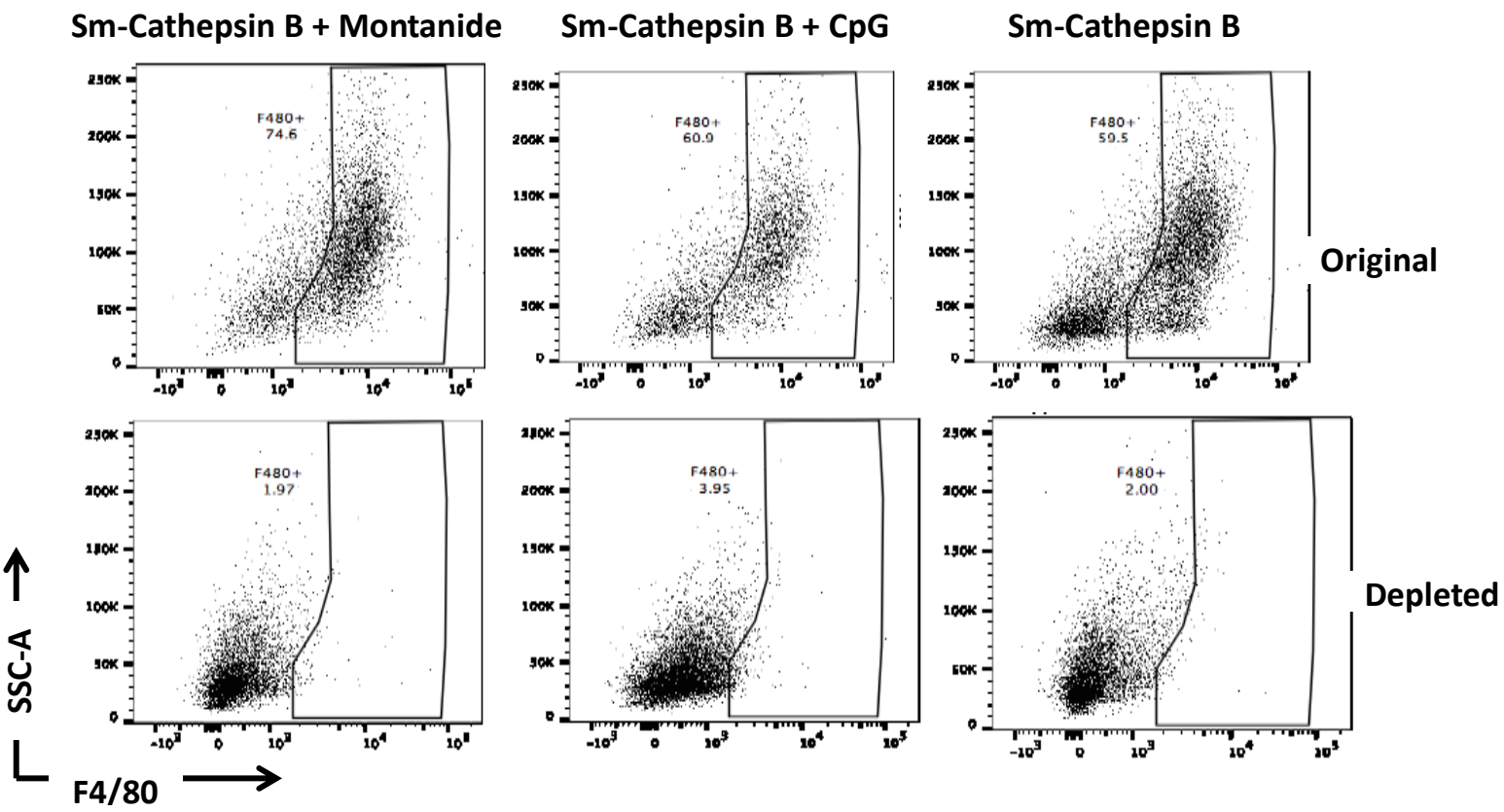
A


Sm-Cathepsin B + Montanide
Sm-Cathepsin B + CpG
Sm-Cathepsin B
CD4+ 12.7
CD4+ 19.5
CD4+ 16.8
Original
CD4+ 1.48
CD4+ 0.89
CD4+ 0.94
Depleted
SSC-A
CD4

B


Sm-Cathepsin B + Montanide
Sm-Cathepsin B + CpG
Sm-Cathepsin B
CD8+ 9.83
CD8+ 21.1
CD8+ 13.5
Original
CD8+ 1.38
CD8+ 2.96
CD8+ 1.37
Depleted
SSC-A
CD8

C


Sm-Cathepsin B + Montanide
Sm-Cathepsin B + CpG
Sm-Cathepsin B
F480+
74.6
F480+
60.9
F480+
59.5
Original
F480+
1.97
F480+
3.95
F480+
2.00
Depleted
SSC-A
F4/80

D

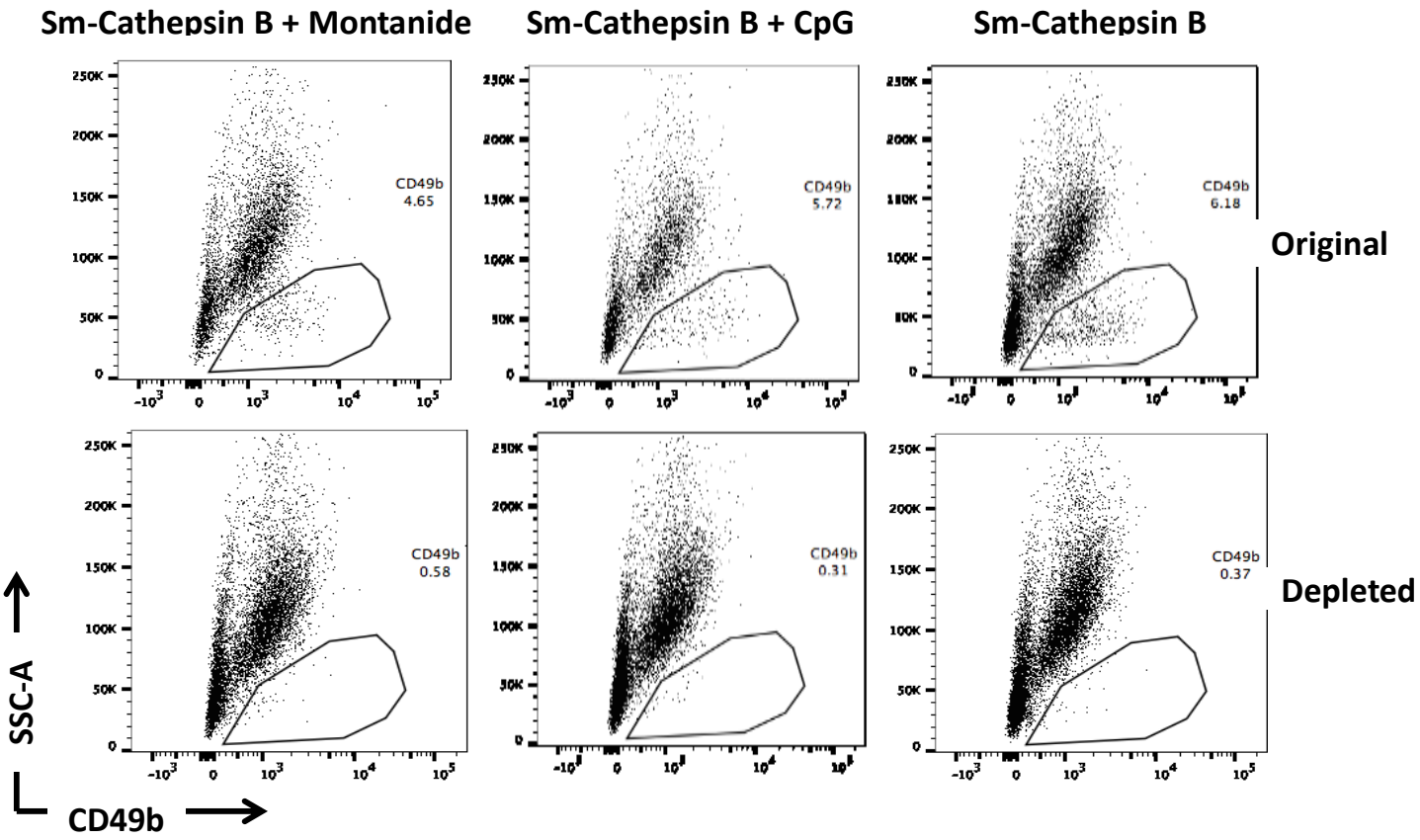
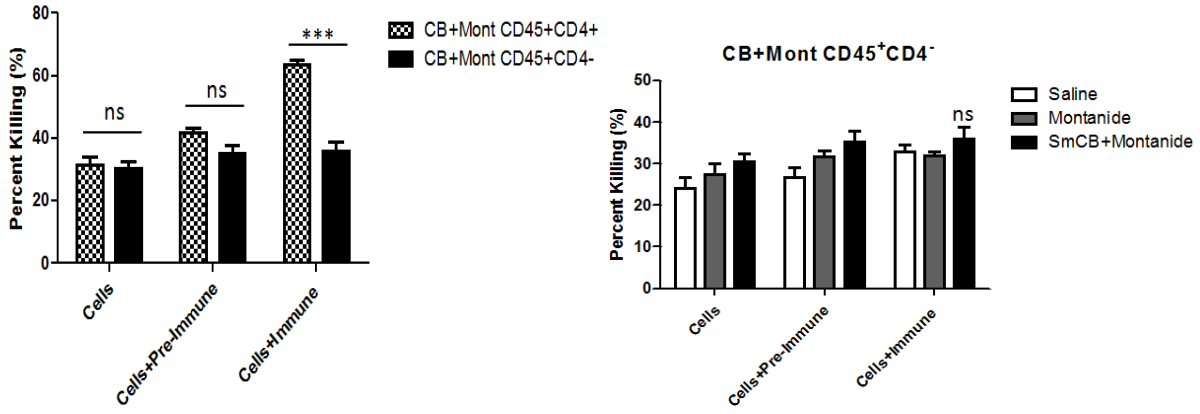


