Developing an attenuated *Salmonella* Typhimurium vector to deliver Cathepsin B as a prophylactic and therapeutic vaccine against *Schistosoma mansoni* in a pre-clinical model

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ABSTRACT

Schistosomiasis is among the most important neglected tropical diseases and *Schistosoma mansoni* threatens hundreds of millions of people in over 50 countries worldwide. Infections occur in fresh water and risk groups include school-aged children as well as adults with occupations or domestic tasks involving contact with infested water. Chronic disease manifestations are attributable to egg deposition with ensuing inflammation in tissues such as the liver and intestine. The larval stage of *S. mansoni* (i.e.: schistosomula) migrate through respiratory tissues and adult worms reside in venules adjacent to the gastrointestinal tract. Praziquantel, an oral anthelminthic that paralyzes the adult worm, is the main drug for treatment of schistosomiasis. However, it does not protect against reinfection and drug resistance is a growing concern. Several candidate vaccines are in pre-clinical and clinical development, but none is designed to elicit both systemic and mucosal responses. We have repurposed an attenuated *Salmonella enterica* Typhimurium strain (YS1646) to produce a vaccine that targets Cathepsin B (CatB), a parasite digestive enzyme important for both juvenile and adult worms. After screening multiple constructs, two YS1646 strains with promising plasmid-based promoter-secretory signal pairings were selected for *in vivo* evaluation (nirB_SspH1_CatB and SspH1_SspH1_CatB). When mice vaccinated using a 3-week oral (PO: dose 1 x 10^9 colony-forming units (cfu)) prime & intramuscular (i.e.: recombinant CatB IM; dose 20 μg) boost regimen were challenged 3 weeks post-vaccination, reduced worm and egg burden were observed in both the intestines and liver 7 weeks post-infection. In the nirB_SspH1_CatB PO + CatB IM group, the worm and intestine/liver egg burdens were reduced 93.1% and 79.5%/90.3%, respectively. Having demonstrated promising prophylactic effects, we next assessed whether these candidate vaccines could act therapeutically. Compared to mock-vaccinated mice, therapeutic vaccination either 2 or 4 months after infection with a combined PO+IM regimen administered over 5 days resulted in significant reductions in all parasitological outcomes (46 – 50%) at 1 month post-vaccination with even greater reductions (64 – 73%) at 2 months post-vaccination. Next, we generated and screened several chromosomally integrated YS1646 strains expressing CatB to produce a viable candidate vaccine for eventual human use (i.e.: more stable expression of the target antigen and removal of plasmid-based antibiotic resistance). Based upon *in vitro* antigen expression, the most promising strain was advanced to *in vivo* testing (nirB_SspH1_CatB). Despite a reduction in CatB gene copy number from 25 – 30 in the plasmid-bearing YS1646 to a single

copy in the chromosomally integrated YS1646 candidate, we observed significant decreases in parasite burden as well as robust systemic and mucosal antibody responses following combined PO+IM (5-day) vaccination. This multimodal vaccination approach led to a mixed T_H1/T_H2 profile in both the humoral and cellular responses. A vaccine having both prophylactic and therapeutic activity would be ideal for use in conjunction with praziquantel mass treatment campaigns, the current 'gold-standard' practice for schistosomiasis control. Overall, the findings in this thesis provide insight into the possible advantages of using a live attenuated bacterial vector expressing a parasite digestive enzyme antigen as a vaccine. These observations make an important contribution to the development of a potential vaccine for schistosomiasis.

RÉSUMÉ

La schistosomiase est l'une des plus importantes maladies tropicales négligées et *Schistosoma mansoni* menace des centaines de millions de personnes dans plus de 50 pays dans le monde. Les infections ont lieu dans l'eau douce et les groupes à risque incluent les enfants d'âge scolaire ainsi que les adultes avec des emplois ou tâches domestiques nécessitant un contact avec de l'eau infectée. Les symptômes de la phase chronique de la maladie sont attribuables à des œufs piégés entraînant de l'inflammation dans des tissus tels que le foie et les intestins. Le stade larvaire de *S. mansoni* (c.-à-d. schistosomules) migre à travers les tissus respiratoires et les vers adultes vivent dans les veinules adjacentes au tube digestif. Le praziquantel, un vermifuge oral qui paralyse le ver adulte, est la drogue principale pour traiter la schistosomiase. Cependant, le médicament ne protège pas contre une réinfection et la résistance au praziquantel est de plus en plus inquiétante. Plusieurs candidats-vaccins sont en phase préclinique et clinique, mais aucun n'est conçu pour déclencher des réponses immunitaires systémique et muqueuse. Nous avons adapté une souche atténuée de *Salmonella enterica* Typhimurium (YS1646) pour produire un vaccin qui cible l'antigène Cathepsine B (CatB), une enzyme digestive du parasite qui est importante pour les vers juvéniles et adultes. Ayant complété un criblage de plusieurs constructions, deux souches YS1646 à base de plasmide avec des combinaisons prometteuses de prometteur et signal de sécrétion ont été choisies pour des tests *in vivo* (nirB_SspH1_CatB et SspH1_SspH1_CatB). Lorsque nous avons vacciné des souris avec une première série de doses orales (PO: dose 1 x 10^9 cfu) suivie d'une dose de rappel intramusculaire (c.-à-d. IM CatB recombinant; dose 20 μg) avec un intervalle de 3 semaines entre les deux, nous avons observé des charges réduites de vers et d'œufs dans les intestins et le foie 7 semaines après l'infection. Dans le groupe nirB_SspH1_CatB PO + CatB IM, les charges de vers et d'œufs dans les intestins et le foie étaient réduites à 93.1%, 79.5% et 90.3% respectivement. Ayant démontré des effets prophylactiques prometteurs, nous avons ensuite évalué si nos candidats-vaccins fonctionnent de façon thérapeutique. Comparée au groupe de contrôle, la vaccination thérapeutique soit 2 ou 4 mois post-infection avec un horaire de vaccination simultanée PO+IM administrée sur 5 jours a engendré des réductions significatives dans tous les résultats parasitologiques (46 – 50%) après un mois post-vaccination. Ces réductions ont augmenté (64 – 73%) 2 mois post-vaccination. Ensuite, nous avons généré et criblé plusieurs souches YS1646 exprimant CatB de façon chromosomique afin de produire un candidat-vaccin

viable pour des éventuels essais cliniques (c.-à-d. une expression plus stable de notre antigène ainsi que l'élimination de la résistance aux antibiotiques dû à un plasmide). Selon l'expression antigénique *in vitro*, la souche la plus prometteuse a avancé pour des tests *in vivo* (nirB_SspH1_CatB). Malgré la réduction dans le nombre de copies du gène CatB de 25 – 30 dans la souche plasmide YS1646 à une seule copie dans le candidat YS1646 avec une intégration chromosomique, nous avons observé des hauts taux de réduction dans la charge parasitaire ainsi que des fortes réponses chez les anticorps dans les systèmes immunitaires systémique et muqueuse après l'horaire de vaccination combinée PO+IM (5 jours). Cette méthode de vaccination multimodale a mené à un profil mixte T_H1/T_H2 dans l'immunité humorale et cellulaire. Un vaccin qui puisse agir de façon prophylactique et thérapeutique serait idéal en utilisation conjointe avec des campagnes de traitement de masse avec le praziquantel qui est le pilier actuel de la lutte contre la schistosomiase. En tout, les découvertes de cette thèse révèlent plus d'informations sur les avantages d'utiliser une souche atténuée d'une bactérie en tant que vecteur pour exprimer une enzyme digestive d'un parasite comme antigène pour un vaccin. Ces résultats sont une contribution importante dans le développement d'un vaccin contre la schistosomiase.

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LIST OF ABBREVIATIONS

Ad5	Adenovirus serotype 5
ADCC	Antibody-dependent cell-mediated cytotoxicity
Amp	Ampicillin
ANOVA	Analysis of variance
ART	Antiretroviral therapy
ASE	Artesunate
ATP	Adenosine 5′-triphosphate
ATRA	All-trans retinoic acid
BATT	β-alanyl-tryptamine
BCA	Bicinchoninic acid
BCG	Bacille-Calmette Guérin
BMDC	Bone marrow-derived dendritic cell
BMGY	Buffered complex glycerol medium
CA	California
CAA	Circulating anodic antigen
CCA	Circulating cathodic antigen
CCL	Chemokine (C-C motif) ligand
CD	Cluster of differentiation
cDNA	Complementary deoxyribonucleic acid
Cfu	Colony-forming unit
CHI-S	Controlled human infection model for schistosomiasis
CHIM	Controlled human infection model
CI	Chromosomally integrated
Cm	Chloramphenicol
CNS	Central nervous system
CO_2	Carbon dioxide
CYP450	Cytochrome P450
D	Day
DALY	Disability-adjusted life year

DAMP	Danger-associated molecular pattern
DAP	Diaminopimelic acid
DAPI	4',6-diamidino-2-phenylindole
DC	Dendritic cell
ddPCR	Digital droplet polymerase chain reaction
DHA	Dihydroartemisinin
DMEM	Dulbecco's Modified Eagle's medium
dmLT	Double mutant heat-labile toxin
eGFP	Enhanced green fluorescence protein
ELISA	Enzyme-linked immunosorbent assay
EN	Endemic normal
ES	Excretory/secretory
ETEC	Enterotoxigenic *Escherichia coli*
FABP	Fatty acid binding protein
FBS	Fetal bovine serum
FGS	Female genital schistosomiasis
FHC	frr_SspH1_Cathepsin B
FLP	Flippase
FMDV	Foot-and-mouth disease virus
GALT	Gut-associated lymphoid tissues
GLA-AF	Glucopyranosyl lipid A in an aqueous formulation
GLA-SE	Glucopyranosyl lipid A in a squalene-in-water emulsion
GM-CSF	Granulocyte macrophage colony-stimulating factor
GMP	Good manufacturing process
H&E	Haematoxylin and eosin
HBV	Hepatitis B virus
HCV	Hepatitis C virus
HD	High dose
HDM	Helminth defense molecule
HIV-1	Human immunodeficiency virus type 1
HRP	Horseradish peroxidase

IFNγ	Interferon gamma
IgA	Immunoglobulin A
IgE	Immunoglobulin E
IgG	Immunoglobulin G
IgM	Immunoglobulin M
IL	Interleukin
IM	Intramuscular
IN	Intranasal
IP	Intraperitoneal
ITZ	Itraconazole
IV	Intravenous
kDa	Kilodalton
Km	Kanamycin
LAMP	Loop-mediated isothermal amplification
LB	Luria/Lysogeny broth
LD	Low dose
LOD	Limit of detection
LPS	Lipopolysaccharide
LT	Heat-labile enterotoxin
M2e	Matrix protein 2
MALT	Mucosa-associated lymphoid tissues
MCP-1	Monocyte chemoattractant protein 1
MD	Maryland
MDA	Mass drug administration
MDR	Multi-drug resistance
MEF	Mefloquine
mg	Milligram
MGS	Male genital schistosomiasis
MHC	Major histocompatibility complex
mM	Millimolar
MIP-1α	Macrophage inflammatory protein 1 α

miRNA	Micro ribonucleic acid
μg	Microgram
μl	Microliter
μm	Micrometer
mL	Milliliter
MO	Missouri
MoDC	Monocyte-derived dendritic cell
MOI	Multiplicity of infection
Mtb	*Mycobacterium tuberculosis*
NAD	Nicotinamide adenine dinucleotide
NHC	nirB_SspH1_Cathepsin B
nirB	Nitrate reductase B
NJ	New Jersey
ng	nanogram
nPCR	Nested polymerase chain reaction
NTD	Neglected tropical disease
NY	New York
ºC	Degree Celsius
OD	Optical density
ODN	Oligodeoxynucleotide
ODT	Orodispersible tablet
OR	Oregon
pagC	phoP activated gene C
PAMP	Pattern-associated molecular pattern
PBMC	Peripheral blood mononuclear cell
PBS	Phosphate-buffered saline
PCR	Polymerase chain reaction
pg	Picogram
PHC	pagC_SspH1_Cathepsin B
PIP	Praziquantel in preschoolers
Pmy	Paramyosin

PO	Per os
PolyI:C	Polyinosinic:polycytidylic acid
PPC	Pediatric praziquantel consortium
PPC	Preferred product characteristics
PRR	Pattern recognition receptor
PspA	Pneumococcal surface protein A
PZQ	Praziquantel
QC	Quebec
qPCR	Quantitative polymerase chain reaction
RA	Radiation-attenuated
RAR	Retinoic acid receptor
RMT	Radiation mutation technology
RNAi	Ribonucleic acid interference
rpm	Revolutions per minute
RPMI	Rosewell Park Memorial Institute medium
RT	Room temperature
SCV	*Salmonella*-containing vacuole
SEA	Soluble egg antigen
SEM	Standard error of the mean
Sh28GST	*Schistosoma haematobium* 28 kDa glutathione S-transferase
SjIR	*Schistosoma japonicum* insulin receptor
SjTPI	*Schistosoma japonicum* triose-phosphate isomerase
Sm-KI-1	*Schistosoma mansoni* Kunitz type protease inhibitor
Sm-p80	*Schistosoma mansoni* calpain
SmAE	*Schistosoma mansoni* asparaginyl endopeptidase
SmCatB/SmCB	*Schistosoma mansoni* cathepsin B
SmCKBP	*Schistosoma mansoni* chemokine-binding glycoprotein
SmHMGP1	*Schistosoma mansoni* high mobility group protein 1
SmNRPS	*Schistosoma mansoni* nonribosomal peptide synthetase
SmTCTP	*Schistosoma mansoni* transcriptionally controlled tumor protein homolog
SmTSP-2	*Schistosoma mansoni* tetraspanin 2

SPI	*Salmonella* pathogenicity island
STH	Soil-transmitted helminth
T3SS	Type 3 secretion system
TAL	Tegument-allergen-like
TB	Tuberculosis
TcdA/TcdB	Clostridium difficile toxin A/B
TDR	Tropical diseases research
Tfh	T follicular helper
TGR	Thioredoxin gluthathione reductase
Th	T helper
TLR	Toll-like receptor
TMB	Tetramethyl benzidine
TPP	Target product profile
TRPM	Transient receptor potential melastatin ion channel
US	United States
UT	Utah
VA	Virginia
w/v	Weight by volume
WASH	Water, sanitation, and hygiene
WHO	World Health Organization
WT	Wild type
x g	Times gravity

CONTRIBUTIONS OF AUTHORS

The candidate has chosen to present a manuscript-based thesis. This thesis contains three original 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, Adam Hassan, 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 4 contains extracts from Winter K[*], **Hassan AS**[*], Dozois CM, Ndao M, Ward BJ. Current applications of *Salmonella enterica* serovar Typhimurium as a vaccine vector. Manuscript prepared for submission to *Frontiers in Immunology*.
[*]KW and ASH are co-first authors.

KW and ASH shared equal contributions in the preparation of the manuscript. CMD, MN, and BJW revised the manuscript.

CHAPTER 2

Hassan AS, Zelt NH, Perera DJ, Xing L, Ndao M, Ward BJ (2019). Vaccination against the digestive enzyme Cathepsin B using a YS1646 *Salmonella enterica* Typhimurium vector provides almost complete protection against *Schistosoma mansoni* challenge in a mouse model. *PLoS Negl Trop Dis* 13(12): e0007490.

This manuscript is reprinted from the journal PLoS Neglected Tropical Diseases with permissions granted by the Creative Commons Attribution (CC BY) license. ASH was involved in all aspects of the study including study design, performing experiments, data analysis and preparation of the manuscript. NHZ and LX designed, constructed, and validated the plasmids. DJP assisted in the design of the snail housing facility and the infection model. BJW and MN supervised all parts of the study and prepared the manuscript.

CHAPTER 3

Hassan AS, Perera DJ, Ward BJ, Ndao M (2021). Therapeutic activity of a *Salmonella*-vectored *Schistosoma mansoni* vaccine in a mouse model of chronic infection. *Vaccine* 39(39): 5580-5588.

This manuscript is reprinted from the journal Vaccine with permission from Elsevier. ASH was involved in all aspects of the study including study design, performing experiments, data analysis and preparation of the manuscript. DJP assisted in the design of the snail housing facility and the infection model. BJW and MN supervised all parts of the study and prepared the manuscript.

CHAPTER 4

Hassan AS, Houle S, Labrie L, Perera DJ, Dozois CM, Ward BJ, Ndao M. Chromosomally integrated gene expression of *Schistosoma mansoni* Cathepsin B in a *Salmonella* Typhimurium vaccine vector protects against murine schistosomiasis. Manuscript under review at *npj Vaccines*.

ASH was involved in all aspects of the study including study design, performing experiments, data analysis and preparation of the manuscript. SH assisted with experimental design of chromosomal integration steps. LL assisted in sample collection & processing from vaccinated animals. DJP provided revisions to the manuscript. CMD supervised the portion of the study related to chromosomal integration experiments and provided revisions to the manuscript. BJW and MN supervised all parts of the study and prepared the manuscript.

The general discussion (Chapter 5**) was written by ASH and edited by BJW & MN.

CONTRIBUTIONS TO ORIGINAL KNOWLEDGE

The work presented in this thesis contributes original knowledge to the fields of schistosomiasis vaccine development and live attenuated vectored vaccines. The specific contributions are as follows:

In **Chapter 2**, we described the development of a prophylactic *S. mansoni* vaccine by repurposing the attenuated *Salmonella* Typhimurium strain YS1646 for plasmid-based expression of Cathepsin B and assessed immunogenicity & efficacy of prime-boost vaccination. We demonstrated that:

1. Oral priming doses with recombinant YS1646 along with an intramuscular boost of recombinant protein leads to mixed T_H1/T_H2 humoral and cellular responses.

2. Vaccination with the recombinant *Salmonella* vector generates potent local mucosal responses characterized by the production of anti-Cathepsin B IgA antibodies.

3. Prime-boost vaccination significantly reduces parasite burden (worms, hepatic eggs, intestinal eggs) in a mouse model of schistosomiasis.

4. Cathepsin B delivered both orally with a vector and intramuscularly induces diminished egg granuloma sizes in the liver and severely impacts the morphology of entrapped eggs.

In **Chapter 3**, we established a chronic infection mouse model for schistosomiasis and examined the therapeutic potential of our recombinant YS1646 vaccine vector. We demonstrated that:

1. A chronic schistosomiasis model in mice is feasible to test the therapeutic applications of a vaccine.

2. Our multimodal vaccination regimen can be reduced from a 3-week schedule to 5 days with similar prophylactic efficacy and significant parasite burden reductions up to 2 months post-vaccination following a period of 2- or 4-months post-infection.

3. Multimodal vaccination was able to skew pre-existing & dominant T_H2 responses elicited by chronic schistosomiasis infection to a more mixed T_H1/T_H2 profile.

4. Vaccination reduced granuloma size and had adverse effects on egg morphology which led to decreased rates of liver fibrosis.

In **Chapter 4**, we designed a chromosomally integrated recombinant YS1646 vaccine vector to express Cathepsin B and evaluated the immunogenicity of such expression using a multimodal vaccination approach. We demonstrated that:

1. Chromosomal integration of a foreign vaccine/antigen has no effect on the fitness of the bacterial vector, and it is possible to integrate other genes in tandem.

2. Chromosomal expression of CatB generates potent humoral and cellular immune responses despite reductions in antigen copy number.

3. Multimodal vaccination with a chromosomally integrated construct yields robust local, mucosal antibody responses along with greater systemic antibody responses than plasmid-based expression.

4. Stable CatB expression in our system generates a mixed $T_H1/T_H2/T_H17$ cellular immune response.

5. Multimodal vaccination significantly reduces parasite burden and impacts egg-associated pathology in a murine challenge model.

Taken together, we have shown that vaccination with a live, attenuated strain of *Salmonella* Typhimurium expressing the parasitic digestive enzyme Cathepsin B can represent an effective vaccination strategy when coupled with intramuscular administration of the antigen in a mouse model of schistosomiasis. These efforts represent a considerable step towards the development of an effective vaccine with both prophylactic and therapeutic effect.

CHAPTER 1

Literature Review and Research Objectives

1.1 SCHISTOSOMIASIS

Human schistosomiasis is a freshwater-borne disease caused by blood flukes of the genus *Schistosoma*. The three main schistosome worm species are *S. mansoni*, *S. haematobium*, and *S. japonicum*. Schistosomiasis is one of the most important parasitic diseases worldwide due to its mortality and morbidity rates[1]. Over 250 million people are affected by this helminthiasis and approximately 800 million are at risk of infection[2]. While over 90% of cases are in sub-Saharan Africa, other geographical foci include South and Central America, Asia, and the Middle East[3,4]. Control measures include snail vector control, improved access to clean water, and mass drug administration (MDA) using praziquantel (PZQ)[5]. Despite these efforts, prevalence rates have not significantly decreased over time[6]. Vaccination campaigns in concert with existing measures as part of a multifaceted approach are likely to be the key to sustainable schistosomiasis control[7]. The following sections will define important aspects of disease burden and existing control measures.

1.1.1 Brief History

The parasite was first described in 1851 by Theodor Maximilian Bilharz and Carl Theodor Ernst while performing an autopsy in Egypt[8]. Dr. Bilharz described the presence of eggs in the patient's bladder and their transmission to freshwater through urine. He also hypothesized a link between the parasite and the clinical symptoms of dysentery and hematuria observed[9]. The disease was later referred to as bilharzia (or bilharziasis) in recognition of Dr. Bilharz. In 1858, the German zoologist David Friedrich Weinland proposed the name *Schistosoma* which means "split body" in Greek due to the worm's physiology[9].

There is evidence of schistosomiasis in antiquity. Dating back over 6000 years, schistosome eggs have been found in pelvic sediment of skeletal remains in Syria[10]. As a result of the importation of monkeys and the slave trade during the fifth dynasty of the pharaohs (~ 2494–2345 BC),

schistosomiasis likely spread to Egypt where it was referred to as the "â-a-â disease" in medical papyri[11,12]. Ancient Egyptians were advised to avoid polluted water and fishermen were asked to wear protective linen in order to prevent the "â-a-â disease"[13]. Finally, schistosomiasis was confirmed in ancient Egypt when, in 1910, *S. haematobium* eggs were identified in two mummies by British professor Marc Armand Ruffer[14].

In the early 20th century, British parasitologist Robert Thomson Leiper elucidated the complete lifecycle of *Schistosoma* spp. and was able to distinguish *S. mansoni* and *S. haematobium* as distinct species due to their morphology, egg type and snail vector[15]. In Asia, clinical manifestations of schistosomiasis infection can be found dating back over 2400 years in ancient Chinese medical texts[16]. In 1904, Japanese pathologist Fujiro Katsurada described hepatic schistosomiasis in a cat and classified the new species as *S. japonicum*[17]. Arrival of schistosomiasis in South America and Europe is thought to have occurred through the slave trade[9,18].

1.1.2 Disease Epidemiology

With more than 200 million people infected and over 800 million more at risk, schistosomiasis is a major global cause of morbidity and mortality[2]. However, with current detection methods, disease prevalence is thought to be greatly underestimated[19,20]. Sources of infection include rivers, lakes, reservoirs, and irrigation channels from tropical and sub-tropical endemic areas[21]. Poverty and poor sanitation contribute to the propagation of the disease. Mortality rates are difficult to assess but are thought to range between 280,000 – 500,000 deaths per year[1]. However, the major impact of the disease lies in its associated morbidity rather than premature death. The global burden of schistosomiasis is estimated to cause the loss of 1.4 – 3.3 million disability-adjusted life years (DALYs)[22,23]. Symptoms of schistosomiasis include anemia, growth stunting, and irreversible organ damage[24].

The World Health Organization (WHO) estimates that schistosomiasis is transmitted in over 78 countries in tropical and subtropical regions[1]. The three major species infecting humans are *S. mansoni*, *S. haematobium*, and *S. japonicum*. Three other species are capable of infecting humans to a lesser degree and are more locally distributed: *S. mekongi*, *S. intercalatum*, and *S. guineensis*[25].

S. mansoni and *S. haematobium* are endemic in Africa and the Middle East whereas only *S. mansoni* is prevalent in South America. *S. japonicum* is geographically restricted to East and Southeast Asia, predominantly found in the Philippines, Indonesia, China, and Japan. *S. mansoni* – *S. haematobium* and *S. bovis* – *S. haematobium* hybrids have been recently discovered in Africa and Europe[26–29]. Hybrid schistosomes are difficult to diagnose and potentially have a wider host spectrum[30,31] (**Figure 1.1**). While many cases are imported to Europe through migrants and travellers[32], the risk of an emergent endemicity in Europe is increased by the detection of *Biomphalaria* and *Bulinus* snail vectors[33,34]. Schistosomiasis infection can also cause significant economic losses represented by the loss of earnings and reduced productivity. *S. bovis* is one of the most important species responsible for infections in cattle and affects approximately 160 million animals in Africa and Asia, thus representing a significant financial burden as a result of livestock schistosomiasis[35,36].

Contact with contaminated water is necessary to contract schistosomiasis. Human waste contamination of local water sources contributes to disease prevalence in affected communities[37]. In some endemic regions, 60 – 80% of school-aged children and 20 – 40% of adults are actively infected[1,25]. The heaviest disease burden occurs in young school-aged children due to the amount of contact with infectious water[1] (**Figure 1.2**). However, recent reports have highlighted the burden on preschool-aged children as well as adolescents and adults, populations that have been often overlooked in monitoring and MDA efforts[38–42]. Adults with occupations that require contact with infectious water such as fishermen, and irrigation workers as well as women performing domestic chores are also at great risk of acquiring infection[1,3].

1.1.3 Clinical Aspects

1.1.3.1 Acute Schistosomiasis

Acute schistosomiasis, also referred to as Katayama syndrome, is a non-specific disease presentation observed in non-immune individuals[43]. Following percutaneous penetration of cercariae, the resulting systemic hypersensitivity is a reaction against migrating schistosomula and can occur from a few weeks to several months after the primary infection[20,44]. It is often characterized by an acute febrile stage (i.e.: fever, chills, myalgias) and may be associated with

pulmonary and intestinal symptoms. However, fever is not always present, and this stage of the disease may be difficult to diagnose[44]. Though travellers and tourists are most affected, people living in endemic areas for *S. japonicum* are susceptible despite a history of previous infections[20]. While most patients recover after 2 – 10 weeks, some develop more serious disease with symptoms including weight loss, hepatomegaly, and generalized rash[43].

1.1.3.2 Hepatic & Intestinal Schistosomiasis

Individuals infected with *S. mansoni* and *S. japonicum* can progress towards a chronic condition known as intestinal schistosomiasis[1]. Clinical manifestations are not due to adult worms, but rather from entrapped eggs in tissues such as the intestines, liver, spleen, lung, or cerebrospinal system[20]. Adult worms reside in the mesenteric veins and most of the eggs produced pass through to the intestinal lumen. However, some of the eggs may become entrapped in intestinal tissues, and this results in gastrointestinal features such as isolated mucosal hyperplasia, pseudopolyposis, microulcerations, and superficial bleeding[45–47]. Venous blood flow can also transport *S. mansoni* and *S. japonicum* eggs from the mesenteric veins to the portal vein where they become trapped in the surrounding periportal tissues[48]. Granulomatous inflammation around the eggs is a cardinal feature of intestinal schistosomiasis pathology[49]. These granulomas can cause significant enlargement of the spleen and liver, characteristic of hepatosplenic schistosomiasis[50,51]. This hepatosplenomegaly is often accompanied by hepatic fibrosis and portal hypertension[49]. Infected individuals are at significant risk of developing ascites, muscular loss, and hepatic failure[52].

1.1.3.3 Urogenital Schistosomiasis

Urogenital schistosomiasis is associated with *S. haematobium* which reside in the pelvic venous plexus. Most of the eggs of this species pass through the urinary bladder wall and are deposited in bladder tissue and genital organs. Continuous inflammatory reaction to the eggs causes inflammation, fibrosis, and fibrotic nodules referred to as sandy patches[53–55]. Blood in the urine (hematuria) is a classical sign of this form of the disease. Tissue inflammation can result in the thickening of the bladder wall and the development of pseudopolyps[48]. In fact, urogenital schistosomiasis is associated with squamous cell carcinoma in the bladder, and *S. haematobium* is classified as a carcinogen[56,57]. Bladder cancer is a frequent complication with an incidence rate of

3 – 4 cases per 100,000[58]. With approximately two-thirds of African cases caused by *S. haematobium*, urogenital pathology is a serious public health threat[59]. Inflammation and granuloma formation may also lead to hydronephrosis due to urine build-up, and thus result in kidney disease[60]. Glomerular disease is also observed in 15% of patients with hepatosplenic schistosomiasis with *S. mansoni* infections[61]. Chronic disease manifestations also include bladder calcification, secondary bacterial infection of the urinary tract, and renal failure[55].

The cervix, seminal vessels, prostate are also susceptible to egg deposition[62,63]. Infected men display increased levels of leukocytes and inflammatory cytokines in semen samples[64]. Both men and women affected by genital schistosomiasis are at higher risk of HIV transmission[65,66]. *S. haematobium* infection greatly impacts women's reproductive health. Female genital schistosomiasis (FGS) causes inflammatory lesions in ovaries, fallopian tubes, cervix, vagina, and vulva[67]. FGS is also associated with neovascularization and friable mucosa which can result in contact bleeding[68]. These symptoms may result in infertility and an increased risk of abortion. Male genital schistosomiasis (MGS) is less well characterized and often underreported. Symptoms include ejaculatory pain, blood in the semen, and infertility[69]. Semen analysis of infected patients may reveal the presence of *S. haematobium* eggs and signs of male infertility such as low or absent sperm counts[70,71]. Contrary to its female genital schistosomiasis, MGS symptoms and complications may resolve following praziquantel treatment[65,72].

1.1.3.4 Neuroschistosomiasis

Although rare, neuroschistosomiasis is the most common form of ectopic egg deposition and is one of the most severe clinical outcomes of schistosomiasis[25,48]. It is caused by inflammatory responses to eggs in the cerebral or spinal venous plexus[73,74]. Due to its smaller egg size, *S. japonicum* is responsible for most cerebral schistosomiasis cases, whereas *S. mansoni* and *S. haematobium* mainly affect the spinal cord[75]. However, CNS involvement occurs in only 2 – 4% of *S. japonicum* infections[21]. Spinal pathology resulting from *S. mansoni* and *S. haematobium* infections cause transverse myelitis or subacute myeloradiculopathy. This can result in paralysis, lumbar or leg pain, muscle weakness, sensory loss, and bladder incontinence[25,74]. *S. japonicum* is associated with cerebral granulomatous lesions and meningoencephalitic symptoms such as headache, vomiting, blurred vision, and epilepsy[20,25,76].

1.1.3.5 Pulmonary Schistosomiasis

Pulmonary schistosomiasis is another rare clinical presentation of the disease and results from ectopic egg deposition in the respiratory system. Due to portacaval shunting, venous blood can bypass the liver and reaches the vena cava through which *Schistosoma* eggs may be transported to the lung capillaries and induce granulomas in the perialveolar area[48]. Egg-associated pathology may result in pulmonary hypertension and cor pulmonale which is a condition that results in failure of the right heart ventricle. Pulmonary hypertension may be a result of direct mechanical obstruction by the eggs or due to vascular remodelling[77]. One form of pulmonary hypertension, pulmonary arterial hypertension, occurs in 5 – 10% of hepatosplenic schistosomiasis patients particularly due to *S. mansoni*. Schistosomiasis-associated pulmonary hypertension is one of the leading causes of pulmonary hypertension worldwide[77,78].

1.1.4 Diagnostics

Microscopic detection of eggs in stool (*S. mansoni* and *S. japonicum*) or urine (*S. haematobium*) samples is the gold standard for schistosomiasis diagnosis[79]. For intestinal schistosomiasis, the Kato-Katz thick smear assay is a quantitative, simple, and inexpensive method to detect eggs from prepared fecal specimen[80,81]. Urogenital schistosomiasis diagnosis requires a urine filtration technique[82]. The level of infection is expressed as eggs per 10 mL of urine or eggs per gram of stool[83]. However, there are several major drawbacks with this diagnostic method. With sensitivity rates less than 50%, low intensity infections and cases in areas of low prevalence are often missed[79,84]. In addition, there is a high degree of day-to-day variability and diurnal variation of egg excretions which renders conventional testing even more difficult[85,86]. The low sensitivity also hinders diagnosis in travellers or individuals who have received praziquantel treatment[48,87]. Modifications to the Kato-Katz technique have been proposed, but the main drawbacks persist[88,89]. Other parasitological procedures include concentration methods to increase the frequency of eggs in stool samples[90–92] or miracidium hatching tests[93,94], however these techniques are laborious, time-consuming, and not optimal for large-scale screening[95].

Routine serology tests measure serum IgG, IgM, or IgE against soluble worm antigen or crude egg antigen[20]. Antibody detection is useful in travellers with no prior history of exposure as many may test positive even 2 years post-treatment[96]. Seroconversion takes place typically from 4 – 8 weeks post-infection but can occur up to 22 weeks[97]. Parasite antigens may also be detected in blood, urine, or sputum. Two main targets are circulating cathodic antigen (CCA) and circulating anodic antigen (CAA)[98]. Both antigens can be detected early after exposure[99] and can be detected in blood and urine samples. Antigen levels are indicative of infection intensity but detection is not sensitive in light infections[100]. Thus, this method is less suitable for areas with low endemicity or for travellers. CCA detection in urine was developed as a rapid lateral flow assay for *S. mansoni* but is less accurate for *S. haematobium* diagnosis[79]. It has been successfully used for mapping *S. mansoni* prevalence before and after MDA campaigns[101] but is not suitable for areas co-endemic for both *S. mansoni* and *S. haematobium*[102]. CAA detection is considerably more sensitive than CCA testing, but it is also more expensive[103]. For *S. japonicum* detection, four circulating antigens have been identified and a highly sensitive and specific sandwich ELISA has been described using chicken egg yolk IgY[104].

In high resource settings, molecular-based diagnostic methods have been implemented for detection in stool and urine[105,106]. Multiplex polymerase-chain reaction (PCR) is particularly advantageous for infections in travellers[107]. Several other PCR-based assays are being developed such as nested PCR (nPCR), real-time quantitative PCR (qPCR), and digital droplet PCR (ddPCR)[108]. Schistosome-specific DNA and RNA targets include egg DNA, circulating cell-free parasite DNA, and circulating microRNAs (miRNAs)[79]. Loop-mediated isothermal amplification (LAMP) is a technique that allows for early detection of *S. mansoni* mitochondrial DNA in stool samples[109]. While promising, molecular methods are currently not a practical tool in the field due to infrastructure requirements in most endemic regions rendering them inaccessible.

1.1.5 Treatment and Control

While schistosomiasis is endemic in many areas around the world, a few countries had success in eliminating the disease through a multifaceted approach combining increased sanitation, targeted vector control, and mass drug administration campaigns. Successful schistosomiasis control

programs include St. Lucia and other Caribbean islands, Morocco, Tunisia, Iran, Japan, and China[110,111]. This demonstrates that schistosomiasis elimination is an achievable goal that will require the integration of various control measures.

1.1.5.1 Water, Sanitation, and Hygiene (WASH)

Many individuals living in endemic regions for schistosomiasis are subject to inadequate access to water and sanitation, poor infrastructure, and unsafe hygiene practices. These factors increase the risk of transmission, and such areas experience high burden of disease[37]. The implementation of WASH practices requires the installation of equipment such as toilets, latrines, and sewage treatments[112]. Barriers include cost, a lack of health professionals and government involvement, as well as a lack of advocacy[113–115]. For example, latrines may be feared, found to be inconvenient, or poorly maintained all of which can reduce their potential impact[116]. Successful implementation requires behavioural changes and educational programs to improve disease knowledge and encourage safe practices[117]. Safe contacts with water also need to be made available as transmission can remain high even with adequate installations[118]. However, schistosomiasis infection rates have been significantly lowered in some countries where WASH has been introduced, and where residents had access to adequate sanitation[119]. Interventions such as proper excreta disposal, access to safe drinking water, and improved handwashing practices helped decrease community transmission in Tanzania and Oman[120,121]. Thus, community participation and intersectoral collaboration are required for the successful implementation and sustainability of WASH interventions to reduce schistosomiasis transmission[122].

1.1.5.2 Vector Control

Freshwater snails are the intermediate hosts of *Schistosoma* spp. The genera *Biomphalaria*, *Bulinus*, and *Oncomelania* are associated with *S. mansoni*, *S. haematobium*, and *S. japonicum* respectively[20]. Elimination of infected snails reduces the risk of reinfection and provides an opportunistic window for treatment campaigns when transmission is low[123]. The use of molluscicides, habitat change, predators (fish and crustacea), and biological competitors are all strategies to reduce host snail populations[25,124]. Another strategy involves the introduction of schistosome-resistant snails into the wild snail population[125]. Most commonly, the molluscicide

niclosamide, a licensed pesticide, is widely used for snail control[126]. The drug kills snails at low concentrations and is non-toxic to humans, however it is toxic to certain freshwater fish and amphibians[127,128]. This may give rise to ecological and economic concerns. The difficulty of such environmental control is increased due to climate change[129]. Regular and long-term treatments are required as snail populations are rarely eliminated and may recolonize their original habitats[20,110]. As niclosamide treatments are expensive and require repeated use, this strategy is not considered rapid or cost-effective[48]. However, long-term management of the snail populations in Morocco and Japan played a considerable role in the elimination efforts in these countries, and it continues to be used in Egypt and China[110]. Therefore, affordable snail control is an important approach to achieve schistosomiasis elimination and should be considered for use in combination with MDA campaigns[130].

1.1.5.3 Praziquantel

Earliest forms of treatment for schistosomiasis were trivalent antimonial compounds such as tartar emetic[131,132]. Antimony potassium tartrate induced muscle paralysis in female *S. mansoni* and *S. haematobium* worms, thus leading to a separation of adult worm pairs in the blood vessels[133,134]. Despite their effectiveness, patients treated with antimonial compounds often presented with severe side effects. Several other drugs such as lucanthone, niridazole, and hycanthone were developed but were later discontinued due to side effects[135]. Metrifonate is a cholinesterase inhibitor which targets the schistosome nervous system and is currently used as an insecticide[136]. It was effective against *S. haematobium*, but the requirement of several doses led to its commercial discontinuation. In the late 1960s, Pfizer developed oxamniquine and it was effective against *S. mansoni* schistosomula and adult worms[134]. The drug can be administered orally as a single dose and damages parasites by disrupting cell division and protein synthesis[1,137]. Oxamniquine is not used to treat any other diseases and remains on the WHO List of Essential Medicines. It was used extensively until the late 1990s in Brazil where only *S. mansoni* is present. However, due to concerns with cost and evidence of drug resistance, it was later replaced by praziquantel as a first-line treatment[138].

Praziquantel (PZQ) was developed in Germany in the mid-1970s in the laboratories of Bayer AG and Merck KGaA, and then later moved into clinical trials and large-scale use a decade later[1]. It is

the cheapest and most effective medication with a reported cure rate of 60 – 90% after a single oral dose (40mg/kg)[139]. PZQ is effective against the adult stage of all three main schistosome species and can also be used to treat veterinary cases[134]. The drug is well-absorbed and is effective as soon as 1 hour after ingestion. However, it can undergo extensive first-pass hepatic clearance wherein approximately 80% of the drug is metabolized by the liver[48]. PZQ causes mild side effects and has low toxicity. It is also safe for use in young children and pregnant women[140,141]. The WHO reported that approximately 76.2 million school-aged children and 19.1 million adults were treated with PZQ in 2018[142]. While increased coverage over the past 20 years has helped to reduce disease prevalence, the COVID-19 pandemic has disrupted preventative chemotherapy treatment and may have impacted the progress made in schistosomiasis control in some countries[143,144].

Praziquantel is thought to induce paralysis in adult schistosomes by altering calcium ion transport in the tegument and increasing cellular permeability[145]. It was hypothesized that β subunits of the voltage-gated calcium channels are responsible[146]. The uncontrolled muscle tension leads to dislodgement of the worms from the mesenteries to the liver where vacuolization and disintegration of the parasite can occur[147,148]. Morphological changes along the tegument also allow for greater antigen expression and the development of protective immune responses[134]. The precise mechanism of action of PZQ was debated and unknown for decades. Through genetic analyses, ligand-based screening, and targeted mutagenesis, the transient receptor potential melastatin ion channel ($TRPM_{PZQ}$) in schistosomes was recently identified as the molecular drug target of PZQ[149,150]. This provides critical information for monitoring drug resistance as $TRPM_{PZQ}$ is responsible for sensitivity to PZQ[149]. These data also explain the lack of effectiveness of PZQ against the liver fluke of the *Fasciola* genus, which is the result of a single amino acid change in the $TRPM_{PZQ}$ binding pocket[150].

Standard praziquantel treatment is difficult in pediatric populations under the age of 6[151]. The anti-schistosomal properties of PZQ are attributable to the (*R*)-praziquantel stereoisomer whereas the (*S*)-praziquantel produces the bitter quality of the drug which makes it difficult for children under 5 – 6 years of age to ingest[152]. Thus, compliance is an important issue in this age group with the currently available 600 mg tablet[153]. Initiatives are being launched to change the synthesis of the drug and remove the (*S*) stereoisomer[154]. In the meantime, PZQ is administered as crushed tablets

mixed with food[155]. Since 2012, the Pediatric Praziquantel Consortium (PPC) has been established to develop a pediatric formulation of the drug suitable for preschool-aged children[156]. As part of the programme, a taste study in Tanzanian children aged 6 to 11 years and a phase II dosage study in *S. mansoni*-infected children in Côte-d'Ivoire have been conducted[157]. This phase II dose-finding study tests two orodispersible tablets (ODTs) developed by the PPC. One contains only the (*R*)-praziquantel isomer (L-PZQ ODT) and the other contains a racemic mixture (rac-PZQ ODT) in a 1:1 ratio. Both tablets are 150 mg which is much smaller than the current form of the drug[158]. Cure rates were highest in the groups receiving L-PZQ ODT 60 mg/kg (89.7%), commercially available PZQ 3x20 mg/kg (89.5%), and L-PZQ ODT 45 mg/kg (86%)[158]. These higher doses correlate with the findings of a pharmacokinetics and pharmacodynamics study which tested higher doses of PZQ (40 mg/kg vs 60 mg/kg) in Ugandan children aged 3 – 8 years. This study suggested that higher doses were needed in younger populations to achieve a cure rate of ≥85%[140]. The praziquantel in preschoolers (PIP) phase II trial is in the recruitment phase to test doses of 80 mg/kg against the standard 40 mg/kg dose in Ugandan children under 4 years of age[159].

While praziquantel is a very effective drug to treat schistosomiasis, there are important limitations to consider. For example, the drug is only effective against the adult worm stage and is unable to kill juvenile worms[160]. Furthermore, PZQ treatment does not prevent re-infection and is ineffective against parasite eggs[161]. Reduced drug sensitivity has been observed in areas of heavy use[162] and diminished susceptibility has been reported in endemic foci for *S. mansoni* in Egypt and Senegal[163–165]. This phenomenon was also reproduced experimentally in both *in vitro* and *in vivo* studies[166,167] and in mice receiving repeated *in vivo* PZQ treatments[168]. A recent study has stipulated that PZQ efficacy is reduced in a low-protein diet mouse model[169]. These findings may have implications in endemic areas for schistosomiasis where protein deficiency and malnutrition are concurrent public health problems, particularly in children. Taken together, overreliance on PZQ should not be overlooked and the development of alternative chemotherapeutic strategies has been encouraged[170].

1.1.5.4 Novel Drug Development

There is a growing need to develop new drugs and to identify novel drug targets for schistosomiasis. Due to its short half-life and inefficacy against larval schistosomes, praziquantel

cannot be administered as a prophylactic drug. The Chinese plant *Artemisia annua* produces the extract artemisinin which has three main derivatives including artemether (ART), artesunate (ASE), and dihydroartemisinin (DHA)[171]. The antimalarial properties of these derivatives have been well established[172] but are also effective against schistosomula in the early stages of infection[173,174]. ART targets several molecular events including glycogen metabolism, Ca^{2+}-ATPase activity, and antioxidant machinery[175–177]. ASE targets the enzyme thioredoxin gluthathione reductase (TGR) which is important for detoxification[178]. DHA affects oviposition by damaging the worms' genital system[179]. Administration of ART, ASE, or DHA can inhibit juvenile parasites independently and in combination[148]. Co-administration of PZQ with ART or ASE provides better efficacy than PZQ treatment alone[180]. Another drug in development is the anti-malarial, mefloquine (MEF), which is an amino alcohol compound. It is effective against all three major *Schistosoma* species. MEF is effective against both juvenile and adult worms and targets the parasite's gastrodermis and reproductive systems as well as the tegument[181]. MEF also reduces glycolytic activity in schistosomula[182]. MEF exhibits synergistic effects with both PZQ and artemisinin derivatives. In animal models, Combinations with either PZQ or ASE were highly effective against *S. mansoni* whereas the MEF-ASE regimen also demonstrated activity against *S. haematobium*[181,183]. MEF has also shown to be effective in pregnant women infected with *S. haematobium*[184]. A recent study in a mouse model has highlighted the synergistic effect of itraconazole (ITZ) with PZQ[185]. ITZ is a cytochrome P450 (CYP450) inhibitor which enhanced PZQ activity against *S. mansoni* infection and alleviated liver pathology. In the liver, praziquantel undergoes extensive first-pass metabolism due to CYP450 enzymes[186]. Therefore, it is suggested that ITZ, like other CYP450 inhibitors, reduces PZQ metabolism and increases systemic exposure[187–189]. Finally, the sequencing of the genomes and transcriptomes of the three major schistosome species has allowed for the identification of novel drug targets[190–192]. New anti-schistosome drugs are being developed by targeting enzymes involved in the apoptotic cell death pathway, tyrosine kinase signalling, and septin structure[148]. The discovery of these targets will aid in the development of novel drugs that could potentially enhance or surpass PZQ activity and lead to novel therapeutic avenues.