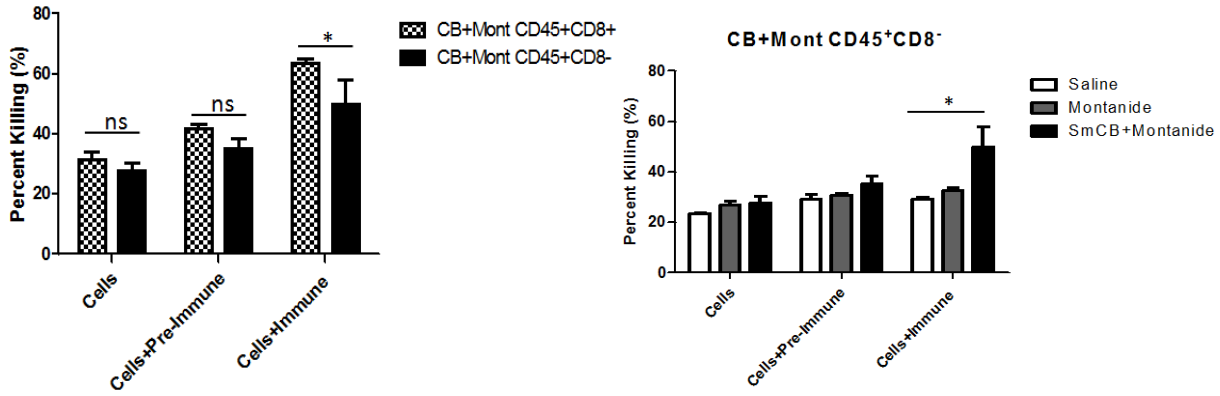
Figure 4.3 Cell population depletion confirmations by flow cytometry.

Lung cell suspensions from the different immunization groups underwent specific depletions followed by $CD45^+$ purification. The depletions were confirmed by flow cytometry. The gates were set to select live $CD45^+$ cells. The top panels represent the original undepleted CD45 purified lung cells taken from Sm-Cathepsin B + Montanide, Sm-Cathepsn B + CpG, and Sm-Cathepsin B immunized mice. The bottom panels represent the lung cell samples from the same immunization groups after the depletions. The depletions were performed and confirmed for **(A)** $CD4^+$, **(B)** $CD8^+$, **(C)** $F4/80^+$, and **(D)** $CD49b^+$ cell populations.

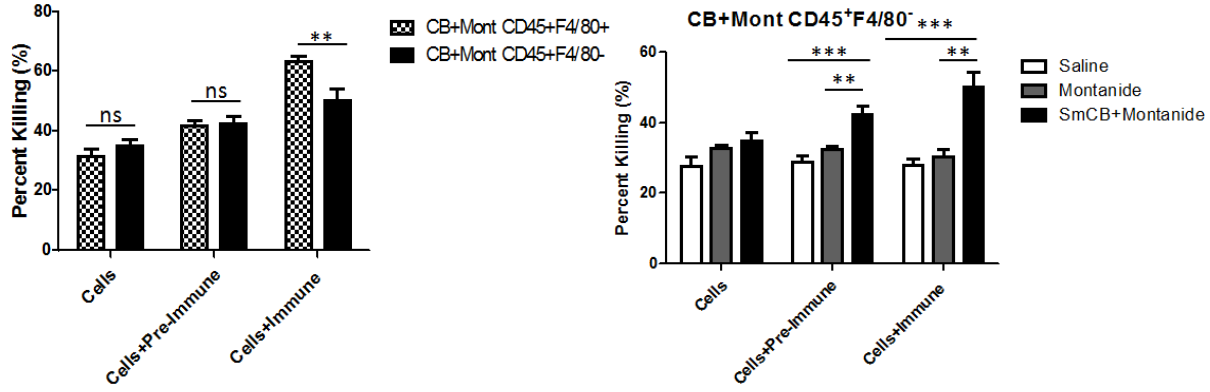
A)


Percent Killing (%)
CB+Mont CD45+CD4+
CB+Mont CD45+CD4-
ns
ns
***
Cells
Cells+Pre-Immune
Cells+Immune
CB+Mont CD45+CD4-
Saline
Montanide
SmCB+Montanide
ns

B)


Percent Killing (%)
CB+Mont CD45+CD8+
CB+Mont CD45+CD8-
ns
ns
*
Cells
Cells+Pre-Immune
Cells+Immune
CB+Mont CD45+CD8-
Saline
Montanide
SmCB+Montanide
*

C)


Percent Killing (%)
CB+Mont CD45+F4/80+
CB+Mont CD45+F4/80-
ns
ns
**
Cells
Cells+Pre-Immune
Cells+Immune
CB+Mont CD45+F4/80-
***
***
**
**
Saline
Montanide
SmCB+Montanide

D)

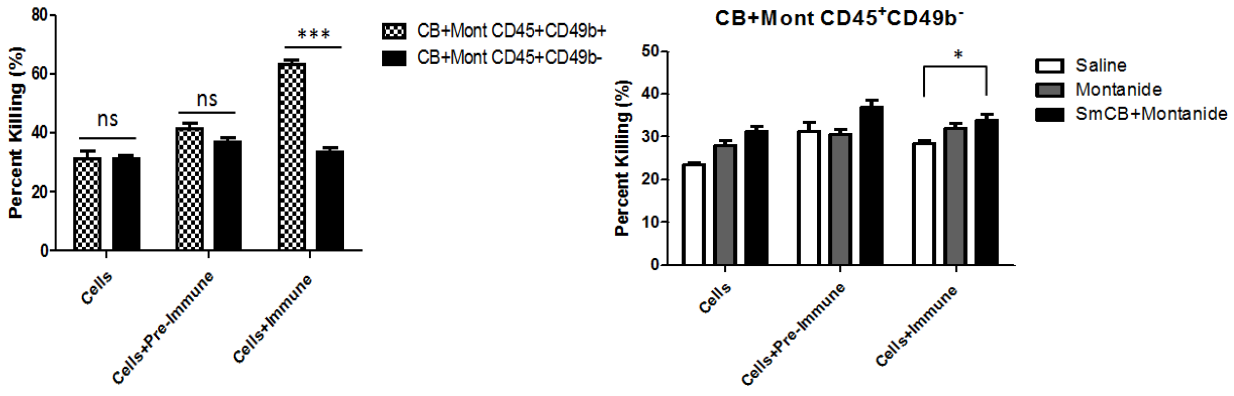
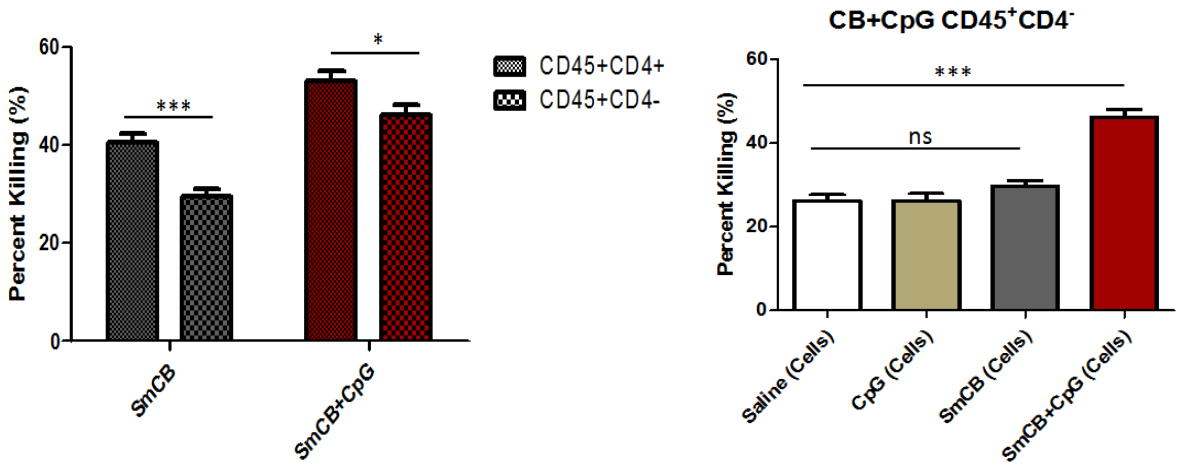


Figure 4.4 Sm-Cathepsin B + Montanide: Parasite killing after the depletion of different cell populations.