1.1.6 Co-Infections

1.1.6.1 Malaria

Malaria is a vector-borne parasitic disease caused by protozoa of the *Plasmodium* genus and transmitted by *Anopheles* mosquitos. Nearly 40% of the world's population is at risk of infection[193]. The sub-Saharan African region accounts for 95% of global cases and over 90% of deaths. Children under 5 years of age and pregnant women make up the most vulnerable populations[194]. Schistosomiasis and malaria are two of the most important parasitic infections and are geographically co-endemic in large parts of sub-Saharan Africa. Co-infections are also common in school-aged children[195]. The impact of *Plasmodium-Schistosoma* co-infections on pathophysiology is not well understood. Pro-inflammatory and type 1 immune responses are necessary to control *Plasmodium* infection, however helminth infections typically downregulate these pathways and modulate the immune system toward anti-inflammatory and type 2 immune responses[196,197]. It remains unclear whether this shift prevents severe pathology or interferes with clearance of *Plasmodium*[198]. Studies conducted in sub-Saharan Africa have been contradictory, and both antagonistic[199–201] and synergistic[202–204] effects on pathology have been reported. Recent studies continue to present contrasting results. A study conducted in Zimbabwe examined co-infections in pre-school aged children[205]. In co-infected children, the progression of severe and complicated malaria was seven times more likely. This finding highlights the need to include pre-school aged children in national schistosomiasis control programmes. However, in a study observing different age populations in Côte-d'Ivoire, no exacerbation of disease was noted in participants aged 5 – 18 years or in adults[206]. These varied observations may be impacted by participant age group, clinical phase of the disease, duration of infection, infection intensity, and the host's immune system[207].

1.1.6.2 Human Immunodeficiency Virus Type 1 (HIV-1)

Sub-Saharan Africa accounts for over 90% of global schistosomiasis cases and more than 68% of the people infected with human immunodeficiency virus type 1 (HIV-1)[208]. Epidemiological studies have shown that 17% of HIV-infected people in sub-Saharan Africa are seropositive for schistosomiasis[209]. Male and female patients with urinary schistosomiasis are at greater risk of

acquiring and transmitting the virus[210]. In Malawi, increased susceptibility in women was suggested to be due to submucosal inflammation surrounding parasite eggs in cervicovaginal biopsy samples[211,212]. In contrast, studies in Kenya and Uganda examining the impact of intestinal schistosomiasis observed down-regulation of anti-HIV immune responses such as decreased cellular production of IL-4 and IL-10 cytokines as well as diminished $CD8^+$ T cell responses[213,214]. Viral transmission is further complicated as intestinal schistosomiasis increases the presence of HIV receptors CCR5 and CXCR4 on $CD4^+$ T cells[215]. Mother-to-child HIV-1 transmission is also increased in mothers co-infected with both HIV and *Schistosoma* spp.[216]. *S. mansoni* egg excretion was found to be decreased in patients co-infected with HIV-1. This effect seems to be correlated with decreased pools of $CD4^+$ T cells but rates of both egg excretion and $CD4^+$ T cell counts were increased following antiretroviral treatment (ART), thus supporting a role for T lymphocytes in egg excretion[208,217,218]. HIV-1 viral load in patients co-infected with schistosomiasis appears to differ over the course of infection. Shortly after infection with the virus, viral load are high but later decrease to levels lower than in patients with HIV-1 alone[219]. A study in Tanzania examining the impact of ART in co-infected patients suggested that ART is the dominant driver of viral load and outweighs the impact of schistosome infection[220]. Finally, HIV-1 infection does not adversely impact praziquantel treatment[221]. However, anti-schistosome treatment may help control HIV-1 infection by reducing viral load, reducing genital lesions in cases of urinary schistosomiasis, and restoring mucosal and/or systemic anti-viral immunity[222].

1.1.6.3 Tuberculosis (TB)

In 2016, tuberculosis (TB) was the leading cause of death due to a single infectious agent[223]. Reports and data on TB control on the African continent are limited despite Nigeria ranking fourth in the world for the highest TB burden[224]. In 2011, a study conducted in Nigeria found that 13.5% of *Mycobacterium tuberculosis* (Mtb) isolates were multidrug-resistant (MDR) which surpassed WHO reports that indicated a 4.3% incidence rate of MDR-TB in the area[224]. A cross-sectional study in Tanzania observed that 34% of positive cases for TB also presented with *S. mansoni* infection[225,226]. With a rise in *S. haematobium* infections in south Corsica and in migrants[227,228], physicians are cautioned to consider both urogenital tuberculosis and schistosomiasis infections[229,230]. Maternal schistosomiasis infection is negatively associated with IFN-γ TB

responses in core blood mononuclear cells but positively associated with cross-placental transfer of TB-specific IgG[231]. A study in Kenya observed that co-infected individuals maintained functional T_H1 responses by $CD4^+$ T cells[232]. *S. mansoni*-infected individuals with latent TB infection had higher frequencies of IFN-γ^+ Mtb-specific $CD4^+$ T cells whereas those with active TB had higher frequencies of $GATA3^+$ $CCR4^+$ Mtb-specific $CD4^+$ T cells expressing T_H1 cytokines[232]. Interestingly, murine studies revealed that *S. mansoni* infection reduces the protective efficacy of BCG vaccination and increases susceptibility to *Mycobacterium bovis* BCG infection[233,234]. However, this same phenomenon was not observed in a Ugandan clinical trial testing a new TB candidate vaccine, MVA85A, in BCG-vaccinated adolescents[235]. *S. mansoni* infection had no impact on T and B cell responses in trial participants[235]. Finally, a longitudinal study in children in Eswatini showed that *S. haematobium* infection induced changes in DNA methylation and immune phenotype that inhibited TB-specific immune control and persisted for at least 6 months after praziquantel treatment[236].

1.1.6.4 Soil-Transmitted Helminths (STH)

Soil-transmitted helminths affect more than a quarter of the global population[237]. In several regions, schistosomiasis is co-endemic with soil-transmitted helminths, such as *Ascaris lumbricoides* and *Trichuris trichiura*, and hookworms, such as *Necator americanus* and *Ancylostoma duodenale*[238]. Polyparasitism for helminth infections are widespread in both animals and humans[239]. Risk factors for such co-infections include low socio-economic conditions, poor hygienic standards, and lack of clean water[240–243]. Moreover, genetic components account for increased accumulation of helminth infections in certain individuals[244,245]. An epidemiological study described that a positive association between *S. mansoni* and hookworm infections is dependent on the socio-economic status of the patient among other factors[246]. Studies conducted in Brazil yielded contrasting results for co-infections. One showed that *S. mansoni* egg counts correlated positively with hookworm infection but negatively with *Ascaris* and *Trichuris* egg counts[247]. However, data from schoolchildren showed that co-infection with *S. mansoni* and *T. trichiura* were associated but not *S. mansoni* and *A. lumbricoides*[248]. Despite a significant degree of antigenic cross-reactivity, humoral responses against *Schistosoma* antigens were not altered in co-infected patients[249]. In addition, peripheral blood mononuclear cells (PBMCs) from hookworm

or *A. lumbricoides* patients responded to stimulation by schistosome antigens[250]. Nonetheless, co-infections can have negative effects in affected individuals. A study conducted in the Philippines observed that children co-infected with hookworm and *S. japonicum* suffered from higher levels of anemia compared to single infections[251]. Children are a vulnerable population as STH infections can occur in the first year of life and schistosomiasis causes high disease burden in school-aged children[238]. More work is needed to study these co-infections and their impact on health.

1.1.6.5 Hepatitis

Schistosomiasis, hepatitis B virus (HBV), and hepatitis C virus (HCV) co-infections are reported in many countries where schistosomiasis is endemic[252]. Globally, the combined prevalence rate of HBV and HCV infection exceeds 50% in most Asian and African regions[253], and these two viral pathogens are of particular interest in concomitant infections with *Schistosoma* spp. since all three infections can cause liver damage[254]. In rural populations of China, it was found that 25% of *Schistosoma* seropositive individuals were co-infected with HBV compared to 0.6% in seronegative patients[255]. In Egypt, prevalence of schistosomiasis and HCV infection is high, and in a study of 200 chronic HCV patients, 12.8% were seropositive for both *Schistosoma* and HBV[252,256]. Furthermore, HCV and *S. mansoni* antibodies were detected in 40.2% of Egyptian wastewater treatment plant workers[257]. The presence of one disease does not necessarily predispose for the other[254]. The higher incidence rate of hepatitis in *Schistosoma*-infected individuals is associated with modes of transmission for HBV and HCV such as parenteral schistosomiasis treatment using unsterile syringes, blood transfusions for anemia and endoscopic interventions[258–261]. Community-wide treatment campaigns previously consisted of intravenous tartar emetic administration and led to a large reservoir of HCV in countries such as Egypt[262]. Chronic schistosomiasis infection induces liver damage, and this is further complicated by HBV infection in which liver fibrosis can progress towards cirrhosis[263]. However, HBV vaccines are immunogenic in *Schistosoma*-infected individuals despite reduced responses to vaccination[264]. Vaccination against HBV would at least prevent triple infection with *Schistosoma*-HBV-HCV[254]. Meanwhile, *S. mansoni* infection increases HCV morbidity by increasing viral RNA titers, histological activity, the incidence of cirrhosis and hepatocellular carcinoma, and mortality rates in patients[265]. Certain HBV therapies have a positive effect on schistosomiasis infection and improve liver fibrosis markers[263]. Additionally, an Egyptian clinical trial examining the effect of

pegylated interferon (PegIFN-a2a) as a treatment for HCV was not affected by schistosomiasis infection[266].

1.2 SCHISTOSOMIASIS INFECTION & HOST IMMUNITY

The *Schistosoma* lifecycle is complex and has several distinct stages. Schistosomiasis has evolved and spread in parallel with human evolution and migration over thousands of years and many of the clinical features of the disease are caused by host immune responses to different stages of the helminth's lifecycle. However, these responses are necessary to contain the infection, despite failing to eradicate it, and achieve a balanced host-parasite inter-relationship. In order to develop vaccines and treatments to eliminate the parasite, a better understanding of the infection and host immune responses is needed. The following sections outline the parasite lifecycle, its interactions with the host immune system, and features of protective immunity.

1.2.1 *Schistosoma* Lifecycle

Schistosome species are transmitted by aquatic snails in freshwater sources[267]. The intermediate hosts of *S. japonicum* are *Oncomelania* snails of the Popamiopsidae family. These snails have differentiated sexes and are capable of breathing under water. Conversely, *S. mansoni* and *S. haematobium* target Planorbidae snails of the genera *Biomphalaria* and *Bulinus* respectively. The planorbid snails are air-breathing and hermaphroditic[1]. The lifecycle starts when parasite eggs are excreted into freshwater from infected hosts and hatch to release free-living ciliated miracidia[25]. Egg hatching is induced by changes in osmotic pressure and the release of an egg-hatching enzyme[268]. Miracidia are one of two non-feeding larval stages and depend on glycogen stores as an energy source while searching for a snail host[269]. Miracidia have a short lifespan and their infectivity and motility decrease with age[270]. Upon recognition of snail mucus, they can increase their angular velocity to increase the odds of finding a suitable host[271]. Within the snail, the parasite undergoes asexual replication through mother and daughter sporocyst stages. This stage of the lifecycle requires 4 – 6 weeks prior to the release of thousands of infectious cercaria[25]. Cercaria also depend on glycogen stores which can be depleted within hours despite a lifespan of 2 – 3 days[272]. This free-living parasite stage possesses a powerful bifurcated tail to search for hosts and

acetabular glands that provide secretions needed for host skin penetration[1]. Linoleic acid and arginine present on the skin serve as chemical attractants[273]. Upon penetration of the skin, cercaria undergo morphological and physiological changes to adapt to the new environment and transform into schistosomula which remain in the skin for a short period before entering the blood vasculature. A migration study performed in hamsters showed that the majority of schistosomula entered circulation between 60 – 80 hours post-exposure[274]. The schistosomula then migrate passively to the lung where they undergo more morphological changes in the capillary beds[79,275]. The migration time to the lung differs between *Schistosoma* species[276]. From the lungs, the elongated schistosomula flow through the blood in the pulmonary veins and eventually reach the liver where they will complete their maturation into adult worms. The parasite doubles in weight every 2.3 days for 2 weeks[277]. At this stage, the sex of the worms can be determined. Before exiting the liver, male and female worms lie *in copula* where the female fits within the gynecophoric canal of the male. These worm pairs are then able to migrate to their final anatomical locations such as the mesenteric veins for *S. mansoni* and *S. japonicum* and the vesical vein plexus of the bladder for *S. haematobium*[1]. Interestingly, the adult worm pairs are necessary for proper reproductive development of the female. The male schistosome is responsible for transducing signals to stimulate the development of mature females[278,279]. Recently, it was found that, under the control of transcription factor GLI1, a male-derived nonribosomal peptide synthetase (Sm-NRPS) is induced in paired male worms. Sm-NRPS then produces the dipeptide β-alanyl-tryptamine (BATT) to induce female sexual development[280]. After 4 – 6 weeks post-infection, mature female schistosomes continuously produce eggs at varying rates depending on the species (150 eggs/day for *S. haematobium*, 300 eggs/day for *S. mansoni*, and 1000 eggs/day for *S. japonicum*)[1,79]. Eggs can traverse through the blood vessel wall by releasing enzymes and enter either the intestinal or bladder lumen from which they will be excreted back into freshwater to perpetuate the lifecycle[276,281]. However, up to two thirds of these eggs are carried into the circulation and are deposited in multiple organs such as the liver and bladder, thus causing chronic pathology[281,282] (**Figure 1.3**).

1.2.2 Immunopathology

Schistosomiasis murine models have been extremely valuable to decipher mechanisms of immunopathology. The acute phase of the disease initiates a moderate T_H1 response to parasitic antigens[210,283]. This is characterized by the release of pro-inflammatory cytokines such as TNF-α, IFN-γ, IL-1, and IL-6[207,284]. Schistosome eggs stimulate the immune system through the release of soluble egg antigens (SEA) which induce the activation of resident macrophages. These macrophages release inflammatory cytokines that recruit leukocytes, neutrophils, and monocytes[285]. The T_H1-type cytokines released during the acute phase mediate the establishment of early granulomas[283]. This is later followed by a shift in the immune response towards a T_H2 phenotype. Egg antigens such as omega-1 condition dendritic cells to prime type-2 responses[286]. This shift in the pattern of the host immunity is necessary to dampen the inflammatory responses induced by SEA. Mouse models with impaired T_H2 immunity experienced severe disease and premature death due to an unchecked T_H1 response[287–289]. Therefore, SEA mediate a shift towards T_H2 responses which exert anti-inflammatory effects and regulate T_H1-mediated immunopathology. Classical T_H2 cytokines, IL-4, IL5, and IL-13, are upregulated and promote granuloma formation and help establish chronic infection[290]. IL-13 is responsible for granulomatous inflammation and liver fibrosis[291,292]. Alternatively activated macrophages and the regulatory cytokine IL-10 serve to dampen the T_H2 responses and limit granulomatous inflammation[288,293]. These immunomodulatory mechanisms serve to limit granulomatous formations and reduce fibrosis of newly deposited eggs in later stages of the infection[294]. Both T_H1 and T_H2 phases are regulated by IL-10 production by macrophages and T cells[295] (**Figure 1.4**).

1.2.3 Immunomodulation

In some cases, schistosome parasites are able to survive within immunocompetent hosts for over 30 years[25,296]. Host immunity is dominated by T_H2 and regulatory responses[297,298] which are driven by diverse parasite immunomodulators to promote survival[299]. All *Schistosoma* lifecycle stages produce immunoregulatory molecules.

1.2.3.1 Cercaria and Schistosomula in the Skin

The cercarial larval stage establishes infection after skin penetration through the release of excretory/secretory (ES) molecules[300,301]. These ES products promote T_H2 responses in the skin by predisposing bone marrow-derived dendritic cells (BMDCs) to upregulate expression of major histocompatibility complex (MHC) class II, CD40, and CD86, and secretion of IL-4 by $CD4^+$ T cells[302,303]. *S. mansoni* cercaria can mediate histamine release via the transcriptionally controlled tumor protein homolog (SmTCTP)[304]. This schistosome protein helps control blood flow and histamine is a vasodilator and capable of inducing IL-10 production[299,303,305]. Skin-stage schistosomula ES products can lead to apoptosis of $CD4^+$ and $CD8^+$ T lymphocytes via the Fas/FasL pathway[306]. *S. mansoni* and *S. japonicum* cercaria express the proteins Sm16 and Sj16, trematode-specific helminth defense molecules (HDMs)[307,308]. Sm16 induces the production of IL-1 receptor antagonist a (IL-1ra) which competes with IL-1α and IL-1β to block IL-1-mediated inflammatory responses in the skin[309]. Sj16 has also shown immunomodulatory effects[310]. *S. mansoni* cercaria induce the production of prostaglandin PGE2 by keratinocytes which stimulates the production of IL-10[311,312].

1.2.3.2 Schistosomula and Adult Worms in the Bloodstream

Schistosome worms reside in the bloodstream of their mammalian hosts and therefore must evade immunological pressure from their surrounding environment. The tegument surface is a major site for host-parasite interactions and plays an important role in parasite survival and immunomodulation[299]. Interestingly, there are no signs of overt inflammation around intravascular schistosomes[313]. This can be achieved through the control of danger-associated molecular patterns (DAMPs). Adenosine triphosphate (ATP) is a potent pro-inflammatory mediator when released in the extracellular environment[314,315]. The *S. mansoni* ATP diphosphohydrolase, SmATPDase1, cleaves exogenous ADP and ATP[316,317]. SmNPP5 is an essential protein for schistosome survival that cleaves nicotinamide adenine dinucleotide (NAD) and blocks NAD-induced T cell apoptosis[318]. The parasite worms express other tegumental proteins such as the proteases SmCalp1 and SmCalp2 which are responsible for cleavage of host blood proteins and allow for unimpeded movement within the vessels[319,320]. Other schistosome proteins serve to evade the host's complement system[321,322]. Aside from tegumental proteins, gastrointestinal and ES molecules are also involved in immune evasion. The cysteine protease cathepsin B1 has immune-skewing

properties and protects mice from lethal injection of LPS by preventing the release of several inflammatory mediators and blocking TLR3 and TLR4 signalling[323]. Glycolytic and redox enzymes as well as heat shock proteins and cyclophilins also exert immunomodulatory effects on host immune cells[299].

1.2.3.3 Eggs in Tissue

Most eggs are not excreted by the host but rather become trapped in tissues where they are responsible for chronic pathology. These eggs induce T_H2-type responses that lead to the formation of granulomas[49]. Analysis of SEA revealed the protein IL-4-inducing principle of schistosome eggs previously denoted as alpha1 (α1) (now IPSE/α1)[324]. IPSE/α1 is a glycoprotein enriched inside the eggshell and secreted to surrounding host tissue. It is recognized by human antibodies as a major immunogenic component of SEA[325]. IPSE/α1 attenuates inflammation by stimulating IL-4 & IL-13 production, blocking chemokine activity, and translocating to the nucleus to modulate transcription[326–328]. IL-4 and IL-13 production in basophils helps generate anti-inflammatory, alternatively activated macrophages and can inhibit the release of pro-inflammatory cytokines[326]. IPSE/α1 may also play a role in the generation of regulatory B cells[329]. Another important egg-stage protein is omega-1 (ω1). This secreted glycoprotein drives human monocyte-derived dendritic cells (MoDCs) to polarize naïve $CD4^+$ T cells towards a T_H2 phenotype. Omega-1 also downregulates CD83 and CD86 expression on DCs and inhibited IL-12 production[330]. It is also recognized by human antibodies[331]. Other egg antigens such as kappa-5, chemokine-binding glycoprotein (SmCKBP), and high mobility group protein 1 (SmHMGP1) modulate the host immune system to favour parasite survival[299].

1.2.4 Protective Immunity

In endemic regions, people are repeatedly exposed to infectious cercaria putting them at risk for both super-infection and reinfection post-treatment. While children remain susceptible to recurrent infections, adults gradually become resistant[332–334]. Indeed, protective immune responses to schistosomes can develop slowly over a period of 10 – 15 years in chronically-exposed individuals[48]. Anti-*Schistosoma* immunity is thought to be responsible for this resistance to reinfection in adulthood rather than a reduced contact with water[334]. While resistance in mice is

characterized by T_H1 responses, resistance to reinfection in humans is associated with T_H2 immune responses mediated by parasite-specific IgE, eosinophils and the cytokines IL-4 and IL-5[335–337]. In contrast, susceptibility to reinfection is correlated with IgG4[338]. However, this resistance is partial and sterile immunity is not observed. Praziquantel treatment increases these correlates of resistance and helps achieve a partial acquired immunity[339,340]. Interestingly, the balance between IgG4 and IgE is affected differentially in children and adults[341]. Following treatment, IgG4 levels in adults decrease while IgE levels remain stable or increase whereas children experience increases in both antigen-specific IgE and IgG4. Studies suggest that natural or chemical worm death causes the release of parasitic antigens that sensitize the host to future, migrating schistosomula[342]. In addition, successive treatments can increase the interval between reinfections[343]. One hypothesis for the difference between age groups for resistance and the long development of partial immunity is that children are exposed to fewer dying worms which have an average lifespan of 3 – 10 years[48]. *S. mansoni* adult worm-associated tegument-allergen-like (TAL) proteins have been suggested to be potential targets of protective IgE antibodies[344]. IL-4-producing T follicular helper (Tfh) cells may mediate these effector functions[345].

It was first observed in experimental models that infected hosts develop an 'immunity' to subsequent cercarial challenges[346–348]. Adult worms might protect their hosts against new infections and this phenomenon was termed concomitant immunity[349,350]. This immunity was directed against skin and lung-stage schistosomula and mediated by the adult worm population to control the infection intensity within the host. Larval worms are more susceptible to host immune responses compared to their adult counterparts that possess several immune evasion mechanisms[351]. Thus, adult worms may help direct an immune response against migrating schistosomula to reduce intraspecific competition[350].

Whereas partial immunity can be gained over a long period either through chemotherapy or a history of repeated infections, natural resistance has been observed in an endemic region of Brazil[352,353]. Stool samples were egg-negative despite exposure to infectious water[354]. These naturally resistant individuals are termed 'endemic normal' (EN)[355]. PBMCs from EN individuals mount both T_H1 and T_H2 responses to stimulation with parasite antigens characterized by elevated IFN-γ secretions, thus classifying them as an immunologically distinct group[353]. They also produce

higher levels of IgE against schistosomula tegument than drug-induced resistant individuals[356]. IgG4 levels to tegument and egg antigens are also low[354]. Many factors may explain this natural resistance such as prenatal tolerance induction, low-intensity infections, single sex infections, aborted infections prior to worm maturation, or self-cure[355]. Interestingly, serum analysis of EN individuals aided in the identification of two schistosomiasis vaccine candidates Sm-TSP-2 and Sm29, and this distinct group may help guide future vaccine discovery[357,358].

1.3 SCHISTOSOMIASIS VACCINES

In order to achieve control and elimination of schistosomiasis, an effective vaccine is necessary as part of a multifaceted approach including the different control measures highlighted earlier[6,7]. For the past few decades, there has been substantial effort made to develop schistosomiasis vaccine candidates, however very few have progressed to clinical trials and none has proceeded to licensure[95,359]. Schistosomiasis has ranked among the top ten diseases for which the need for a vaccine has been deemed urgent by a panel of experts[360]. Early work with a radiation-attenuated cercaria (RA) vaccine showed promise and led the development of several molecular vaccine candidates[361]. Murine immunization with these gamma-irradiated cercaria induced high levels of protection but this approach could not be translated to humans[362]. However, these experiments provided evidence that optimal protection requires both T_H1 and T_H2 associated responses[363]. The following sections outline clinical and pre-clinical development of schistosomiasis vaccines as well as novel approaches to accelerate the vaccine pipeline.

1.3.1 Vaccines in Clinical Development

1.3.1.1 Schistosoma haematobium *28-kDa Glutathione S-Transferase (ShGST28)*

The glutathione S-transferase (GST) protein is involved in the reduction of parasite oxidative stress[364]. It also plays a role in fatty acid metabolism and prostaglandin D_2 synthesis[359]. The antigen is distributed in the cytosol and along the genital organs, tegumental surface and parenchyma[365]. Moreover, inhibition of GST activity affects parasite muscle function and worm fecundity[366,367]. The ShGST28 vaccine was adjuvanted with aluminum hydroxide and named Bilhvax®. A phase Ia study in healthy French adult men indicated that the vaccine was well tolerated, did not cross-

react with human GST, and elicited a strong T_H2-biased response[368]. A phase Ib trial in healthy Senegalese children showed that it was also well tolerated in a pediatric population[369]. Co-administration of Bilhvax and PZQ was safe in *S. haematobium*-infected adults and children in a phase II study[367]. However, despite seroconversion in vaccinated individuals, a phase III trial in Senegalese children reported relatively low efficacy which trial investigators attributed to interference by the repeated PZQ treatments[367].

1.3.1.2 Schistosoma mansoni *14-kDa Fatty Acid Binding Protein (Sm14)*

Fatty acid binding proteins (FABPs) are expressed at all *Schistosoma* lifecycle stages and participate in the uptake of host-derived fatty acids and sterols[370]. These enzymes are present in the cytosol and in tissues such as the tegument and muscle layers[371]. Recombinant protein alone or adjuvanted with Freund's complete adjuvant provided 50 – 68% protection in mice and 89% in rabbits[372]. Interestingly, vaccination also provided cross protection against *Fasciola hepatica*[373]. Naturally resistant individuals display a T_H1-type immune response to Sm14, and the antigen reduces liver pathology in mice[374,375]. Sm14 adjuvanted with glucopyranosyl lipid adjuvant-stable emulsion (GLA-SE) was tested in a phase I trial in healthy Brazilian adult men[376]. The vaccine was well tolerated and highly immunogenic. Sm14/GLA-SE was also safe in a phase IIa study in Senegalese adults and resulted in 92% seroconversion. The vaccine has advanced to a phase IIb trial in Senegalese school-aged children, but results are still pending[6].

1.3.1.3 Schistosoma mansoni *Tetraspanin-2 (Sm-TSP-2)*

Tetraspanins (TSPs) are surface membrane and scaffolding proteins and are involved in tegument formation[377,378]. TSP-2 is present on schistosomula and adult worms, and is an essential protein for nutrient acquisition, waste excretion and immune evasion. Additionally, IgG1 and IgG3 antibodies in the sera of naturally resistant individuals recognize TSP-2[357]. Immunization with TSP-2 in mouse models resulted in 57% and 62% reductions in worm and hepatic egg burdens respectively[357]. In healthy and infection-naïve adults, Sm-TSP-2 adjuvanted with aluminum hydroxide (Sm-TSP-2/Alhydrogel) and co-administered with or without glucopyranosyl lipid A (GLA-AF) was safe and well tolerated[379]. A phase Ib study testing Sm-TSP-2/Alhydrogel with or without AP 10-701 in healthy adults in endemic Brazil has been completed but results are still

pending[6]. Phase I dose-escalation and phase IIb studies in healthy adults are currently recruiting participants in Uganda and are expected to be completed by early 2025[6].

1.3.1.4 Schistosoma mansoni *Calpain (Sm-p80)*

Sm-p80 is the large subunit of the calcium-activated cysteine protease calpain. It is located on the parasite's surface epithelial syncytium and is responsible for tegument biogenesis for immune evasion[380,381]. Sm-p80 is a promising vaccine candidate and exhibits prophylactic, therapeutic, anti-pathology, and transmission-blocking effects[382]. Vaccination with the antigen also induces cross-protection against all three main schistosome species[381]. A double-blind baboon study reported 93% reduction in adult female worms and 90% reduction in tissue egg excretion when formulated with GLA-SE[383]. Robust antigen-specific IgG, IgG1, IgG2a, and IgM levels were observed in baboons and lasted 5 – 8 years[384]. The vaccine is named SchistoShield® and is currently recruiting infection-naïve, healthy adults in the United States. A phase Ib dose-escalation trial is then planned in healthy African adults[6,385].

1.3.2 Pre-Clinical Candidates

1.3.2.1 Tegumental Proteins

The *Schistosoma mansoni* 29 kDa protein (Sm29) is expressed on the tegument of lung-stage schistosomula and adult worms[386,387]. IgG1 and IgG3 antibodies from the sera of naturally resistant individuals recognize Sm29[358]. Immunization with recombinant protein elicits a mixed cellular and humoral response in mice with an increase in antigen-specific $CD4^+$ T cells and memory cells[387,388]. In a murine reinfection model, Sm29 can induce a protective response[388]. Sm29 formulated with the adjuvant alum induced protection in mice ranging from 29 – 37%[389]. A fusion protein combining Sm14 and Sm29 significantly reduced hepatic egg burden by 74% unadjuvanted and by 83% when adjuvanted with polyinosinic-polycytidylic acid (Poly I:C)[390]. Another candidate antigen is paramyosin (Pmy) which is a myofibrillar protein expressed on the tegument of lung-stage schistosomula and in the glands of penetrating cercaria[391]. Pmy peptides are detected by T cells in naturally resistant individuals[392]. In a mouse model, Pmy vaccination reduced worm and intestinal egg burdens by 44% and 59% respectively, and elicited IgG1 and IgG2 antibodies[393]. Lastly, *S. mansoni* Kunitz type protease inhibitor (Sm-KI-1) is an excretory/secretory product

expressed on the tegument of adult worms and sub-shell region of eggs[394]. Mouse immunization moderately reduces parasite burden but is able to induce a mixed T_H1/T_H2 cellular response[395].

1.3.2.2 Digestive Enzymes

Schistosoma mansoni expresses gut-associated proteases to degrade host blood macromolecules for nutrition. These functions are necessary for parasite survival. To date, cysteine peptidases asparaginyl endopeptidase (SmAE/Sm32) and cathepsin B1 (Sm-CatB/SmCB1) have been identified[359,396]. SmAE hydrolyzes pro-proteins and provides an amino acid source for the parasite. Inhibition of this protease results in reduced oviposition of adult worms[397]. An SmAE encoding DNA vaccine resulted in a 37% reduction of egg-laying in female worms, thus suggesting that this antigen exerts an anti-fecundity and anti-pathology effect[398].

Cathepsin B1 is the most abundant protease in the parasite gut and degrades hemoglobin as an energy source[399]. Vaccination with Sm-CatB increases the secretion of T_H2-associated cytokines and induces high titers of IgG, IgG1, and IgG2b antibodies in mice[400]. The Ndao lab has chosen Sm-CatB as a target for murine vaccination studies. These studies consisted of three doses of recombinant protein adjuvanted with either CpG dinucleotides[401], Montanide ISA 720 VG[402], and AddaVax, an MF59-like squalene adjuvant, as well as sulfated lactosyl archaeal archaeosomes (SLA)[403] in a murine vaccination/challenge model. In these studies, the effectiveness for parasitologic outcomes ranged from 54 – 87%. Additionally, expression of Sm-CatB by human adenovirus serotype 5 (Ad5) with recombinant protein boosts reduced worm burden by 72% and hepatic/intestinal egg burden by 69%/76% and ameliorated egg-induced liver pathology[404].

1.3.2.3 Schistosoma japonicum *Vaccine Development*

Schistosoma japonicum vaccine development must consider the zoonotic transmission of the parasite. Livestock animals such as bovines are particularly affected[405]. Therefore, a *S. japonicum* vaccine would need to be administered in reservoir hosts to reduce transmission in humans. Paramyosin (Sj97) is a preclinical vaccine candidate tested in mice and water buffalo[406]. Other important candidates are Sj26GST and Sj28GST[407]. Further examples of *S. japonicum* vaccine targets include the glycolytic pathway enzyme triose-phosphate isomerase (SjTPI), insulin

receptors (SjIRs), fatty acid binding protein (Sj14), a member of the tetraspanin family (Sj23)[359]. The type of preclinical vaccine candidate varies between recombinant protein vaccines, DNA vaccines, and attenuated vectored vaccines[359].

1.3.3 Controlled Human Infection Model

Controlled human *Schistosoma* infection models represent a new and valuable tool to test new vaccine candidates, define correlates of protection, and identify novel vaccine targets[408]. Controlled human infection model (CHIM) studies are cost-effective and can provide preliminary efficacy data in a shorter time frame than field studies dependent on natural infection[409]. CHIM studies do not replace large-scale clinical trials but serve as proof-of-concept studies to validate vaccine candidates. For example, initial efficacy of the malaria RTS,S vaccine was obtained in a CHIM study and later confirmed in a phase III clinical trial[408,409]. These studies recruit a small number of healthy volunteers and are controlled in terms of dose, route, and pathogen strain[362,410]. Recently, a controlled human infection model for schistosomiasis (CHI-S) was put into place in the Netherlands[411]. Healthy Dutch volunteers were infected with either 10, 20, or 30 single-sex, male cercaria to avoid egg-associated pathologies[412]. Sex is determined by multiplex, real-time PCR[413]. Infection was confirmed by detection of circulating anodic antigen (CAA) in 82% of volunteers at 3 – 10 weeks post-exposure in the absence of schistosome egg production[411]. Infection with 20 cercaria led to adverse events in 18% of volunteers. Participants reported symptoms of acute schistosomiasis such as fever, myalgia, and headache. Among volunteers from endemic regions, CHI-S could be utilized to better define responses associated with clinical protection and distinguish them from immune responses acquired from prior exposure[414]. The model could also aid in investigating the effects of repeated cercaria exposure. The single-sex CHI-S model differs from natural infection due to the absence of eggs and is not able to replicate egg-induced immune responses with their known impact on immunomodulation. However, it provides a unique opportunity to rapidly test vaccine candidates and speed up the development of schistosomiasis vaccines and therapeutics[408].

1.4 *SALMONELLA*-BASED VACCINES

The licensed *S.* Typhi oral vaccine, Ty21a, is well tolerated, immunogenic, and easy to administer[415,416]. This has led to the investigation of attenuated *Salmonella* vectors to deliver heterologous recombinant antigens to induce protective responses against several different pathogens[417–419]. Such vaccines can elicit mucosal, humoral, and cellular immunity at a low cost[420]. Wild-type *S.* Typhimurium can cause disease in humans but is limited to the gastrointestinal tract[421]. The following sections outline *Salmonella* Typhimurium infection, resulting host immune responses, and applications of *S.* Typhimurium as a vaccine vector for infectious diseases.

1.4.1 *Salmonella* Infection & Host Immune Responses

1.4.1.1 Salmonella *Biology*

Salmonella spp. are enteric Gram-negative and facultatively anaerobic bacteria of the Enterobacteriaceae family[422]. The genus *Salmonella* is divided into two species: *Salmonella enterica* and *Salmonella bongori*. The species *S. enterica* is further divided into six subspecies and *S. enterica* subspecies I contains the serovars Typhi and Typhimurium[423]. *Salmonella* Typhi causes typhoid fever in humans whereas *S.* Typhimurium causes mild gastroenteritis[424]. Infection naturally occurs after the consumption of contaminated food or water through the oral-gastric route. Bacteria access the mucosa-associated lymphoid tissue (MALT) via invasion of M cells and then proceed to replicate in the Peyer's patches[425]. Only 10% of genes differ between *S.* Typhi and *S.* Typhimurium and this difference lies in their virulence factors which are encoded on the *Salmonella* pathogenicity islands (SPIs)[426]. *Salmonella enterica* contains two type III secretion systems (T3SSs) that play an important role in bacterial virulence. The first one (T3SS1) is encoded by SPI-1 and is crucial for cell invasion and the activation of pro-inflammatory responses[421]. Upon cell entry, the bacterium is contained in a modified phagosome called the *Salmonella*-containing vacuole (SCV). *Salmonella* replicates within the SCV and secretes effector proteins through the T3SS2 which is important for intracellular survival in macrophages[427]. Interestingly, for *S.* Typhi, SPI-2 T3SS is not required for survival in human macrophages but may play a role in modulating the host immune system and infecting other cell types like dendritic cells or natural killer cells[428]. In addition, some *Salmonella* can escape the SCV, replicate in the cytosol,

and serve as a reservoir for dissemination[429]. Following invasion of the M cells, the bacteria translocate to the intestinal lymphoid follicles and the draining mesenteric lymph nodes where they can later multiply within mononuclear phagocytic cells of the lymphoid follicles, liver, and spleen[430].

1.4.1.2 Salmonella *and the Immune System*

Salmonella spp. express several pathogen-associated molecular patterns (PAMPs) that trigger responses by the host innate immune system. The T3SS, fimbriae, flagella, LPS, and bacterial DNA are all recognized by pattern recognition receptors (PRRs)[421]. *Salmonella* LPS and flagellin trigger TLR4 and TLR5 activation respectively[431,432]. Signalling cascades lead to the activation and recruitment of neutrophils, dendritic cells, and macrophages as well as upregulate the production of pro-inflammatory cytokines such as IL-1β, IL-6, TNF-α, and IFN-γ[421]. IL-18 contributes to host resistance by triggering the release of IFN-γ which is required for controlling the infection[433,434]. Inflammasomes play a central role in innate immune responses to *Salmonella* infection. Upon detection of flagellin by the NRLC4 inflammasome, caspase-1 triggers pyroptotic macrophage death to clear infection independently of IL-1β or IL-18[435]. Therefore, the innate immune system is crucial for early *Salmonella* control but requires the aid of the adaptive immune system to clear infection[436].

$CD4^+$ T cells play an important role in host immunity to *Salmonella* infection. *Salmonella* antigens are presented by MHC class II molecules to $CD4^+$ T cells[437]. Knockout mouse models for MHC class II and CD4 demonstrated high susceptibility to infection[438]. The flagellar filament protein FliC is a natural *Salmonella* antigen and is recognized by memory $CD4^+$ T cells in both mice and humans. *S.* Typhimurium-infected murine macrophages and dendritic cells present FliC epitopes which triggers the proliferation of $CD4^+$ T cells and IFN-γ secretion[439]. IFN-γ knockout mice are also susceptible to disseminated *Salmonella* infection[440]. On the other hand, *Salmonella*-specific $CD8^+$ T cells are induced as well following infection or vaccination in both mice and humans[441–443]. Their induction is triggered by bystander dendritic cells that present antigens of *Salmonella*-infected apoptotic cells[444,445]. Ty21a vaccination induces both $CD4^+$ and $CD8^+$ T cell responses to a variety of *Salmonella*-specific antigens, and it also upregulates expression of mucosal homing markers on circulating $CD4^+$ and $CD8^+$ T lymphocytes[446].

Salmonella infection elicits both systemic and mucosal humoral responses as well[447]. B cell deficient mice are more susceptible to infection and the passive transfer of serum or B cells can protect against infection[448,449]. *Salmonella* porins induce long-lived bactericidal antibody responses in mice[450]. At mucosal sites, secretory IgA correlates with resistance to infection[424]. IgA and IgG likely help control *Salmonella* infection in mucosal and systemic compartments whereas T cells act in both. Interestingly, T cells can migrate between and offer protection at different mucosal sites[451,452]. Taken together, *Salmonella*-based vaccines offer great potential for protection against other infectious diseases.

1.4.2 *Salmonella* as Vaccine Vectors for Infectious Diseases

Excerpt adapted from Winter K*, **Hassan AS***, Dozois CM, Ndao M, Ward BJ. Current applications of *Salmonella enterica* serovar Typhimurium as a vaccine vector. Manuscript prepared for submission to *Frontiers in Immunology*.
*KW and ASH are co-first authors.

The use of *Salmonella* as a vaccine vector for other viral, bacterial, and parasitic organisms has many advantages. One of these is the ability of *Salmonella* to induce both local and systemic immune responses, and this may be effective for the expression of heterologous antigens. In addition, *S.* Typhimurium directly targets intestinal microfold (M) cells overlying the gut-associated lymphoid tissues (GALT), has a large carrying capacity, and are easy to manipulate in both laboratory and industry settings[420]. Finally, as mentioned earlier, *Salmonella* Typhimurium also contains a multitude of PAMPs which may serve to enhance immune responses elicited by the heterologous antigen being expressed by the vector.

1.4.2.1 Bacterial Diseases

Salmonella vectors have also been used to target other bacterial pathogens such as Streptococcus pneumoniae. Construction of an attenuated strain capable of expressing pneumococcal surface protein A (PspA), a highly immunogenic protein, was reported[453]. Oral immunization of mice led to a strong Th1 bias based on IgG isotype profile and elicited antigen specific mucosal IgA

responses. Vaccination with recombinant *Salmonella* strains protected against challenge by three different routes (IP, IV, and IN).

Furthermore, studies have been conducted in developing vaccines for *Staphylococcus aureus*. The delivery of SaESxA and SaESxB through the SPI-1 T3SS generated serum IgG and mucosal IgA titers in mice[454]. ELISpot data also revealed that vaccination led to antigen specific IFN-γ and IL-17A splenocytes. While this approach lengthened survival in mice, vaccinated animals still succumbed to infection.

The Ward lab has recently demonstrated the protective capabilities of the YS1646 strain of S. Typhimurium against murine *Clostridioides difficile* infection[455]. A multimodal approach consisting of IM and PO doses on day 0 followed by additional PO doses on days 2 and 4 led to 100% protection against lethal challenge by several candidate strains. This multimodal schedule would be relatively simple to administer and reduce clinic visits. In practice, only the intramuscular dose along with the first oral dose would be supervised and then the following oral doses may be taken autonomously. Combining oral and intramuscular dosing enables the induction of both local and systemic immune responses. Attenuated strains were developed to express either the receptor binding domain of toxin A (TcdA) or toxin B (TcdB). Interestingly, PO administration alone of a combination of TcdA and TcdB strains led to 82% survival in a lethal challenge model (30% survival in the control group). Significant antigen-specific serum IgG and intestinal IgA titers were elicited as well. These titers were maintained for 6 months after vaccination (Winter, unpublished). PO vaccination induced long lived cell-mediated responses in the spleen and protected mice from lethal challenge 6 months after vaccination (~90% survival compared to 33% in the control group).

1.4.2.2 Viral Diseases

Recently, the *Salmonella* vaccine vector system was adapted for the development of next generation foot-and-mouth disease virus (FMDV) vaccines[456]. Through radiation mutation technology (RMT), the research group created the attenuated strain of *Salmonella* Typhimurium KST0666 to express FMDV VP1 protein under a stress-inducible system. Mice immunized orally showed an increase in fecal IgA, serum IgG and serum IgM titers compared to the non-recombinant *Salmonella* control group. The group also observed an increase in activated CD8+ T cells in

vaccinated animals as well as an increase in the production of antigen-specific IFN-γ, IL-5 and IL-17A, demonstrating that their delivery system led to a mixed profile of cellular immune responses. Vaccinated mice were also protected against *Salmonella* challenge, thus suggesting the possibility of dual protection against FMDV and *Salmonella*.

The mucosal immunity generated by *Salmonella* vectors is particularly interesting in the case of HIV vaccines. The virus enters at mucosal surfaces and oral vaccination would improve responses at these tissues and lead to a first line of defense at an early stage of infection. HIV-1 Gag is a conserved structural antigen and a prime target for the development of a vaccine. A number of studies have been conducted with recombinant *Salmonella* expressing HIV-1 Gag, Tat and Nef proteins either in the bacterial cytoplasm or as a secreted antigen[424,457]. A murine study demonstrated that oral immunization led to the expansion of CD4+ T cells and the expression of IFN-γ, IL-2, and TNF-α by Th1 cells as well as IL-4 and IL-5 from Th2 cells[419].

In addition, an attenuated *Salmonella* Typhimurium vector has been used to protect against H1N1 and H5N1 influenza infection in mice[458]. Mice orally vaccinated were protected against lethal challenge with both viruses and vaccination induced significant increases in anti-HA serum IgG and mucosal IgA titers. ELISpot data revealed an increase in anti-HA IFN-γ production in T cells as well. Another study reported the construction a *Salmonella* strain capable of expressing H1N1 Ha and the matrix protein 2 (M2e)[459]. This study examined the IgG profile of vaccinated animals and reported an increase in both IgG1 and IgG2a titers. Interestingly, IM and IP immunization strategies were more effective than PO in protection from lethal challenge (100% for both vs 66.6% for PO). mRNA induction of IFN-γ and IL-4 were observed for all three routes of administration along with IL-10 expression for IM and IP only.

1.4.2.3 Parasitic Diseases

S. Typhimurium vectors have been generated to address both visceral and cutaneous leishmaniasis. Oral delivery of gp63 by an attenuated *S.* Typhimurium vector has been shown to induce resistance in mice whereas another group used *in silico* approaches to produce two *Salmonella* vectors that reduced visceralization of *L. major* and induced resistance to *L. donovani in vivo*[460–463].

The study of *Salmonella*-vectored vaccines targeting helminths is particularly interesting given their size and complexity. One group has described the potential of intranasal administration of *S.* Typhimurium SL3261 for an antigen-derived epitope of *Trichinella spiralis*[464]. Parasite reduction of 61.8% was observed upon challenge and vaccination induced high titers of IgG1 specific antibodies as well as high concentrations of IL-5. The same strain was used in vaccination studies for *Echinococcus granulosus*[465]. Mice were immunized both intravenously and orally against the fatty acid binding protein EgDf1. The group observed that oral administration of the vector was a superior approach and led to the induction of IgG1, IgG2a and IgA antibodies. Splenocytes restimulated *ex vivo* also led to high production of IFN-y, IL-2 and IL-5 cytokines. Furthermore, a *Salmonella* vector expressing TSOL18, an oncosphere antigen of *Taenia solium* has been generated[466]. Vaccinated mice demonstrated high and sustained antibody titers as well as an increase in CD4+ and CD8+ T lymphocytes. High antibody production was also observed in immunized pigs[466]. Finally, the YS1646 strain of *S.* Typhimurium has been used for immunization studies targeting *S. japonicum*[467]. Chen *et al.* showed reduced parasite burden and increased IgG2a production as well as an increase in IL-12 and IFN-y expression[467].

1.4.3 *Salmonella* Typhimurium YS1646

The *Salmonella enterica* serovar Typhimurium strain YS1646 (also VNP20009) is an attenuated strain developed as an anti-cancer treatment[468]. The strain is a purine and xylose auxotroph (deletion of the *purI* and *xyl* genes), has reduced septic shock potential due to modification of lipid A (deletion of the *msbB* gene), and is susceptible to antibiotics[469]. YS1646 was derived from the *msbB*$^{+}$ strain YS72. It can infect a wide variety of solid tumor types and a strong safety profile[469,470]. The strain was originally designed to deliver therapeutic proteins to the tumor microenvironment but proved to be effective in inhibiting tumor growth in mice[470–472]. The anti-tumor effect is suggested to correlate with SPI-2 gene expression and does not depend on the presence of T or B cells[468,473]. A phase I study in patients with metastatic melanoma and renal cell carcinoma was conducted testing intravenous administration of VNP20009[474]. Tumor colonization was only observed in two patients receiving doses of 3 x 10^8 colony-forming units (cfu)/m^2 and 1 x 10^9 cfu/m^2. The former dose was the maximum tolerated in the study and resulted in low toxicity.

However, no patients experienced tumor regression. This lack of efficacy is believed to be due to limited bacterial colonization and rapid clearance of the bacteria from the bloodstream[474]. Despite these results in human trials, subsequent mouse studies indicated that, similar to systemic infection, oral administration of VNP20009 led to high tumor regression and induced effective anti-tumor immune responses as high as doses of 10^9 cfu[475,476]. Recently, VNP20009 continues to be studied as an anti-cancer tool with a focus on improving tumor infiltration and the delivery of therapeutic anti-tumor cargoes[477,478]. Therefore, while this attenuated strain failed to inhibit tumor growth in humans in its first clinical trial, its well-documented safety profile and ability to deliver heterologous antigens makes it an interesting candidate for oral vaccination.

1.5 RATIONALE & RESEARCH OBJECTIVES

For nearly 50 years, schistosomiasis control efforts have relied primarily on mass administration of praziquantel (PZQ)[479]. While resistance to the drug has been induced experimentally[166,480] and field reports have hinted at reduced susceptibility[164,481], widespread drug resistance has yet to be proven and isolated cases of therapeutic failure may be due to other factors[163,482,483]. Despite the success of PZQ, the facts remain that it is less active against juvenile worms and does not protect against reinfection[160,484–486]. As a result, vaccines will likely be needed to ensure long-lasting control and elimination of the disease.

In recent years, many gains have been made in the field of schistosomiasis vaccine development. Several candidate vaccines are in pre-clinical development, and a small number have progressed to human clinical trials[6,487]. While most of these candidates focus on tegumental proteins of the adult worm, our group has targeted the cysteine protease *Schistosoma mansoni* Cathepsin B (Sm-CatB)[401–404,488]. Sm-CatB is an important digestive enzyme of both migrating larvae and adult worms for nutrient acquisition via the breakdown of host blood macromolecules[489]. Recent successes with different adjuvant formulations led us to consider the attenuated *Salmonella* Typhimurium strain YS1646 as a bacterial vector for Sm-CatB. Oral administration may prove more effective in endemic areas, and the YS1646 vector allows for the generation of both systemic and mucosal immune responses. This thesis addresses the central hypothesis that using the live attenuated YS1646 strain of *S.* Typhimurium as a vector for Sm-CatB will stimulate the host's

immune system and lead to greater parasite burden reductions than observed with previous vaccine formulations.

The first objective of this thesis was to construct recombinant YS1646 strains and characterize prophylactic protection against challenge using a prime-boost vaccination strategy in a murine model (**Chapter 2**). We tested 13 different strains *in vitro* and progressed with 2 for *in vivo* evaluation. One of these two strains generated substantial humoral IgG and local IgA antibody responses as well as systemic cell-mediated responses when mice were primed orally and then received an intramuscular boost 3 weeks later. This approach provided almost complete protection against *S. mansoni* challenge. These findings then prompted us to consider the therapeutic benefits of our vaccine. Thus, the second objective of this thesis was to determine the therapeutic efficacy of our multimodal vaccination strategy in a mouse model of chronic infection (**Chapter 3**). We developed a chronic infection model where mice were vaccinated either 2- or 4-months post-infection, and later sacrificed either 1- or 2-months post-vaccination. Parasite burden was decreased by 1-month post-vaccination and relative reductions continued to increase at the 2-month mark. This approach also resulted in a shift in the immune profile of vaccinated animals from a skewed T_H2 bias to a more balanced pattern of response. The success of our candidate vaccine both prophylactically and therapeutically encouraged further development and the generation of a chromosomally integrated strain as opposed to our previous plasmid-based system. Therefore, the third objective of this thesis was to chromosomally integrate the Cathepsin B antigen into the YS1646 genome and evaluate the immunogenicity and protective efficacy of this new vaccine strain (**Chapter 4**). Through chromosomal integration, we successfully removed any risk of antibiotic resistance which was present in our previous plasmid-based strains, and we were able to observe strong systemic humoral and cellular responses as well as robust mucosal immunity. Parasite burden was significantly decreased following multimodal vaccination and egg-associated liver pathology was reduced. The findings outlined in this thesis highlight the potential for oral vaccination with a bacterial vector that delivers a parasite digestive enzyme. The work presented herein suggests that this novel approach may contribute significantly to schistosomiasis vaccine development.

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1.7 FIGURES AND LEGENDS

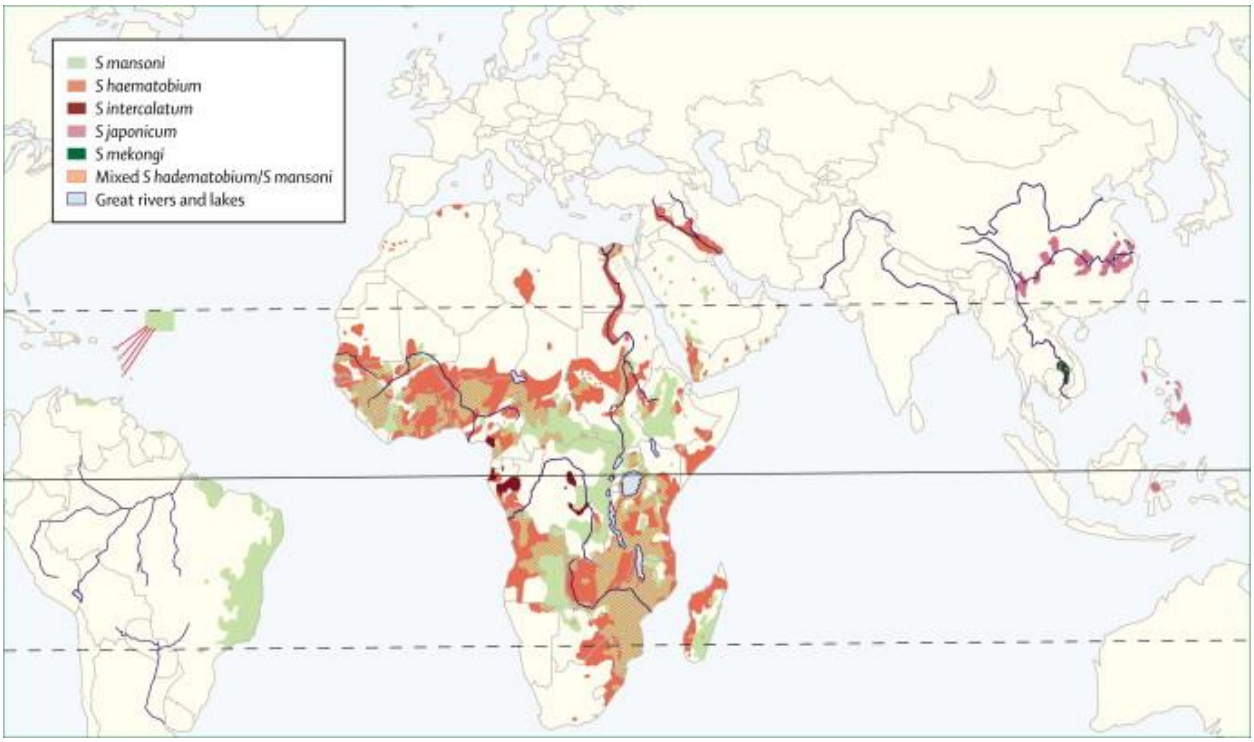

Figure 1.1. Geographic distribution of schistosomiasis. Main foci represented for *S. mansoni*, *S. haematobium*, *S. intercalatum*, *S. japonicum*, and *S. mekongi* infections along with *S. haematobium*/*S. mansoni* co-infections. S. haematobium infections in Corsica are not represented.

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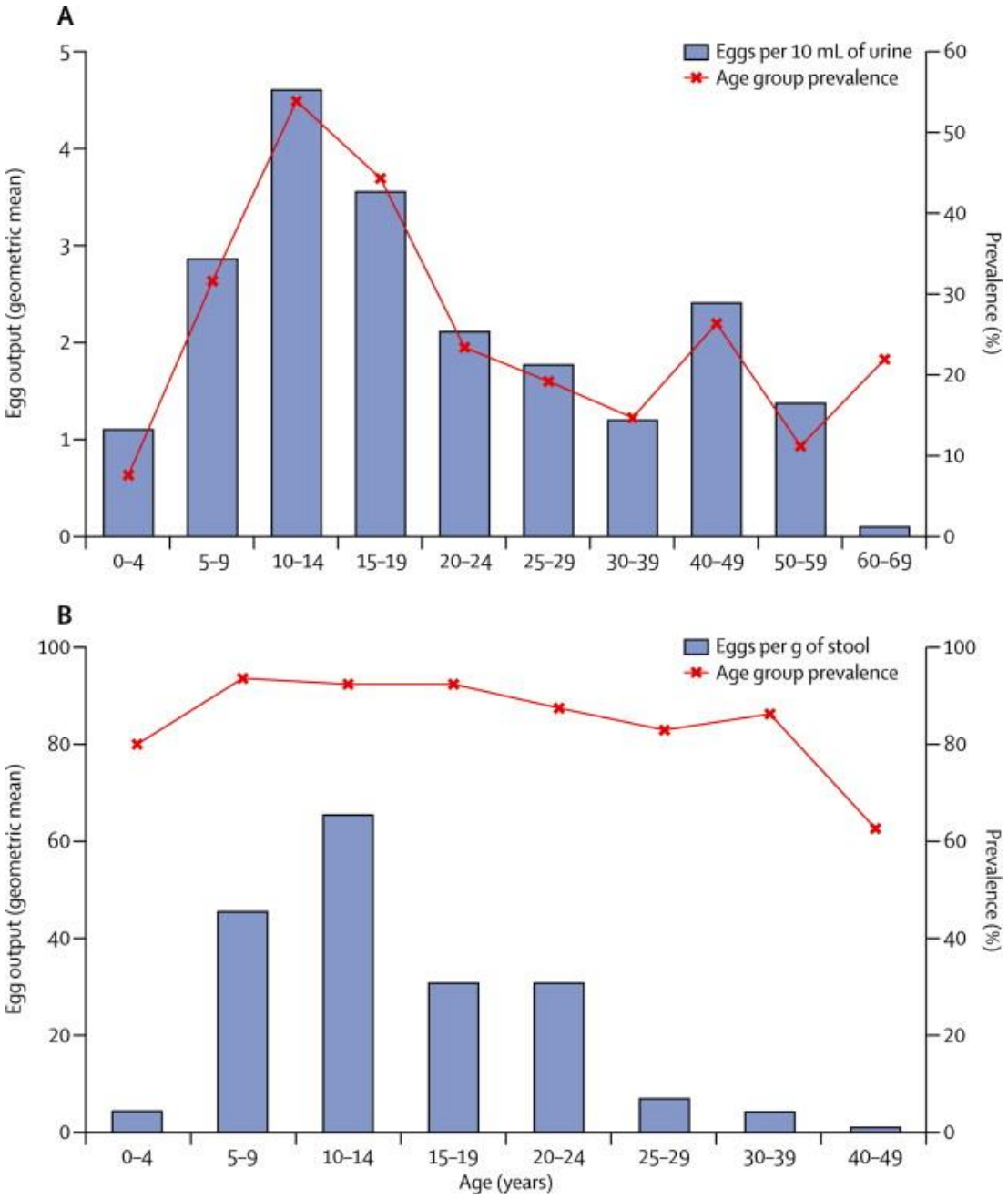

Figure 1.2. *S. haematobium* (A) and *S. mansoni* (B) infection intensity across different ages. Highest burdens of infection occur in school-aged children and infection intensity reduces with age.

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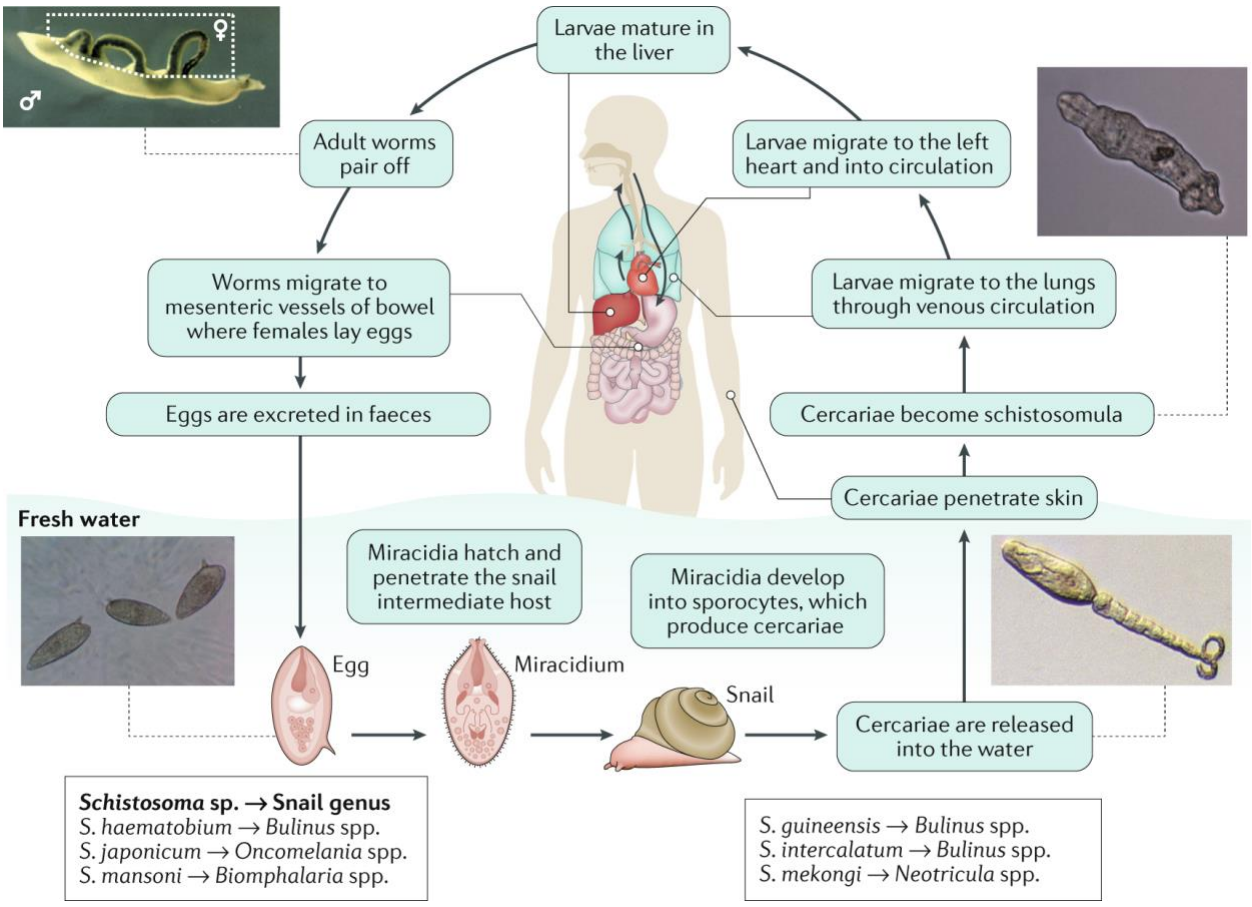

Figure 1.3 *Schistosoma* lifecycle. Eggs hatch in freshwater and release miracidia that will infect their respective snail intermediate host. Within the snail, miracidia replicate and produce cercaria that will infect their definitive host through skin penetration. Cercaria then transform into migrating schistosomula which will travel through venous circulation and eventually reach the liver where they will mature into adult worm pairs. Depending on the species, worms then migrate to their respective anatomical locations and lay eggs that will either be excreted to propagate the lifecycle or retained in host tissues.

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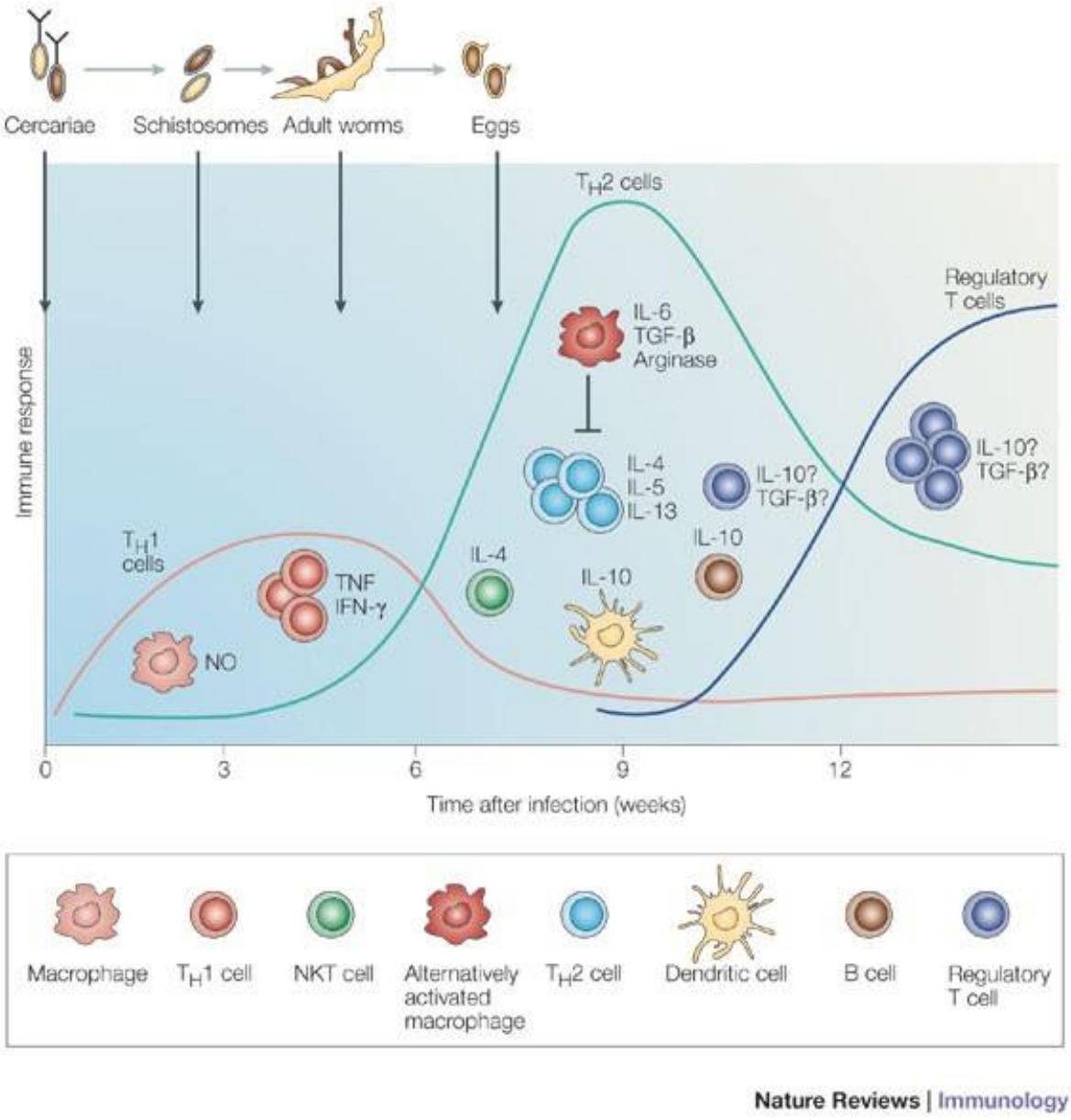

Figure 1.4 Host immune responses to *Schistosoma* infection. Initial immune responses are dominated by a T_H1 phenotype. After oviposition by mature adult worm pairs, there is a shift towards T_H2 immunity mediated by the release of soluble egg antigens. Both T_H1 and T_H2 responses are later regulated by IL-10-secreting regulatory T cells.

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PREFACE TO CHAPTER 2

There are currently no available schistosomiasis vaccines in endemic regions. Mass drug administration campaigns with praziquantel are effective, but the anthelmintic drug has key limitations such as its ineffectiveness at treating larval stages of the parasite lifecycle as well as its inability to provide long-term prophylaxis. Current candidate vaccines are limited to an intramuscular approach targeting surface membrane proteins. Our group has previously demonstrated the immunogenic and protective effects of intramuscular immunizations with adjuvanted Cathepsin B. This cysteine protease is most abundant in the parasite gut, and it is essential for nutrient acquisition and worm development. We believed that existing vaccine formulations with Cathepsin B could be further improved and adapted to alternative delivery systems. Therefore, we sought to develop an oral vaccine repurposing the live attenuated *Salmonella enterica* Typhimurium strain YS1646 that could stimulate both systemic and mucosal immune responses. Mice were immunized following a prime-boost regimen involving a series of oral recombinant *Salmonella* doses and an unadjuvanted intramuscular dose of recombinant Cathepsin B. Vaccinated animals were then exposed to cercarial challenge. The following chapter describes the immune response generated by such vaccination strategies by evaluating serum and intestinal antibody responses as well as cytokine secretion levels. The chapter also describes the protection efficacy of the vaccine using a mouse model of schistosomiasis.

This chapter was adapted from the following manuscript: Vaccination against the digestive enzyme Cathepsin B using a YS1646 *Salmonella enterica* Typhimurium vector provides almost complete protection against *Schistosoma mansoni* challenge in a mouse model. **Hassan AS,** *et al. PLoS Negl Trop Dis* 13(12): e0007490 (2019).

CHAPTER 2

Vaccination against the digestive enzyme Cathepsin B using a YS1646 *Salmonella enterica* Typhimurium vector provides almost complete protection against *Schistosoma mansoni* challenge in a mouse model

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2.1 ABSTRACT

Schistosoma mansoni threatens hundreds of millions of people in >50 countries. Schistosomula migrate through the lung and adult worms reside in blood vessels adjacent to the intestinal mucosa. Current candidate vaccines aren't designed to elicit a mucosal response. We have repurposed an attenuated *Salmonella enterica* Typhimurium strain (YS1646) to produce such a vaccine targeting Cathepsin B (CatB), a digestive enzyme important for parasite survival. Promoter-Type 3 secretory signal pairs were screened for protein expression *in vitro* and transfected into YS1646 to generate candidate vaccine strains. Two strains were selected for *in vivo* evaluation (nirB_SspH1 and SspH1_SspH1). Female C57BL/6 mice were immunized twice, 3 weeks apart, using six strategies: i) saline gavage (control), ii) the 'empty' YS1646 vector orally (PO) followed by intramuscular (IM) recombinant CatB (20μg IM rCatB), iii) two doses of IM rCatB, iv) two PO doses of YS1646-CatB, v) IM rCatB then PO YS1646-CatB and vi) PO YS1646-CatB then IM rCatB. Serum IgG responses to CatB were monitored by ELISA. Three weeks after the second dose, mice were challenged with 150 cercaria and sacrificed 7 weeks later to assess adult worm and egg burden (liver and intestine), granuloma size and egg morphology. CatB-specific IgG antibodies were low/absent in the control and PO only groups but rose substantially in other groups (5898-6766ng/mL). The highest response was in animals that received nirB_SspH1 YS1646 PO then IM rCatB. In this group, reductions in worm and intestine/liver egg burden (vs. control) were 93.1% and 79.5%/90.3% respectively (all $P<.0001$). Granuloma size was reduced in all vaccinated groups (range 32.86–52.83 $x10^3\mu m^2$) and most significantly in the nirB_SspH1 + CatB IM group (34.74 ± 3.35 $x10^3\mu m^2$vs. 62.22 ± 6.08 $x10^3\mu m^2$: vs. control $P<.01$). Many eggs in the vaccinated animals had abnormal morphology. Targeting CatB using a multi-modality approach can provide almost complete protection against *S. mansoni* challenge.

2.2 INTRODUCTION

Schistosomiasis is caused by a number of *Schistosoma spp*. These trematodes currently infect >250 million people worldwide and more than 800 million are at risk of infection[1]. The World Health Organization (WHO) considers schistosomiasis to be the most important human helminth infection in terms of mortality and morbidity[2]. Of the three main human schistosome species, *S. mansoni* is very widespread; causing a significant burden of disease in South America, Sub-Saharan Africa, and the Caribbean[3].

The current treatment of schistosomiasis relies heavily on the drug praziquantel (PZQ). This oral anthelminthic paralyzes the adult worms and has a reported efficacy of 85-90%[4]. The availability of only one effective drug is a precarious situation however and praziquantel resistance has been observed both experimentally[5,6] and reduced PZQ cure rates have been observed in the field[7,8]. Furthermore, praziquantel treatment does not prevent re-infection. There is a clear need for a vaccine that can be used in conjunction with mass drug administration (MDA) and vector control efforts.

The WHO Special Program for Research and Training in Tropical Diseases (TDR/WHO) has encouraged the search for a vaccine that can provide ≥40% protection against *S. mansoni*[9]. Despite this relatively 'low bar', few candidate vaccines have achieved >50% protection in murine or other animal models[10] and even fewer have progressed to human trials[11]. Our group has previously demonstrated 60-70% protection in a *S. mansoni* murine challenge model by targeting Cathepsin B using intramuscular (IM)-adjuvanted formulations[12,13]. Cathepsin B (CatB) is a cysteine protease found in the cecum of both the migratory larval form of *S. mansoni* (i.e.: the schistosomula) and in the gut of the adult worm. CatB is important for the digestion of host blood macromolecules such as hemoglobin, serum albumin and immunoglobulin G (IgG)[14]. Suppression of CatB expression using RNA interference (RNAi) has a major impact on parasite growth and fitness[15]. Because the schistosomula migrate through the lungs and the adult worms reside in mesenteric veins adjacent to the gut mucosa, we wished to determine if a vaccination strategy that targeted induction of both mucosal and systemic responses to CatB could improve protection.

YS1646 is a highly attenuated *Salmonella enterica* serovar Typhimurium carrying mutations in the msbB (lipopolysaccharide or LPS) and purI (purine biosynthesis pathway) genes that was originally developed as a possible cancer therapeutic[16]. Although its development was halted when it failed to provide benefit in a large phase I trial in subjects with advanced cancer, it was well-tolerated when administered intravenously at doses of up to $3.0x10^8$ colony-forming units/m^2 [16]. We are seeking to repurpose YS1646 as a novel vaccination platform and reasoned that a locally-invasive but highly attenuated *Salmonella* vector might induce both local and systemic responses to CatB. The flagellin protein of *S.* Typhimurium has been proposed as a general mucosal adjuvant through its action on toll-like receptor (TLR) 5[17]. Other *Salmonella* products such as LPS would be expected to further enhance immune responses by triggering TLR4[18,19]. Indeed, live attenuated *Salmonella* have multiple potential advantages as vaccine vectors and have been used to express foreign antigens against infectious diseases and cancers[20–22]. They directly target the intestinal microfold (M) cells overlying the gut-associated lymphoid tissues (GALT)[21,23–26], have large 'carrying' capacity[27] and are easy to manipulate both in the laboratory and at industrial scale. Although there is considerable experience with the attenuated *S. typhi* vaccine strain (Ty21a: Vivotif™) in the delivery of heterologous antigens[21,28], far less is known about the potential of other *Salmonella* strains. Of direct relevance to the current work, Chen and colleagues used YS1646 to express a chimeric *S. japonicum* antigen that induced both strong antibody and cellular responses after repeated oral dosing and provided up to 62% protection in a murine challenge model[29].

In this work, we exploited *Salmonella* type-III secretion systems (T3SS) and both T3SS-specific and constitutive promoters to generate a panel of YS1646 strains with plasmid-based expression of enhanced green fluorescent protein (eGFP) or full-length CatB. This panel was screened for protein expression in monomicrobial culture and murine RAW 264.7 murine macrophages and the most promising constructs were advanced to *in vivo* testing in adult female C57BL/6 mice. Animals were vaccinated with the two most promising strains using several strategies and then subjected to cercarial challenge. Herein we report that a two-dose, multimodality schedule starting with oral (PO) gavage of YS1646 bearing the nirB_SspH1_CatB plasmid followed by intramuscular (IM) recombinant CatB (rCatB) was able to reduce both worm and tissue egg burden

by 80-90%. Such reductions are among the best ever reported for any *S. mansoni* candidate vaccine in a murine model.

2.3 METHODS

2.3.1 Ethics statement

All animal procedures were conducted 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).

2.3.2 Plasmids

Gene segments of the *pagc* promoter as well as the *sopE2*, *sspH1*, *sspH2*, *sptP*, *steA*, *steB* and *steJ* promoters and secretory signals were cloned from YS1646 genomic DNA (American Type Culture Collection, Manassas, VA) and the *nirB* and *lac* promoters were cloned from *E. coli* genomic DNA (strain AR_0137) (ThermoFischer Scientific, Eugene, OR). *S. mansoni* CatB complementary DNA (cDNA) was sequence-optimized for expression in *S. enterica* Typhimurium [Java Codon Optimization Tool (jcat)], synthesized by GenScript (Piscataway, NJ) and inserted into the pUC57 plasmid with a 6x His tag at the 3' end. Promoter-T3SS pairs were cloned upstream of the CatB gene and inserted separately into pQE30 (Qiagen, Hilden, Germany). Parallel constructs were made with CatB gene replaced by eGFP to produce expression plasmids used for imaging studies. See **Figure 2.1** for the general plasmid map and **Table 2.1** for a summary of the expression cassettes produced. All plasmids were sequenced to verify successful cloning (McGill Genome Centre, Montreal, QC). *S. enterica* Typhimurium YS1646 (Cedarlane Labs, Burlington, ON) was cultured in Lysogeny broth (LB) media and strains bearing each construct were generated by electroporation (5ms, 3kV: Biorad, Hercules, CA). Successfully transformed strains were identified using LB agar containing 50 μg/mL ampicillin (Wisent Bioproducts, St-Bruno, QC). Aliquots of each transformed strain were stored in LB with 15% glycerol at -80ºC until used in experiments.

2.3.3 Western blotting

Recombinant YS1646 strains were grown in LB broth with 50 μg/mL ampicillin at 37ºC in a shaking incubator under aerobic or low oxygen (sealed twist-cap tubes) conditions. Bacterial lysates were prepared by centrifugation (9,000xg for 5 min) then boiling the pellet (100ºC x 10 min). Proteins from the culture supernatant were precipitated with 10% trichloroacetic acid for 1 hour on ice followed by centrifugation (9,000xg for 2 min) and removal of the supernatant. Protein pellets were resuspended in NuPAGE LDS sample buffer and NuPAGE reducing agent according to the manufacturer's instructions (Thermo Fisher). Immunoblotting was performed as previously described[12]. Briefly, samples were run on a 4-12% Bis-Tris PAGE gel and transferred to nitrocellulose membranes (Thermo Fisher). Membranes were incubated in blocking buffer (5% skim milk in PBS [pH 7.4; 0.01M phosphate buffer, 0.14 M NaCl]) for 1 hour at room temperature (RT) with gentle agitation then washed three times in wash buffer (PBS [pH 7.4; 0.01M phosphate buffer, 0.14 M NaCl], 0.1% Tween 20 (Sigma-Aldrich, St. Louis, MO). Membranes were incubated with a murine, monoclonal anti-polyhistidine primary antibody (1:2,500; Sigma-Aldrich) in blocking buffer overnight at 4ºC with gentle shaking. Membranes were washed three times in wash buffer then incubated with a goat, anti-mouse IgG-horseradish peroxidase secondary antibody (1:5000; Sigma-Aldrich) in blocking buffer for 1 hour at RT with gentle agitation. Membranes were washed three times followed by addition of Supersignal West Pico chemiluminescent substrate (Thermo Fisher) as per the manufacturer's instructions and developed using an autoradiography cassette and the X-OMAT 2000 processor system (Kodak, Rochester, NY).

2.3.4 *In vitro* macrophage infection

Murine macrophage-like cells (RAW 264.7: ATCC-TIB 71) were seeded at 10^6 cells/well in 12-well plates in Dulbecco's Modified Eagle's medium (DMEM) (Wisent Bioproducts) supplemented with 10% fetal bovine serum (FBS: Wisent Bioproducts). Transformed YS1646 were diluted in DMEM-FBS to give a multiplicity of infection of 100 and centrifuged onto the monolayer (110xg for 10 min) to synchronize the infection. After 1 hour at 37ºC in 5% CO_2, plates were washed three times with phosphate buffered saline (PBS: Wisent Bioproducts) and replaced in the incubator with DMEM-FBS containing 50 μg/mL gentamicin (Sigma-Aldrich) to kill any extracellular

bacteria and prevent re-infection. After 2 hours, the cells were washed with PBS three times and the gentamicin concentration was lowered to 5 μg/mL. After 24 hours, the cells were harvested, transferred to Eppendorf tubes and centrifuged (400xg for 5 min). Pellets were prepared for western blotting as above. For imaging experiments, RAW 264.7 cells were seeded into 6-well chamber slides at 10^4 cells/well and cultured as above. After 24 hours, the cells were stained with 4',6-diamidino-2-phenylindole (DAPI) (Thermo Fisher), fixed with 4% paraformaldehyde in PBS and incubated for 10 min at RT. Images were obtained using a Zeiss LSM780 laser scanning confocal microscope and analyzed using ZEN software (Zeiss, Oberkochen, Germany).

2.3.5 Purification of recombinant cathepsin B

S. mansoni CatB was cloned and expressed in *Pichia pastoris* as previously described[12]. Briefly, the yeast cells were cultured at 28ºC with shaking in buffered complex glycerol medium (BMGY) (Fisher Scientific, Ottawa, ON). After two days, cells were pelleted (3,000xg for 5 min) and resuspended in fresh BMMY to induce protein expression. After 3 further days of culture, cells were harvested (3,000 xg for 5 min) and supernatants were collected and purified by Ni-NTA affinity chromatography. Immunoblotting for the His-tag (as above) confirmed successful expression of CatB. Protein concentration was estimated by Pierce bicinchoninic acid assay (BCA) (Thermo Fisher) and aliquots of the rCatB were stored at -80ºC until used.

2.3.6 Immunization protocol

Female 6–8-week-old C57BL/6 mice were purchased from Charles River Laboratories (Senneville, QC). All animals received two doses three weeks apart (See **Figure 2.2** for experimental design). Oral dosing (PO) was accomplished by gavage three times every other day (200 μL containing $1x10^9$ colony-forming units (CFUs)/dose). Intramuscular (IM) vaccinations were administered using a 25g needle in the lateral thigh (20μg rCatB in 50μL PBS). Each experiment included six groups with 8 mice/group: i) saline PO twice (Control or PBS) ii) YS1646 transformed with 'empty' pQE30 vector (EV) PO followed by rCatB IM (EV→IM), iii) CatB-bearing YS1646 PO twice (PO→PO), iv) rCatB IM twice (IM→IM), v) CatB-bearing YS1646 PO followed by rCatB IM (PO→IM), and vi) rCatB IM followed by CatB-bearing YS1646 PO

(IM→PO). The number of animals that were used for each study outcome at each time-point is indicated in the figure legends.

2.3.7 Intestine processing for IgA assessment

Four weeks after the second vaccination, the animals were sacrificed, and 10 cm of the proximal small intestine was collected. Tissue was weighed and stored in a protease inhibitor cocktail (Sigma Aldrich) at a 1:5 dilution (w/v) on ice until processed. Tissue was homogenized (Homogenizer 150; Fisher Scientific), centrifuged at 2500xg at 4ºC for 30 minutes and the supernatant was collected. Supernatants were stored at -80ºC until analyzed by ELISA.

2.3.8 Humoral response by enzyme-linked immunosorbent assay (ELISA)

2.3.8.1 Serum IgG and intestinal IgA

Blood was collected from the saphenous vein at baseline (week 0) and at 3 and 6 weeks in microtainer serum separator tubes (BD Biosciences, Mississauga, ON, Canada). Cleared serum samples were obtained following the manufacturer's protocol and stored at −20ºC until used. Serum CatB-specific IgG and intestinal CatB-specific IgA levels were assessed by ELISA as previously described[30]. Briefly, U-bottom, high-binding 96-well plates (Greiner Bio-One, Frickenhausen, Germany) were coated overnight at 4ºC with rCatB (0.5 μg/mL) in 100 mM bicarbonate/carbonate buffer at pH 9.6 (50 μL/well). Each plate contained a standard curve with 2-fold dilutions of purified mouse IgG or IgA (Sigma Aldrich, St. Louis, MO) starting at 2,000 ng/mL. The plates were washed three times with PBS (pH 7.4) and incubated with blocking buffer (2% bovine serum albumin (Sigma-Aldrich) in PBS-Tween 20 (0.05%; Fisher Scientific)) at 37ºC for 1 hour. The plates were washed three times with PBS and diluted serum samples (1:50 in blocking buffer) were added in duplicate (50 μL/well). Blocking buffer was added to the standard curve wells. After 1 hour at 37ºC, the plates were washed with PBS four times and horseradish peroxidase-conjugated anti-mouse IgG or horseradish peroxidase-conjugated anti-mouse IgA (Sigma Aldrich) diluted 1: 20,000 (1:10,000 for IgA) in blocking buffer was added for 30 min (IgG) or 1 hour (IgA) at 37ºC (75 μL/well). Plates were washed with PBS six times and 3,3′,5,5′-Tetramethyl benzidine (TMB) substrate (100 μL/well; Millipore, Billerica, MA) was used for

detection followed by 0.5 M H_2SO_4 after 15 min (50 µl/well; Fisher Scientific). Optical density (OD) was measured at 450 nm with an EL800 microplate reader (BioTek Instruments Inc., Winooski, VT). The concentration of CatB-specific IgG and IgA were calculated by extrapolation from the mouse IgG or IgA standard curves.

2.3.8.2 Serum IgG1 and IgG2c

Serum CatB-specific IgG1 and IgG2c levels were assessed by ELISA as previously described[12]. Briefly, Immulon 2HB flat-bottom 96-well plates (Thermo Fisher) were coated overnight at 4ºC with rCatB (0.5 µg/mL) in 100 mM bicarbonate/carbonate buffer at pH 9.6 (50 µL/well). The plates were washed three times with PBS-Tween 20 (PBS-T: 0.05%; Fisher Scientific) and were blocked as above for 90 min. Serial serum dilutions in duplicate were incubated in the plates for 2 hours. Control (blank) wells were loaded with PBS-T. After washing three times with PBS-T, goat anti-mouse IgG1-horseradish peroxidase (HRP) (Southern Biotechnologies Associates, Birmingham, AL) and goat anti-mouse IgG2c-HRP (Southern Biotechnologies Associates) were added to the plates and incubated for 1 hour at 37ºC. After a final washing step, TMB substrate (50 µL/well; Millipore, Billerica, MA) was used for detection followed by 0.5 M H_2SO_4 after 15 min (25 µl/well; Fisher Scientific). Optical density (OD) was measured at 450 nm with an EL800 microplate reader (BioTek Instruments Inc.). The results are expressed as the mean IgG1/IgG2c ratio of the endpoint titers ± standard error of the mean. Endpoint titers refer to the reciprocal of the highest dilution that gives a reading above the cut-off calculated as previously described[31].

2.3.9 Cytokine production by multiplex ELISA

In some experiments, some of the animals were sacrificed 4 weeks after the second vaccination. Spleens were collected and splenocytes were isolated as previously described with the following modifications[13]. Splenocytes were resuspended in 96-well plates (10^6 cells/well) in RPMI-1640 (Wisent Bioproducts) supplemented with 10% fetal bovine serum, 1 mM penicillin/streptomycin, 10 mM HEPES, 1X MEM non-essential amino acids, 1 mM sodium pyruvate, 1 mM L-glutamine (all from Wisent Bioproducts), 0.05 mM 2-mercaptoethanol (Sigma-Aldrich). The cells were incubated at 37ºC in the presence of 2.5 µg/mL of rCatB for 72 hours after which the supernatant cytokine levels of IL-2, IL-4, IL-5 IL-10, IL-12p70, IL-13, IL-17, IFNγ, and TNF-α were

measured by QUANSYS multiplex ELISA (9-plex) (Quansys Biosciences, Logan, UT) following the manufacturer's recommendations.

2.3.10 *Schistosoma mansoni* challenge

Biomphalaria glabrata snails infected with the *S. mansoni* Puerto Rican strain were obtained from the Schistosomiasis Resource Center of the Biomedical Research Institute (Rockville, MD) through NIH-NIAID Contract HHSN272201700014I for distribution through BEI Resources. Mice were challenged three weeks after the second immunization (week 6) with 150 cercaria by tail exposure and were sacrificed seven weeks post-challenge as previously described[32]. Briefly, adult worms were counted after perfusion of the hepatic portal system and manual removal from the mesenteric veins. The livers and intestines were harvested from each mouse, weighed and digested in 4% potassium hydroxide overnight at 37ºC. The next day, the number of eggs per gram of tissue was recorded by microscopy. A small portion of each liver was placed in 10% buffered formalin phosphate (Fisher Scientific) and processed for histopathology to assess mean granuloma size and egg morphology (H&E staining). Granuloma area was measured using Zen Blue software (version 2.5.75.0; Zeiss) as previously reported[33,34]. Briefly, working at 400x magnification, the screen stylus was used to trace the perimeter of 6 – 8 granulomas with a clearly visible egg per mouse which the software converted into an area. Mean areas were presented as $x10^3$ μm^2 ± SEM. Eggs were classified as abnormal if obvious shrinkage had occurred, if internal structure was lost or if the perimeter of the egg was crenelated and are reported as a percent of the total eggs counted (± SEM).