Sixty mechanically transformed schistosomulae were incubated 24 hours at 37 ºC, 5% CO_2 under different conditions which included: cells, cells + pre-immune serum, and cells + immune serum. Lung cells and serum were collected from the different immunized mouse groups: saline, Montanide ISA 720 VG, and Sm-Cathepsin B + Montanide ISA 720 VG. The different immune cell depletions were as follows: **(A)** parasite killing was compared between Sm-Cathepsin B + Montanide $CD45^+CD4^-$ and Sm-Cathepsin B + Montanide $CD45^+CD4^+$; n=14 for all groups. Parasite killing was also analyzed for the control groups and the experimental group after CD4 cell depletion; Saline n=10, Montanide n=10, Sm-Cathepsin B + Montanide n=14. **(B)** parasite killing was compared between Sm-Cathepsin B + Montanide $CD45^+CD8^-$ and Sm-Cathepsin B + Montanide $CD45^+CD8^+$; $CD45^+CD8^-$ n=5, $CD45^+CD8^+$ n=14. Parasite killing was also analyzed for the control groups and the experimental group after CD8 cell depletion; n=5 for all groups. **(C)** parasite killing was compared between Sm-Cathepsin B + Montanide $CD45^+F4/80^-$ and Sm-Cathepsin B + Montanide $CD45^+F4/80^+$; $CD45^+F4/80^-$ n=11, $CD45^+F4/80^+$ n=14. Parasite killing was also analyzed for the control groups and the experimental group after F4/80 cell depletion; Saline n=7, Montanide n=7, Sm-Cathepsin B + Montanide n=11. **(D)** parasite killing was compared between Sm-Cathepsin B + Montanide $CD45^+CD49b^-$ and Sm-Cathepsin B + Montanide $CD45^+CD49b^+$; $CD45^+CD49b^-$ n=11, $CD45^+CD49b^+$ n=14. Parasite killing was also analyzed for the control groups and the experimental group after CD49b cell depletion; Saline n=7, Montanide n=7, Sm-Cathepsin B + Montanide n=11. ns: $p > 0.05$, *: $p \leq 0.05$, **: $p \leq 0.01$, ***: $p \leq 0.001$.

A)


Percent Killing (%)
60
40
20
0
***
*
SmCB
SmCB+CpG
CD45+CD4+
CD45+CD4-
CB+CpG CD45+CD4-
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Saline (Cells)
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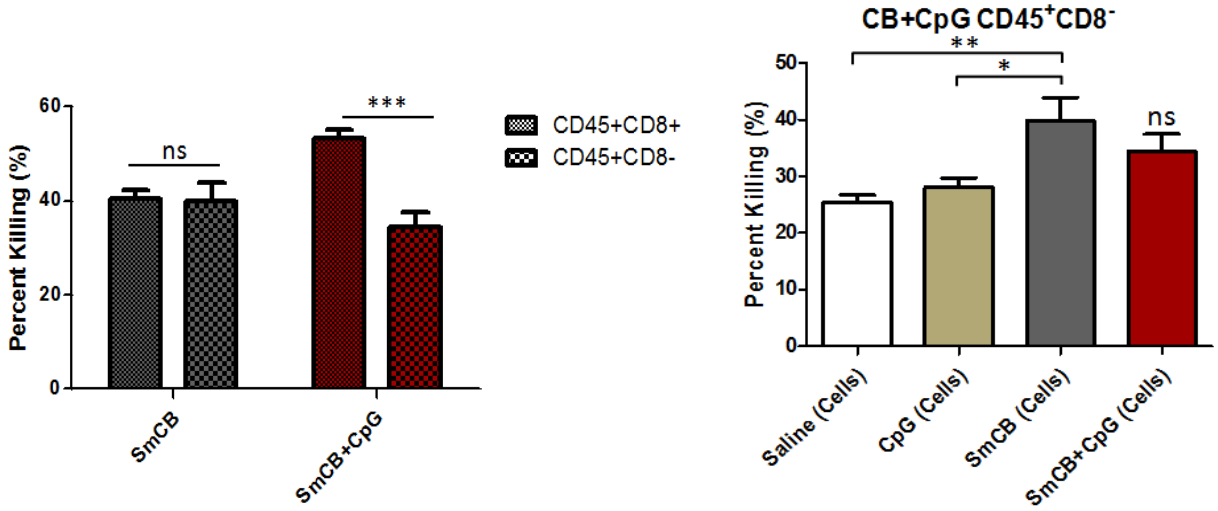
SmCB (Cells)
SmCB+CpG (Cells)

B)


Percent Killing (%)
60
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SmCB
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Saline (Cells)
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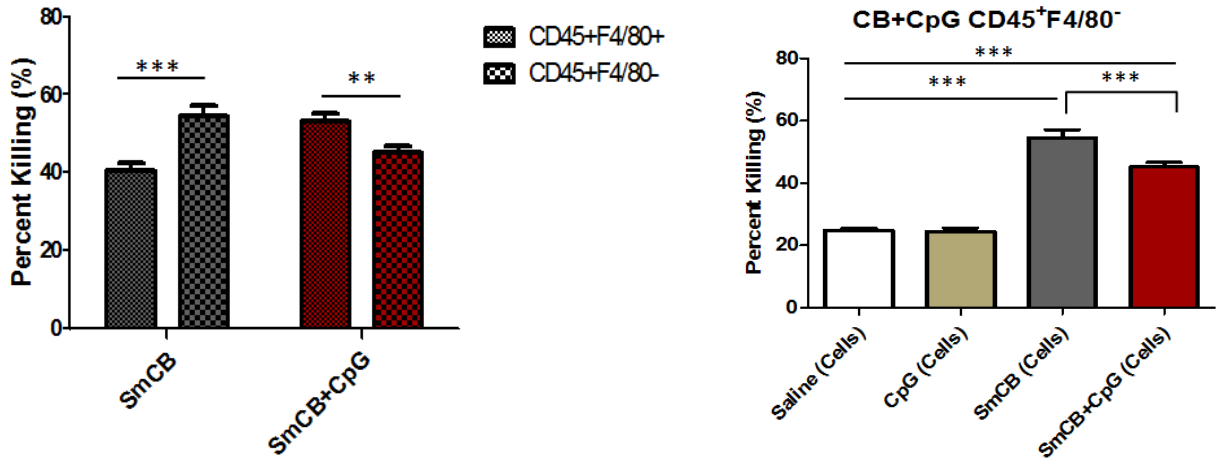
SmCB (Cells)
SmCB+CpG (Cells)

C)


Percent Killing (%)
80
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40
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SmCB
SmCB+CpG
CD45+F4/80+
CD45+F4/80-
CB+CpG CD45+F4/80-
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Saline (Cells)
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SmCB (Cells)
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D)

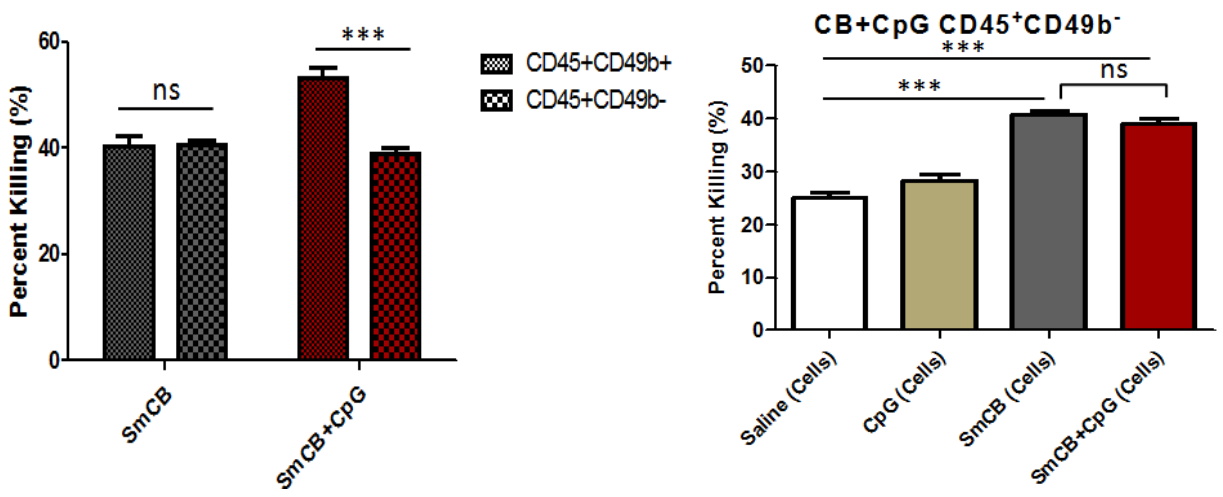
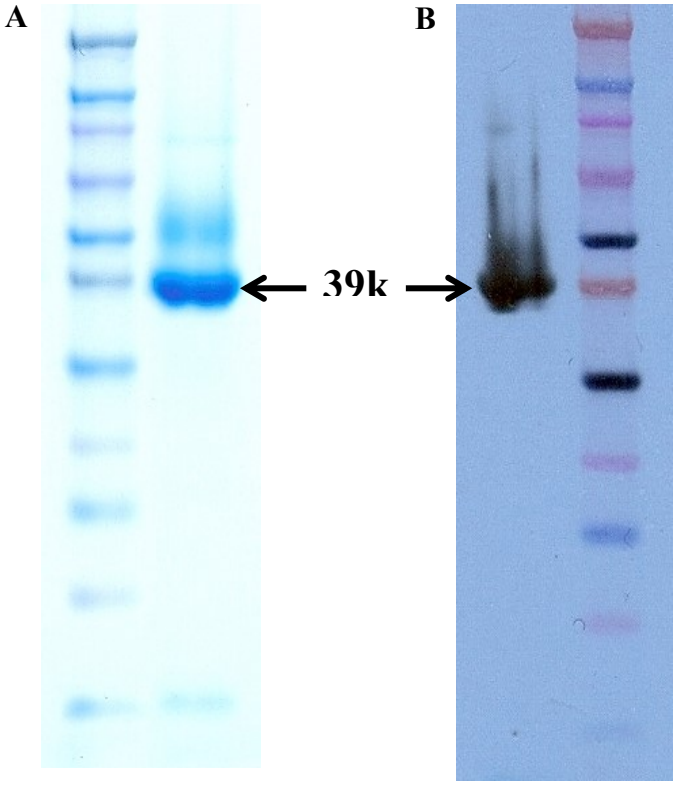


Figure 4.5 Sm-Cathepsin B alone & Sm-Cathepsin B + CpG: Parasite killing after the depletion of different cell populations.