2.3.11 Statistical analysis

Statistical analysis was performed using GraphPad Prism 6 software (La Jolla, CA). In each experiment, reductions in worm and egg burden were expressed relative to the saline control group numbers. Results are represented from two separate experiments. Data were analyzed by one-way ANOVA and multiple comparisons were corrected using Tukey's multiple comparison procedure. P values less than 0.05 were considered significant.

2.4 RESULTS

2.4.1 *In vitro* Expression and Secretion of CatB by Transformed YS1646 Strains

Thirteen expression cassettes were built and the sequences were verified (McGill University Genome Quebec Innovation Centre) (**Table 2.1**). The promoter/T3SS pairs were inserted in-frame with either *S. mansoni* CatB or eGFP. In monomicrobial culture, CatB expression was effectively driven by the nirB_SspH1, SspH1_SspH1 and SteA_SteA plasmids (**Figure 2.3A**) with the greatest production from the nirB promoter in low oxygen conditions as previously reported[29]. Secreted CatB was detectable in the monomicrobial culture supernatants only with YS1646 bearing the SspH1_SspH1 construct (**Figure 2.3A**). In infected RAW 264.7 cells, all of the constructs produced detectable eGFP by immunofluorescence (**Figure 2.3B**) but only the YS1646 bearing the nirB_SspH1 and SspH1_SspH1 constructs produced CatB detectable by immunoblot (**Figure 2.3C**). These constructs also led to the greatest eGFP expression in the RAW 264.7 cells and so were selected for *in vivo* testing.

2.4.2 Antibody Response to YS1646-vectored Vaccination

None of the groups had detectable anti-CatB IgG antibodies at baseline and the saline control mice remained negative after vaccination. Mice in the PO→PO group also had very low serum CatB-specific IgG antibody levels even after the second vaccination (395.7 ± 48.9) (**Figure 2.4A**). In contrast, all animals that had received at least 1 dose of rCatB IM had significantly higher IgG titers at 6 weeks (i.e.: 3 weeks after the second immunization) (**Figure 2.4A**). Mice that received nirB_SspH1 PO followed by an IM boost had the highest titers (6766 ± 2128 ng/mL, $P<.01$ vs. control) but these titers were not significantly different from groups that had received either one (EV→IM) or two doses of rCatB (IM→IM) (5898 ± 1951 ng/mL and 6077 ± 4460 ng/mL respectively, both $P<.05$ vs. control). IgG antibody titers were generally lower in all groups that received the YS1646 strain bearing the SspH1_SspH1 construct (range 333.5 – 3495 ng/mL; $P<.05$, $P<.01$, $P<.001$ vs control) (**Figure 2.4B**). Because the SspH1_SspH1 construct will not be carried forward into more advanced studies, we did not measure the IgG subtypes or the intestinal IgA levels for these experimental groups.

Control mice had no detectable anti-CatB antibodies and were arbitrarily assigned an IgG1/IgG2c ratio of 1. The PO→PO mice had a ratio of 0.9 (**Figure 2.4C**). The EV→IM and the PO→IM groups had IgG1/IgG2c ratios of 2.2 and 2.5 respectively while the highest ratios were seen in the IM→IM and IM→PO groups (10.2 and 7.8 respectively).

Intestinal IgA levels in the saline, EV→IM, and IM→IM groups were all low (range 37.0 – 148.0 ng/g of tissue) (**Figure 2.4D**). Although the data are variable, groups that received at least one dose of nirB_SspH1 YS1646 PO had increased IgA levels compared to the control group that reached statistical significance in the PO→PO group (402.7 ± 119.7 ng/g) and the PO→ IM group (i.e.: nirB_SspH1 PO then rCatB IM: 419.6 ± 95.3 ng/g, both $P<.01$). The IM→PO group also had higher intestinal IgA titers than controls, but this increase did not reach statistical significance (259.8 ± 19.4 ng/g).

2.4.3 Cytokine Production in Response to YS1646-vectored Vaccination

There was only modest evidence of CatB-specific cytokine production by antigen re-stimulated splenocytes immediately prior to challenge (4 weeks after the second dose). There were no significant differences in the levels of IL-2, IL-4, IL-10, IL-12p70, IL-13, IL-17 or TNF-α between vaccinated and control groups (**Supplementary Table 2.1**). Compared to the control group, the levels of IL-5 in splenocyte supernatants were significantly higher in mice that received two doses of rCatB (IM→IM) (475.5 ± 98.5 pg/mL, $P<.01$) and the nirB_SspH1 PO→IM group (364.4 ± 85.2 pg/mL, $P<.05$) whereas the control group was below the limit of detection at 63.1 pg/mL (**Figure 2.5A**). Only the PO→IM group had clear evidence of CatB-specific production of IFNγ in response to vaccination (933 ± 237 pg/mL vs. control 216.4 ± 62.5 pg/mL, $P<.05$) (**Figure 2.5B**).

2.4.4 Protection from *S. mansoni* Challenge from YS1646-vectored Vaccination

At 7 weeks after infection, the mean worm burden in the saline-vaccinated control group was 25.2 ± 4.3 and all changes in parasitologic and immunologic outcomes are expressed in reference to this control group. Relatively small reductions in worm burden were observed in the EV→IM (9.4%) and IM→IM groups (20.5%) across all studies. Overall, protection was better with

nirB_SspH1_CatB schedules compared to SspH1_SspH1_CatB schedules. In the SspH1_SspH1 animals, reductions in worm numbers were similar to the IM→IM group: 17.2% with oral vaccination alone (PO→PO) and only 17.8% and 24.7% in the PO→IM and IM→PO groups respectively. In contrast, the PO→PO group vaccinated with the nirB_SspH1 YS1646 strain had an 81.7% (P<.01) reduction in worm numbers and multi-modality vaccination with this strain achieved 93.1% (P<.001) and 81.7% (P<.01) reductions in the PO→IM and IM→PO groups respectively. (**Figure 2.6A**).

Overall, the reductions in hepatic and intestinal egg burden followed a similar pattern to the vaccine-induced changes in worm numbers. The hepatic and intestinal egg burden in the saline-vaccinated control mice ranged from 1,994 – 13,224 eggs/g and 6,548 – 24,401 eggs/g respectively. Reductions in hepatic eggs in the EV→IM and IM→IM groups were modest at 18.9% and 32.7% respectively. Reductions in intestinal eggs followed a similar trend: 15.4% and 43.6% respectively. In the groups that received the SspH1_SspH1 YS1646 strain, PO→PO immunization did not perform any better with 11.6% and 18.3% reductions in hepatic and intestinal egg numbers respectively. Somewhat greater reductions in hepatic and intestinal egg burden were seen in the PO→IM (51.3% and 60.9% respectively) and IM→PO groups (17.7% and 29.8% respectively). These apparent differences in egg burden between the two multi-modality groups did not parallel the reductions in worm numbers or the systemic anti-CatB IgG levels. Groups that received the nirB_SspH1 strain had more consistent and greater reductions in egg burden: the PO→PO group had 73.6% and 69.2% reductions in hepatic and intestinal egg numbers respectively (both P<.001). The greatest impact on hepatic and intestinal egg burden was seen in the nirB_SspH1 multi-modality groups: 90.3% (P<.0001) and 79.5% (P<.0001) respectively in the PO→IM group and 79.4% (P<.001) and 75.9% (P<.0001) respectively in the IM→PO group (**Figures 2.6B** and **2.6C**).

Hepatic granulomas were large and well-formed in the PBS-treated control mice (62.2 ± 6.1 x10^3 μm^2) and essentially all of the eggs in these granulomas had a normal appearance. The EV→IM and IM→IM groups had slightly smaller granulomas (52.0 ± 6.9 x10^3 μm^2 and 52.8 ± 10.4 x10^3 μm^2 respectively) with modest numbers of abnormal-appearing eggs (i.e.: loss of internal structure, crenellated edge) (**Table 2.2**) but these differences did not reach statistical significance. Groups that received the SspH1_SspH1 strain had granuloma sizes ranging from 47.3–55.0 x10^3 μm^2 with

30.5% of the eggs appearing abnormal in the PO→IM and 28.6% IM→PO groups (both $P<.05$). In the groups that received the nirB_SspH1 strain, both the purely oral (PO→PO) and multi-modality strategies (PO→IM and IM→PO) resulted in even smaller granulomas (32.9 ± 2.0 μm^2, 34.7 ± 3.4 $x10^3$ μm^2 and 39.2 ± 3.7 $x10^3$ μm^2: $P<.01$, $P<.01$ and $P<.05$ respectively). The large majority of the eggs in these granulomas had disrupted morphology (75.9 ± 7.6%, 79.4 ± 4.2% and 71.9 ± 6.0% respectively: all $P<.0001$). Overall, the greatest and most consistent reductions in both adult worm numbers and egg burdens in hepatic and intestinal tissues were seen in the animals that received oral dosing with the YS1646 bearing the nirB_SspH1_CatB construct followed 3 weeks later by IM rCatB.

2.5 DISCUSSION

In the mid-1990s, the Tropical Diseases Research (TDR) committee of the World Health Organization (WHO) launched the search for a *S. mansoni* vaccine candidate capable of providing ≥40% protection[9]. This initiative targeted reduced worm numbers as well as reductions in egg burden in both the liver and the intestinal tissues. *S. mansoni* female worms can produce hundreds of eggs per day[35]. While the majority are excreted in the feces, some are trapped in host tissues where they cause most of the pathology associated with chronic infection[36]. Eggs trapped in the liver typically induce a vigorous granulomatous response that can lead to fibrosis, portal hypertension and death while egg-induced granulomas in the intestine cause local lesions that contribute to colonic polyp formation[37]. Reducing the hepatic egg burden would therefore be predicted to decrease *S. mansoni*-associated morbidity and mortality while reducing the intestinal egg burden would likely decrease transmission.

Our group has previously described the protective efficacy of CatB-based vaccines delivered IM with adjuvants[12,13]. Using CpG dinucleotides to promote a Th1-type response, vaccination resulted in a 59% reduction in worm burden after challenge with 56% and 54% decreases in hepatic and intestinal egg burden respectively compared to adjuvant-alone control animals[12]. Parasitologic outcomes were slightly better in the same challenge model when the oil-in-water adjuvant Montanide ISA 720 VG was used to improve the antibody response: 56-62% reductions in worm numbers and the egg burden in tissues[13]. These results were well above the 40% threshold

suggested by the TDR/WHO and provided proof-of-concept for CatB as a promising target antigen. Based on this success, we expanded our vaccine discovery program to explore alternate strategies and potentially more powerful delivery systems. The availability of the highly attenuated *Salmonella enterica* Typhimurium strain YS1646 that had been used in a phase 1 clinical cancer trial at doses up to $3x10^8$ IV was attractive for many reasons. Although *S. enterica* species replicate in a membrane-bound host cell compartment or vacuole[38], foreign protein antigens can be efficiently exported from the vacuole into the cytoplasm using the organism's T3SS. Like all *Salmonella enterica* species, YS1646 has two distinct T3SS located in *Salmonella* pathogenicity islands 1 and 2 (SPI-I and SPI-II)[39] that are active at different phases of infection[40]. The SPI-I T3SS translocates proteins upon first contact of the bacterium with epithelium cells through to the stage of early cell invasion while SPI-II expression is induced once the bacterium has been phagocytosed[41]. These T3SS have been used by many groups to deliver heterologous antigens in *Salmonella*-based vaccine development programs[22,42].

In this study, we report the protective efficacy of CatB delivered by the attenuated strain YS1646 of *Salmonella enterica* serovar Typhimurium in a heterologous prime-boost vaccination regimen. Compared to infected controls, vaccination with CatB IM followed by YS1646 bearing the nirB_SspH1 strain resulted in an 93.1% reduction in worm numbers and 90.3% and 79.5% reductions in hepatic and intestinal egg burdens respectively compared to the control group. These results not only surpass the WHO's criterion for an effective *S. mansoni* vaccine by a considerable margin, they are a marked improvement on our own work using CatB delivered IM with adjuvants and are among the best results ever reported in similar murine models[12,13]. For example, in the pre-clinical development of two candidate vaccines that subsequently entered clinical trials[43,44], IM administration of the fatty acid binding protein Sm-14 with the adjuvant GLA-SE led to a 67% reduction in worm burden in mice[10] while IM vaccination with the tegumental protein TSP-2 with either Freund's adjuvant or alum/CpG reduced worm numbers by 57% and 25% and hepatic egg burden by 64% and 27% respectively[45,46]. Another vaccine candidate targeting the tegumental protein Sm-p80 that is advancing towards clinical testing achieved 70 and 75% reductions in adult worm numbers and hepatic egg burden respectively when given IM with the oligodeoxynucleotide (ODN) adjuvant 10104[47]. It is noteworthy that these other vaccine candidates were all administered IM, a route that typically results primarily in systemic immunity. Although there are reports of

vaccines delivered IM that can induce some level of mucosal immunity[48], particularly with the use of adjuvants, intramuscular injection is less likely to elicit a local, mucosal response than the multimodality approach taken in our studies.

To what extent the surprising reductions in worm and egg burdens that we observed with the YS1646 can be attributed to the systemic or the local antibody response is currently unknown although it is likely that both contributed to the success of the combined schedules (i.e.: IM→PO and PO→IM). Oral administration of *Salmonella*-vectored vaccines clearly leads to higher mucosal IgA responses than IM dosing[49] and the protective potential of IgA antibodies has been demonstrated in schistosomiasis[50]. The migrating schistosomula likely interact with the MALT during their week-long passage through the lungs. It is therefore possible that IgA produced by the respiratory mucosa interferes with parasite development at this stage in its lifecycle. The importance of the local response is strongly suggested by the fact that PO dosing alone with YS1646 bearing the nirB_SspH1_CatB construct still provided substantial protection (81.7% and 73.6%/69.2% for worms and hepatic/intestinal eggs) despite the almost complete absence of a detectable systemic response (**Figure 2.4A**). Indeed, IgA titers were readily detectable in the intestinal tissues of mice receiving the nirB_SspH1 YS1646 vaccine PO →PO and in mice the received PO→IM dosing (402.7 ng/g and 419.6 ng/g respectively) (**Figure 2.4D**). On the other hand, the importance of IgG antibodies in the protection against schistosomiasis has been reported by many groups[51,52]. Administered IM, rCatB alone consistently elicited high systemic antibody responses and provided a modest level of protection without any measurable mucosal response. Chen and colleagues have also used YS1646 as a vector to test single- and multi-modality approaches for a bivalent vaccine candidate (Sj23LHD-GST) targeting *S. japonicum* in a similar murine model[29]. Although some authors have promoted so-called 'prime-pull' strategies to optimize mucosal responses (i.e.: 'prime' in the periphery then 'pull' to the target mucosa)[53], it is interesting that both the Chen group and our own findings suggest that PO→IM dosing may be the optimal strategy. In the *S. japonicum* model targeting the long hydrophobic domain of the surface exposed membrane protein Sj23LHD and a host-parasite interface enzyme (glutathione S-transferase or GST), the PO→IM vaccination schedule led to important reductions in both worm numbers (51.4%) and liver egg burden (62.6%)[29].

In addition to the substantial overall reductions in worm numbers and egg burden in our animals that received multimodality vaccination, there were additional suggestions of benefit in terms of both hepatic granuloma size and possible reduced egg fitness (**Table 2.2**). The size of liver granulomas is determined largely by a Th2-deviated immune response driven by soluble egg antigens (SEA)[54]. Prior work with CatB vaccination suggests that IM delivery of this antigen alone tends to elicit a Th2-biased response that can be shifted towards a more balanced Th1/Th2 response by CpG or Montanide[12,13,55]. The reduction in the anti-CatB IgG1/IgG2c ratio between the IM→IM only and multimodality groups (IM→PO, PO→IM) supports the possibility that combined recombinant CatB with YS1646 bearing CatB can induce a more 'balanced' pattern of immunity to this antigen and, at least in a limited sense, that the YS1646 is acting as a Th1-type adjuvant (**Figure 2.4C**). Although no adjuvants were included in the current study, the YS1646 vector might reasonably be considered 'auto-adjuvanted' by the presence of LPS, even in an attenuated form, and flagellin which can act as TLR-4 and TLR-5 agonists respectively. It was still surprising however, that the average hepatic granuloma size was significantly smaller in our multi-modality groups than in the IM alone group since no CatB is produced by the eggs (**Table 2.2**). This observation raises the interesting possibility that the YS1646-based vaccination protocol may be able to influence the *overall* pattern of immunity to *S. mansoni* and/or reduce the fitness of the eggs produced (as suggested by the abnormal egg morphology observed). Such effects could significantly extend the value of the combined PO→IM vaccination strategy, i.e.: more durable impact, reduced transmission, etc. Furthermore, prior work with IM vaccination with CatB alone revealed a Th2-type pattern of cytokine response in splenocytes (eg: IL-4, IL-5, and IL-13)[55]. In the current work we observed increases in both IFNγ and IL-5 in the multimodality PO→IM group (**Figure 2.5**), suggesting that YS1646 vaccination can induce more balanced Th1-Th2 immune response. Finally, this study did not consider the possible role of other immune mechanisms in controlling *S. mansoni* infection after YS1646 infection and we have previously shown that $CD4^+$ T cells and anti-schistosomula antibody-dependent cellular cytotoxicity (ADCC) contribute to protection after CatB immunization (± adjuvants)[56]. Studies are underway to examine these possibilities with the multi-modality YS1646-based vaccination protocols. It is also intriguing that the apparent efficacy of either one or two IM doses of rCatB differed considerably between the EV→IM and IM→IM groups with the latter schedule eliciting significantly greater protection for all parasitologic outcomes despite the fact that these groups had similar levels of serum anti-CatB

IgG at the time of challenge (**Figure 2.4**). Future studies will address whether or not there are qualitative differences in the antibodies induced (i.e.: avidity, isotype, competence to mediate ADCC) and/or differences in other immune effectors (i.e.: $CD4^+$ or $CD8^+$ T cells).

Although the findings presented herein are promising, this work has several limitations. First, immune protection is likely to be relatively narrow when only a single schistosome antigen is targeted. In the long term, this limitation could be easily overcome by adding one or more of the many *S. mansoni* target antigens that have shown promise in pre-clinical and/or clinical development (e.g.: GST, Sm23, Sm-p80, etc.) to generate a 'cocktail' vaccine. In this context, an attenuated *Salmonella* vector like YS1646 might be ideal because of its high 'carrying capacity' for foreign genes[57]. Second, our current findings are based on plasmid-mediated expression and pQE30 contains a mobile ampicillin resistance gene that would obviously be inappropriate for use in humans[58]. Although chromosomal integration of our nirB_SspH1_CatB gene is an obvious mitigation strategy, expression of the CatB antigen from a single or even multiple copies of an integrated gene would likely be lower than plasmid-driven expression. Finally, the degree to which a vaccination schedule based on the YS1646 vector would be accepted by regulators is currently unknown. Attenuated *Salmonella* have a good safety track-record in vaccination: e.g.: the Ty21a *S. typhi* vaccine and a wide range of candidate vaccines[57] despite their ability to colonize/persist for short periods of time[59]. Although the total clinical exposure to YS1646 to date is limited (25 subjects with advanced cancer in a phase 1 anti-cancer trial), the available data are reassuring since up to $3x10^8$ bacteria could be delivered intravenously in these vulnerable subjects without causing serious side effects[16]. Finally, these experiments were designed to test the simplest prime-boost strategies based on the YS1646 vaccine so no adjuvants were used with the recombinant protein dose. Experiments are on-going to determine whether or not the inclusion of an adjuvant with either the prime or boost dose of the recombinant protein can further enhance protection.

In summary, this work demonstrates that a YS1646-based, multimodality, prime-boost immunization schedule can provide nearly complete protection against *S. mansoni* in a well-established murine model. The protection achieved against a range of parasitologic outcomes was the highest reported to date for any vaccine. These observations strongly support the continued development of this candidate vaccine for prophylactic and possibly even therapeutic use in the

many hundreds of millions of people in low- and middle-income countries at risk of or already infected by *S. mansoni*.

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2.7 AUTHORS' CONTRIBUTIONS

ASH was involved in all aspects of the study including study design, performing experiments, data analysis and preparation of the manuscript. NHZ designed, constructed and validated the plasmids. DJP assisted in the design of the snail housing facility and the infection model. BJW and MN supervised all parts of the study and prepared the manuscript.

2.8 COMPETING INTERESTS

ASH, MN and BJW are named as inventors on a provisional patent application for a YS1646-based *S. mansoni* vaccine.

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2.10 FIGURES AND TABLES

Table 2.1. Recombinant *Salmonella* constructs.

Plasmid	Promoter	Secretory Signal	Protein	
Lac_SopE2	Lac	SopE2	Sm-Cathepsin B	eGFP
nirB_SopE2	nirB			
pagC_SopE2	pagC			
SopE2_SopE2	SopE2			
Lac_SspH1	Lac	SspH1		
nirB_SspH1	nirB			
pagC_SspH1	pagC			
SspH1_SspH1	SspH1			
SspH2_SspH2	SspH2			
SteA_SteA	SteA			
SteB_SteB	SteB			
SteJ_SteJ	SteJ			
SptP_SptP	SptP			

Each plasmid construct was cloned to express *S. mansoni*-Cathepsin B (Sm-CatB) or enhanced green fluorescent protein (eGFP) fused with a type-3 secretory signal from *S. enterica* Typhimurium and driven by promoters from *E. coli* or *S. enterica* Typhimurium. Construct nomenclature = 'Promoter_Secretory Signal_Protein of Interest'.

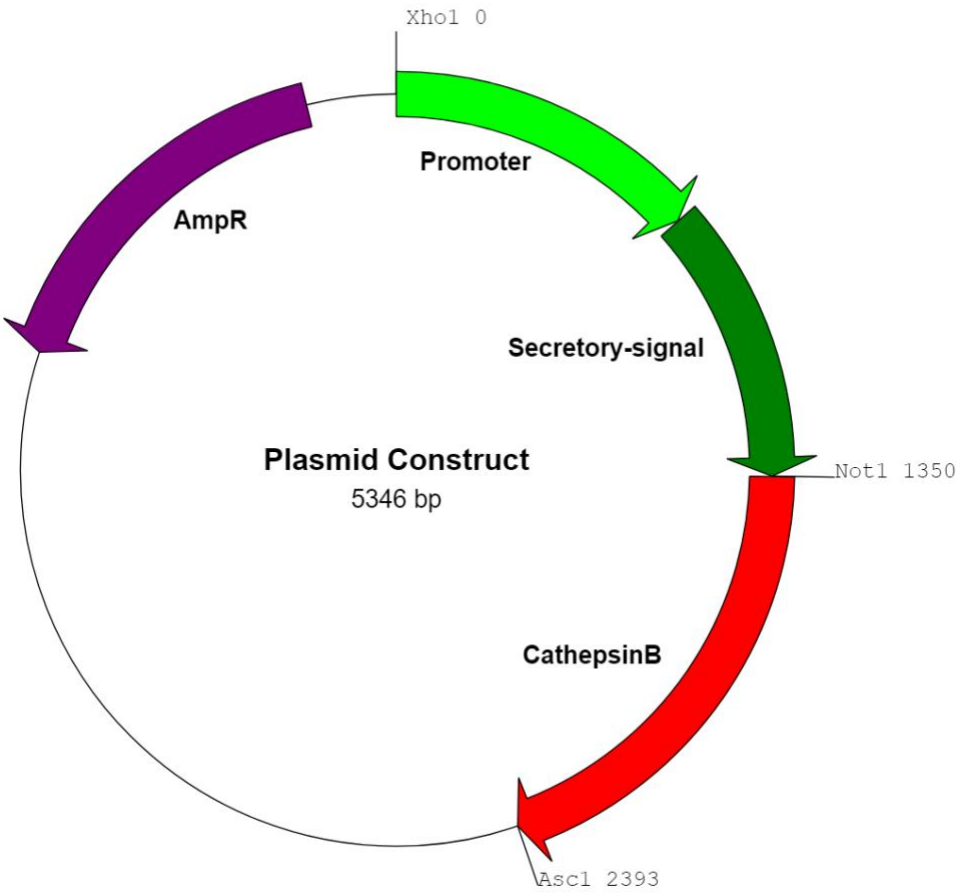


Figure 2.1. Plasmid map for recombinant YS1646 strains. The pQE-30 plasmid served as a backbone. The promoter and secretory signal were inserted between the Xho1 and Not1 restriction sites. The full-length Cathepsin B gene was inserted between the Not1 and Asc1 sites. An ampicillin resistance gene was used as a selectable marker.

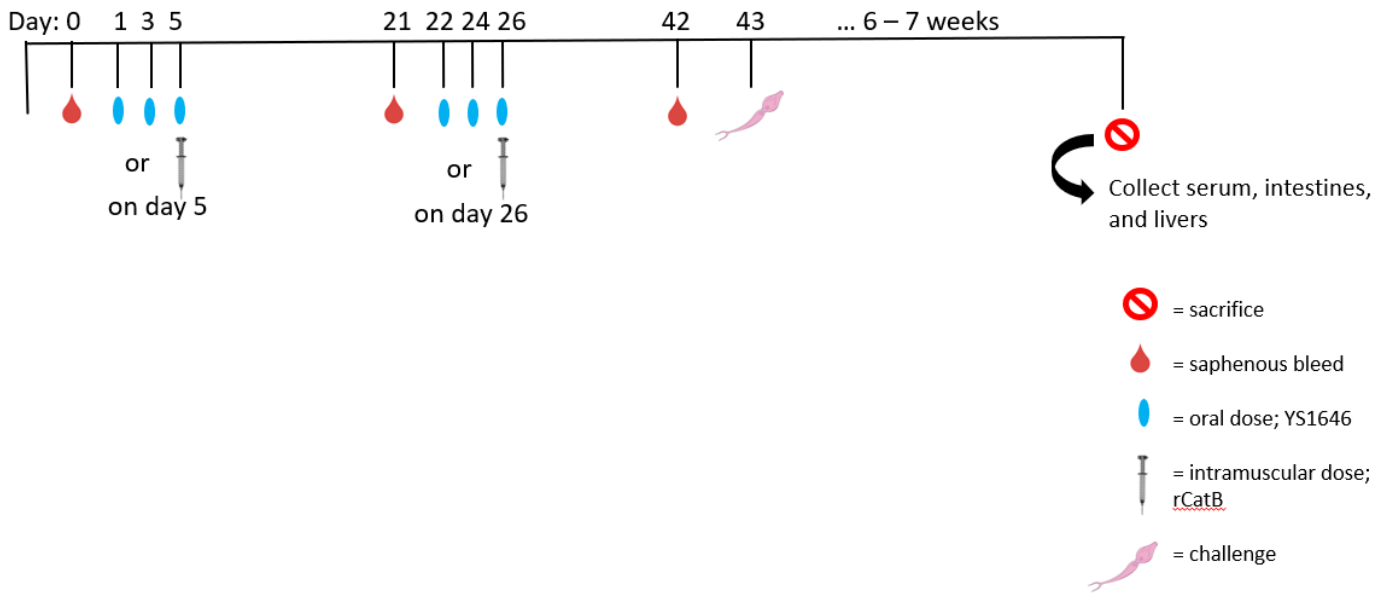


Figure 2.2. Immunization schedule. Baseline serum was collected on day 0 for all mice. Each group consists of either a saline control, EV→IM, PO→PO, IM→IM, PO→IM, and IM→PO for the nirB_SspH1 and/or the SspH1_SspH1 construct. Mice receive 3 oral doses of YS1646 ($1x10^9$ cfu/dose) or PBS every other day while others receive an intramuscular dose of 20 μg of CatB on day 5. Mice were bled and underwent a second round of vaccination three weeks later before being challenged with 150 *S. mansoni* cercaria by tail penetration. All animals were sacrificed 6 – 7 weeks post-infection.

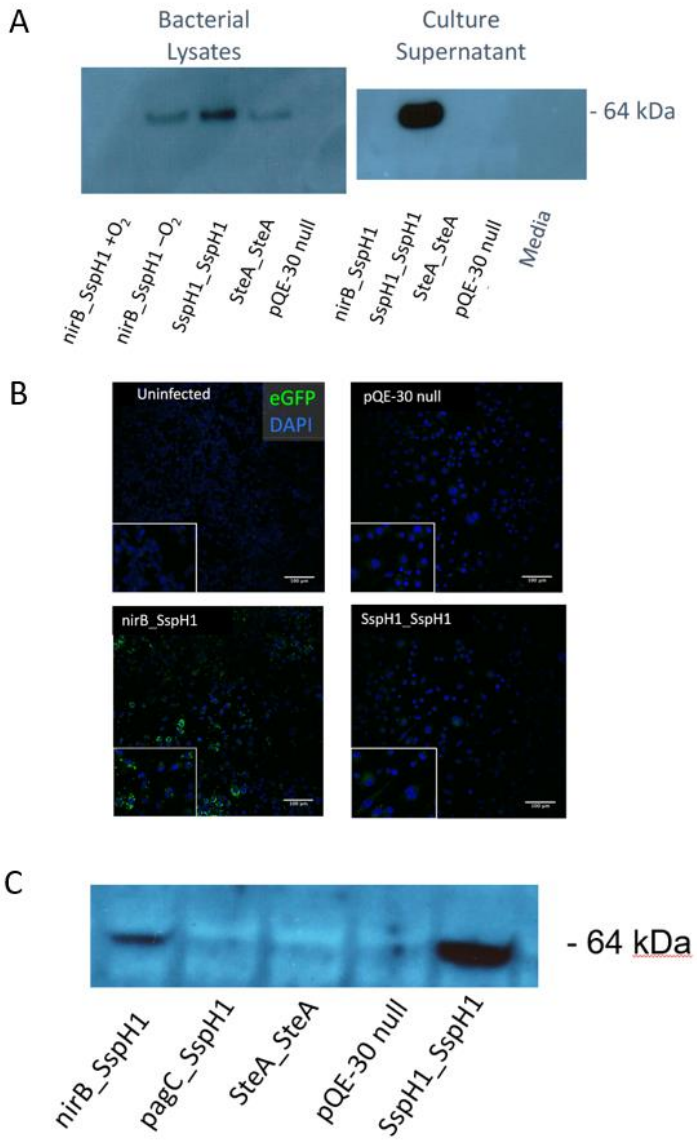


Figure 2.3. Expression of recombinant cathepsin B. A) The plasmids nirB_SspH1, SspH1_SspH1 and SteA_SteA were transformed into *Salmonella* strain YS1646. Whole bacteria lysates and monomicrobial culture supernatants were examined for the presence of CatB by western blot. B) The mouse macrophage cell line RAW 264.7 cells were infected with transformed YS1646 strains expressing eGFP as a marker for the capacity of promoter-T3SS pairs to support expression of a foreign protein. DAPI nuclear stain is represented in blue and eGFP is shown in green. Scale at 100 μm. C) Mouse macrophage cells line RAW 264.7 cells were infected with selected plasmids from Table 1 and the presence of CatB protein was determined by western blotting.

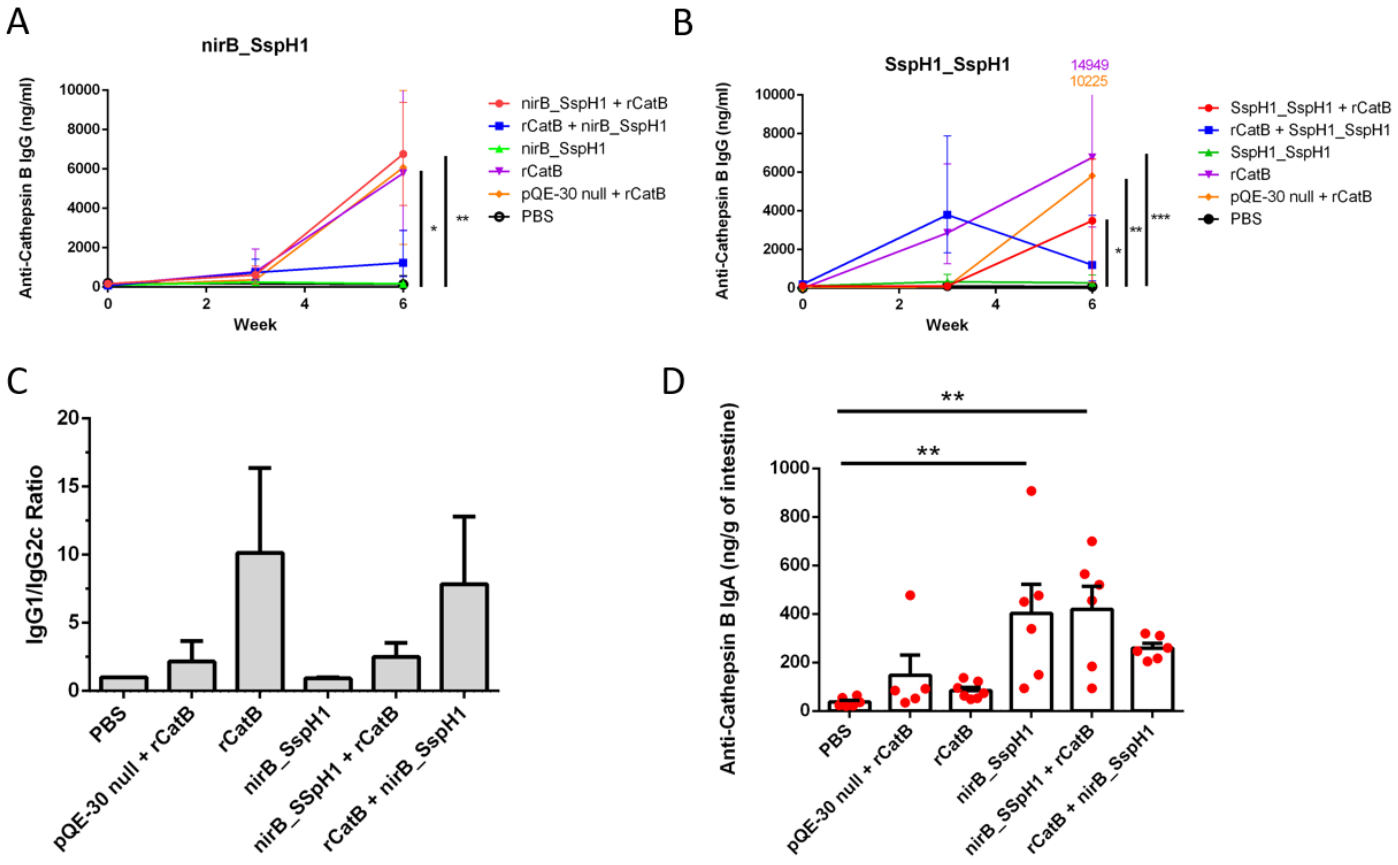


Figure 2.4. Production of Sm-Cathepsin B specific antibodies prior to challenge. Serum anti-CatB IgG was measured by ELISA at weeks 0, 3 and 6 for groups that received the nirB_SspH1 construct (A) or the SspH1_SspH1 construct (B). Each group consists of either a saline control, EV→IM, PO→PO, IM→IM, PO→IM, and IM→PO for the nirB_SspH1 and/or the SspH1_SspH1 construct. These results represent between 8 – 16 animals/group from 2 independent experiments and are reported as the geometric mean with 95% confidence intervals. Significance bars for A and B are to the right of each graph. C) Serum anti-CatB IgG1 and IgG2c were measured by endpoint-dilution ELISA and expressed as the ratio of IgG1/IgG2c. D) Intestinal anti-CatB IgA in intestinal tissue was measured by ELISA and is reported as mean ± standard error of the mean ng/gram. These results represent 5 – 7 animals per group. (*P < .05, **P < .01, ***P < .001 compared to the PBS group).

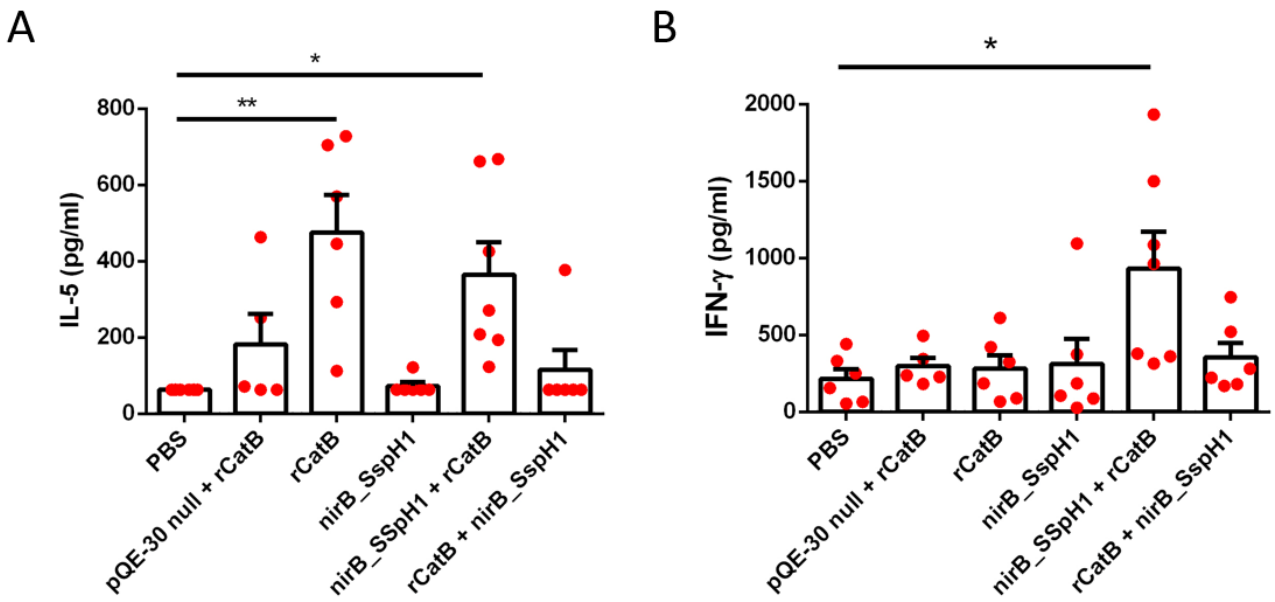


Figure 2.5. Cytokine production prior to challenge. Supernatant IL-5 (A) and IFN-γ (B) levels after stimulating splenocytes with rCatB for 72 hours were measured by QUANSYS multiplex ELISA. Each group consists of either a saline control, EV→IM, PO→PO, IM→IM, PO→IM, and IM→PO for the nirB_SspH1 and/or the SspH1_SspH1 construct. These results represent 5 – 7 animals per group. Results are expressed as the mean ± the standard error of the mean. (**P* < .05, ***P* < .01 compared to the PBS group).

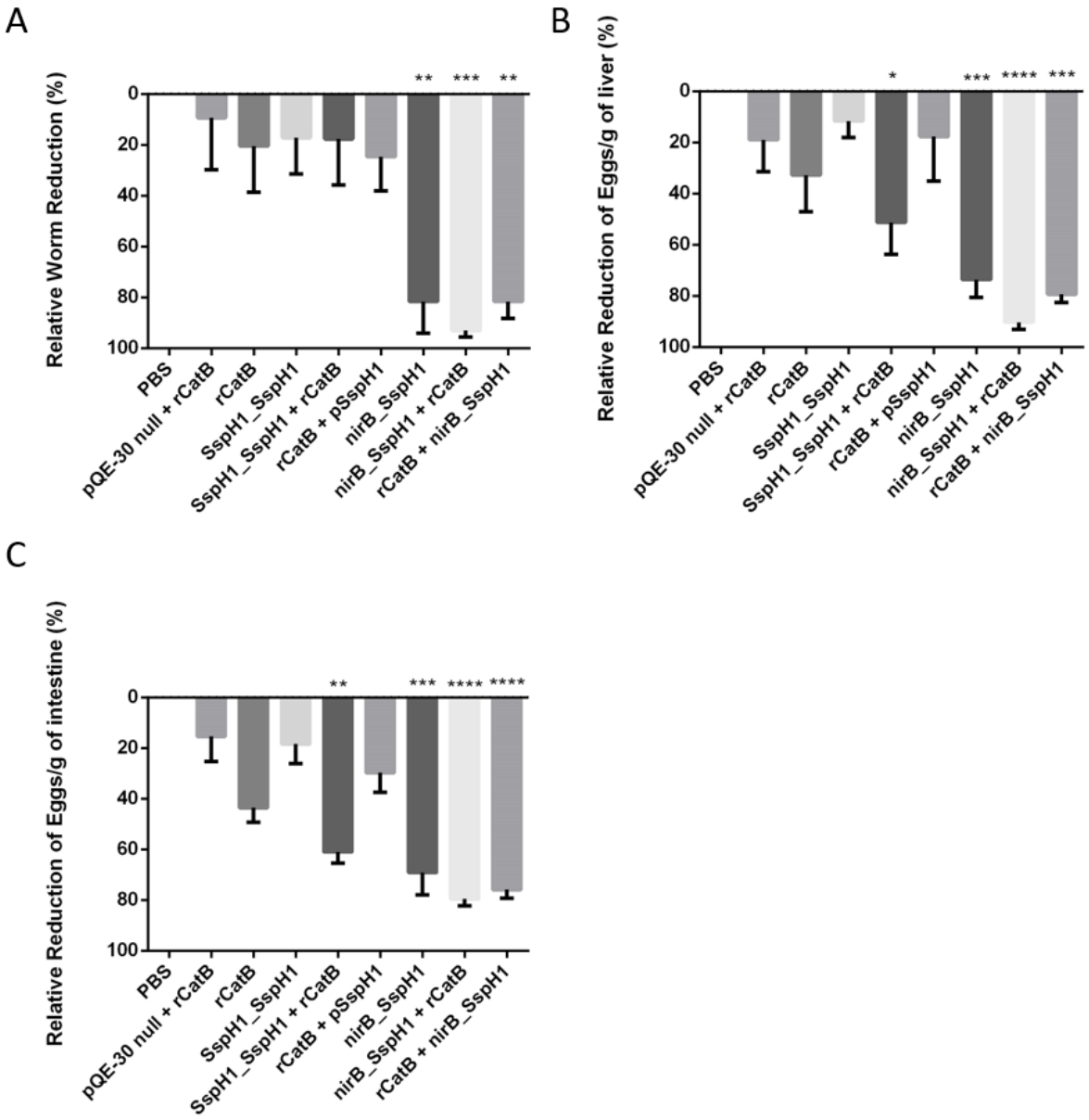


Figure 2.6. Parasitologic burden. The reduction in worm counts (A) as well as the reduction in egg load per gram of liver (B) or intestine (C) are represented for mice in each group consisting of either a saline control, EV→IM, PO→PO, IM→IM, PO→IM, and IM→PO for the nirB_SspH1 and/or the SspH1_SspH1 construct. Worm and egg burdens were determined 7 weeks after cercarial challenge. These results represent between 8 – 16 animals/group from 2 independent experiments. (**P* < .05, ***P* < .01, ****P* < .001, *****P* < .0001 compared to the PBS group).

Table 2.2. Granuloma size and egg morphology.

Group	Granuloma size (x 10^3 μm^2) ± SEM	Abnormal egg morphology (%) ± SEM
PBS	62.2 ± 6.1	0
pQE-30 null + rCatB	52.0 ± 6.9	18.9 ± 3.9
rCatB	52.8 ± 10.4	12.6 ± 5.1
SspH1_SspH1	55.0 ± 8.5	25.0 ± 6.2
SspH1_SspH1 + rCatB	47.3 ± 4.4	30.5 ± 7.7*
rCatB + SspH1_SspH1	49.8 ± 14.3	28.6 ± 6.8*
nirB_SspH1	32.9 ± 2.0**	75.9 ± 7.6****
nirB_SspH1 + rCatB	34.7 ± 3.4**	79.4 ± 4.2****
rCatB + nirB_SspH1	39.2 ± 3.7*	71.9 ± 6.0****

Liver granuloma area (x10^3 μm^2) and egg morphology (i.e.: loss of internal structures, shrinkage, crenelated periphery) were assessed. Each group consists of either a saline control, EV→IM, PO→PO, IM→IM, PO→IM, and IM→PO for the nirB_SspH1 and/or the SspH1_SspH1 construct. SEM represents the standard error of the mean. (*P<.05, **P<.01, ****P<.0001 compared to the PBS group).

2.11 SUPPLEMENTARY MATERIAL

Supplementary Table 2.1. Other cytokine production prior to challenge.

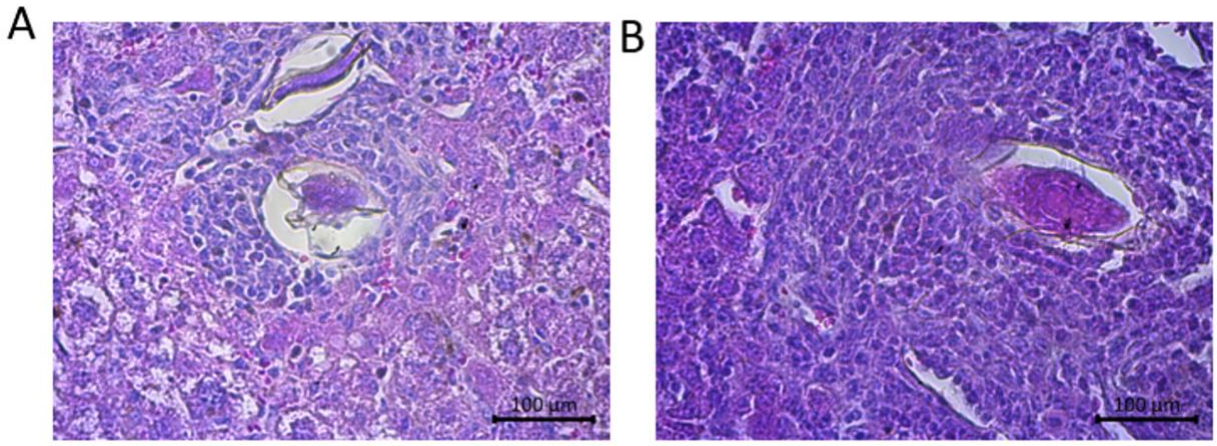
Cytokine (pg/mL)	PBS	pQE30-null + rCatB	rCatB	NirB_SspH1	NirB_SspH1 + rCatB	rCatB + NirB_SspH1
IL-2	424.5 ± 57.9	190.8 ± 62.3	426.9 ± 149.7	174.1 ± 23.5	324.6 ± 52.7	174.8 ± 62.0
IL-4	20.6 ± 2.2	27.5 ± 6.4	18.0 ± 3.2	35.4 ± 7.6	22.8 ± 3.6	10.3 ± 1.7
IL-10	10.2 ± 0.9	23.2 ± 4.3	29.7 ± 5.9	21.6 ± 2.4	21.9 ± 1.5	16.0 ± 3.1
IL-12p70	34.5 ± 12.1	21.5 ± 5.8	16.5 ± 0.8	15.8 ± 0[#]	16.2 ± 0.4	15.8 ± 0[#]
IL-13	23.0 ± 7.1	22.9 ± 8.7	75.1 ± 17.4	16.9 ± 6.1	68.8 ± 33.4	13.1 ± 2.4
IL-17	19.4 ± 5.3	14.1 ± 0[#]	25.3 ± 11.0	14.1 ± 0[#]	14.5 ± 0.4	14.3 ± 0.2
TNFα	26.7 ± 6.2	36.1 ± 6.8	24.0 ± 5.9	17.1 ± 3.0	26.1 ± 3.8	32.0 ± 5.3

Supernatant levels of different cytokines after stimulating splenocytes with rCatB for 72 hours were measured by QUANSYS multiplex ELISA. These results represent 5 – 7 animals per group. Results are expressed as the mean ± the standard error of the mean.

[#]Values were below the limit of detection.

Supplementary Table 2.2. Primers used in construct design.

	Forward Primer (5' → 3')	Reverse Primer (3' → 5')	Source
SopE2 promoter and secretory signal	CCGCTCGAGTAAAAATGTTCCTCGATAAA	CATGGTAGTTCTCCTTTTAG	YS1646
SptP promoter and secretory signal	CGCCTCGAGTTTACGCTGACTCATTGG	CATTTTTCTCTCCTCATACTTTA	YS1646
SseJ promoter and secretory signal	CGCCTCGAGACATAAAACACTAGCACT	CGCCTCGAGACATAAAACACTAGCACT	YS1646
SspH1 promoter and secretory signal	CGCCTCGAGCGCTATATCACCAAAAC	CTCTGCGGCCGCGGTAAGACCTGACGCTC	YS1646
SspH2 promoter and secretory signal	CGCCTCGAGGTTTGTGCGTCGTAT	CTCTGCGGCCGCATTCAGGCAGGCACGCA	YS1646
SteA promoter and secretory signal	CGCCTCGAGGTTTCGCCGCATGTTG	CTCTGCGGCCGCATAATTGTCCAAATAGT	YS1646
SteB promoter and secretory signal	CGCCTCGAGCGCTCCAGCGCTTCGA	CTCTGCGGCCGCTCTGACATTACCATTT	YS1646
Lac promoter	CGCCTCGAGCATTAGGCACCCCAGGCTTTACACTTTATGCTTCCGGCTCGTATGTTGTGTGGAATTGTGAGCGGATAA	GTGGAATTGTGAGCGGATAACAATTTCACACAGGAAACAGCTATGACCATGACTAACATAACACTATCCAC	Sequence is in the primers
nirB promoter	CGCCTCGAGTTGTGGTTACCGGCCCGAT	CGCGCGGCCGCCGGATCTTTACTCGCATTAC	DH5α *E. coli*
pagC promoter	CGCCTCGAGGTTAACCACTCTTAATAA	AACAACTCCT TAATACTACT	YS1646
SopE2 Secretion Signal	GGCGGTAATAGAAAAGAAATCGAGGCAAAAATGACTAACATAACACTATCCAC	AAGTCGCGGCCGCCGGATCTTTACTCGC	YS1646
SspH1 Secretion Signal	GGCGGTAATAGAAAAGAAATCGAGGCAAAAATGTTTAATATCCGCAATACACAACCTT	CTCTGCGGCCGCGGTAAGACCTGACGCTC	YS1646
Cathepsin B	CGCGCGGCCGCGCACATCTCTGTTAAAAACGAA	AGTCGGCGCGCCGTGGTGGTGGTGGTGGTGCGG	*S. mansoni*
eGFP	CGCGCGGCCGCGGTGAGCAAGGGCGAG	AGTCGGCGCGCCTTACTTGTACAGCTCGTC	pEGFP_C1



Supplementary Figure 2.1. Histological staining of liver granulomas. Representative images of H&E staining of granulomas from livers of vaccinated mice (A) and saline control mice (B). Panel A represents the PO → IM group for the nirB_SspH1 construct. Scale is set to 100 µm.

PREFACE TO CHAPTER 3

In **Chapter 2**, we described the development of a prophylactic schistosomiasis vaccine using a prime-boost strategy with oral doses of attenuated *Salmonella* Typhimurium strain YS1646 and intramuscular dosing of recombinant Cathepsin B. We demonstrated that priming with plasmid-mediated expression of Cathepsin B from the nirB_SspH1 construct followed by an unadjuvanted protein boost was capable of eliciting both systemic and mucosal humoral responses. IgG antibody subtypes indicated a mixed T_H1/T_H2 immune profile which was corroborated by cytokine secretions in *ex vivo* restimulated splenocytes. Most importantly, prime-boost vaccination led to significant parasite burden reductions in a mouse model of infection, far surpassing established WHO thresholds along with other pre-clinical data in similar models. These findings encouraged further research into the capabilities of our candidate vaccine. To date, only one other schistosomiasis vaccine has been tested for therapeutic activity and this study was performed in a non-human primate animal model. Therefore, in the following chapter, we sought to examine whether our vaccine displayed any therapeutic effect in a mouse model of chronic schistosomiasis. This chapter describes the efficacy of a shortened vaccination schedule for both prophylaxis and disease treatment. It also describes changes in the immune landscape of chronically infected mice following therapeutic vaccination with the nirB_SspH1 construct using a multimodal approach.

This chapter was adapted from the following manuscript: Therapeutic activity of a *Salmonella*-vectored *Schistosoma mansoni* vaccine in a mouse model of chronic infection. **Hassan AS,** *et al.* *Vaccine* 39(39): 5580-5588 (2021).

CHAPTER 3

Therapeutic activity of a *Salmonella*-vectored *Schistosoma mansoni* vaccine in a mouse model of chronic infection

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3.1 ABSTRACT

Schistosomiasis is an important fresh-water-borne parasitic disease caused by trematode worms of the genus *Schistosoma*. With >250 million people infected worldwide and approximately 800 million people at risk, the World Health Organization considers schistosomiasis to be the most important human helminth infection. Several prophylactic non-living vaccines are in pre-clinical and clinical development, but only one has been assessed for therapeutic effect in an animal model with modest results. Live attenuated *Salmonella* have multiple potential advantages as vaccine vectors. We have engineered an attenuated *Salmonella enterica* Typhimurium strain (YS1646) to produce a vaccine that targets the parasite digestive enzyme Cathepsin B (CatB). A multi-modality immunization schedule was used in chronically infected mice that included three oral (PO) doses of this CatB-bearing YS1646 strain on days one, three, and five as well as an intramuscular (IM) dose of recombinant CatB on day one. Parasite burden (worm count, intestinal and liver egg numbers) were 46.5 – 50.3% lower than in control animals 1 month post-vaccination and relative reductions further increased to 63.9 – 73.3% at 2 months. Serum anti-CatB IgG increased significantly after vaccination with the development of a more balanced T_H1/T_H2 pattern of response (i.e.: a shift in the IgG1:IgG2c ratio). Compared to control animals, a broad and robust CatB-specific cytokine/chemokine response was seen in splenocytes isolated 1 month post-vaccination. A vaccine that has both prophylactic and therapeutic activity would be ideal for use in conjunction with mass treatment campaigns with praziquantel in schistosome-endemic countries.

3.2 INTRODUCTION

Schistosomiasis is a parasitic disease caused by several species of the fresh-water helminth *Schistosoma*. Currently, there are over 250 million affected individuals worldwide and approximately 800 million individuals at risk of infection[1]. Infections with *S. mansoni* are the most widespread, with several endemic countries in Africa, the Middle East, South and Central America and parts of the Caribbean[2]. The symptoms of chronic disease are attributable to prolonged egg deposition with ensuing inflammation in tissues such as the liver and intestine[3]. The chronic phase of schistosomiasis is characterized by sequestration of eggs within inflammatory granulomas and T_H2-dominated immunity caused by the release of soluble egg antigens.

The only treatment for schistosomiasis at the current time is the broad-spectrum anti-helminthic praziquantel (PZQ) that paralyzes the adult worms[4]. Although PZQ has reported cure rates varying from 60 – 90% and egg reduction rates of 80 – 95% at a single dose of 40 mg/kg, there are obvious concerns about over-reliance on a single drug[4]. This is particularly true since reinfection in endemic areas is common, leading to repeated courses of therapy with the same drug. While not yet observed clinically, PZQ resistance has been induced experimentally both *in vitro* and in murine studies[5]. Unfortunately, inadequate infrastructure and resources in many endemic regions have limited the long-term effectiveness of mass drug administration (MDA) programs and other control measures[6].

The need for a schistosomiasis vaccine is therefore urgent. A small number of prophylactic vaccines have entered clinical trials in recent years and several others have shown promise in preclinical studies[2] including the multi-modal approach based on an attenuated *Salmonella enterica* Typhimurium (YS1646) that is the focus of this work[7]. While most other candidate vaccines target the outer surface of the adult worms (i.e.: tegument), we chose a digestive enzyme, Cathepsin B (CatB) that is crucial for the parasite's ability to digest host blood macromolecules at multiple life stages (i.e.: migrating schistosomula, adult worms) and targeting of CatB by RNA interference has led to stunting of parasite growth[8,9]. Used prophylactically in a 3-week prime-boost regimen, this approach led to significant reductions in parasite burden of up to 90% in a mouse challenge model[7]. Given its novel delivery method, it was of obvious interest to determine whether or not this vaccine could also be used therapeutically. The therapeutic potential of only

one other *S. mansoni* vaccine candidate, Sm-p80, has been assessed with modest results in a non-human primate model[10].

In the current work, we report that vaccination of chronically infected C57BL/6 mice resulted in a significant reduction in parasite burden compared to control animals that appeared to progress over time. Vaccination also resulted in a shift in the overall pattern of humoral and cell-mediated immunity response to the targeted antigen. To our knowledge, this is the first report of a candidate therapeutic *S. mansoni* vaccine in a murine model of chronic infection.

3.3 MATERIALS AND METHODS

3.3.1 Ethics statement

All animal procedures were conducted 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) as well as the Canadian Council on Animal Care.

3.3.2 Chronic *Schistosoma mansoni* murine infection

Biomphalaria glabrata snails (PR-1 strain) infected with *S. mansoni* were obtained from the Schistosomiasis Resource Center of the Biomedical Research Institute (Rockville, MD) through NIH-NIAID Contract HHSN272201700014I for distribution through BEI Resources. Female 6 – 8-week-old C57BL/6 mice were purchased from Charles River Laboratories (Senneville, QC). Naïve mice were infected with 150 cercaria by tail exposure as previously described[7] and left to develop chronic infection for a period of two or four months prior to therapeutic vaccination. All animals were sacrificed either one or two months post-vaccination as previously described[7]. For prophylactic vaccination studies, mice were challenged 3 weeks post-vaccination and sacrificed 7 weeks post-infection. Briefly, parasite burden was assessed by manual counting of adult worms in the portal system and mesenteric veins as well as egg burden calculation through enzymatic digestion of liver and intestinal segments.

3.3.3 Mouse vaccination

Oral immunization (PO) was performed using recombinant *S. enterica* Typhimurium YS1646 containing the pQE-30 plasmid with the promoter *nirB* and the secretory signal SspH1fused to the full-length *S. mansoni* cathepsin B gene. Construction of the *S.* Typhimurium vector has been described previously[7]. The recombinant *Salmonella* vector used for oral dosing is nirB_SspH1_CatB which will be abbreviated to nirB_SspH1. Intramuscular (IM) immunization was performed using a purified recombinant *S. mansoni* cathepsin B (rCatB) cloned and expressed in *Pichia pastoris* as previously described[11]. A multimodal vaccination schedule previously demonstrated to be effective in a vaccination/challenge mouse model of *C. difficile* was first verified in our prophylactic *S. mansoni* vaccination/challenge model[12]. This approach consisted of PO dosing by gavage every other day (200 μL containing $1x10^9$ colony-forming units (cfu)/dose) for three days (D1, D3, D5) and intramuscular (IM) dosing once on D1 (20 μg rCatB in 50 μL PBS) (**Figure 3.1**). Each experiment included a PBS control group. This schedule was utilized for both prophylactic and therapeutic vaccination experiments. Additionally, the preliminary prophylactic vaccination/challenge experiments included PO only (nirB_SspH1) and IM only (pQE-null + rCatB) dosing groups as controls. Animal numbers used for each outcome at each time point ranged from 8-10 and are indicated in the Figure legends.

3.3.4 Serum antibody production by enzyme-linked immunosorbent assay (ELISA)

3.3.4.1 Serum CatB-specific Total IgG

Cathepsin B-specific IgG was assessed by ELISA as previously described[7]. Briefly, blood was collected via saphenous bleed pre-infection, pre-vaccination and upon sacrifice in microtainer serum separator tubes (BD Biosciences, Mississauga, ON, Canada). Serum was obtained following the manufacturer's instructions and frozen at –20°C in aliquots until used in assays. Plates were coated overnight at a concentration of 0.5 μg/mL of rCatB in 100 mM bicarbonate/carbonate buffer at pH 9.6 (50μL/well) at 4°C. Each ELISA plate contained a standard curve of murine total IgG (Sigma Aldrich, St. Louis, MO) diluted 2-fold from a starting concentration of 2,000 ng/mL. Each sample was run individually and in duplicate. Serum samples were diluted 1:50 and HRP-conjugated anti-mouse IgG (Sigma Aldrich) was diluted 1:20,000. Optical density (OD) was

measured at 450 nm with an EL800 microplate reader (BioTek Instruments Inc., Winooski, VT). Concentrations of CatB-specific total IgG were calculated by extrapolation from the standard curve. These results are expressed as ng/mL.

3.3.4.2 Serum CatB-specific IgG1 and IgG2c

CatB-specific IgG1 and IgG2c titres were assessed as above by using standard curves of either purified murine IgG1 (Sigma Aldrich) or an IgG2c isotype control (Southern Biotechnologies Associates, Birmingham, AL) also diluted 2-fold starting at 2,000 ng/mL. ELISA were completed using goat anti-mouse IgG1-HRP or goat anti-mouse IgG2c-HRP (Southern Biotechnologies Associates), diluted 1:20,000. The concentrations of IgG subtypes were calculated by extrapolation from the standard curves. These results are presented as a ratio of IgG1/IgG2c.

3.3.4.3 Serum total IgE

Total serum IgE levels were determined by ELISA using the BD OptEIA™ Set Mouse IgE Kit (BD, San Diego, CA) following the manufacturer's directions as previously described[13]. Briefly, Immunolon 2HB plates were coated with anti-mouse IgE capture antibody in coating buffer at a 1:250 dilution at 4°C overnight. Plates were then washed three times (3x) with PBS-Tween 20 (0.05%; Fisher Scientific) and incubated with blocking buffer (10% fetal bovine serum (Wisent Bio Products) in PBS) for 1 hour at room temperature (RT). After washing (3x), standards and serum samples were added and left to incubate for 2 hours at room temperature. After washing (5x), biotinylated anti-mouse IgE and streptavidin-HRP were mixed and added to the plates for 1 hour at RT. After a final washing (7x), 3,3',5,5'-tetramethyl benzidine (TMB) substrate (100 μL/well; Millipore, Billerica, MA) was used for detection followed by 0.5 M H_2SO_4 after 15 minutes (50 μL/well; Fisher Scientific). OD_{450} was measured and concentration of total IgE titres was calculated by extrapolation from the standard curve. These results are expressed as ng/mL.

3.3.5 Cell-mediated response by multiplex ELISA

CatB-specific cytokine and chemokine production were assessed in mice 1 month post-vaccination. Splenocytes were isolated as previously described [7] and restimulated *ex vivo* with 2.5

μg/mL of rCatB for 72 hours. Supernatant cytokine/chemokine levels were measured using the Q-plex Mouse Cytokine-Screen (16-plex) multiplex ELISA following the manufacturer's recommendations (Quansys Biosciences, Logan, UT). This assay included IL-1α, IL-1β, IL-2, IL-3, IL-4, IL-5, IL-6, IL-10, IL-12p70, IL-17, IFNγ, TNFα, MCP-1 (CCL2), MIP-1α (CCL3), GM-CSF, and RANTES (CCL5).

3.3.6 Histopathological assessment

3.3.6.1 H&E staining

Liver sections (1 per mouse) were harvested and placed in 10% buffered formalin phosphate (Fisher Scientific) and processed for histopathology to assess mean egg granuloma size and morphology using Zen Blue Software (version 2.5.75.0; Zeiss) as previously described [7]. Mean areas are presented as $x10^3$ μm^2 ± standard error of the mean (SEM). Egg morphology was classified by an operator blinded to group assignment (AH) as abnormal if there was loss of internal structure or peripheral crenelation. Abnormal eggs are reported as a percent of the total egg count (± SEM) per histopathological sample.

3.3.6.2 PicroSirius Red staining

Slides were processed similarly as mentioned above for all steps prior to staining. Slides were hydrated and then stained with haematoxylin for 10 seconds. After washing under running water for 2 minutes, slides were stained with 0.1% PicroSirius Red solution for one hour (Direct Red 80; Sigma Aldrich, St. Louis, MO). 6 – 8 visual fields per mouse were imaged using Zen Blue Software (version 2.5.75.0; Zeiss) and the percentage of fibrotic area over the total area was measured using ImageJ software (National Institutes of Health). Mean percentages are presented ± standard error of the mean (SEM).

3.3.7 Statistical analysis

Statistical analysis was performed using GraphPad Prism 6 software (La Jolla, CA). Results are represented by at least two independent experiments (see Figure legends for details). Parasite

burden efficacy outcomes (worm number, egg counts in liver and intestinal tissues) following prophylactic vaccination were analyzed by one-way ANOVA followed by Tukey's correction for multiple comparisons. All other outcomes were analyzed by the Mann-Whitney U test. P values less than 0.05 were considered significant.

3.4 RESULTS

3.4.1 Efficacy of rapid, multimodal vaccination in a prophylactic model

We first sought to determine the efficacy of the multimodal vaccination schedule which differed substantially from our previous work that used a 3-week prime-boost regimen[7]. Mean worm, hepatic egg and intestinal egg burden were assessed 7 weeks post-infection in all groups and relative reductions were compared to the PBS-vaccinated control group. Mice that received multimodality vaccination were substantially protected from *S. mansoni* challenge. All parasitologic outcomes were generally comparable to those seen with the longer, prime-boost schedule [7]: compared to the control animals, there were significant reductions in adult worms 77.8 ± 7.9% (7.3 ± 4.2 vs. control 32.9 ± 5.4, $P < 0.0001$), hepatic eggs 75.7 ± 17.2% (1985 ± 786.5 eggs/g vs. control 8168.7 ± 603, $P < 0.0001$), and intestinal eggs 75.1 ± 7.6% (2111 ± 373.8 eggs/g vs. control 8445.2 ± 257.1, $P < 0.0001$) (**Figure 3.2**). When compared to either IM or PO immunization alone, most of the effect of vaccination was attributable to the YS1646 oral component of the multimodality schedule but the greatest impact on all parasitologic outcomes was consistently seen in the animals exposed to CatB by both IM and PO (**Figure 3.2**). The administration of non-recombinant *Salmonella* combined with IM recombinant CatB had no significant effects, therefore eliminating the possibility of non-specific YS1646-mediated protection. Thus, a shortened, multimodal vaccination schedule was also effective in reducing parasite burden in our model.

3.4.2 Parasite burden after therapeutic vaccination

The multimodal approach (IM + PO) was selected as the most optimal treatment for all therapeutic vaccination experiments. Compared to the control group, the chronically infected mice had a significantly lower worm burden (46.5 ± 4.9%) at one month after vaccination (10.6 ± 3.3 worms

vs. control 22.8 ± 5.4, $P < 0.01$), as well as 46.7 ± 8.7% and 50.3 ± 15.3% fewer eggs in the liver (4383.6 ± 332.5 eggs/g vs. control 9386.7 ± 639.1, $P < 0.01$) and intestines respectively (4462.9 ± 560.3 eggs/g vs. control 8987.5 ± 420.2, $P < 0.01$) (**Figure 3.3 A-C**). At 2 months post-vaccination, the vaccinated mice had even greater relative reductions in worm numbers (69.6 ± 8.1%, 6.1 ± 2.0 worms vs. control 20.1 ± 3.6, $P < 0.01$) as well as hepatic (73.3 ± 6.0%; 3053.3 ± 619.1 eggs/g vs. control 11433.5 ± 820.8), and intestinal egg burdens (63.9 ± 3.1%; 3604.9 ± 533.6 eggs/g vs. control 9986.2 ± 793.1) (**Figure 3.3 D-F**). These data reflect the 2-month infection time-point. The same trend was observed in the 4-month infection time-point as well (**Supplementary Figure 3.1**). Histopathologic examination of the livers revealed a larger mean granuloma size in the control mice compared to the vaccinated group (35.8 ± 3.0 x 10^3 μm^2 vs. 19.7 ± 2.0 x 10^3 μm^2, $P < 0.001$) (**Figure 3.4A**). Vaccinated mice also had a significantly higher percentage of abnormal eggs compared to the PBS control group (61.6 ± 16.2 vs. control 12.5 ± 9.7, $P < 0.05$) (**Figure 3.4B**). Liver fibrosis was assessed by PicroSirius Red staining to measure the impact on collagen deposition. Vaccinated mice had a significantly lower percentage of fibrotic surface area compared to the PBS control group (13.7 ± 1.9% vs. control 30.2 ± 3.0%, $P < 0.01$) (**Figure 3.4C**).

3.4.3 Humoral responses after therapeutic vaccination

Serum samples were collected prior to vaccination and upon sacrifice. Mice had no detectable serum anti-CatB IgG antibodies (below the limit of detection (LOD) of the ELISA used) prior to infection but titres rose significantly with infection (14206.8 ± 1101.9 ng/mL and 13864.6 ± 1524.8 ng/mL at 2- and 4-months post-infection respectively: both $p<0.001$). Serum anti-CatB levels of the PBS-treated mice did not change after mock vaccination but rose significantly following vaccination in both the 2- and 4-month infected animals: 14206.8 ± 1101.9 ng/mL to 17866.8 ± 1051.7 ng/mL (**Figure 3.5A**) and 13864.6 ± 1524.8 ng/mL to 18870 ± 1778 ng/mL (**Figure 3.5B**) respectively (both $P < 0.05$). In contrast to the relatively modest changes in total anti-CatB antibody titres in vaccinated animals, the IgG isoform distribution of this response changed remarkably 1 month following vaccination. In the mice that had been infected for 2 months, the IgG1/IgG2c ratio was dominated by a strong IgG1 response prior to and following PBS treatment as well as pre-vaccination for the experimental group: 3.0 ± 0.1, 2.6 ± 0.1, and 2.7 ± 0.2 respectively. Following vaccination, the increase in total anti-CatB IgG was largely attributable to

an increase in IgG2c that resulted in a marked decrease in the IgG1/IgG2c ratio in the animals vaccinated after 2 months of infection: 1.16 ± 0.04 (**Figure 3.5C**).

Following a 2-month infection period, serum total IgE was measured for control and experimental mice pre-vaccination and 1 month post-vaccination. We observed trends towards an increase over time for both groups. PBS control mice total IgE titres increased from 12569.8 ± 2796.2 ng/mL to 20559.2 ± 3248.2 ng/mL whereas vaccinated mice titres increased from 10458.9 ± 1397.7 ng/mL to 15917.7 ± 3327.6 ng/mL. We observed no significant difference between control and experimental mice prior to and following vaccination (**Figure 3.5D**).

3.4.4 Cytokine and chemokine responses

Cytokine and chemokine levels in splenocyte supernatants were analyzed by Quansys multiplex ELISA following *ex vivo* restimulation with rCatB for control and experimental mice 1 month post-vaccination. We observed significant increases in T_H1-associated cytokine levels in vaccinated animals compared to the control group. Of particular note, the levels of several pro-inflammatory cytokines were significantly increased: IL-1α (23.3 ± 3.3 pg/mL vs. control 7.2 ± 0 pg/mL, $P < 0.001$), IL-1β (99.2 ± 11.7 pg/mL vs. control 21.5 ± 2.4 pg/mL, $P < 0.001$), IL-6 (2000.5 ± 192.0 pg/mL vs. control 282.7 ± 65.4 pg/mL, $P < 0.001$), and TNF-α (59.4 ± 5.4 pg/mL vs. control 36.8 ± 2.8 pg/mL, $P < 0.01$). We also observed a strong increase in IFNγ levels (1931.8 ± 388.8 pg/mL vs. control 204.0 ± 71.6 pg/mL, $P < 0.001$). Interestingly, IL-2 levels in splenocyte supernatants were significantly lower in the vaccinated group (64.1 ± 16.7 pg/mL vs. control 176.7 ± 37.7 pg/mL, $P < 0.01$) (**Figure 3.6**).

Vaccination also resulted in substantial changed in some T_H2-associated and regulatory cytokine levels in the re-stimulated splenocyte supernatants: IL-5 (299.0 ± 35.4 pg/mL vs. control 167.1 ± 18.5 pg/mL, $P < 0.01$) and IL-10 (30.8 ± 2.5 pg/mL vs. control 15.1 ± 1.3 pg/mL, $P < 0.001$) (**Figure 3.6**).

The impact of vaccination on the supernatant chemokine levels was variable. We observed a slight decrease in CCL2 (MCP-1) levels (2361.5 ± 106.4 pg/mL vs. control 2595.1 ± 59.6 pg/mL, $P <$

0.05) and a much greater reduction in CCL3 (MIP-1α) (33.5 ± 20.1 pg/mL vs. control 1279.8 ± 226.2 pg/mL, $P < 0.0001$). In contrast, supernatant CCL5 (RANTES) levels were increased in the vaccinated group (424.4 ± 46.2 pg/mL vs. control 255.8 ± 40.3 pg/mL, $P < 0.05$) (**Figure 3.6**). There were no significant differences between groups for IL-3, IL-4, IL-12, IL-17, and GM-CSF levels.

3.5 DISCUSSION

The *S. mansoni* digestive enzyme Cathepsin B (CatB) is needed for nutrient acquisition and maturation of the parasite, making it a promising candidate vaccine antigen. We have previously demonstrated the potential of recombinant CatB as a traditional prophylactic vaccine delivered IM with a number of adjuvants including CpG dinucleotides[11], Montanide ISA 720 VG[14], and AddaVax, an MF59-like squalene adjuvant, as well as sulfated lactosyl archaeal archaeosomes (SLA)[13] in a murine vaccination/challenge model. In these studies, the effectiveness for parasitologic outcomes ranging from 54 – 87%. Most recently, we demonstrated that delivery of CatB using an attenuated *Salmonella* Typhimurium strain (YS1646) in a 30-day prime-boost strategy can achieve reductions in parasite burden as high as 93% in the same model[7]. In the current work, we sought to determine whether or not a YS1646-based vaccination strategy could be shortened to only 5 days and could be used therapeutically in a model of chronic schistosomiasis. The evaluation of therapeutic vaccine efficacy is intrinsically more complex than assessing prophylaxis, particularly in a chronic infection like schistosomiasis in which eggs are deposited and accumulate in the tissues of the infected host continuously once the adult females reach maturity. Nonetheless, when the parasite burden was assessed in our mouse model 1- or 2-months post-vaccination, we observed significant impact on all parasitologic outcomes, ranging from 46 – 50% after 1 month and increasing to 55 – 70% at 2 months post-vaccination relative to control animals. In terms of absolute parasite burden, we observed a trend towards a progressive decrease in worm and tissue egg burden over time after vaccination. While we are unable to determine if this therapeutic vaccination regimen would lead eventually to a cure, these observations suggest a durable effect of vaccination on the host immune response that appears to be detrimental to the parasite.

It is also plausible that our vaccine had an anti-fecundity effect on the female worms, arresting or interfering with the egg-laying process. Indeed, in our prior work, we observed a high rate of morphologically abnormal eggs as well as a significant decrease in egg granuloma size in animals challenged after prophylactic vaccination[7]. These same effects were observed once more in our chronic infection model after therapeutic vaccination. Granuloma formation is mediated by the release of soluble egg antigens (SEA) and serves as a defense mechanism for the host[1,3]. Since CatB is not expressed by *S. mansoni* eggs, the morphologic changes and smaller granuloma size we observed may reflect a change in the host immune response and/or reduced viability of the eggs due to decreased fitness of the female worms. Although the profile of the immune response was clearly altered by therapeutic vaccination, these changes would be predicted to result in more rather than less inflammation. It is therefore of interest that immature and dead *S. mansoni* eggs have recently been reported to induce a weak granulomatous response and that cellular recruitment into granulomas is intrinsically linked to egg antigen secretion[15]. These results raise the possibility that the YS1646-based therapeutic vaccine could also help to interrupt transmission through its impact on fecundity and egg viability.

The use of *Salmonella* Typhimurium YS1646 as a vaccine vector is attractive for several reasons. Live attenuated bacterial vectors provide an in-built adjuvanticity to the antigens they carry. Specifically, the YS1646 vector can stimulating the host immune system through TLR-4 and TLR-5 signalling via its attenuated LPS and flagellin respectively[16,17]. We have previously demonstrated that recombinant CatB-bearing YS1646 strains are capable of inducing more balanced T_H1/T_H2 humoral and cellular responses to CatB[7]. In the current study, we observed a vaccine-induced reversal of the typical parasite-driven IgG1-dominated IgG1/IgG2c ratio. This shift to a more balanced immunity in response to vaccination and an increase in the T_H1 immune response could explain the observed reductions in parasite burden and may help to explain why the predominantly IgG1 anti-CatB response seen during natural infection do not appear to be effective in controlling the infection.

Mouse models have been extensively used to study the immunopathogenesis of schistosomiasis and have deepened our understanding of the human immune response to this family of helminths [1]. A large body of preclinical work has established that T_H1 responses are key to the induction of

a successful immune response against schistosomiasis in murine models[11,18,19] while T_H2-type responses may play the larger prominent role during chronic infection and reinfection in humans[20]. However, both exaggerated T_H1- and T_H2-biased responses can lead to severe pathology and death[1]. Therefore, the optimal T cell immunity in chronic schistosomiasis is likely to be a balanced T_H1/T_H2 response. Infected mice treated with our vaccine displayed an increase in several T_H1-associated cytokines such as IL-1α, IL-1β, IL-6, IFNγ and TNFα compared to unvaccinated controls. These cytokines mediate inflammatory responses and may activate immune cells to counter the existing, non-protective T_H2-skewed immune responses. Indeed, the T_H2-biased and regulatory responses that characterize chronic schistosomiasis may be a compromise that favours the survival of both the host and the parasite long-term[21]. We and others have shown that recombinant Sm-CatB alone skews the overall immune response towards a T_H2 pattern while prophylactic prime-boost vaccination with YS1646 elicits a more mixed T_H1/T_H2 pattern response[7,22]. After therapeutic vaccination in our chronic infection model, we also observed increases in anti-inflammatory cytokines IL-5 and IL-10 in the supernatants of splenocytes re-stimulated with CatB. Together, these observations suggest that our multimodal vaccination strategy was able to redirect the parasite-driven T_H2-skewed immune profile towards a more balanced response. These observations are consistent with the work of Karmakar et al. which showed that vaccination with adjuvanted Sm-p80 increases both T_H1 and T_H17 cytokine expression in baboon peripheral blood mononuclear cells (PBMCs) and T_H2 cytokine expression in lymph node cells[10]. Thus, while YS1646-based therapeutic vaccine showed considerable promise in our mouse model, the possible success of this strategy will only be proved in human trials due to differences in the protective mechanisms between mice and humans.

Although the preponderance of data favours a pivotal role for cellular immunity in both the immunopathology associated with chronic schistosomiasis and protection against *S. mansoni* reinfection[1,20], several studies have suggested a possible supportive role for antibodies as well[23,24]. Indeed, recent studies have reported antibody-mediated resistance in rhesus macaques to infection with *S. mansoni* and *S. japonicum* with detrimental effects to adult worms which appear physiologically damaged[25,26]. It has been demonstrated that Sm-CatB is utilized by schistosomula and adult worms to digest serum IgG as well[9]. Mice in our model had high titres of anti-CatB IgG prior to vaccination (primarily IgG1), but these titres increased following vaccination with a

marked shift towards IgG2c. Since these antibodies target a digestive enzyme, it is possible they result in decreased fitness or starvation of the adult worms leading to the observed changes in egg morphology. In addition to a possible supportive role of the humoral response in chronic schistosomiasis, the tendency of helminth antigens to elicit IgE responses has recently raised concerns about the induction of IgE responses by anti-helminth vaccines. Indeed, participants enrolled in a hookworm vaccine clinical trial suffered from allergic-like responses due to prior exposure to the vaccine antigen[27]. It is therefore reassuring that we observed no significant change in total IgE production between vaccinated and control animals after therapeutic vaccination.

The current state of our candidate YS1646 vaccine strategy is not yet optimal. In particular, the expression of Sm-CatB in the transformed vector was plasmid-mediated (i.e.: pQE-30) which is likely to be present in multiple copies and contains an ampicillin-resistance gene. The integration of our construct into the genome of *S.* Typhimurium would permit removal of the antibiotic resistance gene as well as lead to more stable and predictable expression of our antigen[28]. Of course, chromosomal integration of one or a small number of CatB gene copies could potentially reduce the amount of antigen produced by the YS1646 vector and limit its effectiveness *in vivo*. Differences between persistence in mice and humans might also limit the effectiveness of this strategy. In the early 1990s, YS1646 advanced to phase I clinical testing in subjects with unresponsive cancer as a possible treatment but was subsequently abandoned due to rapid clearance from the bloodstream after intravenous administration[29]. Although this background substantially increases the likelihood that YS1646 can be administered orally with a large margin of safety, the limited survival of this bacterium in humans is very different from its biology in mice in which *S.* Typhimurium disseminates widely and can persist for weeks[30]. While possibly limiting exposure to the vaccine antigen, short-lived local invasion of the gastrointestinal tract might be ideal to induce immunity without risk of long-term *Salmonella* persistence. Ongoing experiments include the chromosomal integration of single- and multi-copy constructs. Future studies will also examine the effects of oral or intramuscular only regimens including non-recombinant *Salmonella*. Finally, mature Cathepsin B is well conserved cysteine protease with 50 – 60% homology between schistosomal and mammalian enzymes[31]. Although this homology raises some concerns about off-target effects, mammalian CatB is restricted to the cell lysosome[32] and therefore relatively unlikely to be accessible to antibodies induced by vaccination even if cross-reactivity were to be present.

In 2013, global experts met to discuss the development of schistosomiasis vaccines[33] and established both a desirable target product profile (TPP) and preferred product characteristics (PPC) for a prophylactic vaccine. It was determined that the acceptable standard of vaccine efficacy would be a reduction of 75% in egg output and worm burden for at least one schistosome species[33]. Furthermore, they indicated that an ideal vaccine would be capable of exerting a therapeutic effect on existing disease as well. Over 250 million people are infected with schistosomiasis and a dual-purpose vaccine would greatly enhance the drive towards elimination in combination with existing mass drug administration campaigns. Although a number of schistosomiasis vaccines are at various stages of pre-clinical and clinical development[2], only one has been assessed for therapeutic use. This vaccine targets the cysteine protease subunit of calpain Sm-p80 and, when chronically infected baboons received 3 intramuscular doses adjuvanted with an oil-in-water emulsion of glucopyranosyl lipid A (GLA-SE)[10], worm numbers, tissue eggs and fecal eggs were reduced approximately by 33 - 54% compared to the adjuvant control. Together with our current CatB observations, their findings strongly support the targeting of cysteine proteases in chronic *S. mansoni* infection. The potential synergistic effects of praziquantel treatment with our vaccine remain to be evaluated.

In summary, we report a multimodal YS1646-vectored vaccine expressing Sm-CatB that is capable of reducing parasite burden in chronically infected mice. To our knowledge, this is the first report of an effective therapeutic vaccine in a murine model of schistosomiasis. The observed reductions in parasite burden were associated with modification of the existing immune profile towards a more balanced T_H1/T_H2 response and increased secretion of T_H1-type cytokines but no evidence of immunopathology or IgE induction. These data support the further development of this potentially dual-purpose *S. mansoni* vaccine.

3.6 ACKNOWLEDGEMENTS

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3.7 AUTHORS' CONTRIBUTIONS

ASH was involved in all aspects of the study including study design, performing experiments, data analysis and preparation of the manuscript. DJP assisted in the design of the snail housing facility and the infection model. BJW and MN supervised all parts of the study and prepared the manuscript.

3.8 COMPETING INTERESTS

ASH, MN and BJW are inventors on a patent application for a YS1646-based *S. mansoni* vaccine.

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3.10 FIGURES AND LEGENDS

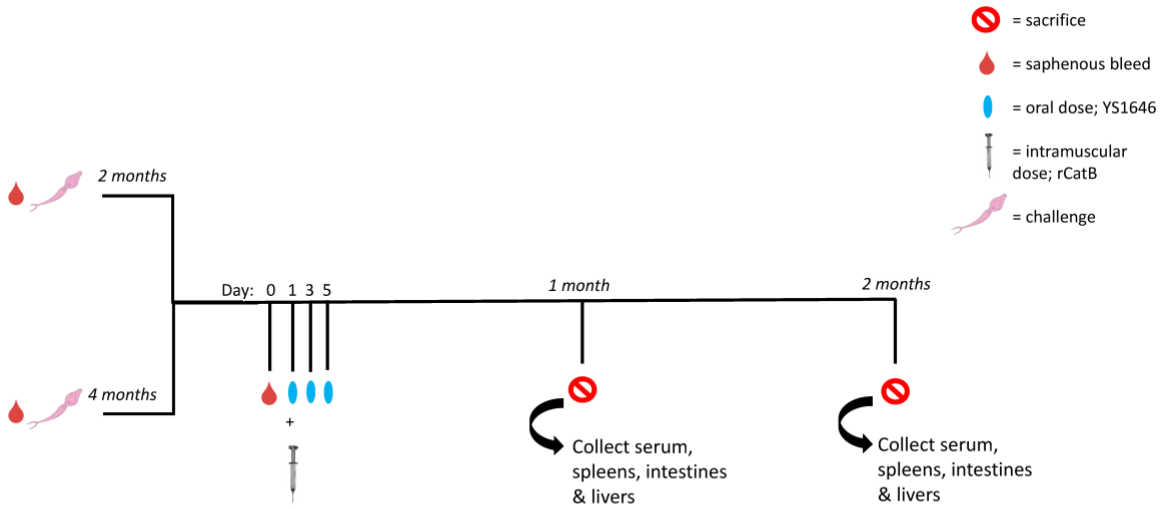


Figure 3.1. Experimental study timeline. Baseline serum is collected prior to infection. Mice are infected with 150 cercaria for either a period of 2 or 4 months. Mice then undergo multimodal vaccination which consists of simultaneous doses of intramuscular rCatB and oral YS1646 on day 1 followed by two additional oral doses on days 3 and 5. Mice are then sacrificed either 1- or 2-months post-vaccination.

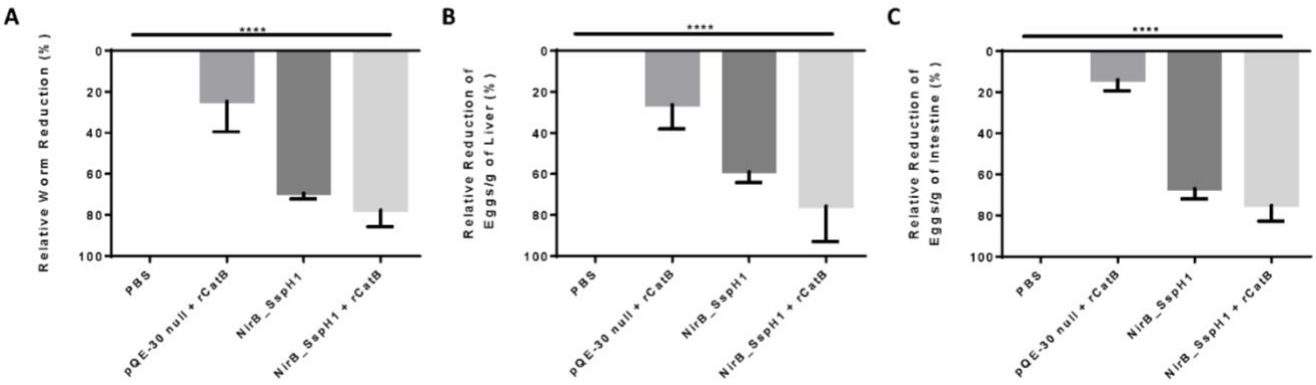


Figure 3.2. Effectiveness of shortened vaccination schedule in prophylactic model. The reduction in worm counts (A) as well as the reduction in egg load per gram of liver (B) or intestine (C) are represented for each group. Groups consist of PBS control, IM only (pQE-30 null + rCatB), PO only (nirB_SspH1), and multimodal vaccination (nirB_SspH1 + rCatB). Worm and egg burdens were determined 7 weeks after cercarial challenge. Each independent experiment consisted of 8 animals per group. Each experiment was performed twice. Data are shown as mean ± SEM (****$P < .0001$ compared to the PBS group).

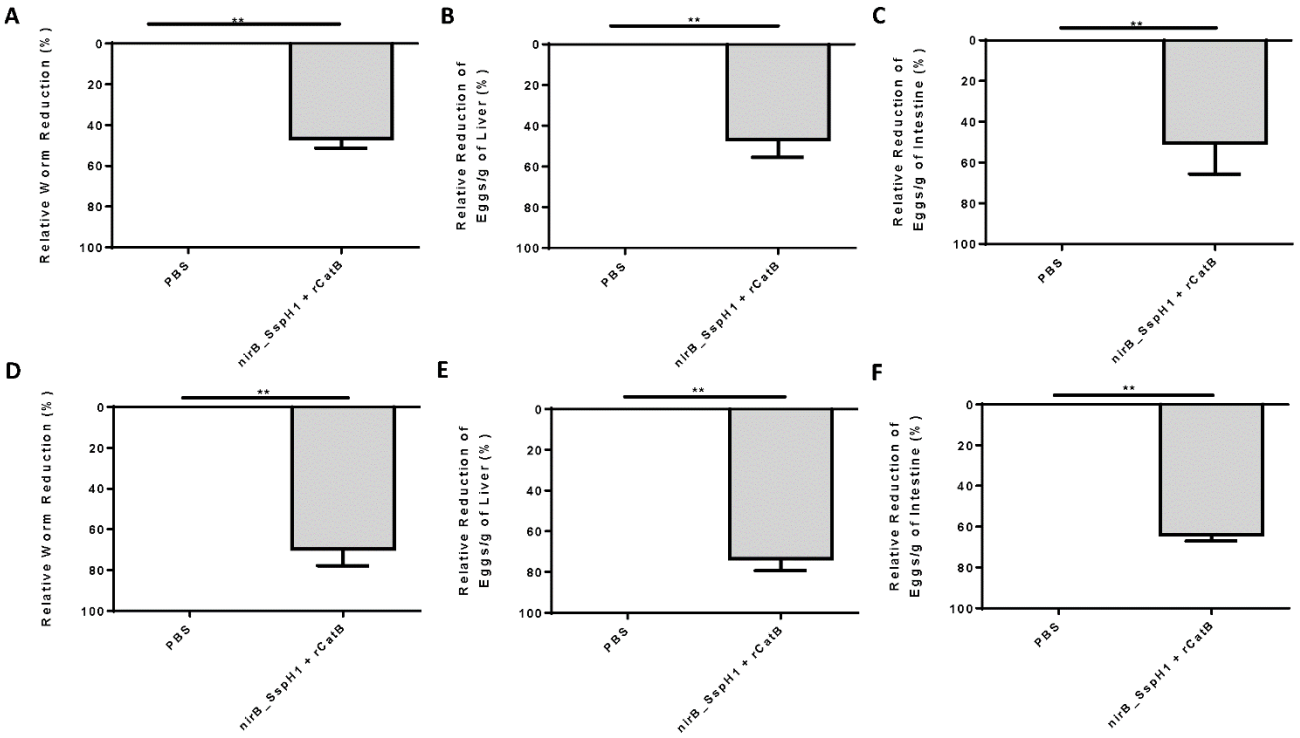


Figure 3.3. Parasite burden reductions after therapeutic vaccination. Worm reduction (A), hepatic egg reduction (B) and intestinal egg reduction (C) were assessed 1 month post-vaccination. Worm (D), hepatic egg (E) and intestinal egg (F) reductions were assessed 2 months post-vaccination. All are expressed as relative to the PBS control. Each independent experiment consisted of 10 animals per group. Each experiment was performed twice. Data are shown as mean ± SEM (**$P < 0.01$).