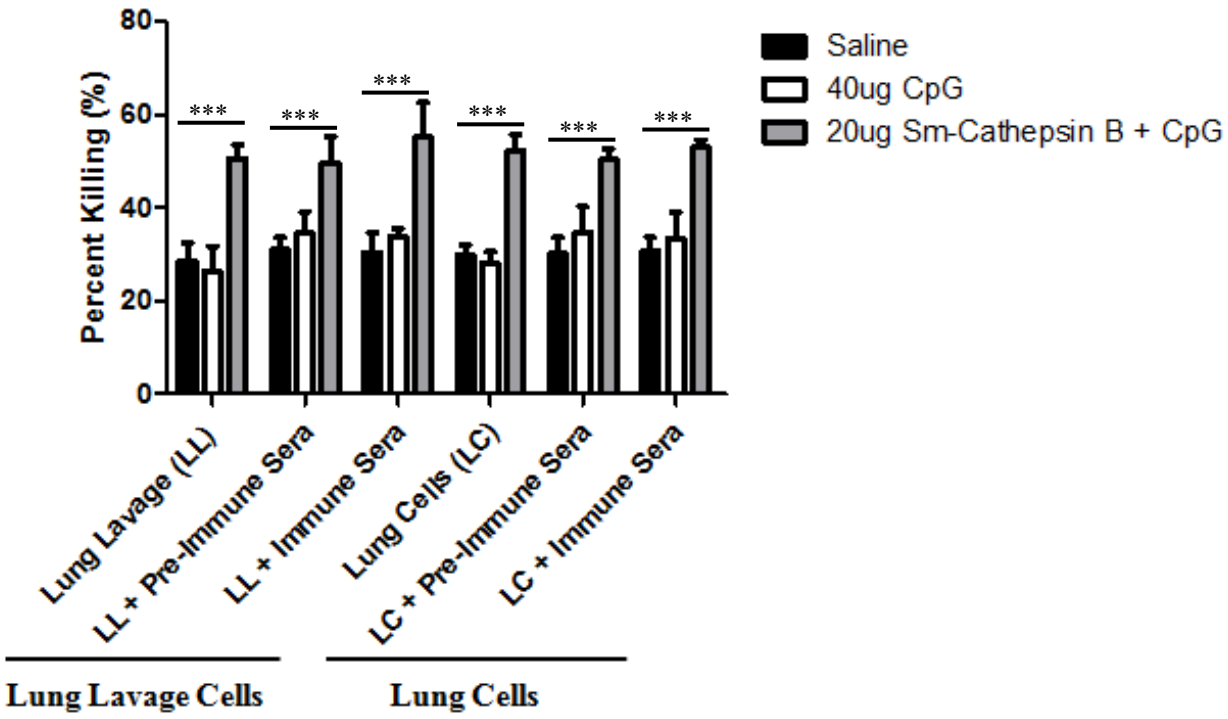
Sixty mechanically transformed schistosomulae were incubated 24 hours at 37 ºC, 5% CO_2. Lung cells were collected from the different immunized mouse groups: saline, CpG dinucleotides, Sm-Cathepsin B, and Sm-Cathepsin B + CpG. The different depletions and comparisons were as follows: **(A)** parasite killing was compared between $CD45^+CD4^-$ and $CD45^+CD4^+$ groups; $CD45^+CD4^+$ groups n=14, Sm-Cathepsin B + CpG $CD45^+CD4^-$ n=14, Sm-Cathepsin B $CD45^+CD4^-$ n=10. Parasite killing was also analyzed for all groups after CD4 cell depletion; Saline n=10, CpG. n=10, Sm-Cathepsin B n=10, Sm-Cathepsin B + CpG n=14. **(B)** parasite killing was compared between $CD45^+CD8^-$ and $CD45^+CD8^+$ groups; $CD45^+CD8^-$ n=5, $CD45^+CD8^+$ n=14. Parasite killing was also analyzed for all groups after CD8 cell depletion; n=5 for all groups. **(C)** parasite killing was compared between $CD45^+F4/80^-$ and $CD45^+F4/80^+$ groups; $CD45^+F4/80^+$ n=14, Sm-Cathepsin B + CpG $CD45^+F4/80^-$ n=11, Sm-Cathepsin B $CD45^+F4/80^-$ n=7. Parasite killing was also analyzed for all groups after F4/80 cell depletion; Saline n=7, CpG n=7, Sm-Cathepsin B n=7, Sm-Cathepsin B + CpG n=11. **(D)** parasite killing was compared between $CD45^+CD49b^-$ and $CD45^+CD49b^+$ groups; $CD45^+CD49b^+$ n=14, Sm-Cathepsin B + CpG $CD45^+CD49b^-$ n=11, Sm-Cathepsin B $CD45^+CD49b^-$ n=7. Parasite killing was also analyzed for all groups after CD49b cell depletion; Saline n=7, CpG n=7, Sm-Cathepsin B n=7, Sm-Cathepsin B + CpG n=11. ns: not significant, $p>0.05$, *: $p\leq 0.05$, **: $p\leq 0.01$, ***: $p\leq 0.001$.

4.11 Supplemental figures



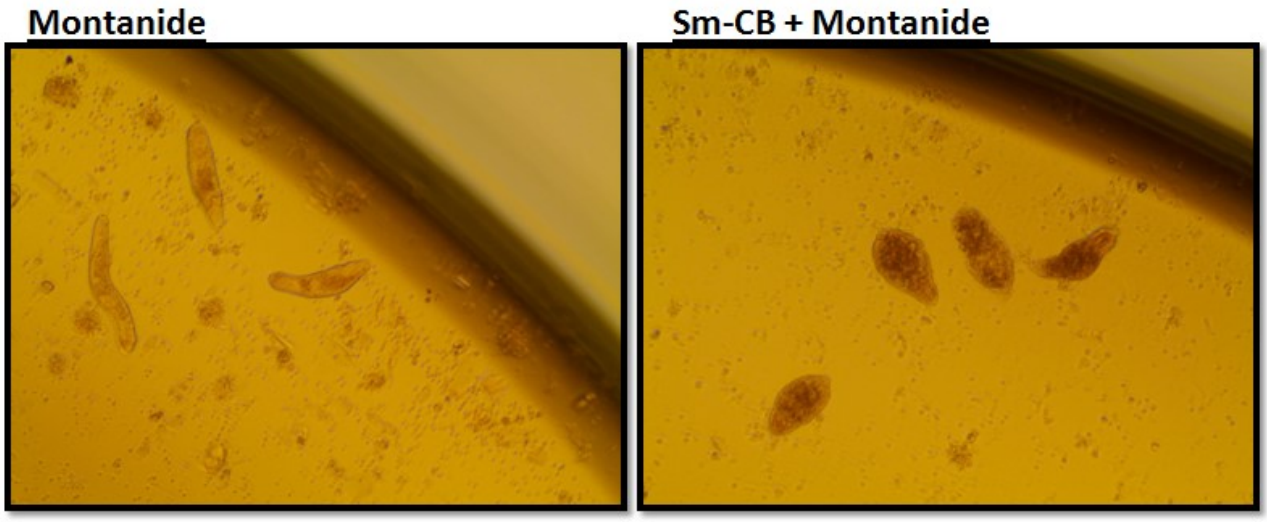
Supplemental Figure 4.1 Expression of recombinant Sm-Cathepsin B

Recombinant Sm-Cathepsin B was expressed using the PichiaPink^{TM} expression system and purified by Ni-NTA chromatography. Protein expression was analyzed by Coomassie blue staining of polyacrylamide gel **(A)** and western blot **(B)**. The expected band at 39kDa can be observed.



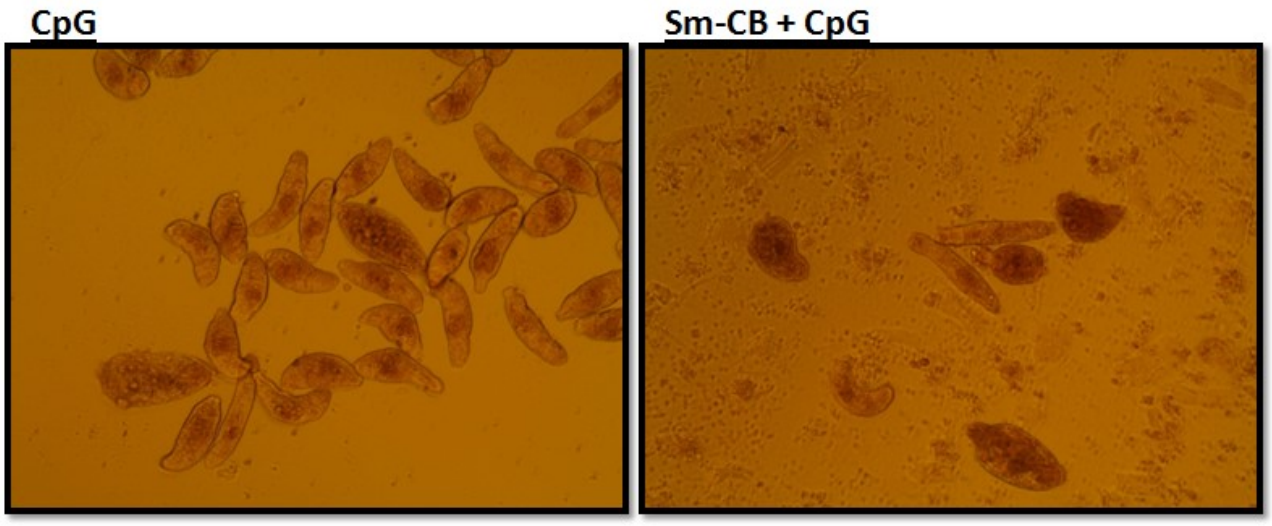
Supplemental Figure 4.2 Schistosomulae death caused by lung lavage/lung cells taken from mice vaccinated with Sm-Cathepsin B + CpG

Schistosomulae were incubated for 24 hours at 37 ºC, 5% CO_2 with serum and cells taken from mice immunized with saline alone, CpG alone, or Sm-Cathepsin B + CpG. Incubations with lung lavage cells were compared to those with whole lung cells. Significant parasite killing is observed with cells taken from the Sm-Cathepsin B + CpG immunized mice, compared to the saline and adjuvant control group mice, in the presence or absence of serum and with lung lavage as well as whole lung cells. n = 5. Statistical analysis was performed by 2-way analysis of variance. ***: $p \leq 0.001$.