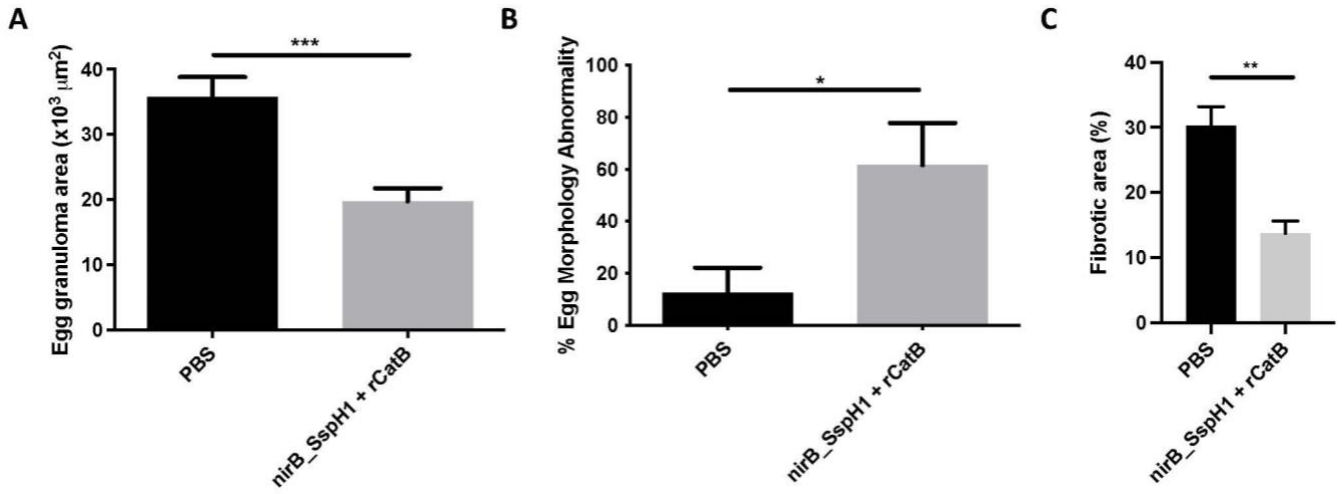


Figure 3.4. Histopathological analysis of hepatic eggs. Liver granuloma area (x10^3 μm^2) (A) and egg morphology (i.e.: loss of internal structure, shrinkage, crenelated periphery) (B) were assessed using Zen Blue software. Each independent experiment consisted of 6 – 8 granulomas per animals per group. Each experiment was performed twice. Percentage of fibrotic area by PicroSirius Red staining of liver sections (C) analyzed by ImageJ software. Data are shown as mean ± SEM (*P < 0.05, **P < 0.01, ***P < 0.001).

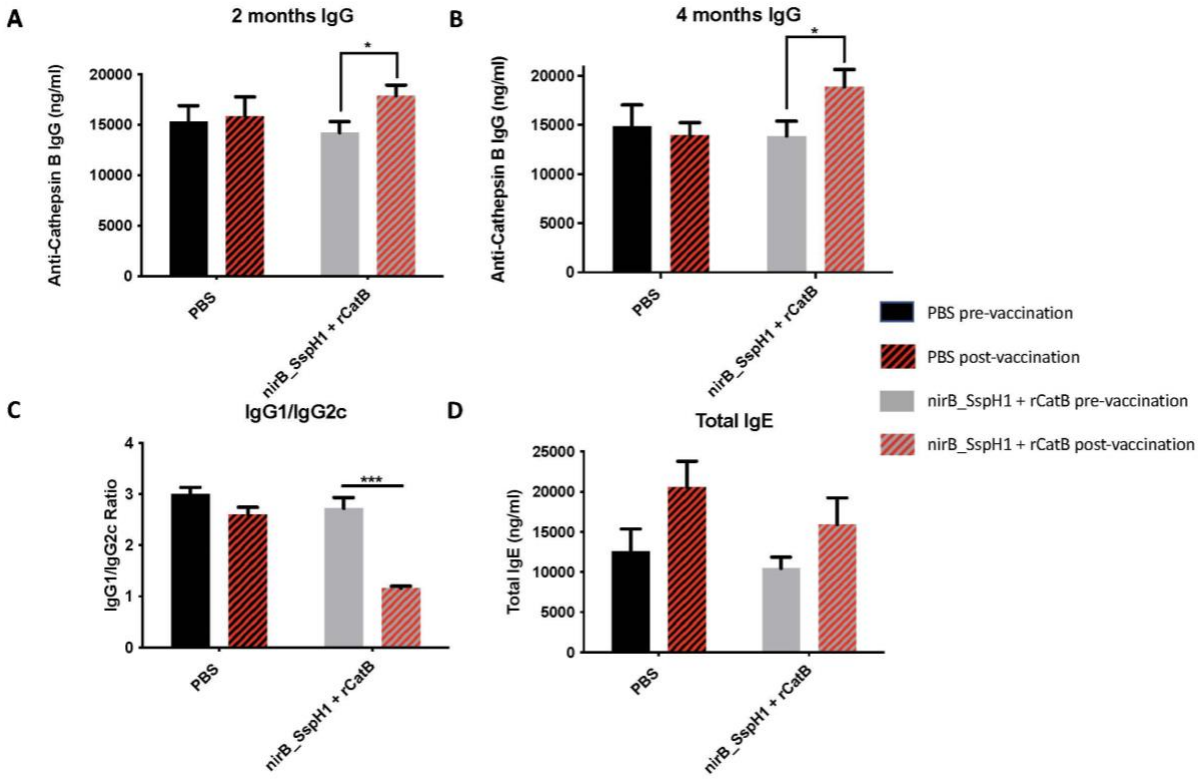
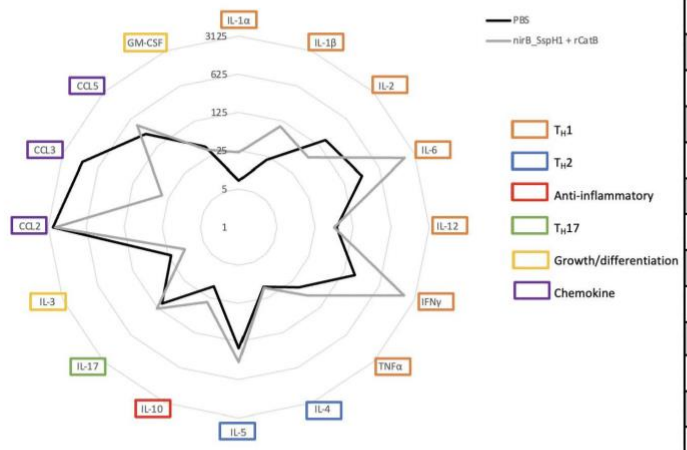


Figure 3.5. Humoral responses. Anti-Cathepsin B-specific IgG combined titres for 1- and 2-months post-vaccination after 2 months (A) and 4 months (B) infection periods. Anti-Cathepsin B-specific IgG1 and IgG2c expressed as a ratio of IgG1/IgG2c 1 month post-vaccination (C). Total IgE 1 month post-vaccination (D). Plain bars represent pre-vaccination titres and bars with red diagonal lines represent post-vaccination titres for both groups. Each independent experiment consisted of 10 animals per group. Each experiment was performed twice. Data are shown as mean ± SEM (*$P < 0.05$, ***$P < 0.001$).

A

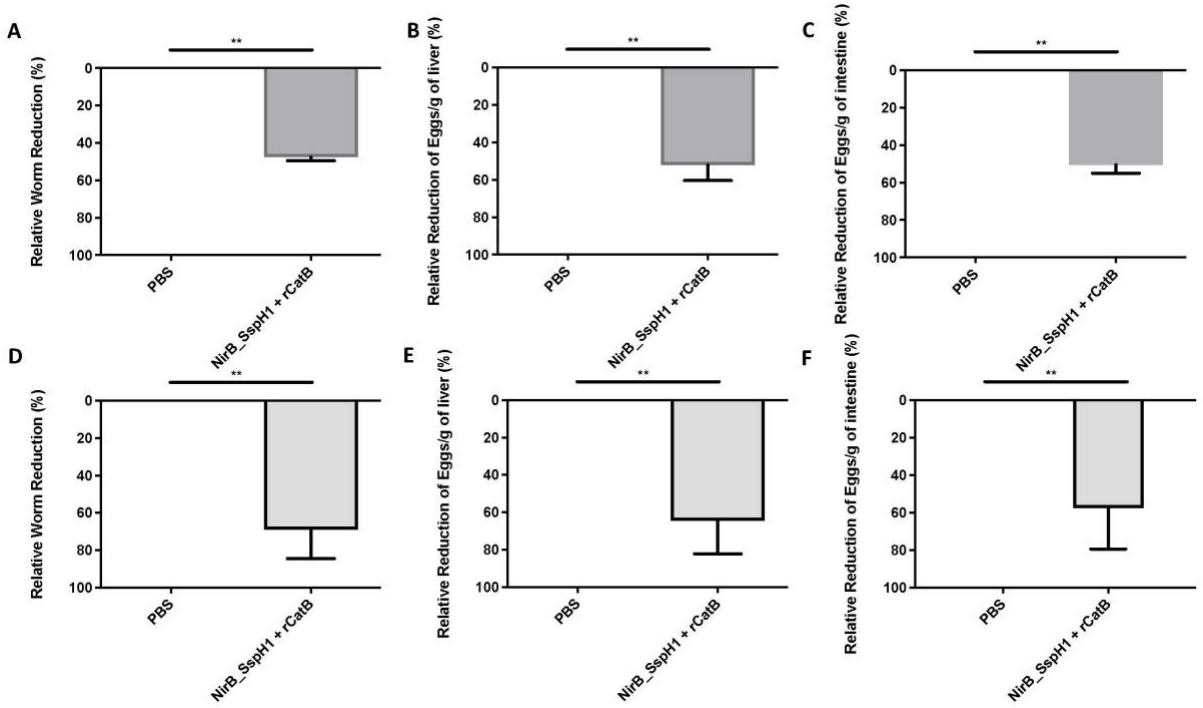


B

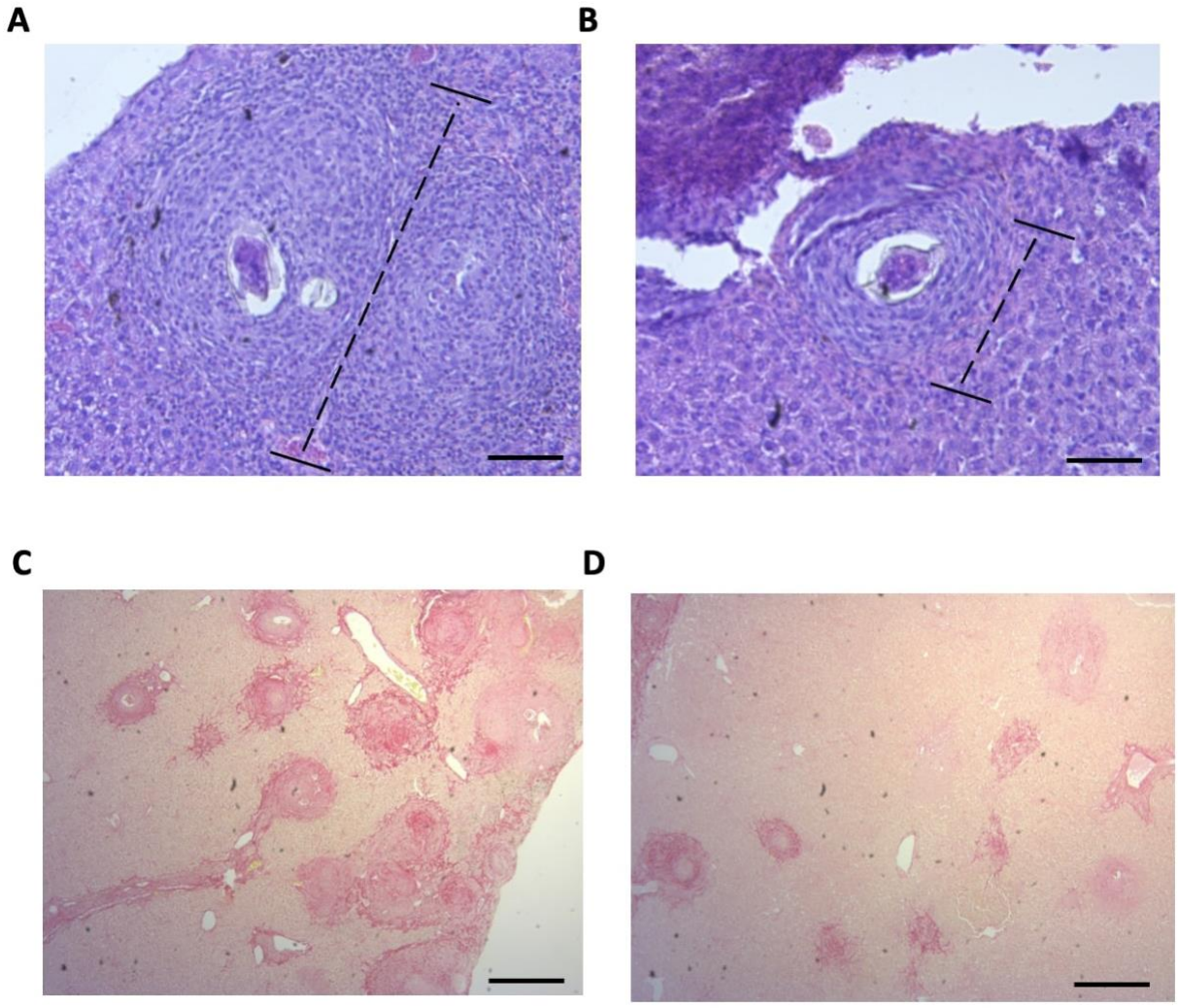
Cytokine/chemokine	Mean fold change relative to control
IL-1α	3.2***
IL-1β	4.6***
IL-2	0.4**
IL-6	7.1***
IL-12	0.9
IFNγ	9.5***
TNFα	1.6**
IL-4	1.0
IL-5	1.8**
IL-10	2.0***
IL-17	1.3
IL-3	0.5
CCL2	0.9*
CCL3	0.03****
CCL5	1.7*
GM-CSF	0.9

Figure 3.6. Cytokine and chemokine secretion levels. Mean secretion levels (pg/mL) of cytokines and chemokines 1 month post-vaccination produced from splenocytes restimulated *ex vivo* with recombinant CatB after 72 hours depicted by radar plot on log scale (A). PBS control and vaccinated group are represented by the black and grey lines respectively. Mean fold changes in secretion levels for each cytokine and chemokine relative to the PBS control (B). Each independent experiment consisted of 8 animals per group. Each experiment was performed twice. (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, ****$P < 0.0001$).

3.11 SUPPLEMENTARY MATERIAL



Supplementary Figure 3.1. Parasite burden reductions after therapeutic vaccination 4 months post-infection. Worm reduction (A), hepatic egg reduction (B) and intestinal egg reduction (C) were assessed 1 month post-vaccination. Worm (D), hepatic egg (E) and intestinal egg (F) reductions were assessed 2 months post-vaccination. All are expressed as relative to the PBS control. Each independent experiment consisted of 8 animals per group. Each experiment was performed twice. Data are shown as mean ± SEM (**$P < 0.01$).



Supplementary Figure 3.2. Histopathological staining of hepatic sections. Representative images of H&E staining of liver egg granulomas of control mice (A) and vaccinated mice (B) 1 month post-vaccination. Representative images of PicroSirius Red staining of liver fibrosis in control mice (C) and vaccinated mice (D) 1 month post-vaccination. Scale is set to 100 μm.

PREFACE TO CHAPTER 4

In **Chapter 2** and **Chapter 3**, we described the prophylactic and therapeutic efficacy of plasmid-mediated expression of Cathepsin B in a live attenuated YS1646 strain of *Salmonella* Typhimurium in combination with intramuscular administration of recombinant antigen in either prime-boost or shortened multimodal vaccination schedules. Expression of our antigen using the pQE-30 plasmid bears limitations if we hope to move forward toward human clinical trials. First, the presence of an antibiotic resistance gene on a mobile genetic element could not be introduced to the patient's gastrointestinal tract. Second, plasmid-based expression is less stable, and the bacterial vector may be subject to losing the plasmid in the absence of selective pressure in the animal model. Therefore, to mitigate these pitfalls, we next sought to develop a novel YS1646 vaccine vector through chromosomal integration. The following chapter describes the cloning experiments performed to generate novel YS1646 vaccine strains. It also describes the immunogenicity conferred by these strains using the shortened multimodal schedule by measuring both humoral and cellular immune responses by ELISA and flow cytometry. Finally, the chapter describes the protection efficacy of multimodal vaccination using a chromosomally integrated vaccine strain.

This chapter was adapted from the following manuscript: Chromosomally integrated gene expression of Schistosoma mansoni Cathepsin B in a Salmonella Typhimurium vaccine vector protects against murine schistosomiasis. **Hassan AS** *et al.* (under review at *npj Vaccines*).

CHAPTER 4

Chromosomally integrated gene expression of *Schistosoma mansoni* Cathepsin B in a *Salmonella* Typhimurium vaccine vector protects against murine schistosomiasis

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4.1 ABSTRACT

Schistosomiasis threatens hundreds of millions of people worldwide. The larval stage of *Schistosoma mansoni* migrates through the lung and adult worms reside adjacent to the colonic mucosa. Several candidate vaccines are in pre-clinical development, but none is designed to elicit both systemic and mucosal responses. We have repurposed an attenuated *Salmonella enterica* Typhimurium strain (YS1646) to express Cathepsin B (CatB), a digestive enzyme important for juvenile and adult stages of the *S. mansoni* life cycle. Previous studies have demonstrated the prophylactic and therapeutic efficacy of our plasmid-based vaccine. We have generated chromosomally integrated (CI) YS1646 strains that express CatB to produce a viable candidate vaccine for eventual human use (stability, no antibiotic resistance). 6–8-week-old C57BL/6 mice were vaccinated in a multimodal oral (PO) and intramuscular (IM) regimen, and then sacrificed 3 weeks later. The PO + IM group had significantly higher anti-CatB IgG titers with greater avidity and mounted significant intestinal anti-CatB IgA responses (all $P < 0.0001$). Multimodal vaccination generated balanced T_H1/T_H2 humoral and cellular immune responses. Production of IFNγ by both $CD4^+$ and $CD8^+$ T cells was confirmed by flow cytometry ($P < 0.0001$ and $P < 0.01$). Multimodal vaccination reduced worm burden by 80.4%, hepatic egg counts by 75.2%, and intestinal egg burden by 78.4% (all $P < 0.0001$). A stable and safe vaccine that has both prophylactic and therapeutic activity would be ideal for use in conjunction with praziquantel mass treatment campaigns.

4.2 INTRODUCTION

Trematode worms of the *Schistosoma* genus are responsible for schistosomiasis, a fresh-water-borne disease that affects over 250 million people worldwide. Approximately 800 million people are at risk of infection caused primarily by three human *Schistosoma* spp.: *S. mansoni*, *S. haematobium*, and *S. japonicum*. As the most widespread, *S. mansoni* is prevalent in Sub-Saharan Africa, South and Central America, the Middle East, and parts of the Caribbean[1]. Although school-aged children are most affected, all age groups can suffer from schistosomiasis symptoms including debilitating disease[2,3].

The oral anthelmintic, praziquantel (PZQ), is the cornerstone of schistosomiasis control in endemic regions. National or regional deworming campaigns typically deliver a single dose of 40 mg/kg and cure rates are reported to vary between 60 – 90%[4]. However, overreliance on PZQ has led to concerns about drug resistance which has been observed experimentally in both *in vitro* and *in vivo* studies[5,6]. While periodic deworming and vector management are useful to control schistosomiasis, vaccines will likely be necessary to achieve elimination[7]. Several candidate vaccines are in pre-clinical development, and few have entered clinical trials in recent years[7]. Most of these candidate vaccines are based on repeated intramuscular injection of one or more recombinant proteins with an adjuvant. In contrast, our group has established a vaccination platform based on a live attenuated *Salmonella enterica* serovar Typhimurium YS1646 that can be administered orally[8,9]. Our strategy also differs from most others in targeting a key digestive enzyme of the parasite, Cathepsin B (CatB), rather than external tegumental proteins[10]. This novel approach results in significant reductions in parasite burden when used either prophylactically in a 3-week prime-boost regimen or therapeutically in a shortened 5-day schedule in chronically infected mice.

Previous studies with our recombinant *Salmonella* strains have used plasmid-based expression of CatB[8,9] which would be unacceptable for human use. These strains are intrinsically unstable since bacteria can shed plasmids during cell division in an environment that does not select for their retention[11]. Furthermore, retention of plasmids typically involves the use of one or more antibiotic resistance genes. The inclusion of such resistance genes on a mobile genetic element is clearly inappropriate for human use due to the risk of spreading resistance to enteric bacteria. As a result,

chromosomal integration of the antigen construct is crucial to the advancement of these vaccine candidates to the clinic. An obvious concern with chromosomal integration is a sharp reduction in the CatB copy number. In a plasmid-based system, each recombinant *Salmonella* may contain up to 30 copies of the CatB gene while chromosomal integration typically introduces only a single copy of the gene.

In the current work, we report the construction of a chromosomally integrated, single-copy CatB recombinant YS1646 *Salmonella* Typhimurium strain. Vaccination with this novel vector resulted in significant reductions in parasite burden in a murine model. Multi-modal immunization with these candidates led to robust humoral and cellular immune responses with a balanced T_H1/T_H2 profile. To our knowledge, this is the first report of a chromosomally integrated *Salmonella*-vectored vaccine for schistosomiasis.

4.3 METHODS

4.3.1 Ethics statement

All animal procedures were conducted 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) as well as the Canadian Council on Animal Care.

4.3.2 Bacterial plasmids

Salmonella enterica Typhimurium (*S.* Typhimurium) strain YS1646 (Δ*msbB2* Δ*purI* Δ*Suwwan xyl* negative; ATCC 202165; ATCC, Manassas, VA) was obtained from Cedarlane Labs (Burlington, ON, Canada). Recombinant Tn7 plasmids were produced in *Escherichia coli* DH5α π kindly provided by Dr. Charles Dozois (Thermo Fisher Scientific, Eugene, OR). Conjugation experiments with YS1646 required *E. coli* MGN-617 for donor genetic material. Plasmids were introduced into YS1646 either by conjugation or by electroporation (20 ng of plasmid at 1.8 kV, 200 Ω and 25 μF; ECM 399 Electroporation System, BTX, Holliston, MA, US). Plasmids were introduced into *E. coli* by heat shock. *S.* Typhimurium and *E. coli* were cultured in Luria broth (LB), with the following antibiotics and amino acids when necessary to maintain plasmids: 100 μg/mL ampicillin

(Amp), 50 μg/mL kanamycin (Km), 30 μg/mL chloramphenicol (Cm), and 50 μg/mL diaminopimelic acid (DAP).

4.3.3 Chromosomal integration

The *nirB* and *pagC* promoters as well as the SspH1 and Cathepsin B sequences have been previously described[8,12]. The *frr* promoter was obtained from YS1646 *S.* Typhimurium by PCR. The pGP-Tn7-Cm plasmid backbone[11] was digested using FastDigest restriction enzymes EcoRI and KpnI (Thermo Fisher Scientific). Promoter, secretory signal, and antigen sequences were inserted using the *pEASY* - Uni Seamless Cloning and Assembly kit (TransGen Biotech, Beijing, China). Following the construction of novel Tn7 plasmids, DAP$^-$ *E. coli* MGN-617 were transformed to generate 3 novel strains. The transformed *E. coli* strains were then used for conjugation experiments with a YS1646 strain containing the temperature-sensitive pSTNSK plasmid which encodes the Tn7 transposase system and confers Km resistance[11]. Donor *E. coli* and recipient YS1646 were resuspended in LB supplemented with Km and DAP. 100 μL of the donor strain and 50 μL of the recipient strain were centrifuged together and then resuspended in 10 μL at 30°C for 5 hours. Mixed culture was later grown on LB Km-Cm plates at 37°C, and YS1646 colonies that grew in the absence of DAP, but that had lost resistance to antibiotic markers present on the pSTNSK plasmid but gained Cm resistance associated with the *att*Tn7 targeting sequence were selected. Chromosomal integration at the *att*Tn7 was then confirmed by PCR. The temperature-sensitive pCP20 plasmid, encoding the recombinase flippase (FLP) and conferring Amp and Cm resistance, was transformed into YS1646 strains. The Cm resistance cassette, integrated at the *att*Tn7 site, was flanked by two FRT regions. Loss of the Cm resistance cassette is mediated by FLP-FRT recombination. Transformants were selected on LB-Amp plates at 30°C to maintain pCP20 activity and then serially passaged on LB-Amp at 30°C as well as LB-Cm and LB at 37°C to screen for loss of the pCP20 plasmid and antibiotic susceptibility. Loss of Cm resistance was further confirmed by PCR. Following a similar strategy as outlined above, mCherry-expressing strains were generated for confocal microscopy experiments.

4.3.4 Growth curves

Cultures of wild-type YS1646 and chromosomally integrated constructs were grown overnight at 37°C. The next day, the cultures were diluted 1:100 in LB medium and plated in quadruplicates (n=4) on a 100-well Bioscreen C honeycomb microplate (Growth Curves USA, Piscataway, NJ, USA). The Bioscreen C plate reader measured the optical density of the cultures at a wavelength of 600 nm every 30 minutes over 24 hours with a 30-second shaking period prior to each reading.

4.3.5 Lyophilization of *Salmonella* strains

YS1646-derived vaccine candidates and control strains were first cultured in LB medium and then formulated in a lyophilization buffer consisting of 28% (w/v) sucrose (BioShop, Burlington, Canada) and 1% gelatin (Sigma Aldrich, St. Louis, MO) in 25 mM potassium phosphate (KPO_4) at pH 7[13]. Samples were aliquoted and frozen at -80°C overnight. The following day, the strains were freeze dried using a ModulyoD Freeze Dryer (Thermo Fisher Scientific) for 24 hours. Samples were later stored at room temperature for various times ranging from 2 weeks to 4 months and cultured on LB plates to determine viability through colony-forming unit (cfu) counts. A sample that did not undergo the freeze-drying process was cultured to determine the initial viability of strains (determined by cfu counts) prior to lyophilization.

4.3.6 Confocal microscopy

Murine macrophage-like cells (RAW264.7; ATCC TIB-71) were propagated and cultured for infection with YS1646 strains as previously described[8]. Briefly, cells were seeded in an 8-well chamber slide at a concentration of 10^5 cells/well in Dulbecco's Modified Eagle medium (DMEM) (Wisent Bioproducts, St-Bruno, QC) supplemented with 10% FBS (Wisent Bioproducts). Recombinant YS1646 strains were diluted in DMEM-FBS to achieve a multiplicity of infection (MOI) of 50 or 100. After 1 hour of infection at 37°C and 5% CO_2, wells were washed with PBS (Wisent Bioproducts) and incubated with DMEM-FBS supplemented with 50 μg/mL gentamicin (Sigma Aldrich) for 2 hours. The wells were then washed again with PBS and the gentamicin concentration was lowered to 5 μg/mL for an overnight incubation. Following this, infected cells were fixed with 2% paraformaldehyde in PBS for 10 minutes at room temperature. Cells were

washed with PBS and then stained with a 1:1,000 dilution of 4',6-diamidino-2-phenylindole (DAPI) (Thermo Fisher Scientific) for an additional 10 minutes at RT. Images were obtained using a Zeiss LSM780 laser scanning confocal microscope and analyzed using ZEN blue software (Zeiss, Oberkochen, Germany).

4.3.7 Mouse immunization

Female 6–8-week-old C57BL/6 mice were immunized orally (PO) with recombinant chromosomally integrated (CI) *S. enterica* Typhimurium YS1646 derivative wherein the *nirB* promoter was used for expression and a *Salmonella*-specific type three secretory signal sequence SspH1 was fused to full-length *S. mansoni* Cathepsin B (YS1646::NHC). This construct was selected for further *in vivo* investigation based on preliminary animal studies. PO dosing, consisting of 200 μL containing $1x10^9$ cfu/dose, was administered by oral gavage every other day for three days (D1, D3, D5). The multimodal vaccination schedule also included a simultaneous intramuscular (IM) on D1 of 20 μg recombinant CatB (rCatB) in 50 μL PBS[9] (**Supplemental Figure 4.1**). Briefly, purified recombinant *S. mansoni* CatB was cloned and expressed in *Pichia pastoris* as previously described[14]. All experiments included a PBS control group with IM-only (wild-type (WT) + rCatB) and PO-only (YS1646::NHC) groups as additional controls. The number of animals used at each experimental endpoint is indicated in the Figure legends.

4.3.8 Evaluation of humoral responses by enzyme-linked immunosorbent assay (ELISA)

4.3.8.1 Serum CatB-specific IgG & IgE

Cathepsin B-specific IgG was assessed by ELISA as previously described[8]. Serum was collected by saphenous vein bleed at baseline (week 0) and 3 weeks post-vaccination in microtainer serum separator tubes (BD Biosciences, Missausauga, ON, Canada). Serum samples were obtained following manufacturer's instructions and stored at -20°C until assayed. U-bottom, high binding 96-well plates (Greiner Bio-One, Frickenhausen, Germany) were coated overnight at 4°C with 0.5 μg/mL rCatB in a 100mM bicarbonate/carbonate buffer at pH 9.6. Purified mouse IgG (Sigma Aldrich) was used to set a 2-fold standard curve at a starting concentration of 2,000 ng/mL. All samples were run in duplicate. Serum samples were diluted 1:50 (IgG) or 1:20 (IgE) and HRP-

conjugated anti-mouse IgG (Sigma Aldrich) was diluted 1:20,000 in blocking buffer (PBS + 2% BSA). HRP-conjugated anti-mouse IgE (Thermo Fisher Scientific) was diluted 1:6,000 in blocking buffer. Optical density (OD) was measured at 450 nm with an EL800 microplate reader (BioTek Instruments Inc., Winooski, VT). The concentrations of CatB-specific total IgG were calculated by extrapolation from the standard curve. These results are expressed as ng/mL. CatB-specific IgE results are expressed as OD_{450} values.

4.3.8.2 Serum CatB-specific IgG avidity

Cathepsin B-specific IgG avidity was assessed following a modified version of the IgG ELISA protocol described above. Following the primary antibody incubation step, plates were washed 4x with phosphate-buffered saline (PBS) and a range of urea concentrations (0M – 10M) was applied to each plate for a 15-minute incubation at room temperature (RT). After washing the plates 4x, they were incubated for 1 hour in blocking buffer. Normal IgG ELISA protocol then resumed with the addition of the secondary antibody. The IgG avidity index was calculated by dividing urea-treated bound IgG titers with IgG titers assessed treated with 0M urea.

4.3.8.3 Serum CatB-specific IgG1 and IgG2c

CatB-specific IgG subtypes, IgG1 and IgG2c, were assessed by ELISA as previously described[9]. Purified mouse IgG1 (Sigma Aldrich) and a mouse IgG2c isotype control (Southern Biotechnologies Associates, Birmingham, AL) were used to set the respective standard curves. ELISA protocol was run as above for total IgG. Either goat anti-mouse IgG1 and goat anti-mouse Ig2c, both diluted 1:20,000, were used as secondary antibodies (Southern Biotechnologies Associates). These results are presented as the ratio of IgG1/IgG2c.

4.3.8.4 Intestinal CatB-specific IgA

Intestines were collected three weeks post-vaccination and processed as previously described[8]. Purified mouse IgA (Sigma Aldrich) was used to set a 2-fold standard curve starting at a concentration of 1,000 ng/mL. Tissue samples were previously diluted 1:5 (w/v) during the processing phase. Aliquots were applied neat to the plate and the anti-mouse IgA secondary

antibody (Sigma Aldrich) was diluted 1:2,000. The concentrations of CatB-specific total IgG were calculated by extrapolation from the standard curve. These results are expressed as ng/gram of intestinal tissue.

4.3.8.5 Serum total IgE

The BD OptEIA™ Mouse IgE ELISA Set (BD, San Diego, CA) was used to determine total serum IgE titers following manufacturer's instructions as previously described[9]. Immulon 2HB flat-bottom 96-well plates (Thermo Fisher Scientific) were coated with anti-mouse IgE capture antibody at a 1:250 dilution in coating buffer at 4°C overnight. Standard concentrations were added following the manufacturer's protocol. Serum samples were diluted 1:20. OD_{450} was measured and concentration of total IgE titers was calculated by extrapolation from the standard curve. These results are expressed as ng/mL.

4.3.9 Cell-mediated responses

4.3.9.1 Cellular responses by multiplex ELISA

CatB-specific cytokine and chemokine production were assessed three weeks post-vaccination in splenocytes restimulated *ex vivo* with 2.5 μg/mL of rCatB for 72 hours at 37°C and 5% CO_2 as previously described[9]. Cells were plated 10^6/well in RPMI-1640 (Wisent Bioproducts) supplemented with 10% fetal bovine serum, 1 mM penicillin/streptomycin, 10 mM HEPES, 1X MEM non-essential amino acids, 1 mM sodium pyruvate, 1 mM L-glutamine (all from Wisent Bioproducts), 0.05 mM 2-mercaptoethanol (Sigma-Aldrich) (fancy RPMI, fRPMI). Supernatant cytokine/chemokine levels were measured using the Q-plex Mouse Cytokine-Screen (16-plex) multiplex ELISA following the manufacturer's recommendations (Quansys Biosciences, Logan, UT). This assay included: IL-1α, IL-1β, IL-2, IL-3, IL-4, IL-5, IL-6, IL-10, IL-12p70, IL-17, IFNγ, TNFα, MCP-1 (CCL2), MIP-1α (CCL3), GM-CSF, and RANTES (CCL5).

4.3.9.2 Flow cytometry analysis

CatB-specific $CD4^+$ and $CD8^+$ T cells were identified from freshly isolated splenocytes plated 10^6/well in 96-well round bottom plates (Fisher Scientific) and incubated for 24 hours at 37°C and

5% CO_2 for flow cytometry analysis as previously described with minor modifications[15]. For the last 5 hours of incubation, Golgi Stop and Golgi Plug (BD Biosciences) were added to samples according to manufacturer's instructions to inhibit protein transport. Cells treated with phorbol 12-myristate 13-acetate (PMA) and ionomycin were included as positive controls. All centrifugation steps occurred at 400x*g* for 7 minutes at 4°C until the cells were fixed after which rotor speed was increased to 450x*g*. All incubation steps required light protection. Cells were washed twice with PBS and later stained with Fixable Viability Dye eFluor 780 (eBioscience, Waltham, MA) for 15 minutes at 4°C. Cells were washed 2x and incubated with Fc Block (BD Biosciences) diluted 1:50 in PBS for 10 minutes at 4°C. Cells were then stained for 25 minutes at 4°C with surface markers from the following extracellular cocktail: anti-CD3 FITC (145-2C11, eBioscience), anti-CD4 V500 (RM4-5, BD Biosciences) anti-CD8 PerCP-Cy5.5 (53-6.7, BD Biosciences), anti-CD44 BUV395 (IM7, BD Biosciences) anti-CD62L BUV373 (MEL-14, BD Biosciences). Cells were then washed with PBS and fixed with 1X Fixation Buffer (BD Biosciences) overnight at 4°C. The next day, cells were washed with perm/wash buffer (BD Biosciences) and stained with anti-IFNγ PE (XMG1.2, BD Biosciences) for 25 minutes at 4°C. Cells were washed in perm/wash buffer, then PBS, and later resuspended in PBS for acquisition. All flow cytometry was conducted using a BD LSRFortessa X20 cell analyzer. Data were analyzed using FlowJo software (Treestar, Ashland, OR).

4.3.10 *Schistosoma mansoni* challenge

Biomphalaria glabrata snails infected with the *S. mansoni* Puerto Rican strain were obtained from the Schistosomiasis Resource Center of the Biomedical Research Institute (Rockville, MD) through NIH-NIAID Contract HHSN272201700014I for distribution through BEI Resources. Mice were challenged three weeks following vaccination with 150 cercaria by tail exposure and were sacrificed seven weeks post-challenge as previously described[16]. Briefly, adult worms were counted after perfusion of the hepatic portal system and manual removal from the mesenteric veins. The livers and intestines were harvested from each mouse, weighed, and digested in 4% potassium hydroxide overnight at 37ºC. The next day, the number of eggs per gram of tissue was recorded by microscopy.

4.3.11 Histopathological assessment by H&E staining

Liver sections were harvested and placed in 10% buffered formalin phosphate (Fisher Scientific) and processed for histopathology to assess mean egg granuloma size and morphology using Zen Blue Software (version 2.5.75.0; Zeiss) as previously described[8]. Mean areas are presented as $x10^3$ μm^2 ± standard error of the mean (SEM). Egg morphology was classified by an operator blinded to group assignment (ASH) as abnormal if there was loss of internal structure or peripheral crenelation. Abnormal eggs are reported as a percent of the total egg count (± SEM) per histopathological sample.

4.3.12 Statistical analysis

Statistical analysis was performed using GraphPad Prism 9 software (La Jolla, CA). Results are represented by at least two independent experiments (see Figure legends for details). Data were analyzed by one-way ANOVA and followed by Tukey's correction for multiple comparisons. P values less than 0.05 were considered significant.

4.4 RESULTS

4.4.1 Chromosomal integration of vaccine constructs in YS1646 *Salmonella* Typhimurium

We first generated plasmids bearing different promoters (*nirB, pagC, frr*) with the *Salmonella* SspH1 secretion signal fused to the full-length CatB peptide sequence cloned into the pGP-Tn7-Cm vector[11] (**Figure 4.1A**). A chloramphenicol resistance gene, flanked by two FRT sites, within the Tn7 transposon arms, Tn7L and Tn7R, was used for selection of colonies where Tn7-mediated integration may have occurred. Constructs were inserted using EcoRI and KpnI cut sites. Tn7-mediated integration occurs at the *att*Tn7 site of *S.* Typhimurium downstream of the *glmS* gene.

By FLP-FRT recombination, we removed the chloramphenicol resistance cassette from the region that integrated at the *att*Tn7 site to generate 3 candidate vaccine vectors in which expression was driven by promoters *nirB* (NHC), *pagC* (PHC or *frr* (FHC) (**Figure 4.1B**). Integration of these constructs occurred downstream of the constitutively active gene *glmS* in YS1646 *S.* Typhimurium. *Salmonella* promoters *nirB* and *pagC* are active intracellularly upon induction by

host oxygen and magnesium levels, whereas the *frr* promoter is constitutively active. Prior to removal of the Cm cassette as part of an intermediate validation step, recombinant antigen expression was confirmed in strains containing the *nirB* and *pagC* constructs, but not the *frr* promoter-based construct. All three chromosomal integrations had no obvious impact on bacterial fitness and growth rates were similar to WT YS1646 (**Figure 4.1C**).

The nirB_SspH1_CatB (NHC) construct was selected for further assessment *in vitro* and *in vivo*. Using a RAW264.7 murine macrophage cell line, we infected 10^5 cells per well with a recombinant YS1646 strain expressing mCherry (YS1646::mCherry) at an MOI of 100 and then stained the cells with DAPI. We observed detectable levels of mCherry expression within the macrophages infected with YS1646::mCherry whereas the signal was not detected in our uninfected control (**Figure 4.1D**).

4.4.2 Heterologous vaccination with nirB_SspH1_CatB leads to robust systemic and local humoral responses

We next moved forward with *in vivo* vaccination studies using our integrated NHC construct in a mouse model. None of the groups had detectable anti-CatB IgG levels at baseline (week 0). Three weeks post-vaccination, no anti-CatB IgG was found in the PBS and PO groups. However, we observed high IgG titers in all mice receiving a single dose of rCatB intramuscularly at 3 weeks. The greatest increase in antigen-specific IgG titers was observed in the multimodal vaccination group, NHC + rCatB and these antibody levels were significantly higher than in the IM only group (24605 ± 1309 ng/mL vs. 16783 ± 1397 ng/mL, $P < 0.0001$) (**Figure 4.2A**). Therefore, multimodal administration led to a greater IgG response to Cathepsin B in vaccinated animals compared to PO or IM dosing alone.

Multimodal vaccination resulted in better IgG avidity maturation as well. At a concentration of 6M urea, the IgG avidity index (%) was significantly greater in the multimodal group compared to IM vaccination (52.7 ± 2.7% vs. 29.5 ± 3.1%, $P < 0.0001$) (**Figure 4.2B**). The IgG avidity indexes for the PBS and PO groups were not calculated. Next, we measured the titers of anti-CatB IgG subtypes IgG1 and IgG2c. The IgG1/IgG2c ratio was significantly lower in the multimodal

group compared to the IM control (1.9 ± 0.2 vs. 5.0 ± 1.1, $P < 0.001$) (**Figure 4.2C**). Since IgG1 titers were comparable between groups, the higher total IgG levels and altered ratio were attributable to an increase in antigen-specific IgG2c levels in mice that received multimodal vaccination.

We then looked at local humoral responses by assessing intestinal CatB-specific IgA titers. All mice that received the NHC-bearing YS1646 orally (with or without rCatB IM) had elevated titers of anti-CatB IgA in the intestinal tissues. Both multimodal vaccination (1071.0 ± 161.4 ng/g, $P < 0.0001$) and PO vaccination alone (842 ± 206.4 ng/g, $P < 0.001$) led to a significant increase in titers compared to the PBS control group (15.9 ± 5.1 ng/g) (**Figure 4.2D**). There was no statistical difference observed between the NHC alone and multimodal groups. WT + rCatB vaccination led to IgA titers similar to the PBS control (83.1 ± 33.8 ng/g).

We also examined the serum total IgE titers in our study following vaccination. The highest titers were observed in the PBS control group (653.6 ± 83.6 ng/mL) (**Figure 4.2E**). Groups that received IM-only and PO-only vaccination had total IgE levels of 387.7 ± 57.0 ng/mL and 444.7 ± 36.6 ng/mL respectively. There was no statistical difference observed between our multimodal vaccination group and any other group in our study. The mean serum total IgE titer in the multimodal group was 457.8 ± 86.1 ng/mL. Thus, none of the vaccination schedules tested increased total IgE levels. Furthermore, no animals developed antigen-specific IgE titers in response to vaccination (**Supplemental Figure 4.2**).

4.4.3 Multimodal vaccination induces a mixed T_H1/T_H2 cellular response in *ex vivo* stimulated splenocytes

Cytokine and chemokine levels in splenocyte supernatants were assessed by Quansys multiplex ELISA following *ex vivo* restimulation with CatB 3 weeks post-vaccination. There were no significant differences or trends in the levels of IL-1α, IL-1β, IL-2, IL-3, IL-4, TNF-α, CCL3 (MIP-1α), CCL5 (RANTES), or GM-CSF. However, there were significant increases in T_H1-associated cytokines in the multimodal group compared to the PBS controls. For example, we observed an increase in IL-6 levels (721.3 ± 61.3 pg/mL vs. 489.9 ± 44.3 pg/mL, $P < 0.05$) (**Figure**

4.3A). We also detected increased levels of IL-12 (68.3 ± 3.8 pg/mL) in the multi-modal group compared to both the PBS control (48.7 ± 2.5 pg/mL) and PO alone group (50.0 ± 2.5 pg/mL) (both $P < 0.001$) (**Figure 4.3B**). Of note, the strongest CatB-specific IFNγ response occurred in the multimodal group (1586.6 ± 82.7 pg/mL) compared to the PO group (1236.2 ± 109.0 pg/mL) ($P < 0.05$), the IM group (869.6 ± 65.2 pg/mL) ($P < 0.0001$), and the PBS control (423.6 ± 79.7 pg/mL) ($P < 0.0001$) (**Figure 4.3C**).

In addition to T_H1-associated cytokines, we also observed significant differences in the expression of T_H2, T_H17, and regulatory cytokines, as well as in chemokine expression with the different vaccination regimens. All mice receiving an IM dose of rCatB had elevated levels of IL-5 that were highest in the multimodal group (230.7 ± 56.5 pg/mL vs. PBS control 12.8 ± 2.9 pg/mL, $P < 0.01$) and only slightly lower significant (200.1 ± 66.4 pg/mL) ($P < 0.05$) (**Figure 4.4A**). While not significantly different, there was a trend toward increased IL-10 expression in the NHC + rCatB group (17.2 ± 1.7 pg/mL vs. PBS 11.4 ± 0.8 pg/mL, $P = 0.096$) (**Figure 4.4B**). IL-17 secretion levels were also increased in the multimodal vaccination group (116.9 ± 24.7 pg/mL vs. PBS control 41.8 ± 6.6 pg/mL, $P < 0.01$) (**Figure 4.4C**). The multimodal vaccine response was also higher than that observed in PO (42.9 ± 4.5 pg/mL, $P < 0.01$) and IM (62.6 ± 8.9 pg/mL, $P < 0.05$) groups. Finally, we CCL2 (MCP-1) levels were also higher in the multimodal group compared to the PBS control (2696.1 ± 107.0 pg/mL vs. 1859.7 ± 169.9 pg/mL, $P < 0.01$) (**Figure 4.4D**).

4.4.4 Vaccine-induced IFNγ production is mediated by $CD4^+$ and $CD8^+$ T cells

We next sought to determine whether the increased cytokine levels observed from splenocyte supernatants were generated by T cells. Antigen-specific $CD4^+$ and $CD8^+$ T cells from vaccinated animals were analyzed by flow cytometry following our multimodal vaccination schedule, and restimulation of splenocytes with either rCatB (2.5 μg/mL) or fRPMI medium for 24h. Responding cells were characterized as $CD4^+$ and $CD8^+$ T cells expressing IFNγ following our gating strategy (**Supplemental Figure 4.3**).

The percentage of responding IFNγ$^+$ CD4$^+$ T cells was significantly increased in the multimodal vaccination group (0.08 ± 0.02%) compared to all other groups including the PBS control (5.0 x 10^{-3} ± 2.5 x 10^{-3}%, $P < 0.0001$), the IM group (0.02 ± 9.3 x 10^{-3}%, $P < 0.001$), and the PO group (0.01 ± 3.2 x 10^{-3}%, $P < 0.001$) (**Figure 4.5A**). Similar but overall slightly lower results were observed in the percentage of responding IFNγ$^+$ CD8$^+$ T cells: multimodal (0.03 ± 7.8 x 10^{-3}%), PBS control (6.1 x 10^{-3} ± 2.4 x 10^{-3}%, $P < 0.01$), IM alone (6.7 x 10^{-3} ± 3.1 x 10^{-3}%, $P < 0.01$), and PO alone (7.9 x 10^{-3} ± 3.2 x 10^{-3}%, $P < 0.01$) (**Figure 4.5B**).

4.4.5 Multimodal vaccination significantly reduces parasite burden and egg-associated pathology

At 7 weeks post-infection, we assessed the protective potential of our recombinant *Salmonella* vectored vaccine. Mean worm counts were highest in the PBS group (51.4 ± 2.2 worms). Multimodal vaccination significantly reduced worm burden by 80.4% (10.1 ± 0.7 worms, $P < 0.0001$). Compared to the control group, mean worm burden in the WT + rCatB and NHC groups were reduced by 27.9% (38.7 ± 3.8 worms) and 51.8% (24.8 ± 2.0 worms) respectively. The worm count in the multimodal vaccine group was significantly lower than the WT + rCatB ($P < 0.0001$) and NHC groups ($P < 0.001$) (**Figure 4.6A**). Since female *S. mansoni* worms can lay up to 300 eggs per day[17], we examined the male-female worm ratio. The percentage of female worms was balanced in the PBS (51.3 ± 1.5%) and the WT + rCatB (49.8 ± 1.3%) groups. In contrast, the proportion of female worms was significantly lower in mice that received oral vaccination with NHC alone (38.8 ± 2.4%, $P < 0.001$ versus PBS) or multimodal vaccination (32.6 ± 1.9%, $P < 0.0001$ versus PBS) (**Figure 4.6B**).

Schistosomiasis pathology is strongly associated with egg deposition in tissue. Hepatic egg burden was highest in the PBS control (15,974 ± 984.7 eggs/g). Multimodal vaccination significantly reduced egg load in the liver by 75.2% (3,965 ± 259.5 eggs/g, $P < 0.0001$). Hepatic eggs were reduced by 17.6% (14,041 ± 1394 eggs/g) in the WT + rCatB group and 54.6 % (7,292 ± 658.2 eggs/g) in the NHC group (**Figure 4.6C**). Similar observations were made for intestinal egg burden. Compared to the PBS control (12,766 ± 804.3 eggs/g), intestinal egg load in the NHC + rCatB group was reduced by 78.1% (2,796 ± 250.5 eggs/g, $P < 0.0001$). Intestinal eggs were

reduced by 20.4% (10331 ± 547.4 eggs/g) in the WT + rCatB group and 51.6% (6,185 ± 353.9 eggs/g) in the NHC group (**Figure 4.6D**).

Histopathological assessment of murine livers showed a larger mean granuloma size (56536.2 ± 4727.3 μm^2) in control mice with a low percentage of abnormal morphology (10.9 ± 1.9%). Mice in the WT + rCatB group did not differ greatly from the PBS group in their mean granuloma size (55304.7 ± 4366.9 μm^2) and percentage of abnormal eggs (15.9 ± 1.4%). Oral vaccination alone with YS1646::NHC significantly reduced the size of granulomas (40136.1 ± 2497.6 μm^2, $P < 0.05$) and mouse livers displayed a higher proportion of abnormal eggs (39.6 ± 2.5%, $P < 0.0001$) compared to the PBS control. The greatest impact was observed in the multimodal vaccination group which had the significantly smallest egg granuloma sizes (31620.4 ± 1759.2 μm^2, $P < 0.001$) and the highest rate of morphologically abnormal eggs (51.9 ± 4.8%, $P < 0.0001$) (**Table 4.1**).

4.5 DISCUSSION

Finding the 'right' antigen for a schistosomiasis vaccine has been challenging. Indeed, a recent study that screened 96 cell-surface and secreted recombinant *S. mansoni* proteins for efficacy in a murine vaccination-challenge model failed to yield any particularly promising candidates[18]. As noted above, almost all of the candidate vaccines for schistosomiasis developed to date, including those that have entered early clinical development have targeted surface proteins of adult worms[7], In contrast, CatB is an important digestive enzyme for *S. mansoni* as it is used to break down host blood macromolecules such as hemoglobin, serum albumin, and IgG. This cysteine protease is expressed in the cecum of migrating schistosomula and in the gut of the adult worm[19]. Suppression of CatB expression by RNA interference leads to significant impacts on parasite growth and survival[20]. IN early work, our group has had considerable success targeting CatB with IM formulations of recombinant protein combined with several adjuvants different CpG dinucleotides[14], Montanide ISA 720 VG[21], the MF59-like AddaVax, and sulfated lactosyl archaeal archaeosomes[22]. These studies demonstrated impressive immunological readouts such as high serum IgG antibody titers and the induction of cellular responses. Parasite burden reductions ranged between 54 to 87%, easily surpassing the 40% protection threshold proposed in 1998 by the Tropical Diseases Research (TDR) committee of the World Health Organization (WHO)[23]. In parallel with this work, we have developed an alternate strategy to deliver Cathepsin B to the host

based on an attenuated *Salmonella* Typhimurium strain YS1646 using either PO alone or multimodal vaccination. Unlike traditional IM vaccination approaches, we hoped this approach would elicit both mucosal and systemic responses. Using a prime-boost strategy with an oral YS1646-vectored dose followed 30 days later by an IM dose, reductions of liver and intestinal parasite burden as high as 93% were observed following challenge[8]. We subsequently established the therapeutic efficacy of multimodal YS-1646 vaccination using a shortened, 5-day regimen (PO + IM vaccination on Day 1 followed by 2 PO doses on Days 3 and 5), with efficacy rates of up to 73% in a chronic schistosomiasis infection model[9]. This strategy therefore meets the more recent criteria established by global experts for a desirable schistosomiasis vaccine: i.e.: 75% efficacy when used prophylactically with an additional therapeutic effect[24]. Using the same shortened multimodal vaccination schedule, the chromosomally integrated YS1646::NHC strain generated in our present study also meets these criteria with an 80% reduction in worm burden and a 75%/78% reduction in hepatic/intestinal egg burden.

While suitable for prior proof-of-concept murine studies[8,9], vaccination using plasmid-based *Salmonella* YS1646 would not be suitable for human use. This is due to the risk of spreading antibiotic resistance as well as unintended effects on the microbiota of the host[25,26]. In the field of DNA vaccines, plasmids for live bacterial vectors are recommended to be designed as marker-free[27]. Additionally, with a mid-range copy number[28], the plasmid may be lost during cell division, especially in non-selective conditions[29,30]. Chromosomal integration of our gene construct into the genome of *S.* Typhimurium also allows for more stable and reliable expression[11]. Through Tn7 transposition, we were able to integrate the vaccine at the *att*Tn7 site which is downstream of the constitutively active *glmS* gene, which encodes an essential glucosamine-fructose-6-phosphate aminotransferase[31]. This gene plays an important role in *Salmonella* cell envelope integrity[32]. While the integration of multiple copies would increase Cathepsin B copy number, single copy integration of our constructs was beneficial as it did not affect bacterial viability and resulted in strong humoral and cellular immune responses. As *Salmonella* Typhimurium targets host macrophages as part of its lifecycle[33], signal from infected RAW264.7 cells with mCherry-expressing YS1646 served as a proxy for CatB expression.

The use of a *Salmonella* delivery platform is attractive for several reasons. The natural presence of bacterial LPS and flagellin proteins can serve to stimulate the immune system and act as 'auto-adjuvants' through binding to TLR-4 and TLR-5, respectively[34–36]. The YS1646 strain has previously been used in a phase I clinical trial for treatment of metastatic melanoma and was well tolerated in patients at doses up to 3 x 10^8 CFU intravenously[37]. Live attenuated *Salmonella* vectored vaccines have been designed against other infectious diseases, including a commercial typhoid vaccine (Ty21a) [38] and several pre-clinical candidates for other parasitic infections[39–41]. The bacterium uses its type 3 secretion system (T3SS) to invade specialized gut epithelial cells[42]. Host cell entry is mediated by early effector proteins secreted by the *Salmonella* pathogenicity island I (SPI-I) T3SS whereas the SPI-II T3SS is active to secrete late effector proteins once the bacterium is within its *Salmonella*-containing vacuole (SCV)[43]. The SspH1 secretion signal can mediate the secretion of our target antigen through both SPI-I and SPI-II T3SSs. Overall, the use of living attenuated *Salmonella* as vaccine vectors is a proven strategy. In fact, the *S.* Typhi commercial vaccine (Vivotif) is administered as a set of 4 oral doses within the span of a week which is very similar to the oral component of our multimodal vaccination strategy.

Developing the 'ideal' schistosomiasis vaccine in the context of desired immune responses is complex. While much work has been done to study the immunopathogenesis of the disease, precise correlates of immunity remain to be elucidated. However, a large body of preclinical data has helped determine possible characteristics for an effective vaccine. Rhesus macaques represent a unique animal model due to their ability to self-cure at the onset of egg deposition by mature worms[44]. There has been renewed interest in studying this model to determine what constitutes a protective immune profile. From these studies, it has been postulated that high antibody titers against key target antigens create an environment of sustained immune pressure which results in reduced rates of worm survival[45]. This immunological pressure may lead to physiological damage of the adult worms[46] and may induce the autophagic machinery of the parasite as has been observed under other stress conditions such as starvation or drug treatment[47–49]. In fact, Sm-Cathepsin B IgG antibodies target the parasite's ability to digest host blood macromolecules. It is plausible that the high IgG titers observed in our study lead to decreased worm fitness, which correlates with the observed changes in egg morphology in our prior work[8,9,50]. Indeed, the induction of high titers of anti-CatB IgG by adjuvanted IM vaccination has been previously demonstrated and may correlate

with reduced parasite burden[51]. The supportive role of the humoral response has also been observed in other mouse models[52]. Interestingly, our multimodal vaccination approach with the YS1646-based single-copy recombinant vaccine elicited stronger IgG titers than previously observed in our plasmid-based system despite what is likely to be far fewer copies of the CatB gene/bacterium[8]. We also measured higher IgG avidity maturation in the NHC + rCatB group, suggesting there may be important differences in the antibodies generated by multi-modal vaccination compared to the IM control. Furthermore, multimodal vaccination led to a more balanced IgG subtype profile with higher titers of antigen-specific IgG2c titers. Recombinant CatB tended to elicit a T_H2-skewed response while the *Salmonella* vector caused a T_H1 bias, leading to a more 'balanced' humoral response.

Oral administration of our recombinant YS1646-based vaccine generated a strong local, mucosal response in the gastrointestinal tract. Although schistosomes are not generally considered to be mucosal pathogens, migrating schistosomula transit through the respiratory mucosa of the lungs and adult worms reside in the mesenteric venules of the small intestine. Both parasite life cycle stages interact with mucosal-associated lymphoid tissues (MALT) and the protective potential of IgA antibodies has previously been reported for schistosomiasis[53,54]. Indeed, lung-stage schistosomula represent one of the most vulnerable stages in the life cycle and are subject to attack by eosinophils[55–57]. Secreted IgA antibodies may work in concert with eosinophils in schistosomula killing by mediating multiple effector functions[58], and eosinophils have also been shown to contribute to the maintenance of IgA^+ plasma cells[59]. While systemic immune responses are crucial to protect against schistosomiasis, the induction of local, mucosal responses may be beneficial and represent a currently underutilized strategy. Allergic-like type I hypersensitivity responses have been detrimental in other helminth vaccination studies[60]. As observed in our therapeutic vaccination model[9], total IgE levels were comparable across all groups and multimodal vaccination did not significantly increase IgE titers. The lack of antigen-specific IgE is also a promising feature.

T_H1 cellular responses have been established as a key feature of schistosomiasis vaccine development in murine models[14,61,62]. However, both exaggerated T_H1- and T_H2-biased responses can lead to severe pathology and death[1]. Thus, the optimal T cell immunity would likely be a

balanced and targeted T_H1/T_H2 response. Upon *ex vivo* restimulation of splenocytes from vaccinated animals with rCatB, we measured high levels of several T_H1-type cytokines in supernatants such as IL-6, IL-12 and IFNγ. These T_H1 immune responses are likely due to the influence of the YS1646 vector. IFNγ is generally regarded as a hallmark of schistosomiasis protection based on findings using radiation-attenuated schistosome vaccines[63,64]. Levels of IFNγ were significantly increased in the multimodal group compared to all other groups in our study. The increased production of IFNγ in mice receiving multimodal vaccination is likely due to production by both $CD4^+$ and $CD8^+$ T cells as demonstrated by our flow cytometry experiments. IL-6 and IL-12 may serve to promote a more pro-inflammatory environment. Increases in IL-12 secretion, although small, are quite significant due to the tight control exerted on this regulatory cytokine by the immune system[65]. IL-12 has even been considered as a potential adjuvant in schistosomiasis vaccine studies[66]. In addition, we also observed significant increases in IL-5, IL-17 and CCL2 along with a trend towards an increase for the regulatory cytokine IL-10. IL-5 is implicated in the recruitment of eosinophils as well as playing a role in IgA secretion by plasma cells[67–69]. Together with IL-10, IL-5 may serve to dampen the pro-inflammatory environment induced by T_H1-type cytokines and generate a more balanced cellular response. The T_H17 pathway has been associated with lower worm burdens and IL-17 secretion has been correlated with recruitment of neutrophils which have an impact on migrating schistosomula via extracellular traps[70]. T_H17 cytokine expression in peripheral blood mononuclear cells (PBMCs) has also been demonstrated in a baboon animal model testing a Sm-p80 vaccine[71]. CCL2 is a chemokine responsible for recruitment of monocytes, memory T cells, and dendritic cells which may serve to further promote a T_H1-T_H2 environment[72,73].

Parasite burden reduction rates achieved by the chromosomally integrated YS1646::NHC strain were comparable with our previous plasmid-based prime-boost vaccination strategy[8] despite a much shorter schedule. These candidate vaccines meet the recently developed Preferred Product Characteristics (PPC) of a schistosomiasis vaccine by reaching the 75% target for worm and egg burden reductions[24,74]. Our results surpass those of other vaccines in comparable murine models that have since entered clinical trials[75]. Recombinant Sm-TSP2 achieved 57% reduction in adult worms and 64% reduction in hepatic eggs whereas recombinant Sm14 reduced worm burden by 66%, both adjuvanted with Freund's complete adjuvant[76,77]. Recently, recombinant Sm-p80

adjuvanted with GLA-SE achieved 93% reduction in female worms and 90% in tissue egg load in a non-human primate model[75]. Mouse studies of this vaccine demonstrated a 70% reduction in parasite burden[74]. These vaccine strategies require repeated intramuscular immunizations whereas the schedule used in the present study required only 5 days. A striking result of the chromosomally integrated vaccine was the significant reduction in the percentage of female worms in the multimodal group, suggesting that this approach may have an anti-fecundity effect. This finding is consistent with the reduced egg granuloma sizes and the higher proportion of morphologically abnormal eggs observed as well as in previous Cathepsin B vaccination studies[8,9,22,50]. Therefore, we hypothesize that adult female worms are under higher immune and metabolic pressure in vaccinated animals which leads to decreased fitness and egg integrity.

The focus of this work was the characterization of the protective potential and immune responses generated by a single-copy chromosomally integrated YS1646 vectored vaccine. While the current work represents considerable progress in the development of a *S. mansoni* vaccine more suitable for human use (i.e.: more stable antigen expression and removal of antibiotic resistance), several possible limitations of this approach remain. The stability of our YS1646::NHC strain for long-term storage remains to be evaluated and it is not yet known if a cold chain would be required. To address this concern, we freeze-dried our vaccine following a similar procedure used for the attenuated S. typhi vaccines, Ty21a[13] and measured CFU concentrations over the course of 16 weeks to assess room temperature (RT) stability (**Supplemental Figure 4.4**). Despite an initial drop of 1 log following lyophilization, concentrations remained stable for 8 weeks until a small decrease of a half log at 12 weeks which was then maintained at 16 weeks. Additional experiments will be required to measure antigen expression and retention of immunogenicity in freeze dried preparations at different time points. Furthermore, previous work using rCatB has highlighted the benefit of adjuvants, while our studies with YS1646 to date have included none. The in-built adjuvanticity of the *Salmonella* vector via its TLR agonists likely helped induce an immune response against CatB, however future studies may include an adjuvant for either PO or IM doses to further enhance immunogenicity and protection. Given the local mucosal response conferred by PO doses and protection against challenge previously observed with oral immunization alone, we hope to explore the possibility of a YS1646-derived vaccine without intramuscular rCatB doses, creating a needle-free vaccine. Future studies will include mechanistic experiments to elucidate

correlates of immunity related to our vaccine. Lastly, *S.* Typhimurium can disseminate widely in the murine host and persist for weeks[78]. Phase I clinical studies with YS1646 demonstrated that the bacterium could not survive long in the host's bloodstream due to its susceptibility to physiologic levels of CO_2[79]. As we consider clinical studies, we hope that short-lived local invasion of the gastrointestinal tract will help induce potent immune responses while limiting persistence and dissemination within vaccinees. Finally, there is 60% homology between schistosomal and mammalian Cathepsin B which raises concerns surrounding off-target effects. However, the mammalian homolog is restricted to the cellular lysosomal compartment[80], rendering cross-reactivity of vaccine-induced antibodies less likely.

In summary, we report the development of a chromosomally integrated multimodal YS1646-vectored vaccine expressing *S. mansoni* Cathepsin B that can induce a superior immune response in a mouse model compared to previous plasmid-based formulations with high parasite burden reductions. Oral dosing with *S.* Typhimurium expressing a single copy of our target antigen in combination with a single, intramuscular, unadjuvanted dose of recombinant protein led to strong local and systemic immune responses. The protection achieved against several parasitological outcomes ranges among the best reported in the murine model. As we look toward clinical studies and implementation of the vaccine, we expect that administration of our multi-modal vaccine will be easier in clinical practice compared to other schistosomiasis vaccination strategies that rely on several intramuscular doses delivered over a period of many months. A single doctor's visit would include administration of the IM dose along with the first PO dose. Then, patients could continue their oral vaccine regimen at home similarly to the Ty21a *S.* Typhi vaccine which consists of 4 oral doses[38]. This would greatly facilitate vaccination of those living in endemic regions for schistosomiasis where recurrent visits to the clinic may be challenging[81,82]. Chromosomal integration of CatB in the YS1646 vector also allowed the removal of the plasmid-located antibiotic resistance gene, making our candidate vaccine much safer for eventual clinical use. These data strongly support the continued development of this candidate *S. mansoni* vaccine to aid the hundreds of millions of people presently infected or at risk worldwide.

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4.7 AUTHORS' CONTRIBUTIONS

ASH was involved in all aspects of the study including study design, performing experiments, data analysis and preparation of the manuscript. SH assisted with experimental design of chromosomal integration steps. LL assisted in sample collection and processing from vaccinated animals. DJP assisted with revisions to the manuscript. CMD supervised the portion of the study related to chromosomal integration experiments. BJW and MN supervised all parts of the study and prepared the manuscript.

4.8 COMPETING INTERESTS

ASH, MN and BJW are named as inventors on a patent for a YS1646-based *S. mansoni* vaccine held by Aviex Technologies LLC. SH, LL, DJP and CMD have no competing interests to declare.

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4.10 FIGURES & TABLE

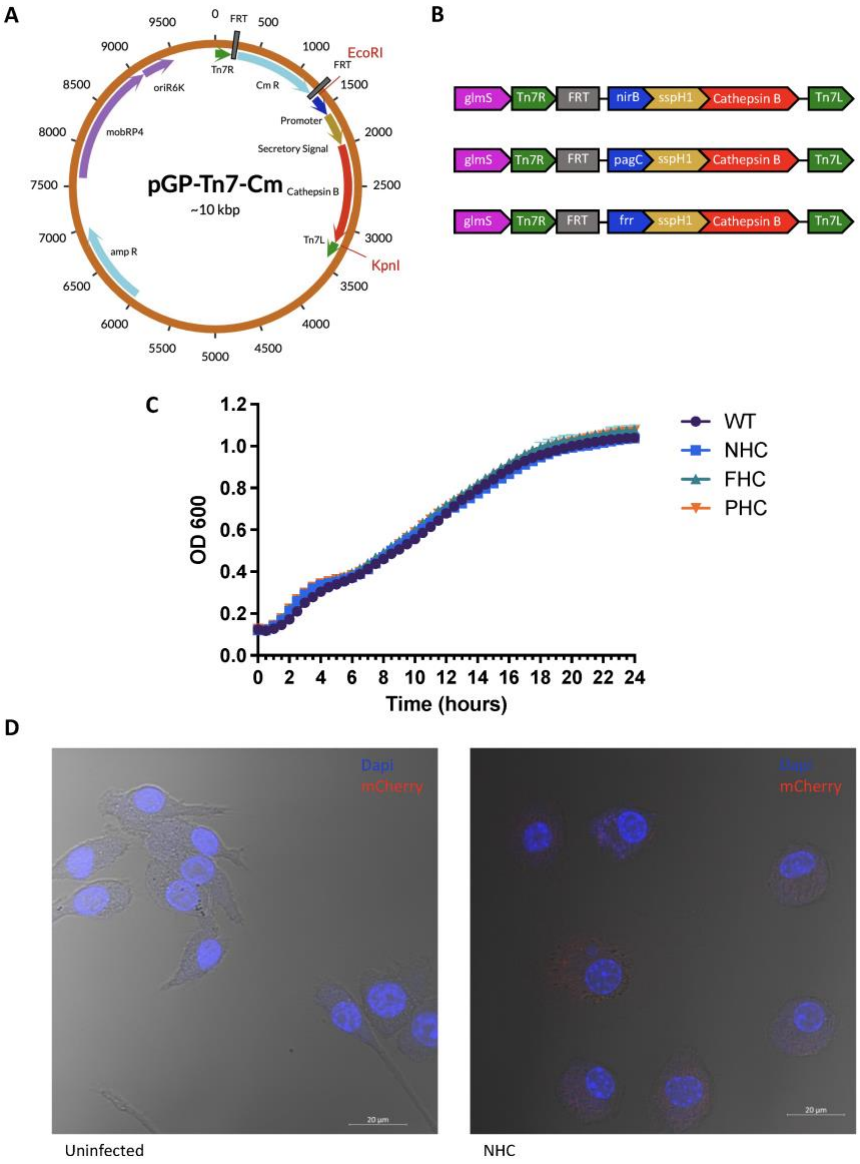


Figure 4.1. Chromosomal integration. Map of the pGP-Tn7-Cm plasmid designed to include promoter, secretory signal, and CatB antigen. Insertion occurred between the EcoRI and KpnI restriction sites (A). Linear integrated sequences for each YS1646 construct following FLP-FRT recombination to remove the chloramphenicol resistance gene (B). Growth curves of WT and recombinant YS1646 strains measured by absorbance at OD_{600} over 24 hours (n = 4 readings per 30 min interval) (C). Confocal microscopy images of uninfected and infected RAW264.7 cells at 63X magnification. DAPI nuclear stain is represented in blue and mCherry expression is shown in red (D).

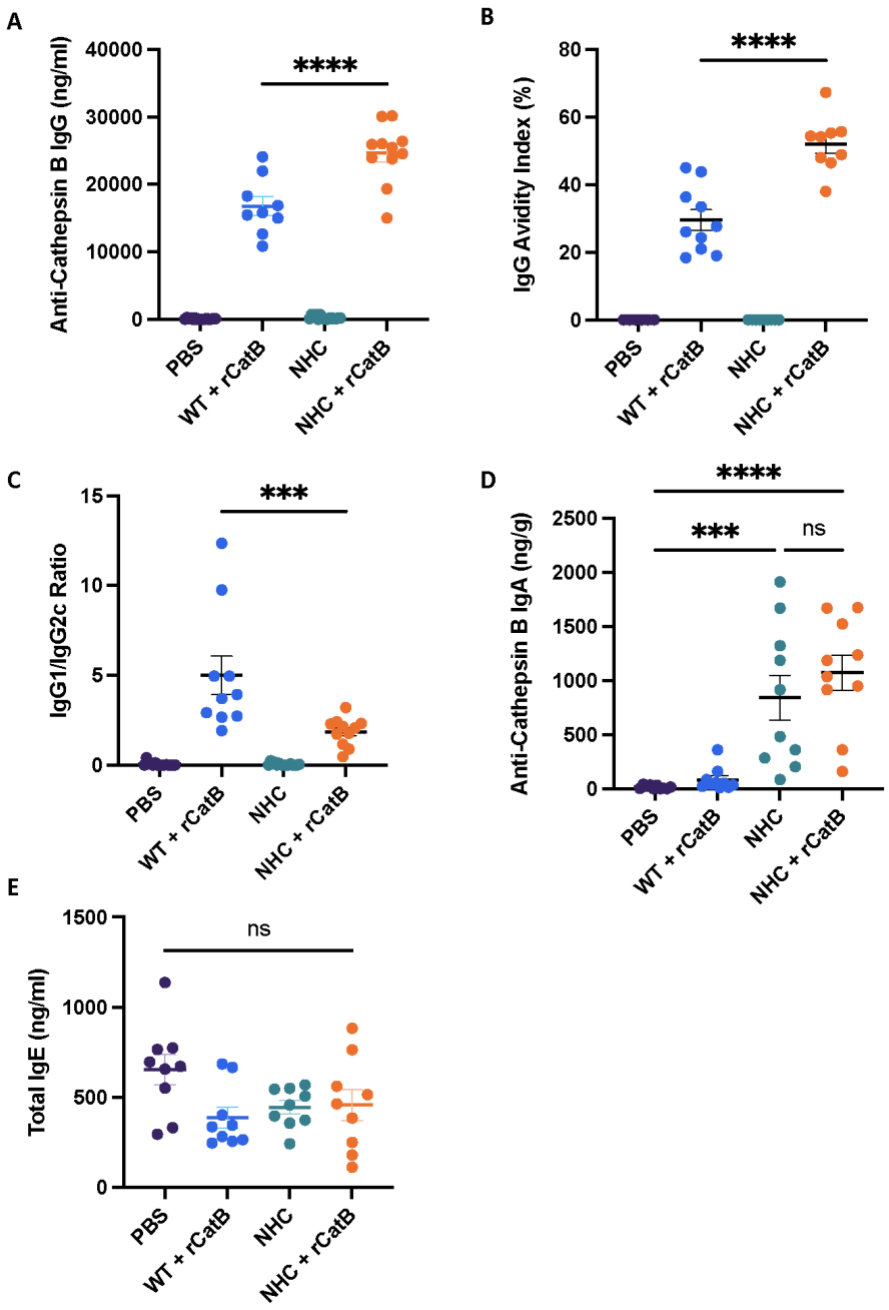


Figure 4.2. Humoral responses. Serum and intestinal samples were collected 3 weeks post-vaccination. Anti-Cathepsin B IgG titers expressed as ng/mL (A). Anti-CatB IgG avidity index (%) (B). Ratio of antigen-specific IgG1 vs IgG2c titers (C). Anti-CatB intestinal IgA titers expressed as ng/g (D). Total serum IgE expressed as ng/mL (E). Each independent experiment consisted of 5 animals per group. Each experiment was performed twice. Data are shown as mean ± SEM (***$P < 0.001$, ****$P < 0.0001$).

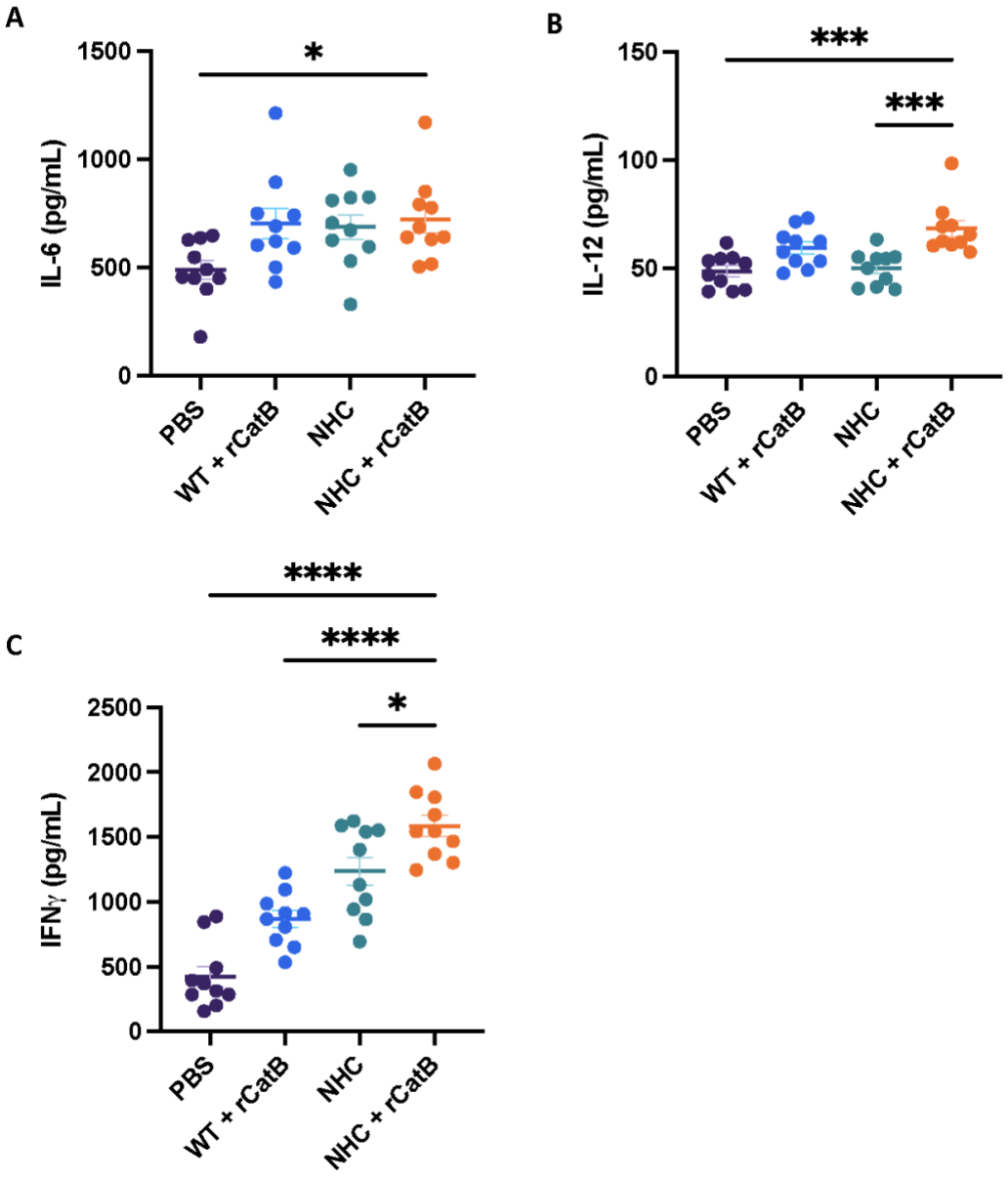


Figure 4.3. T_H1 cytokine secretion levels. Supernatant levels of IL-6 (A), IL-12 (B), and IFNγ (C) after *ex vivo* restimulation of splenocytes with rCatB for 72 hours. Cytokines were measured by Quansys multiplex ELISA and expressed in pg/mL. Each independent experiment consisted of 5 animals per group. Each experiment was performed twice. Data are shown as mean ± SEM (*P < 0.05, ***P < 0.001, ****P < 0.0001).

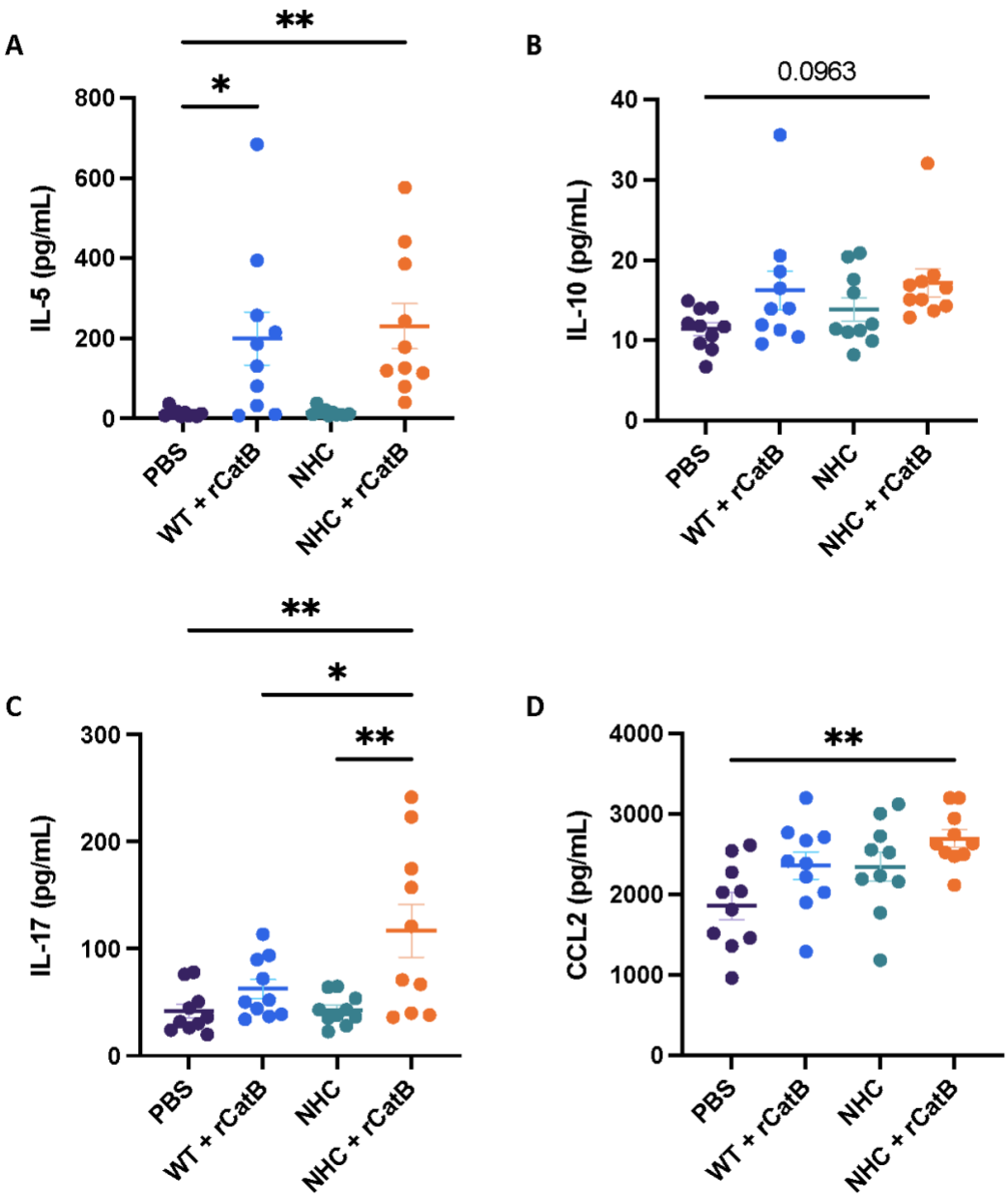


Figure 4.4. Other cytokines and chemokines. Supernatant levels of IL-5 (A), IL-10 (B), IL-17 (C), and CCL2 (D) after *ex vivo* restimulation of splenocytes with rCatB for 72 hours. Cytokines were measured by Quansys multiplex ELISA and expressed in pg/mL. Each independent experiment consisted of 5 animals per group. Each experiment was performed twice. Data are shown as mean ± SEM (*$P < 0.05$, **$P < 0.01$).

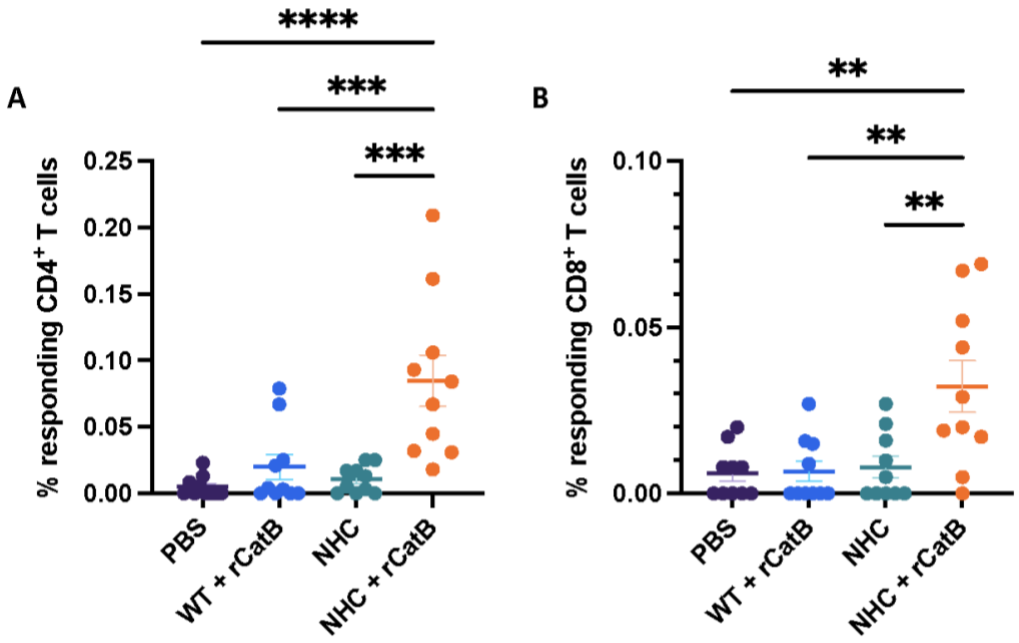


Figure 4.5. Flow cytometric analysis of T cells. The percentage of responding IFNγ$^+$ CD4$^+$ T cells (A) and IFNγ$^+$ CD8$^+$ T cells (B) in *ex vivo* restimulated splenocytes with rCatB after 24 hours were measured by flow cytometry. Each independent experiment consisted of 5 animals per group. Each experiment was performed twice. Data are shown as mean ± SEM of net values after background subtraction with unstimulated cells (**P < 0.01, ***P < 0.001, ****P < 0.0001).

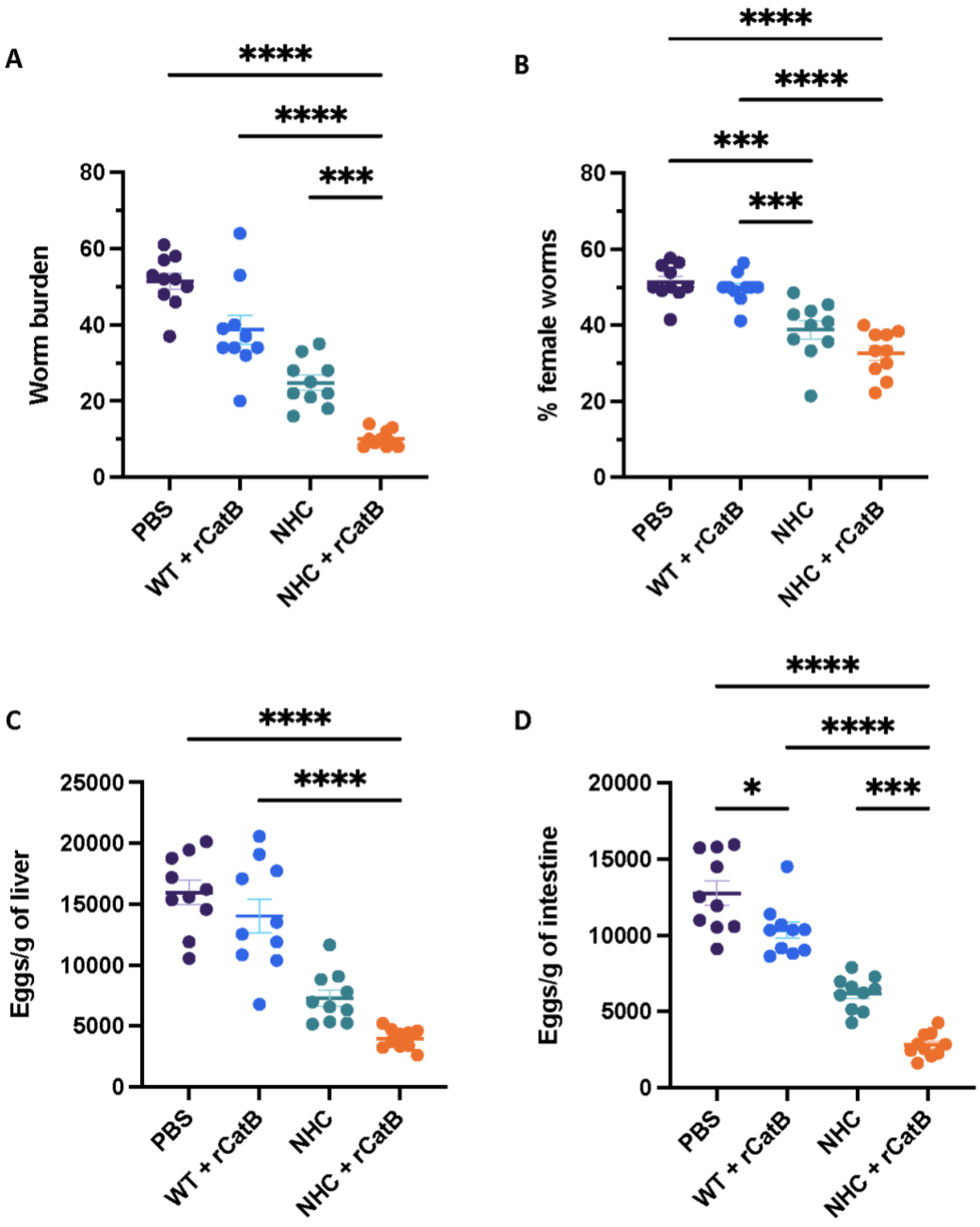


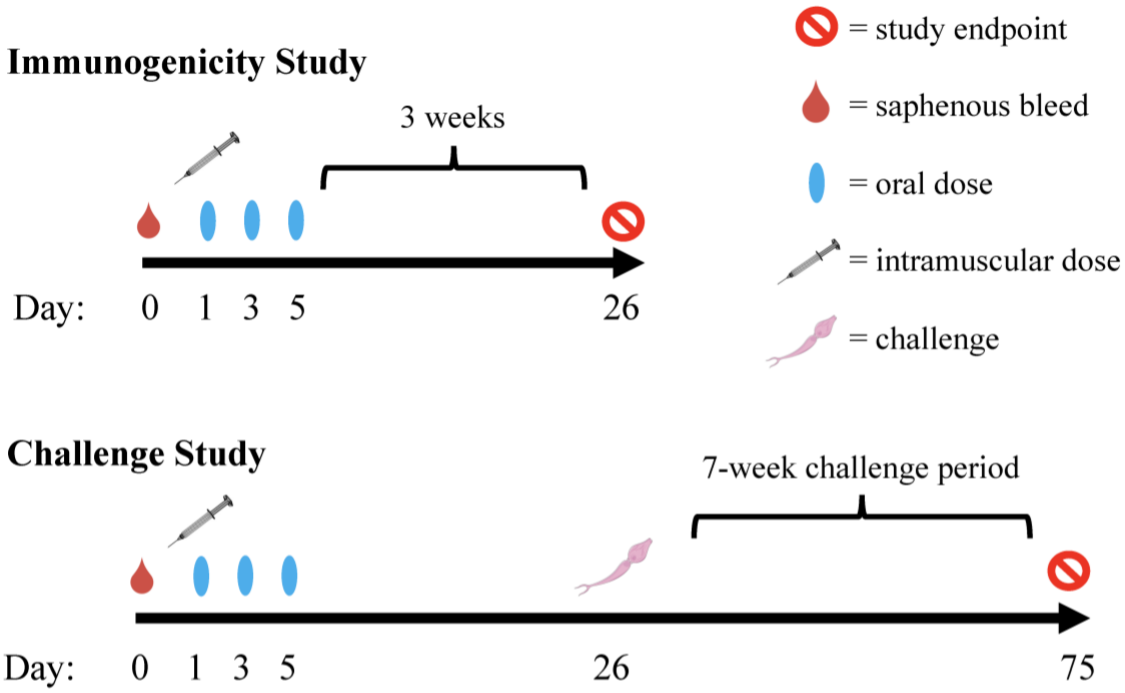
Figure 4.6. Parasite burden. The worm burden (A) and the percentage of female worms (B) were assessed 7 weeks post-challenge per individual mouse in each group. Egg loads were expressed per gram of liver (C) and intestine (D) for each group. Each independent experiment consisted of 5 animals per group. Each experiment was performed twice. Data are shown as mean ± SEM (*P < 0.05, ***P < 0.001, ****P < 0.0001).