Supplemental Figure 4.3 Microscopic examination of schistosomulae death caused by serum and lung cells taken from mice vaccinated with Sm-Cathepsin B + Montanide.

Schistosomulae were incubated for 24 hours at 37 ºC, 5% CO_2 with cells and serum from mice immunized either with Montanide ISA 720 VG or Sm-Cathepsin B + Montanide ISA 720 VG. Parasite viability was determined by microscopic examination of motility, granularity, and shape integrity.



Supplemental Figure 4.4 Microscopic examination of schistosomulae death caused by lung cells taken from mice vaccinated with Sm-Cathepsin B + CpG.

Schistosomulae were incubated for 24 hours at 37 ºC, 5% CO_2 with cells and serum from mice immunized either with CpG or Sm-Cathepsin B + CpG. Parasite viability was determined by microscopic examination of motility, granularity, and shape integrity.

Chapter 5: General Discussion

The numbers defining the current status of human schistosomiasis are still worrisome. Although mass drug administration programs using PZQ have succeeded in dramatically reducing morbidity, they have failed in disrupting disease transmission [1]. In the last twenty years, there has been a push towards schistosomiasis vaccine development. Whether they would be used alone or in combination with chemotherapy or other control measures, vaccines are the missing tool needed to achieve schistosomiasis elimination (schistosomiasis vaccines are reviewed in Chapter 1 section 5). Advancements in molecular technologies as well as the publication of the schistosome genomes have revolutionized and accelerated vaccine research. We have chosen to focus our work on the *S. mansoni* vaccine candidate Cathepsin B. Sm-Cathepsin B is the major cysteine peptidase in the parasite gut. It is essential for nutrient acquisition and proper worm development [2, 3]. The central goal of this thesis work was to evaluate the protective potential of vaccine formulations containing Sm-Cathepsin B. Furthermore, this thesis highlights the importance of understanding the type of immune responses elicited by these different immunizations.

5.1 Main findings

A vaccine candidate is considered to be promising if it consistently elicits protection levels of at least 40% [4]. When determining protection, we considered all forms of parasitological burden: worm, hepatic egg, and intestinal egg numbers. Our collaborator, Dr. John P. Dalton, has demonstrated that recombinant Sm-Cathepsin B possesses inbuilt adjuvant properties [5]. In a mouse challenge model of schistosomiasis, Sm-Cathepsin B immunizations significantly reduced worm and hepatic egg numbers, but had no effect on intestinal egg burden [5]. Furthermore, the immunizations elicited a biased Th2 response [5]. Using a mouse challenge model of schistosomiasis, we investigated the formulation of recombinant Sm-Cathepsin B with CpG dinucleotides. This allowed us to investigate whether the addition of a Th1-biasing adjuvant could increase protection levels and overturn the antigen-driven Th2 response. This formulation significantly reduced all forms of parasitological burden, with protection levels ranging from 54%-59% [6]. The immunizations elicited robust antigen-specific IgG titers as well as a dominant antigen-specific Th1 response. This work was

published in the January 2015 issue of Vaccine and is thoroughly discussed in Chapter 2 of this thesis. Next, we wanted to determine whether the use of a less immune-biasing adjuvant would affect the protective potential of Sm-Cathepsin B. Therefore, we evaluated a new formulation containing the squalene based adjuvant Montanide ISA 720 VG. This adjuvant forms water-in-oil droplets allowing slow antigen release at the injection site. In a mouse model of schistosomiasis, this formulation conferred significant levels of protection, ranging from 56%-62%, when observing all forms of parasitological burden [7]. This formulation containing Sm-Cathepsin B and Montanide ISA 720 VG elicited robust antigen-specific IgG titers and a mixed Th1/Th2 response. This work was published in the March 2016 issue of BMC Infectious Diseases and is detailed in Chapter 3 of this thesis. Building upon these experiments, we wanted to investigate the immune mechanisms mediating vaccine induced protection. The two Sm-Cathepsin B formulations discussed in this thesis conferred similar protection levels in immunized mice. However, they stimulated different immune responses (biased Th1 and mixed Th1/Th2). Additionally, our collaborator demonstrated that a formulation of Sm-Cathepsin B without an adjuvant possesses protective potential and stimulates a biased Th2 response. This suggested that the mechanisms involved in vaccine mediated protection differ between formulations. To further investigate these mechanisms, we performed *in vitro* killing assays using the lung stage parasite as a target. This work is described in Chapter 4 as a manuscript that has been prepared for submission to PLoS Neglected Tropical Diseases. Here, we suggested that protection induced by Sm-Cathepsin B + Montanide immunizations involves the killing of lung stage parasites via ADCC mediated by NK cells. As for the Sm-Cathepsin B + CpG formulation, results from the study suggest that $CD8^+$ T cells are the main effectors targeting the parasite. Lastly, $CD4^+$ T cells were required for the high parasite death observed with cells taken from mice that received unadjuvanted Sm-Cathepsin B immunizations. This work demonstrates that three different formulations containing the same antigen elicit different protection mechanisms in a mouse model of schistosomiasis.

5.2 Future perspectives

5.2.1 Cross-protection

The highest burden of schistosomiasis is found in sub-Saharan Africa where both *Biomphalaria* and *Bulinus* species of snails are present [8]. Due to this overlap in geographic

distribution, urogenital and intestinal schistosomiasis co-occurs in these African regions, and it is not uncommon to find mixed species infections [8-15]. A vaccine that could protect against both *S. mansoni* and *S. haematobium* infection would significantly accelerate disease elimination in regions where both species are endemic. The feasibility of developing a cross-protective vaccine is supported by immunological studies which have demonstrated extensive cross-reactivity among antigenic epitopes from different schistosome species [16]. Tallima *et al.* showed that cathepsin-based vaccinations were effective in protecting mice against a *S. haematobium* challenge [17]. Immunizations with a formulation containing Sm-Cathepsin B and *F. hepatica* Cathepsin L1 resulted in 70% and 60% decreases in *S. haematobium* worm and hepatic egg numbers respectively. The addition of *S. mansoni* recombinant glyceraldehyde 3-phosphate dehydrogenase to the vaccine formulation resulted in a 72% decrease in worm numbers, and no eggs in the livers of immunized animals compared to unimmunized controls [17]. This increase in protection levels exemplifies the benefit of using multi-antigen vaccine formulations.

In order to fully take advantage of the protective capacity of Sm-Cathepsin B, we need to continue to investigate its potential to confer cross-protection against *S. haematobium* infection. In order to continue this research, the vaccine formulations containing Sm-Cathepsin B as well as other promising antigens need to be tested in a better animal model for urogenital schistosomiasis such as the hamster model.

5.2.2 Multi formulations

Schistosomes are complex pathogens. Their lifecycle involves different stages as well as different hosts. Furthermore, they have developed an intricate set of tools to not only survive inside the host, but to actively alter their environment. It has long been argued that multi-antigen vaccine formulations are our best chance at eliciting consistent protection levels above 75%. El Ridi *et al.* investigated different multi-antigen, unadjuvanted vaccine formulations containing the candidate antigen Sm-Cathepsin B [5]. The multi-antigen formulation, which contained Sm-Cathepsin B as well as the recombinant proteins glyceraldehyde 3-phosphate dehydrogenase and peroxiredoxin-multiple antigen peptide, consistently generated the highest levels of protection when assessing all forms of parasitological burden compared to single antigen formulations. Furthermore, as demonstrated by Tallima *et al.*, vaccines composed of

several antigens can confer cross-protection between schistosome species. The benefit of multi-antigen vaccine formulations has been shown for many different pathogens such as influenza, *Staphylococcus aureus*, and *Babesia bovis* [18-20].

The selection of a suitable adjuvant to stimulate an optimal immune response represents a critical step in the development of a successful vaccine. Our results described in Chapter 4 demonstrate the impact of different adjuvants on immune mechanisms mediating vaccine induced protection. Similarly to the multi-antigen approach, there is also a focus on adjuvant biology and multi-adjuvant formulations. The addition of adjuvants to a formulation has shown to be benefitial for vaccines against advanced ovarian cancer and melanoma [21, 22]. Furthermore, the addition of CpG oligodeoxynucleotides to the diphtheria-tetanus-pertussis vaccine, which contains alum and pertussis toxin, improved the immune response in mice [23].

In our work with Sm-Cathepsin B, we have focused on CpG and Montanide ISA 720 VG as adjuvants [6, 7]. Studies have demonstrated promising results with the combination of Montanide adjuvants and different TLR ligands such as LPS, Poly(I:C), imiquimod, and CpG [21, 22, 24]. CpG has also been tested as part of a novel combination adjuvant which contains innate defense regulator peptide and polyphosphazene [24-26]. Since there is an ongoing debate concerning the suitable immune response that should be induced by an antischistosome vaccine, it is important to continue testing different formulations and considering new approaches. However, the development process must be undertaken with great prudence because regulatory agencies require extensive justifications for all components of a vaccine. Therefore, formulation optimization must be coupled with studies aiming to understand the mechanisms.

5.2.3 Potential of other cathepsin peptidases

As explained in Chapter 1 section 1.2.2.3.2, *S. mansoni* Cathepsin L3 (Sm-Cathepsin L3) is a gastrodermal cysteine protease believed to be part of the network of peptidases involved in degrading host blood proteins. This peptidase is part of the metazoan Cathepsin L cluster along with Sm-Cathepsin L2 and Cathepsin L from *Fasciola* species [27]. We carried out the expression of recombinant Sm-Cathepsin L3 using the *Pichia Pastoris* expression

system PichiaPink™ (Invitrogen), similarly to what was done for the expression of Sm-Cathepsin B. Challenge studies using a mouse model of schistosomiasis were performed in order to determine the protective potential of Sm-Cathepsin L3. We tested Sm-Cathepsin L3 without adjuvant as well as a formulation of the recombinant protein in combination with CpG dinucleotides. After three intramuscular immunizations, Sm-Cathepsin L3 without adjuvant reduced numbers of worms, hepatic eggs, and intestinal eggs by 26%, 25%, and 21% respectively compared to saline controls (Figure 5.1). Immunizations with the Sm-Cathepsin L3 formulation containing CpG decreased worm and intestinal egg numbers by 37% and 27% respectively; however, it had no effect on hepatic egg numbers when compared to the adjuvant control group (Figure 5.1). Unfortunately, immunizations using Sm-Cathepsin L3 did not attain the desired 40% protection threshold [4]. Furthermore, preliminary studies indicate that recombinant Sm-Cathepsin L3 on its own is poorly immunogenic. The addition of the CpG adjuvant resulted in increased levels of IL-12 and GM-CSF secretion compared to control animals (Figure 5.2). Future Sm-Cathepsin L3 studies should focus on investigating whether this relatively new parasite peptidase can be an effective component of an upcoming anti-schistosome multi-antigen vaccine.

5.2.4 Novel vaccine methods

5.2.4.1 Oral immunization with attenuated *Salmonella* delivery systems

The Sm-Cathepsin B studies that we have undertaken so far have been successful. However, concerning the immense potential of Sm-Cathepsin B, we have only scratched the surface. We can expand our research to include novel immunization platforms which move away from the traditional approaches focusing on single antigens delivered intramuscularly. We have initiated a novel Sm-Cathepsin B vaccine study supported by a joint Canadian Institutes of Health Research- Industry grant with Aviex Technologies LLC. For this project, we will use the company's attenuated *Salmonella typhimurium* YS1646 strain as a delivery system for Sm-Cathepsin B-based oral immunizations. The use of *Salmonella* strains as "Trojan Horse" vaccine delivery systems has regained popularity. *Salmonella* vectors have been tested for the development of vaccines against pathogens such as HIV and *Trypanosoma cruzi* [28, 29]. The YS1646 strain, developed at Yale University, was originally conceived as an anti-tumor agent targeting melanoma [30]. It underwent toxicity testing and safely made it

all the way through a Phase I clinical trial [31]. Unfortunately the treatment provided no survival advantage to the patients. This was attributed to a deletion which rendered the *Salmonella* acutely sensitive to physiological levels of CO_2 [32]. Now, we are seeking to repurpose the YS1646 strain for the development of a safe and effective vaccination platform.

The feasibility of this project is supported by the work of Chen *et al.* where they successfully used the YS1646 strain as a vaccine delivery system in a mouse model of *S. japonicum* [33]. When using the YS1646 strain alone to deliver their Sj23LHD-GST fusion protein, they obtained 40% protection. However, protection levels were increased to approximately 60% when they adopted a heterologous prime-boost strategy. This involved oral administration of the *Salmonella* strain followed by subcutaneous delivery of the fusion protein; thus, representing a multi-modality vaccination approach. Similarly, we believe that oral delivery of Sm-Cathepsin B using the YS1646 strain will result in significant protection against *S. mansoni* infection in a mouse challenge model. The formulation will not contain an adjuvant as the YS1646 strain is already highly immunogenic as it possesses lipopolysaccharides and flagellin. These components are pathogen-associated molecular patterns, and they act as powerful adjuvants via signalling through TLR-4 and TLR-5. Furthermore, *Salmonella* is known to induce humoral as well as cellular immunity through its invasion of macrophages and dendritic cells. The protective potential of this delivery system will be determined by assessing parasite burden (worms and eggs). Furthermore, immune readouts such as antigen-specific antibody and cytokine production will be evaluated. As done by Chen *et al.*, we will also test a multi-modality approach in order to hopefully significantly increase protection levels. The recombinant *Salmonella* strain expressing Sm-Cathepsin B has been constructed and preparations for the first mouse challenge study have begun (Figure 5.3) (N. Zelt unpublished). If the oral immunizations provide high levels of protection as we expect, the follow-up studies will seek to understand the underlying mechanisms of protection. We believe that delivering the YS1646 strain will activate dendritic cells in gastrointestinal lymphoid tissues and generate both local and systemic antibody and cellular responses. These future immunological studies will be performed in collaboration with Dr. Connie Krawczyk at McGill University, who is an expert in dendritic cell regulation. In the event that protection levels are lower than predicted, a second antigen will be included and a multi-antigen approach

will be evaluated. The carrying capacity of the YS1646 strain allows for the delivery of multiple antigens to the immune system [34].

This novel immunization method using the candidate Sm-Cathepsin B and a *Salmonella* delivery system has the potential to surpass the typical protection levels of 50%-60% seen in pre-clinical models. Furthermore, the vaccine formulation is delivered orally which is advantageous in low resource settings such as schistosomiasis endemic regions. Finally, the information obtained from our Sm-Cathepsin B study will provide a proof-of-concept for the YS1646 antigen delivery system; thus, supporting vaccine development projects for other human infectious diseases.

5.2.4.2 *Schistosoma mansoni* exosomes

Expanding our research on new immunization methods, our lab has undertaken a new project focusing on an exosome based anti-schistosome vaccine. To date, all of the schistosome exosome work focuses on the adult worms with some findings concerning the schistosomulae [35-38]. Our research will investigate cercarial exosomes as no previous work has investigated the biology and function of these vesicles. More specifically, the project will initially seek to isolate and characterize cercarial exosomes. Through methods of cercarial culturing, we were able to isolate exosomes and then identify them by transmission electron microscopy (Figure 5.4). Exosomes contain an array of macromolecules that, in the case of a pathogen, aid in development, host invasion, and immune evasion. The exosomes will be fully characterized with the use of mass spectrometry platforms that will identify the proteins, nucleic acids, and lipids contained within these vesicles. An initial proteomic analysis by liquid chromatography-tandem mass spectrometry identified 55 proteins. The analysis revealed the presence of known schistosome exosome markers such as actin, elongation factor, heat shock protein, tetraspanin, and Rab. We also identified molecules such as elastase and calcium-binding protein that are known to be secreted when cercariae are exposed to human skin lipids [39]. Finally, the proteomic analysis revealed the presence of known vaccine candidates such as Sm-TSP-2 and Venom allergen-like 4; thus, supporting the possibility for a cercarial exosome based vaccine (Table 5.1).

Ultimately, we seek to use the parasite's exosomes to formulate a vaccine. Exosomes are composed of some of the most immunogenic molecules that are also found on the parasite's surface, in its gut, and in its excretory/secretory products. Furthermore, these vesicles are abundantly released during invasion; thus, interact directly with the host immune system. These are favourable characteristics for a vaccine candidate [40]. The protective potential of a cercarial exosome vaccine will be determined by performing a challenge study in a mouse model of schistosomiasis. After being immunized with an exosome formulation, mice would undergo a cercarial challenge. The efficacy of the formulation will be assessed by determining parasite burden in immunized mice compared to unimmunized ones. Along with protective potential, the safety and immune stimulating effects of the formulation will also be examined throughout the study. This initial challenge study will test the exosome formulation without adjuvant. The complex contents of the exosome suggest that this parasite vesicle may have adjuvant properties on its own; thus, simplifying a potential vaccine formulation. Future studies can test different adjuvant classes in order to potentially increase to efficacy of the exosome vaccine.

Immunomodulatory assays will be performed in parallel with the challenge studies in order to understand the immunological effects of delivering parasite exosomes. Cercariae cross the different layers of the skin and ultimately enter into the blood circulation without being detected by the immune system. In order to understand this cercarial advantage, exosome function can be investigated by intradermal delivery of whole exosomes in an *ex vivo* human explant skin model. The immune effects elicited by the presence of cercarial exosomes can be assessed by examining the activation pattern of the main immune cells in the skin, Langerhans cells and dermal dendritic cells, as well as identifying any immune mediating molecules released by the accessory cells in the skin microenvironment [41].

As previously mentioned, there has been no previous work focusing on the characterization of schistosome cercarial exosomes. Furthermore, a cercarial exosome vaccine against schistosomiasis is a novel concept. Positive results from a schistosome challenge study would support further investigation and exploitation of exosome vaccines.

5.3 Concluding remarks

Schistosomiasis still represents an important public health issue in some of the world's poorest countries. The disease, which approximately 700 million people are exposed to, has an estimated global burden of 3.3 million disability-adjusted life years [1, 42]. Schistosomiasis is a chronic disease characterised by years of associated conditions such as pain, anemia, and malnutrition. Furthermore, infected children may experience defects in their growth and cognitive development [9, 43, 44]. Treatment relies solely on PZQ, and the mass distribution of this drug represents the mainstay of schistosomiasis control programs. PZQ is a convenient drug because it is effective, cheap, and well tolerated [45]. However, PZQ does not prevent reinfection; thus, requiring constant drug administration. In endemic areas, where transmission is high, infection prevalence returns to baseline as early as 18 months after PZQ distribution has ceased [46]. MDA programs also struggle to achieve the desired minimum 75% coverage rate encouraged by the World Health Assembly [47]. An evaluation of PZQ coverage performed in 2012 revealed that treatment reaches only 14% of infected individuals [45]. In order to conquer schistosomiasis, we need to alter the approaches currently being used. We cannot continue to rely on MDA programs that are not sustainable, are not reaching the majority of people in need, and are overusing our only effective drug against schistosomiasis. The optimal strategy for successful schistosomiasis elimination needs to be multilayered and use existing methods such as PZQ treatment, snail control, and health/hygiene education. Furthermore, research needs to be focused on developing vaccines as well as new drugs that could be used effectively if PZQ resistance emerges [48, 49]. A vaccine against schistosomiasis could significantly accelerate the disruption of disease transmission.