Table 4.1. Granuloma size and egg abnormality.

Vaccine Group	Granuloma size (μm^2) ± SEM	Abnormal egg morphology (%) ± SEM
PBS	56536.2 ± 4727.3	10.9 ± 1.7
WT + rCatB	55304.7 ± 4366.9	15.9 ± 1.4
NHC	40136.1 ± 2497.6*	39.6 ± 2.5****
NHC + rCatB	31620.4 ± 1759.2***	51.9 ± 4.8****

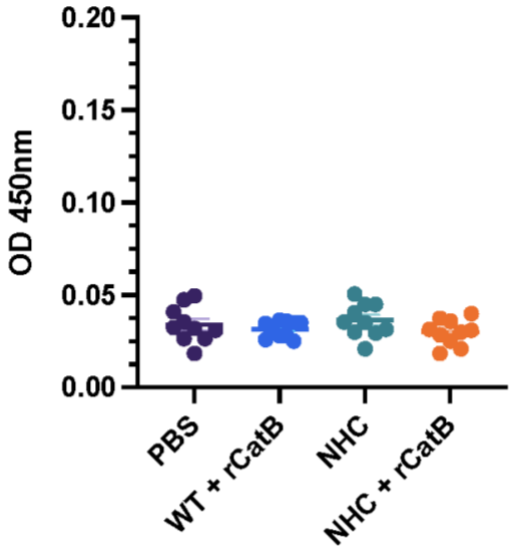
Liver egg granuloma size (μm^2) and egg morphology (i.e.: loss of internal structure, shrinkage, crenelated periphery) were assessed using Zen Blue software. Each independent experiment consisted of 6 – 10 granulomas per animal per group. Each experiment was performed twice. Data are shown as mean ± SEM (*$P < 0.05$, ***$P < 0.001$, ****$P < 0.0001$).

4.11 SUPPLEMENTAL MATERIAL

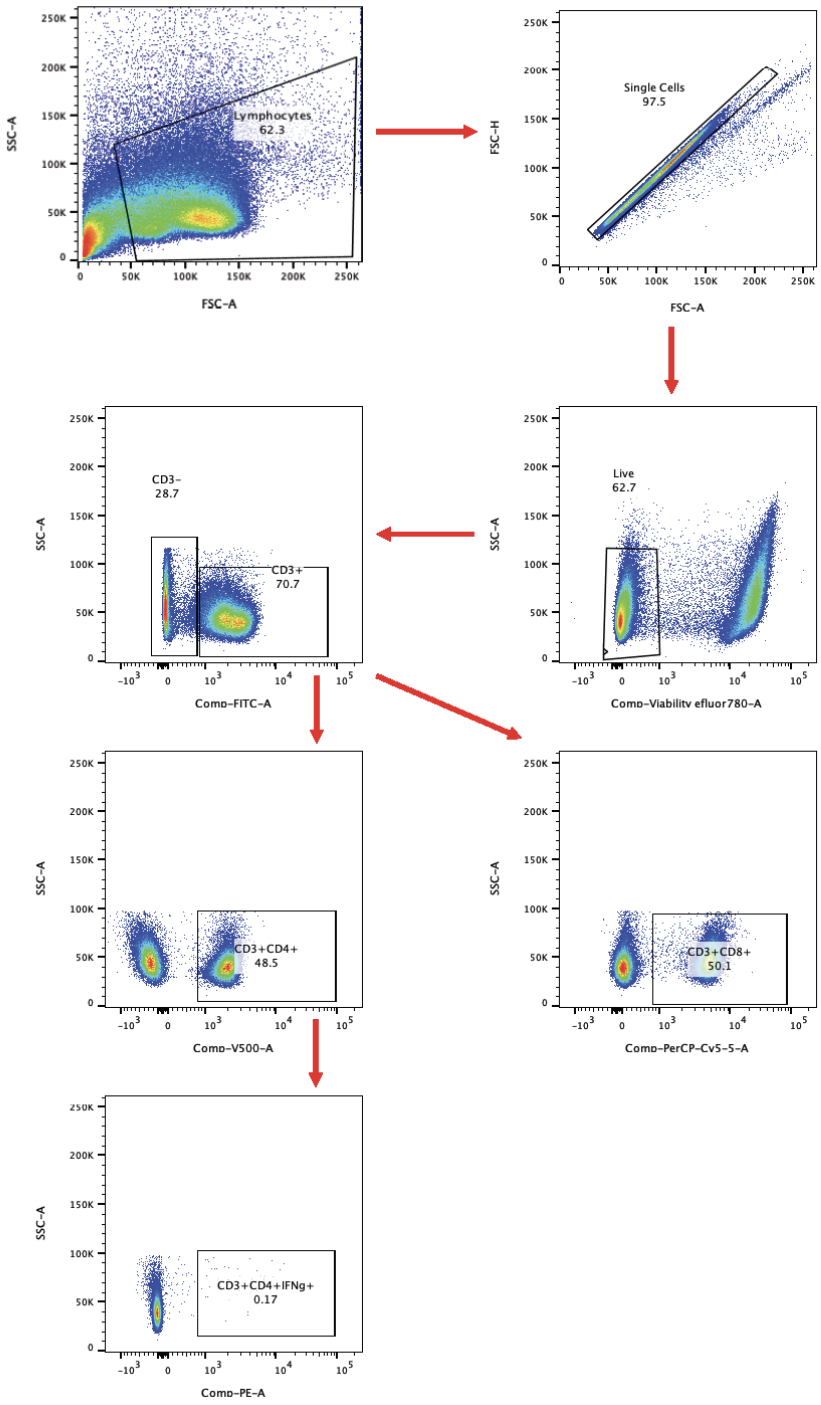


Vaccine Group	Oral Dose	Intramuscular Dose
PBS	PBS	PBS
WT + rCatB	WT YS1646	rCatB
NHC	YS1646::NHC	PBS
NHC + rCatB	YS1646::NHC	rCatB

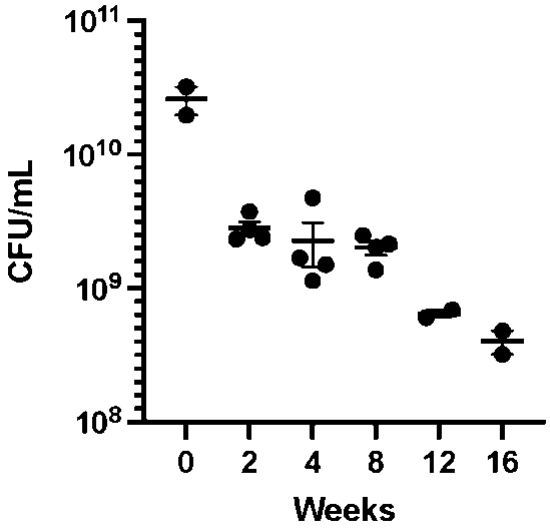
Supplemental Figure 4.1. Study Design. Baseline serum was collected on D0 for all mice. Depending on the experimental group, mice received three oral doses of PBS, WT YS1646 or YS1646::NHC on D1, D3, and D5 (200 µL, 1 x 10^9 CFU/dose). Mice also received an intramuscular dose of PBS or rCatB (50 µL, 20 µg/dose). Mice were then euthanized three weeks post-vaccination for immunogenicity studies. For challenge studies, mice were percutaneously exposed to 150 cercaria and euthanized 7 weeks later to assess parasite burden.



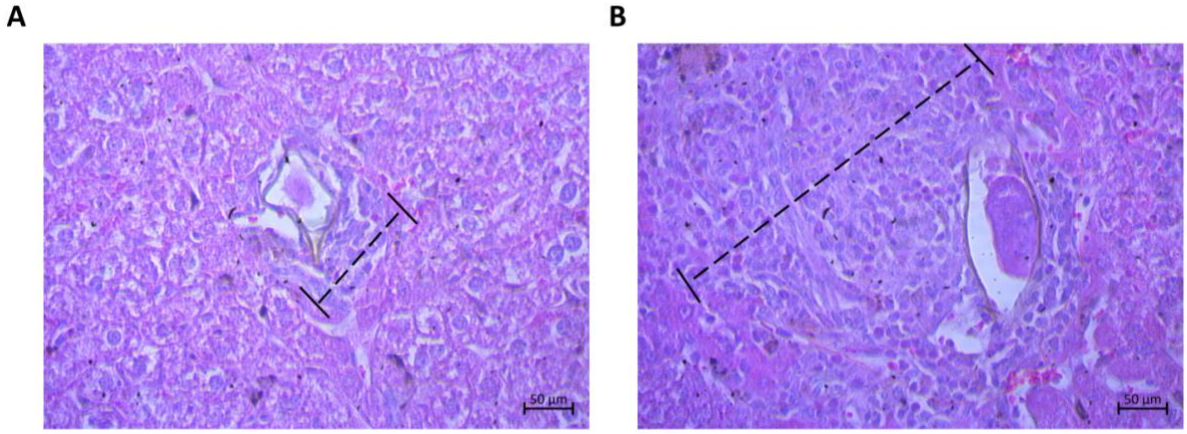
Supplemental Figure 4.2 Antigen-specific IgE. Serum samples were collected 3 weeks post-vaccination. No animals developed antigen-specific IgE in response to vaccination. Each independent experiment consisted of 5 animals per group. Each experiment was performed twice.



Supplemental Figure 4.3. Flow cytometry gating strategy. To identify $CD4^+$ and $CD8^+$ T cells expressing IFNγ, we first gated to exclude debris, cell clusters, and dead cells. After gating for single cells and live cells, T cell subsets were identified from the $CD3^+$ population and $IFN\gamma^+$ cells were identified in each population.



Supplemental Figure 4.4. Viability of lyophilized NHC over time. Recombinant YS1646 strains were freeze dried and stored at room temperature (RT) for 16 weeks. Samples were resuspended and grown on LB plates to assess CFU counts and calculate concentrations. The y-axis is expressed on a log scale. Each time point consisted of either 2 or 4 samples.



Supplemental Figure 4.5. Histopathological staining of hepatic sections. Representative images of H&E staining of liver egg granulomas in control mice (A) and the multimodal vaccination group (B). Scale is set to 50 µm.

CHAPTER 5

General Discussion

Schistosomiasis control efforts have been successfully implemented in parts of the world, but progress towards elimination remains a challenge as global infection numbers surpass 250 million. Praziquantel (PZQ) treatment campaigns have helped to reduce morbidity and mortality rates in endemic regions for the past 40 years. However, as with other neglected tropical diseases (NTDs), the COVID-19 pandemic caused setbacks in mass drug administration (MDA) campaigns, thus increasing the number of untreated cases[1]. To minimize the pitfalls of PZQ (i.e.: lack of efficacy against juvenile worms, no prevention of reinfection, etc.) and provide long-lasting prophylaxis, a vaccine is required[2]. In the mid-1990s, the Tropical Diseases Research committee of the World Health Organization (TDR-WHO) set the bar for an effective schistosomiasis vaccine at ≥40% for parasite burden reductions[3] and several pre-clinical vaccine candidates are in development[4]. More recently, as research progress has advanced, global experts convened to establish preferred product characteristics (PPC) for an eventual vaccine[5]. The new threshold in terms of efficacy (i.e., egg output and/or worm burden) would be ≥75% against at least one *Schistosoma* spp. or against all three as an optimistic target[5]. The PPC propose that an ideal vaccine would also exert therapeutic effects against pre-existing disease. Whereas most vaccines in pre-clinical and clinical development target membrane proteins of the adult worm by intramuscular immunization, our group has chosen the digestive enzyme Cathepsin B (CatB) as a target antigen[6–8]. *S. mansoni* CatB is an important cysteine protease present during both larval and adult stages of the lifecycle, and it is required for digestion of host blood macromolecules and proper parasite maturation[9,10]. The central goal of this thesis work was to evaluate the impact of an attenuated *Salmonella* Typhimurium YS1646 vaccine vector expressing CatB on schistosomiasis vaccine immunogenicity and efficacy. This thesis describes a promising, multimodal strategy for a novel *S. mansoni* vaccine with high prophylactic and therapeutic potential.

5.1 MAIN FINDINGS

Prior experimental work established that *S. mansoni* CatB (SmCatB) is an effective vaccine target when formulated with either CpG dinucleotides[6] or Montanide ISA 720 VG[7] and administered intramuscularly in a murine model. A series of three IM doses over 9 weeks led to parasite burden reductions ranging from 54 – 62%. Both formulations elicited robust IgG antibody titers against Cathepsin B. The use of CpG dinucleotides skewed immune responses towards T_H1-type immunity whereas the squalene-based adjuvant Montanide ISA 720 VG led to a more mixed T_H1/T_H2 response. These studies were vital to establishing the foundation for our efforts to develop a *Salmonella* Typhimurium vectored vaccine to express Cathepsin B. In **Chapter 2**, we describe the *in vitro* experiments performed to successfully clone a pQE-30 plasmid construct harbouring *E. coli* or *S.* Typhimurium promoters, the *Salmonella* secretion signal SspH1 and our full-length antigen. CatB expression was demonstrated in axenic culture and in a murine macrophage cell line for two constructs, nirB_SspH1_CatB (NHC) and SspH1_SspH1_CatB (HHC). Next, we moved forward with these two constructs for *in vivo* immunogenicity and challenge experiments. Even though the HHC construct could cause a systemic immune response, NHC was far more promising. Oral priming doses followed by a single intramuscular boost of recombinant protein 3 weeks later generated considerable local and systemic humoral responses along with mixed T_H1/T_H2 antibody and cellular responses. We observed reductions in adult worm, hepatic egg, and intestinal egg burdens were 93.1%, 90.3%, and 79.5% respectively, thus surpassing recently established thresholds and reported efficacies of other *S. mansoni* vaccine candidates in a murine model.

The work detailed in **Chapter 2**, published in *PLoS NTDs* in December 2019, led us to consider the impacts of vaccination on chronically infected individuals. Previously, only one other vaccine, Sm-p80, has been assessed for therapeutical potential in a non-human primate model[11]. Therefore, in **Chapter 3**, published in *Vaccine* in August 2021, we established a chronic infection mouse model to determine the therapeutic effect provided by our recombinant YS1646 vaccine. Mice were infected for a period of either 2 or 4 months prior to vaccination and then sacrificed either 1- or 2 months post-vaccination. First, we compared a 5-day multimodal schedule and the 3-week prime-boost schedule in a prophylactic vaccination model. The shorter regimen was an effective strategy and protected against parasite challenge. Therefore, this shortened schedule was used for all therapeutic vaccination experiments. At 1 month post-vaccination, parasite burden reductions

ranged from 46.5% - 50.3%. At 2 months post-vaccination, these reductions increased to 63.9% - 73.3% across all parasitological outcomes. Vaccination with recombinant YS1646 shifted the immune landscape from the classical T_H2-dominant environment observed in natural infection to a mixed T_H1/T_H2 response due to the increased secretion of pro-inflammatory T_H1-type cytokines. Interestingly, egg morphology and granuloma sizes were reduced post-vaccination, and liver fibrosis was also decreased following multimodal administration of the vaccine compared to controls. Finally, serum total IgE was not significantly increased in vaccinated animals as had been seen in clinical trials with other helminth vaccination studies where participants suffered from allergic type I hypersensitivity responses[12].

Together, **Chapter 2** and **Chapter 3** demonstrate the efficacy of recombinant YS1646 delivered orally when coupled with intramuscular administration of recombinant Cathepsin B. It is noteworthy that the *Salmonella* vector used to express the antigen CatB in Chapters 2 and 3, was plasmid-based carrying an antibiotic resistance gene cassette which could limit the use of our vaccine in humans. In **Chapter 4**, we redesigned our YS1646 vector to express *S. mansoni* CatB through chromosomal integration to remove the antibiotic resistance gene cassette and ensure more stable expression of the antigen. We evaluated the immunogenicity of this new candidate vaccine and discovered more potent cellular and humoral responses both systemically and locally. We demonstrated that vaccination continued to induce a mixed T_H1/T_H2 response characterized by the increase in antigen-specific IgG2c antibody titers as well as the secretion of both pro- and anti-inflammatory cytokines. We also showed that IFNγ production upon *ex vivo* restimulation of splenocytes with recombinant antigen is mediated by $CD4^+$ and $CD8^+$ T cells. Taken together, we show that Cathepsin B expression by an attenuated *Salmonella* Typhimurium vector is a promising strategy for the development of a schistosomiasis vaccine.

5.2 LIMITATIONS OF THE WORK

Throughout the work presented in this thesis, we have demonstrated the protective capabilities of multimodal vaccination with a *Salmonella* Typhimurium vector. With the aid of chromosomal integration, we have mitigated clear risks associated with plasmid-based expression of our antigen in the live, attenuated YS1646 strain. However, other factors must be considered as these projects

move forward such as limitations with the current animal model as well as challenges with live attenuated vectored vaccination.

5.2.1 Animal model

Throughout this thesis, the C57BL/6 mouse strain has been used as the animal model for all *in vivo* vaccination studies. Inherently, there are several differences between mice and humans in terms of the immune responses elicited and the tropism of our YS1646 vector for example. As we hope to move further toward human clinical trials, these differences should be highlighted. The following section will discuss the mouse model and its application in schistosomiasis challenge studies.

5.2.1.1 Differences in Salmonella *Typhimurium responses in mice vs humans*

Wild-type *Salmonella* Typhimurium is used to replicate typhoid fever in mice. The YS1646 attenuated strain does not cause severe disease or symptoms in our model, however, bacteria may widely disseminate within the host[13,14]. Conversely, in humans, *S.* Typhimurium is confined to the gastrointestinal tract as is common in non-typhoidal salmonellosis[15]. Therefore, responses to vaccination may differ in the human host, and this must be investigated. Initial humoral responses may be similar such as the induction of local IgA and systemic IgG antibodies. However, the extent to which the dissemination of the attenuated bacteria within the murine host contributes to cellular immunity needs to be studied further.

5.2.1.2 Mice are not an ideal schistosomiasis challenge model

Mice represent a cost-effective model and are easy to manipulate. This aids greatly when assessing the immunogenicity and protection of candidate vaccines. However, a recent study has highlighted the pitfalls associated with this model when performing schistosomiasis challenge experiments[16]. Most infection protocols involve exposing mice to 150 – 200 cercaria by percutaneous exposure of the tail. The larva will then migrate through the vasculature to the lungs first before continuing towards the liver in order to mature into adult worm pairs[17]. However, many larval schistosomula may be lost during the lung phase of the migration. While mice are considered to be a permissive

host for schistosomes, few parasites are able to migrate from the skin to the portal system and mature into adult worms. As a result, this may influence parasite burden assessment.

A potential limitation of the murine model of schistosomiasis is centered around the fragility of the pulmonary capillaries[16]. Migrating schistosomula burst into alveoli and their passage through the lung can be impeded unlike in humans or other experimental models including hamsters and non-human primates. Additionally, challenge studies often infect mice soon after the final boosting dose and this may be considered too short of a time interval. The recruitment of immune cells in the lungs is suggested to be due to activation and not by antigen stimulation. Therefore, it is important to examine where parasites are being eliminated when assessing the efficacy of a vaccine candidate. Another consideration would be to delay the challenge until at least 5 weeks after the final vaccination dose. Pre-clinical studies may occur in mice or hamsters for large-scale testing whereas baboons are the best model as a permissive primate.

Other considerations include the difference in acquired infections in natural versus laboratory settings. For example, in an experimental infection, cercaria are introduced to the host as a single exposure whereas infections are typically acquired gradually in endemic regions[18]. Moreover, the intensity of the infection and the *Schistosoma* isolates will vary as well. Finally, the presence of co-infections (i.e., malaria, HIV, hepatitis) may influence the human host.

5.2.1.3 Absence of co-infections in naïve mice

Lastly, naïve mice in our experimental design do not accurately reflect the reality of schistosomiasis endemic regions. Animal test subjects are kept in a clean and controlled environment where the risk of acquiring co-infections is low. In contrast, areas with heavy schistosomiasis burden are also afflicted with other bacterial, viral, and parasitic diseases and the risk of one or more co-infection pathogens is high[19].

Schistosomiasis and malaria share overlapping geographical distribution, and both cause significant disease[20]. Both infections are highly prevalent in children and fishing communities[21]. Acute malaria infections have been shown to cause diminished vaccine responses to several bacterial diseases[22,23]. Malaria infection during pregnancy or infancy may also decrease responses

to measles vaccination[24]. Thus, it may be of interest to test and treat malaria infections before administering a vaccine in an endemic region. In addition, co-infection with soil-transmitted helminths (STHs) can induce immune suppression by diminishing T_H1-type responses and causing a T_H2 bias[25,26]. B and T cell responses may also be affected by anti-helminth treatment[27]. Other co-infections including cytomegalovirus, HIV/AIDS, and tuberculosis are also to be screened during vaccination trials and investigated for possible effects on immunizations[21].

5.2.2 Challenges with live, attenuated vectored vaccines

Live attenuated vaccines have several advantages such as the ability to stimulate the immune system, invade cells to induce cell-mediated immunity, and the possibility to avoid multiple doses or adjuvants. However, they present their own challenges which must be considered. Following a normal course of infection, a live attenuated *Salmonella* Typhimurium vector would remain restricted to the host's gastrointestinal tract until elimination. Nonetheless, there is a risk of persistence of the vector within the host and the safety of immunocompromised individuals must also be considered.

5.2.2.1 Persistence of YS1646 in the host

Due to its nature in the mouse model, the YS1646 strain can widely disseminate within the host, and persist for some time after initial vaccination. The effect of this persistence on maintaining a continued immune response remains to be evaluated. Bacterial shedding is of concern with live vaccines. However, Ty21a vaccination leads to transient shedding and can be resolved by day 4[28]. Other diseases with vaccines known for shedding are cholera which may last for at least 7 days in the stool[29] as well as polio[30]. On the other hand, when YS1646 was introduced as a cancer treatment in a phase I clinical trial, it failed due to rapid clearance in the bloodstream[31]. While bacteria were administered intravenously in this clinical trial, the duration of YS1646 following oral administration within a human host should be investigated.

5.2.2.2 Safety for immunocompromised individuals

Live attenuated vaccination would likely not be recommended for immunocompromised individuals. The Ty21a *Salmonella* Typhi vaccine is contraindicated in this patient population. It is also not recommended for people taking medications such as antibacterial drugs due to possible interference with the bacterium. The Ty21a vaccine is approved in children over the age of 6 years old which coincides with the at-risk age group of 6 – 10 for schistosomiasis[32,33].

5.3 FUTURE PERSPECTIVES

We have demonstrated that a recombinant *Salmonella* vector could deliver a heterologous *Schistosoma mansoni* antigen and protect against challenge. Multimodal vaccination worked effectively in both prophylactic and therapeutic models. However, whether this approach can be used against other *Schistosoma* spp. remains to be evaluated. Furthermore, our current vaccine may be improved using adjuvants or by targeting additional antigens. Future work will also include determining the impact of vaccination in treatment/reinfection models as we move forward toward human studies.

5.3.1 Enhancing mucosal immunity through oral adjuvants

The current state of our multimodal vaccine does not include the use of any adjuvants for either oral or intramuscular doses. Previously, our group tested different formulations of recombinant Cathepsin B in murine models[6–8]. In this work, we have taken advantage of the in-built adjuvanticity of our YS1646 vector to stimulate local and systemic immune responses. Interestingly, oral vaccination alone was shown to protect against *Schistosoma* challenge when administered prophylactically despite the lack of systemic humoral and cellular responses. These findings lead us to consider the benefits of a strictly oral vaccination and whether immune responses can be enhanced through oral adjuvants.

5.3.1.1 Benefits of mucosal immunity for schistosomiasis

The lifecycle of the *Schistosoma* genus involves passage through the lungs where schistosomula will migrate prior to their arrival in the liver for maturation into adult worms[17]. In the case of *S. mansoni*, the paired worms will then reside in the mesenteric veins. Therefore, these are two

anatomical locations where the parasite is in close proximity to the mucosa-associated lymphoid tissues (MALT) and could be subjected to attack by the mucosal immune system. The transit of the schistosomula through the lungs makes them susceptible to immune attack by eosinophils[34–36]. These myeloid cells may bind to circulating secretory IgA antibodies and act in concert with IgA^+ plasma cells[37,38]. Moreover, it is plausible that antigen-sensitized IgA^+ precursor plasma cells from the gut-associated lymphoid tissues (GALT) are disseminated to other mucosa-associated tissues such as the lungs[39]. In fact, lung dendritic cells can induce IgA class switching and generate protective gastrointestinal responses[40,41]. There is more evidence towards considering the mucosal immune system as an integrated system-wide organ and more research is needed in the study of communication between mucosal sites[42].

5.3.1.2 Can oral vaccination alone be sufficient?

The Ty21a vaccine consists of a series of 4 oral doses administered over the course of a week. Oral vaccination would be ideal in endemic regions due to the ease of administration. One possible limitation is that oral vaccination can have lower efficacy in low-and-middle-income countries (LMICs). Children from LMICs may suffer from undernutrition which has been shown to lead to diminished immune responses to oral vaccines[43]. Other risk factors include enteric infections and environmental enteropathy. Moreover, compared to other oral vaccines, Ty21a is an interesting exception. Clinical trials in several LMICs have shown that vaccination induces local intestinal IgA antibodies and robust cellular responses in school-age children[44–46]. It has been suggested that this may be due to the unique biology of *Salmonella* spp. to target M cells and quickly gain access to the GALT. As more *Salmonella*-based vaccines are being developed, it would be of interest to determine if stable immunogenicity is also conferred in this risk population.

In our studies, oral-only administration of the vaccine had some substantial effects on reducing parasite burden despite the lack of a systemic immune response. Local IgA antibodies were induced in this group and may play a role in the decreased parasite load observed. Therefore, adjuvanting the oral doses may lead to better parasite burden reduction and increase systemic and local humoral and cellular immune responses.

5.3.1.3 Preliminary work with mucosal adjuvants: ATRA and dmLT

Due to the proximity of *S. mansoni* to intestinal tissues, we tested the additional protective effect of mucosal adjuvants in our shortened vaccination schedule. We have tested different doses of all-trans retinoic acid (ATRA) and double-mutant heat-labile toxin (dmLT). These adjuvants have shown considerable promise in both preclinical and clinical studies[47,48].

ATRA is a major metabolic derivative of vitamin A and signals through nuclear retinoic acid receptors (RARα, RARβ, RARγ)[49]. ATRA can act on and influence various immune cells such as T cells and dendritic cells[50–53]. The adjuvant can enhance T cell proliferation and mediate homing to the gut mucosa[51,54]. It can also increase homing to other mucosal sites such as the vagina and the lung[49,55]. ATRA also affects B cells and drives their differentiation towards plasma cells and increases IgA production[56]. ATRA has an established safety record in humans as a topical agent[57,58] and has been administered orally for the treatment of acute promyelocytic leukemia[59]. More recently, ATRA has been used as an oral adjuvant for typhoid vaccination with Ty21a where the molecule was well tolerated by subjects and led to an increase in antigen-specific IgA titers[60].

The adjuvant dmLT is derived from heat-labile enterotoxin (LT) of *Escherichia coli* following the substitution of two residues in the A subunit of the molecule[48,61]. Administered through various routes, preclinical studies have demonstrated that dmLT can enhance both IgG and IgA antibody titers as well as T_H1- and T_H17-mediated cellular responses in different animal models[62–68]. The enhanced T_H17 activity allows for IL-17 secretion which can upregulate polymeric Ig receptor levels in epithelial cells and thus increase the transport of secretory IgA antibodies at mucosal sites[69–71]. The adjuvant dmLT is safe and effective for use in humans and has been assessed in numerous completed and ongoing clinical trials, particularly for vaccines targeting enterotoxigenic *E. coli* (ETEC)[48].

Using our recombinant chromosomally integrated nirB_SspH1_CatB (NHC) strain as a control, we tested low (10 μg) and high (20 μg) doses of ATRA and dmLT. Mice were vaccinated following the 5-day multimodality schedule described in Chapters 3 and 4 of this thesis. Three oral doses were administered on days 1, 3, and 5 (D1, D3, D5) along with an intramuscular dose of

recombinant antigen administered on D1. Low (LD) or high (HD) doses of each adjuvant were administered on D1, D3, and D5 together with the oral doses of recombinant YS1646. In a preliminary study, anti-Cathepsin B serum IgG and intestinal IgA antibody responses 3 weeks post-vaccination were measured (**Figure 5.1**).

Serum IgG titers for the LD ATRA (8851.2 ± 2086.2 ng/mL), HD ATRA (16563.2 ± 2666.3 ng/mL) and LD dmLT (15654.8 ± 2813.4 ng/mL) groups were not significantly increased compared to the unadjuvanted NHC group (9804.9 ± 2371.1 ng/mL). The HD dmLT group trended towards an increase (17877.4 ± 520.1 ng/mL, $P = 0.08$) and resulted in anti-CatB IgG titers significantly greater than in the LD ATRA group ($P < 0.05$) (**Figure 5.1A**). Intestinal IgA antibodies were most elevated in the HD ATRA (1081.2 ± 158.5 ng/g) and HD dmLT (1098.8 ± 90.8 ng/g) groups and significantly greater than the unadjuvanted control (453.0 ± 95.1 ng/g) (both $P < 0.01$). The low dose groups for ATRA and dmLT displayed non-significant increases in their IgA titers (864.9 ± 105.4 ng/g and 759.2 ± 44.3 ng/g, respectively) (**Figure 5.1B**). Thus, adjuvanting the oral doses with high doses of ATRA and dmLT (20 μg) increased antigen-specific humoral responses. The effect of these adjuvants on cellular immunity and protection from challenge remains to be investigated.

5.3.2 Vaccination in PZQ-treated subjects

Contrary to naïve travellers, individuals from endemic regions have a history of prior infection. Therefore, it would be beneficial to test candidate vaccines under such conditions. Praziquantel (PZQ) is the main drug available for schistosomiasis infection and mass administration occurs yearly in many endemic regions[72]. Co-administration of schistosomiasis vaccines along with PZQ should be evaluated to better approximate field settings.

Praziquantel is an effective oral medication used to treat several helminth infections. In the case of schistosomiasis, it has a high efficacy rate of 60 – 90%. PZQ works by paralyzing the adult worms through action upon calcium-gated channels along their membrane. PZQ administration exposes the host's immune system to several antigenic proteins as worm surface proteins are exposed[73] and antigen-specific antibody titers increase as a result[74,75]. However, there is a risk of

antigenic competition for single-antigen vaccines. The timing of PZQ administration and vaccination needs to be considered to develop the most efficient approach and to take advantage of possible synergistic effects.

5.3.3 Cross-protection against other *Schistosoma* species

While developing a vaccine against *Schistosoma mansoni* was the primary objective, it is of particular interest to note whether this approach may be used to prevent infection by other *Schistosoma* spp. Along with *S. mansoni*, *S. haematobium* is responsible for 95% of human schistosomiasis cases worldwide[76]. As the most common schistosome species in Africa and the Middle East, *S. haematobium* causes approximately 112 million cases in sub-Saharan Africa[77]. These regions are subject to mixed species infections due to the high geographical overlap[78–80]. Therefore, it would be of great interest to deploy a vaccine capable of reducing the disease burden by both schistosome species.

Cathepsins, specifically Cathepsin B, are also present in both *S. haematobium* and *S. japonicum*. In the case of the *S. japonicum* Cathepsin B (SjCatB), it is highly expressed at the infective cercaria stage to penetrate the host's skin[81,82]. More recent studies have shown a role for SjCatB in the degradation of host blood proteins by adult worms to support nutrient acquisition[82,83]. In fact, all three species release cathepsins in the vomitus of the adult worms[84].

The feasibility of utilizing a cathepsin-based vaccination strategy against *S. haematobium* challenge has previously been demonstrated[85]. When mice were immunized against a mixed formulation of *S. mansoni* Cathepsin B and *Fasciola hepatica* Cathepsin L1, the *S. haematobium* worm burden was reduced by 70% and hepatic egg burden was reduced by 60% in a murine model 12 weeks post-infection. When combined with recombinant *S. mansoni* glyceraldehyde 3-phosphate dehydrogenase (rSG3PDH), worm burden reduction increased slightly to 72% and no eggs were observed in the livers of immunized mice. Therefore, there is evidence that immunization with SmCatB can protect against heterologous challenge with *S. haematobium*.

It remains to be evaluated whether immunization against Cathepsin B using our multimodal vaccination approach could yield similar results in *S. haematobium* murine and hamster models. A combination strategy with multiple antigens may be required to confer sufficient cross-protection.

5.3.4 Targeting other *Schistosoma* antigens

In this work, we targeted the digestive enzyme Cathepsin B which is important for nutrient acquisition for both migrating schistosomula and adult worms. This approach differs from other *Schistosoma* vaccines in pre-clinical and clinical development. The current candidate vaccines in phase I and II clinical trials target outer membrane worm proteins[4]. These antigens are tetraspanin-2 (Sm-TSP2), the 14-kDa fatty acid-binding protein (Sm14), and calpain (Sm-p80). A combination approach may be ideal, and a synergistic effect is possible between these clinical candidates and Cathepsin B.

Other groups have attempted combined regimens involving Cathepsin B for *S. mansoni* and *S. haematobium* vaccination strategies[85,86]. As schistosomes are complex parasites, targeting different life-cycle stages or anatomical locations may have additive effects and lead to higher efficacy rates. One of the advantages of our YS1646 vector is its large carrying capacity. Cloning additional *Schistosoma* antigens would be possible in our expression system and used to vaccinate the host against multiple antigenic targets.

5.3.5 Alternative delivery systems or vaccination approaches for Cathepsin B

While attenuated *Salmonella* vectoring proved to be an effective delivery method of our target antigen, other systems may provide additional benefits and should be explored. In terms of bacterial vectors, live attenuated Bacille Calmette-Guérin (BCG) has been repeatedly repurposed to express heterologous antigens[87,88]. BCG has also been repurposed to design recombinant vectored vaccines for several parasitic diseases, namely malaria[89], toxoplasmosis[90], trypanosomiasis[91], and schistosomiasis[92–94]. As part of a collaboration between the laboratories of Drs. Momar Ndao and Michael Reed, BCG is currently being tested as an adjuvant and a recombinant strain of BCG is being developed as an expression vector for *S. mansoni* Cathepsin

B murine studies. As SmCatB has T_H2-skewing properties[95,96], expression of the antigen using a recombinant BCG vector may lead to mixed T_H1/T_H2 immune responses and yield comparable results to vaccination studies with the *Salmonella* vector described in this thesis.

In addition, recent work in the fight against COVID-19 has highlighted the advantages of adenoviral vectors. The use of these vectors is attractive due to their broad tissue tropism and strong expression of target antigens[97,98]. Their safety record has been proven in clinical trials as most individuals did not produce any severe adverse reactions to vaccination[99–101]. Our group has developed a recombinant human adenovirus serotype 5 (Ad5) vector to deliver *Schistosoma mansoni* Cathepsin B (AdSmCB) intramuscularly[102]. Priming with recombinant adenovirus followed by two recombinant protein boosts led to mixed T_H1/T_H2 immune responses, decreased parasite burden, and decreased liver pathology in vaccinated animals.

Finally, the COVID-19 pandemic has accelerated the pace of the development of mRNA-based vaccines against other infectious diseases. Some of the advantages of mRNA vaccines include the induction of a strong and effective immune response, rapid and easy development, and the possibility of multivalent combinations[103,104]. Such vaccines have been recently developed against a few parasitic diseases such as malaria[105,106], leishmaniasis[107], and toxoplasmosis[108]. The development of an mRNA vaccine encoding for the *S. mansoni* Cathepsin B protease may be particularly successful, especially if coupled with other known target antigens. Therefore, these three approaches are alternative strategies for the delivery of Cathepsin B.

5.3.6 Looking ahead toward human studies

As we hope to move past murine studies, several considerations will have to be made and there will be various hurdles to overcome in order to translate the findings of this thesis into clinical work. Bottlenecks that stall progress towards clinical trials constitute what is known as “the valley of death” which reflects the difficult transition from the laboratory to clinical development and practice[109]. Following pre-clinical proof-of-concept studies, the next step will be manufacturing and the scale-up of the vaccine doses, both oral and intramuscular components, and following good manufacturing process (GMP). The live attenuated YS1646 recombinant strain will need to be

properly formulated and stored at optimum conditions to preserve efficacy and viability[110]. The YS1646 strain has been previously used in a clinical trial study of metastatic melanoma patients[31]. Therefore, the toxicity of the *S.* Typhimurium strain has been tested and it is safe for use in humans. However, this study was conducted by administering the attenuated strain intravenously and it did not produce any heterologous antigen as is the case in our vaccine studies. Thus, short toxicity and dose-escalating studies in another animal model, such as non-human primates, would be required before progressing towards a phase I clinical trial.

Other factors to consider for further studies would include the vaccination schedule. The Ty21a oral vaccine for *S.* Typhi is administered in three doses (four doses in North America) given every other day over the course of one week[111]. Thus, the vaccination schedule may remain the same in clinical trial studies and this would be highly advantageous as it would reduce the number of appointment visits in low-resource areas and be much faster than conventional intramuscular vaccination regimens. Moreover, clinical assays to test the immunogenicity of the vaccine will need to be optimized. Other than assessing serum antibody levels, cellular immune responses may be evaluated by collecting peripheral blood mononuclear cells (PBMCs). Intestinal IgA secretions may be assessed through fecal samples.

Next, following manufacturing processing, assay optimization, and toxicity & efficacy studies in non-human primates, a phase I clinical trial may be implemented to assess safety in healthy adults. The recombinant YS1646 vaccine strain is susceptible to antibiotics in the case of a major adverse event. Dose-ranging studies, as well as safety studies in children, may also be required. Eventually, a phase II study in adults from endemic regions may be implemented to assess vaccine efficacy followed by a phase IIb study in school-aged children from endemic areas as they represent a significant risk group for schistosomiasis. The vaccine may be tested in treated or currently infected individuals to test both its prophylactic and therapeutic effects. The therapeutic arm of the vaccine may include the co-administration of PZQ. As a neglected tropical disease, large amounts of funding from governments as well as philanthropic organizations will be necessary to push a vaccine through the various stages of clinical testing. A large-scale phase III trial would require many resources and the involvement of different stakeholders. Thus, the path for an eventual

schistosomiasis vaccine from pre-clinical testing to licensure will be arduous. Still, it is not outside the realm of possibility if provided with sufficient support.

5.4 CONCLUDING REMARKS

In this work, we explored the use of an attenuated YS1646 strain of *Salmonella* Typhimurium to express the heterologous *Schistosoma mansoni* antigen Cathepsin B as a vaccine vector. Our initial studies suggested high efficacy when delivered in a 3-week prime-boost approach consisting of 3 oral doses followed by a recombinant protein boost administered intramuscularly. These remarkable results lead to the investigation of a therapeutic effect of our vaccine in a chronic challenge model. With success as both a prophylactic and therapeutic vaccine, we then turned towards chromosomal integration of Cathepsin B into our vector to mitigate the risks associated with plasmid-based expression. This newly constructed vaccine proved to be able to elicit robust systemic and local immune responses in a murine model. Considerable work remains to elucidate the mechanisms of action of our vaccine as well as to assess its efficacy in larger animal models, and eventually in humans. Overall, our approach is a promising strategy in the development of a *Schistosoma mansoni* vaccine, and we hope these efforts provide significant contributions to the field.

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5.6 FIGURE & FIGURE LEGEND

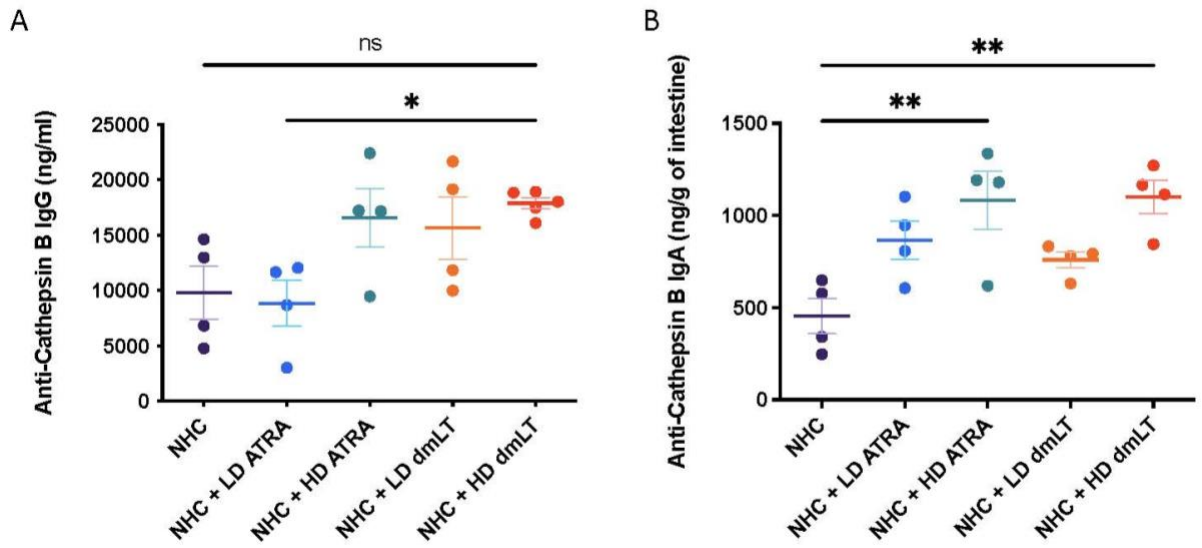

Figure 5.1. Humoral responses. Serum and intestinal samples were collected 3 weeks post-vaccination. Anti-Cathepsin B IgG titers expressed as ng/mL (A). Anti-CatB intestinal IgA titers expressed as ng/g (B). n = 4-5 animals per group. Data are shown as mean ± SEM (**$P < 0.05$, **$P < 0.01$).