Sm-Cathepsin B has been the focus of much research over the years because it is an essential molecule for schistosome development and its immunodominant properties make it a promising target for vaccines as well as diagnostics [3, 5-7, 50-57]. In this thesis, we demonstrated that significant protection levels were obtained with different Sm-Cathepsin B formulations in pre-clinical studies [5-7]. There were no detectable levels of antigen-specific IgE titers in mice immunized with Sm-Cathepsin B, and analysis of sera from patients with patent schistosomiasis revealed low antigen-specific IgE levels [5]. These observations indicate that immunizations with this vaccine candidate do not induce allergenic reactions. This will avoid incidents similar to the *Na*-ASP-2 hookworm vaccine trial whereby the study was halted due to hypersensitivity reactions caused by pre-existing antigen-specific IgE in the endemic

population [58]. Studying the immune responses as well as the underlying protective mechanisms elicited by immunizations could help identify desirable vaccine characteristics and formulate better vaccines. This thesis work therefore has potential applications in vaccine development against schistosomiasis.

The Millennium Development Goals of 2005 displayed a common global health theme which has been a surge in motivation to save the poor. In the field of infectious diseases, this theme was echoed in the slogan prompting the rescue of the bottom billion through the control of neglected tropical diseases [59]. The WHO recognizes seventeen major parasitic and related infections as the neglected tropical diseases [60]. These diseases have a disproportionate impact on the world's poorest populations, and they have the ability to cause as well as perpetuate poverty [61]. Not only is schistosomiasis a member of the neglected tropical diseases, it is also a leader in the number of disability-adjusted life years [42]. As is the case for all other neglected tropical diseases, with the exception of rabies, there is no licensed vaccine for schistosomiasis [61]. The lack of an anti-schistosome vaccine is the result of absent financial incentives and overreliance on PZQ MDA. However, a recent survey of approximately 400 neglected tropical disease experts determined that the currently available strategies and technologies will not eliminate schistosomiasis [62]. In order to achieve schistosomiasis elimination, the development of new integrated tools such as drugs, vaccines, diagnostic tests, and vector control strategies is necessary. A vaccine against schistosomiasis represents a sustainable control tool. Research and development for the optimization of an anti-schistosome vaccine should evaluate production feasibility, protection, elicited immune responses, and delivery. An effective vaccine against schistosomiasis could also be used in vaccine-linked chemotherapy programs in order to prevent reinfection following MDA [63]. Hopefully, further work on promising vaccine candidates such as Sm-Cathepsin B will lead to new control programs, which include immunizations, so that schistosomiasis elimination can be achieved.

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5.5 Table

Table 5.1 Key findings from an initial proteomic analysis of cercarial exosomes

Protein	Molecular mass (kDa)	GenInfo Identifier (gi)
Actin[a]	42	1703114 (+1)
PRX_like2 domain-containing protein[c]	23	353232428
Putative oxalate:formate antiporter	21	360043930
Putative synaptosomal associated protein	23	353229918
Putative elongation factor 1-alpha[a]	51	353230261
Inyadolysin (M08 family)	65	350645238
Putative calmodulin-4 (Calcium-binding protein Dd112)[b]	9	360044081
Putative serpin	44	353228646
Putative merlin/moesin/ezrin/radixin	64	360044613
Putative heat shock protein 70[a]	70	353229993 (+2)
CD63-like protein Sm-TSP-2[a,b]	26	23305772 (+1)
Elastase 2a[b]	29	21217531 (+1)
Glyceraldehyde-3-phosphate dehydrogenase (GAPDH)[a]	36	120709 (+2)
Venom allergen-like (VAL) 4[c]	21	353232981 (+2)
Cofilin	16	350644854
Phosphoglycerate mutase[b]	28	353232181
Rab11, putative[a]	24	350644474
Putative Sm29[c]	21	353230115 (+1)

[a] Known schistosome exosome markers [29-32]

[b] Previously identified cercarial secretion products [33]

[c] Known schistosome vaccine candidates [5, 17, 34, 64]

5.6 Figures and legends

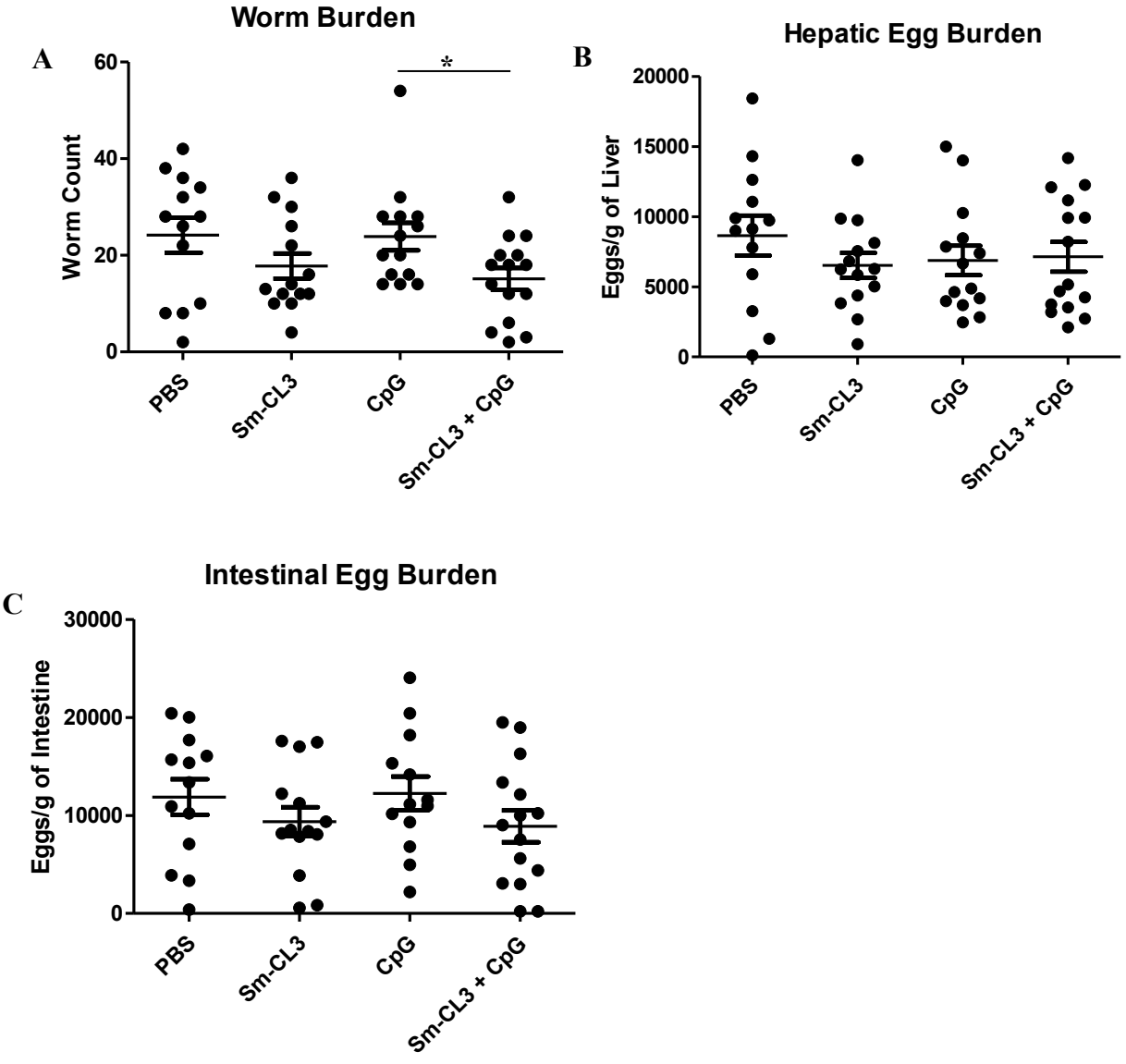

Figure 5.1 Parasite burden of mice immunized with Sm-Cathepsin L3 formulations

The worm counts per individual mouse **(A)** as well as the egg load per gram of liver **(B)** and per gram of intestine **(C)** are represented for the saline as well as adjuvant control mice (PBS and CpG dinucleotides alone), for the antigen alone mice (Sm-Cathepsin L3 no adjuvant), and for the antigen + adjuvant group of mice (Sm-Cathepsin L3 plus CpG dinucleotides). All of the

mice were immunized three times, exposed to a cercarial challenge three weeks post the last immunization, and then sacrificed seven weeks post challenge. The figure demonstrates the mean ±standard error for each group. Methods for animal immunizations and parasite burden assessment are described in Chapters 2 and 3. *: $p \leq 0.05$

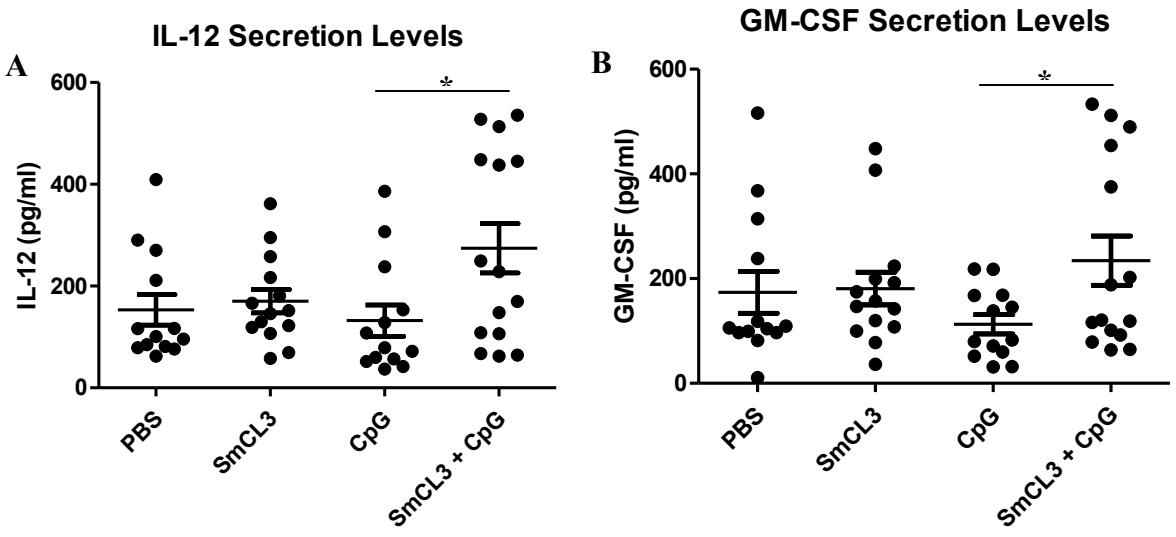

Figure 5.2 Cytokine secretion levels of mice immunized with Sm-Cathepsin L3

Cytokine levels, **(A)** IL-12 and **(B)** GM-CSF, produced 72 hours after stimulating splenocytes *ex vivo* with recombinant Sm-Cathepsin L3. Splenocytes were isolated from every C57BL/6 mice belonging to each group: PBS, Sm-Cathepsin L3, CpG dinucleotides, and Sm-Cathepsin L3 with CpG dinucleotides. Cytokine production was analyzed by QUANSYS multiplex ELISA. The figure demonstrates the mean ±standard error for each group. Methods for mouse splenocyte isolation and QUANSYS multiplex ELISA are described in Chapters 2 and 3. *: $p \leq 0.05$

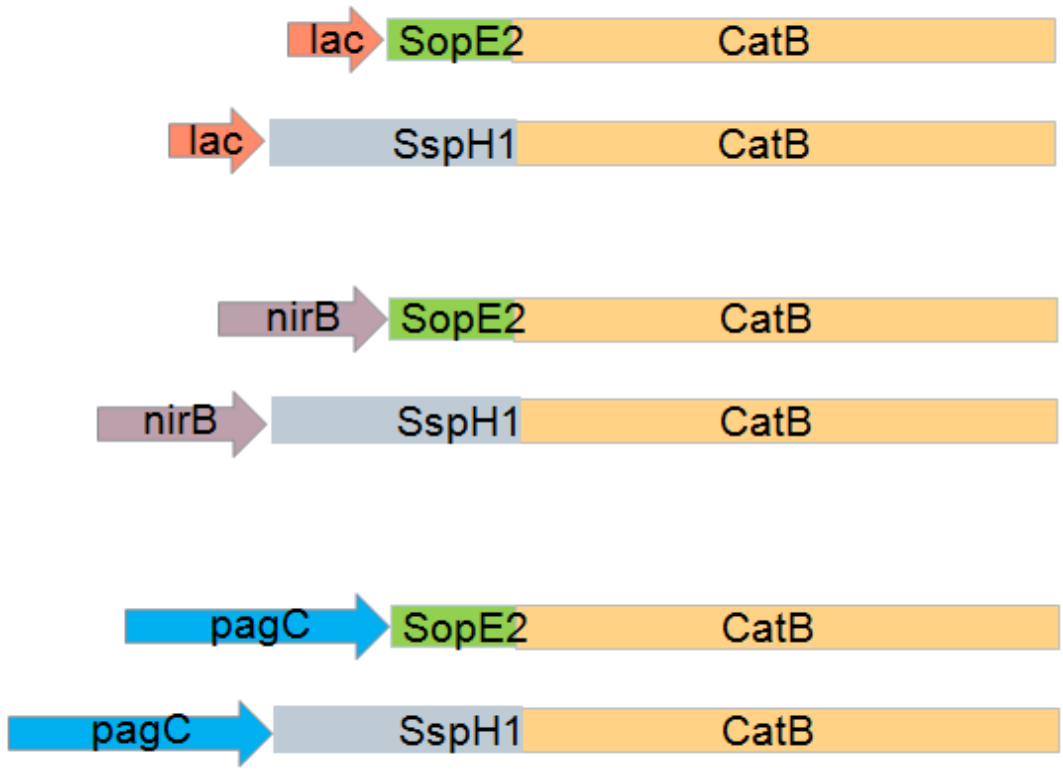

Figure 5.3 Representation of the Sm-Cathepsin B constructs that will be used in the *Salmonella* delivery system study

A series of constructs have been generated in order to allow for the *Salmonella typhimurium* driven expression of chimeric proteins containing a *Salmonella* secretion signal and *Schistosoma mansoni* Cathepsin B (CatB). The chimeric proteins consist of the secretion and translocation signals of *Salmonella* type III secreted proteins *Salmonella* outer protein E2 (SopE2) or E3 ubiquitin-protein ligase SspH1 fused to the vaccine candidate *S. mansoni* Cathepsin B. The lactose operon (*lac*), nitrite reductase B (*nirB*), and phoP activated gene C (*pagC*) gene promoters were used to drive the expression of the chimeric proteins in *S. typhimurium*. The SopE2 secretion signal used for these constructs consists of the DNA fragment encoding the N-terminal amino acids 1-104 of sopE2 and the N-terminal amino acids 1-210 of SspH1. The *S. mansoni* Cathepsin B amino acid sequence used for these constructs is the same as what was used for the studies described in Chapters 2-4.

Contribution of authors: Nicholas Zelt generated the Sm-Cathepsin B based constructs and generated the figure.

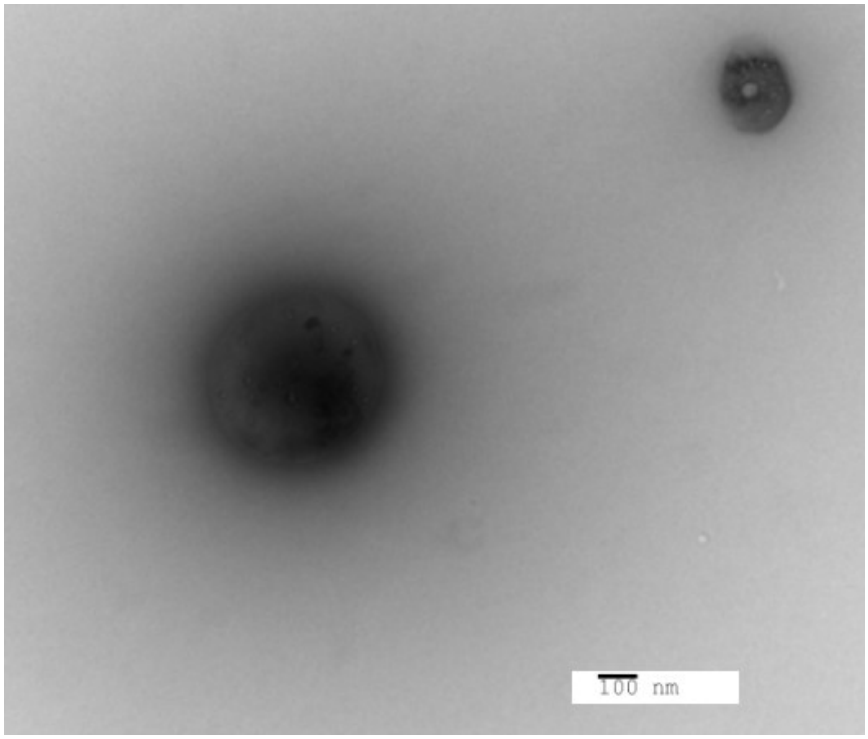

Figure 5.4 *Schistosoma mansoni* cercarial exosome by Transmission Electron Microscopy

Schistosoma mansoni cercariae were incubated overnight at 37 ºC, 5% CO_2 in RPMI 1640 medium (Wisent Bio Products). The sample was then centrifuged at 3,000 rpm for 5 minutes to pellet the parasites. The supernatant was filtered using a 0.45 µm syringe filter followed by a 0.20 µm syringe filter. The filtered supernatant was distributed into ultracentrifuge tubes and the tubes were filled almost up to the top with Exosome Buffer (137 mM NaCl, 20 mM Hepes, pH 7.5). The sample was centrifuged for one hour at 29,000 rpm using the SW32.1 rotor (Beckman Coulter). The supernatant was removed and the remaining pellet was resuspended using Exosome Buffer. The sample was centrifuged once more for one hour at 29,000 rpm. The resulting pellet contains the parasite exosomes. For transmission electron microscopy, 5 µL drops of exosome preparation, at a concentration of 100 to 500 ng/µL in Exosome Buffer, were directly coated on formvar carbon grids for one minute. The grids were fixed with 1% glutaraldehyde in 0.1 M sodium cacodylate buffer for one minute, washed with ultrapure water three times for one minute, and stained with 1% uranyl acetate for one minute. The grids were then dried and observed under the electron microscope.

Contribution of authors: Alessandra Ricciardi isolated the cercarial exosomes and performed the transmission electron microscopy to generate the figure.